Restricted BPA-specific up-regulation of Esrrg mRNA in the mesenchymal component of UGS
In E17 female UGM, the mRNA expression of *Esr1* was up-regulated by both BPA and DES treatment (Figure 5A). At E17, however, the mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the male UGS (Figure 5B). At P1, mRNA expression of *Ar* was up-regulated by both BPA and DES treatment in the female UGS (Figure 5B). In both the male and female, the up-regulation of *Esrrg* mRNA was observed at E17 and restricted in UGM of the BPA-treated group but not in UGE (Figure 5C). In both the male and female UGE, the expression of *Esrrg* mRNA was quite low and not up-regulated, even in the BPA-treated group. At E17, there was no difference in mRNA expression levels when comparing the untreated male UGS with that of the female.

BPA-specific increases of ESRRG-expressing cells in primary cultured UGM
In both the male and female, E17 UGM was primary cultured *in vitro*. Representative pictures of ESRRG-positive cells are shown in Figures 6A–6C. The ESRRG-positive staining was observed in both the nucleus and the cytoplasm of cultured UGM. The number of ESRRG-positive UGM was significantly increased only in the BPA-treated group and showed a 2.2-fold increase in males and a 1.6-fold increase in females (Figure 6D). There was no difference in the rate of positivity of ERRγ when comparing the untreated male UGM with that of the female.

BPA-specific up-regulations of Esrrg and steroidogenic enzyme mRNA in sex hormone-related organs
To investigate the BPA-specific up-regulations of *in situ* steroidogenesis in other organs, we first examined the changes in *Esrrg* mRNA expression in sex hormone-related organs, such as the cerebellum, heart, kidney, ovary, and testis. At P1, the mRNA expression of *Esr1* in the cerebellum, heart, kidney, and ovary, with the exception of the testis, was up-regulated by both BPA and DES treatment (Figure 7A). However, no significant difference in *Ar* mRNA expression was observed in all organs examined (Figure 7B). In the untreated group, the mRNA expression of *Esrrg* was not detected in the testis at E17 and P1 (Figure 7C). The up-regulation of *Esrrg* mRNA was observed at E17 and restricted to the cerebellum, heart, kidney, and ovary (Figure 7C). The BPA-specific up-regulations of *Cyp19a1*, *Cyp11a1*, and *Nr5a1* mRNA were observed only at P1 in the cerebellum, heart, kidney, and ovary, with the exception of the testis (Figure 8).

DISCUSSION
There has been increasing concern about the effects of EDCs such as BPA on human health [24]. Although the majority of EDCs have the potential to alter the functioning of the reproductive and endocrine system, the actual mechanism responsible for such alterations has not been identified thoroughly. BPA is of concern because its chemical structure resembles that of DES. Several studies have reported that BPA can mimic estrogen action: e.g., induction of vaginal cornification, uterine vascular permeability, growth and differentiation of the mammary gland, and synaptic plasticity in the hippocampus [25] [26] [27] [28]. In the prostate, alterations in normal development can produce permanent changes that persist throughout adulthood and may increase the risk of disease in later life [9]. Thus, our objective was to investigate the biological effects of low-dose BPA on the initial development of primary ducts in the fetal prostate.

During prostatic development, alteration of sex steroid hormone synthesis may be responsible for prostatic anomalies associated with fetal exposure to EDCs. In the present study, we showed that fetal exposure to low-dose BPA increased E_2 levels in P1 UGS of both the male and female, while DES-induced changes were not detected. This alteration was also correlated with increased activity of CYP19A1 (aromatase) in UGS at P1, suggesting the unique action of BPA for *in situ* steroidogenesis in UGS. The BPA-specific increase of E_2 levels in UGS at P1 was correlated with the following: (1) mRNA up-regulations of steroidogenic enzymes such as *Cyp19a1* and *Cyp11a1*; and (2) an increased number of aromatase-expressing mesenchymal cells of UGS (UGM). The enzyme CYP19A1 (aromatase) is responsible for *in situ* E_2 production and the crucial T/E_2 balance necessary for normal embryonic and fetal development, even in males. Our data presented here shows that the up-regulation of *Cyp19a1* mRNA in

BPA-treated UGM was comparable to changes in both *in situ* E_2 production and CYP19A1 (aromatase) activity.

In the present study, we demonstrated that the BPA-specific increase in steroidogenic enzyme mRNA and aromatase-expressing cell number were observed in the mesenchymal component of both the male and female UGS. During embryonic development, the mesenchymal component is involved in the induction and organogenesis of various organs including the prostate, mammary gland, lung, kidney, and pancreas. It has been well established that sub-populations of the mesenchymal component are a source of potent molecules that regulate epithelial growth and differentiation [29]. In the prostate, androgen-responsive signals derived from UGM permissively and instructively induce UGE to form primary ducts of the prostate [30].

Comparison between the neonatal male and female UGS shows a similarity in the condensed mesenchyme of the ventral areas: i.e., the ventral prostate mesenchyme (VPM) in the male and the ventral mesenchymal pad (VMP) in the female [31]. In the male, a defined mesenchyme VPM is specifically associated with ductal branching morphogenesis and cytodifferentiation of the ventral prostate. Females do not usually form a prostate. In a tissue recombination model, the female VMP induces prostate development in response to androgen [32], suggesting that cells within the female VMP have prostatic inductive activity. Moreover, an earlier tissue recombination study showed that the ability of the female UGS to respond to androgens in forming prostate was gradually lost between P1 and P5 [33]. These results suggest strongly that androgen-responsive regulatory molecules are expressed constitutively even in the female VMP. Although the female VMP forms in the absence of androgens, AR expression was observed in the neonatal female VMP in a similar pattern to that observed in the male VPM [34]. Therefore, the BPA-specific increase in E_2 levels might interact with the intracellular AR signaling in both the male VPM and female VMP. However, the morphological changes in neonatal female UGS had not yet been investigated.

Our results suggest that BPA has a stimulatory effect on *in situ* steroidogenesis in P1 UGS of both the male and female at low dose exposure levels. Recently, ESRRG is reported to bind strongly with BPA [35]. Susens *et al.* have reported that expression of ESRRG in the mouse is organ-specific, i.e., ESRRG is expressed in the brain, heart, kidney, and skeletal muscle but not in the lung, spleen, and testis [36]. Our findings showed that the up-regulations of *Cyp19a1* and *Cyp11a1* mRNA by BPA treatment were detected only in organs expressing *Esrrg* mRNA. These data suggest that the possibility of a stimulatory effect on *in situ* steroidogenesis by fetal exposure to low-dose BPA may be a concern not only in UGS, but also in organs expressing ESRRG, such as the brain, heart, kidney, and ovary. It is important to note that Takeda *et al.* have recently reported that ESRRG was detected in the human testis, suggesting that the distribution of ESRRG differs slightly between mice and humans [23].

In the present study, our results showed that the BPA-specific up-regulations of steroidogenic enzyme mRNA in UGS, cerebellum, heart, kidney, and ovary were observed only during the neonatal period (i.e., P0 and P1) but not during the prenatal period (i.e., E17 and E18). During pregnancy in rodents, large amounts of estrogens produced in the maternal ovaries are continuously delivered to the fetus through the placenta. After birth, however, the fetus may be released from the maternal high-estrogen environment. Thus, one possibility is that the maternal high-estrogen environment in pregnancy may protect the fetus from the effect of BPA on *in situ* steroidogenesis during the prenatal period. However, we did not investigate the effects of neonatal BPA treatment on *in situ* steroidogenesis.

