Apoptosis and estrogen deficiency in primary Sjögren syndrome

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Purpose of review

Primary Sjögren syndrome is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La. The purpose of this review is to discuss recent advances in the pathogenesis of primary Sjögren syndrome.

Recent findings

Although several candidate autoantigens including α-fodrin have been reported in Sjögren syndrome, the pathogenic roles of the autoantigens in initiation and progression of SS are still unclear. It is possible that individual T cells activated by an appropriate self antigen can proliferate and form a restricted clone. Recent evidence suggests that the apoptotic pathway plays a central role in tolerizing T cells to tissue-specific self antigen, and may drive the autoimmune phenomenon. Cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune response. The studies reviewed imply that Fas-mediated cytotoxicity and caspase-mediated α-fodrin proteolysis are involved in the progression of tissue destruction in Sjögren syndrome. Fas ligand (FasL), and its receptor Fas are essential in the homeostasis of the peripheral immune system. It can be considered that a defect in activation-induced cell death of effector T cells may result in the development of autoimmune exocrinopathy in Sjögren syndrome.

Summary

Although the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear, it is possible that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens.

Keywords

Sjögren syndrome, apoptosis, activation-induced cell death, estrogen deficiency

Curr Opin Rheumatol 16:522–526. © 2004 Lippincott Williams & Wilkins.

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This work was supported in part by Grants-in-Aid for Scientific Research (Nos. 12307040 & 12557022) from the Ministry of Education, Science and Culture of Japan.

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Current Opinion in Rheumatology 2004, 16:522–526

Abbreviations

AICD	activation-induced cell death
FasL	Fas ligand
SS	Sjögren syndrome
TCR	T-cell antigen receptor

1040–8711

Introduction

Organ-specific autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance [1,2]. Sjögren syndrome (SS) is a T-cell-mediated autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La [3,4]. Autoreactive T cells bearing CD4 molecule may recognize unknown autoantigen triggering autoimmunity in the salivary and lacrimal glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) [5]. It is now evident that the interaction of Fas with FasL regulates a large number of pathophysiological processes of apoptosis including autoimmune diseases [6]. Recent studies have now confirmed the observation that apoptotic cells in various cell types are implicated as the source of autoantigen when stimulated with different proapoptotic stimuli [7,8]. Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases [9], accumulated evidences suggest an important role of apoptosis in disease pathogenesis of Sjögren syndrome [10].

Apoptotic cells in Sjögren syndrome

Recent studies have suggested that the Fas-Fas ligand (FasL) system plays a major role on the induction of apoptosis in target organs with autoimmune diseases such as autoimmune gastritis, Hashimoto thyroiditis, and rheumatoid arthritis [11,12]. It has been reported that both Fas and FasL are present in thyrocytes, and their concomitant expression on thyrocytes, independent of infiltrating T cells, is responsible for thyrocyte destruction in Hashimoto thyroiditis [13]. In contrast, expression of Fas by pancreatic β cells has been shown to have a major influence on the susceptibility of tissue destruc-

tion in nonobese diabetic (NOD) mice to diabetes [14,15•]. Since it was reported that Fas expression was observed in the salivary gland cells with human Sjögren syndrome [16], it was likely that Fas-mediated apoptosis may contribute to tissue destruction in the salivary glands with Sjögren syndrome. A cleavage product of 120-kDa α-fodrin was identified as an important autoantigen in human Sjögren syndrome besides NFS/sld murine model for Sjögren syndrome [17]. α-Fodrin is a ubiquitous, calmodulin-binding protein [18] found to be cleaved by calcium-activated protease (calpain) in apoptotic T cells, and by calpain or caspase 3 [19] in anti-Fas-stimulated Jurkat cells or neuronal apoptosis [20]. It was demonstrated that the fodrin α subunit is cleaved in association with apoptosis, and the 120-kDa fragment is a breakdown product of the mature form of 240-kDa fodrin-α subunit [20,21]. Previous studies have demonstrated evidence that caspase 3 is required for α-fodrin cleavage during apoptosis [21]. In Jurkat cells, caspase 3-like proteases have been reported to cleave α-fodrin and poly (ADP-ribose) polymerase [21]. The observation that ubiquitously expressed autoantigens (e.g., α-fodrin, La, and nuclear mitotic apparatus protein) in Sjögren syndrome are specifically cleaved by granzyme B strongly suggests that a common biochemical event (novel autoantigen cleavage during granule-induced epithelial cell death) is responsible for selecting the unconnected group of molecules [22].

In vitro studies demonstrated that apoptotic mouse salivary gland (MSG) cells result in a specific α-fodrin cleavage into 120 kDa, and preincubation with caspase-inhibitor peptides blocked α-fodrin cleavage [23]. A significant increase of TUNEL$^+$-apoptotic epithelial duct cells in the salivary glands was detected in NFS/sld Sjögren syndrome mouse model. MSG cells constitutively express Fas with high proportion, and tissue-infiltrating CD4$^+$ T cells isolated from the salivary gland tissues bear a large proportion of FasL. Importantly, the tissue-infiltrating CD4$^+$ T cells, but not CD8$^+$ T cells, are responsible for tissue destruction as judged by *in vitro* ^{51}Cr release cytotoxic assay against MSG cells *in vitro*. Although it has been reported that Fas-induced apoptosis seems to be the major killing pathway of the CD4$^+$ cytotoxic T cells [24], one mechanism by which activated CD4$^+$ T cells induce cytotoxicity towards salivary gland cells in Sjögren syndrome is Fas based. *In vivo* treatment with caspase-inhibitors, z-VAD-fmk and DEVD-CHO, into murine model results in dramatically inhibitory effects on the development of autoimmune lesions, and in restoration of sicca syndrome [23]. There is increasing evidence that the cascade of caspases is a critical component of the cell death pathway [25,26], and a few proteins have been found to be cleaved during apoptosis. These include poly (ADP-ribose) polymerase, a small U1 nuclear ribonucleoprotein, and α-fodrin, which were subsequently identified as substrates for caspases [27,28]. The development of autoimmune exocrinopathy in Sjögren syndrome appears to be dependent on autoantigen cleavage through caspase cascade, and caspase-inhibitors might provide a new therapeutic option directed at reducing tissue damage.

