Table 3. Continued

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

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

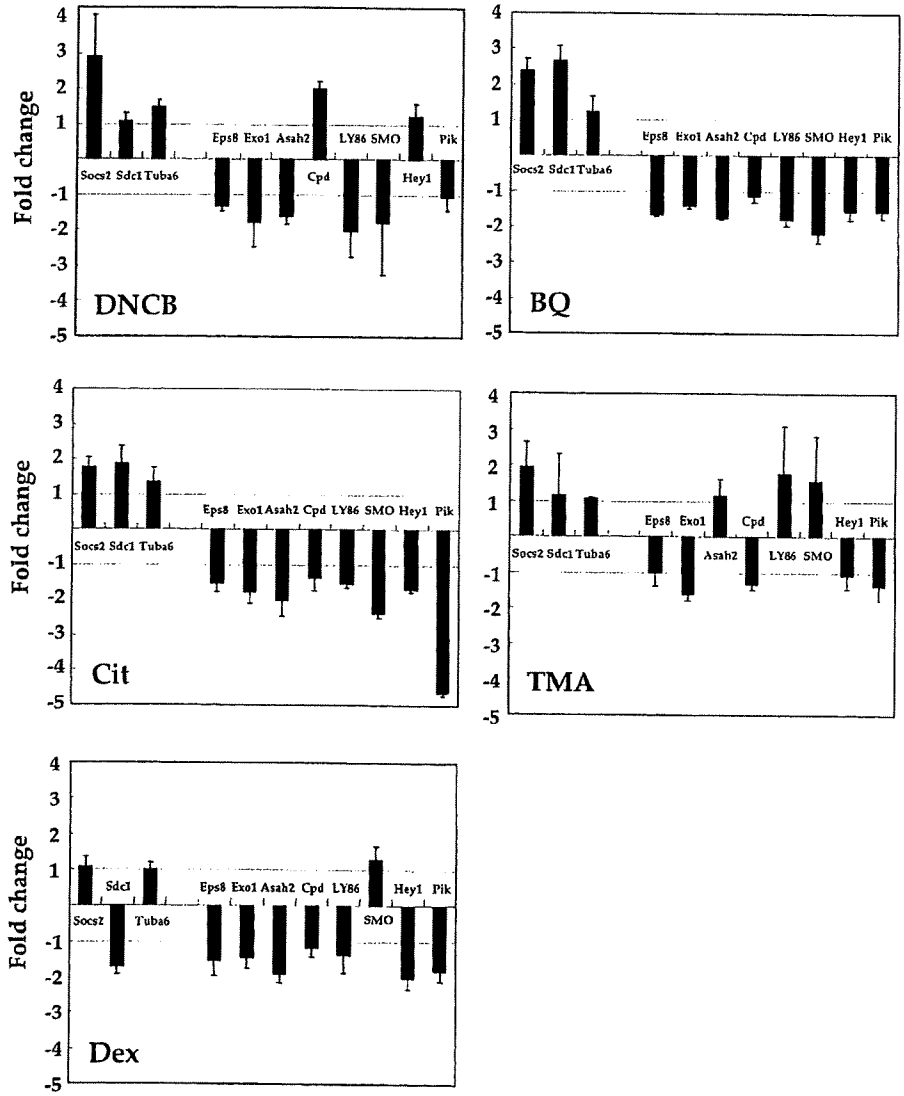

Fig. 2. Gene expression changes in DC2.4 cells induced by DNCB, BQ, Cit, TMA and Dex. DC2.4 cells were exposed to type IV allergy-inducible chemicals, DNCB, BQ or Cit (0.1 μg/m*l*), a type I allergy-inducible chemical, TMA (1.0 μg/m*l*) or a non-sensitizer, Dex (1.0 μg/m*l*) for 24 hr, and the changes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

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

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

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

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

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REFERENCES

1. Aiba, S. and Katz, SI. 1990. Phenotypic and functional characteristics of in vivo-activated Langerhans cells. *J. Immunol.* **145**: 2791–2796.
2. Aiba, S., Terunuma, A., Manome, H. and Tagami, H. 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol.* **27**: 3031–3038.
3. Aiba, S., Manome, H., Yoshino, Y. and Tagami, H. 2000. In vitro treatment of human transforming growth factor-β1-treated monocyte-derived dendritic cells with haptens can induce the phenotypic and functional changes similar to epidermal Langerhans cells in the initiation phase of allergic contact sensitivity reaction. *Immunology* **101**: 68–75.
4. Allan, R. S., Smith, C. M., Beltz, G.. T., van Lint, A. L., Wakim, L. M., Heath, W. R. and Carbone, F. R. 2003. Epidermal viral immunity induced by CD8α^+ dendritic cells but not by Langerhans cells. *Science* **301**: 1925–1928.
5. Ashikaga, T., Hoya, M., Itagaki, H., Katsumura, Y. and Aiba, S. 2002. Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicol. In Vitro* **16**: 711–716.
6. Barak, O., Treat, J. R. and James, W. D. 2005. Antimicrobial peptides: effectors of innate immunity in the skin. *Adv. Dermatol.* 21: 357–374.
7. Basketter, D. A. and Scholes, E. W. 1992. Comparison of the local lymph node assay with the guinea pig maximization test for the detection of a range of contact allergens. *Food Chem. Toxicol.* **30**: 65–69.
8. Braff, M. H. and Gallo, R. L. 2006. Antimicrobial peptides: an essential component of the skin defensive barrier. *Curr. Top. Microbiol. Immunol.* **306**: 91–110.
9. Buehler, E. V. 1985. A rationale for the selection of occlusion to induce and elicit delayed contact hypersensitivity in the guinea pig. A prospective test. *Curr. Probl. Dermatol.* **14**: 39–58.
10. Butala, J. H., David, R. M., Gans, G., McKee, R. H., Guo, T. L., Peachee, V. L. and White, K. L Jr. 2004. Phthalate treatment does not influence levels of IgE or Th2 cytokines in B6C3F1 mice. *Toxicology* **201**: 77–85.
11. Chapuis, F., Rosenzwajg, M., Yagello, M., Ekman, M., Biberfeld, P. and Gluckman, J. C. 1997. Differentiation of human dendritic cells from monocytes *in vitro*. *Eur. J. Immunol.* **27**: 431–441.
12. Cuenda, A. 2000. Mitogen-activated protein kinase kinase 4 (MKK4). *Int. J. Biochem. Cell. Biol.* **32**: 581–587.
13. De Baey, A. and Lanzavecchia, A. 2000. The role of aquaporins in dendritic cell macropinocytosis. *J. Exp. Med.* **191**: 743–747.
14. Enk, A. H. 1997. Allergic contact dermatitis: understanding the immune response and potential for targeted therapy using cytokines. *Mol. Med. Today* **3**: 423–428.
15. Ewens, S., Wulferink, M., Goebel, C. and Gleichmann, E. 1999. T cell-dependent immune reactions to reactive benzene metabolites in mice. *Arch. Toxicol.* **73**: 159–167.
16. Gerberick, G. F., Cruse, L. W. and Ryan, C. A. 1999. Local lymph node assay: differentiating allergic and irritant responses using flow cytometry. *Methods* **19**: 48–55.
17. Grammer, L. C., Harris, K. E., Sonenthal K, R., Ley, C. and Roach, D. E. 1992. Across-sectional survey of 46 employees exposed to trimellitic anhydride. *Allergy Proc.* **13**: 139–142.
18. Heydorn, S., Menne, T., Andersen, K. E., Bruze, M., Svedman, C., White, I. R. and Basketter, D. A. 2003. Citral a fregrance allergen and irritant. *Contact Dermatitis.* **49**: 32–36.
19. Jackson, S. H., Yu, C. R., Mahdi, R. M., Ebong, S. and Egwuagu, C. E. 2004. Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling. *J. Immunol.* **172**: 2307–2315.
20. Kimber, I. and Cumberbatch, M. 1992. Dendritic cells and cutaneous immune responses to chemical allergens. *Toxicol. Appl. Pharm.* **117**: 137–146.
21. Kimber, I., Dearman, R. J., Scholes, E. W. and Basketter, D. A. 1994. The local lymph node assay: developments and applications. *Toxicology* **93**: 13–31.
22. Kimber, I., Cumberbatch, M., Dearman, R. J., Bhushan, M. and Griffiths, C. E. 2000. Mobilization of epidermal langerhans cells. *Br. J. Dermatol.* **143**: 894.
23. Kimber, I. 2002. Reduction, refinement and replacement: putting the immune system to work. *Altern. Lab. Anim.* **30**: 569–

577.