The EDCs-induced alterations of the *in situ* estrogen environment depend on each compound. In addition to atrazine and dioxin, the organotin compound tributyltin (TBT) also increases E_2 production in human placental choriocarcinoma cells [37]. TBT has been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, which is a phenomenon known as imposex [38]. These studies suggest strongly that EDCs might affect fetal development not only by

mimicking the actions of sex steroid hormones but also by alteration of *in situ* steroidogenesis.

In the prostate, AR expressed in mesenchyme is required for directing growth and branching morphogenesis of epithelia, presumably by induction of growth factors [39]. In the present study, we showed that fetal exposure to BPA or DES increased *Ar* mRNA expression in E17 UGM of the male, while *Esr1* mRNA expression was up-regulated in E17 UGM of the female. Recently, Richter *et al.* have reported that *in vitro* BPA treatment stimulates *Ar* and *Esr1* mRNA expression in mesenchymal cells isolated from fetal mouse prostate [40]. Thus, our results support the idea that BPA-induced cell proliferation of the primary prostatic ducts may be caused by inducing *Ar* mRNA expression in the male UGM. In contrast, the induction of *Esr1* mRNA expression by BPA or DES may create a positive feedback loop in the female UGM. In future, further investigation for morphological analysis would be necessary to confirm the effects of up-regulated ESR1 in the female UGS.

In conclusion, we demonstrated the unique action of BPA in the mouse UGS. Specifically, we demonstrated that the increases in E_2 levels and CYP19A1 (aromatase) activity were observed in the BPA-treated UGS but not in the DES-treated UGS. Ricke *et al.* have recently reported that stromal hormone imbalance, a potential source of local E_2 production, may be responsible for prostatic disease such as benign prostatic hyperplasia (BPH) and prostate cancer (PCa) [41]. The data shown in the present study give rise to the concept that the development and differentiation of UGS in mouse fetuses is very sensitive to fetal exposure of low-dose BPA via the mother. Further investigation of various aspects of BPA-specific action is necessary to fully understand the role of BPA as an EDC.

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FIGURE LEGENDS

Figure 1 BPA-specific increases of E_2 levels and CYP19A1 (aromatase) activity in mouse UGS. E_2 levels (A) and CYP19A1 (aromatase) activity (B) were measured in the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS at E17 and P1. *, $p < 0.01$, **, $p < 0.001$ versus control.

Figure 2 BPA-specific up-regulations of steroidogenic enzyme and sex-determining gene mRNA in mouse UGS. The relative mRNA expressions of *Cyp19a1* (A), *Cyp11a1* (B), and *Nr5a1* (C) were determined in the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS between E17 and P1. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control at each time point. †, $p < 0.01$, ††, $p < 0.001$ versus control at E17.

Figure 3 Restricted BPA-specific up-regulations of steroidogenic enzyme and sex-determining gene mRNA in mesenchymal component of UGS. The relative mRNA expressions of *Cyp19a1* (A), *Cyp11a1* (B), and *Nr5a1* (C) were determined for UGE and UGM of the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS at E17 and P1. *, $p < 0.01$, **, $p < 0.001$ versus control.

Figure 4 BPA-specific increases of aromatase-expressing cells in primary cultured UGM. (A-C) Fluorescence signals were detected for the CYP19A1 (aromatase) protein in primary cultured UGM. The nuclei were identified by Ran staining. (D) The number of aromatase-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS, and the percentage of aromatase-positive cells was calculated from at least 10 areas. *, $p < 0.01$ versus control. Scale bar = 100 μm, magnification ×400.

Figure 5 Restricted BPA-specific up-regulation of *Esrrg* mRNA in mesenchymal component of UGS. The relative mRNA expressions of *Esr1* (A), *Ar* (B), and *Esrrg* (C) were determined in UGE and UGM of the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS at E17 and P1. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control.

Figure 6 BPA-specific increases of ESRRG-expressing cells in primary cultured UGM. (A-C) Fluorescence signals were detected for the ESRRG protein in primary cultured UGM. The nuclei were identified by Ran staining. (D) The number of ESRRG-positive cells was counted in primary cultured UGM of the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS, and the percentage of ESRRG-positive cells was calculated from at least 10 areas. *, $p < 0.01$ versus control. Scale bar = 100 μm, magnification ×400.

Figure 7 BPA-specific up-regulation of *Esrrg* mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Esr1* (A), *Ar* (B), and *Esrrg* (C) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis. *n.d.*, not detected. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control.

Figure 8 BPA-specific up-regulations of steroidogenic enzyme and sex-determining gene mRNA in sex steroid hormone-related organs. The relative mRNA expressions of *Cyp19a1* (A), *Cyp11a1* (B), and *Nr5a1* (C) were determined in sex steroid hormone-related organs of the untreated control (open bar), BPA-treated (closed bar), and DES-treated (slashed bar) UGS at E17 and P1. C, cerebellum; H, heart; K, kidney; O, ovary; T, testis. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control.

Table 1 Sequences of oligonucleotide primers used for the real-time PCR analyses

Gene	Primer
Gapdh	F: 5′-AAATGGTGAAGGTCGGTGTG-3′ R: 5′-TGAAGGGGTCGTTGATGG-3′
Cyp19a1	F: 5′-GCCCAATGAATTTACCCTCGAA-3′ R: 5′-AAGCCAAAAGGCTGAAAGTACCT-3′
Cyp11a1	F: 5′-TCGACTCCTCAGAACTAAGACCTG-3′ R: 5′-GTACCCTGGTGTCCTTTATAGCCT-3′
Nr5a1	F: 5′-CCTGGGCTGGCTACCTCTATC-3′ R: 5′-CGAACTAGAGCCAGAGGAGGAC-3′
Esr1	F: 5′-GCACAGGATGCTAGCCTTGTCTC-3′ R: 5′-AATTGTCACCAGCTTGCAGGTTC-3′
Ar	F: 5′-GGCGGTCCTTCACTAATGTCAACT-3′ R: 5′-CTGACTTGTGCATGCGGTACTCAT-3′
Esrrg	F: 5′-CCGAGAGTTGGTGGTTATCATTGG-3′ R: 5′-GGAAGACCCTCGCCGTGC-3′