T cell apoptosis in Sjögren syndrome

Activation-induced cell death (AICD) is a well-known mechanism of peripheral T-cell tolerance that depends upon an interaction between Fas and FasL [29]. AICD plays a central role, especially in killing autoreactive T cells and in preventing autoimmune responses [30]. It has been reported that activation of T-cell clones induces FasL expression, and AICD in autoreactive T cells *in vivo* has been proposed to limit the expansion of an immune response by eliminating effector cells [31••]. Although it can be considered that a defect in AICD of effector T cells may result in the development of autoimmune disease [32], an *in vivo* role of organ-specific autoantigen for AICD is entirely unclear. Because the administration of a soluble form of anti-FasL antibody (FLIM58) results in severe destructive autoimmune exocrinopathy in a murine model of Sjögren syndrome [33], it is possible that an organ-specific autoantigen may play an important role on down-regulation of AICD. A high titer of serum autoantibodies against 120-kD α-fodrin autoantigen was detected in the FLIM58-treated mice, and splenic T-cell culture supernatants contained high level of interferon-γ. FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from the murine Sjögren syndrome model, but not from Fas-deficient MRL/lpr mice and FasL-deficient MRL/gld mice. FasL undergo matrix metalloproteinase–mediated proteolytic processing in their extracellular domains, resulting in the release of soluble trimeric ligands (soluble FasL [sFasL]). In this case, the processing of sFasL occurs in autoantigen-specific CD4$^+$ T cells, and a significant increase in expressions of metalloproteinase-9 mRNA was observed in spleen cells from Sjögren syndrome mouse model [33]. The increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector CD4$^+$ T cells in the murine SS model. Previous studies have demonstrated that CD4$^+$ T cells are susceptible to AICD induced through T-cell receptor–mediated recognition of allogeneic MHC class II molecules, supporting the notion that AICD can be triggered in activated T cells through the T-cell receptor–mediated recognition of antigen [34,35]. Mice or human individuals lacking functional Fas or FasL display profound lymphoproliferative reactions associated with autoimmune disorders [36]. In proteoglycan-induced arthritis, CD4$^+$ T cells proliferate at a high rate in response to proteoglycan stimulation, and exhibit a Th1-type response [37]. These observations have suggested that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of Sjögren syndrome.

Estrogen deficiency in Sjögren syndrome

Sex hormones influence both humoral and cell-mediated immune response, and estrogen is one of the potential factors in this immunologic dimorphism [38,39]. Estrogenic action has been suggested to be responsible for the strong female preponderance of autoimmune diseases including systemic lupus erythematosus and SS [40,41]. Although a number of autoimmune diseases are known to develop in postmenopausal women, the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear. Previous reports indicate that the increase in autoantibody production as a result of estrogen deficiency is mediated by cytokines such as interleukin-6, interferon-γ (interferon-γ, and tumor necrosis factor-α (TNF-α), and that estrogen plays an important role in the regulation of B lymphocyte development in mouse bone marrow and activation of human monocytes [42–44]. Estrogen deficiency induced by ovariectomy accelerates destructive autoimmune lesions, and these lesions were recovered by estrogen administration in an SS mouse model [45]. It was demonstrated that the dysfunction of regulatory T cells caused by estrogen deficiency may play a crucial role in acceleration of organ-specific autoimmune lesions, and that estrogenic action influences target epithelial cells through Fas-mediated apoptosis [45]. It was also demonstrated that interferon-γ-induced Fas expression on these cells was reduced by the addition of estrogens. Previous studies have shown that physiologic concentration of estrogens augmented the activity of the interferon-γ promotor in mitogen-stimulated murine spleen cells [46], and the administration of exogenous estrogens could induce Fas-mediated apoptosis not only in cultured cells but also *in vivo* [47]. Several reports have demonstrated that estrogen may play an inhibitory role on apoptosis in endothelial cells, breast cancer cells, cardiac myocytes, prostate cells, and neuronal cells [48,49].

