toxicity corresponded to a moderate pulp reaction. The authors concluded that cell culture techniques have some value in prediction of toxicity of implant materials. Wennberg et al [25] made similar observations comparing different dental filling materials in different cytotoxicity tests, implantation tests (30-90 days) and usage tests in dental pulp (7-78 days). In this study only few correlations were seen between cytotoxicity tests, usage tests and implantation tests. The outcome of a cytotoxicity test could be altered by conditioning the tested materials in either saline, cell culturing media or saline/bovine serum albumin [27]. Conditioned materials had reduced cytotoxicity compared to unconditioned materials. This indicates that the *in vitro* test design is crucial.. In the dynamic *in vivo* situation the release kinetics is quite different from the *in vitro* model. In the *in vivo* model there is a well-vascularized tissue, which may effectively lower the local concentrations of toxic substances at the interface whereas in the standard *in vitro* test situation the ZDEC compounds could not be effectively removed by the surrounding fluid.

In contrast to some studies demonstrated a correlation between the grade of in vitro cytotoxicity and some in vivo tissue response parameters up to 2 weeks implantation- implantation time [24, 26]. These different results in previous studies only emphasize that the design of both *in vitro* tests and of the *in vivo* situations is of major importance for the outcome of the study. When comparing the results from the water pre-extracted RMs to the RMs that had been implanted in a biological environment, it was apparent that the water pre-extraction method

was not as powerful in washing out the toxic compounds (ZDEC) from the test samples as the *in vivo* situation(see Figs.1 and 5)..

The explanted RMs had released almost all their toxic products during the first week implantation period (see Fig.5), which is most likely the reason for the declining tissue reactions at the ZDEC impregnated implants. The vascularization provides a continuing flow around the inserted implant, including a collection of substances that can elute the toxicity of the implant. For example the toxic compound ZDEC has low solubility in water but is soluble in oil. Body fluids including serum that contains lipid are likely to be more powerful than water to extract lipophilic ZDEC from the hydrophilic polyurethane. In addition, complex-binding proteins may decrease the toxicity. The specific mechanisms are likely to vary with the cytotoxic compound.

No previous studies have analyzed the implant toxicity in the post implantation situation. The results of the present study indicate that an initially inflammtogenic implant material could in long term perspective become biocompatible and the observed tissue response an indicator of the current state of the material than a historical record of past material's properties. As a consequence future material testing should be carried out also *in vivo* even for *in vitro* toxic materials that may have unique clinical use.

In our opinion the *in vitro* cytotoxicity results have to be used with caution when assessing the biocompatibility of a new biomaterial and that initial cytotoxic materials may be used successfully in the clinics, provided that the materials loose their toxicity over time. The *in vitro* models might also be further developed

to mimic the *in vivo* elution situation, either by repeated changes of buffer or by a flow-cell or by optimizing the buffer composition. There may however be instances where the initial tissue trauma is so extensive that the tissue may never recover.

The increased capsule thickness at the ZDEC implants after 1-week implantation could possibly that it be due to an initial tissue edema [28, 29]. In an edema tissue would be extended and the cells should histologically appear more separated. Looking at the numbers of cell nuclei or ED1 positive macrophages, the highest cell densities were present at the one-week time point. This indicates that there was no or very minute edema induced by the implanted materials. Further, there were no differences in cell densities either at the interface or in the capsule between the control material and the toxic materials, supporting the conclusion that ZDEC does not induce edema but an increased cellularity.

**In conclusion**, this study shows that initial cytotoxicity has limited predictive value for long term *in vivo* tissue response. Therefore cytotoxicity tests as used today, may result in the rejection of clinically useful materials. This is in agreement with clinical experience from the use of bone cement that performs well over long time periods despite their initial toxicity. It seems that early tissue events are of minor importance for the long-term biocompatibility.

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## Abbreviated title

Foreign body induction by material toxicity

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The long-term foreign body reaction is not influenced by initial implant material cytotoxicity

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Captions

## Figure legends

*Figure 1*

*Medium-extract method*

In vitro cytotoxicity of positive RMs (0.5% and 1% ZDEC-PU) as a function of pre-extraction times in water.

*Figure 2*

Capsule thickness after 1 and 6 weeks implantation of negative control, 0.5% and of 1% ZDEC-PU implants in rat abdominal wall (n=8). Bars represent the max. and min. values. The crossbar denotes the median value.

*Figure 3*

Microphotographs (objective magnification 20x) of immunohistochemical stained sections taken from toxic and non-toxic implants with adjacent tissue, inserted in rat abdominal wall for 1 or 6 weeks. Implant is always to the *right* with border indicated (arrowheads). ED1 positive cells stain brown (arrows); m = muscle.

a) Negative control after 1 week implantation
b) 1% ZDEC-PU implant after 1 week implantation. Note the foreign body capsule thickness.
c) Negative control after 6 weeks implantation
d) 1% ZDEC-PU implant after 6 weeks implantation

*Figure 4*

Numbers of positive cells in foreign body capsule at negative control, 0.5% and of 1% ZDEC-PU implants after 1 and 6 (n=8). Bars represent the max. and min. value. The crossbar denotes the median value.

*Figure 5*

*Medium-extract method*

In vitro toxicity of explanted test samples (negative controls, 0.5% and 1% ZDEC-PU). $IC_{50}$ =100 denotes a non-toxic material. Note that none of the materials display detectable toxicity after 6 weeks.