24. Krasteva, M., Nicolas, J. F., Chabeau, G., Garrigue, J. L., Bour, H., Thivolet, J. and Schmitt, D. 1993. Dissociation of allergenic and immunogenic functions in contact sensitivity to paraphenylenediamine. *Int. Arch. Allergy Immunol.* **102**: 200–204.
25. Kurosaka, K., Chen, Q., Yarovinsky, F., Oppenheim, J. J. and Yang, D. 2005. Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. *J. Immunol.* **74**: 6257–6265.
26. Magnusson, B. and Kligman, A. M. 1969. The identification of contact allergens by animal assay. The guinea pig maximization test. *J. Invest. Dermatol.* **52**: 268–276.
27. Maurer, D. and Stingl, G., 1999. Dendritic cells and skin immunity. pp. 111–122. *In*: Dendritic Cells (Lotze, M.T. and Thomson, A.W. eds.), Academic Press, San Diego,
28. Morts, C. G. and Andersen, K. E. 1999. Allergic contact dermatitis in children and adolescents. *Contact Dermatitis* **41**: 121–130.
29. Ozawa, H., Nakagawa, S., Tagami, H. and Aiba, S. 1996. Interleukin-1β and granulocyte-macrophage colony-stimulating factor mediate Langerhans cell maturation differently. *J. Invest. Dermatol.* **106**: 441–445.
30. Romani, N., Lenz, A., Glassel, H., Stossel, H., Stanzl, U., Majdic, O., Fritsch, P. and Schuler, G. 1989. Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *Invest. Dermatol.* **93**: 600–609.
31. Ryan, C. A., Hulette, B. C. and Gerberick, G. F. 2001. Approaches for the development of cell-based *in vitro* methods for contact sensitization. *Toxicol. In Vitro.* **15**: 43–55.
32. Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C. R., Qin, S. and Lanzavecchia, A. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* **28**: 2760–2769.
33. Sallusto, F., Palermo, B., Lenig, D., Miettinen, M., Matikainen, S., Julkunen, I., Foster, R., Burgstahler, R., Lipp, M. and Lanzavecchia, A. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* **29**: 1617–1625.
34. Scholes, E. W., Basketter, D. A., Sarll, A. E., Kimber, I., Evans, C. D., Miller, K., Robbins, M. C., Harrison, P. T. and Waite, S. J. 1992. The local lymph node assay: results of a final inter-laboratory validation under field conditions. *J. Appl. Toxicol.* **12**: 217–222.
35. Shen, Z., Reznikoff, G., Dranoff, G. and Rock, K. L. 1997. Cloned dendritic cells can present exogenous antigens on both MHC class I and II molecules. *J. Immunol.* **158**: 2723–2730.
36. Shoeters, E., Verheyen, G. R., Van Den Heuvel, R., Nelissen, I., Witters, H., Van Tendeloo, V. F., Schoeters, G. E. and Berneman, Z. N. 2005. Expression analysis of immune-related genes in $CD34^+$ progenitor-derived dendritic cells after exposure to the chemical contact allergen DNCB. *Toxicol. In Vitro* **19**: 909–913.
37. Shoeters, E., Nuijten, J. M., Van Den Heuvel, R., Nelissen, I., Witters, H., Schoeters, G. E., Van Tendeloo, V. F., Berneman, Z. N. and Verheyen, G. R. 2006. Gene expression signatures in $CD34^-$ progenitor-derived dendritic cells exposed to the chemical contact allergen nickel sulphate. *Toxicol. Appl. Pharmacol.* **216**: 131–149.
38. Shortman, K. and Liu, Y. J. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* **2**: 151–161.
39. Takeyoshi, M., Yamasaki, K., Yakabe, Y., Takatsuki, M. and Kimber, I. 2001. Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2'-deoxyuridine(BrdU) incorporation. *Toxicol. Lett.* **119**: 203–208.
40. Wegrowski, Y., Milard, A. L., Kotlarz, G., Toulmonde, E., Maquart, F. X. and Bernard, J. 2006. Cell surface proteoglycan expresion during maturation of human monocytes-derived dendritic cells and macrophages. *Clin. Exp. Immunol.* **144**: 485–493.
41. Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M. and Suzuki, H. 2003. Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naïve THP-1 cell line. *Toxicol. In Vitro* **17**: 221–228.

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Early embryonic losses in mice induced by diethylstilbestrol.

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Abstract

Estrogens cause embryonic lethality and the disturbance of early placental development in mice. Diethylstilbestrol (DES) at 1, 10, or 100 microg/kg was orally administered to Institute of Cancer Research mice on gestational days (GD) 4 through 8, and the uterus and placenta were examined histopathologically on GD 9. Decidua of DES-treated mice showed insufficient development, and the uterine lumen at the implantation site did not effectively minimize. The trophoblast giant cell layer was not separated from the uterine lumen by the decidua capsularis, and hemorrhage from the denuded trophoblast giant cell layer into the uterine lumen was noted at the peripheral part of the decidua basalis. The results of the present study suggest that decidual hypoplasia and subsequent placental hemorrhage causes fetal death due to the administration of DES during the early stage of pregnancy.

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—Original—

Effects of Transmaternal Exposure to Genistein in Hatano High- and Low-Avoidance Rats

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Abstract: Hatano high- and low-avoidance (HAA and LAA) rats are separated by breeding from Sprague-Dawley rats by high versus low rates of avoidance responses in a shuttle-box task. In addition, compared to HAA rats, LAA rats show lower running-wheel activity, later sexual maturation, 5-day estrous cycling, lower sperm motility, more pronounced immunological reactions, and are generally less reactive to stress. The present study was designed to compare the effects of transmaternal exposure to genistein on these characteristics between HAA and LAA rats. To this aim, litters from both strains were fostered onto Sprague-Dawley rats receiving genistein by gavage with 5 mg/animal/day from day 17 of pregnancy through day 21 of lactation. Inhibited growth after weaning and reduced uterine weight at weaning were observed in the LAA offspring reared by genistein-treated dams. IgM antibody production in response to sheep red blood cells was significantly decreased in the HAA offspring reared by genistein-treated dams. During restraint stress, the plasma concentration of corticosterone was significantly lower in the LAA offspring reared by genistein-treated dams. Strain-related differences were detected in shuttle-box avoidance performance, running-wheel activity, estrous cycling, and sperm motility. The results demonstrate that transmaternal exposure to genistein potentially affects the immunological and stress responses as well as the post-weaning growth of the offspring. It suggests that a comparative study using Hatano rats would be useful for studying the influence of endocrine active chemicals on the whole body systems.

Key words: endocrine disruptor, genistein, inbred strain, offspring

Introduction

From the viewpoint of behavioral genetics, consideration of genetic control is necessary for animal studies of neurobehavioral teratology. Therefore, we have separated two inbred strains from Sprague-Dawley (SD) rats, Hatano high- and low-avoidance (HAA and LAA) rats, which show uniform behavior within the strain, but different baseline behaviors between strains in a shuttle-box avoidance task [27]. The selection criterion is based on the number of avoidance responses obtained during four daily sessions of 60 trials, with HAA rats being

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identified by a high rate of avoidance responses and LAA rats by a low rate. Using these two strains, we have developed new methods for the risk assessment of the toxicological effects of substances on the behavior of the next-generation [28, 30]. Although the Hatano rats are separated by their avoidance performance as mentioned above, characteristic differences between the strains are not only observed in their behaviors but also in their reproductive function [2, 35, 36, 38], immunological reactions [26], and stress responses [1, 3, 31]. For example, compared to HAA rats, LAA rats show lower running-wheel activity, later sexual maturation, 5-day estrous cycling, lower sperm motility, more pronounced immunological reactions, and are generally less reactive to stress. In addition, the data of these two strains falls within the normal range of variation for SD rats, from which the Hatano rats are derived.

In this study, we studied the effects of an endocrine active compound that has the potential to stimulate the normal endocrine system and focused on its effects on the nervous, endocrine, and immune systems. The results of animal studies on endocrine active compounds have often varied study by study, especially in their effects on the next generation. These variations in results may originate in genetic variations in the animals used [39], and we hoped to clarify the situation by using the HAA and LAA inbred strains, which show little inter-individual variation and whose nervous, endocrine, and immune interactions are well characterized.

Genistein (GN) is a typical phytoestrogen. Phytoestrogens are naturally occurring constituents of plants such as soy, and are known to exhibit estrogenic activity in rodent uterotrophic assays [13]. Soy-containing infant formulas and the breast milk of mothers that consume soy-based foods are rich in isoflavones [8, 37]. In animal studies, GN is reported to have had effects on behavior [7, 18], the reproductive system [4, 19, 23, 34], and the immune system [9, 15, 41].

In the present study, we used non-selected SD rats (background strain of Hatano rats) as the foster dams to rear pups of Hatano rats and administered the test compound, genistein, to the foster dams in order to avoid the influence of strain differences in maternal behavior [32].

Materials and Methods

Newborns of ten litters from HAA and LAA strains, maintained at the Hatano Research Institute were used for this experiment. In addition to the newborns, 20 pregnant SD rats purchased from Charles River Laboratories Japan, Inc. were prepared as foster dams for the newborns. The animals were kept in an animal room maintained under a 12-h light-dark cycle (lights on from 07:00 to 19:00), with a room temperature of 22 to 24°C and a relative humidity of 50 to 65%. GN (Purity: minimum 98%) was purchased from Sigma Chemical Co. (St. Louis, MO), and was suspended in corn oil (Nacalai Tesque, Co.) and mixed in a mortar to prepare the dosing sample (5 mg/ml). A stomach tube attached to a syringe was used to orally administer 5 mg/animal/day of GN to ten pregnant SD rats from day 17 of pregnancy through day 21 of lactation. Based on the average body weight of 0.3 kg for a foster dam rat, the dose of 5 mg/animal/day was estimated as being approximately equal to a dose of 16 mg/kg/day. This dose was within the range of human exposure levels. Another ten pregnant SD rats, used as a control group, were administered with 1 ml/animal/day of corn oil in the same manner. All of the pregnant females were housed individually with wood-chip bedding, and free access to food (CE-2, Clea Japan Inc.) and water. On the day after parturition, designated as postnatal day 1 (PD 1), eight newborns (4 males and 4 females where possible) from HAA and LAA dams were fostered onto SD dams receiving GN administration. The ages of the litters reared by the foster dams were within ± 24 h of their own litters. The HAA and LAA offspring were subjected to the tests noted below. The animal experiments in this study were conducted in accordance with the "Guidance for Animal Experiments in Hatano Research Institute, Food and Drug Safety Center".

Body weight and physical development

All offspring were weighed on PDs 1, 4, 7, 14, and 21, and weanlings were further weighed at 4, 5, 6, 7, 8, 9, and 10 weeks of age. Test offspring were examined daily for the following developmental landmarks as previously described: eyelid opening [29] from PD 12, vaginal opening [38] from PD 28, and preputial separa-

tion [36] from PD 35.

Behavioral tests and estrous cycle

Litters weaned at PD 21, were caged in pairs of the same sex and kept in the same animal room as before. Four offspring (2 males and 2 females where possible) from each litter were examined using the following tests as previously described [30]: shuttle-box avoidance and running wheel activity tests at 7 and 8 weeks of age, respectively. From 9 weeks of age, vaginal smears were taken daily from three females of each litter for 3 weeks to determine the stage of the estrous cycle.

