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A statistical method for judging synergism: application to an endocrine disruptor animal experiment

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SUMMARY

This article proposes a statistical method for judging whether or not the combined action of chemicals is synergistic, being focused on the case in which two or more endocrine disruptors are made to act simultaneously. After defining synergism, the synergistic relation of two chemicals is formulated for a higher response than that expected under an exchangeable relation between them. Using this formulation as a basis, we then rationalize the triangular design for an animal experiment in which all dose settings are controlled within a triangle domain that prescribes the sum of doses of simultaneously applied chemicals less than a certain level. In addition, a statistical test is proposed for judging the synergism among chemicals used in animal experiments, i.e. the test evaluates the discrepancy between the observed mean response from simultaneous administration groups of chemicals and an estimated response under the null hypothesis of zero interaction based on data from single administration groups. Finally, test performance is examined using a simulation study and a case study—the rodent uterotrophic assay. The simulation study revealed that the test is not superior in power to the standard analysis of variance test based on a linear model with interaction term, yet robust in the sense that type I errors under variance heterogeneity were better controlled using Welch correction than the analysis of variance test. The application of the proposed statistical test to an animal experiment is considered acceptable based on results. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS: animal experiment; endocrine disruptor; design of experiment; statistical test; synergism

1. INTRODUCTION

To protect people from the harmful effects of chemicals, society has begun regulating environmental pollutants and toxicants at levels having negligible impact. In the past these regulation levels were determined based on the knowledge or toxicity data of a single administration of an individual chemical. Recently, however, synergic effects due to combining chemicals have become apparent and regulations are now considered to be based on the knowledge or data on their combined action. Accordingly, many studies have been carried out to clarify synergism of harmful effects by

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simultaneous administration of chemicals (see, for example, Reif, 1984; Hasegawa *et al.*, 1996). One particular application is that for the synergism of endocrine disruptors.

As one of the authors has been engaged in endocrine disruptor studies underway in Japan (Kanno *et al.*, 2001), it was necessary to determine how to obtain and analyze data from animal experiments concerned with synergism. Under this requirement, here we investigate data collection/analysis allowing evaluation of synergism, applying the devised method to an animal experimental study conducted in Japan.

Section 2 explains the issues elicited in the above-mentioned study, while Section 3 discusses the concept of synergism adopted in the analysis. Sections 4 and 5 subsequently describe the experiment design and statistical test used for analysis of the endocrine disruptor study, after which Sections 6 and 7 respectively present the results of the simulation study, which examines the performance of the proposed test, and a case study. Section 8 provides a conclusion and discussions.

2. ENDOCRINE DISRUPTOR ISSUE

Chemicals that induce a hormonal effect are referred to as hormonally active agents (HAAs)—see, for example, Committee report (1999), EDSTAC (2001) and Solicitation (2001). Endocrine disrupting chemicals (EDCs) are defined as HAAs that induce adverse effects. As most hormonal effects are well known to be mediated by hormone receptors, endocrine disruption can therefore be defined as a 'receptor-mediated adverse effect or toxicity'.

A question arises concerning what are the major differences between traditional toxicity and receptor-mediated toxicity, especially that occurring through nuclear receptors such as estrogen and androgen receptors, or through ligand-inducible transcription factors such as dioxin receptors. It must be realized that the effects are mediated by the 'signal', and that the 'toxicants' do not need to be at the site of the adverse effect. In addition, with regard to the 'redundancy' of the receptor system, such receptors bind a variety of chemicals having various structures. Naturally, then, the affinity is different among chemicals and usually much lower than that of intrinsic natural hormones such as estradiol (see, for example, Yamasaki *et al.*, 2002). However, binding does occur, and if the concentration of the ligand goes above a certain level, then it usually has the capability to transduce the signal just as natural hormones do.

Since the signal transduction system basically amplifies the signal, it is believed that this occurs at a lower dose range than that exhibited in traditional toxicity studies. Expansion of this aspect may indicate that a system exists in which there is no threshold in response. Another aspect of redundancy is that each particular chemical can change the conformation of the ligand-bound receptor molecule according to the shape of each ligand molecule. If true, this may lead to different signaling properties especially when considering interactions with DNA and/or co-factor molecules.

Ligand-bound receptor molecules need to bind to a specific DNA sequence and recruit co-factors and other transcription machinery molecules in order to induce actual biological effects. In this context, the combined effect of multiple chemicals can be slightly different from what we expect from the monitored effect due to a single chemical.

Moreover, because more than one signaling system is present in humans, and because many other nuclear receptors/transcription factors are redundant in such ways, there may be an interaction between different signal pathways which leads to possible synergism for certain biological endpoints. Therefore, the definition currently needed for the expected combined effect is that if two treatments produce the same endpoint, they can be exchanged by any ratio to produce the same magnitude of the

effect. The definition of an unexpected combined effect is that the effect due to such a combination is much larger than the particular effect induced by each treatment alone.

3. DEFINITION OF SYNERGISM

There are numerous discussions on the definition of additivity, synergism, and antagonism (see, for example, Rothman, 1980; Saracct, 1980; Reif, 1984; Berenbaum, 1989; Kodell and Pounds, 1990; Machado and Robinson, 1994; Laska *et al.*, 1997; Gennings *et al.*, 1997; Roy and Estieve, 1998).