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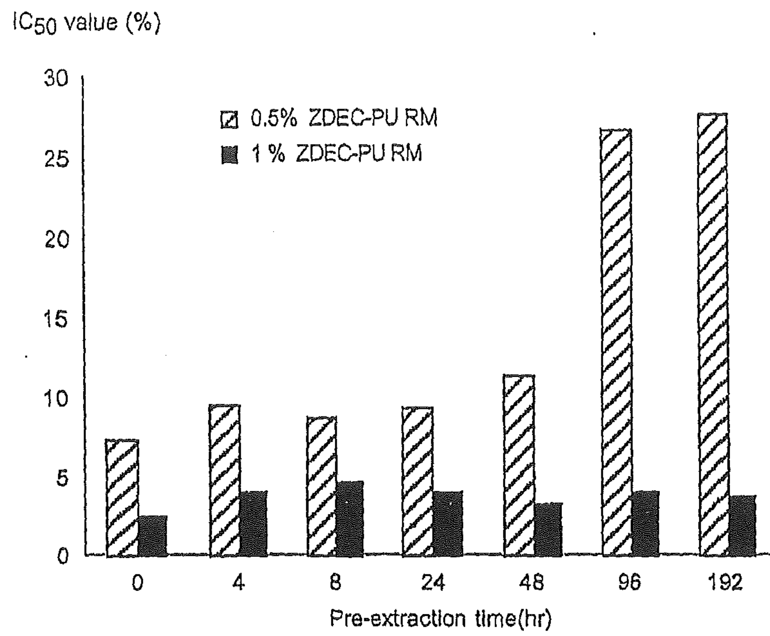
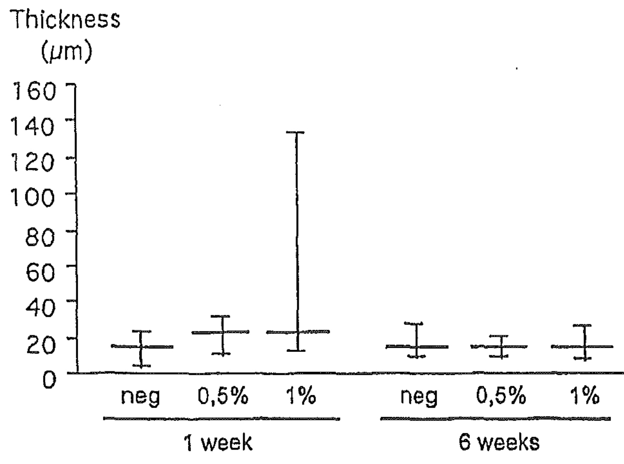



Figure 1. A.Rosengren et al.

Figure 2



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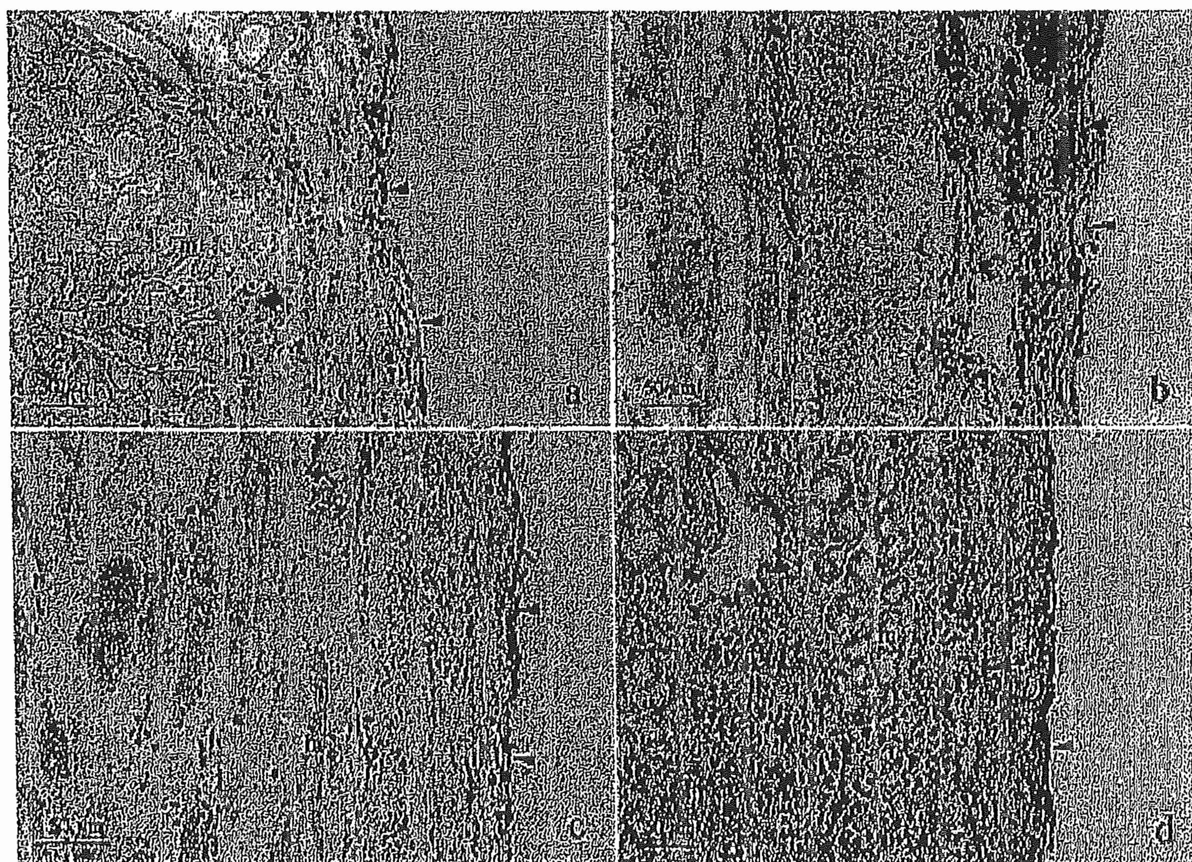
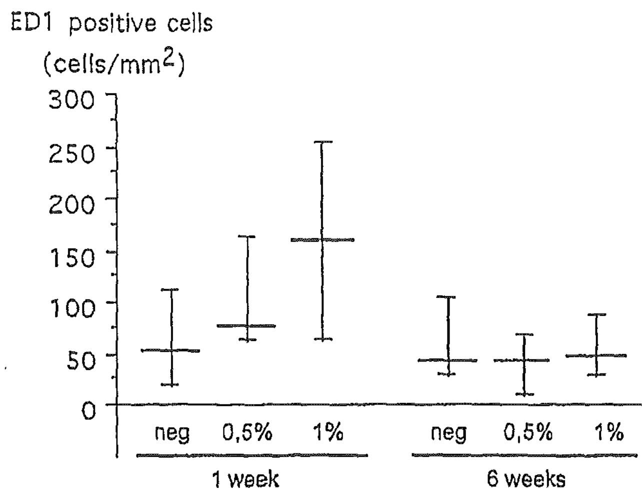


