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Micro two-dimensional electrophoretic analysis of changes in plasma proteins of next-generation rat of the pregnant rats administered to coplanar PCBs

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SUMMARY

Polychlorinated biphenyl (PCB) 126 or PCB 169 was administered to pregnant rats and kinetic changes in plasma protein spots of their next generation (F2 rats) ages 1, 3, 6, and 15 weeks were observed by micro two-dimensional polyacrylamide gel electrophoresis (M2D-PAGE).

In the control group in which age-related changes were observable, changes in the form of the albumin (Alb) spot were recognized at ages 1–3 weeks. Only the Alb spot on the acid side, i.e., non-mercaptoalbumin, was recognized at age 1 week. At age 3 weeks, the Alb spot on the alkaline side, i.e., mercaptoalbumin, also became observable. On the other hand, qualitative changes in the form of Alb, i.e., manifestations of mercaptoalbumin, were recognized at age 1 week by administration of PCB 126. This result suggested that the influence of PCB 126 on the living body is greater than that of PCB 169. In spite of the treatment for toxicity by equivalence of dose control on the basis of toxicity equivalency factor (TEF) 0.1 for PCB 126 (PeCB) and 0.01 for PCB 169 (HxCB), the influence of PCB 126 on the living body was greater than that of PCB 169.

The C3 spot was increased at age 3 weeks in the PCB 126 and the PCB 169. The spot showed a tendency toward increase at age 6 weeks in the PCB 126. The TEF compared PCB169 with PCB126, and distinctly increase of the C3 spot was observed for 15 weeks though the TEF was low. From these, as for the PCB 169, it was suggested appearing remarkably in aging influence of the immunity toxicity.

The observations of the changes in these spots led to the conclusion that the changes in these spots resulted from transfer of the drugs accumulating in the maternal body to the F2 rats of the maternal rats administered PCB 126 and those administered PCB 169 via the breast milk and that these changes are involved with inflammatory proteins, including the target genes influenced by the toxicity of coplanar PCBs.

Key words: coplanar polychlorinated biphenyls (Co-PCBs), next-generation rat (F2 rat), albumin, complement 3, micro two-dimensional polyacrylamide gel electrophoresis.

INTRODUCTION

Some chemical substances observed in the environment to mimic or oppose the *in vivo* syntheses and actions of

endogenous hormones are known generically as environmental hormones (endocrine-disrupting chemicals: EDC). Environmental pollution with dioxins from EDC, which include polychlorinated dibenzo-para-dioxin (PCDD), poly-

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Abbreviations: Co-PCBs, coplanar poly chlorinated biphenyls; M2D-PAGE, micro two-dimensional polyacrylamide gel electrophoresis; α_2 -M, α_2 -macroglobulin; Tf, transferrin; IgG, immunoglobulin G; Alb, albumin; C3, complement 3.

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chlorinated dibenzofuran (PCDF), and coplanar polychlorinated biphenyls (Co-PCBs), is particularly regarded as a problem. Co-PCBs of dioxins are distributed at high concentrations in chimney soot, fixed waste, and soil. They are highly concentrated and rapidly accumulate in the living body, as compared to other dioxins, and they accumulate at a high concentration in the adipose tissue of humans and wild animals¹⁾. Under these circumstances, Co-PCBs have been reported to show a higher relative level of exposure than that for PCDD and to exert a more highly toxic effect on the living body than PCDD²⁾.

On the other hand, there has been a report that the level of dioxins in breast milk is much higher than the tolerable daily intake of dioxins³⁾. Some investigators are apprehensive that the transfer of dioxins from breast milk to suckling newborns may give various hazards to the living body in the subsequent infantile or adult period, because dioxins are transferred to rats of the next generation (F2 rats) via lactation^{3, 4)}. However, there have been few if any reports discussing the observation of kinetic changes in plasma proteins of F2 rats of rats administered to Co-PCBs.

In the present study, 3 µg/kg of 3,3',4,4',5-pentachlorobiphenyl (3,3',4,4',5-PCB: PCB 126) with the highest known PCB toxicity and 30 µg/kg⁵⁾ of 3,3',4,4',5,5'-hexachlorobiphenyl (3,3',4,4',5,5'-PCB: PCB 169) with the second highest toxicity were orally given to pregnant rats once a day for 15 days (at 7–21 days pregnant), and kinetic changes in plasma protein spots of the neonatal (F2) rats, which were administered to these drugs via the maternal body in the fetal period when they show the highest sensitivity to Co-PCBs, were observed at ages 1, 3, 6, and 15 weeks by micro two-dimensional polyacrylamide gel electrophoresis (M2D-PAGE)⁶⁾. In addition, information about these spots was quantitatively determined with a software program for M2D-PAGE analysis⁷⁾.