Figure 1

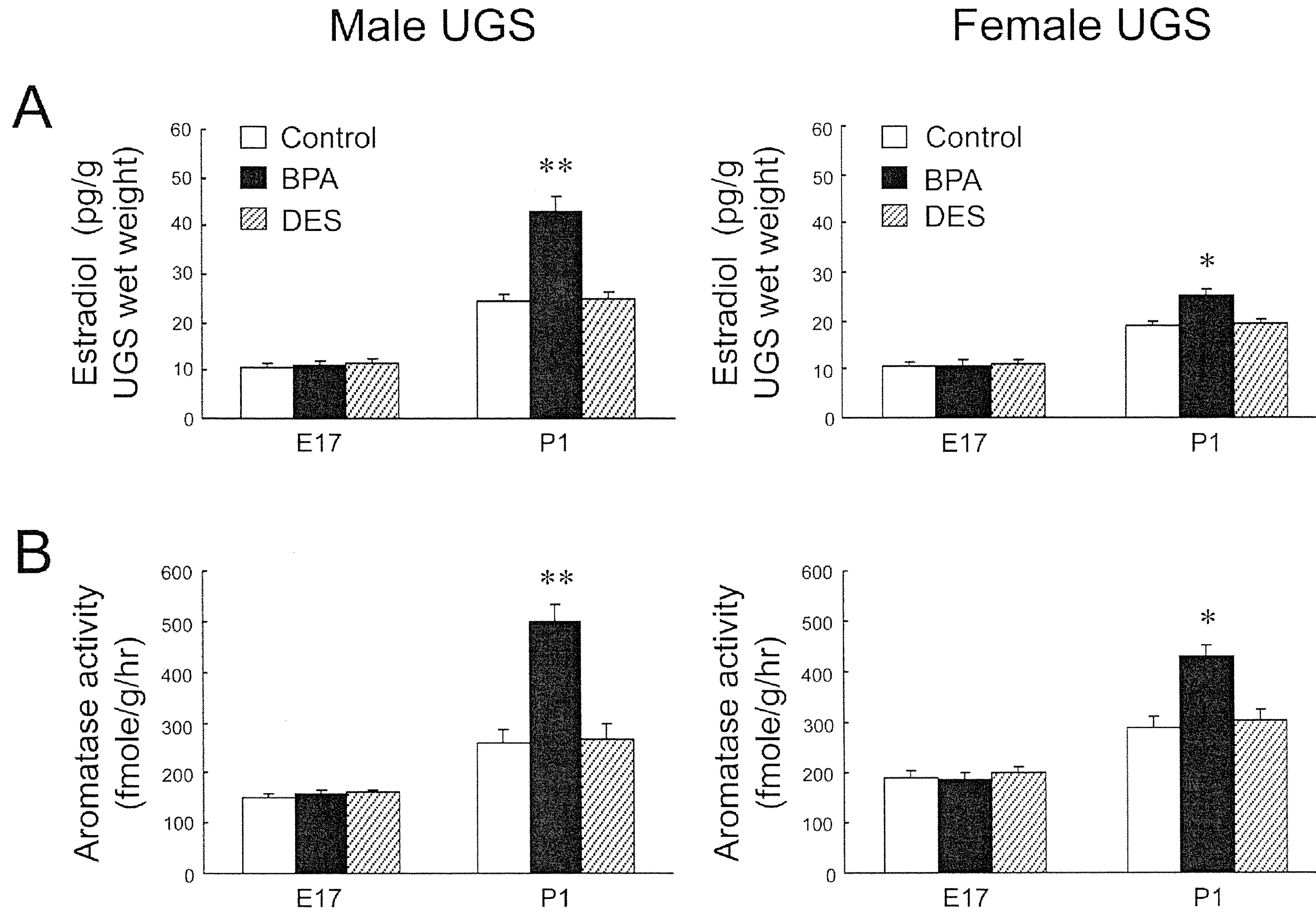


Figure 2

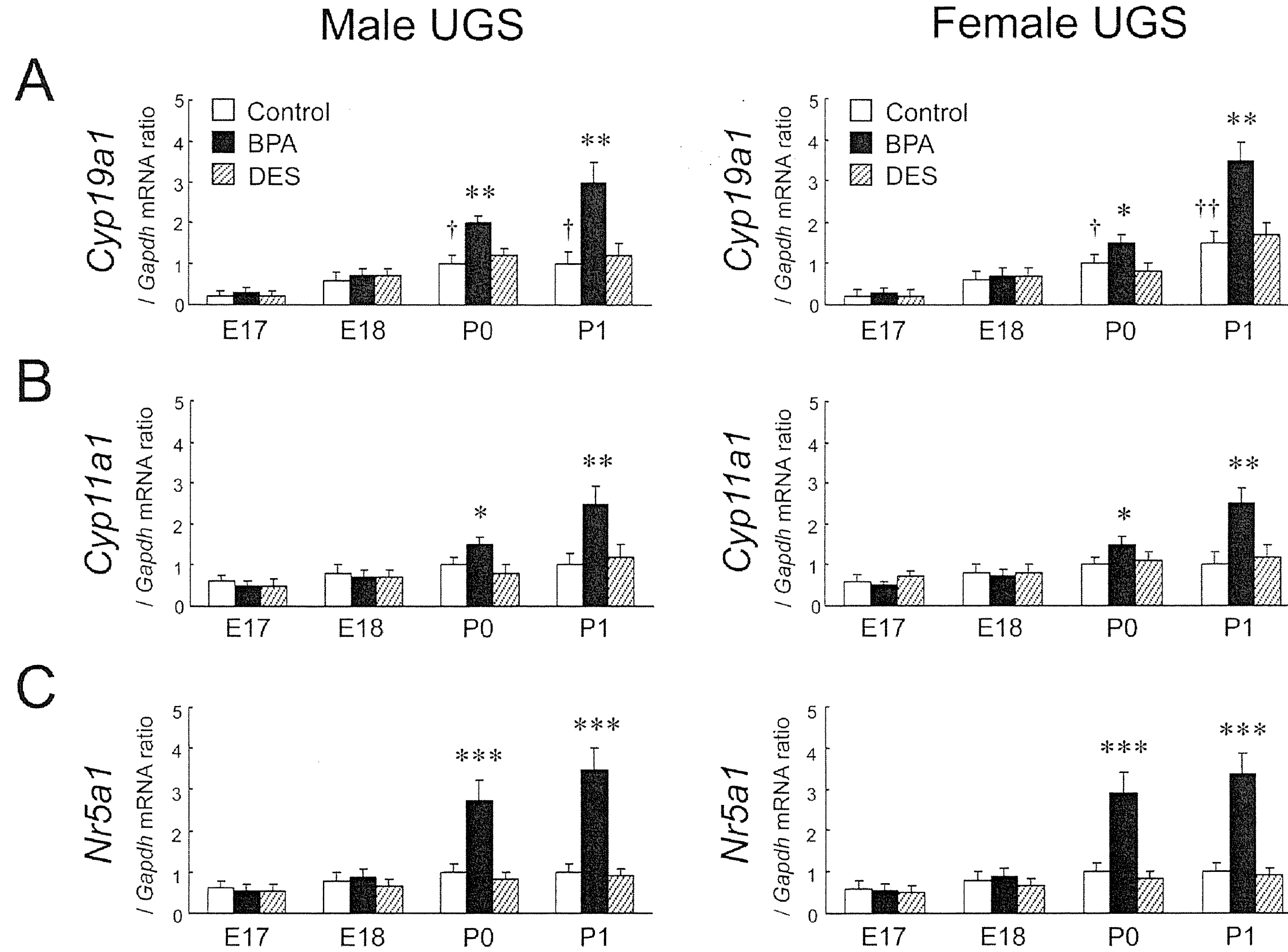