Organ weight, sperm motility, and hormone levels

At PD 21, two offspring (1 male and 1 female where possible) from each litter were sacrificed by exsanguination under sodium pentobarbital anesthesia. The adrenal glands, thymus, testes, epididymides, ovaries, and uterus were weighed.

At 12 weeks of age, four offspring (1 male and 3 female where possible) from each litter were anesthetized with sodium pentobarbital, and blood was collected in heparinized tubes from the posterior vena cava. Females were sacrificed at various times during the estrous cycle (12 and 18 h of pro-estrus, 12 h of estrus). The adrenal glands, spleen, thymus, testes, epididymides, seminal vesicles, ventral prostate, ovaries, and uterus were weighed. The characteristics of motile sperm in the caudal epididymis were determined using a Hamilton-Thorne IVOS analyzer as previously described [35]. Blood collected at necropsy was centrifuged and the plasma was separated and stored at –20°C until determination of progesterone, LH, and FSH [2].

Immune response to sheep red blood cells (SRBC)

At 19 weeks of age, one male from each litter was given a single intravenous injection of 0.7 ml of 1% SRBC four days prior to necropsy. The animals were anesthetized with sodium pentobarbital, and blood was collected from the posterior vena cava, prior to sacrifice. The spleen was weighed, and the spleen cells were subjected to a plaque-formation cell (PFC) assay as previously described [26]. The blood's lymphocyte count was analyzed by an automated hematology analyzer (Cell-Dyn3500, Abbott Laboratories), and then the serum was separated and stored at –80°C until determination of anti-SRBC-IgM [26].

Hormonal response to stress

At 6 months of age, one male from each litter was sacrificed by decapitation following 30 min of immobilization in a plastic bag as previously described [3]. On the day before immobilization, a blood sample was collected from the tail vein as previously described [31] to assess the basal level of hormones. The blood sample was collected in heparinized tubes containing aprotinin and centrifuged. The plasma was separated and stored at –20°C until it was assayed for ACTH, corticosterone and prolactin [3]. The testes, epididymides, and ventral prostate were weighed at necropsy.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) with transmaternal exposure (GN, oil) and strain (HAA, LAA) as between-subject factors, and day as a within-subject (repeated measure) factor. Data were analyzed separately for males and females. Significant interactions were further analyzed using simple-effect ANOVA at each level of interaction to localize the major effects. The offspring data used the litter average as the unit of statistical analysis. Statistical significance was assumed at P values of 0.05 or less.

Results

No effects of GN exposure on the body weight of the offspring during the pre-weaning period were observed in either the HAA or LAA offspring. The body weights after weaning are shown in Figs. 1A to 1D. In the LAA offspring, the post-weaning weights of the GN group were significantly lower than those of the control group in both males [$F(1,48)=6.56$, $P<0.05$] and females [$F(1,54)=5.53$, $P<0.05$]. In the HAA offspring, no significant effects of GN exposure were observed in the post-weaning weights of either sex.

The mean ages of eyelid opening, vaginal opening and preputial separation are shown in Table 1. No influence of GN exposure on eyelid opening was observed in either the HAA or LAA offspring. In the LAA offspring, in which sexual maturation is observed later than HAA

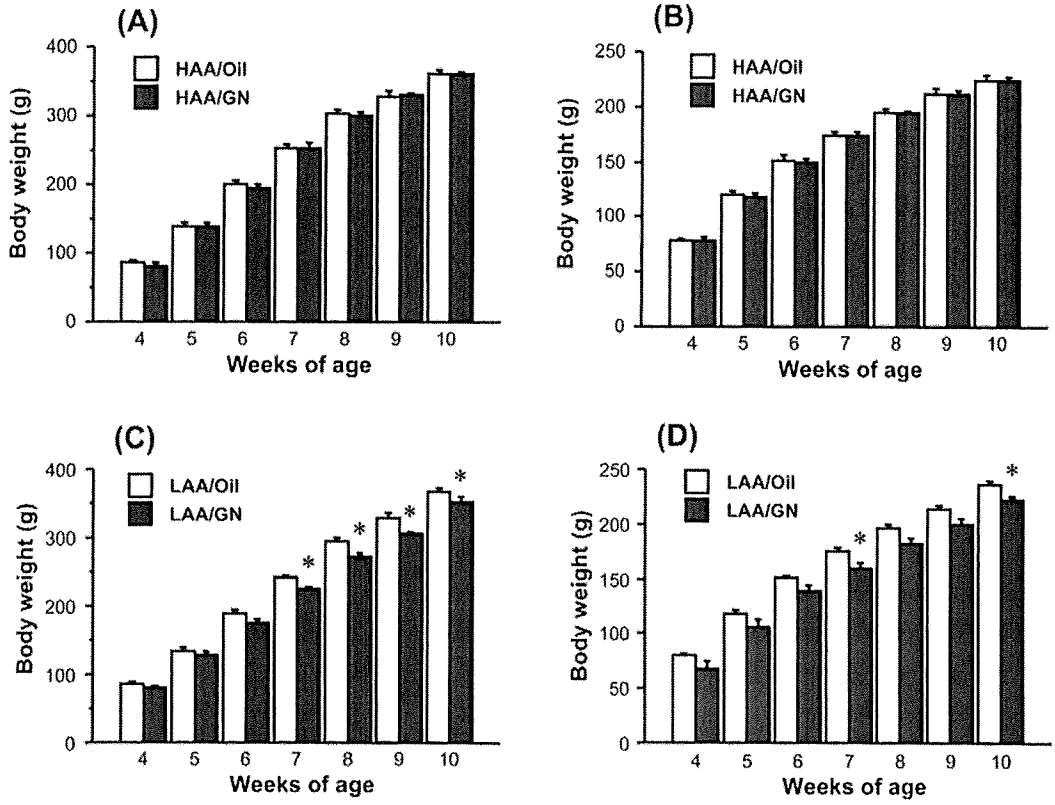

Fig. 1. Body weights of male HAA (A), female HAA (B), male LAA (C), and female LAA (D) offspring from 4 to 10 weeks of age following transmaternal genistein exposure. Data are expressed as the litter mean ± SE. *$P<0.05$ compared to the respective controls.

Table 1. Physical development of HAA and LAA offspring following lactational genistein exposure

Strain	HAA		LAA	
Treatment	Control	Genistein	Control	Genistein
Eyelid opening	14.2 ± 0.2 (32)	14.0 ± 0.1 (39)	14.7 ± 0.2 (48)	14.6 ± 0.2 (39)
Vaginal opening	32.5 ± 0.6 (11)	32.2 ± 0.3 (16)	34.7 ± 0.4 (17)	36.5 ± 0.6* (15)
Preputial separation	40.4 ± 0.9 (13)	41.2 ± 0.5 (12)	46.9 ± 0.4 (19)	48.3 ± 0.7* (13)

Data are expressed as the mean ± SE in postnatal days until the criterion was met. Parentheses show the number of animals examined. *$P<0.05$ compared to the respective controls.

offspring, both vaginal opening [$F(1,30)=6.40$, $P<0.05$] and preputial separation [$F(1,30)=5.93$, $P<0.05$] were further delayed in the GN exposure groups. The differences were marginally significant when individual data were used for analysis, but did not reach significant levels when litter means were used. In the HAA offspring, no influence of GN exposure was observed in either sex.

No significant differences between the control and GN groups were observed in the rate of avoidance responses during 2 days (60 trials per day) of shuttle-box avoidance tests, in spite of clear strain differences between HAA and LAA rats.

The number of revolutions in the running wheel activity test was significantly higher in HAA than LAA offspring over 3 consecutive days. There were, however, no significant effects of GN exposure on the number of revolutions in either HAA or LAA offspring.

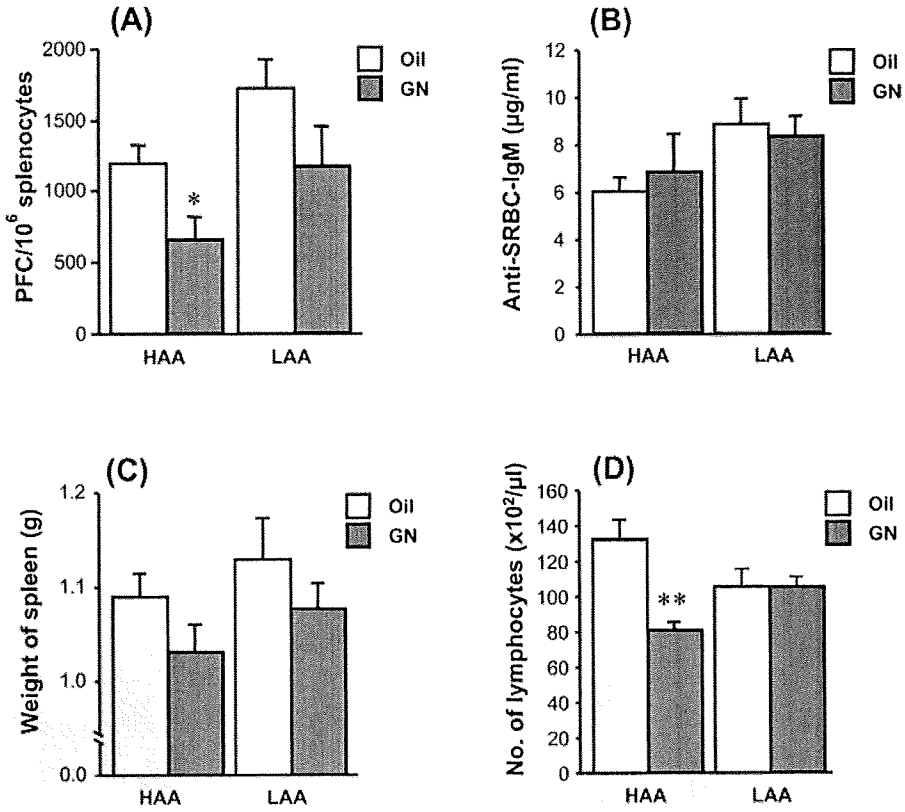

Fig. 2. PFC assay (A), anti-SRBC-IgM (B), spleen weight (C), and the number of lymphocytes (D) in HAA and LAA offspring at 19 weeks of age following transmaternal genistein exposure. Data are expressed as the mean ± SE. $*P<0.05$, $**P<0.01$ compared to the respective controls.