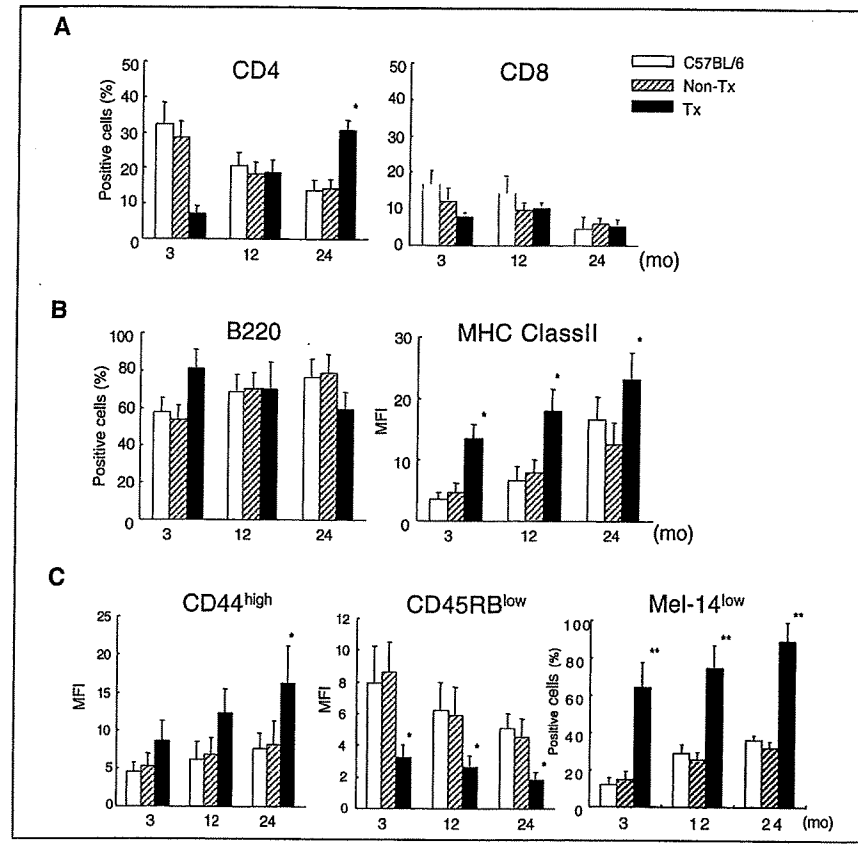
Previous studies concerning gender differences in autoimmunity have suggested that estrogen influences the cytokine production of effector cells and autoantibody production [50,51]. The distinct immune environments in males and females underlie many of the gender-related differences in autoimmunity. These environments are established by the cytokines that are released by immune cells, particularly T helper (Th) lymphocytes. Sex hormones, pituitary hormones including prolactin, and growth hormones, as well as liver-derived insulin-like growth factor-1 affect autoimmune diseases by modulating cytokine productions [52]. Estrogen withdrawal after menopause leads to an increase in the production of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1, interleukin-6, and tumor necrosis factor-α [53]. Although many studies have described the effects of estrogen on cytokine production in effector cells, much less is known about the effect of estrogen deficiency in target organs of postmenopausal women. Recent data demonstrated significant apoptosis associated with α-fodrin cleavage in the salivary gland cells of estrogen-deficient healthy C56BL/6(B6) mice [54••]. Interestingly, inflammatory lesions developed exclusively in the salivary and lacrimal gland after the adoptive transfer with α-fodrin-reactive T cells in both ovariectomized B6 and ovariectomized SCID mice. It has been suggested that α-fodrin cleavage triggered by estrogen deficiency plays an important role in the development of autoimmune exocrinopathy in SS. In *in vitro* studies using primary cultured MSG and human salivary gland cells (HSG), a cleavage product of 120-kD α-fodrin was detected in cells that had undergone tamoxifen (Tam)-induced apoptosis, not in other type of cells including MCF-7 [54••]. Because pretreatment with estrogen inhibits the Tam-induced apoptosis of MSG and HSG cells, estrogen may play a crucial role in the apoptosis-related signal pathway. A recent report by Morkuniene *et al.* [55] has shown that 17β-estradiol prevents calcium-induced release of cytochrome c from heart mitochondria. When we analyzed whether cysteine proteases are involved in Tam-induced apoptosis of HSG cells, we observed a time-dependent increase in the active forms of caspase 1. In addition, the promoter activity of caspase 1 was significantly increased when HSG cells transfected with the promoter-caspase 1 gene were stimulated with Tam.

Conclusion

A cleavage product of 120-kDa α-fodrin was identified as an important organ-specific autoantigen in human SS. The data discussed in this review are strongly suggestive of essential roles of caspase cascade for α-fodrin autoantigen cleavage leading to tissue destruction in autoimmune exocrinopathy in SS. α-Fodrin cleavage by caspases can potentially lead to cytoskeletal rearrangement, and it is of interest to point out that α-fodrin binds to ankylin, which contains a cell death domain [56]. It has been shown that cleavage products of α-fodrin inhibit ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles [57], assuming that a cleavage product of 120 kDa α-fodrin could be a novel component of an unknown immunoregulatory networks such as cytolinker proteins [58]. *In vitro* T-cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from murine SS. The processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells *in vivo*, and a significant increase in expressions of metalloproteinase-9 mRNA was observed in spleen cells from mouse model. These data indicate that the increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cell (Fig. 1). Moreover, antiestrogenic actions have a potent effect on the proteolysis of α-fodrin autoantigen through up-regulation of caspase 1 activity. It has been strongly suggested that α-fodrin fragments induced by estrogen deficiency may

Figure 1. An organ-specific autoantigen may play an important role on down-modulation of AICD

A cleavage product of 120-kD α-fodrin in the target cells could be induced by estrogen deficiency during apoptosis through caspase activation, in particular caspase 1. Activation-induced cell death (AICD) results from the interaction between Fas and FasL, and activated T cells expressing both Fas and FasL are usually killed either by themselves or by interacting with each other. FasL undergo matrix metalloproteinase (MMP)–mediated proteolytic processing in their extracellular domains, resulting in the release of soluble FasL (sFasL). FasL-mediated AICD is down-regulated by autoantigen stimulation, indicating that the increased generation of soluble FasL inhibits the normal AICD process, leading to the proliferation of autoreactive $CD4^+$ T cells. A defect in AICD may result in the development of autoimmune diseases.



play an important role in the development of autoimmune lesions in SS. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are being further investigated.

