Table 3. List of genes that were down-regulated by 24 hr exposure to DNCB and BQ

| Accession no. | Gene name | Gene symbol | Fold-change | |
|---|---|---|---|---|
| | | | DNCB | BQ |
| Cell surface receptor/membrane proteins | | | | |
| Y18365 | CD97 antigen precursor. | Cd97 | -1.6 | -1.7 |
| K027929 | Lymphocyte antigen 86 precursor | LY86 | -2.2 | -2.1 |
| K014556 | epidermal growth factor receptor pathway | Eps8l1 | -1.4 | -1.6 |
| AJ251594 | CD44 antigen | CD44 | -1.3 | -1.3 |
| F263247 | macrophage erythroblast attacher | Maea | -1.4 | -1.3 |
| F033017 | potassium channel. subfamily K. member 1 | Kcnk1 | -1.4 | -1.6 |
| F089721 | smoothened | SMO | -1.4 | -2.4 |
| Transcription factors/activators/repressors | | | | |
| AF172286 | Hairy/enhancer-of-split related with YRPW | Hey1 | -1.8 | -1.8 |
| F004295 | myelin transcription factor 1-like | Myt1l | -1.4 | -1.8 |
| BC048845 | inhibin beta-B | Inhbb | -1.4 | -1.4 |
| BC052697 | splicing factor 3a. subunit 2 | S3A2 | -1.4 | -1.6 |
| BC068268 | POU domain. class 5. transcription factor 1 | Pou5f1 | -1.4 | -1.5 |
| BC057126 | Cbp/p300-interacting transactivator | Cited2 | -1.3 | -1.5 |
| Y10926 | binding protein suppressor of hairless-like | Rbpsuhl | -1.4 | -1.6 |
| AK049260 | RNA polymerase II transcriptional coactivator | Rpo2tc1 | -1.4 | -1.5 |
| U83630 | nuclear antigen Sp100 | Sp100 | -1.4 | -1.4 |
| AK031025 | enhancer of polycomb homolog 2 | Epc2 | -1.4 | -1.4 |
| Cell cycle/proliferation/death | | | | |
| Z26580 | Cyclin A2 (Cyclin A). | Ccna2 | -1.6 | -1.8 |
| AK004355 | cyclin-dependent kinase 2-interacting protein. | NM_026048 | -1.8 | -1.5 |
| AF060246 | zinc finger protein 106 | Zfp106 | -1.3 | -1.3 |
| AF316548 | zinc finger protein 148 | Zfp148 | -1.4 | -1.5 |
| AF068780 | geminin | GEMI | -1.4 | -1.3 |
| BC065144 | meiotic recombination 11 homolog A | Mre11a | -1.3 | -1.5 |
| U52193 | phosphatidylinositol 3-kinase. C2 | Pik3c2a | -1.4 | -1.8 |
| Small molecule transport | | | | |
| AK015400 | ferritin heavy chain 3; mitochondrial ferritin. | NM_026286 | -1.6 | -1.6 |

and thus these genes were subjected to further real-time RT-PCR analysis. The real-time RT-PCR results showed that all up-regulated genes and 6 of 8 down-regulated ones corresponded to microarray analysis data although Sdc1 and Pik3c2a showed only limited changes when compared with microarray analysis data (Fig. 2).

To determine whether any of the significant gene changes observed were due to the reactive nature of the contact allergen, DC2.4 cells were exposed to 1.0 $\mu$g/ml DMSO (solvent used to dissolve chemicals as a control), 0.1 $\mu$g/ml BQ (a type IV allergy-inducible chemical), 0.1 $\mu$g/ml Cit (a type IV allergy-inducible chemical), 1.0 $\mu$g/ml TMA (an irritant on the skin and a type I allergy inducer) and 1.0 $\mu$g/ml Dex (a non-sensitizer on the skin) for 24 hr, and gene expression changes were determined by real-time RT-PCR. As shown in Fig. 2, all 3 up-regulated genes chosen were enhanced, and the 8 down-regulated genes were diminished their expression by BQ- or Cit-treatment for 24 hr although some genes such as Tuba6 and Cpd showed limited changes. When treated with TMA, enhanced expression of LY86 and SMO genes, which are down-regulated after type IV allergy-inducible chemicals, was observed (Fig. 2). Socs2 gene, which was up-regulated after sensitization by type IV allergy-inducible chemicals, was also up-regulated by the TMA-treatment, while the expression levels of other genes were scarcely affected by TMA (Fig. 2). In case of Dex-treated cells, the changes of gene expression levels were seen on some genes; up-regulation of SMO and down-regulation of Sdc1, Eps8, Exo1, Asah2, Hey1 and Pik3c2a (Fig. 2). From these results, up-regulation of Sdc1 gene and down-regulation of SMO gene in DC2.4 cells may be specific for type IV-allergy inducible chemicals.

## DISCUSSION

In the present study, we focused on the induction phase of type IV allergy and attempted to identify the genes specifically changed by type IV allergy-inducible chemicals in DCs. For this purpose, changes of gene expression profile in DCs to hapten exposure were evaluated by microarray analysis, since LCs, a family of DCs, were shown to have central roles on the initiation of immune responses including allergic reactions in the skin [4, 38]. Recently, attempts have been made to develop *in vitro* sensitization tests using DCs derived from peripheral blood mononuclear cells or $CD34^+$ hematopoietic progenitor cells purified from cord

Table 3. Continued

| Accession no. | Gene name | Gene symbol | Fold-change | |
|---|---|---|---|---|
| | | | DNCB | BQ |
| Signal transduction | | | | |
| U85021 | adenylate cyclase 8 | Adcy8 | -1.3 | -1.4 |
| BC012488 | Rho guanine nucleotide exchange | Arhgef1 | -1.3 | -1.3 |
| Y13346 | adenosine A2a receptor | Adora2a | -1.4 | -1.4 |
| BC085270 | RAB11B. member RAS oncogene family | Rab11b | -1.4 | -1.5 |
| L21671 | epidermal growth factor receptor pathway | Eps8 | -1.3 | -1.6 |
| Protein modification/synthesis | | | | |
| D87521 | protein kinase. DNA activated. catalytic | Prkdc | -1.4 | -1.5 |
| M95408 | PTK2 protein tyrosine kinase 2 | Ptk2 | -1.4 | -1.5 |
| Others | | | | |
| AF071316 | COP9 (constitutive photomorphogenic) | Cops7a | -1.9 | -1.5 |
| AJ238213 | exonuclease 1. | Exo1 | -1.5 | -1.7 |
| BC056376 | Myotubularin-related protein 1 | Mtm1 | -1.8 | -1.6 |
| AB037181 | N-acylsphingosine amidohydrolase 2 | Asah2 | -1.7 | -1.5 |
| U94662 | Trk-fused gene. | Tfg | -2.2 | -1.6 |
| AF123502 | DNA polymerase epsilon. catalytic subunit A | Pole | -1.6 | -1.7 |
| BC068143 | DNA-directed RNA polymerase III | Polr3b | -1.8 | -1.5 |
| M94584 | chitinase 3-like 3; eosinophil chemotactic | Chi3l3 | -1.9 | -2.3 |
| AK078888 | interferon-related developmental regulator 1 | Ifrd1 | -1.4 | -1.4 |
| AF045252 | tousled-like kinase 2 (Arabidopsis) | Tlk2 | -1.3 | -1.3 |
| BQ928977 | tumor protein D52 | Tpd52 | -1.3 | -1.5 |
| X84692 | spermatid perinuclear RNA binding protein | Strbp | -1.3 | -1.6 |
| CA478631 | metallothionein 1 | Mt1 | -1.3 | -1.5 |
| AF031939 | RalBP1 associated Eps domain containing | Reps1 | -1.4 | -1.5 |
| X97982 | poly(rC) binding protein 2 | Pcbp2 | -1.4 | -1.4 |
| D85391 | carboxypeptidase D | Cpd | -1.4 | -1.8 |
| NM_007622 | Chromobox homolog 1 (Drosophila HP1 beta) | Cbx1 | -1.4 | -1.5 |
| BC056376 | myotubularin related protein 1 | Mtm1 | -1.4 | -1.6 |
| AF411253 | EF hand calcium binding protein 2 | Efcbp2 | -1.4 | -1.4 |
| BC079642 | abl-interactor 1 | Abi1 | -1.4 | -1.4 |
| BC011246 | hemopexin | HEMO_MOUSE | -1.4 | -1.3 |

blood or bone marrow [1, 36]. However, the use of *in vitro* differentiated primary DCs is difficult due to the nature of these cells such as low numbers in the source and donor-to-donor variability [2]. In addition, treatment with several cytokines is generally applied to obtain DCs from blood or bone marrow cells [3, 11, 30], and this process probably changes the cell reactivity to stimulations. Thus, established cell lines are preferable to standardize the condition among assay. A recently established DC line, DC2.4 cells, was applied as a target cell for this assay, and its reactivity to chemical exposures was addressed by microarray analysis. As the result, many gene expression changes were observed after treatment with two different allergenic chemicals, DNCB and BQ. Overall, the changes seemed to be not so noticeable. It is because of the nature of this cell line since the human monocyte-derived THP-1 cells with the same treatment extensively changed a large number of gene expression profile (data not shown). In addition, similar results to our data were reported in a recent study using primary DCs from peripheral blood after chemical treatment [31, 36, 37], suggesting that the effect of sensitization on the gene expression levels might be relatively mild in DC lineage. We analyzed the two data from DNCB- and BQ-sensitized cells and tried to line up the candidate genes specifically up- or down-regulated by type IV allergy-inducible chemicals. As the results, 26 genes were shown to be up-regulated, and 53 were down-regulated in both groups. Interestingly, some of up-regulated genes were associated with the maturation process of DCs. These include TNF-α (a maturation-inducing cytokine), Sdc-1 (a cell surface proteoglycan induced during the maturation process), Map2k4 (a member of MAP kinase kinase family associated with migration and maturation of DC) and Socs2 (a suppressor of cytokine signaling molecule induced during the maturation process) [12, 19, 40]. In addition, up-regulation was also detected on defensin and cathelin, which were formerly considered to work just as antimicrobial peptides [6, 8] and recently reported to have cell migration activity and to be associated with DC maturation process [25]. In contrast, down-regulation of CD44 was detected, which is reported to be expressed on mature DC and induce adhesion with T cell. This may be explained by that the time point at 24 hr after sensitization is still in the process of maturation. In the previous studies, many other molecules are reported to be associated with DC maturation process; for instance, up-regulation of transcripts for the co-stimulatory molecules

