PERINATAL EXPOSURE OF MICE TO TCDD DECREASES ALLERGIC SENSITISATION THROUGH INHIBITION OF IL-4 PRODUCTION RATHER THAN T REGULATORY CELL-MEDIATED SUPPRESSION

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Abstract
Objective: The 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD) is a widespread, man-made, persistent organic pollutant with high immunotoxic potentials. It suppresses cell-mediated and humoral immune responses through mechanisms dependent on aryl-hydrocarbon receptor expression and immunosuppressive activity of the cells. Most sensitive to TCDD are organisms during fetal and infant life, mostly due to the developmental stage of many biological systems of the host, including immune system. Recent data show that T regulatory cells that have the potential to suppress immune reactions and which develop after TCDD exposure are also responsible for protection from allergy development. Our goal was to investigate if perinatal exposure to TCDD can affect allergic sensitisation and if T reg cells participate in this phenomenon.

Materials and Methods: Mice, Balb/c, were perinatally exposed to TCDD or to the carrier. Six weeks old control or exposed mice were sensitised with ovalbunin. Spleen cells of the animals were used to assess the content of T reg cells by means of flow cytometry. Levels of cytokines were assessed by ELISA technique in supernatants of the cells stimulated with anti-CD3 antibody. As a measure of sensitisation, total IgE and anti-OVA IgE were measured in serum of mice by ELISA method. To assess the function of T reg cells isolated from OVA-sensitised control or TCDD exposed animals we performed transfer studies.

Results: Here we show that perinatal exposure to TCDD decreases allergic sensitisation and that this process is related to inhibition of IL-4 synthesis rather than suppression mediated by T regulatory cells.

Conclusion: We hypothesise that dioxin exposure can be an important environmental modulator of immunological responses that participate in allergic reactions.

Key words: Dioxin, T regulatory cells, Allergic sensitization, Animal model, Perinatal exposure

INTRODUCTION
The 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD) is a widespread, man-made, persistent organic pollutant. This compound is very stable in environment and can accumulate in the tissues of organisms. Together with its already known, deleterious effects on diverse biological systems, including immune system, exposure to TCDD offers possible risk to human health [1–6].

In adult animals, exposure to TCDD has been shown to suppress cell-mediated and humoral immune responses (for review, see [2,3]). However, perinatally exposed offspring is most susceptible to the toxic effects of TCDD. Perinatally exposed animals showed, at the organ level, atrophy of the thymus [7], enhanced apoptosis of thymocytes [8], and alterations in T-lymphopoiesis [9]. The outcome of immunotoxic effects of TCDD on developing immune system in animals was shown to result in exacerbation of
postnatal autoimmune reactions [10,11] suppressed cell-mediated and humoral immune responses [7,12–14]. In studies on Dutch infants [15] prenatal PCB/dioxin exposure was associated with changes in several immunological parameters. At the preschool age of these children [16], an association was found between PCB/dioxin exposure and higher prevalence of infections of middle ear. This association continued to be significant at their school age [17]. Of other clinical effects observed in these children at preschool age, but no more at school age, was decreased susceptibility to allergic sensitisation that authors suggest could be due to skewed immune response toward infectious agents rather than direct effect of dioxin exposure. However, experimental data from studies on adult animals show that humoral immune responses, including antigen-specific antibody production [18], synthesis of proallergic Th2 derived cytokines such as IL-5, IL-4 [19,20] are suppressed due to direct effect of TCDD exposure. Additionally, experimental studies performed in rats showed that exposure to TCDD can directly decrease allergic immune response to house dust mite [21].

TCDD has been shown to induce immunosuppression in mice through mechanisms dependent on the expression of aryl-hydrocarbon receptor (AhR) [22,23] and development of immunosuppressive T cells similar to natural T regulatory cells [24]. Analyses of the characteristics of these cells [25] show that, like natural T reg cells, they suppress responder cells in cell-contact dependent way, do not produce IL-2, suppress early production of this cytokine from responder cells, and exhibit high level of granzyme b gene expression. However, these cells also produce significant amounts of IL-10, a cytokine that is ascribed to other T regulatory cell subpopulation known as Tr1. Natural, CD4CD25foxp3+ and inducible, IL10 (Tr1) T regulatory cells, as well as other subpopulations of suppressor cells, including those which rely on TGF-β (reviewed [26,27]) inhibit processes of allergic sensitisation and/or suppress allergic inflammatory reactions by inhibiting Th2 cell-derived cytokines.

Environmental factors are important elements that affect the development of allergy and asthma [28–30]. Dioxins and PCBs are environmental contaminants, usually present at the very low levels that do not cause apparent adverse health effects. But their accumulation over certain amount of time, especially in fat tissue, may increase the level of exposure during perinatal life, modulate development of immune system and cause changes manifested later in life. We tried to find out if perinatal exposure of mice to TCDD could affect allergic sensitisation and what immunological mechanisms might participate in these reactions.