Figure 3

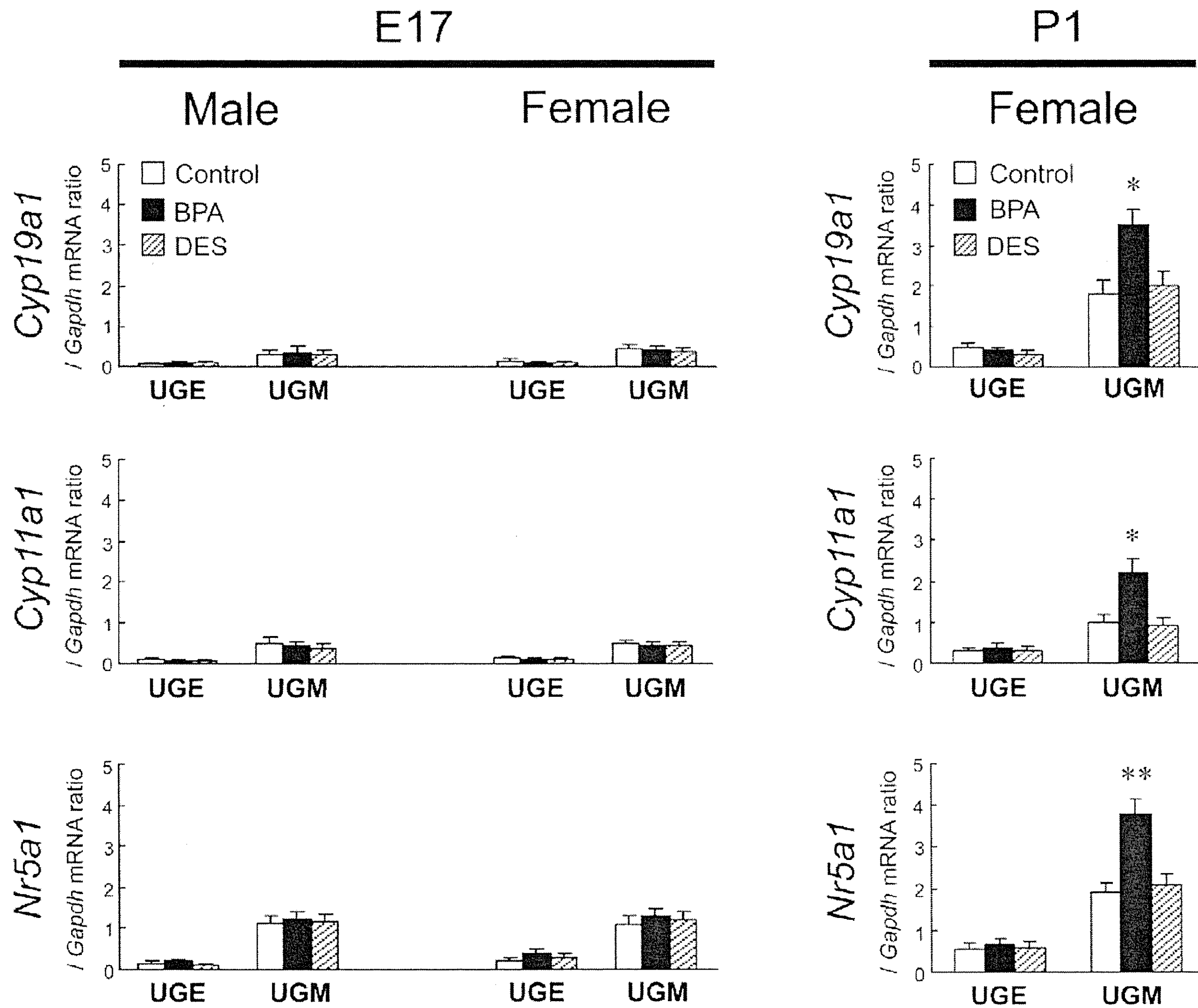
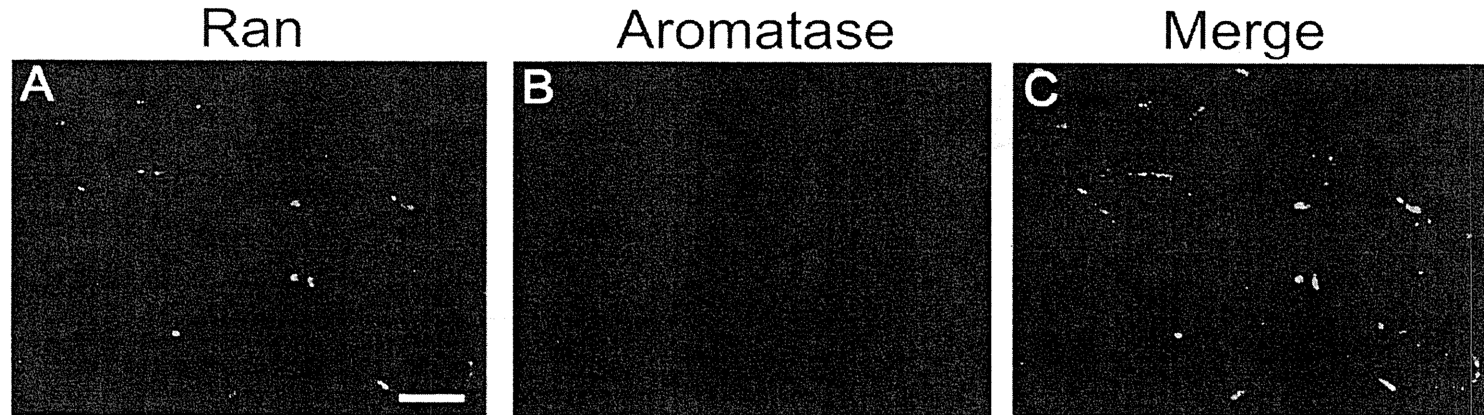


