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Effects of Novel Phenoxazine Compounds, 2-Amino-4, 4α-Dihydro-4α, 7-Dimethyl-3H-Phenoxazine-3-One and 3-Amino-1, 4α-Dihydro-4α, 8-Dimethyl-2H-Phenoxazine-2-One on Proliferation of Phytohemagglutinin- or Anti-Human IgM-Activated Human Peripheral Blood Mononuclear Cells

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Akazawa, M., Koshibu-Koizumi, J., Iwamoto, T., Takasaki, M., Nakamura, M. and Tomoda, A. *Effects of Novel Phenoxazine Compounds, 2-Amino-4, 4α-Dihydro-4α, 7-Dimethyl-3H-Phenoxazine-3-One and 3-Amino-1, 4α-Dihydro-4α, 8-Dimethyl-2H-Phenoxazine-2-One on Proliferation of Phytohemagglutinin- or Anti-Human IgM-Activated Human Peripheral Blood Mononuclear Cells.* Tohoku J. Exp. Med., 2002, **196** (3), 185-192 —— We examined the in vitro effects of 2-amino-4, 4α-dihydro-4α, 7-dimethyl-3H-phenoxazine-3-one(Phx 1)and 3-amino-1, 4α-dihydro-4α, 8-dimethyl-2H-phenoxazine-2-one (Phx 2) on the proliferation of phytohemagglutinin (PHA)- or anti-human IgM-activated human peripheral blood mononuclear cells (PBMC). Phx 1 and Phx 2 inhibited the incorporation of ^{3}H-thymidine of PHA-activated PBMC by as much as 75% and 40%, respectively, at a concentration of 40 μM. The inhibition was dependent on the dose of Phx 1 and Phx 2. These results strongly suggest that Phx 1 and Phx 2 inhibit proliferation of T cells, because PHA specifically activates the T cells among PBMC. On the other hand, when PBMC were activated by anti-human IgM, which specifically stimulates B cells, the incorporation of ^{3}H-thymidine was rather increased in the presence of 15.8 μM Phx 1 or Phx 2. However, at a higher concentration of Phx 1 or Phx 2 (50 μM), the incorporation of ^{3}H-thymidine was increased by Phx 1, but was inhibited by

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Phx 2. These results suggest different effects of Phx 1 and Phx 2 on proliferation of human T and B cells. ——— 2-amino-4, 4α-dihydro-4α, 7-dimethyl-3H-phenoxazine-3-one; 3-amino-1, 4α-dihydro-4α, 8-dimethyl-2H-phenoxazine-2-one; phytohemagglutinin; anti-human IgM; mononuclear cells

Phenoxazines are present in actinomycins (Brockmann et al. 1956) and in some microorganism metabolites (Anzai et al. 1960; Gerber and Lechevalier 1964), and are shown to be biologically synthesized by the reaction of *o*-aminophenol and its derivatives with human and bovine hemoglobin (Tomoda et al. 1991, 1992, 2001). However, the effects of phenoxazines on human cells have attracted little attention. Since the phenoxazine nucleus is an essential constituent of actinomycin D, which shows strong anti-tumor effects (Hollstein 1974), the chemically synthesized phenoxazines were studied for their anti-tumor effects, but little anti-tumor effects were demonstrated on tumor cells, probably due to their poor solubility in water (Motohashi 1983; Motohashi et al. 1991). It has been observed that 2-amino-4, 4α-dihydro-4α, 7-dimethyl-3H-phenoxazine-3-one (Phx 1), produced by the reaction of 2-amino-5-methylphenol with human or bovine hemoglobin, is relatively soluble in water, and exerted in vitro anti-tumor effects by inhibiting the proliferation of a variety of cultured cell lines such as human epidermoid carcinoma cells (Isihda et al. 1996), Meth A tumor cells (Mori et al. 2000), human lung carcinoma cell lines (Abe et al. 2001). The effects of Phx 1 on the proliferation of normal human cells have not been studied hitherto.

