ment schedules. When Bhas 42 cells were seeded at 2×10^3 cells/mL, cells were in growth phase almost until Day 7. In order to treat cells repeatedly during the period of growth phase, change of medium containing MCA or TPA was performed every 2 days (periods 1–3 in Fig. 3). After Day 7, when cells became confluent, the change of MCA- or TPA-containing medium was carried out twice a week during the subsequent period (periods 4–6 in Fig. 3).

Schedules B–E were performed as confirmation test of initiation and promotion assays for MCA and TPA. The number of transformed foci in Schedule F did not increase from that in Schedule B, indicating that the additional treatment with MCA during stationary phase had little effect. In contrast, MCA-treatment during cell-growth phase followed by TPA-treatment during stationary phase clearly enhanced the formation of transformed foci (Schedule G). Repeated treatment with MCA during

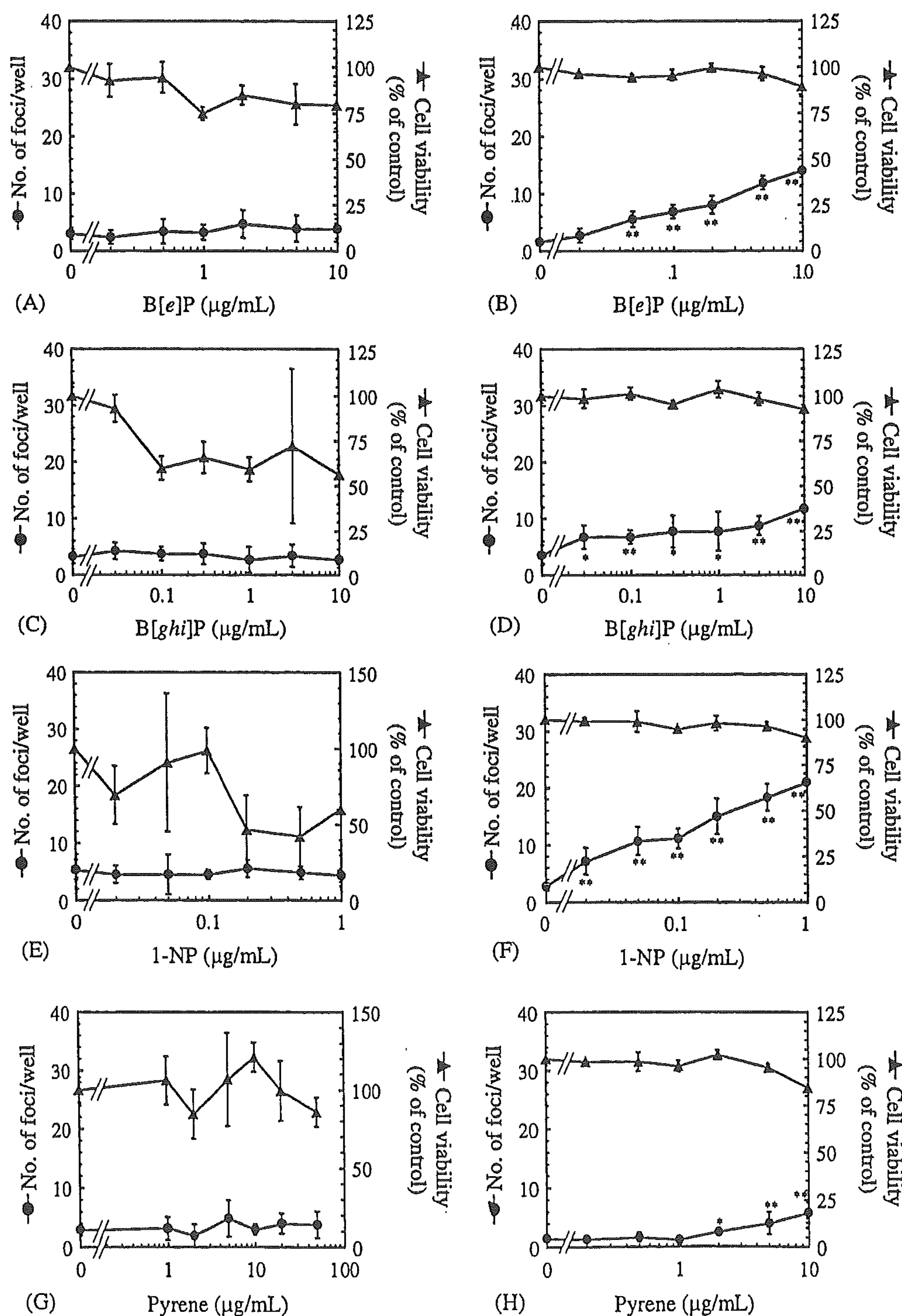

Fig. 5. Results of initiation and promotion assays of B[e]P, B[ghi]P, 1-NP and pyrene (Group II). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. * $p < 0.05$, compared with solvent control. ** $p < 0.01$, compared with solvent control.

ell-growth phase (Schedule H) and the whole period edule J) increased the number of transformed foci. It markable that cell growth was retarded and cells did reach subconfluence on Day 7 because of the cytocity of MCA. Repeated TPA treatment during growth se (Schedule I) was ineffective for inducing transned foci. The number of transformed foci in Schedule TPA treatment during cell-growth phase and stationphase) did not increase from that in Schedule E.

3.5. Initiation and promotion assays of PAHs

Results described above showed that MCA induced foci in the initiation assay without metabolic activation by S9 mix. This means that Bhas 42 cells have the capacity to metabolize PAHs, like the parental cell line BALB/c 3T3. Sixteen PAHs were evaluated for their initiating and promoting activities using the present methods. Response to PAHs in both methods could be categorized into four groups: showing only initiation

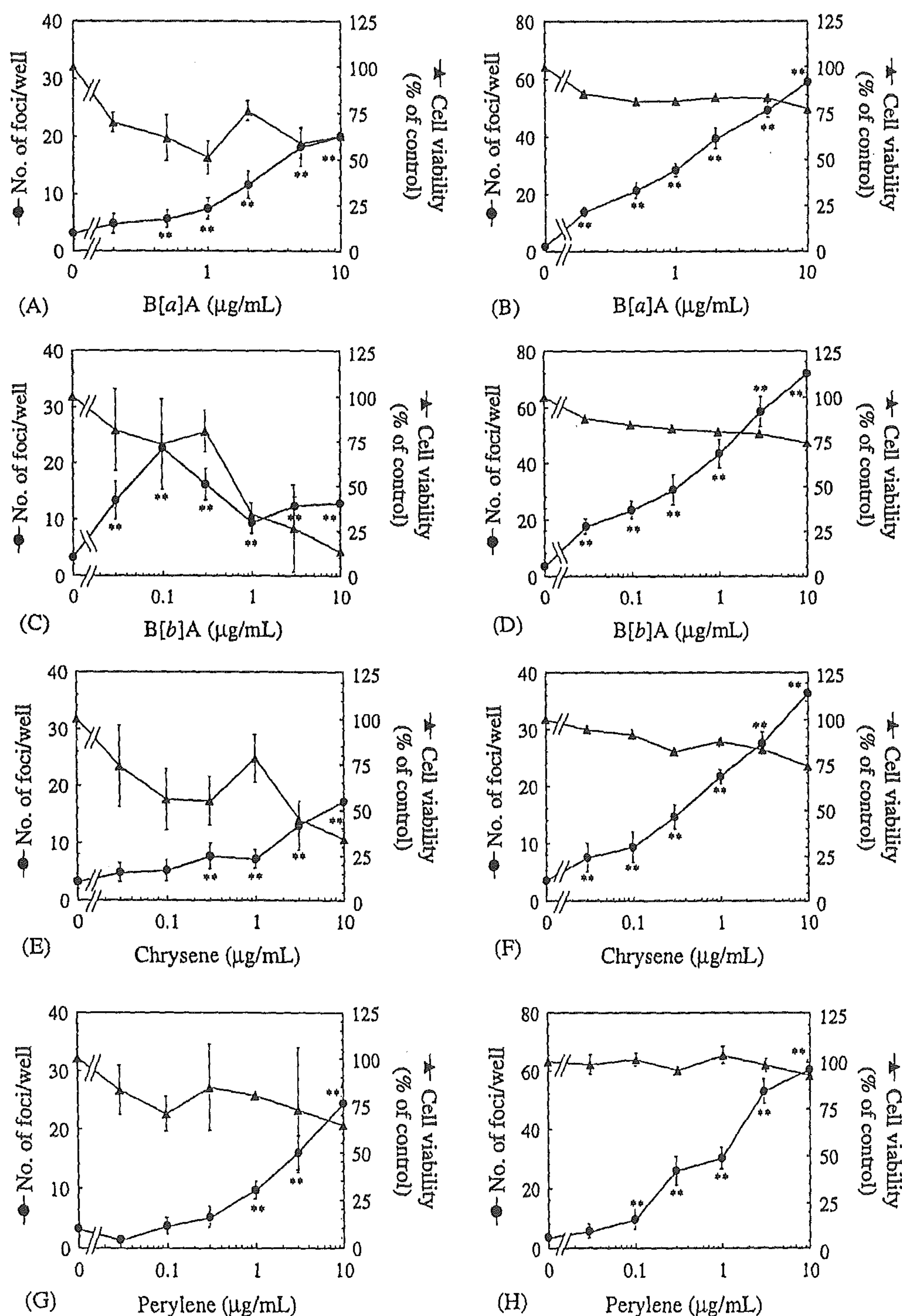

Fig. 6. Results of initiation and promotion assays of B[a]A, B[b]A, chrysene and perylene (Group III). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

