Enhancement of Human T Lymphocyte Colony Formation by 12-O-Tetradecanoylphorbol-13-Acetate (TPA)

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Running Head: TPA & T Lymphocyte Colony

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Summary

The effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on human T lymphocyte colony formation in vitro were investigated. The number of T lymphocyte colonies was increased 4 to 5 times over that of controls by the addition of TPA (10^{-7} to 10^{-9} M) to phytohemagglutinin (PHA)-containing cultures. Few colonies were observed when stimulated with TPA in the absence of PHA. In the cultures containing a sufficient amount of exogenous T cell growth factor (TCGF), the enhancement of T lymphocyte colony formation by TPA was not observed. TPA enhanced TCGF production by peripheral lymphocytes stimulated with PHA. The optimal concentrations of TPA for T lymphocyte colony formation were similar to those for TCGF production. These findings suggest that TPA enhanced T lymphocyte colony formation by stimulating endogenous TCGF production. Interestingly, T lymphocyte colony formation was not inhibited even at high concentrations of TPA that usually inhibit myeloid and erythroid colony formation. This difference may be due to different sensitivities to TPA between T lymphocyte colony-forming cells and myeloid and erythroid colony-forming cells.

Key words: Tumor promoter, T cell, T cell growth factor.

Introduction

12-O-Tetradecanoylphorbol-13-acetate (TPA) has been reported to have diverse effects on the *in vitro* proliferation and differentiation of hemopoietic progenitor cells (1,2). Recently, we have reported that TPA enhanced normal human erythroid burst formation at low concentrations and inhibited it at high concentrations (3), and that normal myeloid colony formation was also suppressed by TPA. Normal human myeloid cells were transformed into macrophage-like cells *in vitro* by treatment with TPA (4).

In lymphocyte culture systems, several effects of tumor-promoting phorbol esters have been reported. For example, they act synergistically with Concanavalin A (Con A) or phytohemagglutinin (PHA) to cause mitogenic response in lymphocytes from mouse (5), cow (6), and human (7). Furthermore, they are mitogenic by themselves for some of these lymphocytes (5,6,7). Recently, TPA has been shown to act synergistically with Con A and stimulates the production of T-cell growth factor (TCGF) by mouse spleen cells as well as by human peripheral blood lymphocytes (8,9).

T lymphocyte colony formation in semi-solid culture is a well-established technique (10,11). However, the nature of T lymphocyte colony-forming cells and the characteristics of the growth factors which promote colony formation are not fully defined. Furthermore, there is little information regarding the effect of TPA on T lymphocyte colony formation. Therefore, we investigated the effect of TPA on human T lymphocyte colony formation *in vitro*. In this report, we show that human T lymphocyte colony formation is markedly enhanced by

the addition of TPA, and discuss the mechanisms of this phenomenon.

Materials and Methods

Cells. Human mononuclear cells were obtained from the peripheral blood of healthy volunteers with their informed consent. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, washed 3 times with α-medium, and then suspended in α-medium supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Maryland).

Adherent cells were removed from the mononuclear cell suspension by incubating it in a 100 mm plastic dish (Falcon Labware, Div. of Becton, Dickson & Co., Oxnard, Calif.) precoated with fetal calf serum for one hour at 37°C in 5% CO_2. Then, the suspension of non-adherent cells was prepared by passing the suspension over a nylon wool-glass head adherent column. The mononuclear cells, after these treatments, consisted of at least 90% E-rosette positive cells. The viability of all suspensions was determined by Trypan blue dye exclusion.

Reagents. Phorbol (Sigma Chemical Co., St. Louis, Mo.) and TPA (Sigma Chemical Co.) were stored as 0.01M stock solutions in dimethyl solfoxide (DMSO) in the dark at -20°C. PHA (Wellcome Reagents Ltd., Backenham, England) was prescreened for its ability to optimally support T lymphocyte colony formation in culture. TCGF was prepared from a monkey spleen cell conditioned medium (Otsuka Assay Lab., Tokyo).