From the 1920s to the 1960s, pharmacologists attempted to classify mechanisms representing the mode of combined action of two chemicals, which is the case considered here. Such trials subsequently generated numerous technical terms such as 'independent joint action,' 'similar joint action,' 'synergistic action,' 'dissimilar joint action,' 'potentiation,' 'depotentiation', and 'augmenter.' Due to the complexity of the concepts and difficulties in actual verification, such mechanistic analyses had virtually ended until a simple definition was introduced (Sakuma, 1996).

It is illustrated in a pharmacology textbook (see, for example, Laurence and Bennett, 1980) as a chart representing a 'Mountain of Happiness', which is an isobolic expression of happiness given after drinking a certain amount of wine followed by coffee. On this chart, a combination of a certain amount of wine and coffee realizes the apex of the response, which cannot be expected by the single administration of wine or coffee, while an excessive administration ends to dullness or sleep. It implies that the pharmacologically useful endpoint is to determine the best combination of two treatments (wine and coffee) regardless of mechanistic considerations. Synergism can be used to express such a peak in an isobologram, which also indicates that too much wine and/or coffee reduces happiness.

In general, toxicologic events are also complex, multi-step phenomena that are not fully understood; hence, it is reasonable to surmise that mechanistic considerations are not established for predicting the combined adverse effect of two chemicals. The definition of synergism regarding hazard identification must therefore be based on a non-mechanistic approach analogous to the Mountain of Happiness, although we are obviously not interested in the best combination of two chemicals that produce the strongest adverse effect. Our interest under the above-mentioned situation concerns the low dosage range in which two chemicals show combined adverse effects at a higher magnitude than that expected when two chemicals are equal in a particular response, i.e. they are exchangeable by any ratio. This viewpoint leads the following formulation adopted here.

Let $\mathrm{f}(d_\mathrm{A}, d_\mathrm{B})$ be the response at the combined dosage $(d_\mathrm{A}, d_\mathrm{B})$ of two chemicals A and B, and D_A and D_B be such that $\mathrm{f}(D_\mathrm{A}, 0) = \mathrm{f}(0, D_\mathrm{B})$ under the assumption that f is a continuously monotone increasing function of either coordinate. If chemicals A and B are exchangeable, then $\mathrm{f}(d_\mathrm{A}, d_\mathrm{B}) = \mathrm{f}(D_\mathrm{A}, 0) = \mathrm{f}(0, D_\mathrm{B})$ is expected for $(d_\mathrm{A}, d_\mathrm{B})$ on the line connecting $(D_\mathrm{A}, 0)$ and $(0, D_\mathrm{B})$. Accordingly, we define the response of the two chemicals to be 'synergistic' if $\mathrm{f}(d_\mathrm{A}, d_\mathrm{B}) > \mathrm{f}(D_\mathrm{A}, 0) = \mathrm{f}(0, D_\mathrm{B})$ for $(d_\mathrm{A}, d_\mathrm{B}), (D_\mathrm{A}, D_\mathrm{B})$ such that

$$\frac{d_\mathrm{A}}{D_\mathrm{A}} + \frac{d_\mathrm{B}}{D_\mathrm{B}} = 1 \tag{1}$$

The case where the equality $\mathrm{f}(d_\mathrm{A}, d_\mathrm{B}) = \mathrm{f}(D_\mathrm{A}, 0) = \mathrm{f}(0, D_\mathrm{B})$ holds, implies 'zero interaction'.

The combined action of two chemicals considered here is, within a certain dose range, the same as the simple similar action for quantal response discussed by Hewlett and Plackett (1959) (see also Piegorsch and Bailer (1997) for summarized explanation), but is slightly different in the sense that it is formulated through an isobolic relation. This formulation is meaningful for proposing a triangular

design, for we need not worry about the combined action of simultaneous administration of chemicals in the dose which is the maximum in the groups with individual chemical administration, while the formulation by Hewlett and Placket was too strict to apply to toxicity evaluation.

4. DESIGN OF EXPERIMENT

From a statistical viewpoint, synergism is examined experimentally using one-sided hypothesis testing for the null hypothesis of zero interaction. Note that a linear model can, without loss of generality, be assumed to express the dose–response relationship under the above-mentioned situation, i.e. in the exchangeable case.

This is true because the dose dependency of the response to both chemicals can be linearized by a suitable scale adjustment and a certain transformation of response, i.e. by the use of a function such as a link function in a generalized linear model that makes the dose–response relationship linear so that the relation $f(d_A, d_B) = \beta_0 + \beta_A d_A + \beta_B d_B$ holds.

While the factorial design shown in Table 1 is most often used for statistically evaluating interaction, it is not appropriate in our case, for the linearization should be confined within a certain dose range. In a two dimensional (2D) plane having coordinates that respectively indicate the dose of each chemical, responses outside the line connecting the maximum dose of the two chemicals do not provide any information on the synergism, so that the dose settings outside this triangle domain are useless for evaluating synergism.