Figure 4



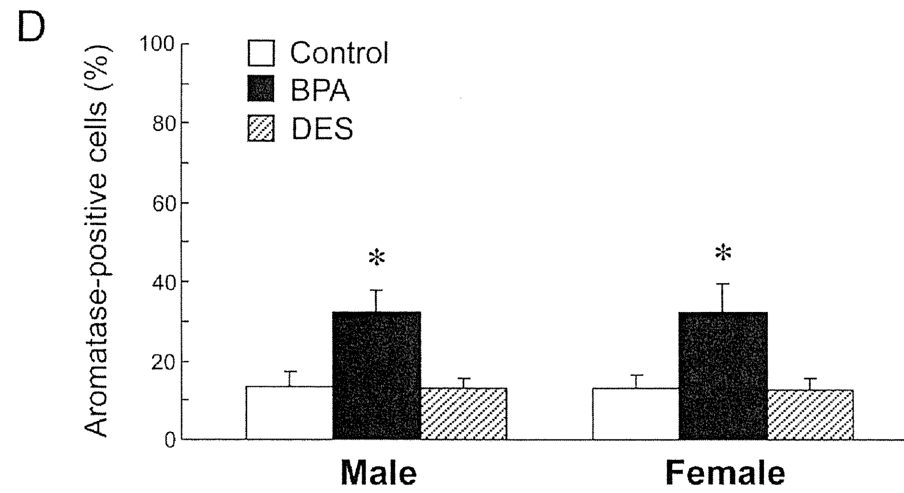


Figure 5

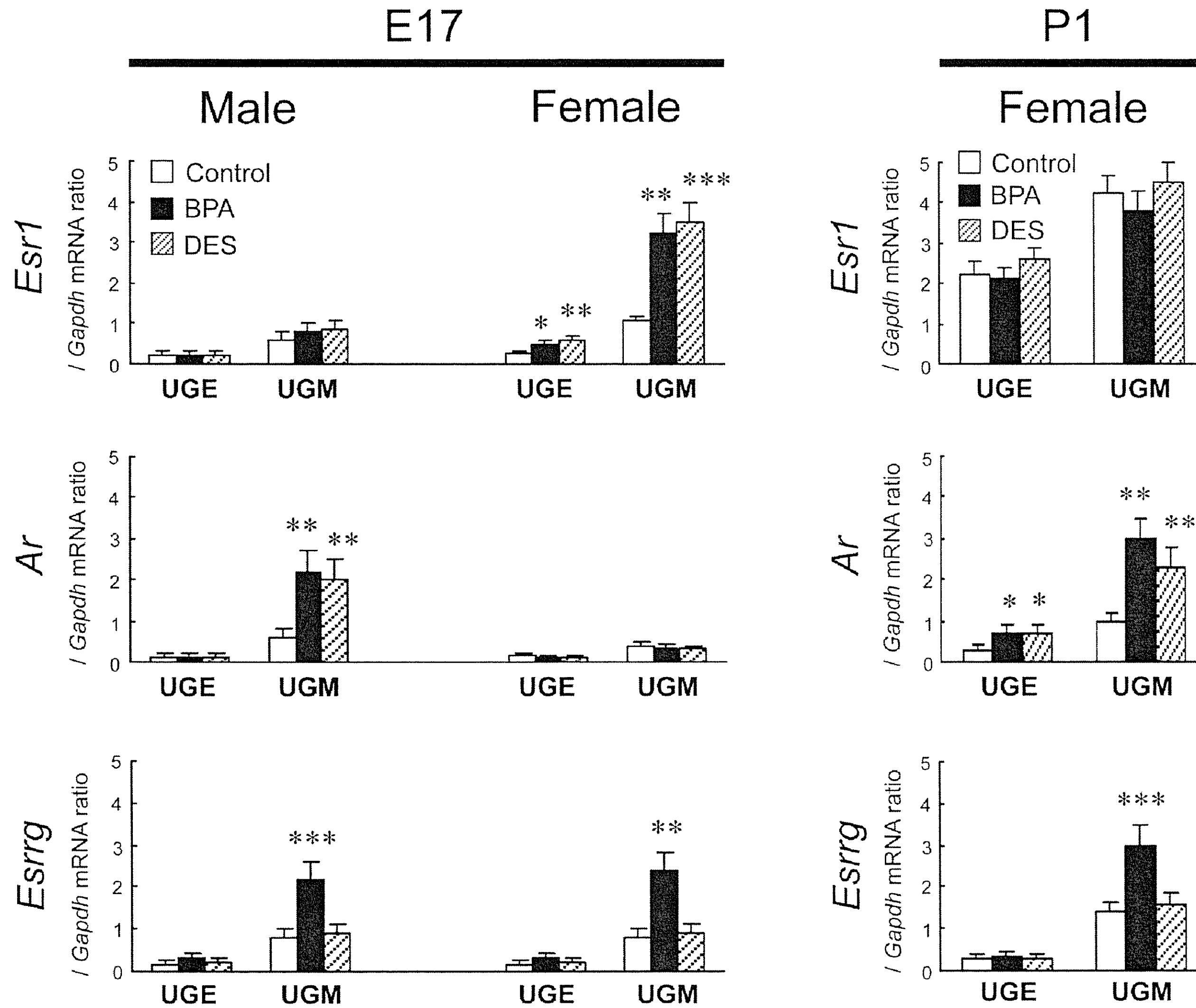
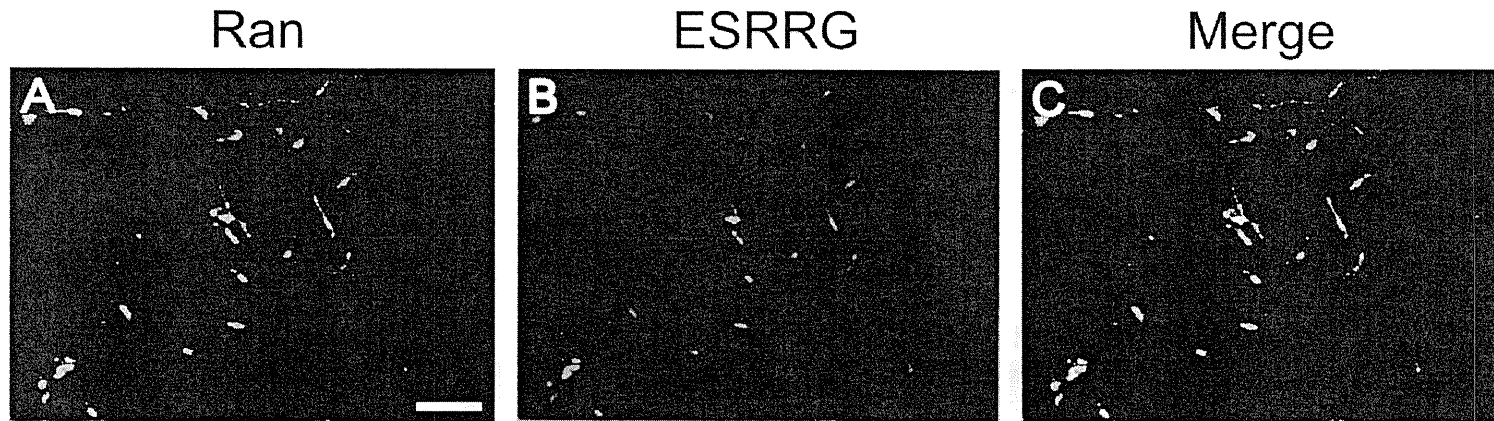