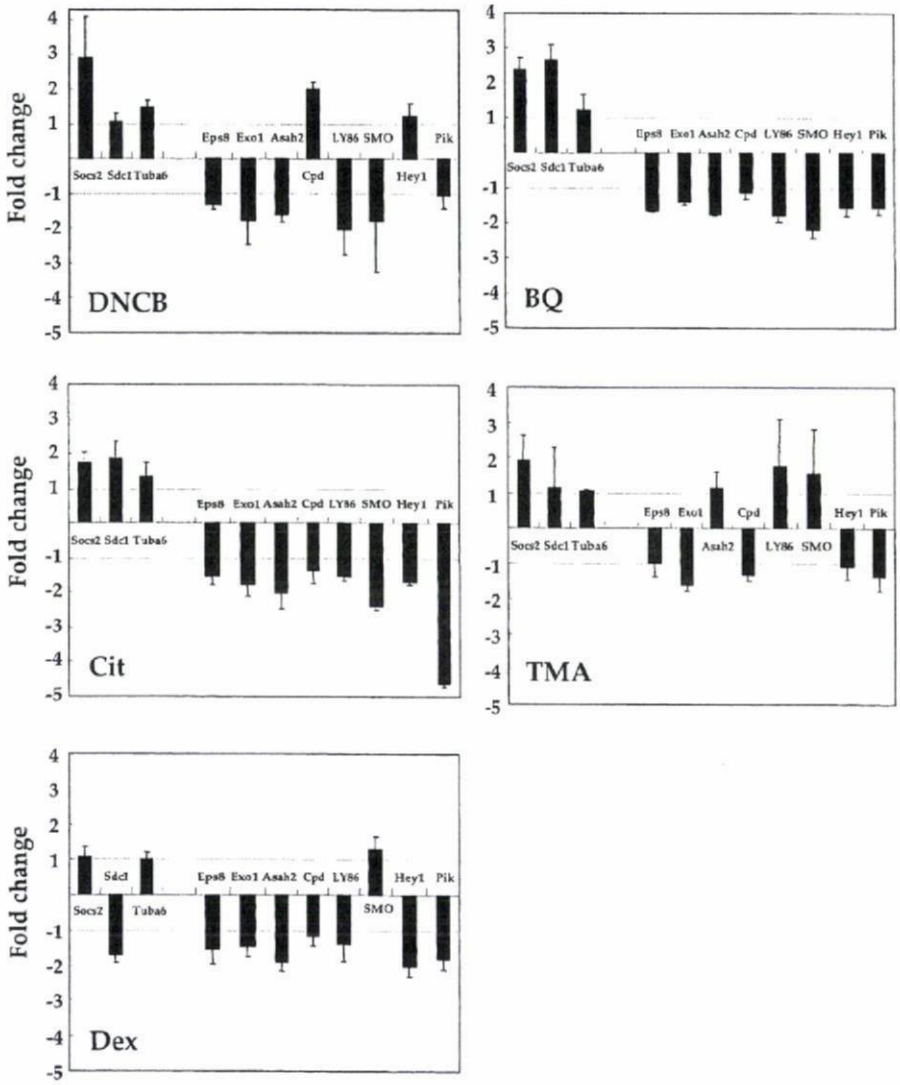


Fig. 2. Gene expression changes in DC2.4 cells induced by DNCB, BQ, Cit, TMA and Dex. DC2.4 cells were exposed to type IV allergy-inducible chemicals, DNCB, BQ or Cit (0.1 $\mu$g/m*l*), a type I allergy-inducible chemical, TMA (1.0 $\mu$g/m*l*) or a non-sensitizer, Dex (1.0 $\mu$g/m*l*) for 24 hr, and the changes of the gene expression were analyzed by real-time RT-PCR. Fold changes were determined based on the gene expression in the cells exposed to solvent DMSO used for solubilization of chemicals.

CD86 [29] and the constitutive chemokine receptor CXCR4 [32, 33], and down-regulation of genes encoding molecules involved in antigen uptake such as the high affinity IgE receptor [27], aquaporin 3 [13]. However, the changes of these molecules were not observed in our study. Assessments of gene expression changes in other time points may detect the up- or down-regulation of these genes. Alternatively, characteristics of DC2.4 cells may give rise to the results.

In order to determine the reproducibility of the gene expression changes observed by microarray analysis, the data from DNCB or BQ-treated DC2.4 cells were compared with that from type IV chemical-exposed mouse ears (data not shown), and 3 up-regulated (Socs2, Sdc1 and Tuba6) and 8 down-regulated (Eps8, Exo1, Asah2, Cpd, LY86, SMO, Hey1 and Pik3c2a) genes in all the experiments were selected for further evaluation by real-time RT-PCR. DC2.4 cells were treated with TMA, an irritant on the skin and type I allergy inducer, and Dex, a non-hazardous chemical on the skin, in addition to type IV allergy inducers, DNCB, BQ and Cit, to identify contact hypersensitivity-specific changes. Although DNCB-induced up-regulation of Sdc1 gene is

limited, other type IV allergy inducible chemicals, BQ and Cit, markedly up-regulated the gene expression as seen in microarray experiments in DNCB or BQ-exposed DC2.4 cells and DNCB-treated mouse tissues. TMA, an irritant on the skin and type I allergy inducer, also up-regulated Sdc1 gene expression in some experiment; however, the changes are neither significant nor reproducible. Thus, these results suggested that up-regulation of Sdc1 gene and especially, down-regulation of SMO gene in DC2.4 cells correlated with type IV allergic reaction (Fig. 2). In the experiment, Dex-treatment induced expression changes of Sdc1, Eps8, Exo1, Asah2, Hey1 and Pik3c2a genes (Fig. 2). Dex is known as a non-sensitizer on the skin; however, we suspected that it had some stimulatory effects on the cells when sensitized directly. Alternatively, uptake of such a high molecular compound with molecular mass of 60,000–90,000 probably initiated DC activation *in vitro*. At present, the function of the proteins derived from Sdc1 and SMO genes on DCs was not well documented although Sdc1 was shown to be a cell surface proteoglycan induced during the maturation process. Further functional analyses may bring interesting information about the role of these proteins on DC maturation and initiation of type IV allergic reaction.

In conclusion, we tried to identify the gene expression changes specifically induced by type IV allergy-inducible chemicals in DCs by microarray and real-time RT-PCR analyses, and 2 possible candidates, Sdc1 and SMO genes, were identified. Thus, up-regulation of Sdc1 gene and down-regulation of SMO gene in DC2.4 cells may be diagnostic markers for the screening of type IV-allergy inducible chemicals. Further analyses of the genes specifically changed by type IV allergy-inducible chemicals are required to clarify the gene expression profiles. The combination of expression changes on several candidate genes may promise reliable results for screening of the allergic chemicals.

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FULL PAPER *Clinical Pathology*

# Development of a Monoclonal Antibody-Based Sandwich ELISA for Detection of Guinea Pig Interleukin-2

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ABSTRACT. Interleukin 2 (IL-2) is a T cell proliferation factor released by Th0- and Th1-type helper T cells and is an essential cytokine for immune responses. In the present study, recombinant glutathione S-transferase (GST)-guinea pig IL-2 (GPIL-2) fusion protein was prepared by *Escherichia coli* (*E. coli*) and by using this protein as an immunogen, monoclonal antibodies (mAbs) against GPIL-2 were produced to establish a basis for a research on immune responses in guinea pigs. Three stable hybridoma cell lines were established, and specific binding of each mAb to recombinant GPIL-2 produced by *E. coli* and insect cells infected with recombinant baculovirus was shown by enzyme linked immunosorbent assay (ELISA) and/or immunoblot analysis. Isotype analyses of these mAbs revealed that all three mAbs were IgG1 and had κ chain. Furthermore, assessment of their epitopes by competition binding assay indicated that the mAbs obtained in this study bound to three different epitopes. Thus, a sandwich ELISA based on the two mAbs specific to different GPIL-2 epitopes was developed for detection of GPIL-2, which had a sensitivity threshold of about 0.3 *ng*/m*l* of GPIL-2.
KEY WORDS: ELISA, guinea pig, IL-2, mAb.

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Cytokines are powerful mediators secreted from a wide variety of cells and regulate a wide spectrum of biological functions. Different production of cytokines by T cells during an immune reaction is shown to have important regulatory effects on the nature of the responses. Initially, it was reported that helper T (Th) cells could be classified into two different subsets according to their cytokine profiles in mice [17]. Later studies showed that these two subsets of Th cells could be applied to those in other species on the basis of the pattern of cytokine production [1, 6, 7, 18]. T helper 1 (Th1) cells are characterized by the production of interferon (IFN)-γ, IL-2 and tumor necrosis factor (TNF)-$\beta$ that induce cellular immune responses, while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 that enhance humoral immunity.

IL-2 plays key roles in T cell differentiation and function, B cell development, and NK cell activation [9, 15]. IL-2 is synthesized and secreted by T cell following stimulation with antigens or mitogens, promotes the growth of T cells [16] and is a potent immune system modulator affecting nearly every facet of host immune response. Although the guinea pig has been used in various immunological experiments and *in vivo* screening test for skin sensitization potential of chemicals for a long time [5], the cytokine responses to allergens have not been well assessed because of the limitation of the experimental tools. In the present study, recombinant GPIL-2 was expressed by *E. coli* and purified as a fusion protein. By using this fusion protein as an immunogen, three monoclonal antibodies (mAbs) specific for GPIL-2 that respectively bound to different epitopes were produced and quantification system of GPIL-2 by sandwich ELISA using these mAbs was developed.