In the monitoring of estrous cycle from 9 to 12 weeks of age, all of the HAA offspring showed a regular 4-day estrous cycle in both the control and GN groups. In the LAA offspring, 24 and 41% of the control rats exhibited regular 4- and 5-day cycles, respectively, as did 7 and 46% of the GN group. The rest of the control (35%) and GN (47%) groups showed repeated 4- and 5-day cycles, and the mean cyclic lengths of the control (4.6 days) and GN (4.7 days) groups were not significantly different.

At weaning of LAA offspring, the mean uterus weight of the GN group (27.0 mg) was significantly lighter [$F(1,10)=6.53$, $P<0.05$] than that of the control group (30.3 mg). There were no significant differences between the control and GN groups in the weights of the adrenal glands, thymus, testes, epididymides, or ovaries in either strain.

In male offspring at 12 weeks of age, the weights of the seminal vehicles, adrenal glands, and thymus were significantly lighter, and the weight of the testes was significantly heavier in LAA than in HAA rats. There were no significant effects of GN exposure on any organ of either strain. The percent of motile sperm was significantly higher in HAA than in LAA rats. The values of VAP, VSL, and VCL, which represent the swimming speed, and ALH, which reflects the oscillation width of a sperm head, were significantly higher in HAA than in LAA rats. There were, however, no significant effects of GN exposure on any of the parameters of sperm motion. A strain difference between HAA and LAA rats was observed in the plasma levels of FSH, but no significant effects of GN on progesterone, LH, or FSH of male offspring were noted.

In female offspring at 12 weeks of age, the weight of the thymus was significantly lighter, and that of the ovaries was significantly heavier in LAA than in HAA rats. No influence of GN exposure was observed in any organ

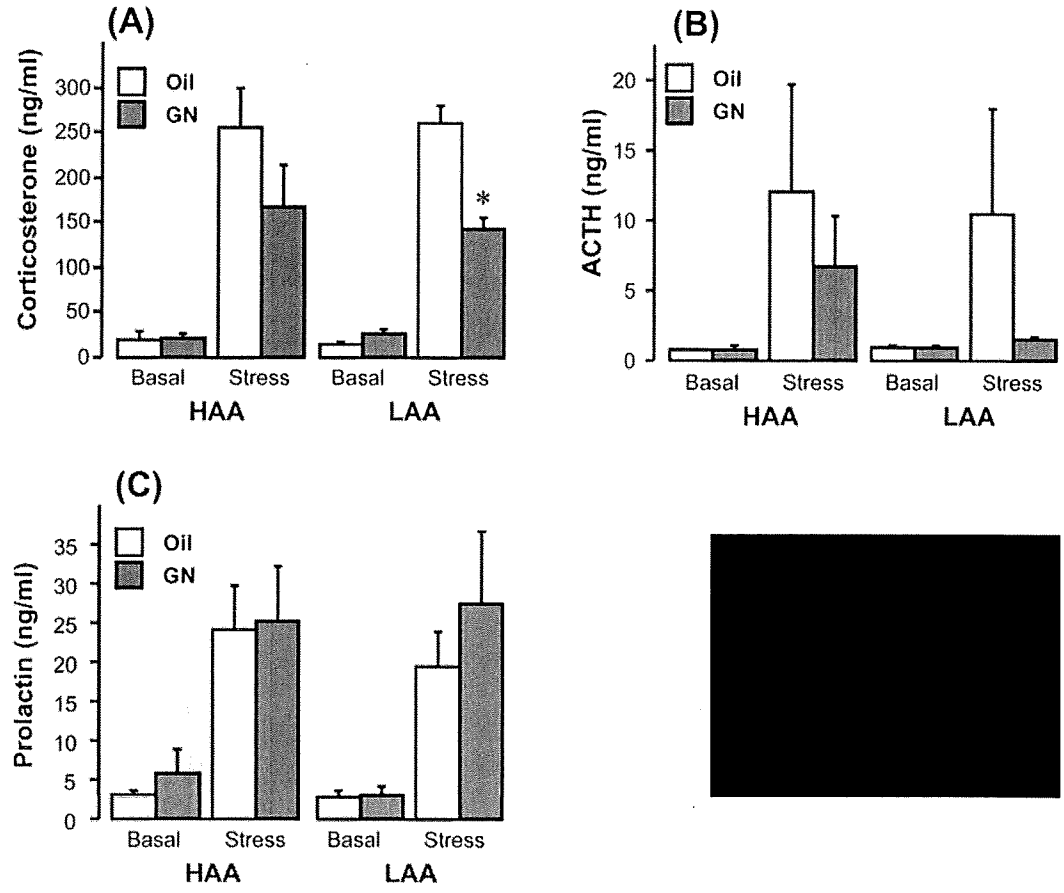

Fig. 3. Plasma levels of corticosterone (A), ACTH (B), and prolactin (C) in HAA and LAA offspring during restraint stress at 6 months of age following transmaternal genistein exposure. Data are expressed as the mean ± SE. **P*<0.05 compared to the respective controls.

of either strain. Strain differences were observed in the plasma levels of progesterone, LH, and FSH, but no significant effects of GN on these hormones were noted.

The results of the PFC assay, the anti-SRBC IgM, spleen weights, and lymphocyte counts are shown in Figs. 2A to 2D. The value of the PFC assay in the HAA offspring was significantly decreased by GN exposure [$F(1,6)=6.42, P<0.05$]. The same tendency was observed in the LAA offspring, but the difference was not significant. The anti-SRBC IgM level was higher in LAA than in HAA offspring. There was, however, no significant effect of GN exposure on anti-SRBC IgM levels. No effect of GN exposure was observed on the spleen weights of either strain. The number of lymphocytes was significantly decreased in HAA offspring reared by GN-treated dams [$F(1,6)=18.92, P<0.01$]. No significant effect of GN exposure was observed in the LAA offspring.

The results of the restraint-stress challenge test are shown in Figs. 3A, 3B, and 3C. During restraint stress, the plasma concentrations of corticosterone, ACTH, and prolactin increased in both strains. A significant effect of GN was found on the corticosterone concentrations of LAA offspring [$F(1,9)=16.53$, $P<0.01$], which were significantly lower in the GN group than in the control group. Similarly, the corticosterone concentrations of the HAA offspring and the ACTH concentrations of both strains tended to decrease in the GN group during stress, but these changes were not significant. There was no significant effect of GN on plasma prolactin concentrations. The weights of the testes and the ventral prostate at 6 months of age were significantly heavier in LAA

than in HAA offspring. However, there were no significant effects of GN on the weights of the testes, epididymides, or ventral prostate at 6 months of age.

Discussion

The influence of transmaternal GN exposure on body weight was observed in both sexes of LAA offspring after weaning. The newborns of SD rats directly given 12.5 mg/kg/day or more of GN by gavage from PD 1 to PD 5 exhibited a decrease in the body weight of both sexes after weaning [23]. It has been reported that in juvenile female mice given GN (20 and 80 mg/kg/day s.c.), fat pad weight decreases dose dependently and that lipoprotein lipase mRNA also decreases [22]. Oral GN treatment (150 and 1,500 mg/kg diet) in ovariectomized mice is also reported to result in reduced body weight and apotosis of adipose tissue [14]. In addition, GN (5 mg/kg) caused a slight increase in blood glucose concentration with a concomitant drop in insulin level in male rats [40]. Although the mechanisms of the body weight reduction induced by transmaternal GN exposure in the present study are not clear, the strain difference in the effect of GN on body weight is interesting, and this difference is probably dependent on the genetic backgrounds of the animals.

Sexual maturation was delayed in both sexes of LAA offspring. This was possibly caused by the body weight effect, because a high dose of GN as well as other estrogenic compounds is expected to accelerate vaginal opening.

No obvious effects of GN were observed on any parameters of shuttle-box avoidance or running-wheel activity for either the HAA or LAA strain. Therefore, transmaternal exposure to GN does not affect avoidance learning or locomotion activity independently of baseline behavior.

When the organ weights were measured at weaning of the LAA offspring, the mean uterine weight in the GN group was significantly lighter than that in the control group. Although postnatal exposure to a high dose of GN is expected to increase uterine weight, decreased uterine weight following lactational exposure was reported at low doses of GN [4, 6]. Low doses of diethylstilbestrol or ethynylestradiol also induced uterine weight reduction by neonatal treatment [5]. Uterine weight reduction may be caused by down regulation of uterine estrogen receptor during neonatal treatment with estrogens.

There were no effects of GN exposure on the estrous cycles of the HAA and LAA offspring. It was shown that irregular estrous cycles occur following neonatal exposure of rats to GN for prolonged periods during estrus [24]. Jefferson *et al.* [12] reported alterations in the estrous cycle of CD-1 mice following neonatal exposure to GN at doses of 0.5 to 50 mg/kg and these were exacerbated more at 6 months than at 2 months of age. The dose of GN used in the present study probably did not affect the estrous cycle of offspring, because no significant effects of GN were observed on reproductive organ weight or hormone levels in adulthood. However, estrous cycle observation in the present study was made only at a young age. Further study at an older age is needed to evaluate the estrous cycle of offspring.

