Chemico-Biological Interactions 206 (2013) 166–174

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

The immunotoxic effects of dual exposure to PCP and TCDD

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ARTICLE INFO

Article history:
Received 7 May 2013
Received in revised form 22 August 2013
Accepted 9 September 2013
Available online 17 September 2013

Keywords:
Pentachlorophenol (PCP)
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)
Immunotoxicity
Splenocyte
Ovalbumin (OVA)

ABSTRACT

Pentachlorophenol (PCP) was a commonly used fungicide, herbicide, insecticide, and bactericide in industrial, agricultural, and domestic settings; however, it was also contaminated with polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). It has been reported that technical grade PCP had immunosuppressive effects and that the immune system was the major target of PCDD/PCDFs toxicity. Although the immune response after exposure to PCP or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been studied, the toxic effects of exposure to both PCP and TCDD have not yet been reported. The aim of this study was to evaluate the effects on immune cells from mice intraperitoneally immunized with OVA and subsequently treated with PCP or TCDD alone or in combination by gavage. The animals were terminated on day 7 and 14, and the spleen and plasma samples were collected for immunotoxicity evaluation. The numbers and populations of splenocytes, T cell-derived cytokines produced by splenocytes, splenocyte-generated cytotoxicity and OVA-specific antibodies in plasma were investigated. Our results indicate that the spleen/body weight ratio and splenocyte number was reduced by TCDD alone; in addition, this reduction was enhanced when TCDD was combined with PCP. Exposure to TCDD alone or in conjunction with PCP suppressed many ovalbumin (OVA)-stimulated cytokines, including IL-2, IFN-γ, IL-4, IL-5, and IL-10. Furthermore, the immunoglobulins IgG and IgM were suppressed in mice administered by PCP alone, but the suppressive effects were greater in mice treated with TCDD alone or in combination with PCP. Co-exposure to PCP and TCDD resulted in an antagonistic effect on TCDD-induced suppression of IFN-γ and IL-10. Our results demonstrate that PCP alone is immunotoxic, regardless of the presence of TCDD. PCP led to mild changes in cytokine secretion, and it compromised splenocyte-generated cytotoxicity and IgM and IgG antibody production on day 7. The finding that PCP antagonizes TCDD-induced IFN-γ suppression could be due to the competitive binding of PCP to AhR (aryl hydrocarbon receptor).

1. Introduction

Pentachlorophenol (PCP) was one of the most widely used pesticides employed as a fungicide, herbicide, insecticide, and bactericide in industrial, agricultural, and domestic settings [1]. The alkaline hydrolysis of chlorobenzenes and the chlorination of phenol in the presence of catalyst are the most commonly used methods for the preparation of PCP. During the manufacture of PCP, the chemical can become contaminated with impurities. These impurities are other chlorophenols, such as tetrachlorophenol (TCP), polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) [2–4]. PCDDs also called 'dioxins', consist of 75 individual isomers that can be found in contaminated sediment, water, soil, and these isomers can accumulate in the adipose tissue of exposed workers due to their structural stability [5]. The most toxic dioxin isomer is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [6,7]. It was indicated that, PCP is not prone to degradation because of its stable aromatic ring system and high chlorine content [8]. PCP contamination has been found in surface and ground-water and in soil surrounding timber treatment sites [9] and deserted manufacturing factories [10]. TCDD has also a very long half-life in the environment. It has been indicated that most of the original sources of

Abbreviations: PCP, pentachlorophenol; TCP, tetrachlorophenol; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; OVA, ovalbumin; LPS, lipopolysaccharide; Th, T helper; Ig, immunoglobulin; AhR, aryl hydrocarbon receptor.

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http://dx.doi.org/10.1016/j.cbi.2013.09.005

dioxin are from industrial activities and human are generally exposed to it due to the inadequate elimination into food, drinking water, soil, dust, smoke and air. In addition, the route of dioxin exposure in general population is exclusively through the consumption of animal foods including meat, fish, and dairy products [6,11]. In Taiwan, the production and application of PCP was prohibited in 1989; however, there are thousands of kilograms of discarded PCP in a deserted PCP factory located in the An-Shun area of Tainan City in southern Taiwan. Due to rainfall, these contaminants were released into the ambient environment surrounding the factory causing inhabitants to be exposed to PCP and TCDD [5,12–15]. Consequently, the potential adverse health effects from exposure to PCP and TCDD remain a great concern.