In fact, even when the response for Groups (10), (12), (13), (14), (15), and (16) in Table 1 is quite high, it cannot be used to evaluate synergism because corresponding zero interaction response to be compared with them cannot be estimated. Consequently, we propose to use the triangular design, which eliminates the above-mentioned groups as shown in Table 2, for an animal experiment under the condition that the number of doses given by the administration of individual chemical is the same between the two chemicals. The number of simultaneous administration groups, which is 3 in Table 2, may well be dependent on the purpose of the experiment, but this is not our principal concern here.

5. STATISTICAL METHOD

The one-sided statistical test for evaluating the discrepancy between the observed response and the response estimated under the null hypothesis of zero interaction is considered reasonable as the statistical method for data analysis.

Table 1. An example of factorial design with 4 dose levels of each chemical. Animals are randomly allocated to each of 16 groups. Groups (1) through (7) correspond to single administration groups, whereas Groups (8) through (16) represent simultaneous administration groups

		Dose of chemical A			
		d_{A1}	d_{A2}	d_{A3}	d_{A4}
Dose of chemical B	d_{B1}	(1)	(2)	(3)	(4)
	d_{B2}	(5)	(8)	(9)	(10)
	d_{B3}	(6)	(11)	(12)	(13)
	d_{B4}	(7)	(14)	(15)	(16)

Table 2. An example of triangular design with 4 dose levels of each chemical. Animals are randomly allocated to each of 10 groups. Groups (1) through (7) correspond to single administration groups, whereas Groups (8) through (10) represent simultaneous administration groups

		Dose of chemical A			
		d_{A1}	d_{A2}	d_{A3}	d_{A4}
Dose of chemical B	d_{B1}	(1)	(2)	(3)	(4)
	d_{B2}	(5)	(8)	(9)	
	d_{B3}	(6)	(10)		
	d_{B4}	(7)			

With the endocrine disruptor issue in mind, we assume that the observed variable y_{ij} of jth individual of ith group is distributed as normal with mean μ_i and variance σ_i^2 and that the ys are independent. Let $\bar{y}$ be the observed mean response for simultaneous administration groups (Groups (8), (9), and (10) in the case of Table 2) of two chemicals and $\hat{y}$ be the estimated response corresponding to $\bar{y}$ using data for groups (Groups (1) through (7) in the case of Table 2) with the administration of individual chemicals under the assumption of zero interaction. Naturally, $\bar{y}$ and $\hat{y}$ are statistically independent.

We propose using the following test statistic:

$$T = \frac{\bar{y} - \hat{y}}{\sqrt{\mathrm{Var}(\bar{y}) + \mathrm{Var}(\hat{y})}} \tag{2}$$

where $\mathrm{Var}(\bar{y})$ and $\mathrm{Var}(\hat{y})$ are the estimated variances of $\bar{y}$ and $\hat{y}$, respectively.

If we assume that all σs are equal, the denominator of the statistic T should be pooled within variance, with the degrees of freedom ν being equal to 'the total number of observations – the number of groups' and the critical value with significance level α is the upper 100α percentage point, $t(\nu, \alpha)$, of a t-distribution with degrees of freedom ν. Else if we assume that σs are homogeneous within simultaneous administration groups or groups with individual chemical administration, but heterogeneous between two classes, the two terms in the denominator of T should be separately estimated as the within-class sum of squares divided by 'the total number of observations of the class—the number of the groups in the class'. In the latter case, the critical value is set at $t(\nu, \alpha)$ with the degrees of freedom ν adjusted by Welch correction (see Welch, 1938, or Satterthwaite, 1946).

In the real situation of toxicity experiments, the variances are likely to be heterogeneous and even the latter assumption may be violated. However, since the heterogeneity of variances cannot be exactly estimated, we propose to use the latter test (Proposed-W) as the statistical method for judging synergism, or the former test (Proposed-T) when the homogeneity of variance is confirmed, the performance of these tests being compared with a regression test in the next section.

Thus, the flow of the proposed method is as follows:

Step 0. Check the linearity of the dose–response relationship for the groups with individual chemical administration. If a non-linear dose–response relationship is observed, transformations that linearize the relation are applied.

Step 1. Fit a linear response plane, i.e.

$$y = \beta_0 + \beta_A d_A + \beta_B d_B \tag{3}$$

to the groups with single chemical administration using a least squares method (assuming zero interaction).
Step 2. Calculate the mean response $\bar{y}$ for simultaneous administration groups and the estimate $\hat{y}$ of expected response corresponding to $\bar{y}$ under zero interaction.
Step 3. Calculate the test statistic T and the critical value $t(\nu, \alpha)$ with the adjusted degrees of freedom ν using the Welch correction, where α is the significance level.
Step 4. If $T > t(\nu, \alpha)$, then the relationship is judged as synergistic with significance level α.

6. SIMULATION STUDY

A simulation study was performed to evaluate the performance of the proposed tests.

6.1. Common setup

Let y_{ij} be the response variable obtained from the jth animal of the ith group, where the number of groups with individual chemical administration is seven, being the same as the triangular design shown in Table 2, whereas that of the simultaneous administration groups is one, two, and three for Cases 1, 2, and 3, respectively. The total number k of groups is therefore eight, nine, or ten, depending on the case. The number of animals was fixed at six to coincide with the number used in the endocrine disruptor experiment for the case study in the next section.

