EFFECTS OF IN OVO EXPOSURE TO PCBs (COPLANAR CONGENER, KANECHLOR MIXTURE, HYDROXYLATED METABOLITE) ON THE DEVELOPING CELL-MEDIATED IMMUNITY IN CHICKENS

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Introduction

Polychlorinated biphenyls (PCBs) are wide spread environmental contaminants and known to cause various adverse effects on health of human and wildlife. Immune system is one of the several targets for toxic effects of PCBs [1] and its normal balance is often disrupted by the exposure of the compounds. For example, PCBs may induce immune suppression and result in increased susceptibility to bacterial and viral infections, or conversely, excessive immune enhancement may cause adverse outcomes including as autoimmune disease and anergy. Therefore immune function is regarded as one of an important endpoint in toxicological risk assessment. There are a number of studies shown that neonatal organisms perinatally exposed to polyhalogenated aromatic hydrocarbons (PHAHs) such as PCBs have severer effects on their immune system than adult [2]. Dioxins and coplanar PCB congeners, structurally planar PHAHs are known to have high affinity for aryl hydrocarbon receptor (AhR). 2,3,7,8-tetrachlorinated dibenzo-*p*-dioxin (TCDD) have the strongest affinity among such compounds and these are considered to act on immune system through AhR. On the other hand, such as non-planar PCB congeners with low affinity for AhR, which are abundantly contained in commercial PCB preparations have non-additive (antagonistic) effects on immune function [3]. Prenatal exposure of TCDD to rodent induced abnormal lymphoid development in the thymus and thymus-dependent immune functions were remarkably disturbed [4,5]. Although several experimental studies in mammals have been carried out on the developmental immunotoxicity of PCBs, there are still limited information available on avian species. Thus in this study, prenatal exposure to low level of PCBs and the effects on the developing immune system were investigated with chicken as a model animal of avian species, especially it is focused on the cell-mediated immune function.

Materials and Methods

Chemicals

In order to evaluate the immunotoxic potential of representative congener and mixture, non-*ortho* coplanar congener or commercial mixture, additionally one hydroxylated metabolite congener were tested. 3,3',4,4',5-pentachlorobiphenyl (CB 126) and 4OH-2',3,3',4',5,5'-hexachlorobiphenyl (4OH-CB159) were purchased from AccuStandard Inc. (New Haven, CT), and Kanechlor (KC) 500, 600 from GL Science (Tokyo, Japan). KC mixture was prepared as KC 500:600 =1:1.

In Ovo Exposure

Chicken egg surface above the air cell was sterilized with 70% ethanol and then a small hole was made using a multi-tool drill. The substances were dissolved in corn oil and injected into air cell by micro syringe for 0.2µl/egg. Two levels of non-lethal concentrations for each substances were given as follows; CB 126: 0.15, 0.30 ng/g egg; KC mixture: 1.5, 3.0 µg/g egg; 4OH-CB159: 2.5, 5.0µg/g egg, respectively. As vehicle control, only corn oil was injected. After injection, the hole was sealed with cellophane tape. The eggs were incubated for 21 days at 37.5 with 60% humidity and automatically turned every hour. Hatched chicks were marked and placed in floor pens equipped with heat lamps. All chicks were raised for seven days. Food and water were provided *ad libitium*.

Immunological Assessment

To evaluate the effects on immune system of neonatal chicks, primary immune organ, thymus and bursa of Fabricius masses, and phytohemagglutinin (PHA), primary T-cell mitogen, induced *in vivo* and *in vitro* cell-mediated immune response were measured. Five chicks for each treatment were tested in triplicate.

PHA-Skin Response

In vivo PHA-skin test was conducted according to Smits *et al* [6]. On the 6th days after hatching, chicks were injected intradermally in the wing web with 50µl of 2mg/ml PHA (Sigma, St. Louis, MO) dissolved in phosphate buffer saline (PBS). The thickness of wing web was measured to the nearest 0.01mm prior to and 24h after the injection of PHA using a dial thickness gauge (Peacock, Ozaki Mfg. Co., Japan) with its spring removed to avoid compressing the soft swollen tissues. PHA-skin response (wing web swelling) was expressed as the difference in wing web thickness between 24h after and before injection.