On the other hand, the proliferation of human lymphocytes, T cells and B cells, is shown to be activated with phytohemagglutinin (PHA) (Fibach et al. 1976; Georgoulias et al. 1984) and anti-IgM, respectively. It was shown that incorporation of ^{3}H-thymidine into the PHA-activated human peripheral blood mononuclear cells (PBMC) was inhibited by emodin (1, 3, 8 trihydroxy-6-methyl-anthraquinone), suggesting that emodin suppressed the proliferation of PBMC and exerted immunosuppressive effects (Huang et al. 1992). Later, Kuo et al. (1997) found that emodin suppressed the proliferation of Raji cells, a human B cell lymphoblastoid cell line. Taking account of the fact that the chemical structure of Phx 1 is similar to that of edmodin in some points, i.e., these compounds are tricyclic chromophores (see Fig. 1), it is conceivable that Phx 1 may exert some effects on the proliferation of PHA- or anti-IgM-stimulated PBMC. As an analogue of Phx 1, Tomoda et al. (1991) found that 3-amino-1, 4α-dihydro-4α, 8-

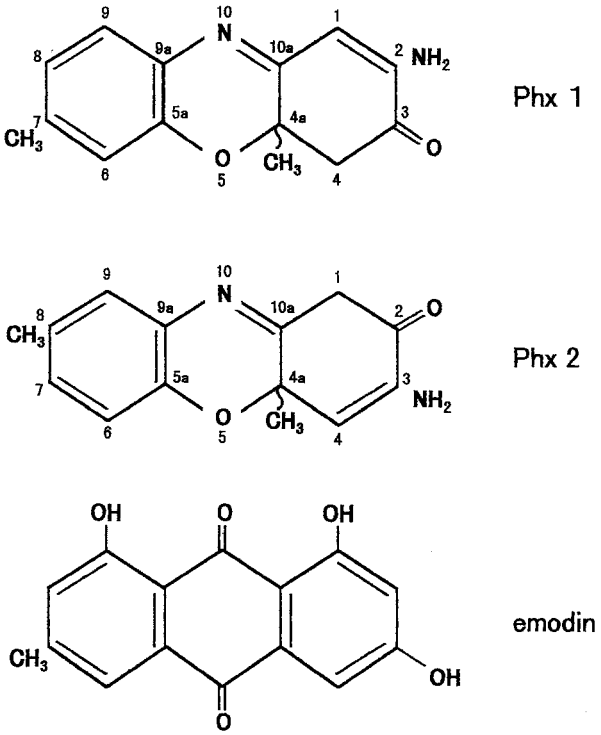

Fig. 1. Chemical structure of Phx 1, Phx 2 and emodin.

dimethyl-2H-phenoxazine-2-one (Phx 2) is produced by the reaction of 2-amino-4-methylphenol with human hemoglobin. It seems to be of significance to study the effects of these phenoxazines, on the proliferation of PBMC, in terms of immunosuppressive effects.

In the present study, we observed the inhibitory or stimulatory effects of Phx 1 and Phx 2, on incorporation of ^{3}H-thymidine into PHA- or anti-human IgM-activated human lymphocytes under various conditions, and suggested that these phenoxazine compounds may affect the proliferation of human T and B cells.

MATERIALS AND METHODS

Materials

2-Amino-5-methylphenol and 2-amino-4-methylphenol were purchased from Tokyo Kasei Chemicals Co., Ltd. (Tokyo). PHA was obtained from Sigma Co., Ltd. (St. Louis, MO, USA). ^{3}H-thymidine was purchased from Pharmacia-Amersham Japan (Tokyo). RPMI 1640 medium and fetal calf serum were purchased from Gibco Laboratories (Grand Island, NY, USA). Affinity-purified goat anti-human IgM heavy chain and goat IgG were purchased from ICN Pharmaceut. Inc., (Aurora, OH, USA) and Chemicon International (Temecula, CA, USA), respectively. Ficoll-Hypaque was obtained from Pharmacia-Amersham Japan.