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## MATERIALS AND METHODS

*Production of recombinant GPIL-2 by E. coli and baculovirus*: In order to obtain recombinant GPIL-2 without signal peptide produced by *E. coli*, GPIL-2 cDNA [20] was amplified by polymerase chain reaction (PCR) with *Eco*R I adaptor-ligated forward primer and *Sal* I adaptor-ligated reverse primer. The primer sequences were 5'-GAA TTC GCA CCT ACG TCA AGC TCT-3' (forward) and 5'-GTC GAC TCA AGT CAG TCT TGA CAT GA-3' (reverse). The amplified fragment was purified and subcloned into *Eco*R I/*Sal* I site of GST fusion plasmid vector, pGEX6P-3 (Amersham Pharmacia BiotechAB, Uppsala, Sweden). *E. coli*, XL-1 Blue cells were transformed with the recombinant plasmid and grown in 2xYTG medium (16 g/*l* tryptone, 10 g/*l* yeast extract, 5 g/*l* NaCl and 20 g/*l* glucose) containing ampicillin (100 $\mu$g/m*l*). Production of GST-GPIL-2 fusion protein was induced by addition of isopropyl $\beta$-D-thiogalactoside (IPTG) to a final concentration of 0.8 mM. These XL-1 Blue cells, which suspended in phosphate buffered saline without divalent cations [PBS(−)], were sonicated and insoluble fusion protein was solubilized by refolding procedure using urea [12]. The GST-GPIL-2 fusion protein was purified with glutathione sepharose 4B (GS4B; Amersham Pharmacia BiotechAB). The GPIL-2 cDNA without signal peptide was also subcloned into *Eco*R I/*Sal* I site of the thioredoxin (Trx) fusion plasmid vector, pET32b(+) (Novagen, Darmstadt, Germany). The Trx-GPIL-2 fusion protein was expressed in *E. coli* strain BL21 (DE3) (Novagen) by using IPTG as described above. The cells suspended in ice-cold binding buffer (5 mM imidazole,

0.5 M NaCl and 20 mM Tris-HCl, pH 7.9) were sonicated and solubilized with 0.5% Triton X-100. The Trx-GPIL-2 fusion protein was purified with His-Bind Resin (Novagen).

For production of recombinant GPIL-2 by baculovirus, AcVAPK6 was used as an expression vector in this study. *Spodoptera frugiperda* (*Sf*9 cell) was infected with GPIL-2 cDNA-inserted AcVAPK6 (AcVAPK6/GPIL-2) baculovirus at a multiplicity of infection (m.o.i) of 10 and added with TC100 medium with 10% FCS. The culture was harvested 96 hr after infection and centrifuged at 2,000 g for 5 min at 4°C. After removal of the supernatant, cells were washed with PBS(–) three times and lysed in triple detergent RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% $NaN_3$, 0.1% SDS, 100 $\mu$g/m*l* PMSF, 1 $\mu$g/m*l* aprotinin, 1% Nonidet P-40 and 0.5% sodium deoxycholate). The samples were kept on ice for 20 min followed by centrifugation at 10,000 g for 5 min at 4°C and then cell lysates containing recombinant GPIL-2 were recovered and used for protein analysis.

*Immunization*: BALB/c mice were injected intraperitonealy (i.p.) with 50 $\mu$g of GST-GPIL-2 fusion protein emulsified in an equal volume of complete Freund's adjuvant (DIFCO, Detroit, MI), and boosted at a two-week intervals with the equal amount of GST-GPIL-2 in incomplete Freund's adjuvant (DIFCO) twice. More than two weeks after the third injection, the mice were injected with 50 $\mu$g of GST-GPIL-2 fusion protein without adjuvant. Three days after the last injection, the blood was collected from the mice and kept for 2 hr at 4°C. Then the antiserum was recovered by centrifugation at 8,500 g for 10 min at 4°C and used for ELISA.

*Hybridoma production*: Single-cell suspensions of spleen from immunized mice were prepared as described previously [21]. Briefly, fresh splenocytes were suspended in NS-1 medium (RPMI1640 containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 IU/m*l* penicillin and 50 mg/m*l* streptomycin) and centrifuged at 400 g for 5 min at room temperature (RT). The cells were treated with 0.8% $NH_4Cl$-Tris (pH 7.6) followed by washing twice with NS-1 medium and resuspended in NS-1 medium.

Cell fusion was performed by the previously established method [8, 10]. In brief, splenocyte and P3U1 mouse myeloma cells were mixed at 5:1 ratio and centrifuged at 400 g for 5 min at RT. After washing with serum-free NS-1 medium, 1 m*l* of 50% polyethylene glycol (MERCK, Darmstadt, Germany) was added with vigorous agitation. Then, 10 m*l* of serum-free NS-1 medium was added and the cells were recovered by centrifugation at 170 g for 5 min at RT. The cells were resuspended into HAT selection medium (NS-1 medium containing 100 $\mu$M hypoxantine, 0.4 $\mu$M aminopterin and 16 $\mu$M thymidine) and seeded in 96-well flat bottom microplate (Falcon, Franklin Lakes, NJ) ($1 \times 10^5$ per well). The cells were cultured at 37°C in a humidified 5% $CO_2$ atmosphere. Supernatants from wells showing hybridoma cell growth were screened for antibodies to GPIL-2 using ELISA described below. The hybridomas in mAb-positive wells were cloned by limiting dilution method and isotype analyses of mAbs were performed by using Mouse Monoclonal Antibody Isotyping Kit (Amersham Pharmacia BiotechAB) according to the manufacturer's instruction.

*Enzyme linked immunosorbent assay (ELISA)*: 96-well U-bottomed microtiterplate (Immuron G; Greiner, Frankfurt, Germany) were coated with 5 $\mu$g/m*l* Trx-GPIL-2 fusion protein solution in 0.1 M carbonate buffer (pH 8.0) by overnight incubation at 4°C. After blocking with 2% gelatin in PBS(–) for 1 hr at RT, hybridoma cell culture supernatants were added to each well. After incubation for 1 hr at RT, peroxidase-conjugated goat IgG fraction against mouse IgG Fc (CAPPEL, West Chester, PA) were used. The color reaction was developed with 0.04% orthophenylene diamine in 0.05 M citric acid/0.1 M $Na_2HPO_4$ buffer containing 0.02% $H_2O_2$. The reaction was stopped by addition of 2.5 M $H_2SO_4$ and absorbance was read by microplate reader (BIO-RAD, Anaheim, CA) at a test wavelength of 490 nm and a reference wavelength of 630 nm.

*Competition binding assay*: Monoclonal Abs were purified from supernatant of the hybridoma cell culture by using mAbTrap Kit (Amersham Pharmacia BiotechAB) according to the manufacturer's instruction. Purified mAbs were biotinylated by ECL Protein Biotinilation Module (Amersham Pharmacia BiotechAB) and used for competition binding assay.

Microplates were coated with 100 *n*g/m*l* of Trx-GPIL-2 fusion protein and saturated or diluted amount of unlabeled mAbs specific for GPIL-2 were added to each well after blocking with 2% gelatin in PBS(–). After incubation for 1 hr at RT, competition among mAbs was assessed by binding of biotinylated mAbs to coated Trx-GPIL-2 fusion protein in the presence of saturated or diluted unlabeled mAbs. For detection, horseradish peroxidase (HRP) labeled streptavidin was used. The color reaction was developed as described above.

*Monoclonal Abs-based sandwich ELISA*: Microplates were coated with unlabeled mAbs overnight at 4°C and serially diluted Trx-GPIL-2 fusion proteins were added to each well after blocking with 2% gelatin in PBS(–). After incubation for 1 hr at RT, biotinylated mAbs were added and further incubation for 1 hr at RT. For detection, HRP labeled streptavidin was used and the color reaction was developed as described above. A standard curve was generated with serial five-fold dilutions of purified Trx-GPIL-2 fusion protein and the concentrations of GPIL-2 were calculated from the molecular mass of GPIL-2 in Trx-GPIL-2 fusion protein. The concentrations of the sample were interpolated from the linear portion of the standard curve.

*Immunoblot analysis*: Cell lysates of *E. coli* transformed with GPIL-2 cDNA-inserted expression plasmid or recombinant baculovirus-infected *Sf*9 cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electorophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene difluoride membranes (MILLIPORE, Bedford, MA). After blocking with 5% skim milk in PBS(–) containing 0.1% Tween 20 (PBS-T) for 1 hr at RT, the membranes

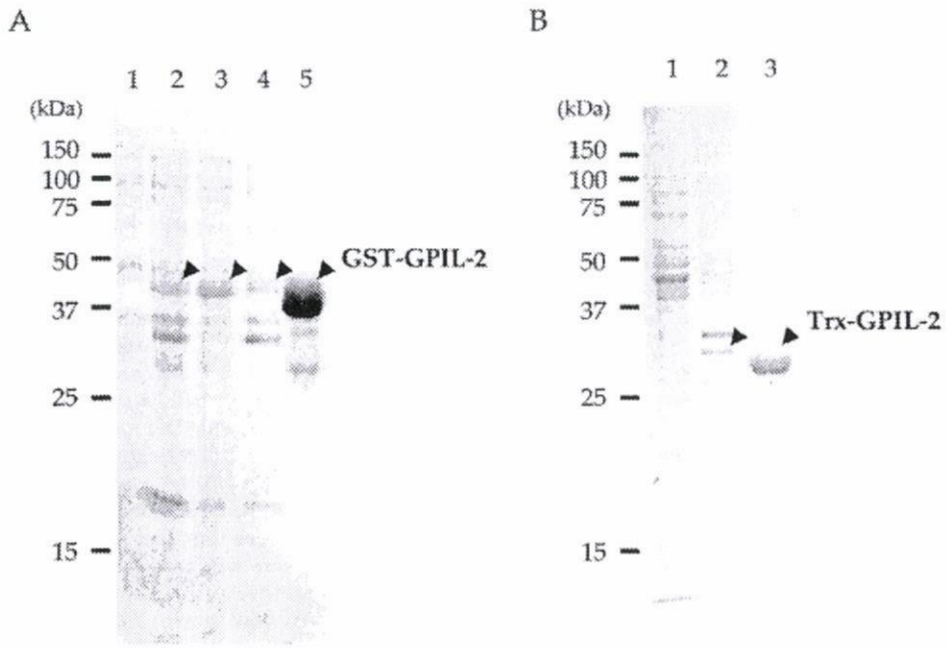



Fig. 1. SDS-PAGE analysis of expressed GPIL-2 fusion proteins in *E. coli*. (A) pGEX-6P-3/GPIL-2: lanes 1 and 2: cell supernatant and pellet after sonication; lanes 3 and 4: cell supernatant and pellet after refolding using urea; lane 5: purified GST-GPIL-2 fusion protein. (B) pET32b(+)/GPIL-2: lanes 1 and 2: cell supernatant and pellet after sonication; lane 3: purified Trx-GPIL-2 fusion protein.

were incubated with purified mAbs against GPIL-2 for 1 hr at RT and washed three times with PBS-T. The membranes were further incubated with alkaline phosohatase-conjugated rabbit anti-mouse IgG antibody (SIGMA, St Louis, MO) for 40 min at RT. After three washes with 0.1% PBS-T, the blots were developed by addition of substrate (Alkaline Phosohatase Conjugate Substrate Kit; BIO-RAD) and visualized.

