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Effects of Benzo[a]pyrene, 2-Bromopropane, Phenol and 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin on Proinflammatory Cytokines Gene Expression by Mice Spleen Cells

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Abstract

The detrimental effects of environmental pollutants on the health of the individual are generally accepted, although the mechanisms of these effects remain to be incompletely understood. In the present study, we examined the effects of B[a]P, 2-BP, phenol and TCDD on proinflammatory cytokine gene expression in mice spleen cells which were stimulated with anti-CD3. 10^{-9}M TCDD increased IFNγ and TNFα gene expression, but suppressed IL-1 gene expression. 10^{-6}M phenol inhibited IL-1, IL-6 and TNFα gene expression, and 10^{-6}M of 2-BP downregulated TNFα gene expression. However, 10^{-6}M of B[a]P did not influence on IL-1, IL-6, IFNγ and TNFα gene expression. These findings suggest that TCDD may impair the immune functions of mice by enhancing proinflammatory cytokines production, whereas phenol and 2-BP may impair the functions by inhibiting the production of these cytokines.

Key words : cytokine, TCDD, benzo[a]pyrene, 2-bromopropane, phenol

Introduction

Recently, the issue of environmental pollution has aroused increasing concern in many countries because of its implications on human health. The immune system responds to many foreign antigens, therefore it is presumed that environmental contaminants may affect immune function. A numerous studies showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo[a]pyrene (B[a]P), and 3-methycholanthrene caused impaired immune function [1-8].

The immune response is regulated by cytokines, which stimulate the diverse cellular responses involved in immunity and inflammation. Most cytokines are multifunctional in nature and more than one cytokine may act on the same target cells and mediates the same or similar function. Examples of cytokines, which shown a wide variety of biological functions, are interleukin-1 (IL-1), IL-6, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα). Because these multifunctional cytokines are characterized as primary mediators of acute inflammatory responses, they are also known as proinflammatory cytokines [9-12]. In this study, we investigated the effects of pollutants on proinflammatory cytokines gene expression in mice splenocytes.

Materials and Methods

Animals

C3H male mice (10-14 weeks of age) were used in the study. Room temperature was maintained at 22±1℃ and a relative humidity between 40 and 60%. Standard laboratory rodent chow and tap water were available *ad libitum*. For any given experiment, pooled spleens from 5-10 mice were used.

Culture conditions

Single cell suspensions from spleens were washed in RPMI 1640 (Gibco BRL, Grand Island, NY), and resuspended at 5 × 106 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT), 200mM L-glutamine (Sigma, St. Louis, MO), 50mM 2-ME (Sigma), and 1mg gentamicin (Gibco BRL)/100ml medium. Cells were either left unstimulated, stimulated with anti-CD3 plus DMSO (Sigma), or treated with anti-CD3 plus various concentrations of benzo[a]pyrene (B[a]P, 10^{-10} to 10^{-3}M, Sigma), 2-bromopropane

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(2-BP, 10-10 to 10-3M, Tokyo Kasei, Toyko), phenol (10-10 to 10-3M, Sigma), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 10-13 to 10-6M, Supelco, Bellefonte, PA), and then incubated at a 37℃ in a humidified incubator in an atmosphere of 5% CO2 for 24 h. The various chemicals employed in these investigations were solved in DMSO and the final vehicle concentration in culture was 10㎕/ml.

Quantification of IL-2

Supernatants from cultured cells were collected, clarified by centrifugation, and stored at -70℃ until the assay for IL-2. The protocol used to quantify immunoreactive murine IL-2 was that described by Schumacher et al. [13]. Anti-IL-2 monoclonal antibodies and murine recombinant IL-2 were obtained from PharMingen (San Diego, CA). Mean IL-2 concentrations for triplicate assays were calculated.