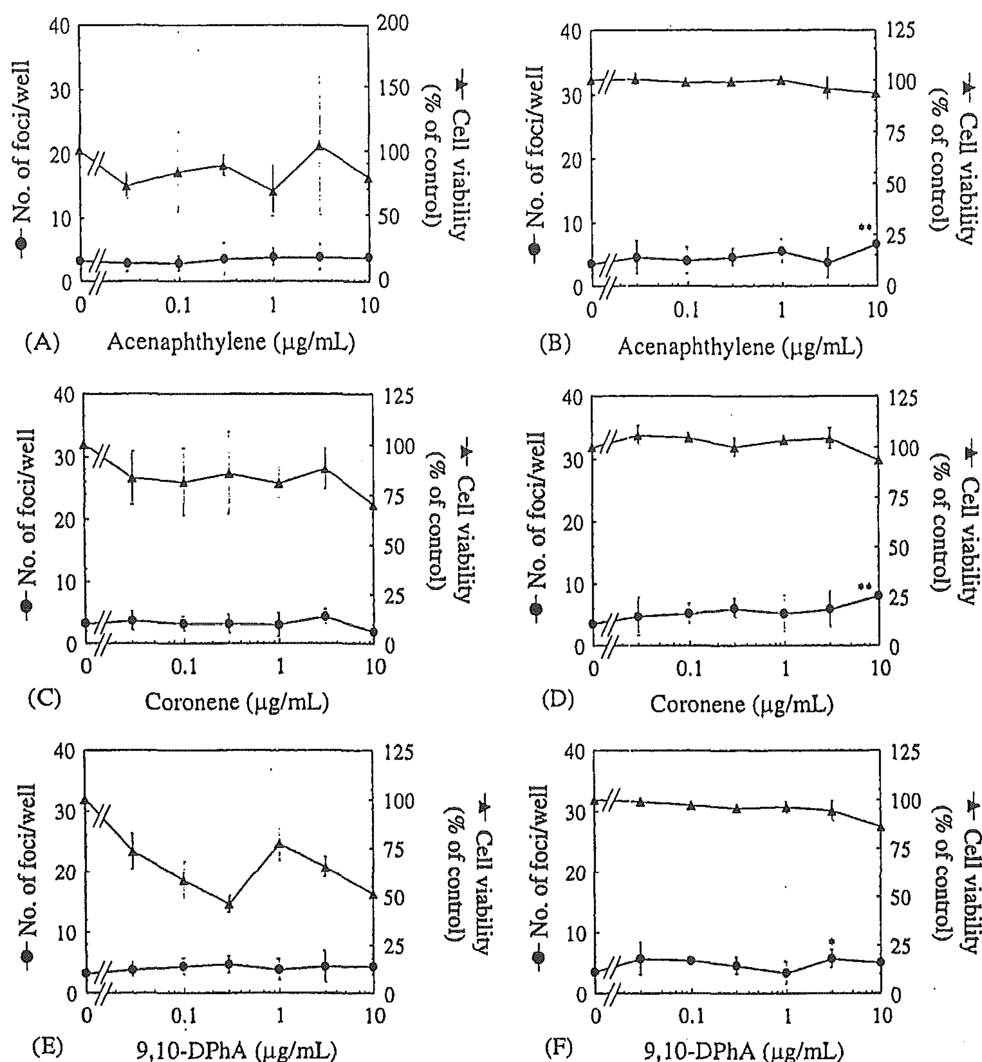

Fig. 7. Results of initiation and promotion assays of acenaphthylene, coronene and 9,10-DPhA (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p<0.05$, compared with solvent control. $^{**}p<0.01$, compared with solvent control.

activity (Group I), only promotion activity (Group II), both initiation and promotion activities (Group III), and equivocal or negative response in both methods (Group IV).

Fig. 4 shows results of two chemicals showing positive responses only in the initiation assay (Group I). B[*a*]P at concentrations of 0.5 and 1 μg/mL (Fig. 4A) and DMBA at concentrations of 10 ng/mL and above (Fig. 4C) induced transformed foci in the initiation assay only. In the case of the promotion assay, no transformed foci were induced at any concentrations tested for B[*a*]P (Fig. 4B) or DMBA (Fig. 4D).

Chemicals classified in Group II consisted of B[*e*]P, B[*ghi*]P, 1-NP, and pyrene, which showed positive responses only in the promotion assay (Fig. 5). No increase of foci was observed in the initiation assay with these chemicals (Fig. 5A, C, E and G). In contrast, they induced significant increase of foci in the promotion assay (Fig. 5B, D, F and H).

Results for Group III are shown in Fig. 6. B[*a*]A at concentrations of 0.5 μg/mL and above (Fig. 6A), chrysene at concentrations of 0.2 μg/mL and above (Fig. 6E) and perylene at concentrations of 1 μg/mL and above (Fig. 6G) induced foci in the initiation assay. In the initiation assay of B[*b*]A, the number of foci increased on increasing the dose up to 0.1 μg/mL, but it decreased at concentrations over 0.2 μg/mL (Fig. 6C). In the promotion assay, a marked increase in focus formation was observed with these chemicals at a non-cytotoxic concentration range from 0.1 to 10 μg/mL (Fig. 6B, D, F and H).

Fig. 7 shows results of initiation and promotion assays of acenaphthylene, coronene, and 9,10-DPhA. No increase of foci was observed in the initiation assay at all concentrations tested for acenaphthylene (Fig. 7A), coronene (Fig. 7C) and 9,10-DPhA (Fig. 7E). These chemicals weakly induced foci in the promotion assay at the highest concentration (Fig. 7B, D and F). Results of initiation and promotion assays for anthracene, naph-

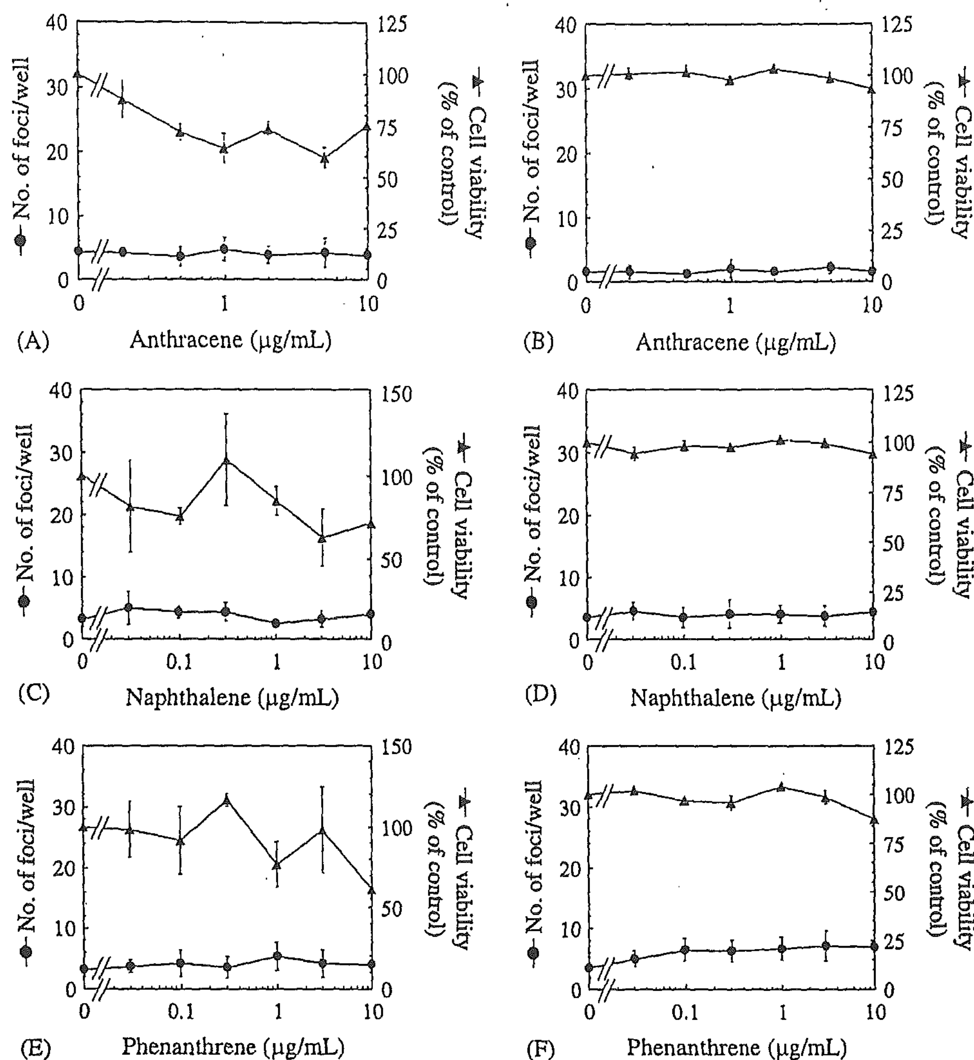

Fig. 8. Results of initiation and promotion assays of anthracene, naphthalene and phenanthrene (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p<0.05$, compared with solvent control. $^{**}p<0.01$, compared with solvent control.

thalene, and phenanthrene are shown in Fig. 8. These chemicals were entirely negative in both initiation and promotion assays at concentrations up to 10 μg/mL.

4. Discussion

In addition to the Bhas promotion assay, we have developed a method for evaluation of initiating activity of chemicals using Bhas 42 cells. In the promotion assay, cells are treated with test chemicals at subconfluent condition, in which further cell division is limited, whereas the initiation assay was designed for cells at a lower cell density to be able to divide several times after treatment with test chemicals. The fact that tumor initiators could transform Bhas 42 cells only under cell proliferating conditions was important in aiding understanding of the two-stage model of chemical carcinogenesis. MNNG and MCA, both tumor initiators, were positive in the initiation assay but negative in the promotion assay. In contrast, TPA, LCA, and okadaic acid, tumor promoters [26–31], were positive in the promotion assay but were negative in the initiation assay.