## RESULTS

*Production and purification of recombinant GST- and Trx-GPIL-2 fusion protein*: To establish recombinant GPIL-2 expression system, we inserted GPIL-2 cDNA fragment without a signal sequence into the eukaryotic expression plasmid vector, pGEX6P-3 and pET-32b(+). Each recombinant plasmid was introduced into *E. coli*, XL-1 Blue and BL21 cells, and expression of fusion proteins was induced by IPTG. Since only a small proportion of fusion protein was obtained in the soluble fraction of each of the cells, the insoluble proteins were dissolved by the refolding procedure using urea and 0.5% Triton X-100, and then purified respectively. Purified GST-GPIL-2 fusion protein had a molecular mass of about 42 kDa and purified Trx-GPIL-2 fusion protein had 36 kDa based on polyacrylamide gel electrophoresis (Fig. 1A and B).

*Production and characterization of mAbs to GPIL-2*: Since GPIL-2 protein could not be obtained in soluble fraction after excision by enzyme digestion, mAbs were prepared using two forms of the fusion proteins. Namely, mice were immunized with GST-GPIL-2 fusion protein and culture supernatants of hybridoma clones were screened by ELISA using Trx-GPIL-2 as an antigen. Supernatants from twenty two hybridoma clones reacted with Trx-GPIL-2 fusion protein. Sixteen out of these clones were shown to produce antibodies that bound to GST-GPIL-2 fusion protein but not to GST only. These hybridoma cells were further cloned twice by limiting dilution and three independent clones referred to 1B-12, 3D-7, and 2H-1 were established. Isotype analyses of mAbs produced by these clones revealed that all three mAbs were IgG1 and had k-light chains (data not shown).

All mAbs were tested their reactivity to GPIL-2 by immunoblot analysis using *E. coli*-derived recombinant protein as an antigen. All the three mAbs reacted with GST- and Trx-GPIL-2 fusion protein but not with GST and Trx protein only, indicating that these mAbs specifically bound to GPIL-2. The representative findings using 3D-7 mAb were shown in Fig. 2. The mAbs were also tested by immunoblot analysis for their reactivity with recombinant GPIL-2 expressed in insect cells by baculovirus. As shown in Fig. 3, all mAbs bound to single protein with an approximate molecular mass of about 15 kDa corresponding to recombinant GPIL-2 protein expressed by baculovirus. These

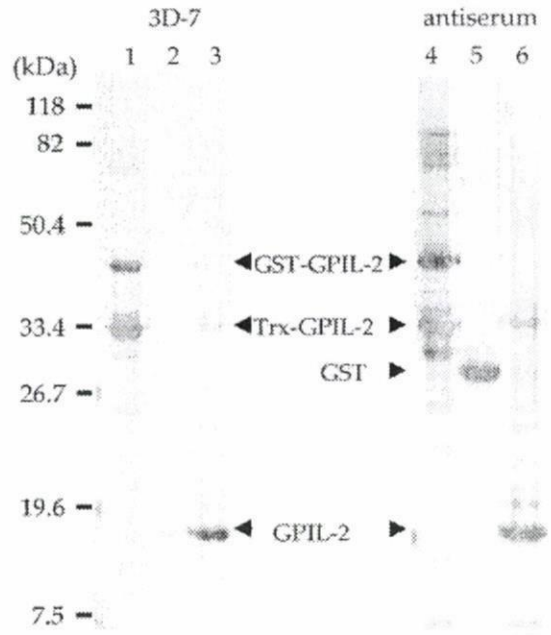



Fig. 2. Immunoblot analysis of GST-GPIL-2, Trx-GPIL-2, GST, Trx, and purified GPIL-2 using the mAb (3D-7) or antiserum. Lanes 1 and 4: GST-GPIL-2 and Trx-GPIL-2 fusion proteins; lanes 2 and 5: GST and Trx tag proteins; lanes 3 and 6: Trx-GPIL-2 digested by enterokinase.

results indicated that three mAbs obtained specifically recognized GPIL-2 protein produced by insect cells as well as *E. coli*.

*Relative affinity of mAbs against recombinant GPIL-2*:

Table 1. Relative affinity of the GPIL-2 mAbs

| GPIL-2-spcific mAb | Relative affinity[a] ($\mu$g/m*l*) |
|---|---|
| 1B-12 | 0.24 |
| 3D-7 | 0.56 |
| 2H-1 | 1.92 |

a) The affinity of GPIL-2 mAbs was represented by the concentration ($\mu$g/m*l*) of purified antibody necessary to obtain an optical density of 1.0 after 5 min of revelation.

Purified mAbs were assessed their titers against GPIL-2 by ELISA (Table 1). Titers were defined as the concentration of antibody required to obtain an optical density of 1.0 after 5 min of revelation. 1B-12 mAb presented the highest titer for the recombinant GPIL-2 (0.24 $\mu$g/m*l*), the next one was 2H-1 mAb (0.56 $\mu$g/m*l*) and 3D-7 mAb presented the lowest reactivity against GPIL-2 (1.92 $\mu$g/m*l*).

*Epitope analysis of mAbs by competition binding assay*: To examine whether the three mAbs obtained in this study could recognize different epitopes on GPIL-2 or not, competition binding assay was performed. In the preliminary study, the amount of each mAb that saturates coated antigen was determined (data not shown). Three mAbs were labeled with biotin and assessed its binding ability to Trx-GPIL-2-coated plate pre-incubated with saturated or diluted amount of unlabeled purified mAbs. As shown in Fig. 4A, biotin-labeled 1B-12 mAb was inhibited its binding to GPIL-2 by the increasing amount of 2H-1 mAb a little but not by 3D-7 mAb. Using a labeled 3D-7 mAb, which was blocked its binding to coated antigen by 2H-1 mAb partly but not by 1B-12 mAb (Fig. 4B). Further, when biotinylated 2H-1 mAb was used, a little inhibition was observed by the other two mAbs (Fig. 4C). These data indicate that 1B-12 and

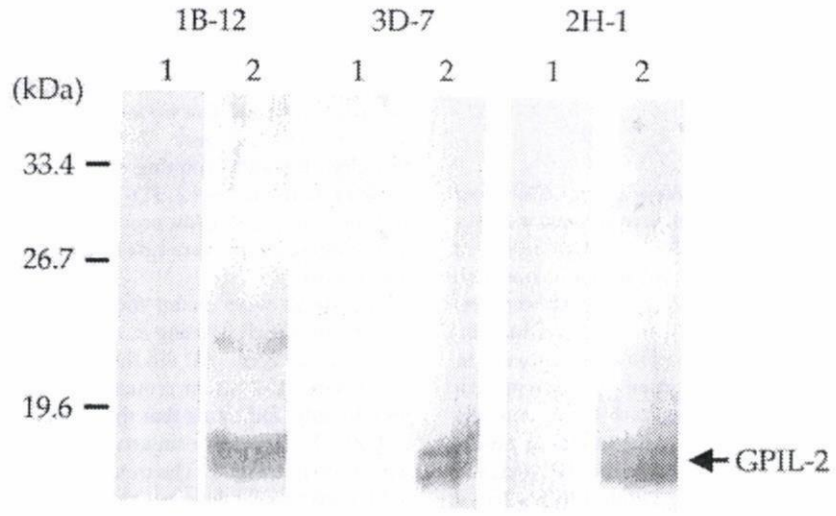



Fig. 3. Immunoblot analysis of MAbs for their reactivity with cell lysate of *Sf*9 cell in the absence (lane 1) or presence (lane 2) of recombinant GPIL-2-expressing baculovirus infection. Blots were proved with mAb 1B-12, 3D-7 or 2H-1.

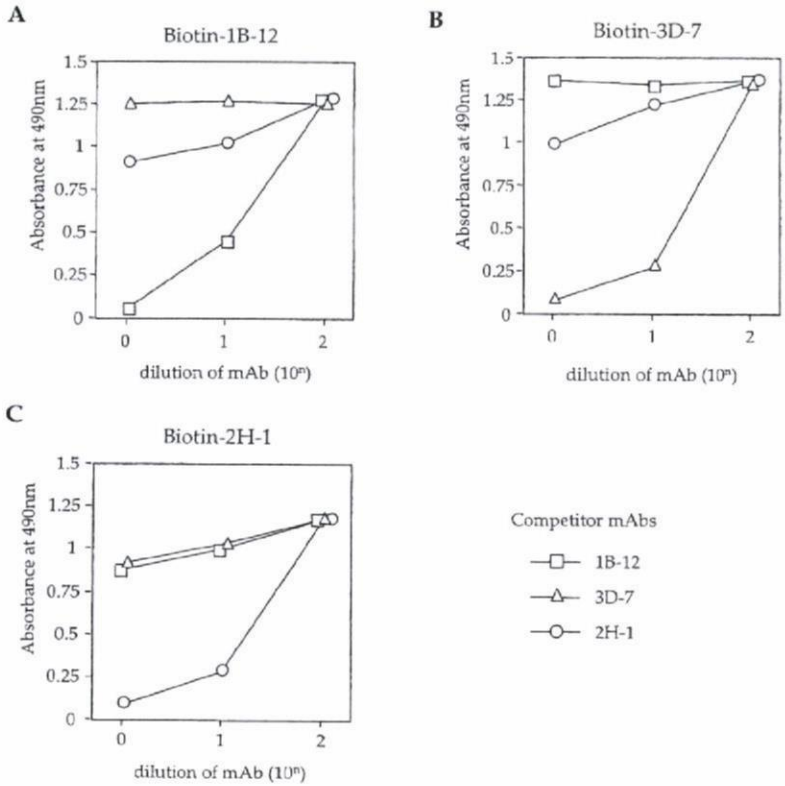



Fig. 4. Competition binding assays among GPIL-2 specific mAbs. Three mAbs, 1B-12 (A), 3D-7 (B) and 2H-1 (C), were labeled with biotin and assessed their binding ability to Trx-GPIL-2-coated microplates pre-incubated with saturated or diluted amount of unlabeled competitor mAbs.

Table 2. Comparison of the different combinations of GPIL-2-specific mAbs in the sandwich ELISA

| Capture antibody | Detection antibody | | |
|---|---|---|---|
| | 1B-12 | 3D-7 | 2H-1 |
| 1B-12 | <0.001[a)] | 1.204 | 1.036 |
| 3D-7 | 2.211 | <0.001 | 0.907 |
| 2H-1 | 1.078 | 0.599 | <0.001 |

a) Absorbance values at 490 nm were determined after 5 min of revelation in the sandwich ELISA using 100 *ng*/m*l* Trx-GPIL-2 as an antigen.