Figure 6



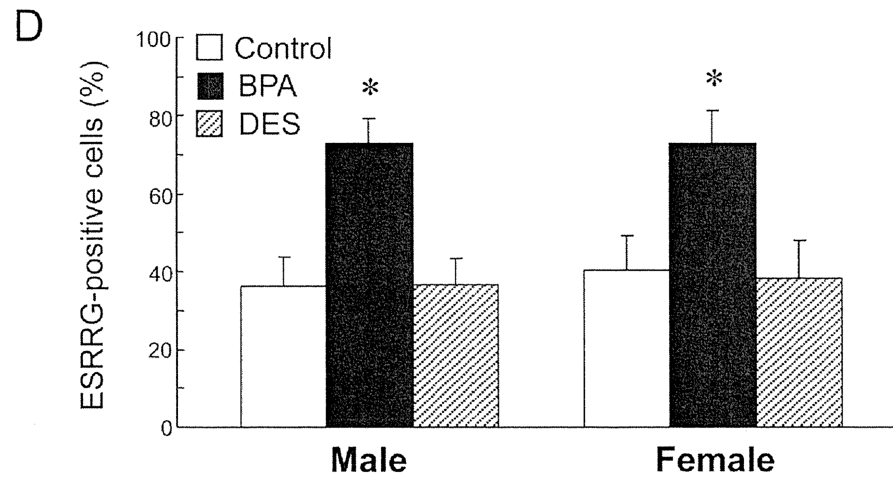


Figure 7

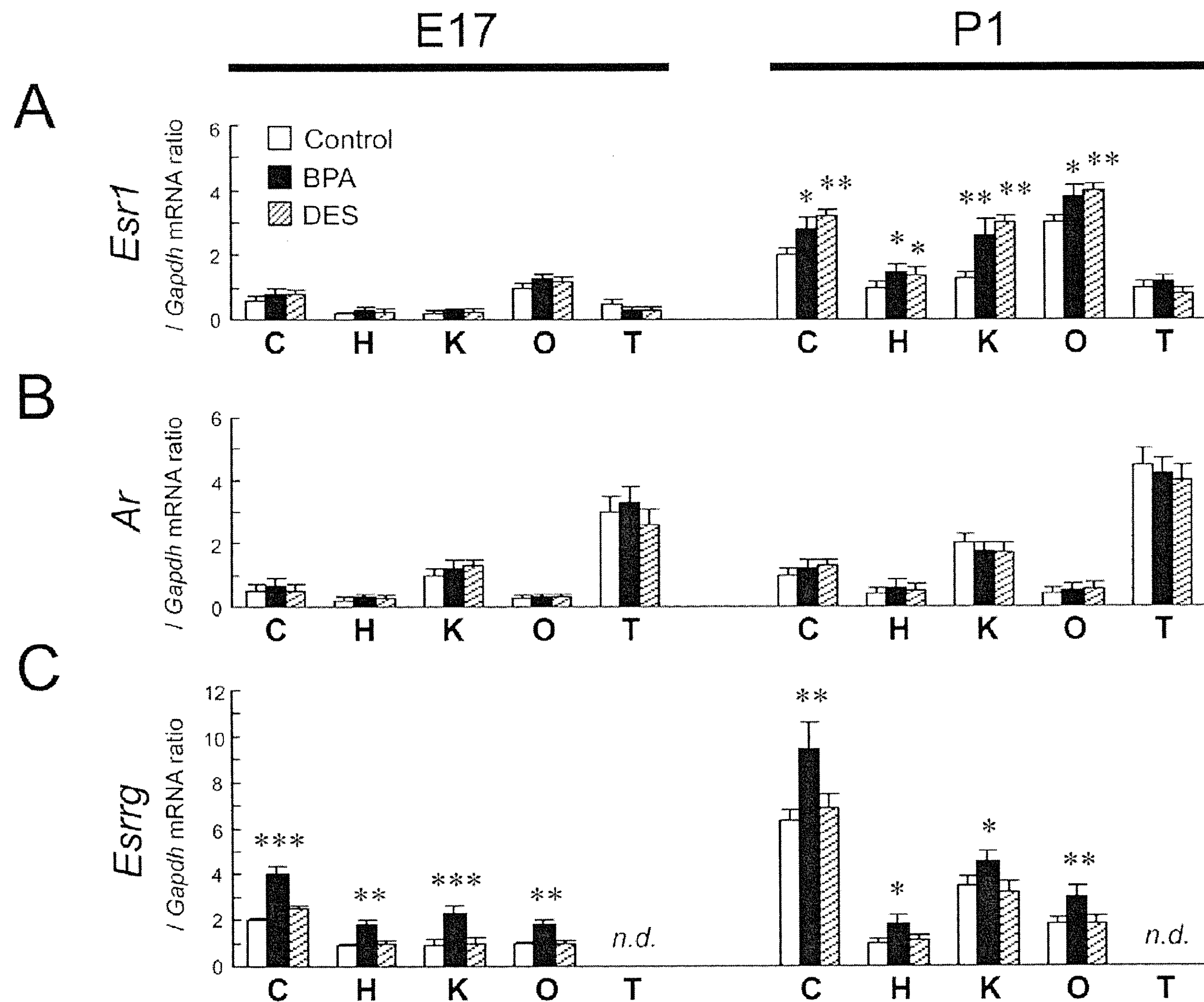
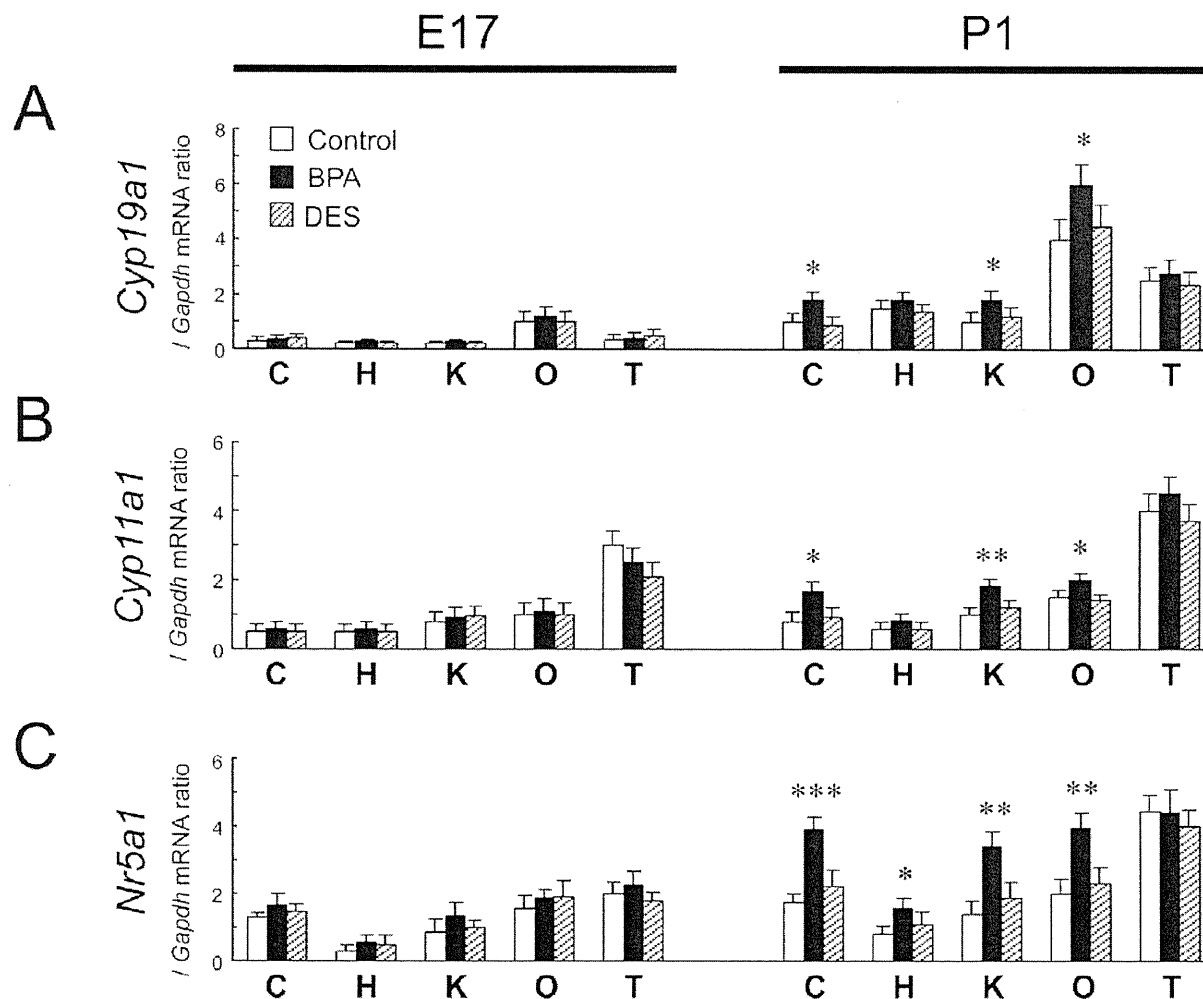


Figure 8



特集 周産期のアレルギー

—周産期の環境と小児アレルギー疾患発症のリスク—

Developmental Immunotoxicology (DIT)

太田　亮　　大沢基保

はじめに

近年，種々の疾患・障害と発生発達期における有害因子の曝露との関連が注目されている。先進工業国でのアレルギーや自己免疫疾患の増加への関心から，免疫毒性学のサブ領域として形成された developmental immunotoxicology (DIT) も，発生発達期の免疫系に対する有害因子曝露とその影響に焦点をあてたものである[1]。DIT は胎生期・周産期での有害因子曝露の免疫影響が主対象であるが，ヒトの免疫機能の成熟過程(10 歳頃までに成人レベルに達するとされる)にあたる乳幼児期曝露の影響をも含めた発生発達免疫毒性学とされる。この時期の免疫毒性による健康障害は，多様かつ長期にわたる可能性が高い。そのため，DIT は発生発達期の免疫毒性(immunotoxicity)そのものを指す意味にも用いられる。

曝露因子としては，生物的物質，化学物質，医薬品，医療器具，物理的因子(紫外線や放射線)などが主であるが，一部の生理的因子の免疫の発生発達過程への影響も対象とされる。これらによる DIT の回避は，胎生・周産期予防医学にとって基本的事項になりつつある。本稿では, DIT の特徴，疾患との関連および前臨床試験法について，これまでの知見を概説し，あわせて内分泌攪乱物質の DIT についての筆者らの経験を紹介する。

免疫系の発生発達過程と化学因子による攪乱

活発な細胞増殖や高度なネットワーク機構を有する免疫系の攪乱である免疫毒性は，感度の高い毒性影響として知られている。さらに，代表的免疫毒性物質の動物実験データから，免疫系の毒性感受性は発生発達段階のほうが成熟段階よりも高いことが示されている[2]。この曝露時期による感受性の差は異なる形態で現れる。通常の免疫毒性指標バッテリー(抗体，細胞性，非特異的の各免疫能と感染抵抗性)を用いて動物の発生発達期曝露と成熟期曝露で比較すると，毒性を発現する曝露量の差〔ラットの四塩化ダイオキシン(TCDD)による免疫毒性は，胎児期曝露では成熟動物に比べ 1/100 の曝露量で発現〕や，免疫影響の持続期間の差(成熟期曝露では影響が一過性でも発生発達期曝露ではより持続的)，免疫毒性スペクトラムの差(発生発達期曝露では有害影響を示す免疫毒性指標が多様で影響の程度も大きい)など，いずれの面でも発生発達期曝露のほうで感受性が増加していた。

