over time. Spearman Rank correlation test was used to analyze correlations between grade of toxicity and capsule thickness or numbers of cells.

In vitro cytotoxicity of explanted RMs

The explanted RMs were subjected to *in vitro* cytotoxicity test, using the TC-insert method (1) and Medium extract method (2), described above.

Results

Cytotoxicity of RMs extracted in vitro

TC-insert method (1)

Negative RMs showed high level of colony formation in similarity to the negative control at the present test condition. No colony formation was detected for the 0.5% and 1% ZDEC-PU film (data not shown). The positive RMs that were subject to water extraction were still toxic in all intervals of extract conditions. This implies that the water extraction is not as efficient as the actual implant situation to elute toxic materials.

Medium-extract method (2)

The colony formation was not inhibited at any conditions for the negative RM group. For the 0.5% ZDEC-PU film group, cytotoxicity decreased with increasing pre-extraction times with water (see Fig. 1). A remarkable decrease of cytotoxicity was noted after 4 and 8 days pre-extractions with water. However, the cytotoxicity did not decrease

for any of the pre-extraction times in the 1% ZDEC-PU film group. The findings indicate that the 1% ZDEC-PU film group has a different kinetic release of toxicity compared to the 0.5% ZDEC-PU film group (see Fig. 1). These results suggest that the elution of the toxic compounds depends on extraction period as well as material toxicity.

In vivo host response

After 1-week implantation the implants containing 0.5% and 1% ZDEC displayed a significantly thicker foreign body capsule compared to the non-toxic controls (p<0008; p<0.0006) (see Figs. 2 and 3a,b). This was also reflected in the number of ED1 positive macrophages as well as in the total number of cells throughout the reactive capsule, which were significantly higher at both the 0.5% (p<0.0078; p<0.0113) and the 1% implants (p<0.0002; p<0.0007) when compared to controls (see Fig. 4).

After 6 weeks implantation the foreign body capsule reactions had subsided. Over time the capsule thickness decreased significantly for the 0.5% and 1% implants (p<0.0287; p<0.0117) to at 6 weeks be indistinguishable from the controls (see Figs. 2 and 3c,d). The ED1 positive cells and the total number of cells in the capsule displayed also a significant decrease over time (p<0.0001; p<0.0004) and at 6 weeks the number of cells became similar for all implants (see Fig.4). Above-mentioned data reflected also as a significant correlation between level of implant toxicity and capsule thickness after 1-week implantation (p<0.0001). The level of toxicity was also correlated to numbers of ED1 positive cells (p<0.0001) and to total cells (p<0.0001) in the foreign body capsule at the 1-

week implantation time point.

To investigate if the increase in capsule thickness at the ZDEC containing implants at 1 week was due to tissue edema the cell densities were analyzed. For all implants (both controls and toxic implants) the highest cell densities were seen at 1 week. This was significant for both the ED1 positive cells and the total numbers of cells in the foreign body capsule ($p < 0.0001$; $p < 0.0001$).

Some animals were solely implanted with negative implants to examine if the toxic implants influenced the tissue response to the adjacent situated negative controls. After 1-week implantation no significant differences in foreign body capsule reactions were seen for implantation with solely negative controls compared to implantation with both toxic and non-toxic implants. This indicates that the inflammatory reaction is strictly localized to the close vicinity of the implant.

In the kinetic study over day 1-8 using negative and ZDEC impregnated implants the number of test samples was insufficient for a statistical analysis. However, a trend was seen where the capsule thickness appeared to correlate to the implant toxicity. The foreign body reaction thus appeared to subside over time and also to be related to the *in vitro* measured toxicity (data not shown).

Cytotoxicity of explanted RMs

TC-insert method (1)

The explanted test samples were primarily tested using the TC-insert method. Both 0.5 and 1 % ZDEC implants were highly cytotoxic when retrieved during the

first week. However, after 8 days the 0.5% ZDEC implants showed a decreasing toxicity.

Medium-extract method (2)

To detect low levels of cytotoxicity the medium-extract method was used. This very sensitive method revealed that the positive RMs showed severe cytotoxicity at implantation. However already after 1 day in the animal the cytotoxicity of the 0.5% ZDEC implants started to subside (see Fig.5). For the 1% ZDEC implants no noticeable decrease in toxicity occurred until 8 days implantation.

After 6 weeks implantation no or very low toxicity was left in both types of ZDEC containing implants (see Fig.5).