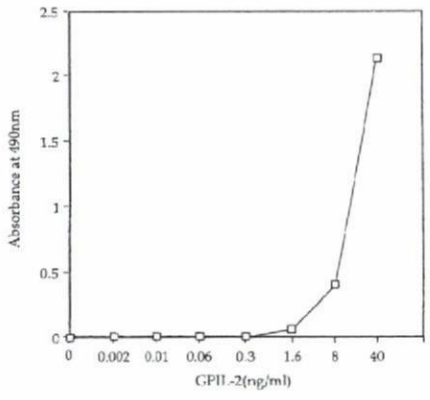
3D-7 mAbs respectively recognize completely different epitopes and 2H-1 mAb has partly overlapped or very similar epitope of GPIL-2 with the other two mAbs.

*Quantification of GPIL-2 using a mAbs-based sandwich ELISA*: A comparison of the combinations of the anti-GPIL-2 mAbs in the sandwich ELISA was performed (Table 2). When the same mAb was employed as both the capture and detection antibody, no binding of detection antibody to recombinant GPIL-2 was confirmed. As expected from the competition studies, the results also suggest that 2H-1 mAb probably recognized overlapping epitope with the other two mAbs since low absorbance values were observed when 2H-1 was used as capture or detection antibody. The combination of mAbs 3D-7 as capture antibody with 1B-12 as detection antibody resulted in the highest absorbance values. Therefore, the standard dose-response curves were obtained by this combination (Fig. 5). The sensitivity or detection limit, defined as the antigen concentration that produced a significant positive signal, was determined to be 0.3 *ng*/m*l*.

## DISCUSSION

In the present study, recombinant GPIL-2 protein was expressed by *E. coli*, purified and used as an immunogen for production of mAbs against GPIL-2. Three stable hybridoma cell lines were established and specific binding of each mAbs to recombinant GPIL-2 produced by eukaryotic cells as well as *E. coli* was shown by ELISA and/or immunoblot analysis. In addition, the mAbs were shown to recognize different epitopes respectively by competition binding assay. Thus, a sandwich ELISA was developed for the quantification of GPIL-2.

The guinea pig is a sensitive animal to several infectious pathogens and Th1- and Th2-mediated allergic reactions




| GPIL-2 (ng/ml) | 0 | 0.002 | 0.01 | 0.06 | 0.3 | 1.6 | 8 | 40 |
|---|---|---|---|---|---|---|---|---|
| Absorbance at 490nm | 0 | 0.005 | 0.005 | 0.007 | 0.011 | 0.06 | 0.405 | 2.131 |

Fig. 5. Dose-response curve in sandwich ELISA of GPIL-2 by the combination of mAbs 3D-7 with 1B-12 as detector. The values shown are the mean of two independent experiments. Concentrations of GPIL-2 were calculated from the molecular mass of GPIL-2 in Trx-GPIL-2 fusion protein.

and has been used as a useful animal model to address immune reactions *in vivo* [2, 11, 13]. In addition, the guinea pig has been used as the most common test animal for appraising skin sensitization potential of chemicals and the results of the skin sensitization test were reported to correlate well with that obtained from humans [3, 4, 13, 14]. Although the assessment of cytokine responses is essential to know the mechanism for evocation of allergic reactions, such analyses have not been done well in guinea pigs because of the limitation of immunological tools for guinea pig studies. Thus, we attempted to obtain monoclonal antibodies to GPIL-2 and develop a sensitive and specific detection system by using these antibodies.

In this study, we expressed recombinant GPIL-2 in *E. coli* to obtain a sufficient amount of immunogen since it is impossible to purify enough amount of natural GPIL-2 for immunization. For this purpose, two popular methods, GST and Trx fusion protein expression system were applied and we successfully produced recombinant fusion protein by the both methods. However, recombinant GPIL-2 protein was precipitated into insoluble fraction for some reason when tag proteins were removed. Therefore, we used GST-GPIL-2 fusion protein for immunization of mice and recombinant Trx-GPIL-2 for screening of culture supernatants from hybridoma clones to determine specific binding to GPIL-2 but not to tag protein. As the result, we obtained 3 independent clones specifically bind to recombinant GPIL-2 produced by *E. coli*. However, eukaryotic proteins expressed in *E. coli* are not always properly modified, and mAbs produced by immunization with *E. coli*-derived recombinant protein may not react with the same protein produced by mammalian cells. Thus, we addressed this issue and showed that all mAbs obtained in this study reacted with modified GPIL-2 protein produced by insect cells infected with recombinant baculovirus, strongly suggesting that these mAbs would recognize natural form of GPIL-2. Epitope analysis of mAbs using competition binding assay showed that mAbs obtained in this study recognized three different epitopes; two of them (1B-12 and 3D-7) bind completely different sites and the rest (2H-1) has overlapped epitope with the other two. Based on these findings, we attempted to establish a sensitive and specific detection system of GPIL-2 by sandwich ELISA. As expected, the highest sensitivity was obtained when two mAbs (1B-12 and 3D-7) that recognize completely different epitopes were used as capture and detection antibodies. In this assay, we determined the detection limit to be about 0.3 *ng/ml*. As far as we know, IL-2 ELISA kits are commercially available only in human, mouse and rat. They are all very sensitive and the detection limits are 15–50 *pg/ml*. Our system is less sensitive when compared to these kits; however, some improvements could be achieved by mAb purification and/or labeling methods to get more sensitivity.

In conclusion, we produced three mAbs to GPIL-2 and developed the detection system of GPIL-2 by a sandwich ELISA. Since the guinea pig is known to be a useful animal model for type I and type IV allergy as well as some infectious diseases [2, 11, 13, 19], this system will be helpful for analysis of the mechanisms of disease development. In addition, mAbs established in this study will be applied for other immunological purpose such as immunohistochemistry and intracellular cytokine staining by flow cytometric analysis.

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ORIGINAL ARTICLE

# Cardiovascular malformations induced by prenatal exposure to phenobarbital in rats

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**ABSTRACT** **The effects of prenatal exposure to phenobarbital (PB) on the cardiovascular system were examined in rat fetuses and pups. PB was administered at a dose of 80 or 120 mg/kg/day by gavage to Sprague Dawley (SD) rats on two consecutive gestational days (GD): 7–8, 8–9, 9–10, or 10–11. Fetuses were examined for cardiovascular malformations on GD 20. In addition, pups were examined for PB-induced cardiovascular malformations. Incidences of ventricular septal defect (VSD), overriding aorta, double outlet right ventricle and transposition of great arteries were significantly increased in the fetuses whose dams were administered PB at 120 mg/kg on GD 8–9, 9–10 or 10–11. GD 8–11 was the critical period for the cardiovascular malformations associated with administration of PB in rats. Various types of cardiovascular malformations were detected in pups from the PB-administered dam. Severe cardiovascular malformations induced by PB caused deaths on early postnatal days. However, slight malformations such as isolated VSD persisted until weaning, and did not affect postnatal viability.**

**Key Words:** cardiovascular malformations, fetus, neonates, phenobarbital, pups, rats

## INTRODUCTION

Anticonvulsant drugs are classified into five different groups; barbiturates (phenobarbital [PB], primidone), hydantoins (phenytoin), succinimides (ethosuximide), oxazolidinediones (trimethadione [TMD]) and a miscellaneous group (valproic acid and carbamazepine). These drugs are used as monotherapy or in combination. It has been recognized that treatment of women of childbearing age with anticonvulsant drugs may cause congenital heart disease, cleft lip/palate, skeletal defects, central nervous system defects, or functional defects in their offspring (Bossi 1983; Finnell *et al.* 1997; Schardein 2000). Prenatal exposure to PB was reported to increase the risk of congenital malformations in human offspring (Holmes *et al.* 2004). In addition, teratologic studies with laboratory animals demonstrated PB-induced cleft palate in mice (Walker & Patterson 1974; Sullivan & McElhatton 1975), skeletal malformation in rats (McColl *et al.* 1963) and skull defects in rabbits (McColl 1967). However, it was reported that PB had a weak ability to induce congenital malformations in mice and humans (Sullivan & McElhatton 1977; Kaneko & Kondo 1995). Only two teratologic studies showed that various cardiovascular malformations were induced in rat fetuses from the dams administered PB (Vorhees 1983; Terada *et al.* 1987).

The objective of this study was to morphologically examine the developmental effects of PB on the fetal cardiovascular system from the standpoints of the critical period for induction of malformations, types of malformations and dose–response relations for the malformations in rats. In addition, we also investigated the postnatal fate of the cardiovascular malformations in pups from dams administered PB.

## MATERIALS AND METHODS

### Animals

Male and female Crj:CD(SD) rats were purchased from Charles River Japan, Inc. (Atsugi) at 9 and 8 weeks of age, respectively. After 2 weeks of quarantine and acclimation, animals were individually housed in stainless-steel wire mesh cages (220 [W] × 270 [D] × 190 mm [H]) in a barrier system animal room and were given food (CA-1, Japan CLEA, Tokyo) and tap water *ad libitum*. Temperature and relative humidity in the animal room were maintained constant at 24 ± 1° and 55 ± 5%, respectively, with 10–15 room air changes/h. Fluorescent lighting was controlled automatically to give a 12 h light (07: 00–19: 00)/dark (19: 00–07: 00) cycle. Female rats were paired with males on a 1:1 basis overnight, until copulation. Every morning, females were checked for the presence of sperm or a plug in the vagina. The day that sperm or a plug was detected was defined as gestation day (GD) 0. The pregnant females were divided into 9 weight-matched groups, each comprising 7–8 animals in the study of fetal anomalies and into 4 weight-matched groups, each comprising 9–10 animals in the study of postnatal fate of cardiovascular malformations.

### PB administration

Phenobarbital (PB) was purchased from Daiichi Pharmaceutical (Tokyo, Japan) PB was suspended in an aqueous solution of 1% carboxymethyl cellulose sodium (CMC-Na; Maruishi Pharmaceutical, Osaka, Japan), adjusting the PB solution to a volume of 5 ml/kg. Pregnant rats were administered PB by gavage at a time between 13.00 and 15.00 hours. A dose level of PB was selected as 80 or 120 mg/kg/day on two consecutive GD 7–8, 8–9, 9–10, or 10–11 in the study of fetal anomalies and as 120 mg/kg/day on two consecutive GD 8–9, 9–10 or 10–11 in the study of postnatal fate of anomalies. The dose levels were selected on the basis of results of a preliminary study in which incidences of cardiovascular malformations were increased by administration of PB to pregnant rats by gavage at a dose of 80 mg/kg/day on GD 7 through 11. The two consecutive days of PB administration were selected to find a critical period for induction of cardiovascular malformations. Control animals were administered 1% CMC-Na at a dose of 5 ml/kg/

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day by gavage on GD 7 through 11 in the study of fetal anomalies or on GD 8 through 11 in the study of postnatal fate of anomalies.