この発生発達期免疫の感受性の高さは，免疫の発生発達過程での感受性時期(critical windows：例えば，胸腺での自己反応性 T 細胞のネガティブ選択期や免疫応答における抗原特異的 T，B 細胞のポジティブ選択期，出生前後の細胞性免疫能の抑制期など)の攪乱に関係する。いくつかの代表的な免疫毒性物質の比較研究から，DIT とその影響の特徴が**表**のように示される[1]が，DIT による免疫影響は免疫抑制，自己免疫やアレルギー反応の誘発，炎症反応の不全などのかたちで現れる。

DIT と疾患の関連

近年増加しつつある疾患の多くに共通することとして，環境リスク因子(化学物質や病原体)への発生発達期曝露は重要な疾患リスクであること，

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表　代表的な化学物質と医薬品による胎生期–周産期の免疫発生発達過程の攪乱（Dietert, 2009より一部改変）[1]

化学物質/混合物	免疫成熟過程の感受性期（Window）	出生後に生じる有害影響	想定される健康リスク
エタノール 重金属 TCDD PCBs	組織中の骨髄単球系細胞の定着と成熟	末梢マクロファージの調節不全 自然免疫の変調 炎症性損傷	・小児感染症（例：中耳炎）の増加 ・小児・成人期の神経疾患の発生 ・感染時の組織損傷の増加 ・疲労関連疾患の発生 ・細菌性う歯（虫歯）
TCDD PCBs ビスフェノールA 水銀 トリクロロエチレン DES たばこ煙	T細胞の分化，選択，末梢移行・定着	T細胞依存性免疫反応の異常 Treg細胞の機能不全 経口寛容の障害	・自己免疫症の発生 ・食物アレルギーの発生 ・インフルエンザ感受性の増大 ・ワクチン接種による疾患 ・小児癌（白血病ほか）
鉛 たばこ煙 エタノール	樹状細胞の成熟と機能発現	樹状細胞の数と機能の変化 Th2への偏向 IgEの過剰産生	・喘息を含むアレルギー性疾患の発生 ・特定の免疫機能の感受性増大

TCDD：2,3,7,8-四塩化ダイオキシン，PCBs：ポリ塩化ビフェニル類，DES：ジエチルスチルベストロール

免疫–炎症系の傷害または機能障害が実際にあること，発症または疾患の早期バイオマーカーの出現がリスク因子の曝露を受けた子どもで目立つことの三つがあげられ，DITの関与が指摘されている[3]。DITと疾患の関連性は，前臨床試験と疫学・臨床的研究により予測や検証がなされてきた。動物実験では周産期曝露が可能であるが，ヒトの場合は臍帯血や胎盤の化学分析により周産期曝露の程度が検索される。DITの関与が想定される主な疾患には小児の感染症や癌，アレルギー，喘息，Ⅰ型糖尿病，アテローム動脈硬化症などがあげられているが，自閉症のような神経症状への関与の指摘もある[1]。

DITによる免疫抑制は，感染や発癌への感受性を高める。一例をあげると，カナダのイヌイットについての疫学調査では，臍帯血中のPCB濃度を測定し，そのコホート（5歳未満児）の急性中耳炎や呼吸器感染症の発生率を調べると，PCBの高濃度曝露群では低濃度曝露群に比して感染症の相対危険度が有意に高かった[4]。インフルエンザ感染などで類似の報告も多い。たばこ煙の妊娠マウスへの曝露実験では，曝露動物からの子マウス（5週齢）で，移植腫瘍細胞に対する抵抗性と腫瘍細胞傷害性T細胞の活性が低下していた[5]。また，カーテンなどの難燃剤である臭素化難燃剤DBDE（decabromodiphenyl ether）の母ラットへの周産期曝露（妊娠10日～分娩後21日まで混餌曝露）実験では，1,000 ppmのDBDE曝露母動物の子ラットで，脾臓中の$CD4^+$T細胞や活性化T，B細胞や末梢血中のNK細胞が有意に減少し[6]，DBDEの間接曝露が弱いながら子動物の免疫系発達を攪乱することが示された。このような免疫抑制が免疫反応の抑制的制御機構（Treg細胞など）に働くと，結果として抑制的な免疫制御が外れてアレルギーや自己免疫反応を併発することもある。

DITは新生児のアトピー性皮膚炎，じん麻疹，喘息などのアレルギー症とも関連づけられてきた。このうち，アレルギー性喘息の早期曝露リスクとしては，母親の喫煙，妊娠時のアセトアミノフェノンの使用，ディーゼル排気などがあげられている。また，Celiac病（麦蛋白のグルテンに対して経口免疫寛容が働かず小腸の過敏性炎症を引き起こす）では，遺伝要因の関与が大きいが，経口免疫寛容の阻害因子（例えば，TCDDやエストラジオール，免疫抑制剤，COX-2阻害薬など）の関与も考えられる。また，塩化水銀を含む飲料水を妊娠マウスに摂取させると，胎児マウスのリンパ組織中にリンパ球の表現型変化と自己免疫性腎炎関連抗原と反応するリンパ球が生じ[7]，出生マウスでは持続的なT細胞の表現型と機能の変化が観察

され[8]，これらの変化はアレルギーや自己免疫症の発症に関連し得るとされている。アレルギー症の発症には，Th1/Th2細胞のバランスの乱れ，免疫反応を制御しているTreg細胞の機能阻害，IgE産生のコード遺伝子の脱メチル化によるIgE産生亢進などが介在している可能性があげられる。

DITの試験法と事例

現在のところDIT試験は，化学物質や医薬品の安全性評価でのルーチン試験にはなっていない。唯一の例外は，米国環境保護庁(EPA)による農薬規制で，通常の免疫毒性試験で陽性知見が得られた時は，DIT試験を行うことが推奨されている。DITの試験法の枠組みは米国トキシコロジー学会(SOT)フォーラムで検討[9]され，WHO/IPCSやOECDでも発生毒性試験の一部として検討がなされた。SOTフォーラムでの主な一致点は，①推奨モデル動物はラット，②DITプロトコルはすでに検証された免疫試験法に基づき，生殖発生毒性の標準プロトコルに組み入れられること，③化学物質と医薬品でDIT探索のアプローチを類似させること，④一つのプロトコルで全critical windowsを試験する研究計画は可能である，などである。しかし，DITの試験法のガイドラインは国内外ともになく，一般には発生毒性(developmental toxicity)の試験プロトコルを用い，成熟動物での免疫毒性試験法が用いられる。そこで，以下に発生毒性試験のプロトコルと免疫毒性試験法を概説し，筆者らが行った内分泌攪乱物質のDIT試験の事例を述べる。

1）発生毒性試験プロトコル：薬物や化学物質の有害影響を受けやすい子どもの発達期は，胎生期と新生児期に分けることができる。有害影響の評価に使用されるげっ歯類の新生児は，ヒトと比べてより未成熟な段階で娩出され，ヒトの胎生末期に相当すると考えられている。胎生期の影響を評価するには，通常薬物を妊娠動物に投与し，経胎盤的に薬物を胎児に移行させて影響を調べる。一方，新生児期の影響を評価するには，出産後の母動物に薬物等を投与し，経母乳的に薬物を新生児に移行させて調べる方法と，新生児に直接投与して調べる方法がある。後者は前者に比べて確実に児動物へ薬物が到達するというメリットはあるが，ヒトのモデルとしては考えにくい経路である。また，新生児の発達は，母親の哺育の良し悪しにも左右されるため，母動物への有害影響による哺育不良のため，新生児に誘発される発達不良と区別するのが難しい。