MATERIALS AND METHODS

Animals

Inbred strain BALB/c mice (Harlan, Netherlands) were used in the experiments. The animals were bred in accordance with the standard procedures and were kept in a room with appropriate 12 h light/dark cycle. They received water and pelleted food (Murigran) ad libitum. Animal experiments were approved by the local Ethical Committee (doc. no. L/BD/285). Analyses of perinatal exposure use data collected from 6-week old male mice. Experiments were repeated twice and the total number of animals was 6. Spleens and blood were not pooled.

Reagents

The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; CAS 1746-01-6, AccuStandard, Inc. USA) was dissolved in acetone and mixed with olive oil. Acetone was removed by evaporation by heating the solution. Further dilutions were done in olive oil. For sensitisation of animals we used ovalbumin (OVA, CAS 9006-59-1, SIGMA). Ovalbumin was dissolved in 1:1 mixture of PBS (Dulbecco’s Phosphate Buffered Saline, SIGMA) and aluminum hydroxide (Alum, Al(OH)₃, 13 mg/ml, CAS 1330-44-5, SIGMA) was used at a final concentration of 20 μg OVA/1 mg Alum.

Perinatal exposure of mice to TCDD

Female and male mice were cohabited and the appearance of a vaginal plug was considered to be the beginning of pregnancy and gestational day (GD) 0. On GD15, mice were given intraperitoneally a single dose of 100 μl of TCDD at concentration of 10 μg/kg of body mass.
or 100 μl of carrier (Olive Oil). Offspring was nursed by their mothers and pups were weaned at 22nd day of age, transferred to one cage and bred in regular conditions. At 6 weeks of age, mice were sacrificed by intraperitoneal injection of overdose of sodium pentobarbital (100 mg/kg body mass) and spleens were removed for further processing.

**OVA sensitisation protocol**
Sensitisation of 6-week old mice perinatally exposed to TCDD or carrier, was performed by 3 intraperitoneal injections of 150 μl of 20 μg OVA/1 mg on day 0, 14 and 21. Nine days after the last dose, mice were sacrificed by intraperitoneal injection of overdose of sodium pentobarbital (100 mg/kg body mass) and blood was collected from orbital sinus and spleens removed.

**Preparation of spleen cells**
Spleens after removal were kept in Petri dishes in cold PBS until ready for cell isolation. Cells were removed by tearing spleens over the surface of 100 μm pore cell strainer (Becton-Dickinson) and washed with PBS. Erythrocytes were removed by haemolysis using RBC Lysis Buffer (BioLegend). Lysis was stopped after 5 min by adding 10 ml PBS. Cell viability was estimated by trypan blue exclusion method and the number of cells was estimated by use of Neubauer haemocytometer.

**In vitro stimulation of spleen cells**
Spleen cells were seeded into 48 well plate at density of 1.0×10^6 cells/well in 1 ml of culture media. Cells were left unstimulated or stimulated with 0.1 mg of anti-CD3 monoclonal antibody (anti-mouse CD3 purified, BD Pharmingen) for 3 days in incubator, at 37°C and in atmosphere containing 5% CO₂. After that time plates were centrifuged and the collected supernatant was stored at -70°C till the time of cytokine analysis.

**Assessment of cytokine concentration in supernatants**
Cytokine supernatant levels were assessed with the use of mouse Th1/Th2 Cytokine Cytometric Bead Array (Becton Dickinson) according to the protocol supplied by manufacturer. Analyses were performed on flowcytometer FACSCantoII (Becton Dickinson).

**Total and anti-OVA specific IgE serum levels assessment**
The concentrations of total IgE in serum were estimated by ELISA (Becton Dickinson) according to the protocol supplied by manufacturer. The sensitivity of the test was 2 ng/ml.

The concentrations of IgE specific to OVA in serum were also assessed by ELISA (MD Biosciences, Switzerland) according to the protocol supplied by manufacturer. The sensitivity of the test was 3.8 ng/ml.

**Cell transfer studies**
CD4+CD25+ cells were isolated from spleens of 6-week old mice perinatally exposed to TCDD or control animals. Isolation of these cells was performed first by negative selection of CD4+ T cells using antibody coated magnetic beads (Invitrogen). In the next steps, CD25+ T cells were isolated from CD4+ T cells using antibody coated beads (Miltenyi Biotec) according to the protocol supplied by manufacturer. The purity of isolated CD4+CD25+ T cells was 99% as assessed by flow cytometry. Next, 0.4×10^6 of these T cells, suspended in 0.2 ml of NaCl were transferred to untreated 6-week old Balb/c mice. Mice that were transferred with CD4+CD25+ T cells were subjected to the protocol of OVA sensitisation. First dose of OVA was given on the day after the cell transfer. Blood isolated on 30th day after the first dose of OVA was collected for serum isolation and the use in assessment of total and specific anti-OVA IgE.