It is assumed that y_{ij}, $i = 1, 2, \ldots, k, j = 1, 2, \ldots, 6$, were distributed independently as normal with mean μ_i and variance σ_i^2 and that the dose–response relationship was linear when each chemical was singly administered. As an alternative to the proposed test, we considered an analysis of variance test for interaction in a regression model with interaction, i.e. the null hypothesis was $H_0 : \beta_{AB} = 0$ for the following model:

$$E\{y_{ij}\} = \beta_0 + \beta_A d_A + \beta_B d_B + \beta_{AB} d_A d_B \tag{4}$$

where d_A, d_B are the doses of chemicals A and B, respectively, administered to the ith group. Robustness was examined by comparing the proposed t-tests with Welch correction (Proposed-W test) and without Welch correction (Proposed-T test) with the analysis of variance test (Regression test). Other common simulation conditions were as follows:

- repetition of simulation, 10 000 times
- dose setting for singly administered groups,

$$(d_A, d_B) = (0,0)\ (0,1)\ (0,2)\ (0,3)\ (1,0)\ (2,0) \text{ or } (3,0).$$

- parameter values: $\beta_0 = \beta_A = \beta_B = 1$
- nominal significance level: 5 per cent.

6.2. Alternative hypothesis

Three cases of simultaneous administration groups were considered, i.e.:

Case 1: One group with $(d_A, d_B) = (1.0, 1.0)$.
Case 2: Two groups with $(d_A, d_B) = (1.0, 1.0), (1.5, 1.5)$.
Case 3: Three groups with $(d_A, d_B) = (1.0, 1.0), (1.0, 2.0), (2.0, 1.0)$.

Table 3. Setup of the strength of synergy in alternative hypothesis. Δ_i represents the strength of synergy on the ith group with simultaneous administration of two chemicals. Model (1) corresponds to the null hypothesis, whereas models (2) through (9) represent alternative hypothesis

		Model								
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Case 1	Δ_1	0.0	0.3	0.5	1.0	1.5	2.0			
Case 2	Δ_1	0.0	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5
	Δ_2	0.0	0.45	0.75	1.5	0.675	1.125	2.25	0.9	1.5
Case 3	Δ_1	0.0	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5
	Δ_2	0.0	0.45	0.75	1.5	0.6	1.0	2.0	0.9	1.5
	Δ_3	0.0	0.45	0.75	1.5	0.6	1.0	2.0	0.9	1.5

The number of simultaneous administration groups is therefore different, depending on the case, and $\bar{y}$ is the mean of the observed responses of 1, 2, or 3 groups, depending on Cases 1, 2, or 3, respectively. The strength of the synergism is represented by the parameters Δ_1, Δ_2, and Δ_3, which are defined as the difference between the expected value of y_{ij} of the simultaneous administration groups and the one under the null hypothesis, i.e. Equation (3). If we adopt Equation (4) as an alternative model such as models (5), (6) and (7) in Table 3, then $\Delta_i = \beta_{AB} d_A d_B$, where d_A, d_B are the doses of A and B of the ith group, respectively.

Δs in Table 3 were selected as the simulation setting. As all Δs are obviously zero for model (1), this implies the null hypothesis. For models (2)–(4) the Δs are proportional to $d_A + d_B$, while for models (5)–(7) the Δs are proportional to $d_A d_B$, being advantageous for the analysis of variance test. Models (8) and (9) use steeper Δs.

6.3. Power under variance homogeneity

Table 4 summarizes the results of the simulation, where all the σ_i^2s are the same. It is theoretically natural that powers in Case 1 are the same between the two tests. In other settings, it is noted that the proposed tests are slightly inferior to the analysis of variance test in power under variance homogeneity.

Table 4. Probability (%) to realize significance. Type I error in model (1) and powers in other models. 'Proposed-W' and 'Proposed-T' are proposed tests with or without Welch correction, respectively

		Model								
Case	Test	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Case 1	Proposed-W	5.4	9.7	17.3	49.9	82.5	96.7			
	Proposed-T	4.9	10.6	20.2	61.0	91.7	99.5			
	Regression	4.9	10.6	20.2	61.0	91.7	99.5			
Case 2	Proposed-W	5.2	18.0	41.6	92.2	26.3	60.6	99.3	37.9	77.4
	Proposed-T	4.9	18.8	44.0	93.7	27.2	63.4	99.4	39.6	80.3
	Regression	5.0	17.9	42.5	93.4	30.1	68.8	99.8	46.9	88.2
Case 3	Proposed-W	4.9	24.1	54.1	98.5	33.5	73.0	100.0	57.3	94.9
	Proposed-T	5.1	24.2	54.4	98.6	34.4	73.8	100.0	58.1	95.2
	Regression	4.8	24.0	54.5	98.5	36.1	75.8	100.0	62.8	97.0

Table 5. Type I error (%) under heteroscedasticity. $\sigma_i^2 = \sigma^2 + \mu_i \times \gamma$. 'Proposed-W' and 'Proposed-T' are proposed test with or without Welch correction, respectively