新生児の発達は，哺育の良し悪し以外に1腹当たりの匹数，離乳までの日数，離乳直後のストレスなどによっても影響を受けるので注意が必要である。例えば，ラットでは生後4日に1腹当たりの匹数を雌雄各4匹に調整し，哺育児数の過剰による発育遅延を防ぐとともに，腹ごとのバラツキを抑えるように努めている。また，離乳時期は，生後21日に統一し，離乳後は個別飼育によるストレスを避けて，1ケージ当たり2匹以上の複数飼育を原則としている。

2）免疫毒性試験：げっ歯類を用いた免疫毒性試験の指標には，胸腺や脾臓の重量，白血球数，免疫細胞プロファイル，抗体産生能，脾細胞の幼若化反応，NK細胞アッセイなどがある。胸腺や脾臓の重量と白血球数の測定は，一般的な毒性指標であるが，胸腺や脾臓の重量はストレスによる影響を受けやすい臓器なので，免疫毒性に起因した変化なのかストレスの現象なのかを区別する必要がある。免疫細胞サブセットのプロファイルはフローサイトメーターで測定される。抗体産生能の測定にはヒツジ赤血球(SRBC)や貝ヘモシアニン(KLH)などを抗原とするT細胞依存性抗体反応(TDAR)が標準的に用いられ，特異抗体産生能を脾細胞中の特異抗体産生細胞を数えるplaque forming cell(PFC)アッセイや血清中の特異抗体価をELISA法で測定する。マイトジェンを用いたリンパ球の幼若化試験は，T細胞とB細胞の機能不全を調べる試験として利用されている。NK細胞アッセイには，NK細胞の腫瘍細胞傷害能と細胞数を測定する方法がある。これらのうち，DIT試験でよく用いられるのは免疫細胞プロファイルとTDARである。

3）DIT試験の事例：発生発達期の曝露因子による免疫系への影響を調べるためには，発生発達毒性のプロトコルと免疫毒性試験を組み合わせた評

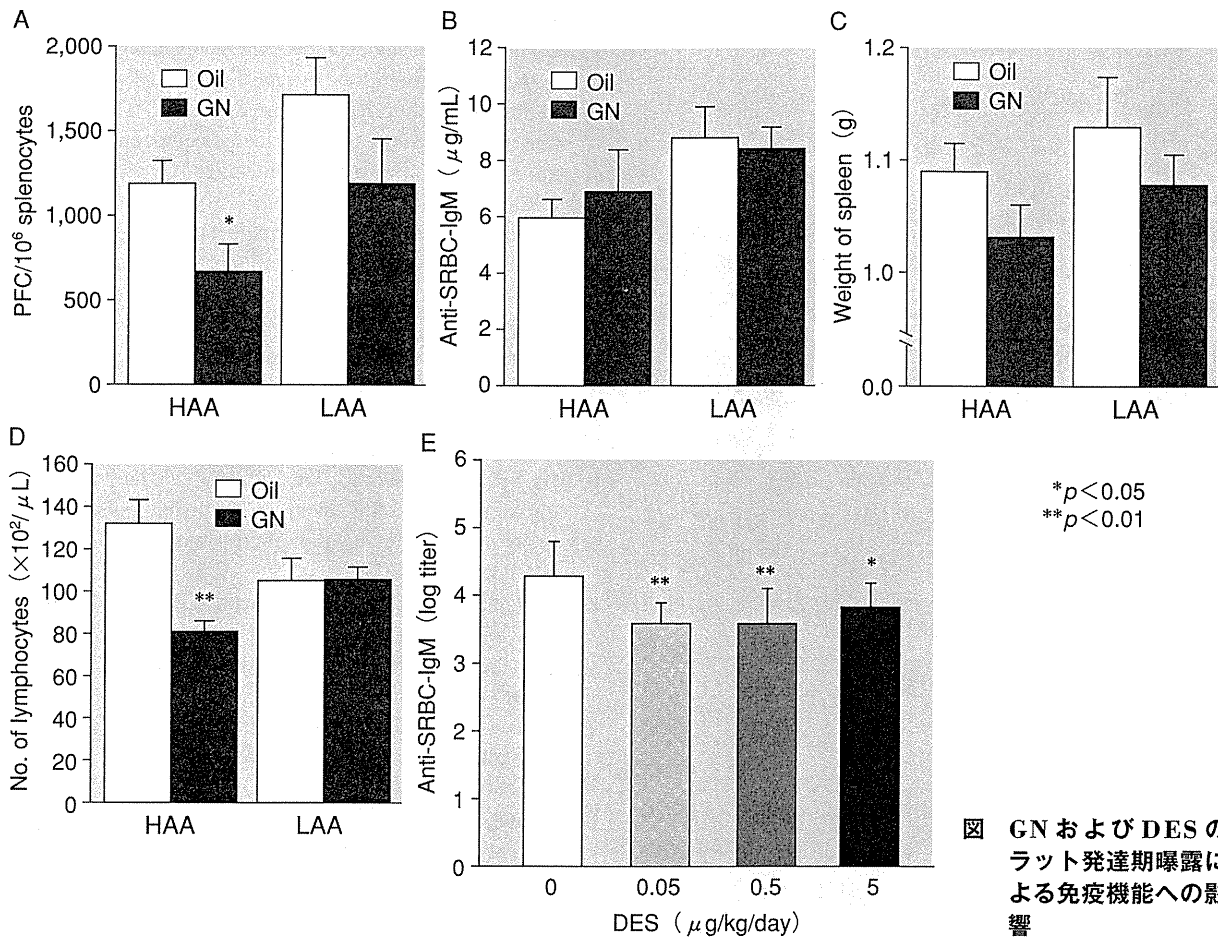

図　GNおよびDESのラット発達期曝露による免疫機能への影響

価法が必要である。この評価法は，子の発生発達期に受けた曝露により，成長後に発現する免疫異常を検索するもので，これまで見過ごされてきた可能性のある潜在的あるいは遅発性の免疫毒性やその影響を明らかにすることを目的としている。

ここで筆者らが行った内分泌撹乱物資についての二つの実験を紹介する。一つは，植物エストロゲンのゲニステイン(GN)を強制的に投与した母ラットに子どもを育てさせ，その子ラットの免疫機能を調べた実験[10]で，もう一つは，合成エストロゲンのジエチルスチルベストロール(DES)をラットの新生児に直接投与して，その後の免疫機能を検査した実験である。

ⅰ）授乳期GN投与実験：筆者らは，SD系ラットから回避学習能の異なる高回避系(HAA)と低回避系(LAA)の2種類の近交系ラットを確立したが，LAAはHAAに比べて免疫機能が高いという特徴がある[11]。そこで，この2系統を使って代表的な植物性エストロゲンであるGNを経母乳的に曝露し，免疫機能への影響が遅発的に誘発されるか否かを調べた。

妊娠期から哺育期にかけてGNを投与したSD系の母ラットと，HAAあるいはLAAの母ラットの間で養母交換を行い，HAAおよびLAAの新生児に経母乳的にGNを曝露させた。その後，新生児は生後21日に離乳させ，生後19週時に1%のSRBCを静脈内投与した。投与の4日後に解剖して脾臓重量を測定後，脾細胞のPFCアッセイを行った。また，血液中のリンパ球数と抗SRBC-IgM(ELISA法)を測定した。

その結果，両系の抗-SRBC-IgM値については，GNの影響は認められなかったが，両系ともGN曝露によりPFCアッセイと脾臓重量で抗体産生能の低下を示唆する変化がみられ，かつHAAのみでリンパ球数の減少がみられた(**図A〜D**)。これらの結果から，母動物のGN摂取