**Flow cytometry analysis**
Half a million of spleen T cells per sample were surface stained with anti-CD3 (Alexa Fluor 488, clone 17A2, Biolegend), CD4 (PE/Cy5, Clone GK 1.5, Biolegend) and CD25 receptors (PE, clone PC 61, Biolegend). For assessment of T regulatory cells, 1×10^6 cells were surface stained with CD4 and CD25 + T cells were subjected to the protocol of OVA sensitisation. First dose of OVA was given on the day after the cell transfer. Blood isolated on 30th day after the first dose of OVA was collected for serum isolation and the use in assessment of total and specific anti-OVA IgE.
The effect of perinatal exposure to TCDD on proallergic inflammatory cytokines produced in vitro by spleen cells of OVA-sensitised or non-sensitised mice

Perm buffer (Biolegend) according to the protocol supplied by manufacturer. Control samples were stained with mouse IgG1 antibodies (Alexa Fluor 488, clone MOPC-21, Biolegend) to control for foxp3 specific staining, and Rat IgG1 antibodies (PE, clone RTK2071, Biolegend) to control for CD25 expression.

Statistical analysis
Statistical analyses were performed by T-test or nonparametric test of Mann-Whitney using GraphPad Prism software.

RESULTS
IgE levels in serum of OVA-sensitised, perinatally TCDD-exposed or non-exposed mice
Six week old mice from perinatal exposure to TCDD or control animals were subjected to the experimental, allergic sensitisation by OVA and serum levels of total IgE and IgE specific for OVA were evaluated. We found that total levels of IgE in serum of mice perinatally exposed to TCDD and OVA-sensitised were significantly higher than in animals not exposed to dioxin (Figure 1). However, the levels of serum IgE specific to OVA were higher in control animals, although the difference was close to the limit of statistical significance (p = 0.063).

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The effect of perinatal exposure to TCDD on proallergic inflammatory cytokines produced in vitro by spleen cells of OVA-sensitised or non-sensitised mice
Allergic sensitisation depends on the release of IL-4, cytokine that switches production of immunoglobulins to IgE class in B cells. Allergic inflammatory processes include participation of such cytokines as IL-5, which is major protein that affects eosinophil activity, and TNF-α that is mainly released from neutrophils and monocytes. We determined the levels of IL-4, IL-5 and TNF-α in supernatants of cells stimulated in vitro with anti-CD3 that were obtained from spleens of OVA sensitised animals perinatally exposed to TCDD or to the carrier. Concentrations of IL-4 in supernatants of anti-CD3 stimulated cells were significantly lower in TCDD exposed mice, whereas IL-5 and TNF-α were at similar levels (Figure 2).

The effect of perinatal exposure to TCDD on IFN-γ and IL-2 cytokines produced in vitro by spleen cells of mice sensitised to OVA
Allergic sensitisation is accompanied by preferential synthesis of cytokines such as IL-4 that belongs to cytokines produced by so called Th2 cells, and inhibition or no effect on cytokines such as IL-2 or IFN-γ that belong to cytokines released by Th1 cells. We determined the levels of IL-2 and IFN-γ in supernatants of cells stimulated in vitro with anti-CD3 that were obtained from spleens of
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production of these cytokines in the same conditions and the same animals in which production of other cytokines was tested. We could show that mice exposed perinatally to TCDD produced significantly (p < 0.05) lower levels of IL-10 than control animals and the TGF-β concentrations were not significantly different (Figure 4). Although the non-stimulated cells did not show detectable levels of IL-10 in any of the groups, TGF-β was present (TCDD group 180±107; control 181±29.4) but not at the amounts significantly different between the groups.

Analyses of CD4+CD25+foxp3+ T regulatory cells in spleen of 6-week old mice with or without perinatal exposure to TCDD

Perinatal exposure of mice to TCDD did not cause significant changes in the percentage of T regulatory cells among spleen cells as determined by expression of CD4CD25foxp3 among all T cells or within CD4+ T cells of spleen (Figure 5).

The effect of transfer of CD4+CD25+ T cells obtained from spleens of mice exposed to TCDD or carrier on IgE anti-OVA serum levels in OVA sensitised animals

To analyse whether T cells of mice perinatally exposed to TCDD could affect the extent of allergic sensitisation we performed cell transfer studies. T cells expressing CD4 and CD25 markers were isolated from spleens of 6 week

** p < 0.01.

Fig. 3. Effect of perinatal exposure to TCDD on anti-CD3 stimulated in vitro production of IL-2 and IFN-γ by spleen cells of mice sensitised to OVA.