Case	Test	$\gamma = 0$	$\gamma = 1$	$\gamma = 2$	$\gamma = 3$
Case 1	Proposed-W	5.4	5.9	5.6	5.7
	Proposed-T	4.9	6.3	6.2	6.1
	Regression	4.9	6.3	6.2	6.1
Case 2	Proposed-W	5.2	6.1	6.1	6.1
	Proposed-T	4.9	7.0	7.6	7.8
	Regression	5.0	8.0	8.2	8.5
Case 3	Proposed-W	4.9	6.4	6.2	6.4
	Proposed-T	5.1	7.4	7.4	7.3
	Regression	4.8	7.6	7.7	7.6

6.4. Robustness against variance heterogeneity

Variance heterogeneity will likely occur in real situations, and we therefore examine the robustness of the proposed test against variance heterogeneity by considering the case in which within-group variance σ_i^2 is as follows:

$$\sigma_i^2 = \sigma^2 + \mu_i \gamma \tag{5}$$

The parameter γ takes values 1, 2, and 3. Simulation results are summarized in Table 5, where, even though all tests appear to be liberal, the proposed test is the most robust one.

7. CASE STUDY

As a case study, we selected an 'ovariectomized rodent uterotrophic assay' containing seven groups with six animals per group, examining the quantitative endpoint of uterine weight gain in response to the estrogenicity of administered test chemicals. The dose setting was $(d_A, d_B) = (0.0, 0.0)$, (10.0, 0.0), (20.0, 0.0), (0.0, 10.0), (0.0, 20.0), (5.0, 5.0), and (10.0, 10.0), i.e. two simultaneous administration groups were included. The major interest was whether or not the combined effect was synergistic. The observed uterine weights were averaged over two simultaneous administration groups and compared with the response for $(d_A, d_B) = (7.5, 7.5)$ estimated under the null hypothesis based on the data from groups with individual chemical administration.

The observed mean response was $\bar{y} = 1606.4$ with standard error $V(\bar{y}) = 59.4$, and the estimated response was $\hat{y} = 1450.0$ with standard error $V(\hat{y}) = 39.7$. The degrees of freedom using Welch correction were 19, and therefore the one-sided p-value corresponding to the observed value $T = 2.190$ was $p = 0.021$. From these results we judged that the combined action was synergistic—a judgment accepted as reasonable by researchers conducting this experiment.

8. CONCLUSION AND DISCUSSIONS

One issue elicited from endocrine disruptor studies is how to judge the occurrence of synergism among chemicals. Here, we considered exchangeable cases in which two chemicals induce the same type of response on animals.

A linear model was assumed regarding the experiment data using a proper transformation of response and/or doses of chemicals, with synergism being defined as the case in which there occurs a higher response for simultaneous administration of chemicals than the corresponding response expected from the linear model without interaction. Based on this consideration, we proposed a triangular design for an animal experiment and a statistical test for judging synergism.

Results of a simulation study indicate that the proposed test is not superior in power to the analysis of variance test in the regression model with interaction, yet it is nevertheless robust against heteroscedasticity for type I error. By applying the proposed test to actual experimental data, a judgment was made that the combined effect of test chemicals was synergistic. Researchers who conducted the experiment in question agreed this was a reasonable finding.

When considering the molecular mechanism of the receptor-ligand system, it is obvious that the simplest *in vitro* system has only a slight possibility to produce a synergistic response because at the ligand binding site the best ligand is always interfered with by a less potent ligand. The occurrence of synergism is always speculated when the system is complex and has multiple signal pathways, most of which are in a 'black box'.

It is for this reason that a study aimed at detecting possible synergism will likely be an *in vivo* experiment. And yet, such *in vivo* studies always have limitations in their size, sometimes due to (i) the limited capacity of the animal facility, or (ii) manpower limitations. This affects the ability to complete each process within a certain timeframe in order to minimize the circadian variables. The proposed experimental design using a limited number of animals together with a robust statistical analysis method is therefore expected to be useful to many researchers for detecting possible synergistic effects in *in vivo* assays.

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Phytoestrogen-Low Diet for Endocrine Disruptor Studies

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Hormonally active chemicals (HACs) that are capable of inducing adverse effects on wildlife as well as human beings are featured as "endocrine disruptors". Various animal studies conducted to clarify the characteristics of HACs, including the uterotrophic assay, are sufficiently sensitive to detect the effect of 17-β-estradiol in micrograms per kilogram of body weight or lower. In such systems, a trace amount of HACs in the dietary pellets may interfere with the test results and thus can be a serious problem for the low-dose issue, which is now a major topic in the field of endocrine disruptor research. Here, the significance of the hormonal effects of phytoestrogen components in the NIH-07 diet is confirmed and a NIH-07-based open formula "phytoestrogen-low diet" (PLD) is proposed, which effectively reduces uterine weight as well as the uterine luminal epithelial labeling index in ovariectomized rats.