Figure 4



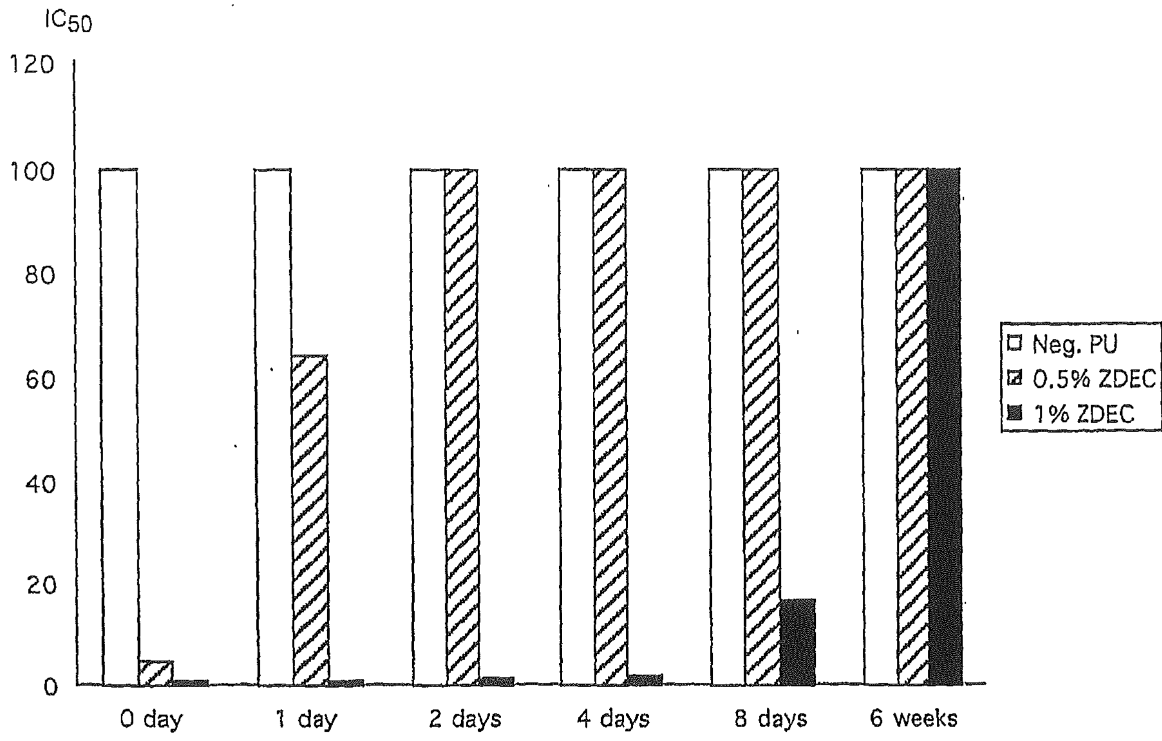



Figure 5. A.Rosengren et al.

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Genetic Toxicology and Environmental Mutagenesis

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# Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-*ras*-transfected BALB/c 3T3 cells (Bhas 42 cells)

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**›stract**

Cell transformation assay using BALB/c 3T3 cells, C3H10T1/2 cells and others, can simulate the two-stage carcinogenesis lized for formation of transformed foci. A sensitive cell transformation assay for tumor initiators as well as promoters has en developed using a v-Ha-*ras*-transfected BALB/c 3T3 cell line, Bhas 42; these cells are regarded as initiated in the two-ıge paradigm of carcinogenesis. To distinguish between initiation and promotion, the initiation assay involves a 2-day treatment low-density cells, obtained one day after plating, with a test chemical, and the promotion assay involves treatment of near-nfluent cells with a test chemical for a period of 12 days (Day 3–14). When Bhas 42 cells were treated with tumor initiators, ·methyl-*N'*-nitro-*N*-nitrosoguanidine and 3-methylcholanthrene, transformed foci were induced in the initiation assay but not the promotion assay. In contrast, tumor promoters, 12-*O*-tetradecanoylphorbol-13-acetate, lithocholic acid and okadaic acid, ıve negative responses in the initiation assay but positive responses in the promotion assay. The results were reproducible with ırious treatment protocols. Sixteen polycyclic aromatic hydrocarbons were examined using both assays. Benzo[*a*]pyrene and 12-dimethylbenz[*a*]anthracene induced focus formation only in the initiation assay. Increase of focus formation was observed in ıe promotion assay with benzo[*e*]pyrene, benzo[*ghi*]perylene, 1-nitropyrene and pyrene. Benz[*a*]anthracene, benz[*b*]anthracene, ırysene and perylene showed positive responses in both initiation and promotion assays. Results of initiation and promotion assays f acenaphthylene, anthracene, coronene, 9,10-diphenylanthracene, naphthalene and phenanthrene were negative or equivocal. he present Bhas assays for the detection of either/both initiating and promoting activities of chemicals are sensitive and of high erformance compared with other cell transformation assays.
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*eywords:* Transformation; Bhas 42 cells; Complete carcinogen; Initiation and promotion

## 1. Introduction

Chemical carcinogens can be divided into two categories, i.e., initiators and promoters, based on the two-stage model of carcinogenesis [1,2]. Most initiators can be detected by various genotoxicity tests, the results

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of which are used for carcinogenicity prediction and for regulatory purposes by authorities [3,4]. In the case of tumor promoters, several methods have been proposed [5], but none of them have been routinely used for regulatory purposes. Therefore, to develop a method for detection of non-genotoxic carcinogens with various mechanisms of action is a major challenge for the safety evaluation of chemicals [5,6]. The utilization of additional screening tests covering a wide range of carcinogenic processes has advantage before contemplating in vivo long-term carcinogenicity experiments for chemical safety assessment.

The cell transformation assays using BALB/c 3T3 cells [7,8] and C3H10T1/2 cells [9,10] can simulate the process of two-stage animal carcinogenesis [11,12]. Formation of transformed foci is the consequence of the complex process of transforming cells to a malignant state. Since these assays can detect both initiating and promoting activities [13,14], their inclusion as screening tools is anticipated to be useful for detection of not only tumor initiators but also tumor promoters such as non-genotoxic carcinogens. In spite of this expectation, cell transformation assays have not been accepted as a routine screening method, because of the laborious and time-consuming procedure compared with the routine genotoxicity assays [15].