T lymphocyte colony formation. T lymphocyte colonies were formed by the methylcellulose monolayer method. Briefly, $3x10^5$ cells were plated on 35 mm-gridded Petri dishes in an α-medium containing 0.8% methylcellulose, one ml of 20% fetal calf serum, 1% PHA, and various

concentrations of TPA. After 7 days of incubation at 37°C in a fully humidified atmosphere with 5% CO_2, the colonies which contained more than 40 cells were scored under an inverted microscope. At least four plates were counted for each experimental point, and the data were expressed as the mean±SEM. In another series of experiments, the influence of TCGF upon T lymphocyte colony formation was assessed. The monkey spleen cell conditioned medium was used as a source of TCGF and added to the culture at a final concentration of 20%.

Assay for TCGF. The activity of TCGF produced by peripheral blood mononuclear cells was assayed as described elsewhere (13). Briefly, peripheral blood mononuclear cells ($1x10^6$/ml) were incubated in one ml culture medium with 1% PHA and various concentrations of TPA at 37°C. The culture supernatant was harvested after 72 hours. TCGF activity in the supernatant was assessed by the ability to induce proliferation of TCGF-dependent human T lymphocytes which had been cultured in a relatively high concentration of TCGF for at least 10 days. $1x10^4$ TCGF-dependent T lymphocytes were incubated with 0.1ml of the culture supernatant to be tested. After incubation for 24 hours at 37°C, the cultures were pulsed with 0.5μCi of ^{3}H-thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) for 12 hours and the amount of ^{3}H-thymidine incorporated into the cells was determined in triplicate. TPA or PHA did not stimulate the proliferation of the TCGF-dependent T lymphocytes, nor did they argument or inhibit the proliferation of TCGF-dependent T lymphocytes stimulated by TCGF.

Myeloid colony formation and erythroid colony formation. Bone marrow specimens were obtained from normal volunteers after written informed consent. Single-layer soft agar cultures were performed for an assay for granulocyte-macrophage progenitors (CFU-C) according to

the method of Robinson et al. (14) with minor modifications (4). Human placental conditioned medium (15) was used as a source of colony-stimulating factor (CSF). Single-layer methylcellulose cultures of primitive erythroid progenitor cells (BFU-E) were performed according to the method of Iscove et al. (16). These cultures for myeloid and erythroid colony formation were performed for 9 and 14 days, respectively. Details are mentioned elsewhere (4,17).

Results

Effects of TPA on T lymphocyte colony formation. The optimal concentration of PHA for T lymphocyte colony formation in our system was determined as 1%(vol/vol) by preliminary experiments. The addition of TPA to the PHA-containing cultures markedly increased the number of T-lymphocyte colonies to 3 to 4 times that of controls (Table 1). In the culture containing TPA alone, only a few T lymphocyte colonies formed ; in the culture containing both PHA and TPA, they were slightly larger in size and more densely packed as compared to those formed in the culture containing PHA alone.

Relationship between the number of T lymphocyte colonies and TPA concentrations (Fig. 1). TPA augmented T lymphocyte colony formation in a dose-dependent fashion from 10 pM to 1 nM. No further increase was noted at greater concentrations. The plateau value was approximately 4 times that of the control which was stimulated by PHA alone. Inhibition of T lymphocyte colony formation was not observed even at higher concentrations of TPA such as 10^{-7}M. The addition of phorbol as a control and the solvent (DMSO) had no significant effect on T

lymphocyte colony formation.

Effects of TPA in cultures with exogenous TCGF. Since TPA is known to enhance the production of TCGF by PHA-stimulated lymphocytes (8), we compared the effect of TPA on T lymphocyte colony formation in cultures with or without the addition of exogenous TCGF (Fig. 2). It was found that in the cultures to which sufficient amounts of exogenous TCGF were added, TPA had little effect on T lymphocyte colony formation (Fig. 2 right).

Effects of the delayed addition of TPA to the cultures. We examined the effect of TPA added on midculture days on T lymphocyte colony formation (Fig. 3). TPA was poured on top of the methylcellulose medium. Delayed addition of TPA on day 1 to day 3 caused almost the same increment in the number of T lymphocyte colonies, as compared to when TPA was added on day 0. When TPA was added on day 4, the increase in the number of T lymphocyte colonies was moderate, about half that of the maximal increase.

Effects of TPA on TCGF production. The effect of TPA on TCGF production by human peripheral mononuclear cells was also examined. Maximum stimulation for TCGF production was observed at concentrations of TPA at 1 nM or greater (Fig. 4). Phorbol had no significant effect on TCGF production at any concentration.