**Observation of congenital malformations for the study of fetal anomalies**

After oral administration of PB, clinical signs were observed and body weights were measured daily in each dam. On GD 20, the dams were sacrificed by ether anesthesia, and the number of corpora lutea was recorded for each dam. The uterus of each dam was examined for the number of implantation sites, resorptions, or live and dead fetuses. All live fetuses were sexed, weighed, and examined for external malformations. Approximately two-thirds of the live fetuses were fixed in 10% formalin solution and then in Bouin's solution and the head were examined by the freehand razor blade sectioning method of Wilson (1965). The visceral and thoracic organs of these fetuses were examined by the microdissection method of Nishimura (1974). The remaining fetuses were fixed in 90% ethanol and processed for skeletal specimens by the method of Dawson (1926), and then examined for skeletal malformations and variations, or degree of ossification.

Overriding aorta, double outlet right ventricle and transposition great arteries were diagnosed according to the following definitions of morphological characteristics, as illustrated in Figure 1. Overriding aorta was defined as the aorta arising from the biventricles. Double outlet right ventricle was defined as both the aorta and the pulmonary artery arising from the right ventricle. Transposition of great arteries was defined as the aorta arising from the right ventricle and the pulmonary artery arising from the left ventricle.

**Observations of cardiovascular malformations for the study of postnatal fate of anomalies**

Body weights of dams were recorded daily. After PB administration, the dams were observed daily for clinical signs and allowed to deliver naturally. The day of parturition was defined as postnatal day (PND) 0. The live neonates were sexed, and examined for external malformations and clinical signs. The litter weight was measured on PND 0, 4, 7, 14 and 21. The live pups were culled to 4 males and 4 females in each litter on PND 4, and then euthanized by ether anesthesia for cardiovascular examination on PND 21. Dead, moribund or culled pups were also examined for cardiovascular malformations, using the same methods described above.

**Statistical analysis**

Incidences of external, cardiovascular and skeletal anomalies, expressed as fetal unit, were analyzed by $\chi^2$ test. Other parameters were analyzed by the following algorithm described by Yamazaki *et al.* (1981) and Hamada *et al.* (1998): Bartlett's test was used to determine whether or not the variance was homogeneous or not. When the variance was homogeneous, the one-way ANOVA was applied. When the variance was not homogeneous, the Kruskal–Wallis rank sum test was performed by arranging all data of the control and exposed groups in descending order. Statistical differences in the means and the rank means among the groups were analyzed by Dunnett's multiple comparison test, and the same multiple comparison test by rank, respectively. Two-sided analysis with a *P*-value of 0.05 or 0.01 was performed.

## RESULTS

**Effects of PB on dams**

No deaths occurred in any dam administered PB at a dose of 80 or 120 mg/kg/day during gestation. Ataxic gait, loss of righting reflex, decreased locomotor activity, salivation and lacrimation were observed after the oral administration of 80 and 120 mg/kg PB. These signs disappeared at least within 48 h after the second day of administration of PB. There was no significant difference in body weight between any PB-administered group and the control (Data not shown). No macroscopic change was observed in any PB-administered dam at cesarean section on GD 20 or at the necropsy on PND 21.

**Effects of PB on fetuses**

Table 1 shows the reproductive parameters of dams administered PB on two consecutive days during gestation. Percent postimplantation loss was significantly increased in the group administered PB at 120 mg/kg on GD 10–11. No significant difference in fetal body weight was found between any PB-administered group and the control.

Table 2 shows the external, visceral, and skeletal anomalies observed in fetuses from the dams administered PB on 2 consecutive days during gestation. No significant increases in the incidences of external malformations were observed in any PB-administered group. Incidences of visceral malformations were sig-

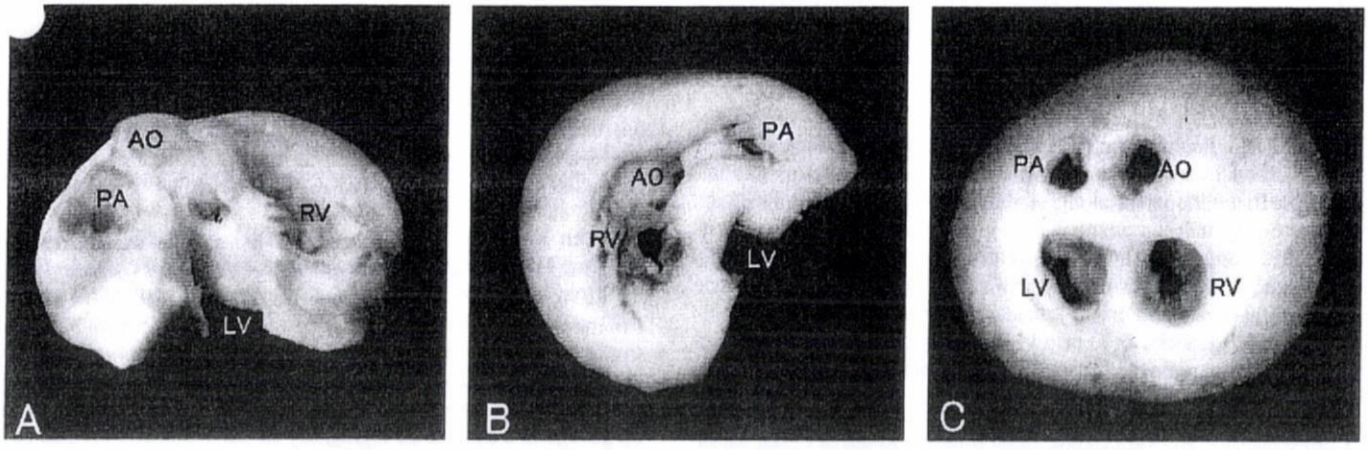


**Fig. 1** Three types of cardiovascular malformations induced in fetuses from dams administered PB at a dose of 120 mg/kg for 2 consecutive days during gestation. (A) Overriding aorta with VSD: the aorta arising from the biventricles. (B) Double outlet right ventricular with VSD: both the aorta and pulmonary artery arising from the right ventricle. (C) Transposition of great artery: the aorta arising from right ventricle and pulmonary artery arising from left ventricle. AO, PA. RV and LV indicate aorta, pulmonary artery, right ventricle and left ventricle, respectively.

**Table 1** Reproductive parameters of dams administered PB by gavage at a dose of 80 or 120 mg/kg/day on two consecutive days during gestation

| Dose (mg/kg/day) | 0 | 80 | | | | 120 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Administration period (GD) | 7–11 | 7–8 | 8–9 | 9–10 | 10–11 | 7–8 | 8–9 | 9–10 | 10–11 |
| No. dams | 8 | 7 | 7 | 7 | 7 | 8 | 8 | 8 | 8 |
| No. corpora lutea (total no.) | 18.9 ±1.6 (151) | 17.9 ±2.5 (125) | 19.4 ±1.6 (136) | 19.1 ±2.0 (134) | 18.4 ±2.2 (129) | 18.0 ±1.9 (144) | 18.8 ±2.4 (150) | 16.9 ±3.4 (135) | 16.8 ±2.2 (134) |
| No. implantations (total no.) | 17.3 ±3.6 (138) | 15.7 ±1.3 (110) | 16.4 ±2.3 (117) | 16.7 ±2.2 (117) | 16.3 ±1.7 (114) | 16.3 ±1.8 (130) | 15.9 ±1.6 (127) | 14.8 ±2.4 (118) | 14.8 ±2.4 (118) |
| No. intrauterine death (total no.) | 0.5 ±0.8 (4) | 1.4 ±1.7 (10) | 1.6 ±1.5 (11) | 0.9 ±0.9 (6) | 0.4 ±0.5 (3) | 1.4 ±1.1 (11) | 2.9 ±5.0 (23) | 1.8 ±0.9 (14) | 3.3 ±3.4 (26) |
| Postimplantation loss no. (%)† | 2.8 ±4.1 | 9.7 ±12.3 | 9.8 ±10.0 | 5.5 ±5.9 | 2.7 ±3.4 | 8.7 ±8.1 | 18.8 ±33.3 | 12.3 ±6.4 | 24.5 ±28.3* |
| No. live fetuses (total no.) | 16.8 ±3.5 (134) | 14.3 ±2.7 (100) | 15.1 ±2.6 (106) | 15.9 ±2.6 (111) | 15.9 ±1.8 (111) | 14.9 ±2.2 (119) | 13.0 ±5.5 (104) | 13.0 ±2.7 (104) | 11.5 ±4.8 (92) |
| Fetal body weight (g) | | | | | | | | | |
| Males (total no.) | 3.11 ±0.23 (65) | 3.24 ±0.21 (49) | 3.08 ±0.31 (53) | 3.05 ±0.13 (56) | 3.08 ±0.22 (49) | 3.18 ±0.28 (57) | 3.05 ±0.26 (49) | 3.07 ±0.41 (60) | 3.13 ±0.53 (53) |
| Females (total no.) | 2.99 ±0.31 (69) | 3.12 ±0.23 (51) | 3.03 ±0.20 (53) | 2.98 ±0.09 (55) | 3.00 ±0.24 (62) | 3.02 ±0.26 (62) | 2.93 ±0.21 (55) | 2.90 ±0.31 (44) | 3.03 ±0.42 (39) |

*Significantly different from the control at $P < 0.05$.
†(No. implantations – No. live fetuses/No. implantations) × 100
Values are expressed as means ±S.D.