KEYWORDS: Phytoestrogen; genistein; genistin; daidzein; daidzin; rodent diet; uterotrophic response

INTRODUCTION

Hormonally active chemicals (HACs) that are capable of inducing adverse effects on reproduction and/or carcinogenesis in wildlife as well as human beings are featured as "endocrine disruptors" (ED). The National Academy of Science (*1*) and the U.S. Environmental Protection Agency (*2*) recommend various animal studies to clarify the characteristics of those HACs. Some methods, including the uterotrophic assay, are sensitive enough to detect the effect of 17-β-estradiol in micrograms per kilogram of body weight or lower. In such systems, a trace amount of HACs, if any, in the dietary pellets may interfere with the test substance. This dietary hormonal effect can even be a serious problem in assays especially aimed at the low-dose issue, which is now a major topic in the field of ED research (*3*). Odum et al. (*4*) recently reported that five different rodent diets containing different amounts of soy/alfalfa-derived isoflavones (β-glucuronidase-treated genistein and daidzein ranging from barely detectable levels to approximately 18 and 11 mg/100 g of diet, respectively) showed different effects on sexual development in rats, indicating that dietary phytoestrogens can be a modifier of certain hormone-sensitive endpoints.

Indeed, NIH standard dietary pellets (NIH-07 open formula) for experimental animals contain a certain amount of phytoestrogens known to originate from soy products as well as alfalfa (*5*−*8*) (**Table 1**). Here we report the significance of the hormonal effects of the NIH-07 diet and, at the same time, propose an open formula "phytoestrogen-low diet" (PLD), a modified NIH-07 diet. Additionally, a uterotrophic assay was performed to test the effect of dietary phytoestrogens on its response.

Table 1. Contents[a] of Genistein/Genistin and Daidzein/Daidzin in Diets and Components

	genistein	genistin	daidzein	daidzin
NIH-07(OY)[b]	1.6	10	1.4	6.4
PLD	nd[d]	nd	nd	nd
PLD-S[c]	2.8	8.8	2.2	5.1
dried skim milk	nd	nd	nd	nd
fish meal (65% protein)	nd	nd	nd	nd
soybean meal (45% protein)	9	91	7.1	60
alfalfa meal	nd	nd	nd	nd
ground shelled corn	nd	nd	nd	nd
corn gluten meal	nd	nd	nd	nd
wheat (flour)	nd	nd	nd	nd
casein	nd	nd	nd	nd
dried skim milk	nd	nd	nd	nd
soy oil	nd	nd	nd	nd
corn oil	nd	nd	nd	nd

[a] Expressed as mg/100 g. Measured by LC; detection level > 0.5 mg/100 g. [b] NIH-07 slightly modified by Oriental Yeast Co., solely due to material availability. [c] For reference, PLD-S was prepared; genistein, genistin, daidzein, and daidzin in the amounts found in NIH-07(OY) were added back to PLD. Slight change in free/conjugated ratio may be due to heating during pellet formation. [d] nd, not detected.

MATERIALS AND METHODS

Measurement of Phytoestrogens in Diet. Geinstein, genistin, daidzein, and daidzin were measured by a liquid chromatography (LC) technique (*9*) at the Japan Food Research Laboratories, Tokyo, Japan. Approximately 2 g of sample diet was mixed with 80% ethanol and

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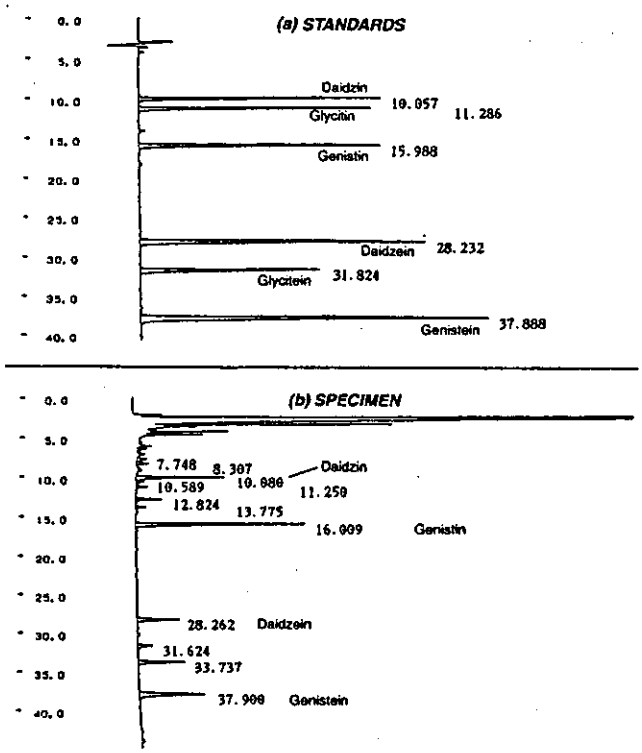

Figure 1. HPLC chromatography of (a) standards and (b) specimen. Elution times for genistein, genistin, daidzein, and daidzin were about 38, 16, 28, and 10 min, respectively.