Synthesis of Phx 1 and Phx 2

Phx 1 and Phx 2 were synthesized by the reaction of 2-amino-5-methylphenol or 2-amino-4-methylphenol with bovine hemolysates, as described previously (Tomoda et al. 2001). The chemical structure of these phenoxazine compounds are shown in Fig. 1, with that of emodin.

Preparation of mononuclear cells

Human peripheral blood was freshly drawn from a normal adult, after obtaining written informed consent. Peripheral blood mononuclear cells (PBMC) were then isolated from the blood, using Ficoll-Hypaque density gradients. PBMC are composed of T cells, B cells, NK cells, and monocytes as described previously (Fibach et al. 1976; Georgoulias et al. 1984).

Preparation of Phx 1 and Phx 2 solution

Phx 1 and Phx 2 were dissolved in hot ethanol, and then diluted with RPMI 1640/10% fetal calf serum. Final concentration of ethanol in culture did not exceed 0.35%, which gave no significant effects in our experiments.

PHA response

PBMC (2×10^{5}/well) were stimulated with 0.25% PHA in RPMJ 1640 containing 10% FCS in 96-well plates for 3 days and the ^{3}H-thymidine (0.5 μCi/well) incorporation was assessed for the last 6 hour. The indicated concentrations of Phx 1 and Phx 2 were added at the start of the cultures or 30 minutes before or after the ^{3}H-thymidine pulse. Then, the cells were harvested using a cell harvester, and the incorporated ^{3}H-thymidine was measured on a scintillation counter. Data are represented as the mean$\pm$S.E.M. of quadriplicate wells. Nonspecific incorporation in the absence of PHA was subtracted from all data.

Anti-IgM response

Each well of 96-well plates was coated with 31.6 μg/ml of goat anti-human IgM antibody or control goat IgG at room temperature for 2 hour. Then, PBMC (4×10^{5}/well) in RPMI 1640 containing 10% FCS were cultured in these wells for 4 days and the incorporation of ^{3}H-thymidine (1 μCi/ well) was assessed for the last 6 hour. The indicated concentrations of Phx 1 and Phx 2 were added at the start of the cultures or 30 minutes after the addition of ^{3}H-thymidine. Then, the cells were harvested and the incorporated ^{3}H-thymidine was measured on a scintillation counter. Nonspecific incorporation in the control IgG-coated wells was subtracted from all data. Data are re-

presented as the mean±S.E.M. of six replicated wells.

Statistical analysis

Data are expressed as the means±S.E.M. Significant difference was tested by Student's t-test. Values of $p<0.05$ were considered significant.

RESULT

In the present study, we investigated the effect of Phx 1 and Phx 2 on the ^{3}H-thymidine incorporation of PHA- or anti-human IgM-activated human PBMC. Fig. 2 shows the incorporation of ^{3}H-thymidine into PHA-activated human PBMC in the presence of 40 μM Phx 1 or Phx 2, expressed as percentages of the untreated control values of the PHA-activated cells. The vehicle control (0.1% ethanol) did not significantly affect the response. When 40 μM Phx 1 and Phx 2 were added at the start of the cultures, ^{3}H-thymidine incorporation was inhibited by as much as 75% and 40%, respectively, compared with the vehicle control.

We next studied the inhibition of ^{3}H-thymidine incorporation into PHA-activated T lymphocytes at various concentrations of Phx 1 and Phx 2 (Fig. 3). The ^{3}H-thymidine incorporation was inhibited by Phx 1 and Phx 2 in a dose-dependent manner. The IC_{50} values were 15 μM and 31.6 μM, for Phx 1 and Phx 2, respectively.