Some tumor initiators are considered to have tumor-promoting activity. This recognition came from the experimental observation that repeated application at subtumorigenic doses can induce tumors in mouse skin without any post-treatment with a promoter [1]. In order to probe why repeated treatment with MNNG or MCA could not induce transformed foci in the Bhas promotion assay, an experiment with various schedules of treatment was conducted (Fig. 3). This demonstrated that MCA was effective only when cells were in a dividing phase, consistent to the current notion for the mechanism of initiator action, i.e., the fixation of genetic damage resulting in genetic alteration, namely mutation, after several cell divisions. On the contrary, the promoter, TPA, induced transformed foci when cell division was limited but it was not effective during the cell-dividing phase. It is well known that tumor promoters inhibit the interaction with neighboring cells (metabolic cooperation) [32–35].

Table 2
Summary of Bhas initiation and promotion assays for 22 tested chemicals, and published carcinogenicity and mutagenicity assay summaries

Chemical	CAS no.	IARC	Bhas 42 assay		In vivo carcinogenicity[a]		Ames' test				E. coli		Metabolic cooperation		MLA		References
			Initiation assay	Promotion assay	Carcinogenicity	Promotion	Frameshift mutation[b]		Missense mutation[c]		S9 (−)	S9 (+)	S9 (−)	S9 (+)	S9 (−)	S9 (+)	
							S9 (−)	S9 (+)	S9 (−)	S9 (+)							
Group I																	
MNNG	70-25-7	2A	+	−	+		+	+	+	+	+	+	+		+	+	[42,44–46,48,50,55]
MCA	56-49-5		+	−	+			+		+	−	−		+			[42,44,45,48,55]
B[a]P	50-32-8	2A	+	−	+		−	+		+			−	+		+	[38,41,42,55,57,61]
DMBA	57-97-6		+	−	+		+	+	+	I	−	−	−	+	−	+	[41,42,44,48,55,60]
AFB1	1162-65-8	1	+[d]	−	+[e]		I			+			−	+			[39,42,55]
Group II																	
TPA	16561-29-8		−	+		+		−		−			+				[1,42,62]
LCA	434-13-9		−	+	−		−	−	−	−	−	−	+		−	−	[41,44,47,60,62]
Okadaic acid	78111-17-8		−	+		+		−		−				I			[30,54,56]
B[e]P	192-97-2	3	−	+	−	+	−	+	−	−	−	−	−				[38,41–44]
B[ghi]P	191-24-2	3	−	+	−	−	−	+									[38,41,49,50]
1-NP	5522-43-0	2B	−	+	+		I	I									[39,49,50,58]
Pyrene	129-00-0	3	−	+	−	I	−	+	−	I	−	−					[38,41,42,44]
Group III																	
B[a]A	56-55-3	2A	+	+	+	+		+					−	+			[38,42,53,55]
B[b]A	92-24-0		+	+			−	+	−	−							[51]
Chrysene	218-01-9	3	+	+	+	−	−	+									[38,41,42,59]
Perylene	198-55-0	3	+	+	−	−	−	+		+					−	+	[38,41,50,52,60]
Group IV																	
Acenaphthylene	208-96-8		−	±													
Anthracene	120-12-7	3	−	−	−		−	+	−	−	−	−			−	+	[38,41,42,44,60]
Coronene	191-07-1	3	−	±	+	−	−	+									[38,41,43]
9,10-DPhA			−	±													
Naphthalene	91-20-3	2B	−	−	−		−	−	−	−	−	−			−	+	[40–42]
Phenanthrane	85-01-8	3	−	−	−	−	−	−	−	−	−	−					[38,41,42,44]

+, positive result; −, negative result; ±, *equivocal result*; I, *inconclusive result*.
[a] Carcinogenicity was tested by skin application to mice and promoting activity was assessed by mouse-skin initiation-promotion assay.
[b] Frameshift mutation was detected with TA97, TA98, TA1537 and TA1538.
[c] Missense mutation was detected with TA100 and TA1535.
[d] Initiating activity of AFB1 required metabolic activation with S9.
[e] Carcinogenicity of AFB1 was shown in liver.

:hiya and Umeda [36] have demonstrated that a sub-luent phase of MNNG-initiated BALB/c 3T3 cells the most sensitive stage for the induction of trans-ıed foci by TPA.

3has 42 cells were considered as initiated cells by ıe of transfection of the v-Ha-*ras* gene. Here, how-:, we demonstrated that the additional treatment with iating chemicals is effective for inducing transformed i. Carcinogenesis is now considered to be a multi-step ·nomenon. Sugimura stated in his review that several tations could lead to tumor formation [37]. Thus, the ults are interpreted as showing that initiator-induced tations of oncogene(s) other than *ras* oncogene in as 42 cells can result in the induction of cell trans-mation.

These discussions may pertain to a specific in vitro perimental assay, but the phenomena support the pothetical mechanism for tumor formation in the ouse skin, where repeated treatment with tumor ini-ıtor causes genetic damage of several oncogenes in e skin cells. The basement cell layer of mouse skin ımprises actively dividing stem cells, wherein genetic ımage can be fixed and result in mutations. There-ıre, we hypothesize that tumor formation from tumor ıitiator exposure does not involve promoting activ-y, such as disturbed interaction between neighboring ells, i.e., loss of metabolic cooperation, but involves xation and accumulation of genetic damage after cell ivisions.

In order to investigate the applicability of Bhas initi-.tion and promotion assays to an in vitro screening for :arcinogenesis, various PAHs and other initiators and ›romoters were evaluated by the assays; these could be :lassified into four groups (Table 2). Group I consists of :hemicals showing positive results in the initiation assay ›ut negative in the promotion assay. MNNG, MCA, \FB$_1$, and carcinogenic PAHs were classified in this group. Group II chemicals are negative in the initiation ıssay but positive in the promotion assay. Tumor promot-ers, TPA, LCA, and okadaic acid, are Group II chemicals. These promoters are also negative in bacterial mutation ıssays. Group III consists of chemicals showing positive esponse in both initiation and promotion assays (B[*a*]A, 3[*b*]A, chrysene and perylene). Group IV consists of :hemicals showing equivocal or negative response in ›oth assays. These results showing that there are various kinds of PAHs with initiating and/or promoting activities suggest carcinogenic risk in mixtures of PAHs such as soot from exhaust gas, and particulate matter in polluted air.

In the present study, a metabolic activation system was not used in the examination of PAHs. Some chemicals negative in the Bhas initiation assay (Groups II and IV) are positive in the bacterial reverse mutation assay in the presence of an exogenous metabolic activation system (Table 2). It is, therefore, necessary to include a metabolic activation system in the Bhas initiation assay. To incorporate the metabolic activation system into the Bhas assay, we tried to modify the present Bhas initiation assay. AFB$_1$, as a model initiator that requires metabolic activation by CYP enzymes such as 2A6, 3A4 and 1A2 [24,25], was used to validate the incorporation of the metabolic activation system. AFB$_1$ did not induce transformed foci in the initiation assay in the absence of the metabolic activation system but it induced transformed foci in its presence.

In summary, Bhas assays for the detection of both initiating and promoting activities of chemicals are more sensitive and economical than other cell transformation assays, and deserve consideration as a promising screening tool. However, the results obtained are still limited, and further studies are necessary to confirm the applicability of the Bhas assays especially in the presence of an exogenous metabolic activation system. In addition, the Bhas assays may be able to play an important role for understanding the mechanism of chemical carcinogenesis.

Acknowledgements

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An Inter-laboratory Collaborative Study by the Non-Genotoxic Carcinogen Study Group in Japan, on a Cell Transformation Assay for Tumour Promoters Using Bhas 42 cells

Kiyomi Ohmori,[1] Makoto Umeda,[2] Noriho Tanaka,[2] Hiroki Takagi,[3] Isao Yoshimura,[4] Kiyoshi Sasaki,[2] Shin Asada,[2] Ayako Sakai,[5] Harumi Araki,[6] Masumi Asakura,[7] Hiroshi Baba,[8] Yuichi Fushiwaki,[1] Shuichi Hamada,[9] Nobuko Kitou,[6] Tetsu Nakamura,[10] Yoshiyuki Nakamura,[11] Hidetoshi Oishi,[12] Satoshi Sasaki,[13] Sawako Shimada,[14] Toshiyuki Tsuchiya,[15] Yoshifumi Uno,[8] Masataka Washizuka,[16] Satoshi Yajima,[13] Yasuhito Yamamoto,[17] Eiji Yamamura[8] and Tomoko Yatsushiro[10]

[1]Kanagawa Prefectural Institute of Public Health, Kanagawa, Japan; [2]Food and Drug Safety Center, Kanagawa, Japan; [3]Aventis Pharma Ltd, Tokyo, Japan; [4]Tokyo University of Science, Tokyo, Japan; [5]National Institute of Health Sciences, Tokyo, Japan; [6]Japan Bioassay Research Center, Kanagawa, Japan; [7]Mitsubishi Pharma Corporation, Chiba, Japan; [8]Mitsubishi Chemical Safety Institute Ltd, Ibaraki, Japan; [9]Toyama Chemical Co. Ltd, Toyama, Japan; [10]Canon Inc, Tokyo, Japan; [11]Sugiyama Jogakuen University, Nagoya, Japan; [12]Dainippon Pharmaceutical Co. Ltd, Osaka, Japan; [13]Takasago International Corporation, Kanagawa, Japan; [14]Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan; [15]Banyu Pharmaceutical Co. Ltd, Ibaraki, Japan; [16]Zeria Pharmaceutical Co. Ltd, Saitama, Japan; [17]LION Corporation, Kanagawa, Japan