We have developed a sensitive cell transformation assay for detecting tumor promoters using Bhas 42 cells [16] that was established by Sasaki et al. [17]. The cells, v-Ha-*ras*-transfected BALB/c 3T3 cells, are considered as initiated in the two-stage transformation paradigm [18]. The assay method has many advantages, e.g., high sensitivity, short experimental period, use of smaller amounts of materials, and simplicity of the procedure.

After establishing the promotion assay using Bhas cells, various chemicals including tumor initiators were examined for their potency of tumor-promoting activity. Some initiators are known to have tumor promotion capacity in animal experiments (complete carcinogens). However, *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and 3-methylcholanthrene (MCA), as examples of initiators, did not show clear positive response in our preliminary study. In the promotion assay, Bhas cells were treated with these chemicals when the cells were near confluence. Since initiators need several cell divisions for fixation of gene mutation [19–21], we designed a new treatment protocol in which cells were seeded at a lower density and allowed to divide several times in culture dishes after treatment with test chemicals.

In the present work, tumor initiators and promoters were examined in the newly developed protocol with various treatment schedules in order to define the most effective protocol for detection of initiators and promoters. The effect of repeated treatment with MCA at initiating and/or promoting period with several time Schedules was compared with that of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a model promoter. In addition, several polycyclic aromatic hydrocarbons (PAHs) were tested with the novel protocol to validate the assay.

## 2. Materials and methods

### 2.1. Cell cultures

Minimum essential medium (MEM) was obtained from Nissui Pharmaceutical, Tokyo, Japan. Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) was purchased from GIBCO Laboratories, Grand Island, NY. Fetal bovine serum (FBS) was obtained from Moregate, Bulimba, Australia.

Bhas 42 cells were routinely cultured in MEM supplemented with 10% FBS (M10F) in a humidified 5% $CO_2$ incubator at 37 °C. The cells were subcultured using 0.25% trypsin (GIBCO) before reaching confluence. For transformation assays, Bhas 42 cells were cultured in DMEM/F12 supplemented with 5% FBS (DF5F).

### 2.2. Chemicals

Acenaphthylene, benzo[*a*]pyrene (B[*a*]P), benzo[*ghi*]perylene (B[*ghi*]P), coronene, 7,12-dimethylbenz[*a*]anthracene (DMBA), lithocholic acid (LCA), MCA, naphthalene, okadaic acid, perylene, phenanthrene and pyrene were obtained from Wako Pure Chemical Industries (Osaka, Japan). Anthracene, benz[*a*]anthracene (B[*a*]A), benz[*b*]anthracene (B[*b*]A) and MNNG were obtained from Kanto Chemical (Tokyo, Japan). Aflatoxin $B_1$ ($AFB_1$), benzo[*e*]pyrene (B[*e*]P) and TPA were obtained from Sigma Chemical Co. (St. Louis, MO). Chrysene was obtained from Avocado Research Chemicals (Lancashire, UK), 1-nitropyrene (1-NP) from Aldrich (St. Louis, MO), and 9,10-diphenylanthracene (9,10-DPhA) from Merck (Rahway, NJ). These chemicals except for coronene were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries); coronene was suspended in 0.5% carboxymethylcellulose sodium salt solution (CMC-Na; Wako Pure Chemical Industries). Final concentrations of these solvents in experimental medium were adjusted to less than 0.1% in the case of DMSO or less than 1% in the case of CMC-Na.

### 2.3. Cell growth assay

Cell growth assays, using the standard crystal violet absorption method [22], were applied to dose range finding for initiation and promotion assays. Additionally, the cell growth assay was performed concurrently with every transformation assay. In the case of initiation assay, cell numbers were adjusted to $2 \times 10^3$ cells/mL in M10F and the cell suspension was seeded onto 24-well microplates at 0.5 mL per well (Day 0). Three wells were prepared for each test concentration. After a 24 h