Previously studies reported that PCP is rapidly absorbed from the gastrointestinal, dermal, and respiratory tracks [16]. PCP can induce toxicity in the liver, kidney, hematopoietic system, bone marrow, and neurological system, and it can increase cancer risk [17]. PCP is indicated as an immunosuppressive agent. This immune dysfunction could explain the chronic infection, fatigue, and hormonal dysregulation in PCP-exposed patients [18]. Such immune alterations in cell-mediated and humoral immunity include functional and numerical shifts of diverse immune cell populations. The functional changes include alterations in natural killer cytotoxicity [19,20], the number of circulating NK cells, T-cell (ovalbumin (OVA) induced) and B-cell (lipopolysaccharide (LPS) induced) activation response, mixed lymphocyte response (proliferation and cytotoxicity), antibody production, and the percentages of specific T cells [21]. Mostly released by activated immune cells, cytokines serve as communication signals critical for cell-to-cell interactions, and their production patterns can change in response to toxicants. T cells, the principle regulators of the immune response, are commonly classified into T helper (Th), T cytotoxic and regulatory T cells [21]. Activated Th cells are the major source of cytokines involved in the regulation of immune response. The cytokines produced by Th1 cells are IFN-γ and IL-2, and these are known to promote cell-mediated immunity and enhance NK cell activity. Th2 cytokines, including IL-4, IL-5, IL-6, IL-9, and IL-10, primarily facilitate humoral immunity [22]. The activated T cells directly interact with B cells and lead to induction of several B cell functions. IL-4, IL-5, and IL-6 play an important role in survival, differentiation and isotype switching of B cells; therefore, inhibition of IL-5 production could be instrumental for the blocking of immunoglobulin (Ig) production [23]. It has been reported that robust humoral immunity requires large amounts of Ig secretion. Thus, dysregulation of cytokine production and impaired Ig secretion has been linked to a number of pathological conditions. TCDD is also a potent immunosuppressive agent in laboratory animals. It has been reported that TCDD can reduce both humoral and cell-mediated immune reposes [24–26]. However, the immunotoxic effects of exposure to both PCP and TCDD in vivo have not yet been reported. Therefore, the aim of this study was to assess the combined immunotoxic effects of PCP and TCDD. Female BALB/c mice were applied in our animal study. It has been reported that female murine was more sensitive than male for most endpoints on the chronic effects of TCDD [27]. In addition, previous studies have also reported that mice possess a sensitive AhR genotype, such as BALB/c or C57BL/6 was more sensitive than the other strains to the immunosuppressive effects by TCDD [7,28,29].

2. Materials and methods

2.1. Animals

All experiments on mice were performed according to the guidelines of our institute (Guide for Care and Use of Laboratory Animals, National Cheng Kung University Medical College). Female BALB/c mice (5–8 weeks of age) were acquired from the animal center of the National Cheng Kung University Medical College. The animals were housed five per cage at 24 ± 2 °C and 50 ± 10% relative humidity under a 12-h light/12-h dark cycle. They were acclimatized to their housing environment for 1 week prior to the start of experiments and fed with a Purina chow diet and water ad libitum.

2.2. Mouse exposure

TCDD (purity, 98%) (Sigma–Aldrich, St. Louis, MO) was prepared at a concentration of 10 μg/ml in toluene. The TCDD/toluene solution was further diluted with olive oil to 2 μg/ml. The total injected volume of toluene was approximately 18–36 μl per mouse. Analytical grade PCP (Sigma–Aldrich, St. Louis, MO) with a purity >99% and with no detectable dioxin impurities was dissolved in olive oil at a concentration of 600 μg/ml. Ovalbumin (OVA) (Grade VII; Sigma–Aldrich, St. Louis, MO) was dissolved in phosphate–buffered saline (PBS) at a concentration of 1 mg/ml. An equal volume of 9% (w/v) $AlK(SO_4)_2$ solution was added to the OVA solution and the pH was adjusted to 6.5 with KOH. After repeatedly washing with PBS, the alum-precipitated OVA (OVA/alum) was resuspended in PBS at 0.5 mg OVA/ml. The mice were randomly divided into different groups (4 or 5 mice per group). Mice were immunized with an intraperitoneal injection of 100 μg of OVA/alum on day 0 and were subsequently administered as following groups by gavage: (1) vehicle (OVA control; olive oil containing 36 μl toluene), (2) a single dose of TCDD (20 μg/kg), (3) PCP (6 mg/kg, tree times per week), (4) a single dose of TCDD (10 μg/kg) combined with PCP (3 mg/kg, tree times per week), (5) a single dose of TCDD (20 μg/kg) combined with PCP (6 mg/kg, tree times per week). The mice without OVA treatment and administrated with only normal diet and water were taken as normal controls. The animals were sacrificed on days 7 or 14, respectively, and the plasma and spleen samples were collected for immunotoxicity evaluation. The experimental timeline was shown in Fig. 1. Our procedure followed the method described by Inouye et al. [30].

2.3. Isolation of splenocytes

Animals were euthanized via CO_2 asphyxiation followed by cervical dislocation, and their spleens were removed aseptically. The spleens were crushed with the end of a syringe plunger in RPMI 1640 medium supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (complete medium) through a stainless-steel mesh. The spleen cells were treated with 5 ml ACK lysis buffer for 5 min at room temperature to eliminate red blood cells and then washed with PBS. Cell numbers were counted with a hemocytometer following staining with trypan blue.

2.4. Splenocyte subpopulations

At days 7 or 14, splenocytes were prepared from the mice. Splenocytes (2×10^5) were stained with fluorescein isothiocyanate, phycoerythrin (PE)-labeled anti-mouse CD3e mAb, (FITC)-labeled anti-mouse-CD4 mAb and PE-CD8 mAb (BD Biosciences, San Jose, CA). The percentages of the splenocyte subpopulations were analyzed by FACScan (BD Bioscience, Franklin Lakes, NJ, USA).

2.5. Measurement of Th1 and Th2 cytokines in splenocytes with an enzyme-linked immunosorbent assay (ELISA)

At days 7 or 14, splenocytes were prepared from the mice. Splenocytes (1×10^6) were incubated in a 96-well plate and then treated with 100 μg/ml OVA for 48 h. The supernatants from the