Although no effect of GN exposure was observed on anti-SRBC IgM levels, the value of the PFC assay was decreased by GN exposure in the HAA offspring. Furthermore, the number of lymphocytes was decreased in the HAA offspring reared by GN-treated dams. These results suggest that GN induces immunosuppression *in vivo*. GN at a dose of 80 mg/kg/day produced impairments in humoral immunity reducing keyhole limpet haemocyanin-specific antibody titers in mice [41]. In ovalbumin-immunized mice, GN at a dose of 20 mg/kg/day suppressed ovalbumin-specific IgG levels [16]. However, an increased splenic T-cell number was observed in SD rats exposed to GN during gestation and lactation [10]. Sakai and Kogiso [34] suggest that the effect of GN on immunity is immune cell-dependent.

Long-Evans rats that were given a high phytoestrogen diet showed decreased anxiety, as expressed in elevated plus maze results [18]. During restraint stress in the present study, the plasma concentrations of corticosterone and ACTH were lower in the GN group than in the control group in both strains. These results seem to agree with the decreased anxiety reported for GN offspring as described above. The opposite result was reported in hooded Lister rats that were fed 150 μg of GN plus daidzein for 14 days [11]. Furthermore, male Long-Evans rats on a lifelong high phytoestrogen diet (600 μg/g of

diet) showed higher plasma ACTH but similar corticosterone levels after stress [17]. In addition, serum corticosterone levels tended to decrease in male Wistar rats that were administered subcutaneously with GN (40 mg/kg/day) for 3 weeks after weaning [25]. These reports suggest that GN alters the negative feedback of stress hormones and/or steriodogenesis in the adrenal gland of rats. However, the perinatal effect of GN on the hypothalamic-pituitary-adrenal (HPA) axis is not clear. Further study is needed to evaluate the effects of GN on the relationship between anxiety stress and the HPA axis.

The dosage of 5 mg/animal/day (approximately 16 mg/kg/day) of GN, that was used in the present study, was chosen to be comparable to the normal range of human exposure levels during lactation [8, 37]. Lewis *et al.* [20] reported that SD rats administered with GN in a single oral dose of 16 mg/kg during lactation had a milk GN level of 0.17 μg/ml, while the plasma level of dams was 1.8 μg/ml. Thus, the amount of GN expressed into milk is low. However, some alterations were detected in offspring reared by GN-treated dams in the present study suggesting that this dose of GN caused some effects in the next generation.

This comparative study using HAA and LAA rats, which have different characteristics between their strains and uniform characteristics within strains, may provide useful information on individual differences in sensitivity to compounds with estrogen activity such as GN. Transmaternal exposure to GN inhibited growth and reduced uterine weight in the LAA offspring. Antibody production was inhibited in the HAA offspring and a reduced stress response was observed in the LAA offspring. These results suggest that the HAA and LAA strains are useful animal models for studying the influence of endocrine active chemicals found in the environment and for estimating their influences on the whole body systems.

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References

1. Asai, S., Ohta, R., Fujikawa, T., Sakai, R.R., Shirota, M., Watanabe, G., and Taya, K. 2006. Gastric ulceration and expression of prolactin receptor in the brain in Hatano high- and low-avoidance rats. *Endocrine* 30: 161–166.
2. Asai, S., Ohta, R., Shirota, M., Sato, M., Watanabe, G., and Taya, K. 2002. Reproductive endocrinology in Hatano high- and low-avoidance rats during the estrous cycle. *Endocrine* 18: 161–166.
3. Asai, S., Ohta, R., Shirota, M., Watanabe, G., and Taya, K. 2004. Differential responses of the hypothalamo-pituitary-adrenocortical axis to acute restraint stress in Hatano high- and low-avoidance rats. *J. Endocrinol.* 181: 515–520.
4. Awoniyi, C.A., Roberts, D., Veeramachaneni, D.N., Hurst, B.S., Tucker, K.E., and Schlaff, W.D. 1998. Reproductive sequelae in female rats after in utero and neonatal exposure to the phytoestrogen genistein. *Fertil. Steril.* 70: 440–447.
5. Branham, W.S., Zehr, D.R., Chen, J.J., and Sheehan, D.M. 1988. Uterine abnormalities in rats exposed neonatally to diethylstilbestrol, ethynylestradiol, or clomiphene citrate. *Toxicology* 51: 201–212.
6. Casanova, M., You, L., Gaido, K.W., Archibeque-Engle, S., Janszen, D.B., and Heck, H.A. 1999. Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors α and β *in vitro*. *Toxicol. Sci.* 51: 236–244.
7. Flynn, K.M., Ferguson, S.A., Delclos, K.B., and Newbold, R.R. 2000. Effects of genistein exposure on sexually dimorphic behaviors in rats. *Toxicol. Sci.* 55: 311–319.
8. Franke, A.A., Custer, L.J., and Tanaka, Y. 1998. Isoflavones in human breast milk and other biological fluids. *Am. J. Clin. Nutr.* 68: 1466S–1473S.
9. Guo, T.L., Auttachoat, W., and Chi, R.P. 2005. Genistein enhancement of respiratory allergen trimellitic anhydride-induced IgE production by adult B6C3F1 mice following in utero and postnatal exposure. *Toxicol. Sci.* 87: 399–408.
10. Guo, T.L., White, K.L.Jr., Brown, R.D., Delclos, K.B., Newbold, R.R., Weis, C., Germolec, D.R., and McCay, J.A. 2002. Genistein modulates splenic natural killer cell activity, antibody-forming cell response, and phenotypic marker expression in F(0) and F(1) generations of Sprague-Dawley rats. *Toxicol. Appl. Pharmacol.* 181: 219–227.
11. Hartley, D.E., Edwards, J.E., Spiller, C.E., Alom, N., Tucci, S., Seth, P., Forsling, M.L., and File, S.E. 2003. The soya isoflavone content of rat diet can increase anxiety and stress hormone release in the male rat. *Psychopharmacology (Berl)* 167: 46–53.
12. Jefferson, W.N., Padilla-Banks, E., and Newbold, R.R. 2005. Adverse effects on female development and reproduction in CD-1 mice following neonatal exposure to the phytoestrogen genistein at environmentally relevant doses. *Biol. Reprod.* 73: 798–806.
13. Kanno, J., Onyon, L., Peddada, S., Ashby, J., Jacob, E., and Owens, W. 2003. The OECD program to validate the rat uterotrophic bioassay. Phase 2: dose-response studies. *Environ. Health Perspect.* 111: 1530–1549.
14. Kim, H.K., Nelson-Dooley, C., Della-Fera, M.A., Yang, J.Y.,

Zhang, W., Duan, J., Hartzell, D.L., Hamrick, M.W., and Baile, C.A. 2006. Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice. *J. Nutr.* 136: 409–414.

15. Klein, S.L., Wisniewski, A.B., Marson, A.L., Glass, G.E., and Gearhart, J.P. 2002. Early exposure to genistein exerts long-lasting effects on the endocrine and immune systems in rats. *Mol. Med.* 8: 742–749.
16. Kogiso, M., Sakai, T., Mitsuya, K., Komatsu, T., and Yamamoto, S. 2006. Genistein suppresses antigen-specific immune responses through competition with 17β-estradiol for estrogen receptors in ovalbumin-immunized BALB/c mice. *Nutrition* 22: 802–809.
17. Lephart, E.D., Galindo, E., and Bu, L.H. 2003. Stress (hypothalamic-pituitary-adrenal axis) and pain response in male rats exposed lifelong to high vs. low phytoestrogen diets. *Neurosci. Lett.* 342: 65–68.
18. Lephart, E.D., Setchell, K.D., Handa, R.J., and Lund, T.D. 2004. Behavioral effects of endocrine-disrupting substances: phytoestrogens. *ILAR J.* 45: 443–454.
19. Levy, J.R., Faber, K.A., Ayyash, L., and Hughes, C.L.Jr. 1995. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc. Soc. Exp. Biol. Med.* 208: 60–66.
20. Lewis, R.W., Brooks, N., Milburn, G.M., Soames, A., Stone, S., Hall, M., and Ashby, J. 2003. The effects of the phytoestrogen genistein on the postnatal development of the rat. *Toxicol. Sci.* 71: 74–83.
21. Lund, T.D., West, T.W., Tian, L.Y., Bu, L.H., Simmons, D.L., Setchell, K.D., Adlercreutz, H., and Lephart, E.D. 2001. Visual spatial memory is enhanced in female rats (but inhibited in males) by dietary soy phytoestrogens. *BMC Neurosci.* 2: 20.
22. Naaz, A., Yellayi, S., Zakroczymski, M.A., Bunick, D., Doerge, D.R., Lubahn, D.B., Helferich, W.G., and Cooke, P.S. 2003. The soy isoflavone genistein decreases adipose deposition in mice. *Endocrinology* 144: 3315–3320.
23. Nagao, T., Yoshimura, S., Saito, Y., Nakagomi, M., Usumi, K., and Ono, H. 2001. Reproductive effects in male and female rats of neonatal exposure to genistein. *Reprod. Toxicol.* 15: 399–411.
24. Nikaido, Y., Yoshizawa, K., Danbara, N., Tsujita-Kyutoku, M., Yuri, T., Uehara, N., and Tsubura, A. 2004. Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring. *Reprod. Toxicol.* 18: 803–811.
25. Ohno, S., Nakajima, Y., Inoue, K., Nakazawa, H., and Nakajin, S. 2003. Genistein administration decreases serum corticosterone and testosterone levels in rats. *Life Sci.* 74: 733–742.
26. Ohta, R., Kanazawa, Y., Shindo, T., Furuya, M., Shirota, M., and Kojima, K. 2006. Immunological characteristics of Hatano high-and low-avoidance rats. *Exp. Anim.* 55: 369–374.
27. Ohta, R., Matsumoto, A., Hashimoto, Y., Nagao, T., and Mizutani, M. 1995. Behavioral characteristics of rats selectively bred for high and low avoidance shuttlebox response. *Cong. Anom.* 35: 223–229.
28. Ohta, R., Matsumoto, A., Hashimoto, Y., Nagao, T., and Mizutani, M. 1997. Behavioral characteristics of micrencephalic rats in high and low shuttlebox avoidance lines. *Neurotoxicol. Teratol.* 19: 157–162.
29. Ohta, R., Matsumoto, A., Nagao, T., and Mizutani, M. 1998. Comparative study of behavioral development between high and low shuttlebox avoidance rats. *Physiol. Behav.* 63: 545–551.
30. Ohta, R., Matsumoto, A., Sato, M., Shirota, M., Nagao, T., Tohei, A., and Taya, K. 2000. Postnatal behavior in Hatano high- and low-avoidance rats following prenatal exposure to low-dose methylazoxymethanol. *Neurotoxicol. Teratol.* 22: 405–413.
31. Ohta, R., Shirota, M., Adachi, T., Tohei, A., and Taya, K. 1999. Plasma ACTH levels during early, two-way avoidance acquisition in high- and low-avoidance rats (Hatano strains). *Behav. Genet.* 29: 137–144.
32. Ohta, R., Shirota, M., Tohei, A., and Taya, K. 2002. Maternal behavior, milk ejection, and plasma hormones in Hatano high- and low-avoidance rats. *Horm. Behav.* 42: 116–125.
33. Sakai, T. and Kogiso, M. 2008. Soy isoflavones and immunity. *J. Med. Invest.* 55: 167–173.
34. Santell, R.C., Chang, Y.C., Nair, M.G., and Helferich, W.G. 1997. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J. Nutr.* 127: 263–269.
35. Sato, M., Ohta, R., Kojima, K., and Shirota, M. 2002. Differences in sperm motion between high- and low-shuttlebox avoidance rats (Hatano strains). *J. Androl.* 23: 250–258.
36. Sato, M., Ohta, R., Kojima, K., Shirota, M., Koibuchi, H., Asai, S., Watanabe, G., and Taya, K. 2002. A comparative study of puberty, and plasma gonadotropin and testicular hormone levels in two inbred strains of Hatano rats. *J. Reprod. Dev.* 48: 111–119.
37. Setchell, K.D., Zimmer-Nechemias, L., Cai, J., and Heubi, J.E. 1998. Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. *Am. J. Clin. Nutr.* 68: 1453S–1461S.
38. Shirota, M., Sato, M., Kojima, K., and Ohta, R. 2004. Minor involvement of somatic growth in the onset of puberty of Hatano high- and low-avoidance rats. *Reproduction* 127: 389–395.
39. Spearow, J.L., Doemeny, P., Sera, R., Leffler, R., and Barkley, M. 1999. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. *Science* 285: 1259–1261.
40. Szkudelska, K., Nogowski, L., KAczmarek, P., Pruszynska-Oszmalek, E., Szkudelski, R., Sieczka, A., and Szkudelski, T. 2003, Hormonal and metabolic effects of genistein and daidzein in male rat. *J. Anim. Feed Sci.* 12: 839–847.
41. Yellayi, S., Naaz, A., Szewczykowski, M.A., Sato, T., Woods, J.A., Chang, J., Segre, M., Allred, C.D., Helferich, W.G., and Cooke, P.S. 2002. The phytoestrogen genistein induces thymic and immune changes: a human health concern? *Proc. Natl. Acad. Sci. U.S.A.* 99: 7616–7621.