MATERIALS AND METHODS

1. The drugs used

The PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and PCB 169 (3,3',4,4',5,5'-hexachlorobiphenyl) used were PCB 126 and PCB 169 reference standards, respectively, which were purchased from AccuStandard Inc.

2. The animals used and housing conditions

Female Sprague-Dawley SPF rats (Japan S.L.C. Inc.), which were 15 weeks (W) old at mating, were used. They were maintained in the animal room set up at 21±2°C and humidity of 55±5%. The animals were given food pellets of CE-2 (CLEA Japan Inc.) and tap water *ad libitum*.

3. Experimental methods

The daily dose of PCB 126 used for administered was 3 µg/kg, and the daily dose of PCB 169 used for administered was 30 µg/kg. The animals were orally administered

to the drugs. The solution of each PCB was prepared for administered by dissolving each PCB in corn oil. The animals were administered to each PCB solution once a day for 15 days when they were 7–21 days pregnant. Other normal rats, which were administered to corn oil in the way similar to that for the drug groups, were assigned to the control group. The situation of delivery was observed at the time of delivery. The day when delivery was confirmed was defined as delivery day 0 (0 day after birth). Based on this definition, the F2 rats were subjected to weaning on the delivery day 21 (21 days after birth).

The F2 rats were dissected 7 days (at age 1 week) 21 days (at age 3 weeks), 42 days (at age 6 weeks), and 105 days (at age 15 weeks) after birth. In this study, 4 male and 4 female rats among children derived from the mother rat having more than 8 children were chosen 4 days after birth. Experimental chose three of them as sample manufacture with each group among those four male rats at random and held an autopsy.

Blood samples collected from the F2 rats under ether anesthesia on autopsy were kept still at room temperature for 5 minutes, and centrifuged at 2,500×g for 15 minutes. The supernatant fluid was used as specimens, which were stored at -45°C before use. Saturated sucrose solution was added to the same quantity of plasma specimens, then 1.5 µL of the solution was analyzed.

4. Reagents

•M2D-PAGE

The amphoteric carriers for isoelectric electrophoresis were Ampholines at two pH ranges (pH 3.5–10 and 3.5–5.0) from Amersham-Pharmacia. Acrylamide, N,N,N',N'-tetramethylethylene-diamine, and ammonium persulfate were special grade reagents for electrophoresis from Wako Pure Chemical Industries. Special grade N,N'-methylenebisacrylamide, Tris-hydroxymethyl aminomethane, and glycine were from Wako Pure Chemical Industries and electrophoresis grade Coomassie Brilliant Blue R-250 (CBB) was from Nakarai Chemicals. The transfer membrane used in immunostaining was PVDF Membrane (Bio-Rad).

•Immunoblotting

The primary antisera

Goat anti-human α₂M macroglobulin and rabbit anti-human IgG/Fc were from Cappel. Sheep anti-human complement 3 c was from Dako. Rabbit anti-rat transferrin and rabbit anti-rat albumin was from Inter-Cell Technologies, Inc.

The secondary antisera

Immunoglobulin/HRP [Goat polyclonal anti-rabbit], immunoglobulin/HRP [rabbit polyclonal anti-sheep] and immunoglobulin/HRP [rabbit polyclonal anti-goat] was from Dako.

Staining Kit

Vecter-DAB Substitut Kit (Funakoshi Co., Ltd.) were used.

5. M2D-PAGE

M2D-PAGE (type I) and blotting were performed as described previously^{6,8}. However, the second dimensional gel was change to 4–15% linear gradient gel.

6. Immunostaining

Immunostaining were performed as described previously⁶.

7. Image processing system

Images of gel specimens were performed as described previously⁶.

8. Analysis with a software program for M2D-PAGE analysis

Kinetic changes in the plasma protein spots were analyzed by means of Phoretix 2D Full (Ver. 5.01) (Nonlinear Co.) with reference to the method established by Mizuma, et al⁷.