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Exp. Anim. 53(2), 171–173, 2004

—Note—

Differences in Responsiveness of Mouse Strain against p-Benzoquinone as Assessed by Non-Radioisotopic Murine Local Lymph Node Assay

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Abstract: *The non-radioisotopic modification of murine local lymph node assay (LLNA) by using 5-bromo-2'-deoxyuridine (BrdU) was conducted to investigate the strain-related difference of the responsiveness of mice to* p*-benzoquinone (PBQ) with BALB/cAnN, CBA/JN and CD-1 mouse strains. Strain and dose related differences were analyzed by two-way analysis of variance (two-way ANOVA). CBA/JN was considered to be the highest responsive strain to PBQ, and interaction was detected between CD-1 and each of the other inbred strains. These results support the recommendation in the OECD test guideline 429 and the skin sensitization test guideline of US-EPA with regard to the selection of mouse strain for LLNA.*

Key words: *local lymph node assay, responsiveness,* p*-benzoquinone*

Contact dermatitis caused by chemicals is a serious health problem, and a prediction of the skin sensitizing potential of chemicals is necessary to secure safe handling of chemicals. The guinea pig maximization test and the Buehler test have been widely used for predicting the skin sensitizing potentials of chemicals for regulatory purposes for a long time [1, 6]. Recently the murine local lymph node assay (LLNA) has been recognized as a new stand-alone sensitization test which can be used for regulatory purposes [3–5], and it is based upon consideration of the induced proliferative responses in lymph nodes draining the site of topical exposure to the test chemical. In the standard LLNA, cell proliferation is measured using the incorporation of radiolabeled thymidine or uridine into draining lymph node cells, and this requires specific facilities and handling conditions. We previously developed a non-radioisotopic alternative method for the LLNA which uses 5-bromo-2'-deoxyuridine (BrdU) incorporation in place of radioisotopes [9, 10]. The responsiveness of mouse strains against antigen is known to vary with their H-2 haplotypes. We report here the difference of responsiveness of three mouse strains in the modified murine local lymph node assay against *p*-benzoquinone, a known potent contact allergen to human.

p-Bebzoquinone (BZQ, Lot No. 012D2294, Kanto Chemical Co., Tokyo, Japan) was dissolved in acetone:olive oil (AOO; 4:1). 5-Bromo-2'-deoxyuridine (BrdU, Nacalai Tesque, Kyoto, Japan) was dissolved in

(Received 22 September 2003 / Accepted 25 December 2003)

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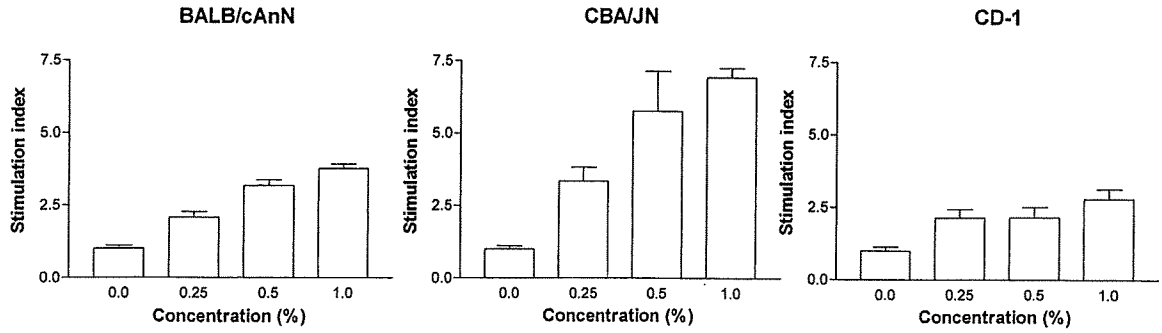

Fig. 1. Dose responses in the modified local lymph node assay with *p*-benzoquinone.
*Data are represented as the mean ± standard error.

physiological saline at a concentration of 10 mg/ml. Female CBA/JN, BALB/cAnN and CD-1 mice were obtained from Charles River Japan Ltd. (Kanagawa, Japan). Mice were housed in animal rooms maintained at a temperature of 23 ± 2°C and relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10 to 15 cycles/h, and lighted artificially for 12 h daily.

Mice were randomly allocated to 4 groups (4 mice/group) per each strain of mouse. A 25 µl volume of PBQ in AOO was applied to the dorsum of both ears of the mice daily for three consecutive days. A single intraperitoneal injection (5 mg/mouse/injection) of BrdU was made on day 4. On day 5, auricular lymph nodes were removed, weighed, and stored at –20°C until analysis by an ELISA to measure BrdU incorporation.

BrdU incorporation into the lymph node cells was determined using a commercial cell proliferation assay kit (Boehringer Mannheim Corp., Indianapolis, IN, USA, Cat. No. 1647229). Lymph nodes were crushed, and after passage through a #70 nylon mesh, the cells were suspended in 15 ml of physiological saline. The cell suspension (100 µl) was added to the wells of a flat-bottom microplate (Coster 3595, Corning Inc., NY, USA) in quadruplicate. After centrifugation (3000 × g, 10 min), the supernatants were removed, 200 µl of Fix-Denat solution was added to each well, and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 µl, Boehringer Mannheim Corp.) was added to each well, and after rinsing 3 times with washing buffer (phosphate-buffered saline), 100 µl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined as the BrdU labeling index with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. Means and standard errors for the labeling indices were calculated for each treatment group, and the stimulation index (SI) was calculated by dividing the labeling indices in each test group by that in the concurrent vehicle control group. Then, two-way analysis of variance (Two-way ANOVA) was performed with regard to dose and strain as factors.