Comparison of the effects of TPA on T lymphocyte colony formation and myeloid and erythroid colony formation (Fig. 5). Previously, we reported the effect of TPA on myeloid colony and erythroid burst formation (3,4). In those studies, relatively high concentrations (10^{-9} -10^{-8}M) of TPA strongly inhibited both types of colony formation. However, T lymphocyte colony formation was not suppressed at these

concentrations.

Discussion

Studies reported here were undertaken to determine whether TPA, a potent tumor promoter, has any effect on T lymphocyte colony formation or on myeloid colony and erythroid burst formation. It is known that TPA has effects on various cell lineages of hematopoietic systems. In this study T lymphocyte colony formation was enhanced by the addition of TPA to the PHA-containing cultures. TPA enhanced not only the colony number but also the colony size. It was shown that TPA acts synergistically with PHA to enhance T lymphocyte colony formation. Similar synergistic actions of phorbol esters were previously reported in mitogen activated bovine lymphocytes (6), and it was concluded that phorbol esters influenced the cellular responsiveness to endogenous or exogenous mitogens.

TPA is known to enhance the production of TCGF by PHA-stimulated lymphocytes. However, the target cell of the TPA action has not been fully clarified (8,18). There may be two different hypotheses to explain the enhancing effects of TPA on T lymphochte colony formation. One would be that TPA acts directly on progenitor cells of the T-cell lineage. The other would be that TPA augments T colony formation indirectly by stimulating endogenous TCGF production. To clarify this point, we compared the effects of TPA on T lymphocyte colony formation in cultures with and without exgenous TCGF. In cultures containing sufficient amounts of exogenous TCGF, TPA had little effect on T lymphocyte colony formation when compared with the controls. Moreover, the

concentrations of TPA required for the maximum stimulation of TCGF production were similar to the optimal concentrations of TPA for T lymphocyte colony formation. These findings suggest that TPA acts on cells which produce TCGF and that TPA augments T lymphocyte colony formation mainly by the stimulation of endogenous TCGF production. Delayed addition of TPA showed that at least 4 days' treatment with TPA was required for the full enhncement of T lymphocyte colony formation. This culture period may be required for TPA to stimulate the endognous TCGF production in the cultures.

This indirect action of TPA on T lymphocyte colony formation suggests a mechanism to account for the effects of TPA on colony formation of different hemopoietic cell lineages. It was reported that myeloid and erytroid colony formations are enhanced by low concentrations of TPA (3,19,20). The mechanism involved in this phenomenon might be based on the TPA-mediated stimulation of the production of colony-stimulating factor (CSF) or burst-promoting activity (BPA) in cultures, similar to the stimulation of exogenous TCGF production in T lymphocyte colony formation. Additionally, relatively high concentrations (10^{-9}-10^{8}M) of TPA strongly inhibited myeloid colony and erythroid burst formation (1,3,4), although T lymphocyte colony formation was not inhibited by such high concentrations of TPA. This may be due to differing sensitivities of these progenitor cells to TPA.

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Table 1. Effects of PHA and TPA on human T lymphocyte colony formation

Stimulator	Number of T lymphocyte colonies per $3x10^5$ peripheral blood mononuclear cells[a]
None	0
1% PHA	1008.0 ± 29.0
$1.6x10^{-8}$M TPA	29.3 ± 5.3
1% PHA + $1.6x10^{-8}$M TPA	4200.0 ± 202.8

[a]Mean ± SEM

Figure legends

Figure 1. Correlation between the concentration of TPA and human T lymphocyte colony formation. o——o : TPA was added to the PHA-containing cultures. •——• : Phorbol was added instead of TPA as a control. Ordinate: number of T lymphocyte colonies. Abscissa: the concentration of TPA or phorbol.

Figure 2. Effects of TPA (1.6×10^{-9}M) on T lymphocyte colony formation in the culture with or without exogenous TCGF. Left column shows the result of the cultures containing PHA alone. Right column shows the result of the cultures containing PHA and sufficient amout of exgenous TCGF. The monkey spleen cell conditioned medium was used as a source of TCGG, and added to the culture at a final concentration of 20%. Colonies were counted after 7 days' culture. Each column represents the mean (±SEM) of quadruplicate experiments.