It is possible that the inhibition of ^{3}H-thymidine incorporation into PHA-activated T lymphocytes was caused by the inhibition of transport of of ^{3}H-thymidine through the cell membrane. In order to address this possibility, we examined the effects of Phx 1 and Phx 2 when added 30 minutes before or after the addition of ^{3}H-thymidine (Fig. 4). A in Fig. 4 shows the incorporation of ^{3}H-thymidine when

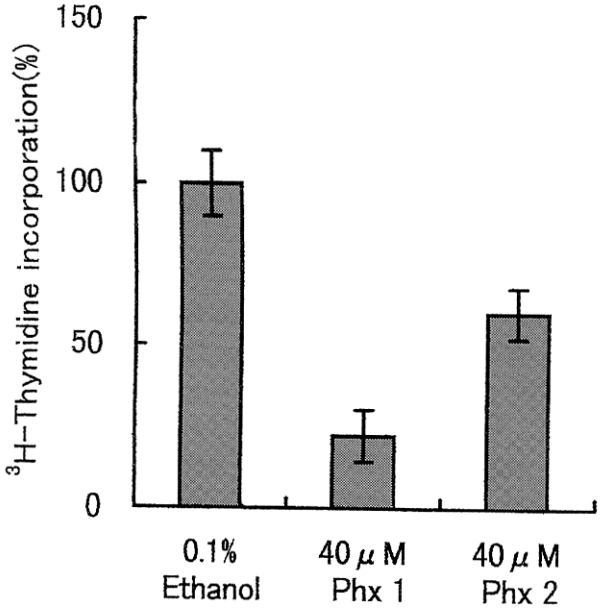

Fig. 2. Effect of Phx 1 and Phx 2 on the incorporation of ^{3}H-thymidine into PHA-activated human PBMC. Human PBMC were incubated with 0.25% PHA, in the presence of 40 μM Phx 1 or Phx 2, for 3 days. Six hr before, ^{3}H-thymidine was pulsed, and the incorporation of ^{3}H-thymidine into PBMC was estimated.

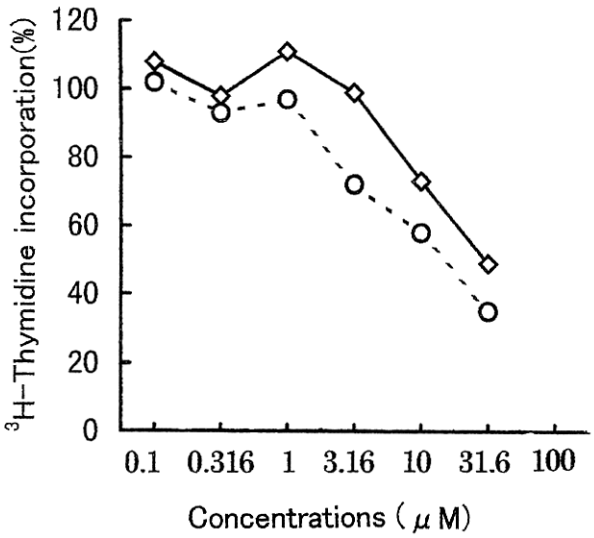

Fig. 3. Effects of various concentrations of Phx 1 and Phx 2 on the incorporation of ^{3}H-thymidine into PHA-activated human PBMC. Human PBMC were incubated with 0.25% PHA, in the presence of various concentrations of Phx 1 or Phx 2, for 3 day. Six hour before, ^{3}H-thymidine was pulsed, and the incorporation of of ^{3}H-thymidine into PBMC was estimated.
○−○, Phx 1 (+); ◇−◇, Phx 2 (+).