Summary — The Bhas promotion assay is a cell culture transformation assay designed as a sensitive and economical method for detecting the tumour-promoting activities of chemicals. In order to validate the transferability and applicability of this assay, an inter-laboratory collaborative study was conducted with the participation of 14 laboratories. After confirmation that these laboratories could obtain positive results with two tumour promoters, 12-O-tetradecanoylphorbol-13-acetate (TPA) and lithocholic acid (LCA), 12 coded chemicals were assayed. Each chemical was tested in four laboratories. For eight chemicals, all four laboratories obtained consistent results, and for two of the other four chemicals, only one of the four laboratories showed inconsistent results. Thus, the rate of consistency was high. During the study, several issues were raised, each of which were analysed step-by-step, leading to revision of the protocol of the original assay. Among these issues were the importance of careful maintenance of mother cultures and the adoption of test concentrations for toxic chemicals. In addition, it is suggested that three different types of chemicals show positive promoting activity in the assay. Those designated as T-type induced extreme growth enhancement, and included TPA, mezerein, PDD and insulin. LCA and okadaic acid belonged to the L-type category, in which transformed foci were induced at concentrations showing growth-inhibition. In contrast, M-type chemicals, progesterone, catechol and sodium saccharin, induced foci at concentrations with little or slight growth inhibition. The fact that different types of chemicals similarly induce transformed foci in the Bhas promotion assay may provide clues for elucidating mechanisms of tumour promotion.

Key words: *Bhas 42 cells, cell transformation assay, inter-laboratory collaborative study, tumour promoter.*

Address for correspondence: *K. Ohmori, Chemistry Division, Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa 253-0087, Japan.*
E-mail: *ohmori.n4yf@pref.kanagawa.jp*

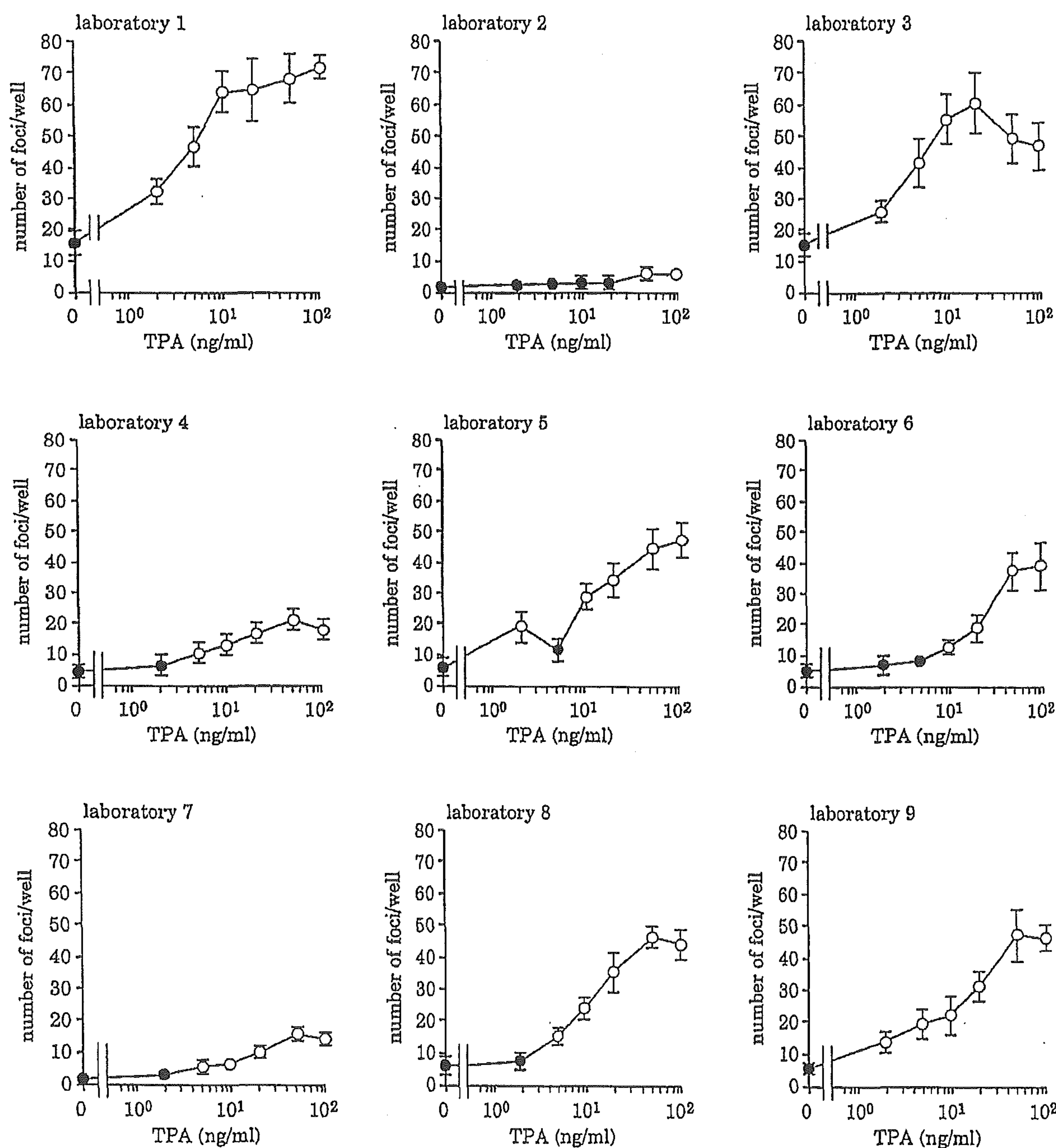
Introduction

Chemicals active in carcinogenesis according to the two-stage model can be divided into two categories, initiators and promoters (1). Most initiators can be detected by using various genotoxicity tests, some of which are used for carcinogenicity evaluation of chemicals by regulatory authorities (2). In the case of tumour promoters, several detection methods have been reported (3–13), but none of them have been accepted for regulatory purposes. Meanwhile, the existence of non-genotoxic carcinogens has presented a major challenge for the toxicity screening of chemicals. Since tumour promoters are considered to be one kind of non-genotoxic carcinogen, the development of reliable and applicable screening methods for the detection of tumour promoters is an important goal.

In vitro cell transformation tests, involving BALB/c 3T3 cells or C3H10T1/2 cells, can simulate the process of two-stage animal carcinogenesis, and can

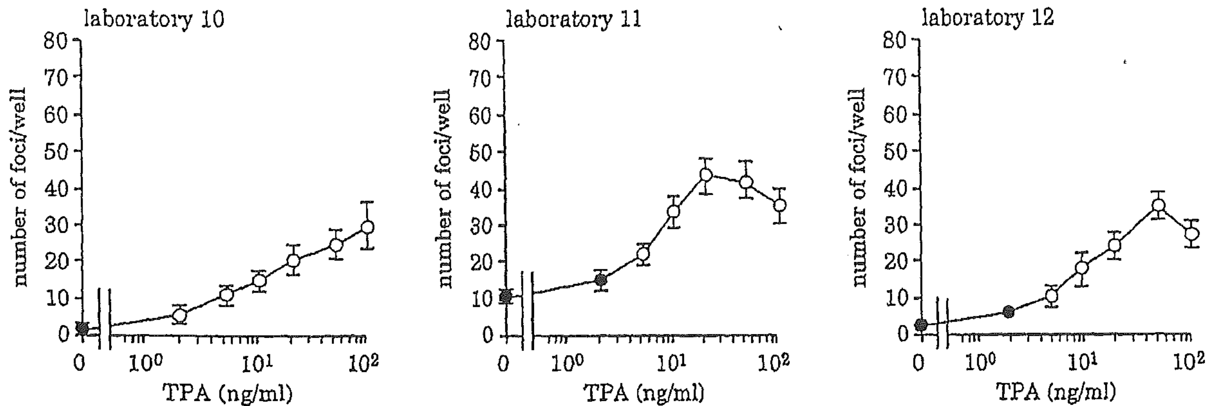
detect both the initiating and the promoting activities of chemicals. Recently, Ohmori *et al.* have developed an *in vitro* cell transformation assay for tumour promoters, by using Bhas 42 cells, a v-*Ha*-ras-transfected BALB/c 3T3 cell line, which are considered to be initiated cells in the two-stage transformation model of carcinogenesis (14). This cell transformation assay has many advantages: a) treatment with an initiating agent and subsequent cultivation for an expression period can be omitted; b) the experimental period is

Figure 2: Transformation frequencies with TPA in the Bhas promotion assay



○ = *significant point compared to control.*

Figure 2: continued



○ = *significant point compared to control.*

shortened from 4–6 weeks to 2.5–3 weeks after cell inoculation; and c) the transformation frequency is high, so only three to six wells in 6-well plates are required for each dose, instead of 10–20 6cm dishes. In order to gain wider awareness of this method, it was necessary to confirm its applicability and transferability between laboratories.

The aim of this report is to describe an inter-laboratory collaborative study on the Bhas promotion assay. Fourteen laboratories, belonging to the Non-Genotoxic Carcinogen Study (NGCS) Group in the Environmental Mutagen Society of Japan, participated in the project. Here, we describe four consecutive studies, during which the method originally reported by Ohmori *et al.* (14) was examined and improved after the analysis of each stage of the project.