Proliferation assay

Cultured cells were either left unstimulated, or treated with anti-CD3 plus DMSO, 10-6 M B[a]P, 10-6 M 2-BP, 10-6 M phenol or 10-9 M TCDD, in 96 well tissue culture plate and then incubated at 37℃ in a humidified incubator in an atmosphere of 5% CO2 Following incubation for 8 h, cell proliferation was determined using the MTT assay [14]. Comparisons between control and each experimental groups were made using the Student's t-test; $p<0.05$ was considered significant. Data are presented as means ±SD.

DNA agarose gel electrophoresis

Splenocytes were either left unstimulated, or treated with anti-CD3 plus DMSO, 10-6 M B[a]P, 10-6 M 2-BP, 10-6 M phenol or 10-9 M TCDD for 8 h. They were harvested and incubated with lysis buffer (50mM Tris-HCl, 20mM EDTA, 1% NP-40, pH 7.5) on ice for 30 min, and supernatants were obtained by centrifugation. Supernatants were incubated with 1% SDS and RNase A (Sigma) for 2 hours at 50℃ and then further incubated with proteinase K (Sigma) for 2 hours at 37℃. Following the incubation, supernatants were precipitated with ice-cold ethanol (2 times v/v) and ammonium acetate (0.2 times v/v) at -70℃. After centrifugation, the DNA pellet was washed twice with 70% ethanol, briefly dried for 5-10 min and then dissolved in TE (10mM Tris, 1mM EDTA, pH 8.0) buffer. DNA was electrophoretically separated in a 1.5% agarose gel containing 1㎍/ml ethidium bromide.

Extraction of cellular RNA and comparative RT-PCR

Following incubation for 18 h, total cellular RNA was extracted using TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. cDNA was synthesized from total cellular RNAs by reverse transcription using TaKaRa RNA PCR kit (TaKaRa Shuxo, Biomedical Group, Otsu, Shiga, Japan). PCR amplifications were performed with cDNA derived from RNA by using a DNA thermal cycler (Perkin Elmer). A 100㎕ of final reaction volume contained 20㎕ from the reverse transcription reaction, 63.5 ㎕ of H2O, 0.5㎕ of 5U/㎕ DNA Tag polymerase, 6㎕ of 25mM MgCl2, 8l of 10×PCR buffer, 1㎕ of each primer (100pmoles). PCR conditions were: 30 cycles of 30 s at 94℃, 30 s at 60℃ and 90 s at 72℃ for β-actin, IL-1β and TNF α, 25cycles of 30 s at 94℃, 30 s at 56℃ and 30 s at 72℃ for IL-6 and IFN γ. The sequences of primers used are shown in Table 1. PCR products were analyzed by agarose gel electrophoresis and visualized using ethidium bromide under UV light. A 100 bp DNA ladder (Promega Co., Madison, WI) was used as a size maker during electrophoresis. The relative density of each band was determined by scanning with a laser Computing Densitometer and ImageQuant (version 3.3) (Molecular Dynamics, Sunnyvale, CA).

Results

Dose response effects of pollutants on IL-2 production

IL-2, the major growth factor of T cells, is mainly produced by CD4+T cells, and the quantity of IL-2 synthesized by activated T cells is an important determinant of the magnitude of immune responses [9]. Therefore, to determine the reference dosage for further experimentation we first

Table 1. Primers for PCR amplification of cytokines

Primer	Sequence	Size(bp)
β-actin	5′-ATGGTGGGAATGGGTCAGAAG 3′-GGAAGATGTTACTCGACGAGC	169
IL-1β	5′-TGACCCATGTGAGCTGAAAG 3′-GACTTGGCAGAGGACAAAGG	499
IL-6	5′-CCACCCACAACAGACCAGTA 3′-GAGCATTGGAAGTTGGGGTA	498
TNF α	5′-GCTCCCTCTCATCAGTTCCA 3′-CGGAGAGGAGGCTGACTTTC	501
IFN γ	5′-GCGGCTGACTGACTGAACTCAGATTGTAG 3′-GGGATATGTCGACTTTTGACACTG	306

investigated the effect of pollutants on a dose-r esponse plot of IL-2 production by splenocytes. As shown in Figure 1, the addition of 10-6M B[a]P, 2-BP, phenol or 10-9M TCDD to cultures resulted in maximum suppression compared to that of the vehicle-added control. Thus, these concentrations were adopted as reference values.