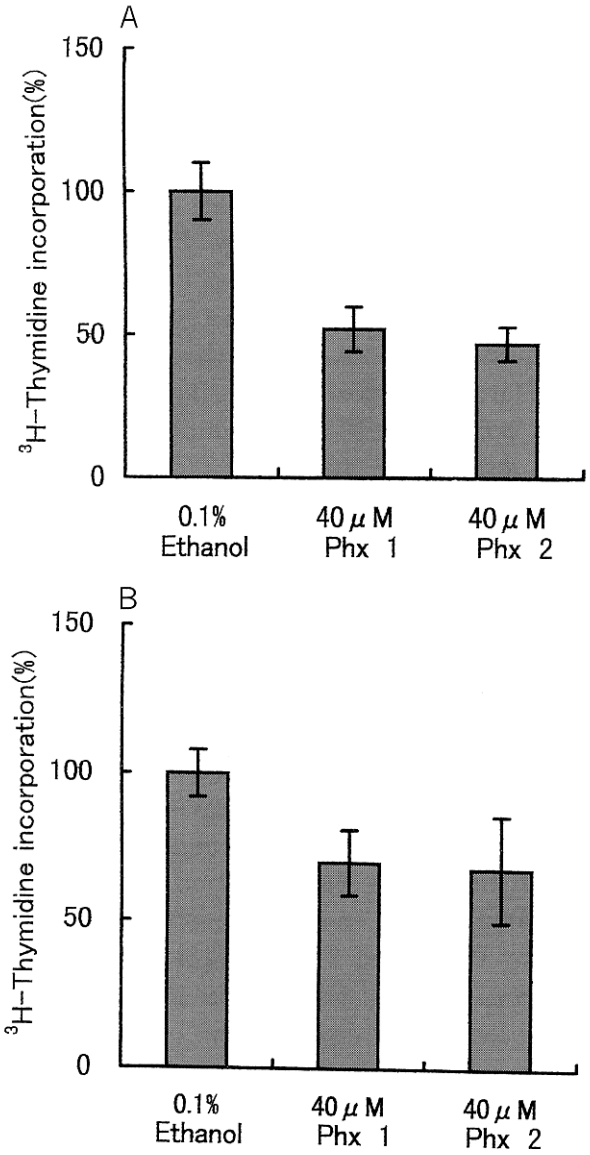

Fig. 4. Incorporation of ^{3}H-thymidine into PHA-activated human PBMC when Phx 1 and Phx 2 was added 30 minutes before or after the addition of ^{3}H-thymidine. A: Phx 1 or Phx 2 was administered 30 minutes before the addition of ^{3}H-thymidine. B: Phx 1 or Phx 2 was administered 30 minutes after the addition of ^{3}H-thymidine.

40 μM Phx 1 or Phx 2 was added 30 minutes before. The incorporation rate was 52±8% and 47±6% of the untreated control for Phx 1 and Phx 2, respectively. B in Fig. 4 shows the incorporation of ^{3}H-thymidine when 40 μM Phx 1 or Phx 2 was added 30 minutes after. The incorporation rate was 70±11% and 68±18% of the untreated control for Phx 1 and Phx 2, respectively. It is likely that the inhibition rate was somewhat larger when Phx 1 or Phx 2 was added 30 minutes before the ^{3}H-thymidine pulse than when added 30 minutes after. However, by the statistic analysis of these values, there was no significant differences between these two treatments ($p<0.05$). These results suggest that the uptake process of ^{3}H-thymidine through the cell membrane of PHA-activated human PMBC was not affected by Phx 1.

We next studied the ^{3}H-thymidine incorporation into PBMC stimulated with anti-human IgM in the presence or absence of Phx 1 and Phx 2 for 3 days (Table 1). When 0.1% ethanol alone was added, the incorporation of ^{3}H-thymidine was not significantly affected. The ^{3}H-thymidine incorporation was increased to 165.7±22.3% and 123.9±6.3% of the control in the presence of 15.8 μM Phx 1 and Phx 2, respectively. However, the ^{3}H-thymidine incorporation was increased by Phx 1 to 165.2±40.0% but was reduced by Phx 2 to 84.2±13.4% at the dose of 50 μM. In order to confirm this inhibitory effect of Phx 2, we studied the ^{3}H-thymidine incorporation into PBMC which were pre-activated by anti-human IgM in the absence of Phx 1 and Phx 2 for 3 days.

Table 1. *Incorporation of ^{3}H-thymidine into the DNA of human PBMC after stimulation with anti-human IgM in the presence or absence of Phx 1 and Phx 2*

	Concentrations		
	0	15.8 μM	50 μM
Phx 1	100±14.8	165.7±22.3	165.2±40.0
Phx 2	100±14.8	123.9± 6.3	84.2±13.4

Human mononuclear cells were incubated in wells coated with goat IgG or anti-human IgM for 3 days in the presence or absence of Phx 1 or Phx 2 (15.8 μM and 50 μM) ($n=6$). Then, ^{3}H-thymidine was added to wells, the cells were incubated for one more day. Data are represented as % relative to the 0.1% ethanol vehicle control.