I

its of initiation and promotion assays for initiators and promoters

| ical | Concentration | S9 mix | Initiation assay | | Promotion assay | |
|---|---|---|---|---|---|---|
| | | | Number of foci/well | Cell viability | Number of foci/well | Cell viability |
| NG | 0 μg/mL | – | 5.3 ± 1.8 | 100.0 | 1.5 ± 0.5 | 100.0 |
| | 0.002 μg/mL | – | N.E. | | 1.5 ± 1.2 | 96.4 |
| | 0.005 μg/mL | – | N.E. | | 1.2 ± 1.0 | 96.1 |
| | 0.01 μg/mL | – | N.E. | | 1.3 ± 1.5 | 95.8 |
| | 0.02 μg/mL | – | 4.0 ± 1.8 | 90.6 | 1.2 ± 0.4 | 98.4 |
| | 0.05 μg/mL | – | 5.8 ± 2.9 | 90.8 | 1.0 ± 1.3 | 97.4 |
| | 0.1 μg/mL | – | 5.5 ± 1.9 | 88.7 | 1.7 ± 1.2 | 93.1 |
| | 0.2 μg/mL | – | 6.7 ± 2.9 | 62.1 | | tox. |
| | 0.5 μg/mL | – | 10.0 ± 2.6 | 69.6 | | tox. |
| | 1 μg/mL | – | 12.0 ± 1.5 | 57.0 | | tox. |
| A | 0 μg/mL | – | 3.0 ± 1.1 | 100.0 | 1.5 ± 0.5 | 100.0 |
| | 0.002 μg/mL | – | N.E. | | 1.0 ± 0.9 | 93.6 |
| | 0.005 μg/mL | – | N.E. | | 1.3 ± 0.5 | 89.7 |
| | 0.01 μg/mL | – | N.E. | | 1.2 ± 0.4 | 85.5 |
| | 0.02 μg/mL | – | 2.7 ± 2.0 | 61.6 | 1.8 ± 0.8 | 88.7 |
| | 0.05 μg/mL | – | 3.7 ± 2.0 | 65.6 | 1.5 ± 1.0 | 84.7 |
| | 0.1 μg/mL | – | 6.2 ± 2.6 | 62.7 | 2.0 ± 1.3 | 69.2 |
| | 0.2 μg/mL | – | 8.2 ± 1.9 | 79.6 | | tox. |
| | 0.5 μg/mL | – | 9.7 ± 2.1 | 45.2 | | tox. |
| | 1 μg/mL | – | 11.8 ± 4.0 | 35.8 | | tox. |
| FB1 | 0 μg/mL | – | 3.0 ± 1.1 | 100.0 | 1.5 ± 0.5 | 100.0 |
| | 0.02 μg/mL | – | N.E. | | 1.5 ± 0.8 | 95.9 |
| | 0.05 μg/mL | – | 2.5 ± 1.5 | 64.2 | 0.7 ± 1.2 | 91.5 |
| | 0.1 μg/mL | – | 1.8 ± 1.2 | 48.8 | 0.7 ± 0.5 | 85.2 |
| | 0.2 μg/mL | – | 3.0 ± 1.7 | 49.3 | 0.5 ± 0.8 | 87.4 |
| | 0.5 μg/mL | – | 3.5 ± 1.5 | 44.7 | 0.7 ± 0.5 | 86.0 |
| | 1 μg/mL | – | 2.5 ± 0.5 | 24.5 | 1.3 ± 0.8 | 73.4 |
| | 2 μg/mL | – | 0.8 ± 1.0 | 12.5 | | tox. |
| FB1 | 0 μg/mL | + | 3.2 ± 1.5 | N.E. | N.E. | |
| | 0.05 μg/mL | + | 3.7 ± 1.6 | N.E. | N.E. | |
| | 0.1 μg/mL | + | 4.2 ± 1.5 | N.E. | N.E. | |
| | 0.2 μg/mL | + | 4.8 ± 3.3 | N.E. | N.E. | |
| | 0.5 μg/mL | + | 4.7 ± 1.8 | N.E. | N.E. | |
| | 1 μg/mL | + | 14.3 ± 2.4 | N.E. | N.E. | |
| | 2 μg/mL | + | 11.7 ± 2.8 | N.E. | N.E. | |
| PA | 0 ng/mL | – | 4.3 ± 1.0 | 100.0 | 2.8 ± 0.4 | 100.0 |
| | 2 ng/mL | – | 2.3 ± 0.5 | 97.5 | 9.0 ± 1.3 | 100.0 |
| | 5 ng/mL | – | 2.7 ± 1.2 | 92.8 | 12.7 ± 1.0 | 92.7 |
| | 10 ng/mL | – | 2.5 ± 1.0 | 93.8 | 17.2 ± 2.2 | 95.9 |
| | 20 ng/mL | – | 1.3 ± 0.8 | 97.6 | 26.0 ± 3.0 | 105.5 |
| | 50 ng/mL | – | 2.2 ± 0.8 | 96.8 | 28.7 ± 3.3 | 111.1 |
| | 100 ng/mL | – | 0.8 ± 1.3 | 90.0 | 27.3 ± 2.9 | 112.2 |
| CA | 0 μg/mL | – | 3.0 ± 1.1 | 100.0 | 4.2 ± 0.8 | 100.0 |
| | 0.5 μg/mL | – | 2.5 ± 2.2 | 91.2 | 6.5 ± 1.9 | 99.3 |
| | 1 μg/mL | – | 3.2 ± 1.6 | 84.1 | 7.8 ± 1.0 | 94.7 |
| | 2 μg/mL | – | 2.2 ± 1.8 | 89.4 | 7.2 ± 2.2 | 94.0 |
| | 5 μg/mL | – | 2.0 ± 1.3 | 125.7 | 11.7 ± 1.4 | 101.1 |
| | 10 μg/mL | – | 2.8 ± 1.2 | 117.7 | 16.5 ± 1.0 | 91.0 |
| | 20 μg/mL | – | 3.8 ± 1.9 | 109.7 | 31.8 ± 3.1 | 79.7 |
| kadaic acid | 0 ng/mL | – | 4.3 ± 1.4 | 100.0 | 4.2 ± 0.8 | 100.0 |
| | 0.2 ng/mL | – | 2.0 ± 1.1 | 87.5 | N.E. | |
| | 0.5 ng/mL | – | 3.0 ± 0.6 | 96.0 | N.E. | |
| | 1 ng/mL | – | 3.3 ± 2.2 | 105.5 | 4.8 ± 2.5 | 97.1 |

Table 1 (*Continued*)

| Chemical | Concentration | S9 mix | Initiation assay | | Promotion assay | |
|---|---|---|---|---|---|---|
| | | | Number of foci/well | Cell viability | Number of foci/well | Cell viability |
| | 2 ng/mL | – | 3.5 ± 1.2 | 114.5 | 5.8 ± 1.6 | 90.1 |
| | 4 ng/mL | – | N.E. | | 4.3 ± 1.9 | 70.7 |
| | 5 ng/mL | – | 3.0 ± 1.4 | 89.0 | N.E. | |
| | 6 ng/mL | – | N.E. | | 7.0 ± 3.1 | 56.8 |
| | 8 ng/mL | – | N.E. | | 7.7 ± 1.5 | 40.3 |
| | 10 ng/mL | – | 3.5 ± 2.4 | 98.0 | 12.5 ± 1.4 | 37.0 |

N.E., not experimented; tox.: toxic.

cultivation, cells were treated with a fresh medium containing a test chemical. The cells were fixed with a 10% formalin on Day 3 and stained with a 0.1% crystal violet (CV) solution. CV was extracted from stained cells in each well with 0.5 mL of a solution containing 0.02 mol/L hydrochloric acid in 50% ethanol. Optical density of CV extracted from stained cells was measured at 540 nm, and the results were expressed as percentage of absorbance compared to the solvent control culture.

In the growth testing for the promotion assay, cell numbers were adjusted to $2 \times 10^4$ cells/mL in DF5F and seeded onto 24-well microplates at 0.5 mL per well. After a 3-day cultivation, medium was replaced with the one containing a test chemical. Cells were then fixed and stained on Day 7, and optical density of CV extracted from stained cells was measured as above.