RESULTS AND DISCUSSION

The spots observed in the present study included complement 3 (C3) (Fig. 1-B) and transferrin (Tf) (Fig. 1-C), which were identified with anti-human serum, in addition to four molecular species of α_2 M (α_2 M-1, α_2 M-2, α_2 M-3, and α_2 M-4) (Fig. 1-A), which the authors have previously reported⁶) (Fig. 1). Then, immunoglobulin G (IgG) and albumin (Alb) were added to the spots observed in this study, in comparison with the M2D-PAGE pattern in humans⁸) showed five spots (α_2 M, C3, Tf, IgG, and Alb) (Fig. 2). Moreover, changes in four (α_2 M, C3, Tf, and IgG) of the spots in the rats (3 rats in each group) were quantitatively determined with a software program for M2D-PAGE analysis (Figs. 3 and 4).

Prior to screening of changes in spots which were attributed to administration of Co-PCBs, kinetic changes in spots

of 1-, 3-, 6-, and 15-week old F2 rats of the control rats were observed by M2D-PAGE with the aim of confirming age-related changes.

Changes in configuration of the Alb spot were also recognized in the rats at ages 1 to 3 weeks, and the spot of Alb on the acid side, non-mercaptoalbumin, was markedly recognized at age 1 week (Fig. 2-1). The Alb spot on the alkaline side, i.e., mercaptoalbumin, also became observable at age 3 weeks (Figs. 2-1 and 2-2).

The IgG spot was slightly recognized at age 1 week in the control group (Fig. 2-1), but it was distinctly recognized at age 3 weeks (Fig. 2-2). It was decreased at age 6 weeks (Fig. 2-3). Based on these observations, the frequency of IgG synthesis is considered to be still low in F2 rats, and it is known that IgG is eliminated from plasma by catabolism of IgG derived from the maternal body. IgG was assumed to be eliminated from plasma at approximately age 6 weeks in rats. In the control group, α_2 M-1 and α_2 M-4 were recognized at all ages (Figs. 2-1, 2-2, 2-3, and 2-4), while α_2 M-2 and α_2 M-3 were not recognized at age 1 week. α_2 M-2 and α_2 M-3 were recognized only slightly at age 3 weeks. At age 6 weeks, they were slightly recognized (Figs. 2-3 and 4).

In the PCB 126 and PCB 169 groups, kinetic changes in spots of the 1-, 3-, 6-, and 15-week-old rats were compared with those of control rats (Figs. 2-1, 2-2, 2-3, and 2-4, respectively), then spots of the rats in the PCB 126 group (Figs. 2-5, 2-6, 2-7, and 2-8, respectively), and spots of the rats in the PCB 169 group (Figs. 2-9, 2-10, 2-11, and 2-12, respectively) were observed; the spots of the rats in the PCB groups, which were increased as compared to those in the control group, are shown by solid line arrows ($\leftarrow$).

As for the Alb spot, changes in the configuration of Alb on the alkaline side were recognized and the spot was increased at age 1 week in the F2 rats of the PCB 126 group (Fig. 2-5). This suggests that PCB126 administration had an influence on the change in the form of Alb, i.e., hepatic toxicity accompanying the increase in mercaptoalbumin⁹).

The C3 spot was increased at age 3 weeks in the PCB 126 group and the PCB 169 group (Figs. 2-6 and 2-10). The

Fig. 1. Identification of rat plasma proteins shown as spots of anti-human α_2 M, C3, and Tf.

A. α_2 M-1: α_2 -macroglobulin-1, α_2 M-2: α_2 -macroglobulin-2, α_2 M-3: α_2 -macroglobulin-3, α_2 M-4: α_2 -macroglobulin-4, α_1 AT: α_1 -antitrypsin, B. C3: complement 3, C. Tf: transferrin.

Fig. 2. Polychlorinated biphenyl (PCB) 126 or PCB 169 was administered to pregnant rats and kinetic changes in plasma protein spots of their next generation (F2 rats) ages 1, 3, 6, and 15 weeks were observed by micro two-dimensional polyacrylamide gel electrophoresis (M2D-PAGE).

Top: Control rats (1–4). Middle: Administered rats in the PCB 126 group (5–8). Bottom: Administered rats in the PCB 169 group (9–12). The spots shown by solid line arrows in the rats of the PCB groups were markedly increased, as compared to those in the control group. The Alb spot shown by a dotted line arrow on the alkaline side were recognized and the spot was increased (5). α_2 M-1: α_2 -macroglobulin-1, α_2 M-2: α_2 -macroglobulin-2, α_2 M-3: α_2 -macroglobulin-3, α_2 M-4: α_2 -macroglobulin-4, C3: complement 3, Tf: transferrin, IgG: immunoglobulin G, Alb: albumin.

spot showed a tendency toward increase at age 6 weeks in the PCB 126 group (Fig. 2-7). Toxicity equivalency factor (TEF) compared PCB169 with PCB126, and distinctly increase of the C3 spot was observed for 15 weeks though the TEF was low (Figs. 2-12 and 3).