**Table 2** Types and incidences of external, visceral and skeletal anomalies in fetuses from the dams administered PB by gavage at a dose of 80 or 120 mg/kg/day on two consecutive days during gestation

| Dose (mg/kg/day) | 0 | 80 | | | | 120 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Administration period (GD) | 7–11 | 7–8 | 8–9 | 9–10 | 10–11 | 7–8 | 8–9 | 9–10 | 10–11 |
| No. dams | 8 | 7 | 7 | 7 | 7 | 8 | 8 | 8 | 8 |
| External observations | | | | | | | | | |
| No. fetuses examined | 133 | 100 | 106 | 111 | 111 | 119 | 104 | 104 | 92 |
| No. fetuses with malformations | 0 | 0 | 0 | 1 (0.9) | 1 (0.9) | 0 | 0 | 2 (2.0) | 0 |
| Visceral observations | | | | | | | | | |
| No. fetuses examined | 86 | 65 | 68 | 72 | 72 | 76 | 67 | 66 | 59 |
| No. fetuses with malformations | 1 (1.2) | 3 (4.6) | 1 (1.5) | 2 (2.8) | 2 (2.8) | 3 (3.9) | 24 (35.8**) | 31 (47.0**) | 14 (23.7**) |
| Isolated ventricular septal defect (VSD) | 0 | 1 1.5 | 1 (1.5) | 1 (1.4) | 1 (1.4) | 1 (1.3) | 7 (10.4**) | 13 (19.7**) | 5 (8.5*) |
| Overriding aorta | 0 | 2 (3.1) | 0 | 0 | 0 | 0 | 3 (4.5) | 6 (9.1*) | 1 (1.7) |
| Double outlet right ventricle | 0 | 0 | 0 | 0 | 0 | 0 | 6 (9.0*) | 2 (3.0) | 1 (1.7) |
| Transposition of great arteries | 0 | 0 | 0 | 1 (1.4) | 0 | 0 | 4 (6.0) | 9 (13.6**) | 0 |
| Branching of the right subclavian artery from aorta | 1 (1.2) | 0 | 0 | 0 | 0 | 0 | 9 (13.4**) | 4 (6.1) | 2 (3.4) |
| Retroesophageal subclavian artery | 0 | 1 (1.5) | 0 | 0 | 0 | 0 | 0 | 2 (3.0) | 5 (8.5*) |
| Common atrioventricular canal | 0 | 0 | 0 | 0 | 0 | 2 (2.6) | 2 (3.0) | 0 | 0 |
| Supernumerary azygos vein | 0 | 0 | 0 | 0 | 0 | 1 (1.3) | 0 | 0 | 0 |
| Atrial septal defect | 0 | 0 | 0 | 0 | 1 (1.4) | 2 (2.6) | 1 (1.5) | 0 | 1 (1.7) |
| Persistent truncus arteriosus | 0 | 1 (1.5) | 0 | 1 (1.4) | 0 | 0 | 0 | 1 (1.5) | 0 |
| Others† | 0 | 0 | 0 | 0 | 0 | 2 (2.6) | 1 (1.5) | 1 (1.5) | 3 (5.1) |
| No. fetuses with variations | 10 (12.8) | 9 (13.8) | 4 (5.9) | 9 (12.5) | 7 (9.7) | 10 (13.2) | 10 (14.9) | 20 (30.3*) | 25 (42.4**) |
| Thymic remnant in the neck | 10 (12.8) | 8 (12.3) | 4 (5.9) | 6 (8.3) | 6 (8.3) | 9 (11.8) | 6 (9.0) | 15 (22.7) | 24 (40.7**) |
| Persistent left umbilical artery | 0 | 0 | 0 | 0 | 0 | 1 (1.3) | 3 (4.5) | 6 (9.1) | 1 (1.7) |
| Dilated renal pelvis | 0 | 1 (1.5) | 0 | 3 (4.2) | 1 (1.4) | 0 | 2 (3.0) | 1 (1.5) | 0 |
| Skeletal observations | | | | | | | | | |
| No. fetuses examined | 47 | 35 | 38 | 39 | 39 | 43 | 37 | 38 | 33 |
| No. fetuses with malformations | 0 | 0 | 0 | 0 | 0 | 1 (2.3) | 0 | 0 | 0 |
| No. fetuses with variations | 0 | 2 (5.7) | 5 (13.2*) | 6 (15.4*) | 8 (20.5*) | 10 (23.3**) | 26 (70.3**) | 33 (86.8**) | 18 (54.5**) |
| Splitting of ossification centers of thoracic vertebral bodies | 0 | 0 | 0 | 2 (5.1) | 3 (7.7) | 3 (7.0) | 8 (21.6**) | 16 (42.1**) | 7 (21.2**) |
| Dumbbell shape of ossification centers of thoracic vertebral bodies | 0 | 1 (2.9) | 0 | 1 (2.6) | 3 (7.7) | 1 (2.3) | 8 (21.6**) | 18 (47.3**) | 6 (18.2**) |
| Extra 14th ribs | 0 | 0 | 0 | 0 | 0 | 0 | 1 (2.7) | 3 (7.9) | 0 |
| Rudimentary 14th ribs | 0 | 0 | 5 (13.2*) | 3 (7.7) | 2 (5.1) | 3 (7.0) | 17 (45.9**) | 14 (36.8**) | 7 (21.2**) |
| Others‡ | 0 | 1 (2.9) | 0 | 0 | 0 | 3 (7.0) | 4 (10.8) | 2 (5.3) | 1 (3.0) |

*Significantly different from the control at $P < 0.05$.

**Significantly different from the control at $P < 0.01$.

†Included cor triloculare, small left ventricular chamber, double aortic arch, right-sided aortic arch, vascular ring, situs invs. and abnormal lung lobation; ‡included splitting of ossification centers of lumbar vertebral bodies, dumbbell shape of ossification centers of lumbar vertebral bodies, rudimentary cervical ribs and asymmetry of the sternebrae.

The percent value is indicated in parenthesis.

nificantly increased in the groups administered PB at 120 mg/kg on GD 8–9, 9–10 and 10–11. Almost all the malformations observed in the fetuses from the PB-administered dams originated from the cardiovascular system. The malformations observed at high frequency included the isolated ventricular septal defect (VSD), overriding aorta, double outlet right ventricle, transposition of great arteries, branching of the right subclavian artery from the aorta and retroesophageal right subclavian artery. Three representative types of cardiovascular malformations, overriding aorta, transposition of great arteries and double outlet right ventricle are illustrated in Figure 1. These malformations were mostly accompanied by VSD. The incidence of visceral variations was also increased in the groups administered PB at 120 mg/kg on GD 9–10 and 10–11. The increased type of variation was thymic remnant in the neck.

Although the incidence of skeletal malformations was not increased in any PB-administered group, the incidences of skeletal variations were increased in fetuses from the dams administered PB at 80 mg/kg on GD 8–9, 9–10 and 10–11, and from all the dams administered PB at 120 mg/kg. The principal skeletal variations included splitting or dumbbell shape of ossification centers of thoracic vertebral bodies and rudimentary 14th rib. In addition, delayed ossification of interparietal, maxilla and cervical vertebra were observed at high frequency in fetuses from the PB-administered dams (Data not shown).

**Cardiovascular malformations observed in pups**

Table 3 shows the effects on pups from the dams administered PB at a dose of 120 mg/kg/day on 2 consecutive days during gestation. No developmental parameters of pups were affected by prenatal PB exposure when observed on PND 0. However, both the number of live pups and viability tended to decrease in the groups administered PB on GD 9–10 or 10–11, when observed on PND 4.

Table 4 shows the incidences of cardiovascular malformations in pups from the dams administered PB at 120 mg/kg/day on two consecutive days during gestation. Numbers of dead or moribund pups bearing cardiovascular malformations were significantly increased in the groups administered PB on GD 8–9, 9–10 or 10–11. The malformations observed at high frequency included overriding aorta, double outlet right ventricle, transposition of great arteries and patent ductus arteriosus. The number of pups bearing cardiovascular malformations culled on PND 4 was significantly increased in the group administered 120 mg/kg PB on GD 10–11. The isolated VSD was the most commonly observed malformation. Incidences of cardiovascular malformations observed on PND 21 were significantly increased in the groups treated with PB on GD 8–9, 9–10 and 10–11. Those increased incidences were primarily attributed to the increased number of isolated VSD.

## DISCUSSION

**Maternal toxicity of PB**

Two-day administration of PB by gavage at a dose of 80 or 120 mg/kg/day to pregnant rats did not affect body weight gain but induced clinical signs such as loss of righting reflex, ataxic gait and decreased locomotor activity of dams. Terada *et al.* (1987) reported that administration of PB to pregnant rats at 80 mg/kg/day by gavage on GD 7–17 induced neurotoxicologic signs and retarded body weight gain. Vorhees (1983) reported that the body weights of pregnant rats administered PB at 80 mg/kg/day on GD 7–18 were decreased, whereas a daily dose of 125 mg/kg for 12 days caused deaths of the pregnant rats. It is considered therefore that the present dose levels and repetitions of PB did not induce systemic toxicologic signs such as retarded body weight gain in the dams, but elicited pharmacologic actions of PB that caused the clinical signs.

**Table 3** Developmental effects on pups from the dams administered PB by gavage at a dose of 120 mg/kg/day on two consecutive days during gestation

| Dose (mg/kg/day) | 0 | 120 | | |
|---|---|---|---|---|
| Administration period (GD) | 8–11 | 8–9 | 9–10 | 10–11 |
| No. dams with live pups | 9 | 9 | 10 | 9 |
| Gestation length (day) | 22.0 ±0.0 | 22.0 ±0.0 | 22.2 ±0.4 | 22.1 ±0.3 |
| No. implantations (total no.) | 15.0 ±2.6† (135) | 14.9 ±1.8 (134) | 13.9 ±4.7 (139) | 14.2 ±3.5 (128) |
| PND 0 | | | | |
| No. neonates born (total no.) | 14.3 ±2.3 (129) | 13.2 ±1.8 (119) | 13.0 ±4.3 (130) | 13.0 ±3.2 (117) |
| No. live neonates (total no.) | 14.2 ±2.4 (128) | 12.9 ±1.9 (116) | 12.1 ±3.9 (121) | 12.6 ±3.2 (113) |
| Pups weight (g) | 6.3 ±0.8 | 6.1 ±0.5 | 5.9 ±0.8 | 5.9 ±0.5 |
| PND 4 | | | | |
| No. live pups (total no.) | 12.9 ±3.0 (116) | 11.6 ±3.0 (104) | 9.4 ±4.2 (94) | 9.3 ±3.0 (84) |
| Viability (%)† | 90.8 ±15.6 | 88.8 ±16.3 | 78.6 ±37.1 | 77.6 ±24.2 |
| No. pups after culling (total no.) | 8.0 ±0.0 (72) | 7.8 ±0.7 (70) | 6.5 ±1.7 (65) | 7.1 ±1.2 (64) |
| Pups weight after culling (g) | 10.1 ±1.6 | 9.4 ±1.1 | 9.2 ±1.9 | 8.9 ±1.7 |
| PND 21 | | | | |
| No. live pups (total no.) | 8.0 ±0.0 (72) | 7.8 ±0.7 (70) | 6.5 ±1.7 (65) | 7.0 ±1.1 (63) |
| Viability (%)‡ | 100.0 ±0.0 | 100.0 ±0.0 | 100.0 ±0.0 | 98.6 ±4.2 |
| Pups weight (g) | 56.7 ±5.5 | 55.0 ±3.0 | 53.6 ±6.1 | 52.1 ±10.0 |

†(No. live pups on PND 4/No. live neonates on PND 0) × 100.
‡(No. live pups on PND 21/No. of live pups on PND 4) × 100.
Values are expressed as means ±SD.