Effect on cell proliferation and DNA fragmentation

We investigated whether the reference dosage of each pollutant affected the proliferation of anti-CD3-stimulated T cells. Although the stimulating effect of pollutants on cell proliferation was evident compared to the baseline level of anti-CD3-unstimulated group, no significant differences were apparent between the pollutant-treated and the vehicle-treated groups (Fig. 2).

Next, we addressed the question as to whether the administration of each pollutant at the reference dosage caused variations in apoptotic response. Exposure to B[a]P, 2-BP, phenol and TCDD resulted in a characteristic DNA ladder formation within 8 h, whereas DNA fragmentation was absent in unstimulated cells and substantially less in anti-CD3 stimulated cells. As shown in Figure 3, the DNA cleavage variations of the different pollutants were not clear.

Effect of pollutants on IL-1, IL-6, IFN and TNF gene expression

The expression of proinflammatory cytokines genes were studied by comparative RT-PCR on RNA derived from cultured splenocytes (Fig. 4 and 5). In mice spleen cells, B[a]P plus anti-CD3 caused no significant change in cytokines gene expression. In contrast to B[a]P, 2-BP plus anti-CD3 decreased TNF α gene expression (21.14%), and phenol plus anti-CD3 diminished IL-1, IL-6 and TNF α gene expression versus vehicle-treated cells (77.92%, 45.75%, 38.2%, respectively). TCDD plus anti-CD3 showed a tendency to enhance IFN γ and TNF α gene expression (120.80%, 131.58%, respectively), but to inhibit the IL-1 gene expression (79.34%).

Discussion

In this study, to investigate the in vitro effects of pollutants on cytokines production, we cultured splenocytes with pollutants in the presence of anti-CD3. The present investigation shows that exposure of murine splenocytes to 10-9M of TCDD induces IFN γ and TNF α gene expression, and suppresses IL-1 expression. 10-6M phenol suppresses IL-1, IL-6 and TNF α gene expression, and 2-BP downregulates TNF α gene expression. However, B[a]P did not alter IL-1, IL-6, IFN γ and TNF α gene expression in these cultures. Based on the fact that cell proliferation and DNA fragmentation assays did not showed significant differences between the pollutant- and the vehicle-treated groups, we thought that differences in the patterns of cytokines gene expression in each exposed group were due mainly to the different amounts of induced genes in the cultured cells.

Previous investigations have shown that B[a]P only slightly diminished the viability of con A-stimulated human peripheral blood T cells by 10-7 to 10-6M [15], reduced IL-1 production by mouse macrophages [4, 15], and reduced IL-2 production by mouse T cells [5, 15], but increased IL-6 production by mouse lymph node cells at 10-5M [2]. However, we observed that B[a]P did not alter proinflammatory cytokine gene expression by spleen cells at a concentration of 10-6M; however, the cell proliferation assay showed no significant difference between the B[a]P- and the vehicle-treated group. We believe that this discrepancy may due to the different cells used and T cell stimulants, such as, anti- CD3.