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内分泌攪乱性確定試験としてのラット一生涯試験の試み

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Rat One-lifespan Test as a Definitive Test for Endocrine Disruptors

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In order to establish a definitive test protocol for endocrine disruptors, a one-lifespan test was performed using rats and the aging process of reproductive function was observed. Neonates of Sprague-Dawley rats received forced oral administration of diethylstilbestrol (DES) at doses of 0 (vehicle), 0.05, 0.5 and 5 μg/kg for 5 days after birth. Sexual maturation (vaginal opening and preputial separation), estrous cycles (from 8 to 49 weeks of age), mating (at 12, 23, 34, 56 and 68 weeks) and litter size (of the 1st to 3rd parturitions) were observed. Each half of the males were examined for sperm counts and organ weights at 26 and 52 weeks of age. In half of the females, hCG induced ovulation and organ weights were examined at 54 weeks of age. Then the observation of remaining animals was terminated at 101 weeks and survival rate were determined.

Vaginal opening in the group received DES at 5 μg/kg was significantly earlier than the vehicle control group. Normal estrous cycles were observed in no animals of 5 μg/kg DES group throughout the study, and in less than 10% of 0.5 μg/kg DES group at 28 weeks and on. Fertility rate of 12 week-old females of the 5 μg/kg DES group was 0%, and that of 23 week-old females of the 0.5 μg/kg group was 33.3%. Mating rate of 0.05 μg/kg females of this age was reduced to 60%. Influence of neonatal DES exposure was not observed in the first delivery in any group, but in the second parturitions litter size was reduced significantly in the 0.5 μg/kg group. Organ weights of 54 week-old females showed dose-related significant increase of pituitary weight in the 0.05 to 5 μg/kg groups. Adrenal weight was increased in the 0.5 and 5 μg/kg groups. Weight of ovaries was lowered significantly in the 0.5 and 5 μg/kg groups. Testing of induced ovulation with hCG revealed lack of influence of DES on number of shed oocytes. No effects of neonatal DES exposure in males were observed on preputial separation, fertility, sperm counts and organ weights. The lower survival rate was observed in the 5 μg/kg group females.

These results showed that early life exposure of low doses of DES potentially cause precocious sexual maturation, and decreases in reproductive function such as estrous cyclicity, fertility or litter size in female rats. These effects were considered to cause through disruption of hypothalamo-pituitary system, not through direct disturbance on ovarian function. The effects of DES observed in this study indicate the usefulness of one-lifespan test as a definitive test protocol for endocrine disruptors.

緒言

現在，内分泌攪乱化学物質（環境ホルモン，EDC）研究の焦点は，化学物質の内分泌攪乱性を確定する試験法の開発にある．ホルモン活性を有する化学物質が環境中にも存在することは既知の事実で，化学物質のホルモン活性の有無を検討する方法はEDCのスクリーニング試験となり得る．しかし，ホルモン活性を有する化学物質が，生体に有害な影響すなわち内分泌攪乱性を示すか否かを判定する試験法は確立されていない．実

1　毒性部遺伝学研究室
2　毒性部毒性学第1研究室
3　研究顧問

際，これら外因性ホルモン活性物質よりはるかに強力な受容体結合性を持つ内因性ホルモンの影響が，従来の生殖発生毒性試験（多世代繁殖試験）では確認されないにもかかわらず，ジエチルスチルベストロール（DES）のような物質では内分泌系など高次調節系の遅発性の異常が臨床的に起っている．つまりEDCは実際に存在し，それを試験する方法が求められている．このような理由から，現在，EDCの確定試験として従来の多世代繁殖試験に代る「一生涯試験」が考案された．

本研究ではエストロゲン活性を有するDESをSprague-Dawley（SD）ラットの新生児期に投与し，児の発達，成熟および老化に至る各段階において生殖器系機能の変化を検索する「ラット一生涯試験」を試みた．本研究では，新生児期DES投与が引き起こす遅発性の生殖機能異常を検索するために，雌は8週齢から49週齢まで性周期を観察し，12，23および34週齢で交配実験を行った．雄については，26および52週齢で精子検査を行い，12，23，34，56および68週齢で交配実験を行った．

材料および方法

試験には，日本チャールス・リバーから8週齢で入手したCrl:CD（SD）雌雄ラットを使用した．SD系ラットは，毒性試験において一般的に用いられている系統であり，生殖毒性に関する背景データが豊富で，Wistar系ラットに比べて性周期の加齢性変化が早期に起り易いことが知られている[1]．11週齢時に交配し，交尾が確認された雌を1群12匹以上からなる4群に振分けた．動物は温度22～25℃，湿度50～65％，照明12時間（7時～19時点灯）に調節された飼育室で，固型飼料（CE-2，日本クレア）と水道水を自由摂取させて飼育した．妊娠雌は，紙パルプ製チップを入れた金属製ケージに1匹ずつ収容した．全ての実験操作は，「財団法人食品薬品安全センター秦野研究所 動物実験に関する指針」に基づいて実施した．

EDCには子宮内あるいは新生児期の曝露での影響が指摘されていることから，DESの投与経路は新生児への強制経口投与を選択した．投与量は，内分泌攪乱化学物質に対する厚生労働省の試験スキーム[2]を考慮し，子宮肥大試験の結果をもとに設定した．すなわち，0.05～15 μg/kg/dayのDESを卵巣摘出マウスに3日間反復経口投与し，最終投与の約24時間後に子宮重量を測定した結果，5 μg/kg/day以上を投与した群で子宮重量が有意に増加したことから，5 μg/kg/dayを確実影響量として一生涯試験の最高用量に設定し，無影響量と考えられる0.5 および0.05 μg/kg/dayをそれぞれ中用量および低用量に設定した．投与液は，DES（Sigma-Aldrich, St. Louis, MO）20 mgを1 mLのエタノールに溶解し，コーン油で段階希釈して調製した．

新生児は，生後1日（分娩日を生後0日とする）に性別および外表奇形の有無を検査し，異常のない雌雄各5匹を1腹毎に選抜し，四肢の皮下に墨汁を注入して個体識別した．投与は生後1日から生後5日まで1日1回，マイクロシリンジおよび新生児用カテーテル[3]を用いて行い，投与液量は10 mL/kgとした．投与終了後は同腹児数を雌雄各4匹に調整し，生後21日に離乳させた．離乳後は，金属製金網床ケージに2匹ずつ収容した．体重は，生後0～5日（毎日），7，14および21日に測定し，離乳後は週1回，10週齢以降は隔週1回，26週齢以降は4週間毎に測定した．