PHA-Lymphocyte Blastogenesis Response

In vitro PHA-lymphocyte blastogenesis was determined using a modified whole blood assay based on the method by Lee [7]. After the skin test, 7 days old chicks were weighted and anesthetized with ether and blood was drawn from the heart by heparinized syringe. Heparinized blood was diluted 1:20 in RPMI-1640 medium supplemented with 2mM L-glutamine, 10mM HEPES buffer, 100U/ml penicillin, 100µg/ml streptomycin, without fetal bovine serum. 100µl aliquots of the diluted blood was dispensed triplicate into 96well U-bottom microtiter plates containing 100µl of PHA in medium (final concentration: 100µg/ml). Plates were incubated for 72h at 39 , 5% CO_2. 8 hours before terminating incubation, [^{3}H]thymidine were added to each well (0.5µCi/well). The cells were harvested onto glass fiber filter using multiple cell harvester. Hemoglobin pigments on the filter discs were bleached by adding 25µl of 30% hydrogen peroxide to avoid color quenching. After the filter discs were dried, placed into glass vials with scintillation cocktail. The [^{3}H]thymidine incorporation rate was measured by Beckman liquid scintillation counter. The blastogenesis response was expressed as stimulation index (S.I.): count per minutes (cpm) of PHA stimulated well / cpm of unstimulated well.

Immune organ mass

After the euthanasia by cardiac bleeding, right robe of thymus and bursa of Fabricius ware removed and weighted. Results were evaluated as ratio to body mass (somatic index).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. Differences between control and treatment groups were considered to be statistically significant when $P<0.05$. Data were presented as means±standard errors.

Results and Discussion

Immune Organ Mass

The results of thymus and bursa of Fabricius somatic index are given in Table 1.

Thymus index was unaffected by any treatment. Coplanar PCBs are known to cause the thymus atrophy [8,9], but it appears that the dosed concentrations in this study could be below the threshold level for affecting thymus of chickens. It was reported that relative thymus mass of day 20 chick embryos were unaffected by exposure to 0.32ng/g egg of CB126 *in ovo* at prior to incubation [9].

The higher dose of CB126 (0.30ng/g egg) significantly reduced the bursa of Fabricius index compared with the control group. The higher dose of KC mixture (3.0μg/g egg) and 4OH-CB159 (5.0μg/g egg) were slightly lower than control, but not significant. It was consistent that bursa of Fabricius is more sensitive to coplanar PCBs than thymus as reported on other investigators [8,9].

Table 1. Thymus and bursa of Fabricius somatic index of 7 days old chicks exposed to CB126, KC mixture, 4OH-CB159 *in ovo*.

Treatment		Thymus	Bursa of Fabricius
Vehicle Control		2.30 ± 0.07	3.17 ± 0.11
CB126 (ng/g)	0.15	2.26 ± 0.06	3.01 ± 0.14
	0.30	2.16 ± 0.08	2.60 ± 0.10*
KC mixture (μg/g)	1.5	2.24 ± 0.05	3.14 ± 0.11
	3.0	2.27 ± 0.06	2.90 ± 0.12
4OH-CB159 (μg/g)	2.5	2.19 ± 0.07	3.03 ± 0.09
	5.0	2.20 ± 0.06	2.90 ± 0.07

Somatic index are expressed as organ/body mass (g) x 1000.
Each result represent mean ± S.E. for three independent experiments.
Total sample size: 15 chicks/treatmet. *Significantly different from control values ($p<0.05$).

PHA-Skin Response

The higher dose of CB126 significantly suppressed the PHA-skin response (Fig. 1). A trend toward decreased response was observed in KC mixture and 4OH-CB159, but the change was small. It was found that *in vivo* cell-mediated immunity such as

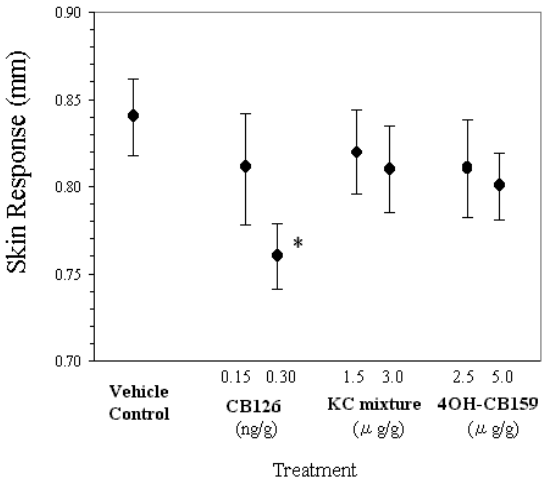

Fig. 1. PHA-induced wing web skin response of 7days old chicks exposed to CB126, KC mixture, 4OH-CB159 *in ovo*. Each bar represent mean ± S.E. for three independent experiments. Total sample size: 15 chicks/treatmet. *Significantly different from control values ($p<0.05$).

delayed-type hypersensitivity (DTH) response was very sensitive to prenatal TCDD exposure in rats experiments [5]. In field study, Caspian tern (*Sterna caspia*) chicks environmentally exposed to PHAHs in Great Lakes significantly suppressed the PHA-skin response with increasing plasma level of PCBs, and observed stronger negative correlations with mono-*ortho* coplanar congeners than di-*ortho* non-planar congeners [10].