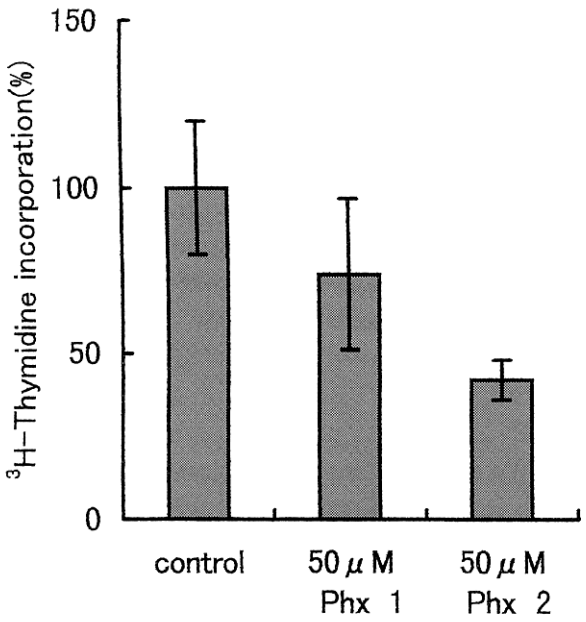

Fig. 5. Effect of Phx 1 or Phx 2 on incorporation of ^{3}H-thymidine into anti-IgM-stimulated human PBMC. Human PBMC were incubated in the wells coated with goat IgG or anti-human IgM for 3 days. Then, ^{3}H-thymidine was added. After 30 minutes, Phx 1 or Phx 2 (50 μM) was added to each well, and the cells were incubated for one more day. A a vehicle control, 0.1% ethanol was added in the place of Phx 1 and Phx 2.

Thirty min after the addition of ^{3}H-thymidine, 50 μM Phx 1 or Phx 2 was added to the cells. As shown in Fig. 5, the ^{3}H-thymidine incorporation was inhibited by as much as 58% in the presence of 50 μM Phx 2, but was not significantly affected in the presence of 50 μM Phx 1, compared with the control. From these results, it was suggested that proliferation of anti-IgM-activated B cells was accelerated by Phx 1, while it was accelerated by a lower concentration (15.8 μM) of Phx 2, and was inhibited by a higher concentration (50 μM) of Phx 2.

Discussion

We found that the incorporation of ^{3}H-thymidine into PHA-activated PMBC was inhibited by phenoxazines such as Phx 1 and Phx 2 (Fig. 2), and in a dose-dependent manner (Fig. 3). This result suggests that the process of DNA synthesis in PHA-activated human T lymphocytes was suppressed by Phx 1 and Phx 2, being consistent with the finding by Ishida et al. (1996) that the DNA synthesis was significantly suppressed in the human epidermoid carcinoma cell line by Phx 1. In the mechanism for the inhibition of ^{3}H-thymidine incorporation, the enzymes related with DNA synthesis may be involved. Recently, Yoshida and Marinela showed that purified mammalian DNA polymerase α was inhibited by 0% and 25%, respectively, at 12.5 μM Phx 1 and Phx 2, 20% and 70%, respectively, at 40 μM Phx 1 and Phx 2, and 4% and 80%, respectively, at 50 μM Phx 1 and phx 2 (Personal communications), though the action manner of these phenoxazines was different. From their results, it may be explained, in part, that the inhibition of the ^{3}H-thymidine into PHA-activated PBMC by Phx 1 and Phx 2 may be caused by the suppression of DNA polymerase α activity. However, since we observed that the extent of inhibition of ^{3}H-thymidine incorporation into PHA-activated PBMC by Phx 1 was far larger than that by Phx 2 (Fig. 2) at the dose of 40 μM, the unknown mechanisms may be concerned with inhibition of the incorporation.