Materials and Methods

Media, cells and culture conditions

Minimal Essential Medium (MEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan),

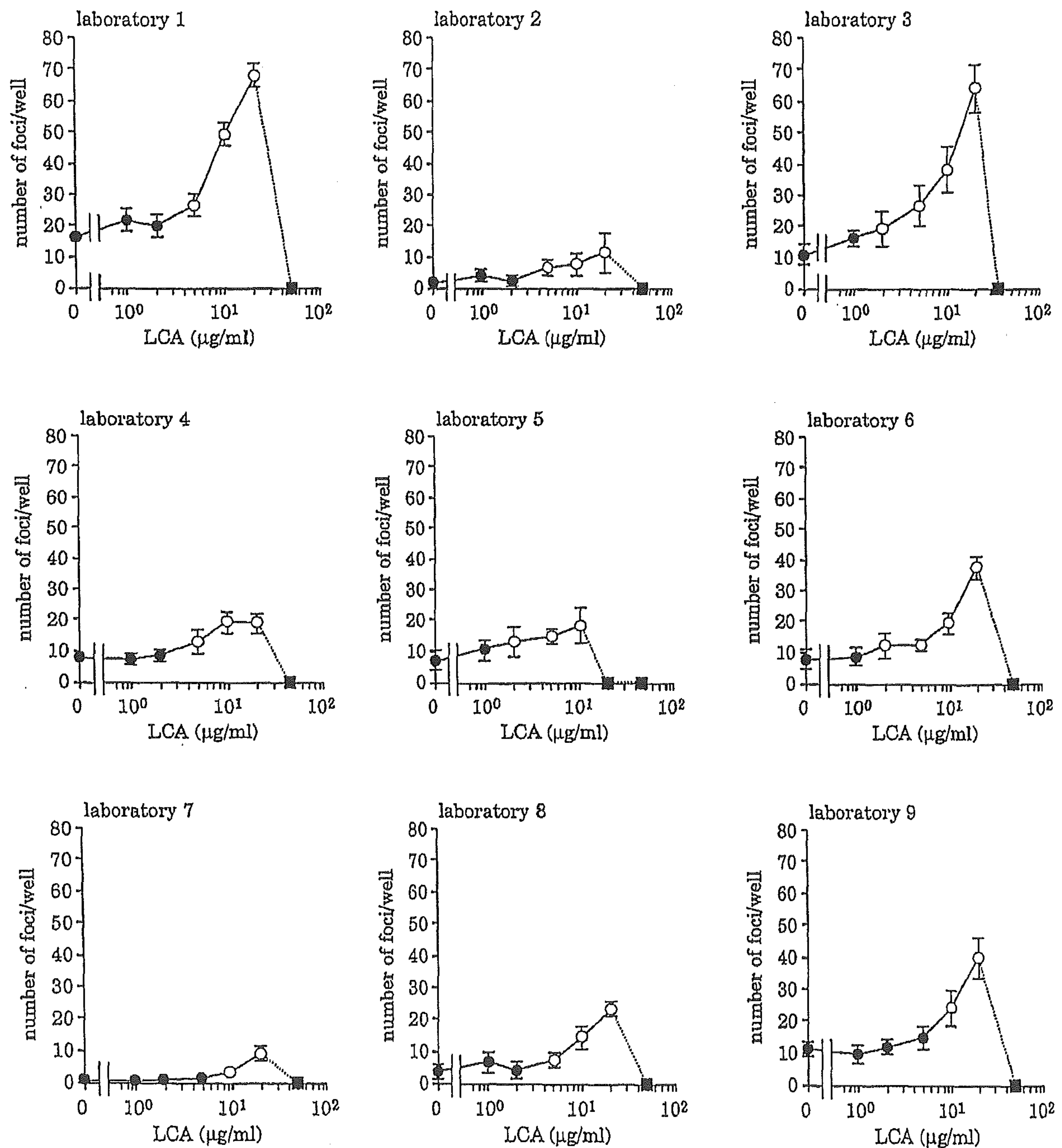
Table 1: Evaluation of the promotion assay for TPA, based on four criteria for transformation (Study II)

Lab No.	1: No. of concentrations with significant effect	2: Significant at consecutive concentrations	3: No. of concentrations showing a 3-fold increase	4: No. of concentrations showing a 2-fold increase	Judgement based on	
					1 + 2 + 3	1 + 4
1	6	yes	4	6	+	+
2	2	yes	2	5	+	+
3	6	yes	4	5	+	+
4	5	yes	3	5	+	+
5	5	yes	4	6	+	+
6	4	yes	3	5	+	+
7	5	yes	5	6	+	+
8	5	yes	4	5	+	+
9	6	yes	5	6	+	+
10	5	yes	6	6	+	+
11	5	yes	4	5	+	+
12	5	yes	5	6	+	+

and Dulbecco's Modified Eagle's Medium/Ham's F12 Medium (1:1; DMEM/F12) was the product of GIBCO Laboratories (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Moregate (Bulimba, Queensland, Australia).

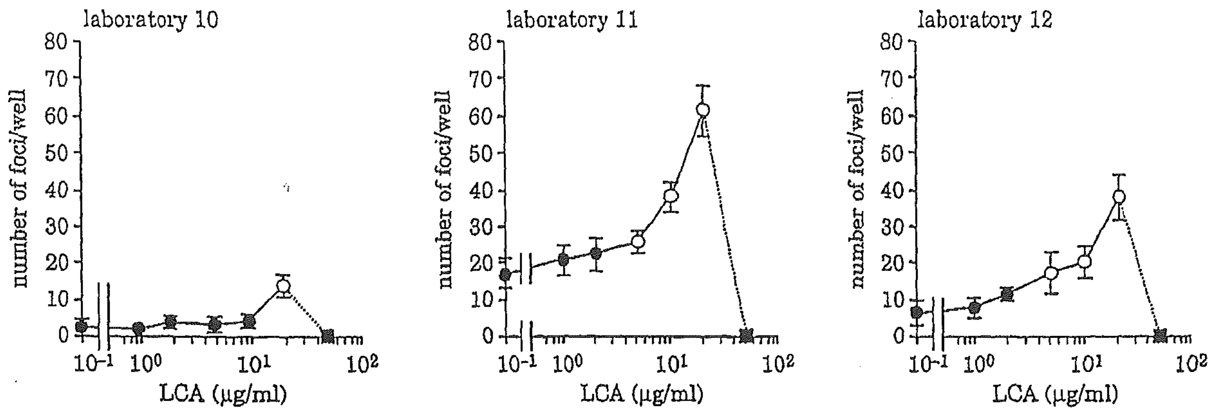
Bhas 42 cells were routinely cultured in a medium consisting of MEM supplemented with 10% FBS (M10F) at 37°C, in an atmosphere of 95% air and 5% CO_2. The cells were subcultured by using 0.25% trypsin (Wako Pure Chemical

Figure 3: Transformation frequencies with LCA in the Bhas promotion assay



○ = *significant point compared to control;* ■ = *point of cell death because of toxicity.*

Figure 3: continued



○ = *significant point compared to control;* ■ = *point of cell death because of toxicity.*

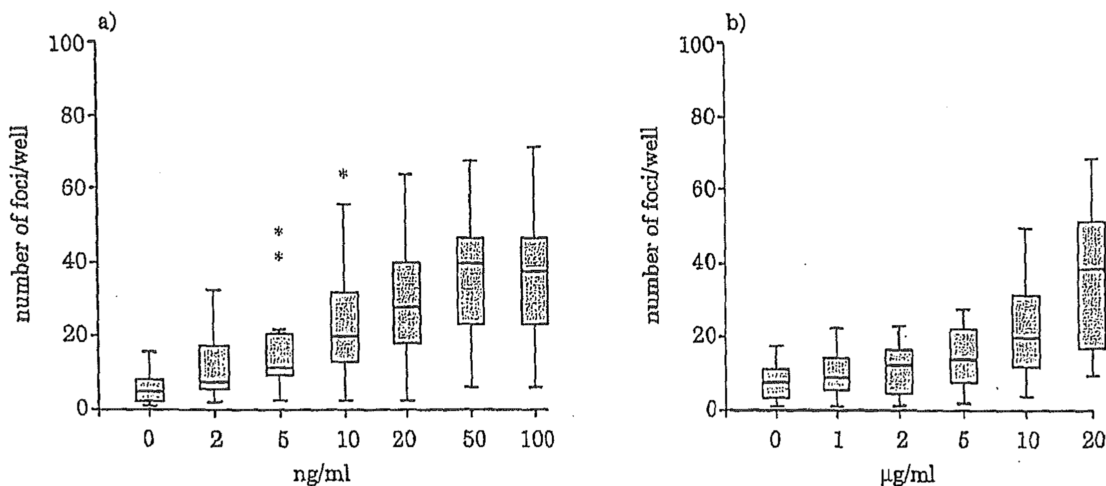
Industries, Osaka, Japan). It is necessary to keep the cell density at no more than 60–70% confluence. For the collaborative study, Bhas 42 cells were distributed to each laboratory by the management team. After the expansion of the number of cells in each laboratory, aliquots of the cells were kept frozen at -80°C. Each experiment was performed by using an aliquot of these stock cells.

Plastic culture dishes and plates were obtained from Sumitomo Bakelite (Tokyo, Japan), Corning Inc. (New York, NY, USA), Nalge Nunc International (Denmark) and Iwaki (Tokyo, Japan).