### 2.4. *Transformation assay for initiating activity (Bhas initiation assay)*

The procedure for the initiation assay using BALB/c 3T3 cells [23] was adopted for the present Bhas initiation assay. Cell numbers were adjusted to $2 \times 10^3$ cells/mL in M10F and the cells were seeded onto each well of six-well microplates in 2 mL amounts (Day 0). Six wells were prepared for each test concentration. After a 24 h cultivation, cells were treated with a fresh medium containing a test chemical. Culture medium was replaced with DF5F without test chemical on Day 3; thereafter, culture medium was changed twice a week with fresh DF5F without test chemical. On Day 24, the cells were fixed with methanol and stained with a 5% Giemsa solution. Transformed foci were judged from morphological characteristics: deep basophilicity, dense multilayering of cells, and random orientation of cells at the edge of foci.

### 2.5. *Transformation assay for promoting activity (Bhas promotion assay)*

Bhas promotion assay was conducted according to the procedure reported by Ohmori et al. [16]. Cell numbers were adjusted to $2 \times 10^4$ cells/mL in DF5F and seeded onto each well of six-well microplates in 2 mL amounts (six wells per test concentration) (Day 0). Medium was replaced with fresh medium containing a test chemical on Day 3, Day 7 and Day 10, and then with fresh medium without the test chemical on Day 14. On Day 21, the cells were fixed with methanol and stained with a 5% Giemsa solution for focus counting.

### 2.6. *Metabolic activation in initiation assay*

In the case of metabolic activation of $AFB_1$, cultures of Bhas 42 cells were started under the same conditions as those of the initiation assay. Three wells were prepared for each test concentration. After a 24 h incubation, cells were treated with $AFB_1$ together with S9 mix which contained 5% rat S9 (Kikkoman, Chiba, Japan), 2 mM HEPES (Sigma Chemical Co.), 5 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphatase (Sigma Chemical Co.) and 4 mM β-$NADP^+$ (Oriental Yeast, Osaka, Japan). After a 3 h treatment, all plates were washed with phosphate-buffered saline and provided fresh M10F. The culturing was continued until Day 24 and the cells were fixed and stained for focus counting.

### 2.7. *Transformation assay with various schedule of treatments*

Cells were seeded onto six-well microplates as with the initiation assay. MCA (100 ng/mL) or TPA (50 ng/mL) was added at various time schedules during cell-growth phase (initiation stage) and/or stationary phase (promotion stage). Three wells were prepared for each set of conditions. Cells were cultivated until Day 24, and then fixed and stained for focus counting.

### 2.8. *Statistical analysis and criteria of judgment*

Results of initiation and promotion assays were evaluated as follows. After *t*-test analysis, chemicals showing significant increase ($p < 0.05$) of focus number at more than two consecutive concentrations were judged positive. Chemicals showing statistically significant effect at only one concentration were considered equivocal. Negatives were those which induced no statistically significant increase of transformed foci at any concentrations tested.

## 3. Results

### 3.1. Establishment of initiation assay

Preliminary examination with MNNG and MCA in Bhas promotion assay revealed little response in the formation of transformed foci. Then, an experimental procedure was designed in which Bhas 42 cells were seeded at one-tenth lower cell density ($2 \times 10^3$ cells/mL) than in the promotion assay ($2 \times 10^4$ cells/mL), a procedure that will permit cells to divide several times after the treatment with test chemicals. With the protocol described in Section 2.4, MNNG and MCA induced a significant number of foci (Fig. 1A and C).