**Table 4** Incidence of cardiovascular malformations found in pups from the dams administered PB by gavage at a dose of 120 mg/kg on two consecutive days during gestation

| Dose (mg/kg/day) | 0 | 120 | | |
|---|---|---|---|---|
| Administration period (GD) | 8–11 | 8–9 | 9–10 | 10–11 |
| No. dams | 9 | 9 | 10 | 9 |
| No. pups examined (A + B + C) | 123 | 116 | 122 | 105 |
| Pups with cardiovascular malformations | 0 (0) | 24 (20.7)** | 36 (35.2)** | 33 (31.4)** |
| No. pups died or sacrificed for moribund during breast-feeding (A) | 3 | 12 | 31 | 23 |
| Pupa with cardiovascular malformations | 0 (0) | 12 (100)** | 27 (87.1)** | 19 (82.6)** |
| Isolated ventricular septal defect (VSD) | 0 | 0 | 4 (12.9) | 0 |
| Overriding aorta | 0 | 3 (25.0) | 11 (35.4) | 10 (43.5) |
| Double outlet right ventricle | 0 | 2 (16.7) | 3 (9.7) | 5 (21.7) |
| Transposition of great arteries | 0 | 2 (16.7) | 5 (16.1) | 0 |
| Patent ductus arteriosus | 0 | 2 (16.7) | 6 (19.4) | 7 (30.4) |
| Persistent atrioventricular canal | 0 | 1 (8.3) | 1 (3.2) | 0 |
| Coarctation of aorta | 0 | 2 (16.7) | 0 | 0 |
| Branching of the right subclavian artery from aorta | 0 | 0 | 1 (3.2) | 1 (4.3) |
| Retroesophageal subclavian artery | 0 | 0 | 0 | 2 (8.7) |
| Persistent truncus arteriosus | 0 | 1 (8.3) | 0 | 0 |
| Atrophy left atrial chamber | 0 | 0 | 1 (3.2) | 0 |
| Vascular ring | 0 | 0 | 0 | 1 (4.3) |
| Supernumerary azygos vein | 0 | 1 (8.3) | 0 | 0 |
| No. pups alive during breast-feeding (B + C) | 120 | 104 | 91 | 82 |
| Alive neonates with cardiovascular malformations | 0 (0) | 12 (11.5)** | 9 (9.9)** | 14 (17.1)** |
| No. pups culled at PND 4 (B) | 48 | 34 | 26 | 20 |
| Pups with cardiovascular malformations | 0 (0) | 4 (11.8) | 2 (7.7) | 3 (15.0)* |
| Isolated VSD | 0 | 1 (2.9) | 1 (2.2) | 3 (15.0)* |
| Overriding aorta | 0 | 1 (2.9) | 0 | 0 |
| Double outlet right ventricle | 0 | 0 | 1 (6.6) | 0 |
| Branching of the right subclavian artery from aorta | 0 | 1 (2.9) | 0 | 0 |
| No. pups at PND 21 (C) | 72 | 70 | 65 | 62 |
| Pups with cardiovascular malformations | 0 (0) | 8 (11.4)** | 7 (10.8)** | 11 (17.7)** |
| Isolated VSD | 0 | 7 (8.6)* | 4 (6.2) | 8 (12.9)** |
| Overriding aorta | 0 | 0 | 1 (1.5) | 0 |
| Double outlet right ventricle | 0 | 0 | 0 | 1 (1.6) |
| Patent ductus arteriosus | 0 | 0 | 1 (1.5) | 1 (1.6) |
| Right-sided aortic arch | 0 | 0 | 0 | 1 (1.6) |
| Vascular ring | 0 | 0 | 0 | 1 (1.6) |
| Branching of the right subclavian artery from aorta | 0 | 1 (1.4) | 0 | 0 |

*Significantly different from control group at $P < 0.05$.
**Significantly different from control group at $P < 0.01$.
The percent value is indicated in parenthesis.

**Fetal toxicity of PB**

Maternal exposure to PB at a dose of 120 mg/kg/day on two consecutive days during gestation increased postimplantation loss, whereas a dose of 80 mg/kg did not affect the viability of fetuses. The present finding of no increased postimplantation loss in the dams administered PB at 80 mg/kg/day is compatible with the results of the two previous studies by Vorhees (1983) and Terada *et al.* (1987).

Cardiovascular malformations were increased in fetuses from the dams administered PB at 120 mg/kg/day on GD 8–9, 9–10 and 10–11, but not in any fetus from the dams administered PB at 80 mg/kg/day. The malformations observed in the present study were

characterized by overriding aorta, double outlet right ventricle and transposition of great arteries. Terada *et al.* (1987) reported that maternal exposure to PB 80 mg/kg/day on GD 7–17 caused VSD, overriding aorta and double outlet right ventricle in rat fetuses but did not induce transposition of great arteries. On the other hand, Vorhees (1983) reported that maternal exposure to PB at 80 mg/kg/day on GD 7–18 marginally increased the incidences of incomplete ventricular septum and ringed aorta in rat fetuses. The difference between the two studies and the present study might be attributable to differences in dose levels and the administration period of PB. In addition, the differences between Vorhees's (1983) study and the present one can be attributed partly to a methodologic difference in the detection of cardiovascular malformations. Wilson's (1965) method which Vorhees (1983) used for detection of cardiovascular malformations was to prepare 1 mm-thick transverse slices of fetal trunk by freehand razor blaze sectioning in the regions from the shoulder joint through the thoracic and abdominal cavities and organs. On the other hand, Nishimura's method used in the present study was to examine the great vessels, the heart and its transverse sections with a dissecting microscope after fixation with Bouin's solution. Nishimura (1974) argued that Wilson's method was not suitable for the detection of subtle cardiovascular malformations, because of the freehand transverse sectioning of the fetal trunk.

Severe cardiovascular malformations, such as transposition of the great arteries, the double outlet right ventricle and the overriding aorta, induced at high frequency by the PB administration on GD 8–10 in the present study, are in agreement with the types and severities of cardiovascular malformations which were reported to be induced at high frequency in mouse fetuses (Davis & Sadler 1981; Irie *et al.* 1990; Yasui *et al.* 1995; Shoji *et al.* 2005) and in hamster fetuses (Taylor *et al.* 1980) from the dams administered retinoic acid. These severe cardiovascular malformations induced by maternal exposure to retinoic acid appeared to be similar to those induced in rat fetuses from the dams administered PB, as reported by Terada *et al.* (1987) and in the present study.

Furthermore, Shoji *et al.* (2005) reported that excessive exposure of mouse dams to tretinoin (retinoic acid) induced craniofacial anomalies in fetuses, such as micrognathia and sacral caudal anomalies which were termed as DiGeorge–Velocardiofacial Syndromes in the human case. Teratologic studies with laboratory animals demonstrated PB-induced cleft palate in mice (Walker & Patterson 1974; Sullivan & McElhatton 1975), skeletal malformation in rats (McColl *et al.* 1963) and skull defects in rabbits (McColl 1967). The types of retinoic acid-induced skeletal anomalies (Shoji *et al.* 2005) were found to be different from those of the skeletal anomalies in fetuses from the PB-administered dams reported in the rat studies by Terada *et al.* (1987) and McColl *et al.* (1963, 1967) and in the present study, as well as in the mouse studies by Walker and Patterson (1974) and Sullivan and McElhatton (1975). Further study will be needed to explore any causative factor to elucidate possible differences and similarities in types of anomalies between retinoic acid and PB, and to further clarify the mechanisms underlying the PB-induced cardiovascular malformations and skeletal anomalies, as suggested by a cellular hypothesis for formation of the retinoic acid-induced cardiovascular malformations proposed by Yasui *et al.* (1995).

**Postnatal fate of cardiovascular malformations**

The tendency toward decrease in viability during the period from PND 0–4 in the PB-administered groups found in the present study was consistent with the results of Vorhees's (1983) study. Vorhees reported that the number of postnatal deaths before weaning increased in rat pups from the dams administered PB on both GD 7–10 and GD 11–14, but not in pups from those treated with PB on GD 15–18. The period of GD in which on the increased number of postnatal deaths was observed in Vorhees's study was similar to the critical period for induction of the life-threatening cardiovascular malformations in fetuses found in the present study. However, Vorhees did not further examine the types of cardiovascular malformations that caused the increased mortality in the prenatal and preweaning offspring. Since the neonates were found to suffer from overriding aorta, double outlet right ventricle and transposition of great arteries in the present study, it can be inferred on the basis of the present study that the postnatal deaths resulted from the life-threatening cardiovascular malformations.

On the other hand, isolated VSD was not observed frequently in the dead pups from the PB-administered dams. It has been reported that VSDs did not affect postnatal survival in the rat pups of TMD 400 mg/kg-treated dams, and that the VSDs closed spontaneously during the neonatal period (Solomon *et al.* 1997). However, the postnatal fate of the PB-induced cardiovascular malformations was characterized by persistence of isolated VSD. The present results are in good agreement with those of Fleeman *et al.* (2004) who demonstrated that TMD-treatment-induced VSDs were closed postnatally, and the timing of closure and survivability depended on the severity of the VSDs.

VSD, overriding aorta, double outlet right ventricle, transposition of great arteries and persistent truncus arteriosus found in the present study may be considered to be part of an anatomical continuum in order of increasing severity, resulting from deficiency in the conotruncal region of the fetal heart (Daft *et al.* 1986; Veuthey *et al.* 1990). Severity of these cardiovascular malformations was reported to be based on varying degrees of malrotation of the great vessels with subsequent channeling of blood in improper pathways and on the degree of conotruncal deficiency (Daft *et al.* 1986). Recently, Hoffman and Kaplan (2002) reported an attempt to categorize congenital heart disease into three forms, minor, moderately severe and severe, in order to estimate the variations in reported incidences of congenital heart disease.

The present findings are compatible with a previous report showing that human pregnancies with exposure to phenobarbital monotherapy are associated with increased risk of infant malformations, including coarctation of the aorta with abnormal valves, ventricular septal defect and tetralogy of Fallot (Holmes *et al.* 2004). Although the teratogenic PB dose found in the present study was much higher than the reported human therapeutic dose (Kaneko 1991; Moore *et al.* 2000), the present findings would provide an animal-experimental basis for assessing the increased risk of congenital heart disease in human offspring from mothers who use PB as monotherapy or in combination.

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