Discussion

The implication of using materials, which have tested positive for *in vitro* cytotoxicity for implant device purpose is unclear. The host response to implanted materials with controlled toxicity has previously been reported [23-25], but the cytotoxicity after explantation was not analyzed. Often multiple samples are implanted in the same animal for testing purposes and the interference between samples becomes an issue. This study indicates that there is no interference of a toxic implant to the tissue response at a neighboring non-toxic implant. Some clinically widely used implant materials have tested positive in *in vitro* cytotoxicity tests but have proved to be successful in long term clinical

implantation. Examples of such materials are bone cements and dental filling materials [6, 25]. Some authors consider both the initial toxic effects as well as the initial thermal effects of bone cement of no importance for long-term biocompatibility [26]. For bone cements the host tissue appears to recover or be resilient to the released toxic compounds.

In the present study a significantly thicker foreign body capsule as well as higher number of cells surrounded ZDEC containing implants after 1-week implantation (see Figs.2 and 3a,b and 4). However after six weeks implantation the inflammatory response had declined, which was reflected in the thickness of the capsule and in the numbers of cells (see Figs.2 and 3c,d and 4). These results indicate that the early events in the tissue adjacent to the cytotoxic implants are not reflected in the long term tissue response. Another interesting finding was that the *in vitro* extraction with water did not elute toxic compounds to the same extent as the *in vivo* conditions.

reports on correlations between *in vitro* tests and *in vivo* results are scarce, indicating that the complex biological situation is hard to mimic with a single *in vitro* experiment. Such a comparative study was however done by Mjör et al [23], evaluating dental filling materials. The study showed that the cell culture technique (24 hours) correlated poorly with the soft tissue implantation test (7-90 days) or to the usage-test (7-45 days) in the dental pulp. Materials that displayed severe toxicity in cell culture induced small or no reactions in the implantation or in the pulp reaction tests. The opposite was also seen: very slight cell culture

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.

Acknowledgements

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.
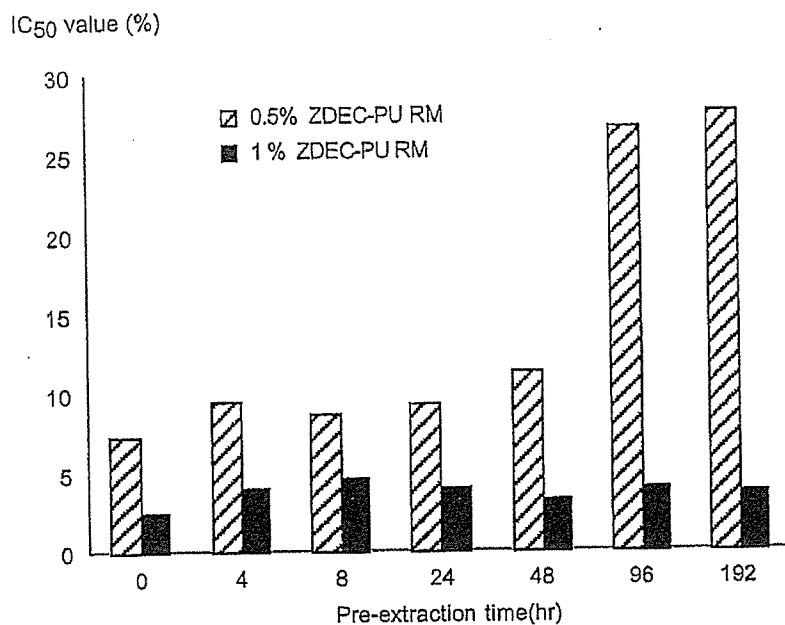
Figure 1

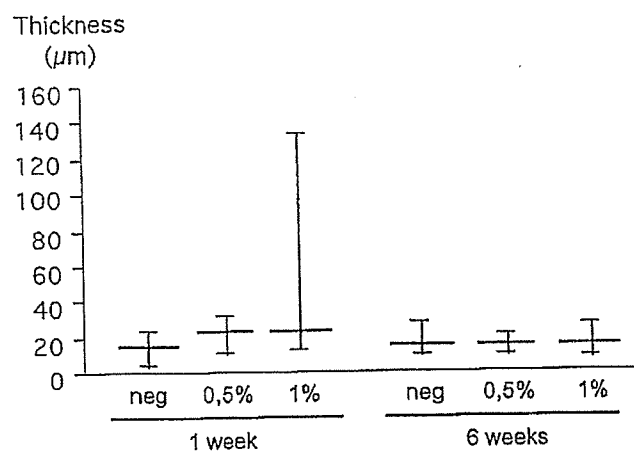Figure 1. A.Rosengren et al.