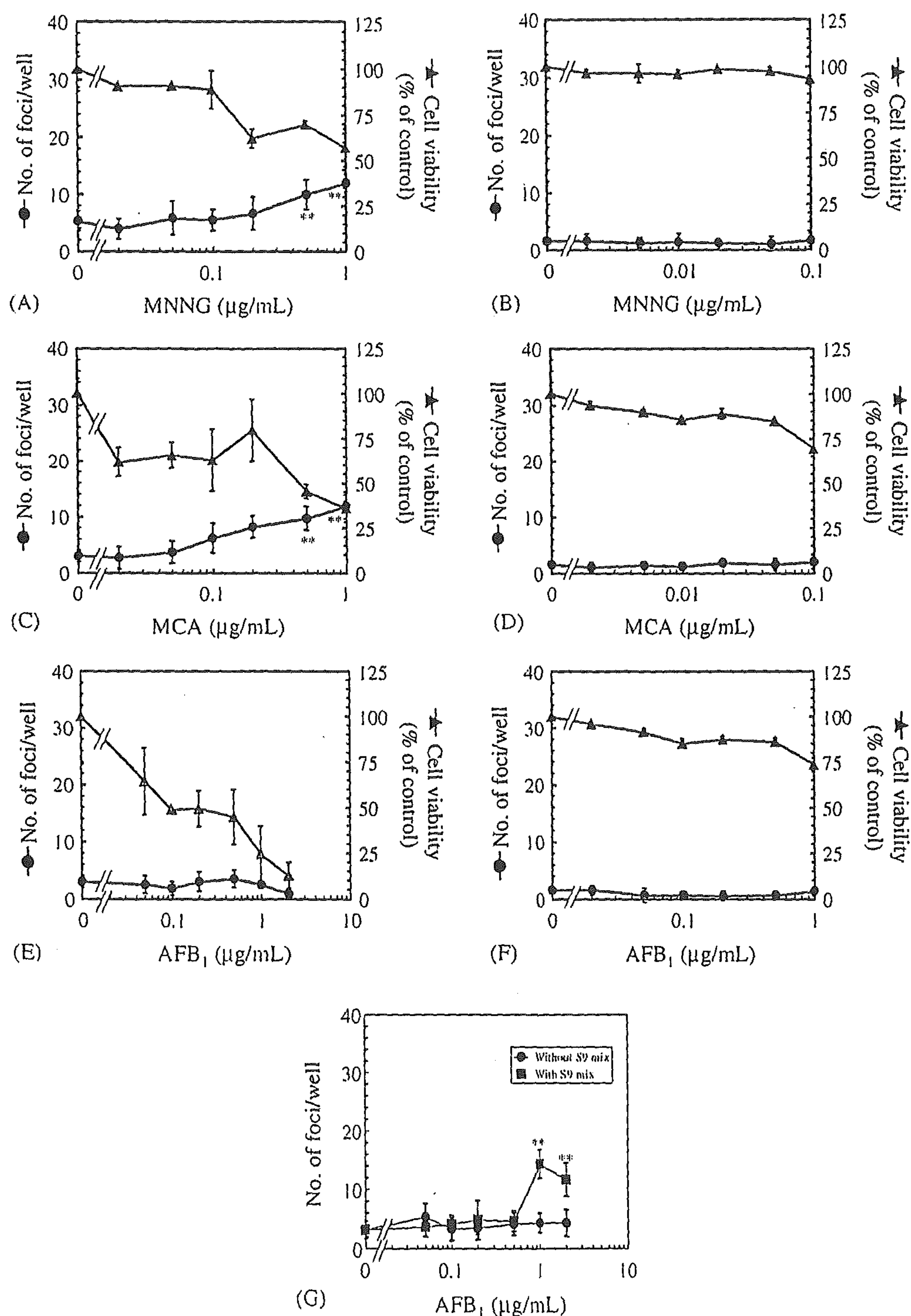


Fig. 1. Results of transformation assays of initiators. A, C and E show results of initiation assay and B, D and F show results of promotion assay. G shows result of metabolic activation in AFB$_1$ initiation assay. $^{*}p<0.05$, compared with solvent control. $^{**}p<0.01$, compared with solvent control.

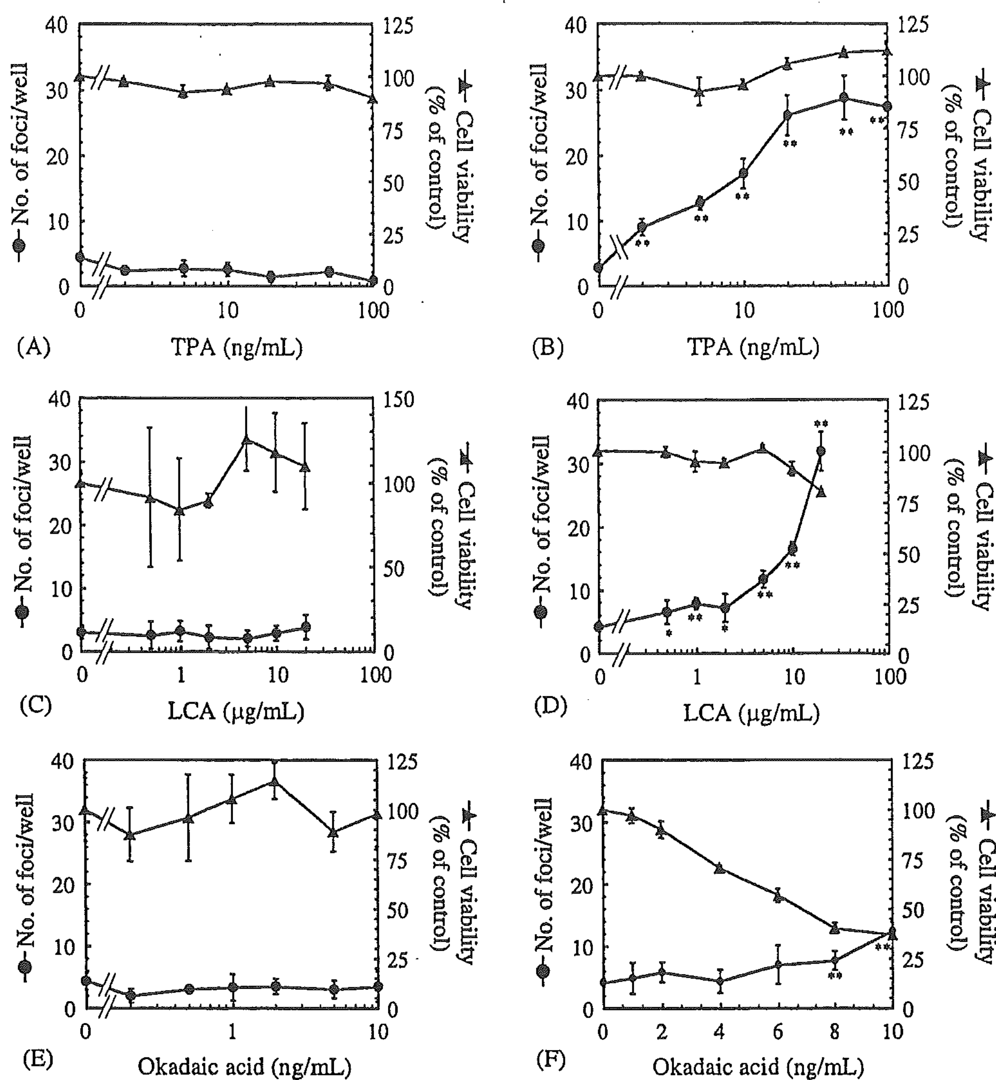


Fig. 2. Results of transformation assays of promoters. A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