Figure 3. Effects of the delayed addition of TPA (1.6×10^{-9}M) to the cultures. Colonies were counted after 7 days' culture. TPA was poured on top of the methylcellulose medium. Ordinate: number of colonies. Abscissa: the culture day of TPA addition. Each column represents the mean (±SEM) of quadruplicate value.

Figure 4. Effects of TPA on the TCGF production by peripheral mononuclear cells stimulated with PHA. o——o treated by TPA. •——• treated by phorbol as a control. Ordinate: ^{3}H-thymidine incorpration by TCGF dependent cells. Abscissa: Concentrations of TPA or phorbol.

Each point represents the mean (±SEM) of quadruplicate value.

Figure 5. Effects of TPA on the proliferation of hemopoietic precursors. ○—○ T lymphocyte colony formation (T-CFC) in the cultures without exogenous TCGF. □—□ T lymphocyte colony formation in the cultures with sufficient amount of exogenous TCGF. ▲—▲ Granulocyte-macrophage colony formation (CFU-C). ●—● Erythroid burst formation (BFU-E). Ordinate: number of colonies or bursts expressed percentage of control that is the value in the culture without TPA. Abscissa: the concentration of TPA.

Figure 1

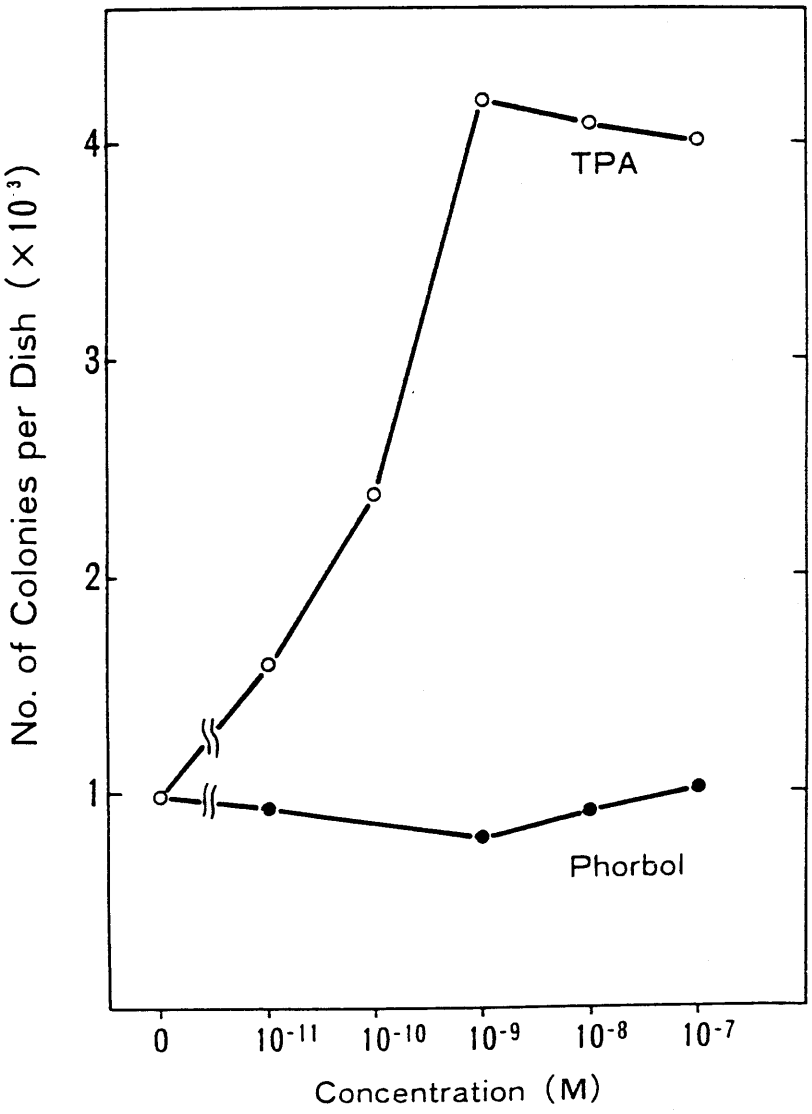

No. of Colonies per Dish (×10³)
4
3
2
1
TPA
Phorbol
0
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
Concentration (M)