OVA sensitised mice perinatally exposed to TCDD or to the carrier. Concentrations of IL-2 in supernatants of anti-CD3 stimulated cells were significantly (p < 0.01) higher in TCDD exposed mice, whereas IFN-γ release was not significantly different between the groups (Figure 3).

The effect of perinatal exposure to TCDD on in vitro production of IL-10 and TGF-β by spleen cells of mice sensitised to OVA

IL-10 and TGF-β belong to regulatory factors that suppress immune responses. We estimated the levels of

** p < 0.01.

Fig. 4. Effect of perinatal exposure to TCDD on anti-CD3 stimulated in vitro production of IL-10 and TGF-β by spleen cells of mice sensitised to OVA.

Fig. 5. Effect of perinatal exposure to TCDD or carrier (control) on the frequency of T regulatory cells in spleens.
from development of allergies. The mechanisms of such a protection were recently postulated to be dependent on enhanced development of maternal T regulatory cells and thus enhanced immunotolerance to environmental antigens in neonates [32]. Recently, another environmental factor, in this specific case man made toxic contaminant (TCDD) has been shown to modulate the immune responses through affecting T regulatory cells in experimental animal models [25].

Dioxins and the chemicals similar in structure and toxicity, polychlorinated bi-phenyls (PCB), at the levels commonly found in environment, do not pose direct threat to health [6]. However, most health related concerns come from the fact that TCDD is wide-spread in environment, can accumulate in the tissues of organisms [33,34] and affect directly the health status during foetal life and later on in infants due to its transfer through the placenta and during lactation period [35,36]. The outcome of immunotoxic effects of TCDD on developing immune system in animals was shown to result in exacerbation of postnatal autoimmune reactions [10,11], suppressed cell-mediated and humoral immune responses [7,12–14].

Studies performed on Dutch cohort of infants and preschool age children have shown the correlation of perinatal exposure to TCDD and dioxin-like PCB with increased incidence of middle ear infections and decreased incidence of allergy [15,16]. The authors suggest the decreased incidence to be the result of infections that skew the immune response away from those related to development of Th2-cytokine proallergic reactions. Experimental data show that TCDD can directly suppress production of antigen-specific antibodies [18] and synthesis of proallergic cytokines such as IL-5 and IL-4 [19,20]. Additionally, experimental studies performed in rats showed that exposure to TCDD could directly decrease allergic immune response to house dust mite [21]. However, whether such an effects can take place in animals after perinatal exposure to TCDD and what immunological mechanisms are involved has not been answered yet.

Here we demonstrate that mice perinatally exposed to TCDD show lower serum levels of allergen-specific IgE, in comparison to non-exposed control animals, after

**DISCUSSION**

Allergy and asthma development depends on interplay of many genetic factors with those of environmental origin. According to the “hygiene hypothesis” [28,29,31], contact with farm animals in prenatal time and thereafter protects old mice perinatally exposed to TCDD or carrier. Next, these cells were transferred to mice that were sensitised to OVA and serum levels of anti-OVA specific IgE were evaluated. Direct isolation of T reg cells is not possible due to the fact that the marker of these cells is located in intracellular compartments. We isolated CD4+CD25+ T cells that, in addition to natural and inducible T regulatory cells, included also activated T cells. However, considering that there were no significant differences in the number of CD4+CD25+ T cells between the studied groups, we could assume that any differences in inhibitory effect of transferred cells on sensitisation could come through the effect on T regulatory cells without interference of contaminating CD4+CD25+ effector T cells. In Figure 6 we show that transfer of selected cells in fact reduces serum concentrations of anti-OVA specific IgE in comparison to the levels of this antibody found in serum of animals without cell transfer. However, the degree of this reduction was not different between the studied groups.
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might be responsible for decreased synthesis of anti-OVA IgE in animals exposed to TCDD. However, flow cytometry analysis performed in animals perinatally exposed to TCDD or carrier did not show differences in the frequency of CD4+CD25+Foxp3+ T cells, and the transfer of CD4+CD25+spleen T cells from these animals did not cause significant differences in extent of inhibition of anti-OVA IgE serum concentrations.

Thus, we show that not only direct but also perinatal exposure of animals to TCDD may contribute to lower allergic sensitisation. The mechanisms of such an effect of TCDD may be related to reduced production of IL-4 rather than immunosuppression mediated by T regulatory cells. This effect could contribute to decreased incidence of allergies observed in studies of Dutch infants and pre-school age children [15,16]. Whether exposure to dioxins and dioxin-like compounds can significantly contribute to the modulation of immune responses that lead to suppression of allergy development and how it may contribute to other environmental factors known to exert such an effect cannot be answered now.

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