It has been shown that emodin, a derivative of anthraquinone obtained from a Chinese herb, inhibits the incorporation of ^{3}H-thymidine into PHA-activated human mononuclear cells (Huang et al. 1992), suggesting that this compound inhibits the PHA-activated proliferation of T cells. The chemical structure of Phx 1 and Phx 2 are similar to that of emodin in some points, i.e., these compounds are tricyclic chromophores, and Phx 1 and Phx 2 have the methyl group at the 7th and 8th position of phenoxazine nucleus, respectively, while emodin has the methyl group at the 6th position of anthraquinone nucleus. The position of the methyl group of Phx 1 and emodin are the same (Fig. 1). Therefore, the inhibitory effects of Phx 1 and Phx 2 on PHA-activated human

mononuclear cells may be related with the chemical structure of these compounds.

According to Huang et al. (1992), the inhibition of ^{3}H-thymidine into human mononuclear cells by emodin was partly mediated by active oxygens such as superoxide and hydrogen peroxide which were generated by the reaction of oxygen and the hydroxyl groups of the anthraquinone nucleus in emodin. The hydroxyl group containing anticancer drug such as adriamycin is shown to produce active oxygens and exert cytotoxic effects (Bates and Winterbourn 1982). However, Phx 1 and Phx 2 do not include the hydroxyl groups in the phenoxazine nucleus and do not produce active oxygens. These facts are consistent with the results by Mori (2000) that Phx 1 causes little myelo-suppressive effects in mice. Judging from these facts, it may be plausible that the inhibitory effects of Phx 1 and Phx 2 on the incorporation of ^{3}H-thymidine into activated human T cells are caused by the mechanisms related with the tricyclic structure and the methyl group in the phenoxazine nucleus.

Palmer et al. (1988) suggested that the tricyclic chromophore exerts anticancer effects after intercalating with DNA. Therefore, the question whether Phx 1 and Phx 2 may exhibit their inhibitory effects through the binding to DNA may arise. However, we observed that Phx 1 does not intercalate with DNA (Ishida et al. 1996).

We also found that the ^{3}H-thymidine incorporation into the anti-IgM-activated B cells was affected by Phx 1 and Phx 2, though the action manner of these compounds was diverse at different concentrations (Table 1, and Fig. 5). We expected that the ^{3}H-thymidine incorporation into the anti-IgM-activated B cells would be inhibited by Phx 1 and Phx 2, as was observed for the PHA-activated T cells (Fig. 3). However, at a lower concentration (15.8 μM) of Phx 1 and Phx 2, and at a higher concentration (50 μM) of Phx 1, it was accelerated (Table 1). The mechanism for this acceleration is obscure. It was demonstrated that ^{3}H-thymidine incorporation into the anti-IgM activated B cells was rather suppressed at a higher concentration (50 μM) of Phx 2 (Table 1). Plausible explanation for this inhibition may be that the reduced activity of DNA polymerase α caused by Phx 2 as communicated by Yoshida and Marinela, might be directly reflected to the suppression of ^{3}H-thymidine incorporation into the cells. Present results suggest that Phx 1 and Phx 2 show stimulatory and inhibitory effects on the proliferation of the anti-IgM-activated B cells, at different concentration, but the detailed mechanism should be clarified by further investigation.

The vasorelaxing and immunosuppresive activity of emodin, whose chemical structure is similar to Phx 1 and Phx 2 (Fig. 1), was demonstrated by Huang et al. (1991). They suggested that emodin may be more clinically useful than cyclosporin A that exerts immunosuppressive effects but causes hypertension (Xue et al. 1987). Present results suggest that Phx 1 and Phx 2 may exert immunosuppressive effect, though the extent of the effect may be smaller than that of emodin, judging from the fact that the IC_{50} of Phx 1 (15 μM) and Phx 2 (31.6 μM) for the PHA-stimulated T cells (Fig. 3) is smaller than that of emodin (7 μM) (Huang et al. 1991). We also found that Phx 1 and Phx 2 have vasorelaxing activity (unpublished data). Phx 1 and Phx 2, therefore, may be clinically useful as immunosuppressive agents with vasorelaxant effects.

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