Dose responses against PBQ for each mouse strain are shown in Fig. 1. The SI values for 0.25%, 0.5% and 1% PBQ were 3.4, 5.8 and 6.9 in CBA/JN, 2.1, 3.2, and 3.8 in BALB/cAnN, and 2.1, 2.2 and 2.8 in CD-1, respectively. The SI values increased in a dose dependent manner in all mouse strains. Positive responses (SI>3) were noted ≥0.25% in CBA/JN, ≥0.5% in BALB/cAnN and >1.0% in CD-1. As the results of two-way ANOVA, CBA/JN showed the highest responsiveness to PBQ, and interaction was noted between CD-1 and each of the other inbred strains (Table 1). Consequently, CBA/JN was considered to be the highest responder strain to PBQ, and CD-1 is not a preferable strain for LLNA. In the OECD and US-EPA guidelines [2, 8], and the ICCVAM validation report for LLNA [7], CBA/Ca or CBA/J mouse are recommended for selection of animal species. In this study, the CBA/JN mouse showed the highest responsiveness to PBQ among three mouse strains tested. Our result supports the animal selection described in the test guidelines and the review article mentioned above.

Table 1. Probabilities detected in two-way analysis of variance (two-way ANOVA)

Source of Variation	CBA/JN vs. BALB/cAnN	CBA/JN vs. CD-1	BALB/cAnN vs. CD-1
Interaction	0.2209 ns	0.0076 **	0.0125 *
Strain	0.0385 *	0.0001 ***	<0.0001 ***
Dose	<0.0001 ***	<0.0001 ***	<0.0001 ***

Asterisks indicate significance levels (*: P<0.05, **: P<0.01, ***: P<0.001). ns: not significant.

Acknowledgments

We wish to thank Ms. Nobuko Mitoma, Ms. Naoko Kuga and Ms. Satoko Sakamoto for their skillful technical assistance.

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JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. **24**, 77–81 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jat.951

Assessment of the Skin Sensitization Potency of Eugenol and its Dimers using a Non-radioisotopic Modification of the Local Lymph Node Assay

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Key words: eugenol, local lymph node assay, non-radioisotopic, potency, sensitization.

Allergic contact dermatitis is a serious health problem. There is a need to identify and characterize skin sensitization hazards, particularly with respect to relative potency, so that accurate risk assessments can be developed. For these purposes the murine local lymph node assay (LLNA) was developed. Here, we have investigated further a modification of this assay, non-radioisotopic LLNA, which in place of tritiated thymidine to measure lymph node cell proliferation employs incorporation of 5-bromo-2′-deoxyuridine. Using this method we have examined the skin sensitizing activity of eugenol, a known human contact allergen, and its dimers 2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA) and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB). Activity in the guinea pig maximization test (GPMT) also measured. On the basis of GPMT assays, eugenol was classified as a mild skin sensitizer, DHEA as a weak skin sensitizer and DHEB as an extreme skin sensitizer. In the non-radioisotopic LLNA all chemicals were found to give positive responses insofar as each was able to provoke a stimulation index (SI) of ≥3 at one or more test concentrations. The relative skin sensitizing potency of these chemicals was evaluated in the non-radioisotopic LLNA by derivation of an EC_3 value (the concentration of chemical required to provoke an SI of 3). The EC_3 values calculated were 25.1% for eugenol, >30% for DHEA and 2.3% for DHEB. Collectively these data suggest that assessments of relative potency deriving from non-radioisotopic LLNA responses correlate well with evaluations based on GPMT results. These investigations provide support for the proposal that the non-radioisotopic LLNA may serve as an effective alternative to the GPMT where there is a need to avoid the use of radioisotopes. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

Allergic contact dermatitis is an important occupational and environmental health problem and there is a continuing need to identify accurately potential skin sensitization hazards and to assess effectively the likely risks to human health. Various methods have been developed for the assessment of skin sensitization potential, including those using guinea pigs, such as the guinea pig maximization test (GPMT) (Magnusson & Kligman, 1969) and Buehler's occluded patch test (Buehler, 1995), and more recently the murine local lymph node assay (LLNA) (Kimber *et al.*, 1994, 1995; Loveless *et al.*, 1996; Gerberick *et al.*, 2000). In the GPMT and the Buehler's occluded patch test the skin sensitizing potential is determined as a function of challenge-induced reactions in previously sensitized guinea pigs, whereas the LLNA is based upon consideration of induced proliferative responses in lymph nodes draining the site of topical exposure to the test chemical. In addition to hazard assessment, attention has focused more recently on evaluation of the relative skin sensitization potency as a first step in the risk assessment process. The view is that the LLNA is particularly suited to this application, not least because it is known that the vigour of lymphocyte proliferative responses induced in skin-draining lymph nodes correlates closely with the extent to which sensitization will develop.