雌は生後25日から膣開口を，雄は生後35日から陰茎包皮分離[4]を性成熟の指標として毎日観察した．各腹の雌2匹は，8週齢から49週齢まで2週間間隔で連日2週間，膣垢を採取し，性周期を観察した．膣垢像は発情前期，発情期，発情休止期に分類し，渡辺らの報告[1]と同様に性周期の型を分類した．各腹の雌雄各2匹は，12，23および34週齢から2週間を限度に，兄妹交配を避けて1：1で群内交配させた．群内交配で交尾が確認されなかった場合，雄は無処置雌と，雌は交尾が確認された同群の雄と，いずれも2週間を限度に再交配させた．雄は，さらに56および68週齢から2週間を限度に無処置雌と交配させた．群内交配で交尾が確認された雌は自然分娩させ，妊娠日数および産児数を確認し，哺育0および4日の哺育児体重を測定した．無処置雌は妊娠13日以降に帝王切開し，妊娠の有無を確認した．

雄は26，52および101週齢時，雌は54および

101週齢時にペントバルビタールナトリウム麻酔下で採血し，剖検した．雌雄とも101週齢以外の剖検時には，脳，下垂体，甲状腺，肝臓，脾臓，腎臓，副腎，精巣，精巣上体，前立腺（腹葉），精嚢（凝固腺を含む），卵巣，子宮の重量を測定した．

26および52週齢の剖検時に雄から採取し，凍結保存した精巣上体尾部および精巣を用いて精子数および精子頭部数を測定した．精巣上体尾部および精巣は，解凍後，ホモジナイズした精子懸濁液をModified IDENT STAIN Kit（Hamilton-Thorne）により染色し，HTM-IVOSにより，精巣上体尾部および精巣重量当たりの精子数および精子頭部数を求めた[5]．

各腹とも雌1匹は，54週齢時に排卵可能な卵胞の有無を確認するため，剖検16～17時間前にヒト絨毛性性腺刺激ホルモン（hCG，Sigma）を10 IU尾静脈内投与し，剖検時に卵管内の誘起排卵数を数えた[1]．

離乳前の児に関するデータは腹単位，離乳以降のデータは個体を標本単位として解析した．体重，器官重量，産児数および精子数のデータは，一元配置型の分散分析を行い，群間に有意差が認められた場合はDunnett法による多重比較検定を行った．性成熟，妊娠日数および生存率のデータは，Kruskal-Wallisの順位検定を行い，群間に有意差が認められた場合には，順位化した値を用いてDunnett法による多重比較を行った．交尾率および受胎率の差は，Fisherの直接確率法による検定を行った．有意水準は5％および1％とした．

結果

体重：生後0日から離乳まで，および離乳後から26週齢までの体重は，各群とも順調に増加し，雌雄ともDES投与の影響は認められなかった．また，26週齢以降の体重推移についても，46週齢から50週齢にかけて対照群の雌の体重が低下した以外に異常は認められなかった．

性成熟：雌の膣開口時期（平均±S.D.，日）は，5 μg/kg投与群（29.8 ± 2.2）で対照群（32.9 ± 1.7）より有意に早まったが，雄の陰茎包皮分離時期にはDES投与の影響はみられなかった．膣開口時期が早まった5 μg/kg投与群では，雌の全例で尿道開口部の過剰開裂[6]が観察された．

性周期：正常な性周期を示した雌の割合を図1に示した．5 μg/kg投与群では，観察を開始した8週齢から正常な性周期を示す雌は認められなかった．0.5 μg/kg投与群では8週齢から13週齢にかけては80％以上の雌が正常な性周期を示したが，20週齢から25週齢時には約50％，28週齢以降は10％未満となった．0.05 μg/kg投与群は対照

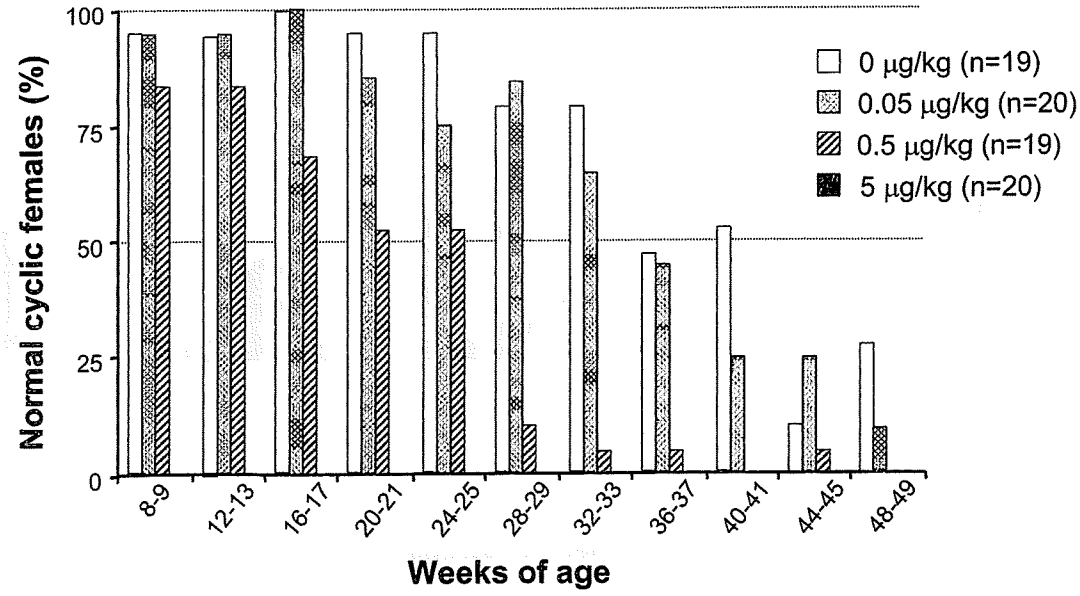

図1　新生児期にDESを投与したSD系雌ラットの性周期（正常性周期の割合の推移）

群とほぼ同様に推移し，正常な性周期を示す雌が29週齢までは80％以上，36週齢以降は50％未満となった．性周期を型別（図2）に見ると，5 μg/kg投与群で早期にみられた異常周期の型は連続発情であったのに対し，0.5 μg/kg以下の用量で加齢に伴って増加した異常周期の型は不規則周期や無発情であった．

交尾率・受胎率：交配結果を表1に示した．雄は12，23および34週齢のいずれの交配時期においても，交尾率および受胎率にDES投与の影響は認められなかった．また，56および68週齢の無処置雌との交配においても，DES投与の影響を示唆する変化は認められなかった．雌は12週齢の交配では，5 μg/kg投与群の交尾率は90％であったが，受胎率は0％となったため同群雌の23週齢以降の交配は中止した．23週齢の交配では，0.05 μg/kg投与群の交尾率が60％に低下し，0.5 μg/kg投与群の受胎率が33.3％に低下した．34週齢の交配では，対照群を含む各投与群の交尾率および受胎率が低下した．

分娩・哺育：分娩した雌の哺育成績を表2に示した．初回分娩では5 μg/kg投与群で産児が得られなかった以外にDES投与の影響は認められなかった．2産目では，0.5 μg/kg投与群の産児数が対照群より有意に減少し，0.05 μg/kg投与群の妊娠日数が対照群より延長する傾向にあった．3産目については，対照群を含む各投与群で受胎率が低下したことから，産児数の評価はできなかった．

雄の精子数および器官重量：26週齢および52週齢で精子数と器官重量を調べたが，いずれの時期においても，精巣上体尾部の精子数，精巣重量当りの精子頭部数，ならびに生殖器を含むいずれの器官重量にもDES投与の影響を示唆する変化は認められなかった．

雌の器官重量：54週齢の雌の器官重量を図3に示した．雌では，全てのDES投与群で下垂体重量が対照群より有意に増加し，5および0.5 μg/kg投与群で副腎重量が，5 μg/kg投与群で甲状腺重量が有意に増加した．また，5および0.5 μg/kg投与群で卵巣重量が対照群より有意に低下した．剖検時には，皮下に乳汁が貯留している例が0.05 μg/kg投与群で20例中2例，0.5 μg/kg投与群で18例中3例，5 μg/kg投与群で19例中9例みられた．その他，血中ホルモン濃度の測定では，0.05 μg/kg以上の投与群でプロラクチン濃度の上昇が，0.5 μg/kg以上の投与群でLH濃度の上昇