Test chemicals

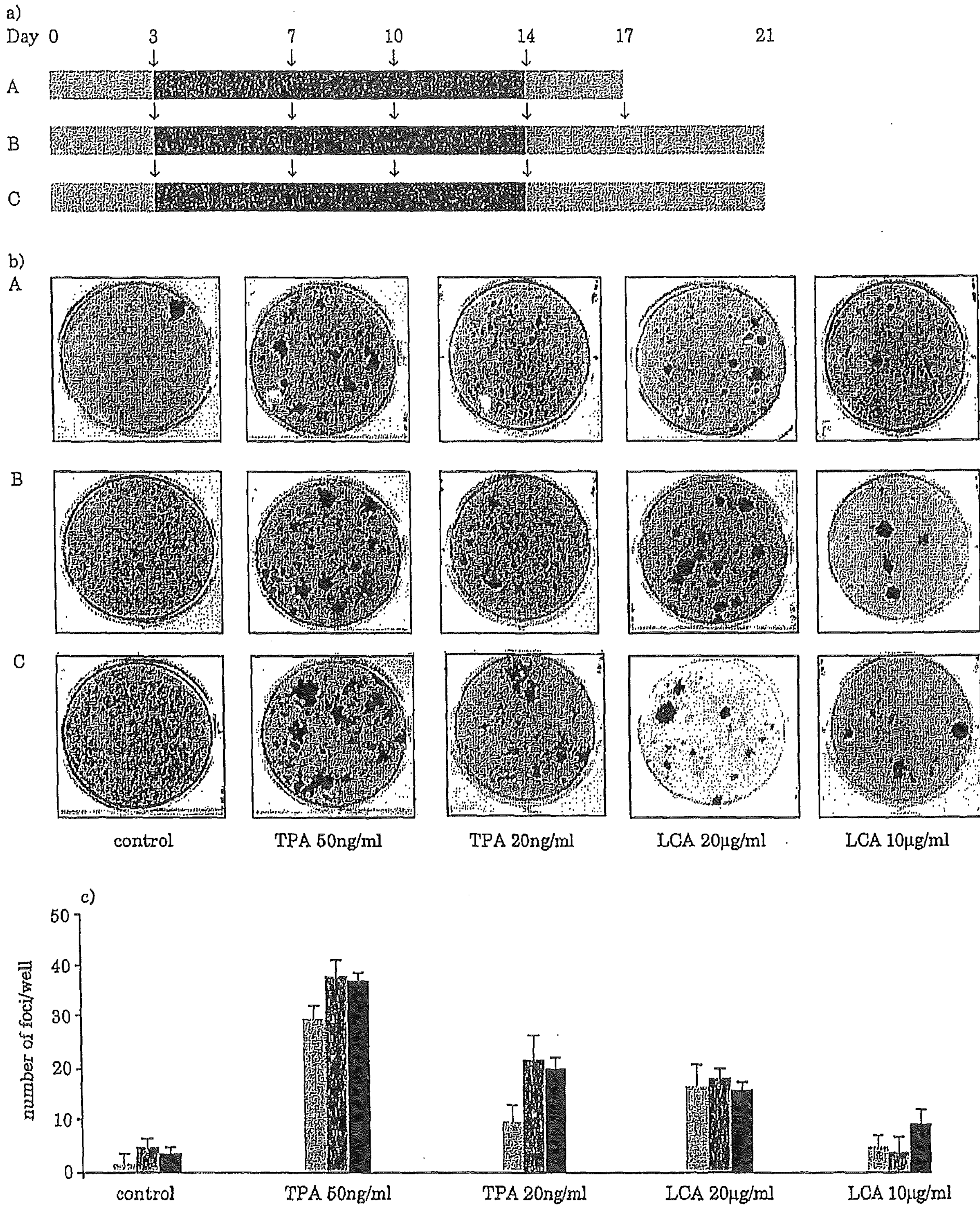
Catechol, dexamethasone, diethylstilboestrol (DES), lithocholic acid (LCA), okadaic acid, sodium saccharin and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 17β-oestradiol, insulin,

Figure 4: Box-whisker plots of transformation frequencies



From assays with a) TPA and b) LCA, conducted by 12 laboratories.

Figure 5: Effects of culture period on transformation frequencies with TPA and LCA



a) time schedules of Bhas promotion assay (dark grey boxes denote period of culture with medium containing TPA or LCA; arrows denote medium changes); b) Giemsa-stained wells of 6-well plates showing transformed foci; c) transformation frequencies induced with various time schedules.
= schedule A; = schedule B; = schedule C.

mezerein, phorbol 12,13-didecanoate (PDD), 4α-phorbol and progesterone were obtained from Sigma-Aldrich (St. Louis, MO, USA), and anthralin from Tokyo Kasei Kogyo (Tokyo, Japan). The purity level of 10 of the test chemicals was greater than 97%, that of LCA and TPA was greater than 95%, that of okadaic acid was greater than 85%, and that of insulin was 27USP units/mg.

The test chemicals, except insulin and sodium saccharin, were dissolved in dimethyl sulphoxide (DMSO, greater than 99.9%). Insulin was dissolved in 0.1mol/l hydrochloric acid, which, after more than 500 times dilution in the medium, had little effect on the cells. Sodium saccharin was dissolved directly in the medium. Chemical solutions were prepared by the management team and divided into aliquots, then distributed to the respective laboratories.

Cell growth assay for the determination of test concentrations

The crystal violet (CV) method was employed, as described previously (14). On Day 0, a cell suspension (0.5ml) of Bhas 42 cells in DMEM/F12 + 5% FBS (DF5F) at 2×10^4 cells/ml, was distributed into 24-well plates at 1×10^4 cells/well. On Day 3, the medium in each well was replaced with the medium containing the test chemicals. Three wells were used for each dose group. On Day 7, the cells were fixed with 10% formalin for 30 minutes, then washed with water, and stained with 0.1% CV solution for 30 minutes. After a thorough rinsing with water, the plates were dried. The stained CV in each well was extracted with 0.5ml of a solution consisting of 0.02mol/l HCl-50% ethanol. The optical density of each extract was measured at 540–570nm.

The chemical concentrations for transformation experiments were determined by using the results of the cell growth assays. Chemicals were classified into three types, according to their effects on cell growth. The first type of chemical (designated as T-type) exhibited marked enhancement of cell growth; the second type (L-type) showed cytotoxicity without growth enhancement; and the third type (M-type) promoted focus formation at concentrations having little effect on cell growth. TPA was included in the first group, and test concentrations for these chemicals were selected to cover from little effect on cell growth to growth enhancement. The L-type chemicals included LCA, and the M-type group comprised miscellaneous chemicals. With the latter two types, test concentrations were selected ranging from those exhibiting below 70% growth level to those having little effect on cell growth.

In the inter-laboratory collaborative study, the management team conducted trial cell-growth assays in advance for the test chemicals at tentative concentrations, set up according to the above principles. From these results, the test concentrations to be used by the participating laboratories were specified by the management team.

Bhas promotion assay

The original Bhas promotion assay protocol (14) was employed, with several modifications, as follows. Although M10F medium was used for routine cell culture, DF5F medium was used, not only for transformation experiments, but also for the mother cultures. In addition, it was critically important to use mother culture cells at around 60–70% confluence. A cell suspension of 2×10^4 cells/ml was prepared from a

Table 2: Evaluation of the promotion assay for LCA, based on four criteria for transformation (Study II)

Lab No.	1: No. of concentrations with significant effect	2: Significant at consecutive concentrations	3: No. of concentrations showing a 3-fold increase	4: No. of concentrations showing a 2-fold increase	Judgement based on	
					1 + 2 + 3	1 + 4
1	3	yes	1	2	+	+
2	3	yes	3	3	+	+
3	4	yes	2	3	+	+
4	3	yes	0	2	±	+
5	3	yes	0	3	±	+
6	4	yes	1	2	+	+
7	2	yes	2	2	+	+
8	3	yes	2	3	+	+
9	2	yes	1	2	+	+
10	1	no	1	1	±	+
11	3	yes	1	2	+	+
12	3	yes	2	3	+	+

Table 3: Evaluation of the promotion assay of various chemicals, based on two criteria (Study III, Figure 6 results)

Compound	Lab No.	1: No. of concentrations with significant effect	4: No. of concentrations showing a 2-fold increase	Judgement based on 1 + 4
Mezerein	3	5	4	+
	7	5	2	+
	8	3	2	+
	10	3	4	+
4α-Phorbol	3	0	0	–
	7	0	0	–
	8	0	0	–
	10	0	0	–
PDD	2	6	4	+
	9	4	4	+
	11	4	3	+
	12	5	4	+
17β-Oestradiol	2	0	0	–
	9	0	0	–
	11	0	0	–
	12	0	0	–
Okadaic acid	1	0	0	–
	4	2	1	+
	5	2	2	+
	6	1	1	+
Dexamethasone	1	3	0	±
	4	0	0	–
	5	2	2	+
	6	0	0	–

mother culture, and 2ml was distributed into each well of 6-well plates (4×10^4 cells/well). In the test chemical assays, each dose group consisted of 6 wells. After culture for three days, the medium was replaced with fresh medium containing the test chemical. The final concentration of organic solvents in the medium was less than 0.1% in the case of DMSO and ethanol, and less than 0.5% for acetone. The cultures were again provided with fresh medium containing test chemicals on Day 7 and 10, and then with fresh DF5F medium alone on Day 14. On Day 21, the cells were fixed with methanol for 10 minutes and then stained with 5% Giemsa solution for 30 minutes. Transformed foci were characterised by using the following morphological criteria: deep basophilic staining, dense multi-layering of cells, random orientation of cells at the edge of foci, and more than 50 cells within a focus.

Concurrent cell growth assay was performed by preparing additional 6-well plates and treating the cells as in the transformation assay. On Day 7 the cells were fixed and stained as described in the cell growth assay for dose-determination.