TCDD had little or no effect on the viability and cell growth of murine macrophages exposed in vitro at 10-9M, and induced TNF α production by peritoneal macrophages when stimulated with LPS [6]. Several reports on altered IL-1 production by exposure to TCDD have shown different results, which were attributed to the model systems used for the experiments, these varied from the enhancement of induction to no change [1, 7, 16-18]. In terms of the effects on IL-6 and TNF α production, exposure to TCDD resulted in the similar patterns as observed for IL-1 production [3, 6, 16, 18, 19-21]. These previous reports demonstrated that the effects of TCDD on cytokines production appear to be very complex and sensitive to experimental differences and the methods of stimulation used by the different groups. The present study has extended some of these previous findings, as it shows that TCDD plus anti-CD3 upregulates IFN γ and TNF α gene expression, and downregulates IL-1 gene expression. However, further studies are necessary to classify the causes of the many different results obtained. TCDD exposure enhances the inflammatory and systemic manifestations of TNF α, but has little effect on TNF's tumoricidal properties, because it decreases the stability of the membrane-bound portion of the TNF molecule [6]. In addition, our results show that IFN gene expression is induced by treating spleen cells with TCDD plus anti-CD3, although previous experiments implied that TCDD decreased its expression [3, 18, 21]. It is generally assumed that anti-CD3 can stimulate the production of IL-2 by T cells and in turn promote IFN γ production [9]. The production of IFN γ is generally considered to be strictly controlled, and significant amounts of this cytokine are only found after specific stimulation or in the course of certain pathologic conditions [for review see 23]. Since studies using anti-CD3 indicated that TCDD activated T cells [19, 21, 24], we believe that TCDD plus anti-CD3 can induce IFN γ gene expression.

Although several studies showed that phenol and 2-BP have a toxic potential on the immune system because the number of white blood cells was significantly decreased when rats or workers were exposed [25-27], little is known about the effects of phenol and 2-BP on cytokines production