Because neonatal chicks are immature to produce antibody as other animals, humoral immunity during the some weeks posthacth maybe depend on transferred immunoglobulin (Ig) from the parent hens, while cellular immunity gradually matured depend on their own ability during the first week posthatch [11]. Thus the cellular immunity during the early developmental stages plays important role in their physiological defense system. Suppression of cell-mediated immunity may cause serious impact on chicks that have still lowered resistance to infections.

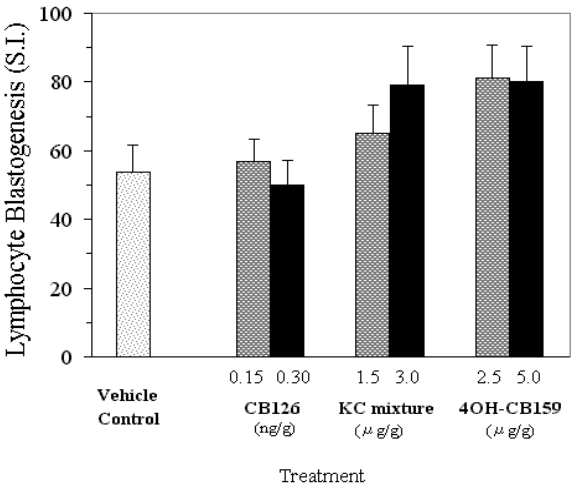

Fig. 2. PHA-induced whole blood lymphocyte blastogenesis of 7days old chicks exposed to CB126, KC mixture, 4OH-CB159 *in ovo*. Each bar represent mean ± S.E. for three independent experiments. Total sample size: 15 chicks/treatmet. S.I. = cpm of stimulated well / cpm of unstimulated well.

PHA-Lymphocyte Blastogenesis

PHA-lymphocyte blastogenesis was unaffected by CB126 compared to control, while enhanced in dose-dependent manner but not significant by KC mixture treatment (Fig. 2). The elevated response of PHA-lymphocyte blastogenesis had been observed in other animals such as rats [12] and ducks [13] dosed PCB mixture Aroclor1254 corresponding to KC500.

Although such mechanism is not clear, it is assumed that PCBs might affect regulatory T cell numbers or functions, or disrupt chemical messengers such as cytokine.

Interestingly, 4OH-CB159 also increased as KC mixture. Since most OH-PCBs are exhibited to not interact with AhR [14], the enhanced response seems likely to be caused by AhR independent mechanism.

There were differences between the results of *in vitro* and *in vivo* response of cell-mediated immunity. It seems likely to reflect that *in vitro* PHA-lymphocyte blastogenesis is T cell proliferation and differentiation, while *in vivo* PHA-skin response is one of the inflammation reactions, which concerned complex interactions of cells not only lymphocytes but also macrophages and basophils.

Although it should be noted that the sensitivity to PCBs much differ between chickens and wild

avian species [15], the dose levels of CB126 and PCBs (as KC mixture) in this study were found in the egg samples of wild birds. Kannan and co-workers [16] reported that the levels of CB126 and Total PCBs in eggs of fish-eating birds such as herring gulls (*Larus argentatus*) and double-crested cormorants (*Phalacrocorax auritus*) from Great Lakes in 1998 were 0.14-4.4ng/g egg, 1.0-7.9μg/g egg, respectively. Moreover dominant residual congeners in the egg of these wild birds [16] were unmetabolizable di-*ortho* congeners such as CB138, 153, 180, which are considerably contained in highly chlorinated KC mixture used in this study. On the other hand, it was reported that OH-PCBs levels in wild bird eggs were very low (less than 1/1000 of Total PCBs) [17]. Immunotoxicity of OH-PCBs have been little reported until now. Therefore further research for low level of exposure test is needed.

In conclusion, prenatal exposure to environmentally relevant concentrations of CB126 and PCB mixture, and considerably high levels of OH-PCBs could cause some immunomodulatory effects mediated by Ah receptor dependent and/or independent on chickens during early post-hatching. As bursa of Fabricius is considered relatively sensitive target organ, humoral immunity as well as cellular immunity in juvenile period could be influenced. It is important to elucidate the adverse effects of PCBs, especially mixture extracted from environmental samples and their metabolites on the developing immune system of avian species.

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