Figure 2

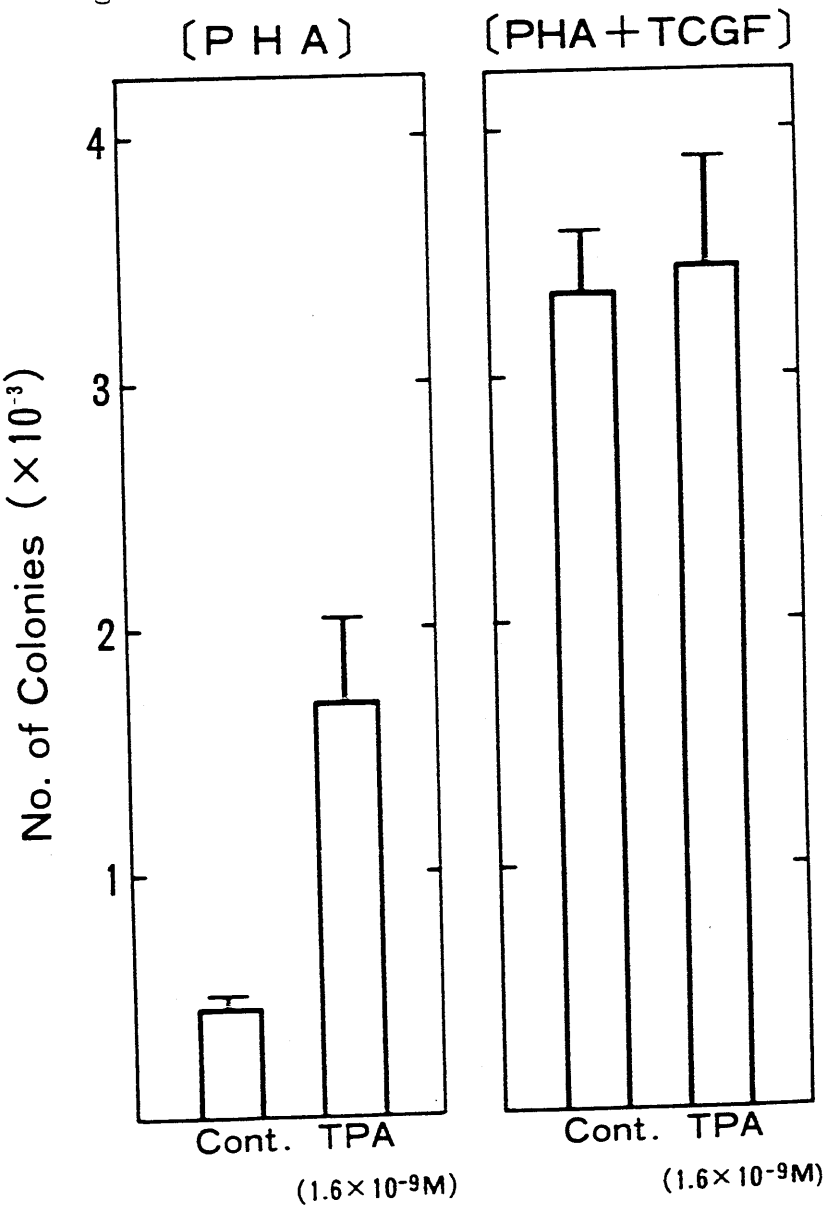
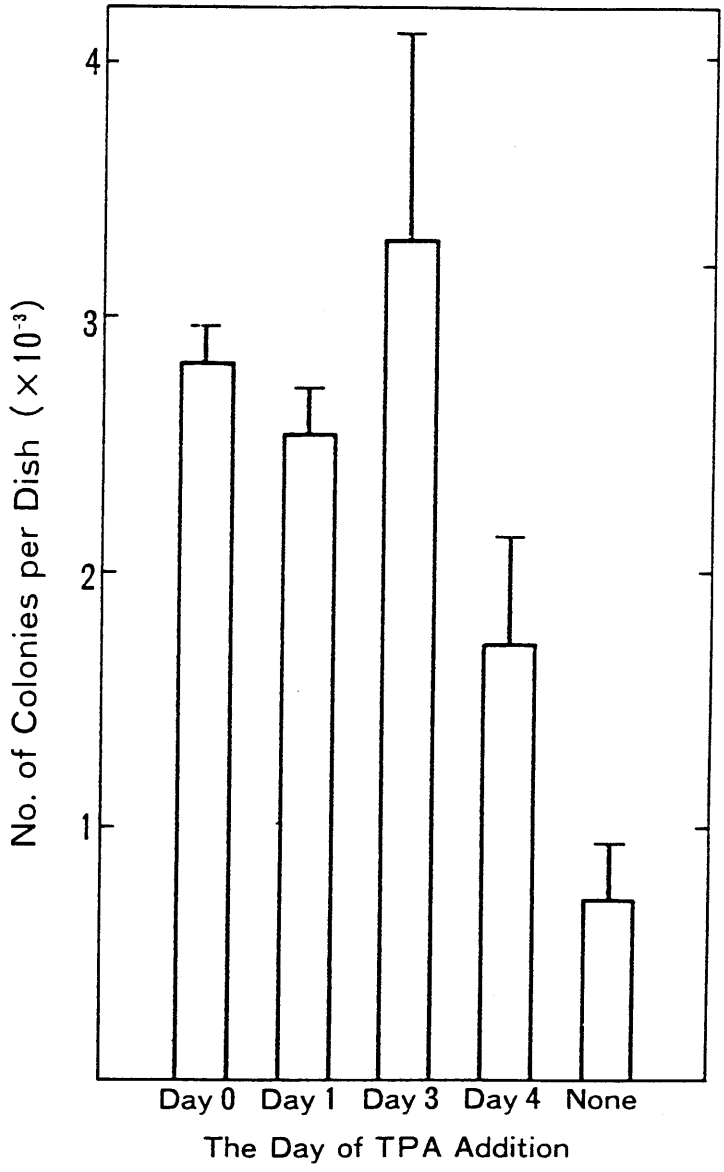