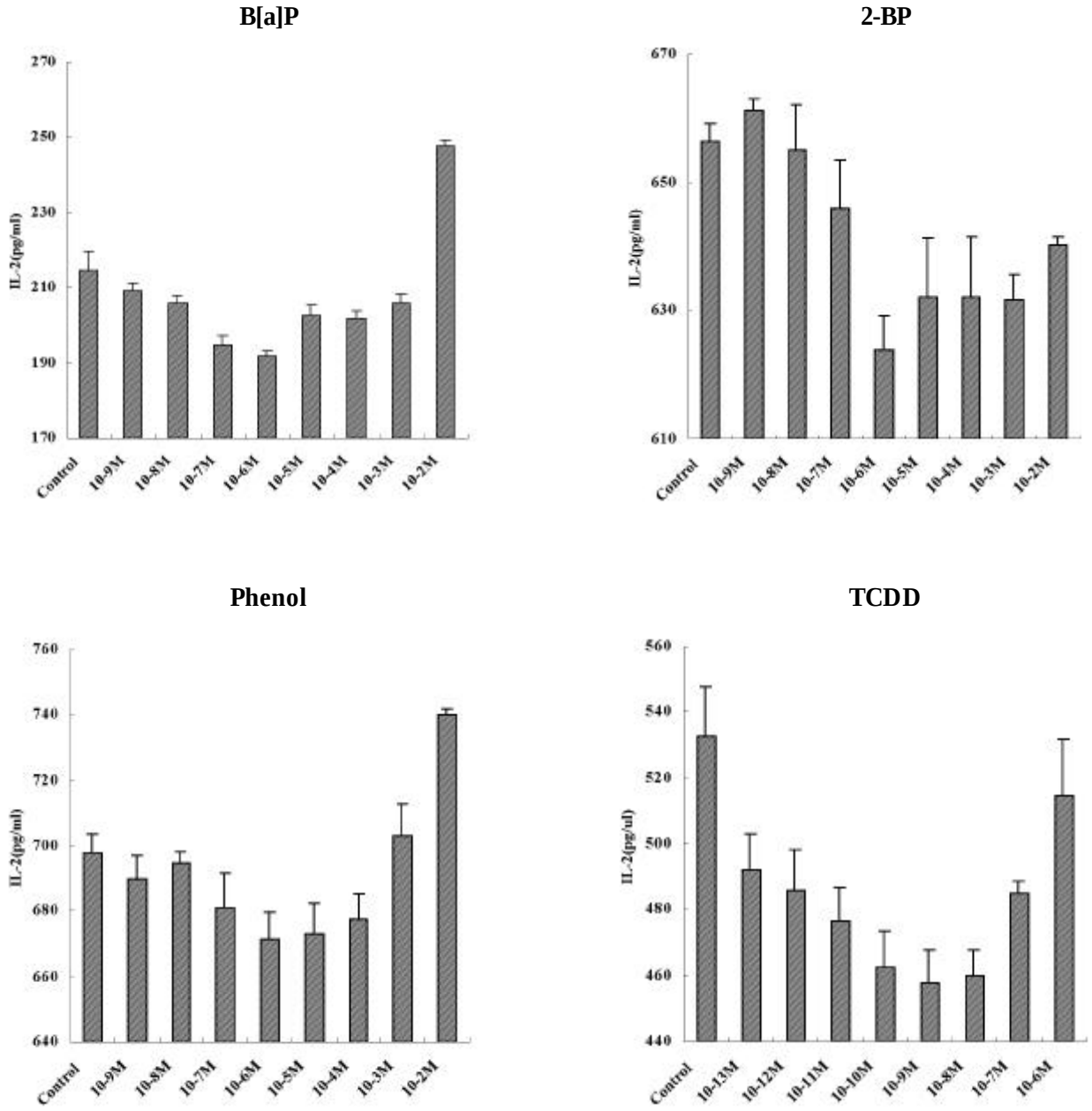


Fig. 1. Effect of different concentrations of pollutants on IL-2 production by spleen cells. Cells were incubated with anti-CD3 plus vehicle, DMSO, or treated with anti-CD3 plus B[a]P, 2-BP, phenol or TCDD at the concentrations shown. The columns represent the means of triplicate cultures, and the bar on the columns represent the means±SD of triplicate cultures.

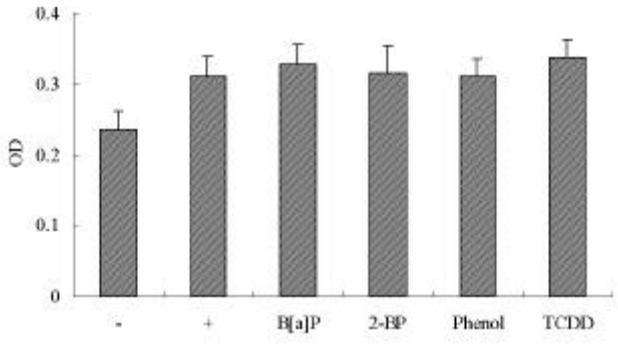


Fig. 2. Effect of pollutants on cell proliferation. Cells were treated for 8h with anti-CD3 plus DMSO (+), 10^{-6}M B[a]P plus anti-CD3, 10^{-6}M 2-BP plus anti-CD3, 10^{-6}M phenol plus anti-CD3, 10^{-9}M TCDD plus anti-CD3, or were left unstimulated (−). Values shown are means±SD of three individual experiments.