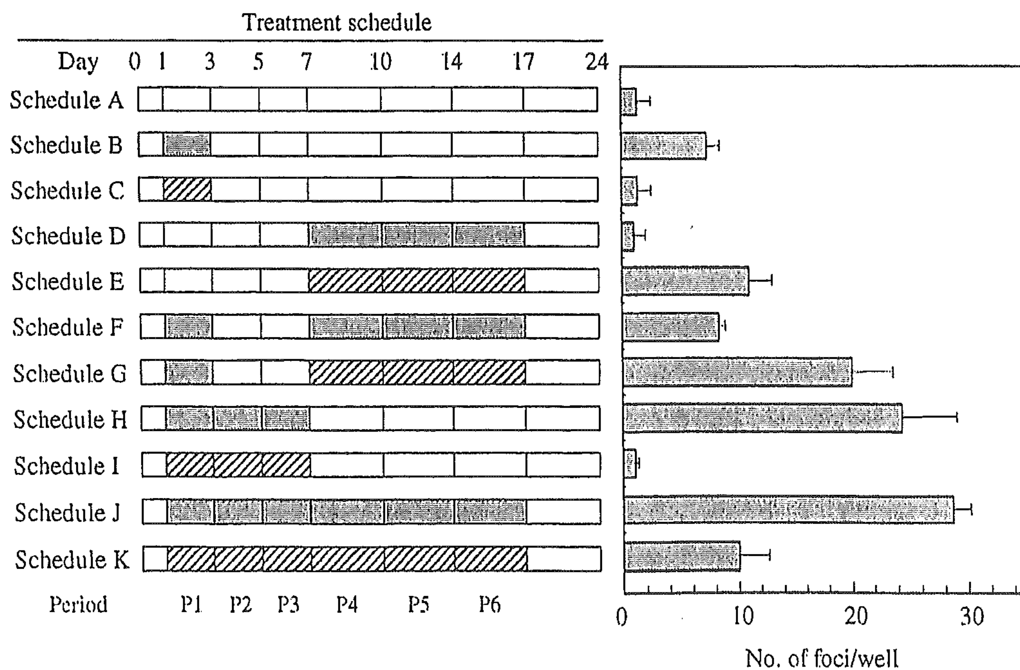


Fig. 3. Result of transformation assay under various time schedules. In treatment schedule, solid blocks represent treatment with 100 ng/mL MCA and diagonal blocks represent treatment with 50 ng/mL TPA.

Transformation assays of initiators

e results are summarized in Table 1. Fig. 1A and B s the results of Bhas initiation and promotion assays NNG, respectively. Significant increase of focus ation was observed in the initiation assay at the entrations of 0.5 μg/mL and above. As mentioned e, MNNG did not induce transformed foci in the notion assay up to 0.1 μg/mL. With concentrations 0.2 μg/mL in the promotion assay, cell flattening enlarged cell size and without increased cell num- was observed after triple dosing with concentrations 0.2 μg/mL in the promotion assay.

Results of the initiation and promotion assays for A are shown in Fig. 1C and D. MCA showed a ponse similar to that with MNNG. MCA induced nsformed foci in the initiation assay at concentrations 0.5 μg/mL and higher. In the case of the promotion ay, no focus formation was observed at concentrations to 0.1 μg/mL and severe cytotoxicity was observed at ncentrations over 0.2 μg/mL.

$AFB_1$ showed negative results in both initiation and omotion assays (Fig. 1E and F). In the case of pro- otion assay, 1 μg/mL of $AFB_1$ was the maximum con- ntration tested because of its cytotoxicity. Metabolic tivation of $AFB_1$ by cytochrome P450 (CYP) 2A6, A4 and 1A2 is necessary to exert mutagenic and car- nogenic activities [24,25]. Fig. 1G shows results of the itiation assay of $AFB_1$ metabolically activated by S9 mix, and there was significant foci induction at 1 and 2 μg/mL.

### 3.3. Transformation assays of tumor promoters

Initiation and promotion activities of three tumor promoters were also examined and the results are summarized in Table 1. Fig. 2A and B shows results on TPA. No increase of focus number was observed in the initiation assay up to 100 ng/mL (Fig. 2A). In contrast, significant increase of focus formation was observed in the promotion assay at all concentrations tested up to 100 ng/mL (Fig. 2B).

Fig. 2C and D shows results on LCA. As in the case of TPA, transformed foci were not induced in the initiation assay at all concentrations tested (Fig. 2C), whereas transformed foci were observed in the promotion assay at all concentrations tested (Fig. 2D).

Okadaic acid, up to 10 ng/mL, did not induce transformed foci (Fig. 2E) in the initiation assay. A significant increase of focus formation was observed in the promotion assay at 8 and 10 ng/mL at which high cytotoxicity was shown (Fig. 2F).

### 3.4. Transformation assays with various schedules of treatment

Fig. 3 shows results of treatment with MCA (100 ng/mL) and/or TPA (50 ng/mL) under various treat-

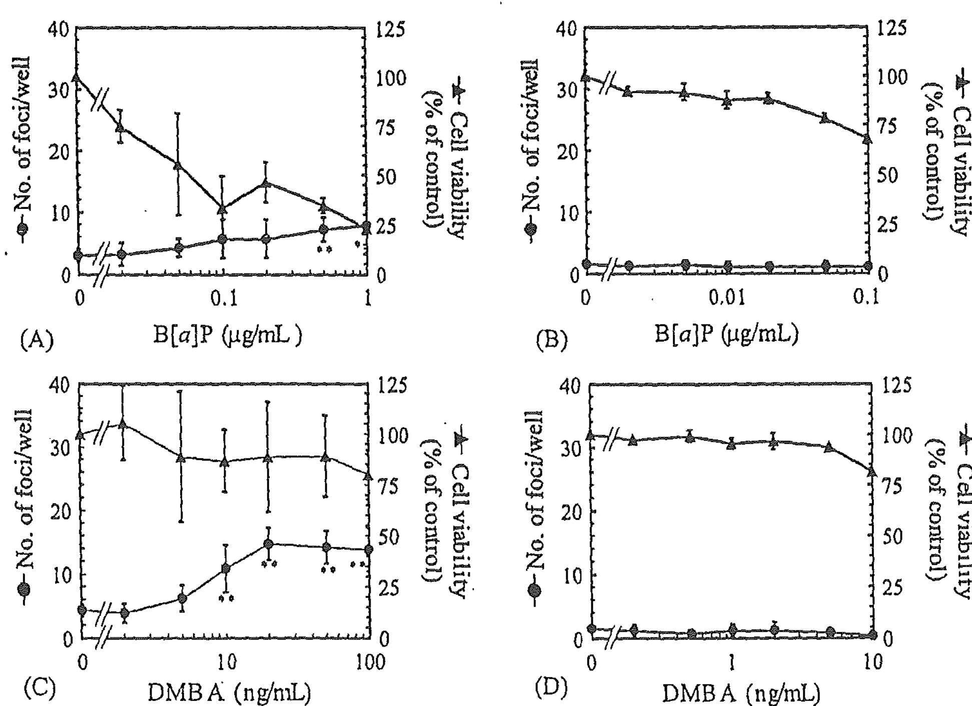


Fig. 4. Results of initiation and promotion assays of B[a]P and DMBA (Group I). A and C show results of initiation assay and B and D show results of promotion assay. $^{*}p<0.05$, compared with solvent control. $^{**}p<0.01$, compared with solvent control.