Table 2. Ingredients of the Diets

ingredient	NIH-07 (original)	NIH-07(OY)[a] (used in this study)	PLD (used in this study)
dried skim milk, %	5.0	5.0	
fish meal (60% protein), %	10.0		
fish meal (65% protein), %		10.0	14.0
soybean meal (49% protein), %	12.0		
soybean meal (45% protein), %		11.75	
alfalfa meal, %	4.0	4.0	
corn gluten meal, %	3.0	3.0	
ground shelled corn, %	24.5	24.5	8.0
ground hard winter wheat, %	23.0		28.5
wheat middlings, %	10.0		
wheat (flour), %		32.87	40.62
casein, %			
brewer's dried yeast, %	2.0	2.0	2.0
dried molasses, %	1.5		
molasses, %		0.75	0.75
soy oil, %	2.5	2.5	
corn oil, %			2.5
salt, %	0.5	0.33	0.33
dicalcium phosphate, %	1.25		
ground limestone, %	0.5		
premixes, %	0.25		
mineral premixes,[b] %		1.05	1.05
vitamin premixes,[b] %		1.0	1.0
total	100.0	100.0	100.0

[a] NIH-07 slightly modified by Oriental Yeast Co., solely due to material availability. [b] Adjusted to original NIH-07.

extracted twice for 1 h. The extract was filtered, concentrated, and measured by an LC-10ATvp/SPD-10AVvp liquid chromatograph (Shimadzu Corp., Kyoto, Japan), using a YMP-Pack ODS-A A-312 f 6 mm × 15 cm column (YMC Co., Ltd., Kyoto, Japan). Standard phytoestrogens were purchased from Funakoshi Co. Ltd., Tokyo, Japan. Standards were dissolved in 80% ethanol (v/v) at 0.25−20 μg/mL, and standard curves were drawn. The conditions for LC were as follows: mobile phase A, 2.5% acetic acid/acetonitrile/methanol, 85+10+5 (v/v); mobile phase B, 2.5% acetic acid/acetonitrile/methanol, 58+19+23 (v/v/v); a gradient program of (A) to (B) in 45 min, linear gradient; flow rate, 1.0 mL/min; column oven, 50 °C; detection, 260 nm; injection volume, 10 μL; measurement interval, 66 min.

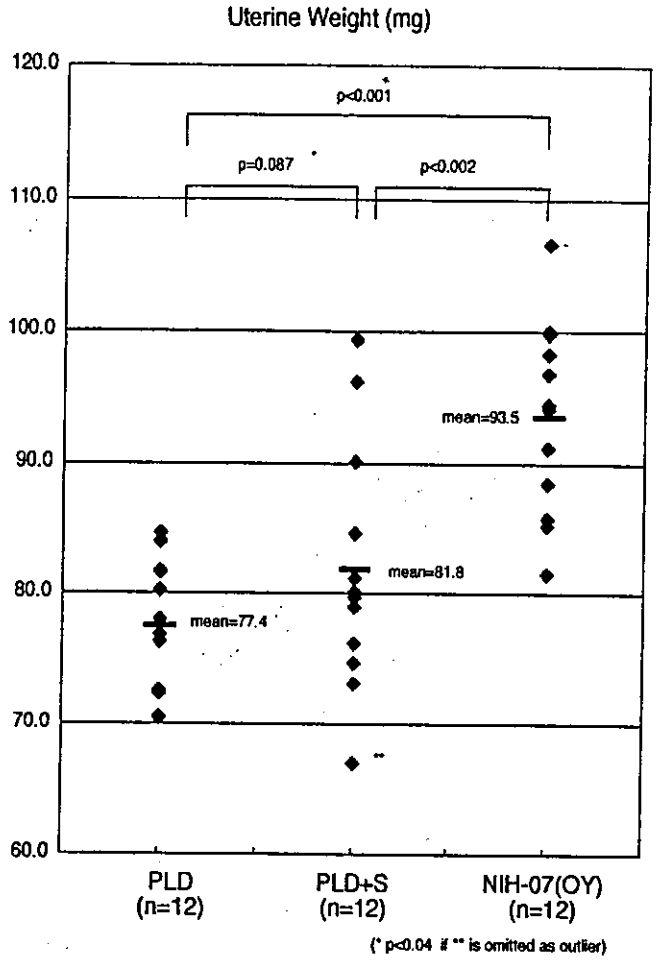

Figure 2. Uterine weight of ovariectomized rats maintained with NIH-07 and PLD: ovariectomized Crj:CD(SD) IGS female rats were used. In the PLD group, animals were maintained on PLD for 4 weeks; in the PLD-S group, animals were given PLD for 2 weeks and then given PLD-S for another 2 weeks; in the NIH-07(OY) group, animals were given PLD for 2 weeks and then given NIH-07(OY) for another 2 weeks.

Diet. The NIH-07 open formula diet (slightly modified by the Oriental Yeast Co., cf. **Table 1**), the PLD, and the PLD supplemented with geinstein, genistin, daidzein, and daidzin (PLD-S) were obtained from the Oriental Yeast Co., Ltd.

Animals. Female Crj:CD(SD)IGS rats (Charles River Japan, Atsugi, Japan) were used. Rats were kept in SPF condition (23 ± 3 °C, 55 ± 15% humidity).

Uterotrophic Assay. Thirty-six 6-week-old female rats were ovariectomized, fed on PLD for 2 weeks, and divided randomly into three groups of 12 rats each. The first group was given PLD for 2 additional weeks. The second and third groups were fed PLD-S or NIH-07 diet for an additional 2 weeks, respectively. BrdU (4 mg/kg) dissolved in DMSO was injected intraperitoneally 2 h prior to necropsy. Aortic blood was collected under ether anesthesia. Blotted uterine weights were measured. Uteri were fixed with buffered formalin and subjected to histology preparation and immunohistochemical staining for BrdU.