Statistical analysis and judgement criteria

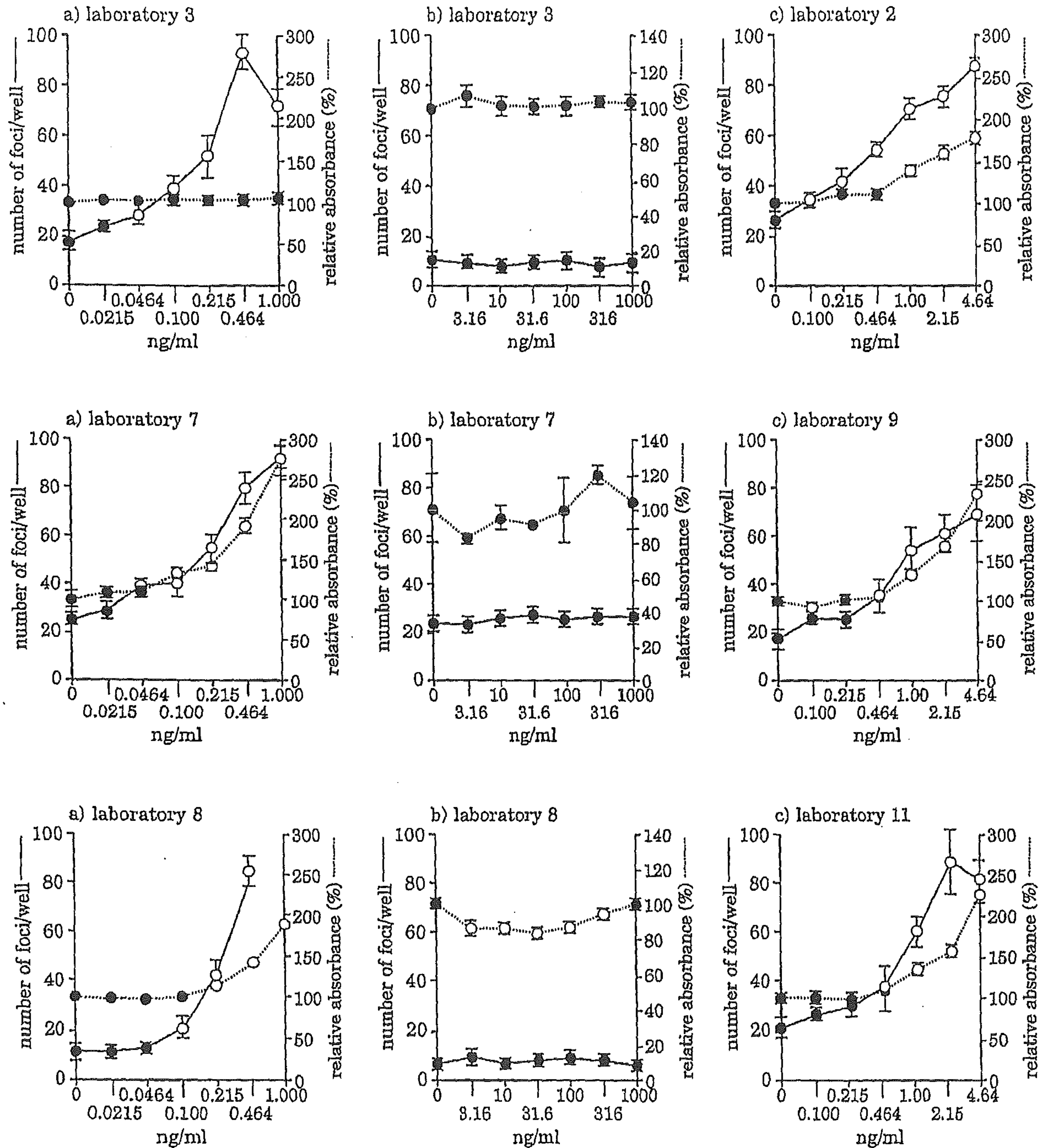
The following criteria were used for the evaluation of transformation results: a) a significant increase in a one-sided Dunnett test, with a significance level of 5% ($p < 0.05$); and b) more than a two-fold increase as compared with the solvent control. Chemicals which satisfied these two criteria were judged to be positive (+). Chemicals which met only the first criterion, but not the second, were considered equivocal (±), while negative chemicals (–) were those which induced no statistically significant increase in transformed foci at any concentration.

Results and Discussion

Cell growth assay (Study I)

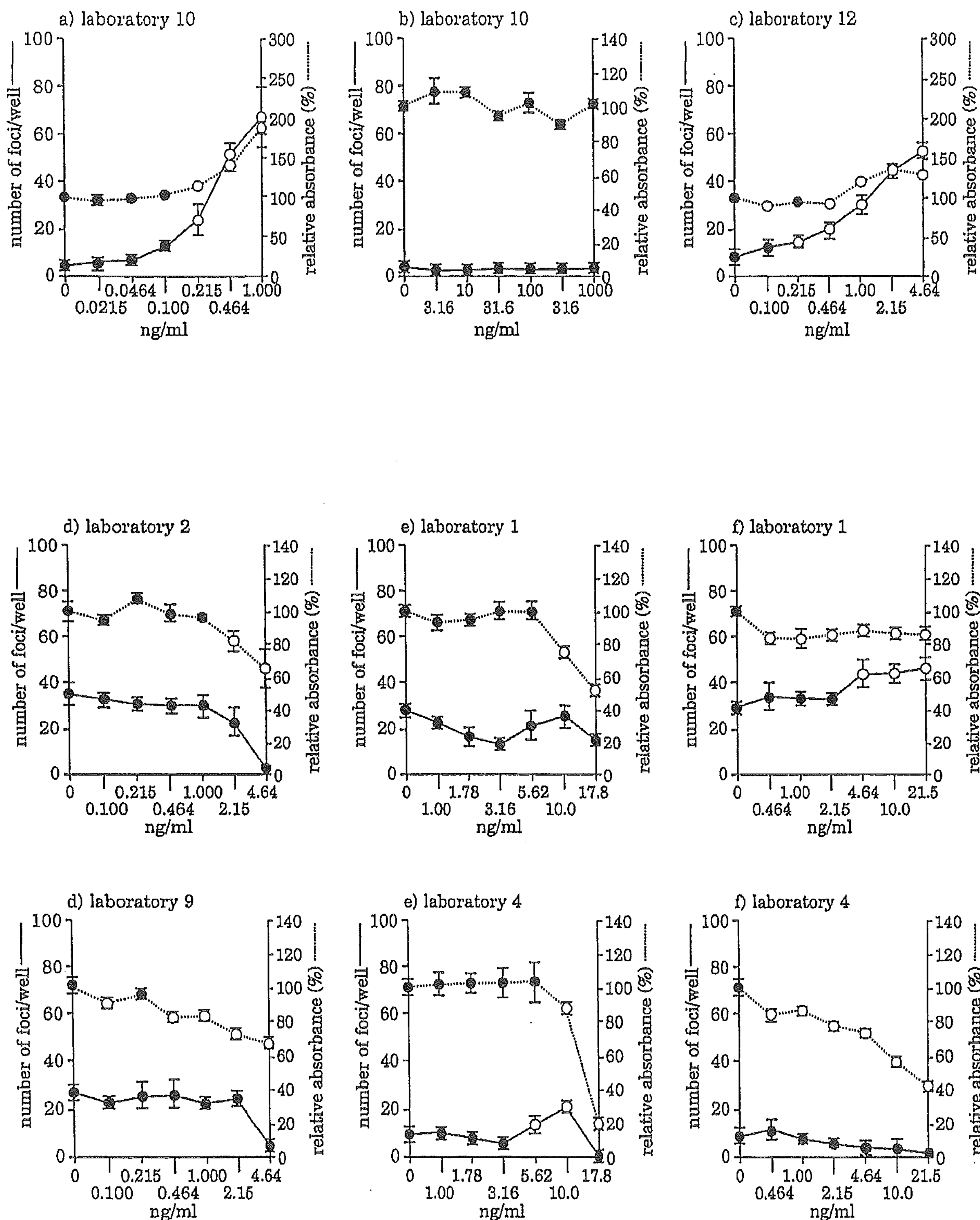
In order to confirm the applicability of the cell growth assay as reported by Ohmori *et al.* (14), LCA was evaluated for its effect on cell growth. Eight laboratories

Figure 6: Transformation frequencies and effects on cell growth with various chemicals (Study III)