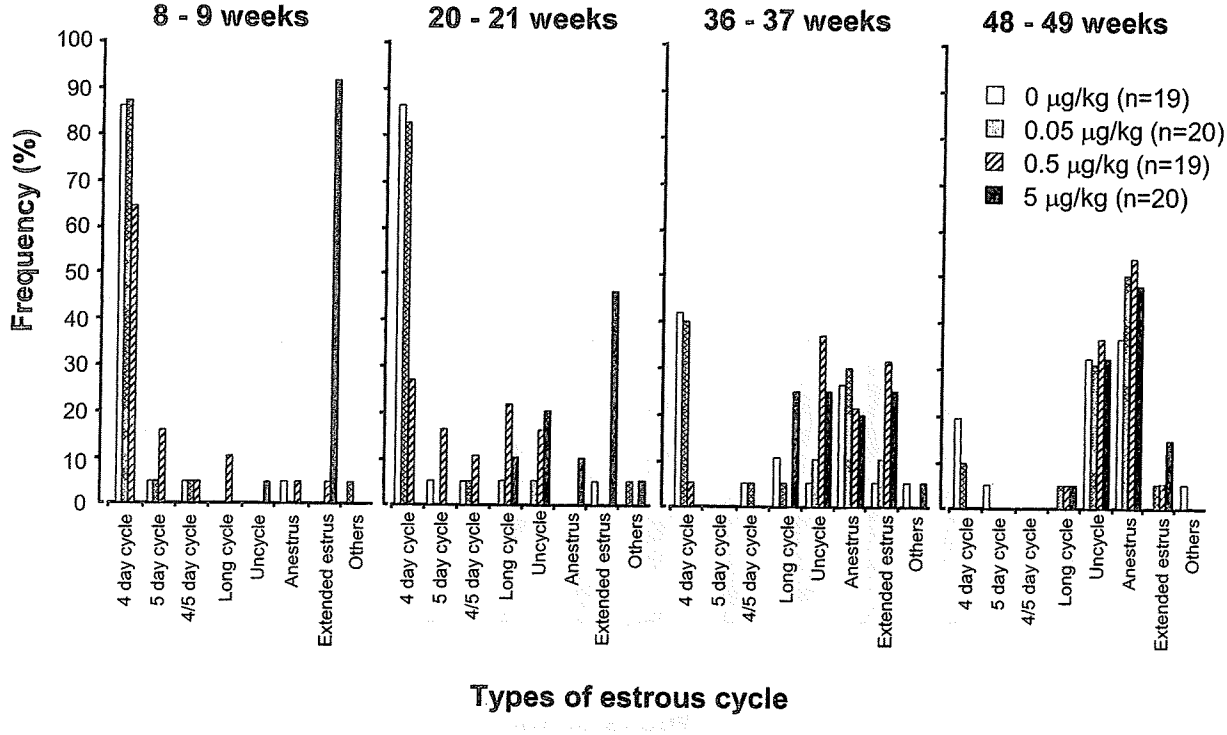

図2　新生児期にDESを投与したSD系雌ラットの性周期（性周期の型別の推移）

表1　新生児期にDESを投与したSD系ラットの交配成績

	Males				Females			
DES (μg/kg)	0	0.05	0.5	5	0	0.05	0.5	5
At 12 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)
Fertility index (%) (No. pregnant/no. copulated)	100.0 (20/20)	84.2 (16/19)	95.0 (19/20)	100.0 (18/18)	90.0 (18/20)	81.3 (13/16)	80.0 (16/20)	0.0 ** (0/18)
At 23 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (17/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	60.0 ** (12/20)	90.0 (18/20)	
Fertility index (%) (No. pregnant/no. copulated)	89.5 (17/19)	100.0 (17/17)	100.0 (20/20)	83.3 (15/18)	80.0 (16/20)	58.3 (7/12)	33.3 ** (6/18)	
At 34 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	55.0 (11/20)	25.0 (5/20)	20.0 * (4/20)	
Fertility index (%) (No. pregnant/no. copulated)	95.0 (19/20)	72.2 (13/18)	90.0 (18/20)	84.2 (16/19)	54.5 (6/11)	60.0 (3/5)	25.0 (1/4)	
At 56 weeks of age								
Copulation index (%) (No. copulated/no. mated)	90.0 (18/20)	60.0 (12/20)	60.0 (12/20)	79.8 (15/20)				
Fertility index (%) (No. pregnant/no. copulated)	72.2 (13/18)	66.7 (8/12)	83.3 (10/12)	66.7 (10/15)				
At 68 weeks of age								
Copulation index (%) (No. copulated/no. mated)	57.9 (11/19)	47.4 (9/19)	55.6 (10/18)	55.6 (10/18)				
Fertility index (%) (No. pregnant/no. copulated)	72.7 (8/11)	66.7 (6/ 9)	80.0 (8/10)	50.0 (5/10)				

*, ** は対照群と比較して有意差（5%および1%）があることを示す.
データには無処置雌との交配結果も含まれる.

表2　新生児期にDESを投与したSD系母ラットの哺育成績

DES (μg/kg)	0	0.05	0.5	5
At the 1st parturition				
Number of dams	18	13	16	0
Gestation length in days	22.1 ± 0.3	22.2 ± 0.7	22.1 ± 0.5	
Number of newborns	13.9 ± 3.4	12.9 ± 3.9	14.1 ± 3.6	
Pup weight (g) Male	6.9 ± 0.3	6.8 ± 0.6	6.7 ± 0.7	
Female	6.5 ± 0.3	6.4 ± 0.6	6.3 ± 0.7	
Viability index on PND 4	99.7 ± 1.5	98.5 ± 3.0	99.0 ± 3.0	
At the 2nd parturition				
Number of dams	16	7	8	0
Gestation length in days	22.3 ± 0.5	22.9 ± 0.4 *	22.5 ± 0.5	
Number of newborns	12.8 ± 3.9	12.7 ± 3.7	7.6 ± 5.8 *	
Pup weight (g) Male	7.2 ± 0.8	6.9 ± 0.7	7.4 ± 0.9	
Female	6.7 ± 0.7	6.8 ± 0.7	6.6 ± 0.6	
Viability index on PND 4	93.3 ± 25.1	100.0 ± 0.0	100.0 ± 0.0	
At the 3rd parturition				
Number of dams	6	3	1	0
Gestation length in days	22.4 ± 0.5	22.7 ± 0.6	22.0	
Number of newborns	12.0 ± 4.3	11.0 ± 6.6	14.0	
Pup weight (g) Male	7.1 ± 0.5	7.2 ± 1.2	6.8	
Female	6.6 ± 0.5	6.8 ± 1.4	6.7	
Viability index on PND 4	96.5 ± 5.9	100.0 ± 0.0	100.0	

* は対照群と比較して有意差（5%）があることを示す. 各値は平均±標準偏差を示す.

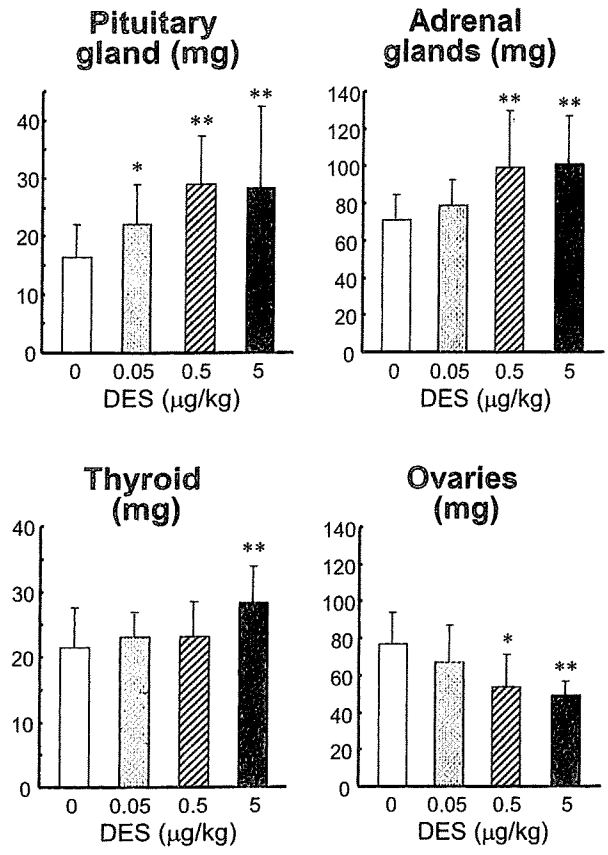

図3　新生児期にDESを投与したSD系雌ラットの54週齢における器官重量

*, ** は対照群と比較して有意差（5%および1%）があることを示す.

対照群（n=16），0.05 μg/kg群（n=20），0.5 μg/kg群（n=19），5 μg/kg群（n=19）

が確認されたが，T_3，T_4およびFSH濃度には，群間の差は認められなかった.

排卵検査：54週齢の排卵検査では，hCG投与により排卵した雌が対照群で8例中7例，0.05 μg/kg投与群で10例中6例，0.5 μg/kg投与群で10例中9例，5 μg/kg投与群で10例中8例みられ，誘起排卵数に群間の差は認められなかった.

生存曲線：雌雄の生存曲線を図4に示した．5 μg/kg投与群の雌では，生存日数が短縮したが，雄の生存日数にDES投与の影響はみられなかった.

考察

性成熟の観察では，5 μg/kg投与群で膣開口時期の早期化がみられ，同群では雌の尿道開口部の過剰開裂も認められた．性成熟の早期化[7]や尿道開口部の過剰開裂[6]を内分泌攪乱物質の生体に及ぼす有害影響と断定するには，さらに慎重に検討すべきであるが，一生涯試験の中では早期に検査できる項目であることから，後に得られる結果とあわせて内分泌攪乱性を判断する材料の一つになると考えられる．陰茎包皮分離時期に関しては，5 μg/kg投与群においても投与の影響はみられなかった．吉村ら[8]は，DESを出生後1～5日に投与したSD系ラットのうち，100 μg/kg以上の投与群で陰茎包皮分離時期の遅延を報告している．したがって，本研究で用いたDESの投与量では，雄の性成熟に影響を及ぼさないと判断される.

5 μg/kg投与群では8週齢から正常な性周期を示す雌動物は認められなかった．この結果から，同群ではDES投与により性成熟前の性腺刺激ホルモンが低下し，androgenizationを起し，膣開口後も排卵はなかったと推察される．一方，0.5 μg/kg投与群でも，16週齢以降に正常な性周期を示す動物の割合が減少した．TCDDの性成熟前投与[9]やビスフェノールAの胎生期投与[10]でも性周期の異常は対照群よりも早く起こることが示されている．これらのことは，内分泌攪乱化学物質の検索において，性周期を長期にわたって観察することの重要性を示している．本研究でみられた異常周期の型は，5 μg/kg投与群では主に連続発情であったのに対し，0.5 μg/kg投与群では不規則周期や無発情などが主であった．このことから，5 μg/kg投与群でみられた異常周期は無排卵に起因したのに対し，0.5 μg/kg投与群でみられた異常は，対照群にみられる加齢性変化が早期に誘発されたものと推察される.

12週齢の交配では，5 μg/kg投与群の雌で交尾が確認されたものの，受胎率は0%であった．これは，前述の排卵を伴わない連続発情を反映した結果と考えられる．一方，0.5 μg/kg投与群の雌では，23週齢の交配で受胎率が低下し，2産目の産児数が減少した．この変化も，同群で早期に加齢性変化，すなわち性周期の乱れを来したことと一致すると考えられ，2産目の産児数の低下は，エストロゲン分泌の低下に起因した排卵数の減少が原因と推定される[11]．なお，23週齢の交配で0.05 μg/kg投与群にみられた交尾率低下の原因は