In the standard LLNA a chemical is classified as a skin sensitizer if at one or more test concentrations it is able to induce a threefold or greater increase in lymph node cell proliferation, i.e. a stimulation index (SI) of ≥3. For the purposes of evaluating relative potency, an EC_3 value is derived mathematically from consideration of LLNA dose responses, EC_3 being the amount of contact allergen necessary to induce an SI of 3. Although the LLNA has proved to be a robust and reliable method for evaluation of

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sometimes limited its application is the need for a radioisotope. In the standard LLNA, lymph node cell proliferation is measured on the basis of incorporation by cells of [^{3}H]thymidine (^{3}HTdR).

We have previously explored the utility of a modified version of the assay in which, in place of radiolabelled thymidine, cell turnover is measured using the incorporation of 5-bromo-2′-deoxyuridine (BrdU) (Takeyoshi *et al.*, 2001). Here we describe investigations to explore further the value of this non-radioisotopic LLNA for the purposes of hazard identification and the determination of relative potency. To this end, responses to three chemicals have been measured: eugenol, a known contact allergen, and its dimers 2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA) and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB). For comparative purposes the activity of each of these three chemicals was also measured using the GPMT.

EXPERIMENTAL

Chemicals and reagents

Eugenol (lot no. EG0704; >95%), 2,2′-dihydroxyl-3,3′-dimethoxy 5,5′-diallyl-biphenyl (DHEA: lot no. DHEA0704; >95%) and 4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB: lot no. DHEB0704; >95%) were kindly donated by Kanebo Cosmetics Company (Odawara, Kanagawa, Japan) (Fig. 1). Eugenol and its dimers were dissolved in olive oil for the GPMT or in acetone–olive oil (AOO, 4 : 1) for the non-RI LLNA. 5-Bromo-2′-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg ml^{-1}.

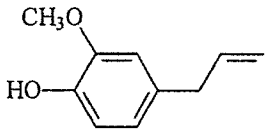
Animals

Female Hartley guinea pigs and CBA/JN strain mice were obtained from SLC Japan Ltd (Shizuoka, Japan) and Charles River Japan Ltd (Kanagawa, Japan), respectively. The animals were housed in animal rooms maintained at a temperature of 22 ± 3 °C and at a relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10–15 cycles per hour and lighted artificially for 12 h daily. Animals were allowed free access to a laboratory diet (RC-4 for guinea pigs and MF for mice; Oriental Yeast Co., Tokyo, Japan) and tap water.

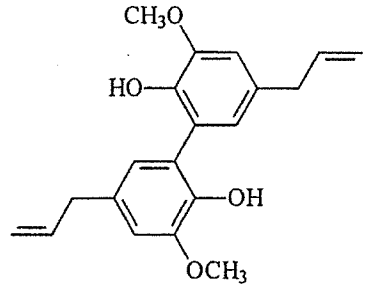
Experimental designs

Guinea pig maximization test. Guinea pigs were allocated randomly to three groups (10 animals per group). The test was conducted according to a method described previously (Magnusson & Kligman, 1969). Guinea pigs received a series of intradermal injections of eugenol or its dimers in the shoulder region to induce sensitization. After 6–8 days, sensitization was boosted by a 48-h occluded patch of the same compound placed over the injection sites. Fourteen days later, the animals were challenged on a shaved flank by a 24-h occluded patch containing the same compound. All induction and challenge concentrations were set at 5% (maximum non-irritant concentration) in olive oil for all compounds in view of preliminary dose-finding tests. All compounds elicited an apparent irritation at 10% in preliminary tests for intradermal injection and topical application, so we decided on induction and challenge concentrations of 5% for all compounds in order to compare the sensitization potency of these three compounds. Chemicals were classified by the sensitization rate for each chemical (0–8%, weak; 9–28%, mild; 29–64%, moderate; 65–80%, strong; 81–100%, extreme) according to the criteria given by Magnusson and Kligman (1969).

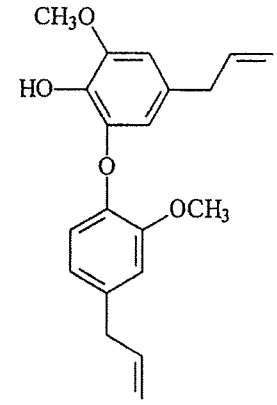
Non-radioisotopic LLNA. Mice were allocated randomly to 11 groups (four animals per group). A 25-µl volume of test chemicals at concentrations of 1%, 6%, 15% or 30% for eugenol, 1%, 6% or 30% for DEHA and 1%, 6% or 20% for DEHB was applied to the dorsum of both ears of the mice daily for three consecutive days. The concentration ranges of each test chemical were decided according to the sensitization potencies classified by the results of GPMT. A single intraperitoneal injection (5 mg per mouse per injection) of BrdU was then given on day 4. On day 5, the draining auricular lymph nodes were



Eugenol



2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA)
- Eugenol dimer 1 -



4,5′-diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB)
- Eugenol dimer 2 -

removed, weighed and stored at –20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure BrdU incorporation. The incorporation of BrdU into lymph node cells was determined using a commercial cell proliferation assay kit (Boehringer Mannheim Corp., Indianapolis, IN, USA; Cat. no. 1647229). The lymph nodes were crushed, passed through a no. 70 nylon mesh and the lymph node cells were suspended in 15 ml of physiological saline individually. The cell suspension (100 µl) was added to the wells of a flat-bottom microplate (Coster 3595; Corning Inc., NY, USA) in triplicate. After centrifugation (3000 *g*, 10 min), the supernatants were removed. A 200-µl volume of Fix-Denat solution was added to each well and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 µl, Boehringer Mannheim Corp.) was added to each well and, after rinsing three times with washing buffer (phosphate-buffered saline), 100 µl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labelling index.