〔P H A〕
〔PHA+TCGF〕
No. of Colonies (×10⁻³)
4
3
2
1
Cont.
TPA
(1.6×10⁻⁹M)
Cont.
TPA
(1.6×10⁻⁹M)

Figure 3



Concentration of TPA : 1.6×10^{-9}M

Figure 4

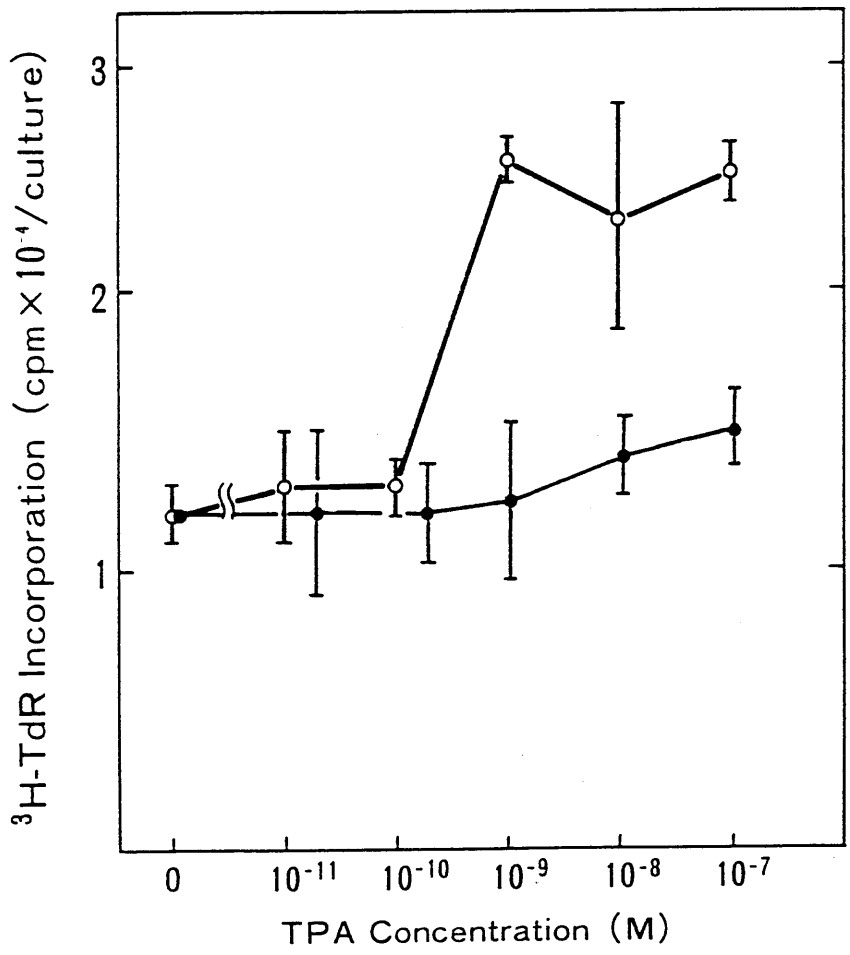
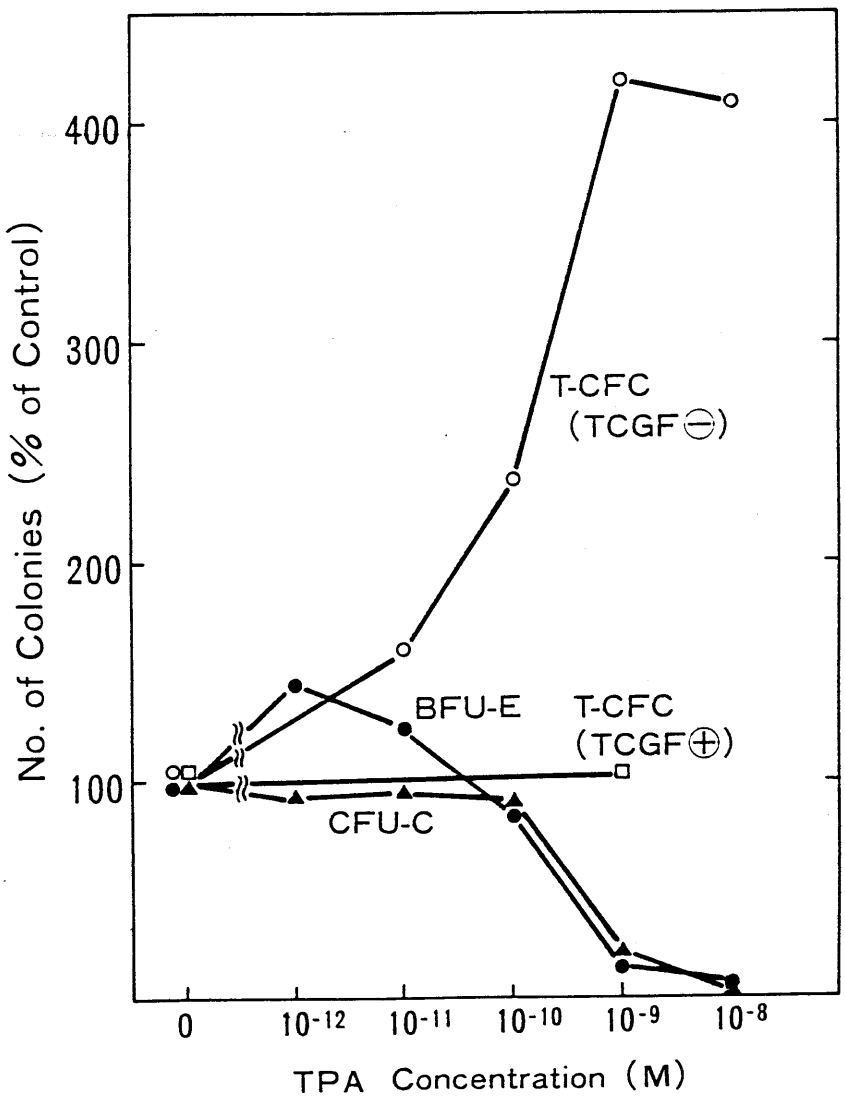

^{3}H-TdR Incorporation (cpm × 10^{-4}/culture)
3
2
1
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
TPA Concentration (M)

Figure 5


400
300
200
100
No. of Colonies (% of Control)
T-CFC
(TCGF⊖)
BFU-E
T-CFC
(TCGF⊕)
CFU-C
0
10-12
10-11
10-10
10-9
10-8
TPA Concentration (M)