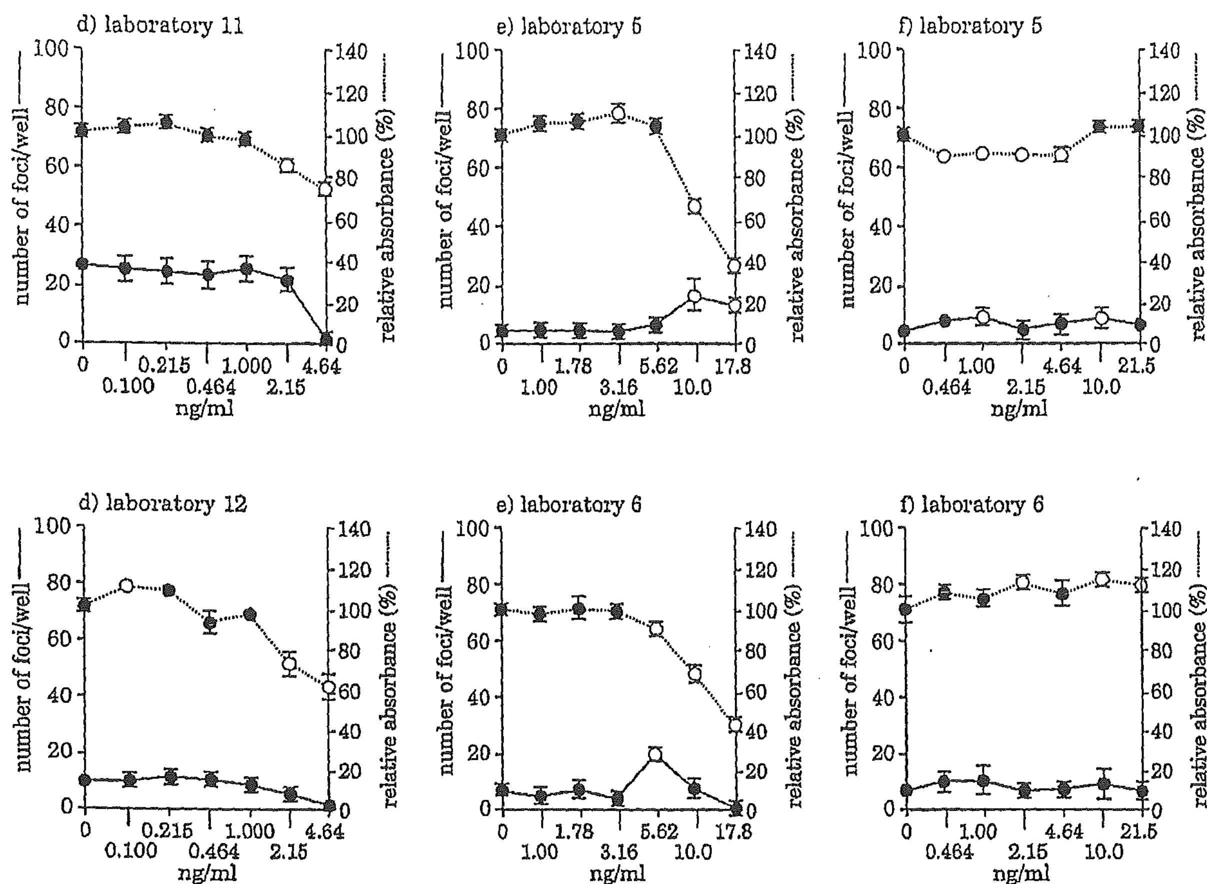
a) mezerein; b) 4α-phorbol; c) PDD. ○ = significant increase of focus number in transformation assay, and significant point in cell growth assay compared to control.

Figure 6: continued



a) mezerein; b) 4α-phorbol; c) PDD; d) 17β-oestradiol; e) okadaic acid; f) dexamethasone. ○ = *significant increase of focus number in transformation assay, and significant point in cell growth assay compared to control.*

Figure 6: continued



d) 17β-oestradiol; e) okadaic acid; f) dexamethasone. ○ = significant increase of focus number in transformation assay, and significant point in cell growth assay compared to control.

participated, but two laboratories did not fully comply with the protocol. The results from the six compliant laboratories are shown in Figure 1a. Most of the laboratories reported slight growth inhibition at 15.8µg/ml, and all observed marked inhibition at 50µg/ml. From these results, the approximate LCA concentration causing 50% inhibition of growth was 30µg/ml. The degree of inter-laboratory variance at each concentration was revealed by a box-whisker plot with SPSS 10.0J (Figure 1b).

These results suggested that the current CV procedure of the cell growth assay was reproducible and transferable.

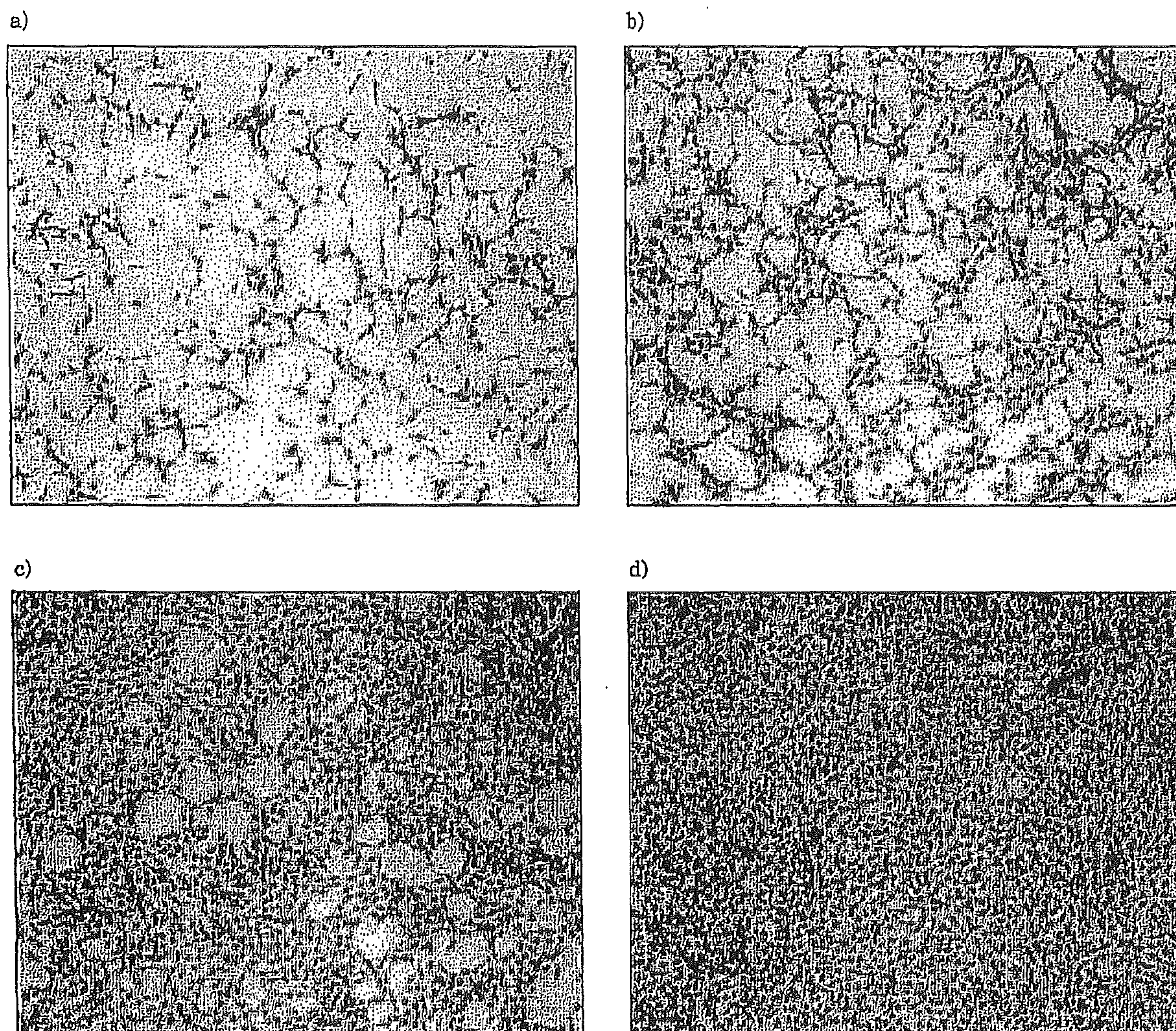
Bhas promotion assay with TPA and LCA (Study II)

TPA and LCA were examined according to the original Bhas promotion assay reported by Ohmori *et al.* (14), whereby cultures were continued for 17 rather than 21 days.

The individual results for TPA treatment in twelve laboratories are depicted in Figure 2. A dose-dependent increase of transformed foci was observed in all the laboratories, but there was variation in the results among the laboratories. The response was highest in laboratories 1 and 3, where many foci were observed in non-treated cultures. In contrast, the response was lowest in laboratories 2 and 7, where few foci appeared in control cultures.

Figure 3 illustrates the data for LCA treatment in the Bhas promotion assay. Test concentrations ranged up to 50µg/ml. A dose-dependent response was observed from 5 or 10µg/ml up to 20µg/ml, but there was one laboratory (laboratory 10) where little response was observed at 10µg/ml. Cytotoxicity was evident at 20µg/ml in laboratory 5, and at 50µg/ml in all the laboratories. Similarly to that with the TPA assays, the response to LCA was

Figure 7: Crystal violet-stained cells of mother cultures



Inoculated at a) 5000 cells/ml; b) 10,000 cells/ml; c) 20,000 cells/ml; d) 40,000 cells/ml, and cultured for three days.

strongly related to the level of spontaneous transformation. The response was high in laboratories 1, 3 and 11, and was low in laboratories 2, 7 and 10.

A box-whisker plot of the results with TPA is shown in Figure 4a. The number of transformed foci showed a dose-dependent increase up to 50ng/ml, and then leveled off at 100ng/ml. Figure 4b shows the box-whisker plot for the LCA data. Here again, a dose-dependent increase of transformed foci was evident.

The original protocol for the collaborative work established three criteria for the judgement of each transformation result: 1) a significant increase, as indicated by Dunnett's test; 2) a significant increase at more than two consecutive concentrations; and 3) more than a three-fold increase as compared with the solvent control.

Table 1 summarises the evaluation in each criterion applied to the results for TPA from each laboratory. The data from all the laboratories satisfied all three criteria, loading positive judgements in all cases (Table 1, column 6).

Table 2 shows the evaluation of the results for LCA. Laboratory 10 obtained a statistically significant increase of foci at only one concentration. The third criterion was not satisfied in laboratories 4 and 5, although a dose-dependent increase of foci was observed in these laboratories. Consequently,