Statistical analysis

Means and standard errors were calculated for the labelling index obtained by ELISA for each treatment group. The SI values relative to the AOO-treated control value were then calculated. Data were analysed simultaneously using the Bartlett test for homogeneity of variance. If the variances were homogeneous at a level of 5% significance, a one-way analysis of variance (one way-ANOVA) was performed. If the one-way ANOVA produced a significant difference, the differences between the control group and each of the experimental groups were analysed using the Dunnett test. If the variances were not homogeneous, the Kruskal–Wallis test was employed. If this test produced a significant difference, the difference between the control group and each of the experimental groups was analysed using the non-parametric Dunnett test (Bruning & Kintz, 1997).

Measurement of EC_3 values

The estimated concentration of a chemical required to induce an SI of 3 relative to vehicle-treated controls (EC_3 value) was derived by linear interpolation as described previously (Basketter *et al.*, 2000). The EC_3 value was calculated by interpolating between two points on the SI axis, one immediately above and one immediately below the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a, b) and (c, d), respectively, then the EC_3 value may be calculated using the following equation:

$$EC_3 = c + [(3 - d)/(b - d)](a - c)$$

RESULTS

Guinea pig maximization test

In the GPMT for eugenol and its dimers, the sensitization response rates were as follows: eugenol, 20%; DHEA, 0%; DHEB, 100%. According to convention (Magnusson & Kligman, 1969) therefore, eugenol was classified as a mild skin sensitizer; DHEA as a weak skin sensitizer and DHEB as an extreme skin sensitizer (Table 1).

Non-radioisotopic LLNA

At application concentrations of 15% or greater, exposure of mice to eugenol caused a significant increase in draining lymph node weight compared with concurrent vehicle-treated controls (see Table 2). A positive response with respect to lymph node cell proliferation was obtained with 30% eugenol (SI = 3.3). Significant increases in the incorporation of BrdU were observed following treatment with both 15% and 30% eugenol, but at the lower concentration this did not translate into a positive response with respect to the stimulation index (SI = 2.3). The DHEA dimer failed to induce a positive response in the nonradioisotopic LLNA at any concentration tested, although at the highest concentration (30%) there was a significant increase in BrdU incorporation compared with vehicle-treated controls. The highest concentrations of DHEA were without effect on draining lymph node weight, although at the lowest concentration of this dimer tested there was a significant increase. Finally, the second dimer (DHEB) provoked clear positive responses in the non-radioisotopic LLNA. Treatment with 6% DHEB resulted in SI = 5.0 and treatment with 20% DHEB produced SI = 7.2. At both of these test concentrations there was also a statistically significant increase in the incorporation by lymph node cells of BrdU compared with controls. At all concentrations of DHEB examined there were significant increases in lymph node weight.

The EC_3 values for eugenol and DHEB were calculated using a standard method and were found to be 25.1% and 2.3%, respectively. Such an approach was not possible with DHEA because at no test concentration was a positive response elicited in the non-radioisotopic LLNA with respect to an SI of ≥3. An EC_3 value for DHEA has therefore been estimated, for the purposes of comparison, by linear extrapolation of the dose–response curve. Although this is somewhat unconventional, it is considered acceptable in

Table 1—Results of the guinea pig maximization test for eugenol and its dimers

Chemical name	Sensitization rate (%)	Classification[a]
Eugenol	20	Mild
2,2′-Dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl (DHEA)	0	Weak
4,5′-Diallyl-2′-hydroxy-2,3′-dimethoxy phenyl ether (DHEB)	100	Extreme

Table 2—Results[a] of non-radioisotopic local lymph node assay with eugenol and its dimers used in this study

Group	Concentration (%)	Lymph node weight (mg)			BrdU labelling index ($A_{370-490}$)			
		Mean	SEM	SI[b]	Mean	SEM	SI[b]	EC_3
Vehicle control (AOO)	—	2.150	0.155	—	0.107	0.010	—	
	1	3.325	0.485	1.5	0.187	0.031	1.7	
	6	3.325	0.578	1.5	0.161	0.031	1.5	
Eugenol	15	5.175*	0.085	2.4	0.251*	0.028	2.3	25.1
	30	5.650**	0.517	2.6	0.355**	0.048	**3.3**	
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-	1	3.400**	0.426	1.6	0.182	0.018	1.7	
diallyl-biphenyl (DHEA)	6	3.050	0.393	1.4	0.183	0.039	1.7	>30
	30	2.675	0.229	1.2	0.242*	0.043	2.3	
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxy	1	4.400*	0.705	2.0	0.244	0.053	2.3	
phenyl ether (DHEB)	6	5.675**	0.612	2.6	0.538**	0.073	**5.0**	2.3
	20	9.800**	0.610	4.6	0.774**	0.057	**7.2**	

[a] Results represent mean and standard errors in four mice. Significant differences from vehicle control: * $P < 0.05$ and ** $P < 0.01$ (Dunnett's test).
[b] The stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group. The cases showing three or greater SI values were defined as positive (in bold type).

the context of these comparative studies. The EC_3 value estimated for DHEA was >30%.