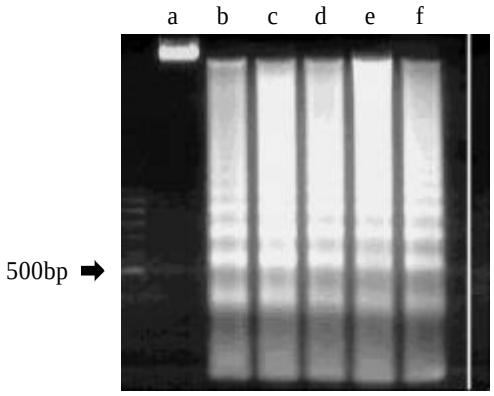
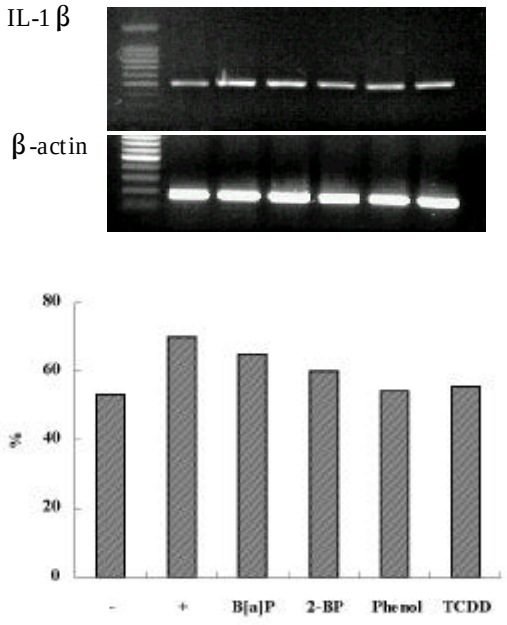
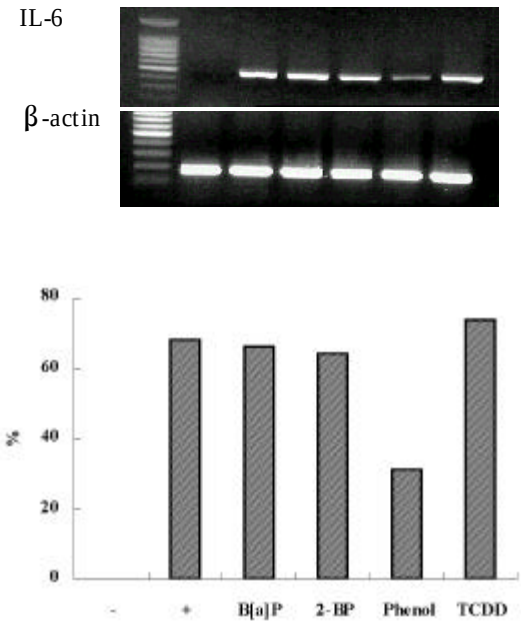


Fig. 3. Effect of pollutants on apoptosis. Mouse spleen cells were exposed for 8 h to anti-CD3 plus DMSO (b), 10^{-6}M B[a]P plus anti-CD3 (c), 10^{-6}M 2-BP plus anti-CD3 (d), 10^{-6}M phenol plus anti-CD3 (e), 10^{-9}M TCDD plus anti-CD3 (f), or were left unstimulated (a). DNA fragments were isolated and visualized by UV transillumination after 1.2% agarose gel electrophoresis. The gel shown is representative of three similar experiments.

A)



B)