Figure 2

Figure 3
Click here to download high resolution image

Figure 4

ED1 positive cells

(cells/mm^2)
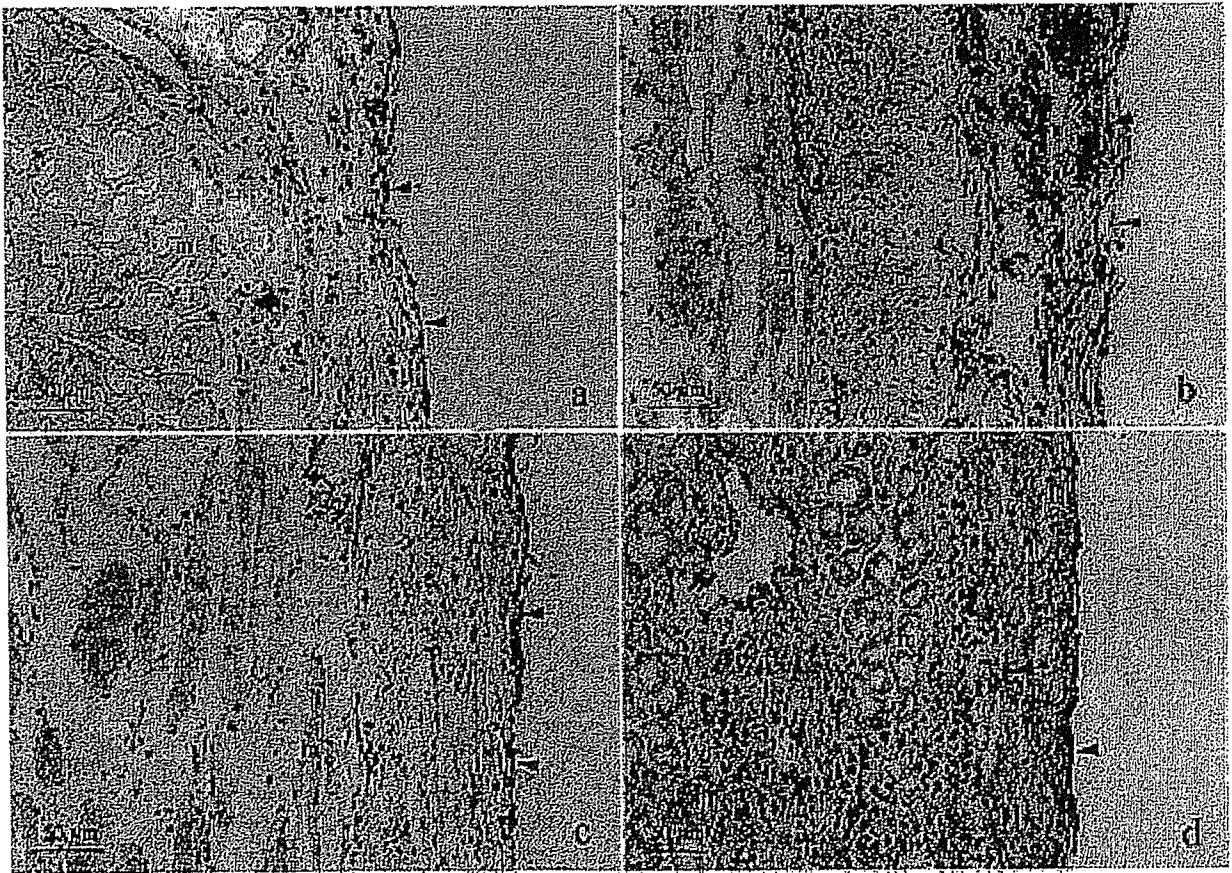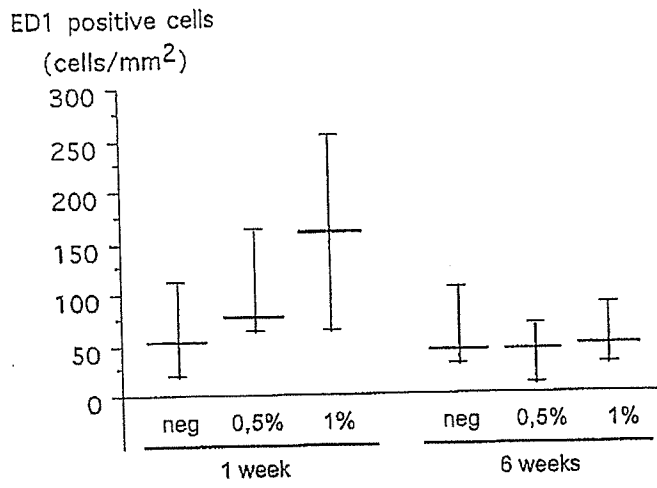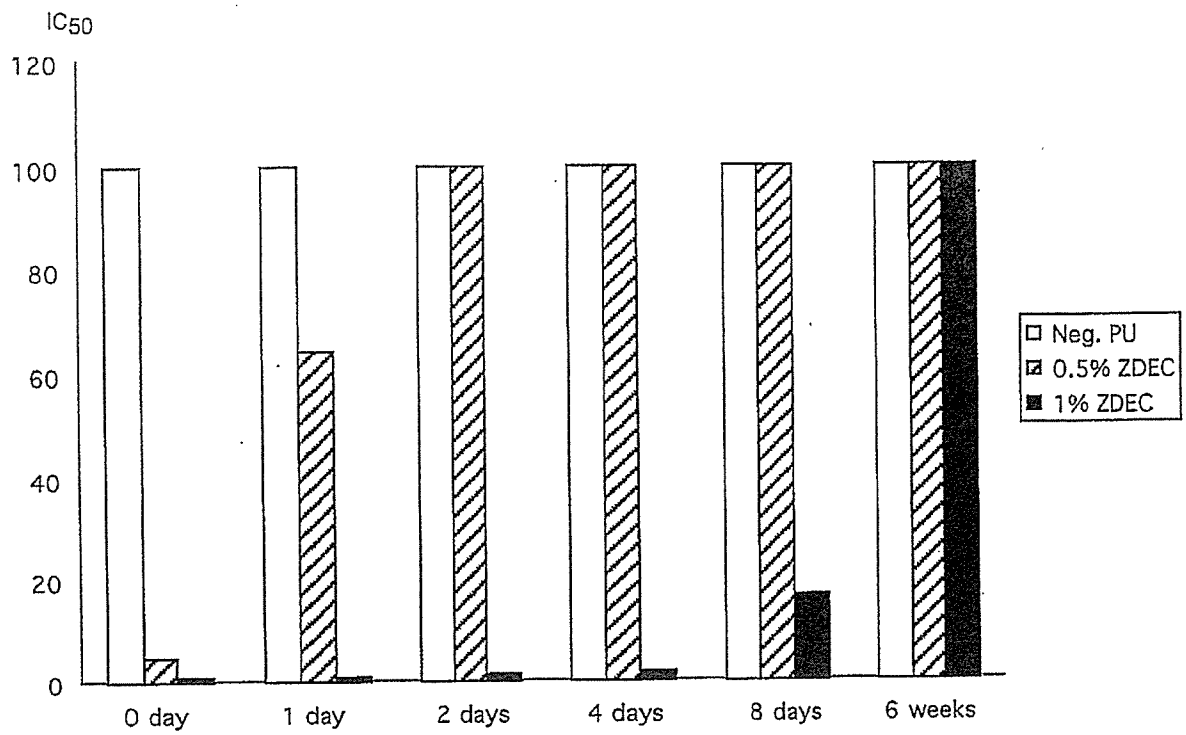
Figure 5