DISCUSSION

Eugenol (a component of clove oil and nutmeg oil) has antibacterial and antioxidative effects and has utility (due to its whitening and antibacterial effects) in the cosmetic and dermatological fields (Yamazaki *et al.*, 1998, 2000). It is regarded as being a relatively weak skin sensitizer in humans and is known to cause allergic contact dermatitis in some subjects (Basketter *et al.*, 2000, 2001; Gerberick *et al.*, 2000). Eugenol has been shown previously to induce positive responses in the standard LLNA and these present investigations have shown that it is also positive in the non-radioisotopic LLNA. The derivation of EC_3 values provides an opportunity to compare the two forms of the assay with respect to sensitivity. In one series of investigations eugenol was recorded as having as EC_3 value of ca. 13% (Basketter *et al.*, 2000). In the non-radioisotopic LLNA reported here, eugenol displayed a somewhat higher EC_3 value that might be indicative of somewhat lower sensitivity (for this chemical allergen at least) compared with the standard method. Colorimetric analysis, such as an ELISA for BrdU employed in this study, has an apparently narrower dynamic range compared with the scintillation counting for radiolabelled thymidine employed in the standard LLNA. The narrow dynamic range of the endpoint in the non-radioisotopic LLNA may be a source of lower sensitivity of this alternative. Notwithstanding this difference, the data suggest that the non-radioisotopic LLNA might have the equivalent sensitivity to the GPMT.

As far as we are aware, the dimers DHEA and DHEB have not been tested in the standard assay. In the non-radioisotopic LLNA, however, they exhibited clear and marked differences in skin sensitizing activity; the former had an estimated EC_3 value of >30% (and in fact failed to elicit a positive response with respect to an SI value of ≥3) whereas the derived EC_3 value for DHEB was 2.3%. It is instructive to compare these data with the results of GPMT analyses. The same ranking order was obtained with DHEB, the chemical displaying the greatest potency (lowest EC_3 value) of the chemicals tested, being classified as an extreme sensitizer in the GPMT. In the GPMT eugenol gave a 20% response rate (classification of mild) and DHEA gave a response rate for 0% (classification of weak). In addition, the results obtained in this study showed that the non-radioisotopic LLNA could detect the sensitization potential of a chemical classified in the lowest sensitizing potency in the GPMT by using the statistical endpoint. This suggests that the statistical endpoint can achieve an improvement in the sensitivity of this alternative. Taken together, therefore, the results obtained with the non-radioisotopic LLNA reflect what can be judged from GPMT data of the relative skin sensitizing potential of these chemicals.

In conclusion, the results reported here reveal that the sensitivity of the non-radioisotopic LLNA may not be very dissimilar from the standard method and that estimations of relative potency based on EC_3 values deriving from non-radioisotopic dose responses appear to provide an accurate picture of ranking. Experience to date suggests that the non-radioisotopic LLNA may be of value in circumstances where there is a need to assess skin sensitization activity without the use of radioisotopes. Moreover, non-radioisotopic LLNA apparently has an advantage over the GPMT with regard to points of animal welfare and it can provide a rapid and cost-effective method for screening sensitizers in the same way as the standard LLNA.

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doi:10.1111/j.1741-4520.2006.00109.x

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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Received October 3, 2005; revised and accepted February 8, 2006.

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 of 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 χ^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-

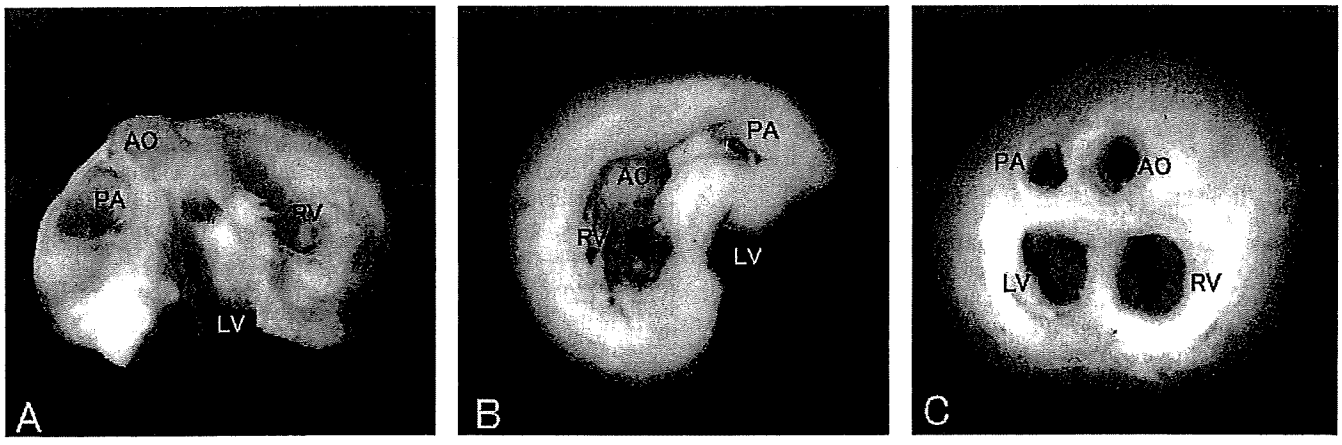

Fig. 1 Three types of cardiovascular malformations induced in fetuses from dams administered PB at a dose of 120 mg/kg for 2 consecutive days during gestation. (A) Overriding aorta with VSD: the aorta arising from the biventricles. (B) Double outlet right ventricular with VSD: both the aorta and pulmonary 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.

ACKNOWLEDGMENTS

We thank Dr T. Matsushima and Dr M. Mizutani for their encouragement and valuable advice in the present study and Dr H. Arito and Dr T. Takeuchi for their review of the manuscript.

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