In the PCB 169 group, Tf also showed a tendency toward increase at age 3 weeks (Figs. 2-10 and 3). In the PCB 126 group, IgG showed a tendency toward increase (Figs. 2-6 and 3) and the α_2 M-1 (169) spot showed a tendency toward increase (Figs. 2-10 and 4).

Thus, the form of Alb was qualitatively changed, i.e., mercaptoalbumin appeared⁹⁾, 1 week after PCB 126 administration. These results suggested that the influence of PCB 126 on the living body was greater than that of PCB 169. The TEF for 126 (PeCB) is 0.1 and that for 169 (HxCB) is 0.01. Based on the values, the dose was controlled with the aim of realizing equivalence of toxicity. Even so, the

influence of 126 was greater than that of 169.

The increase in C3 due to PCB administration indicates that PCB 126 and PCB 169 cause inflammatory diseases. Furthermore, as for the PCB 169, it was observed appearing remarkably in aging influence of the immunity toxicity^{10, 11)}.

Moreover, IgG (126), Tf (169), and α_2 M-1 (169) showed a tendency toward increase 3 weeks after administration. C3 (126) showed the tendency 6 weeks after administration and C3 (169) showed the tendency 15 weeks after administration.

It has been believed that many EDC function like estrogen and exert a toxic action on the liver by binding to estrogen receptors. Co-PCBs and PCDD, which are dioxins, have a marked affinity to aryl hydrocarbon receptor (AhR), and the first step of manifestation of the toxicity is the binding of these dioxins and this receptor in cytoplasm^{12–14)}.

Fig. 3. The quantitation of rats' plasma IgG, C3 and transferrin spot using an image analysis software. Results are presented mean (n=3).

C3: complement 3, Tf: transferrin, IgG: immunoglobulin G.

Fig. 4. The quantitation of rats' plasma four molecular species of α_2 M (α_2 M-1, α_2 M-2, α_2 M-3, and α_2 M-4) spot using an image analysis software. Results are presented mean (n=3).

α_2 M-1: α_2 -macroglobulin-1, α_2 M-2: α_2 -macroglobulin-2, α_2 M-3: α_2 -macroglobulin-3, α_2 M-4: α_2 -macroglobulin-4.

The dioxins bound to AhR are transferred to cell nuclei after dissociation of HSP90, i.e., an AhR-binding molecule. Subsequently, the dioxins bind to AhR nuclear translocator (arnt) with the helix-loop-helix structure to produce het-

erodimers and specifically bind to the XRE sequence in the promoter area of target genes. Based on these functions of the dioxins, some reports have shown that the dioxins facilitate manifestation of the target genes [dioxin-responsive

gene (IL 1 β), plasminogen activator inhibitor (PAI 2), TGF, and EGF] through the increased activation of transcription¹⁵⁻¹⁷). These observations suggested that the changes in plasma α_2 M and C3 spots due to administration of Co-PCB were correlated with inflammation and toxicity expression^{10, 11, 18-21}).

The knowledge of kinetic changes in plasma proteins in the present study is considered useful because the Co-PCB administered accumulates more in the liver of rats than in the adipose tissue of humans²²).

Each Co-PCB, i.e., PCB 126 or PCB 169, which was administered to rats at 7 to 21 days pregnant, is considered to transfer to the fetuses via the placenta before birth. After birth, each Co-PCB accumulating in the maternal body is considered to transfer to the (F2 rats) via milk^{4, 23}). As described above, changes in the manifestations of plasma proteins included the distinct increase in C3 and a tendency toward increase in α_2 M-4, IgG, and Tf at age 3 weeks. As for changes in the manifestation of plasma proteins, α_2 M-4, IgG, and Tf were distinctly increased at age 3 weeks in the F2 rats than in the control group, as described above. Because administration of Co-PCB to maternal rats was continued until the F2 rats reached 3 weeks of age, i.e., the weaning period, these percentage changes in the proteins seem to have shown peaks at age 3 weeks, and thereafter the concentrations of the Co-PCB in the body seem to have been gradually decreased by excretion from the body of the F2 rats and a dilution effect accompanying the growth of F2 rats.

Future tasks for investigating the plasma proteins that showed kinetic changes in the present study include the quantitative determination of the plasma proteins and the quantitative analysis of target proteins in the target cells influenced by the toxicity of Co-PCBs by M2D-PAGE (type II).

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