Figure 5. A.Rosengren et al.

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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Abstract

Cell transformation assay using BALB/c 3T3 cells, C3H10T1/2 cells and others, can simulate the two-stage carcinogenesis utilized for formation of transformed foci. A sensitive cell transformation assay for tumor initiators as well as promoters has been developed using a v-Ha-*ras*-transfected BALB/c 3T3 cell line, Bhas 42; these cells are regarded as initiated in the two-stage paradigm of carcinogenesis. To distinguish between initiation and promotion, the initiation assay involves a 2-day treatment of low-density cells, obtained one day after plating, with a test chemical, and the promotion assay involves treatment of near-confluent cells with a test chemical for a period of 12 days (Day 3–14). When Bhas 42 cells were treated with tumor initiators, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and 3-methylcholanthrene, transformed foci were induced in the initiation assay but not in the promotion assay. In contrast, tumor promoters, 12-*O*-tetradecanoylphorbol-13-acetate, lithocholic acid and okadaic acid, gave negative responses in the initiation assay but positive responses in the promotion assay. The results were reproducible with various treatment protocols. Sixteen polycyclic aromatic hydrocarbons were examined using both assays. Benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induced focus formation only in the initiation assay. Increase of focus formation was observed in the promotion assay with benzo[*e*]pyrene, benzo[*ghi*]perylene, 1-nitropyrene and pyrene. Benz[*a*]anthracene, benz[*b*]anthracene, chrysene and perylene showed positive responses in both initiation and promotion assays. Results of initiation and promotion assays of acenaphthylene, anthracene, coronene, 9,10-diphenylanthracene, naphthalene and phenanthrene were negative or equivocal. The present Bhas assays for the detection of either/both initiating and promoting activities of chemicals are sensitive and of high performance compared with other cell transformation assays.
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Keywords: Transformation; Bhas 42 cells; Complete carcinogen; Initiation and promotion

1. Introduction

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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

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 2×10^3 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