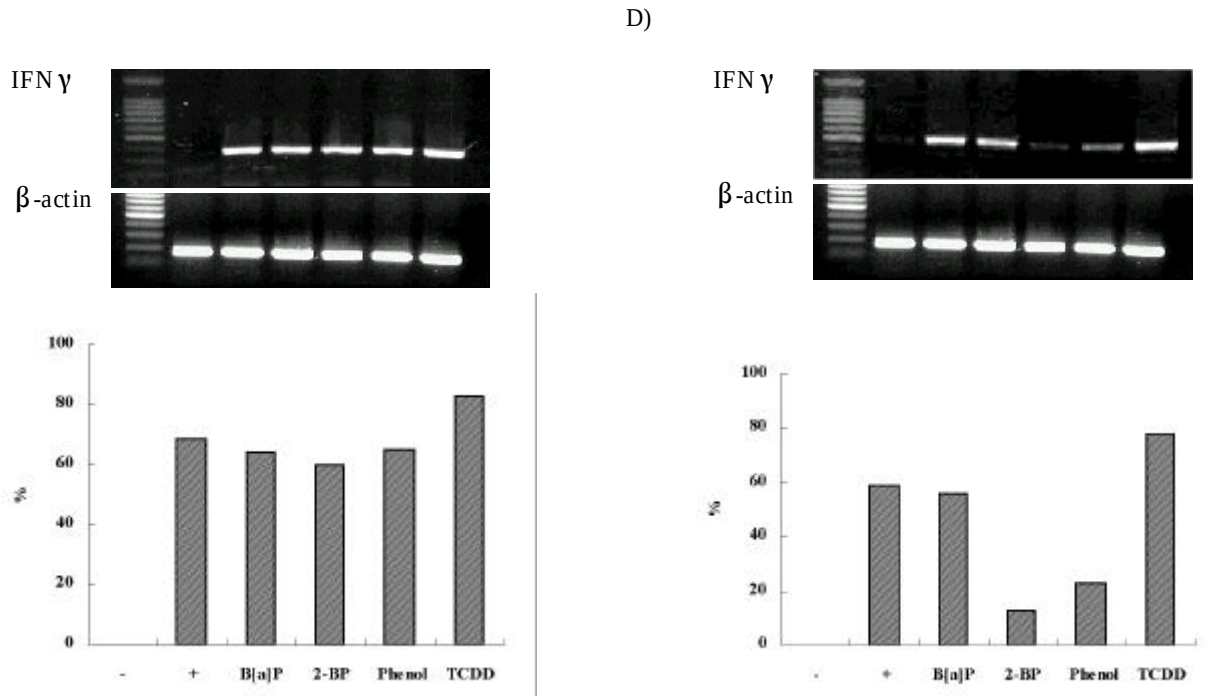


Fig. 4. Cytokines gene expressions in response to pollutants. Cells were left unstimulated (−), stimulated with anti-CD3 plus DMSO (+), exposed to anti-CD3 plus 10^{-6}M B[a]P, 10^{-6}M 2-BP, 10^{-6}6M phenol, or 10^{-9}M TCDD for 18 h. The amounts of IL-1β (A), IL-6 (B), IFNγ (C) and TNFα (D) gene expression were determined by RT-PCR (upper part) and densitometry (lower part). Values are expressed as percentages of the corresponding value of β-actin. Results are representative of three separate experiments.

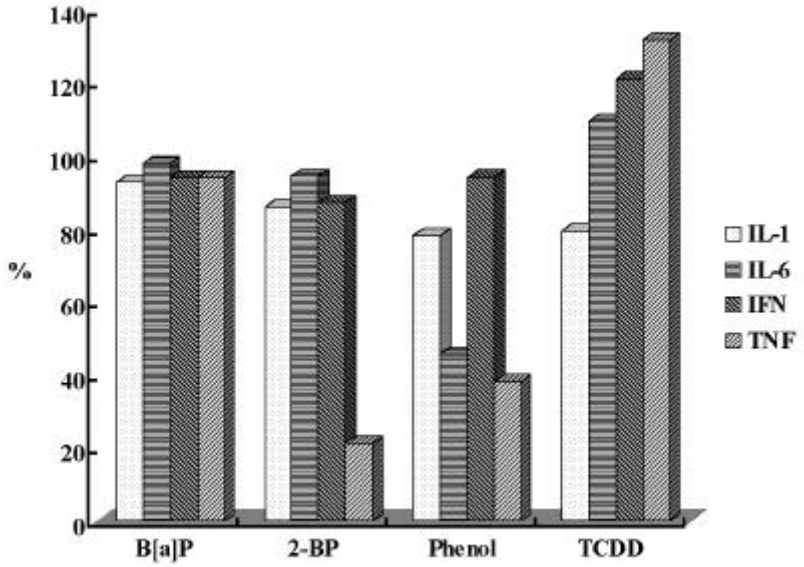


Fig. 5. Effect of pollutants on cytokines gene expression. Based on the Figure 4, the relative inductions of cytokines genes are expressed versus an anti-CD3 stimulated control. Results are representative of three separate experiments.

by mouse spleen cells. Here, we demonstrate that phenol downregulates IL-1, IL-6 and TNF α gene expression, and that 2-BP suppresses TNF α gene expression. We have no explanation for these results, although the results of three separate experiments employing similar experimental conditions showed similar patterns as in Figures 4 and 5. Therefore, further studies are needed to identify the precise mechanism responsible for the suppression of these cytokines genes.

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