RESULTS

The chromatograms of standards and one sample as a representative are shown in **Figure 1**. The elution times for genistein, genistin, daidzein, and daidzin were approximately 38, 16, 28, and 10 min, respectively. The calculated amounts

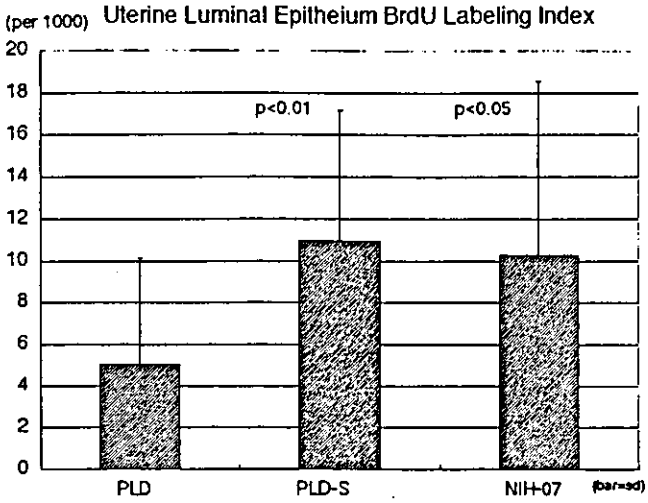

Figure 3. BrdU labeling index of uterine luminal epithelium (number of positive cells per 1000, BrdU 4 mg/kg ip injection 2 h prior to sampling). Both NIH-07 and PLD-S showed significantly high indices when compared to PLD.

of genistein, genistin, daidzein, and daidzin in the NIH-07 diet and its components are shown in **Table 1**. PLD was composed of components free of these four phytoestrogens by current measurements. Although negative for these four phytoestrogens, alfalfa has been reported to include phytoestrogens, and daily products such as dried skim milk and casein, which can contain estrogens of bovine origin, were also removed from the PLD component list. The protein content was adjusted by increasing the percentage of fish meal (**Table 2**). PLD-S, PLD supplemented with genistein, genistin, daidzein, and daidzin at concentrations measured in the NIH-07 diet, was prepared by adding standard phytoestrogen powder to PLD. PLD and PLD-S were also measured for the four phytoestrogens (**Table 1**).

The feeding study using ovariectomized adult female rats showed that the uterine weight, an indicator of estrogenic potency, was significantly lower in rats given PLD than in those given NIH-07 (**Figure 2**). Additionally, the standard deviation of the uterine weight was smallest in the PLD-fed group. The BrdU-labeling index of the luminal epithelium of the uterus was significantly higher in the NIH-07 and PLD-S groups than in the PLD group (**Figure 3**).

DISCUSSION

We have long been aware of HACs such as diethylstilbesterol, a synthetic estrogen, bisphenol A, an industrial chemical, and methoxychlor, a pesticide. Endocrine-disrupting chemicals can be defined as HACs that induce adverse effects in intact organisms, including humans and rodents as our surrogate. Because HACs affect organisms by binding to the hormone receptors, endocrine disruption can be considered a receptor-mediated adverse effect or toxicity.

A characteristic effect of the receptor-mediated toxicity that differs from the traditional toxicity is the effective dose range. Because the endocrine system functions at low concentrations of ligands in the body, subtle hormonal insult by xenoestrogens can induce a cascade of events including altered gene expression. The presence of estrogenic components in the diet, therefore, can be a concern for sensitivity as well as specificity in highly sensitive ED experiments. Our "phytoestrogen-low" diet enhances the sensitivity and reduces the interlaboratory/inter-experimental variations. The latter has often been a focus of discussion in the interpretation of the results generated by low-dose HAC experiments (*4*, *10*).

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Age/sex dependent and non-monotonous dose-response effect of diethylstilbestrol on the immune functions in mice

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Abstract

High amounts of estrogens are known to suppress immune functions in both human and animal models. Our supportive data is that the immune functions were activated by gonadectomy in young adults mice. However, recent reports indicate that estrogen is required for the normal development of thymus. These findings lead us to an assumption that sex hormone status induces much more complex effects on immune functions depending on the host age/sex and dose ranges. Here, low doses of DES (diet containing 0.4 or 4.0 μg/kg diet) were given for 2 months to young adult and aged C57BL/6 mice, and thymic and splenic parameters including flow-cytometric subpopulation analyses, mitogenic responses and NK activity were monitored. This study revealed that the low dose effects of exogenous estrogen are strongly dependent on the age and sex of the recipients and many parameters show non-monotonous dose response effect. In conclusion, effect of exogenous estrogens on immune functions should be assessed for all age and sex, and in expectation of non-monotonous dose-response relationship.

Keywords: Endocrine disrupting chemicals; Diethylstilbestrol; Immune function; Aging; Mouse; Low dose effect

1. Introduction

The immune system is closely related to endocrine system and many hormones can influence functions of various cell types of immune system through hormone receptors (Weigent and Blalock, 1987) including estrogen receptor alpha. Thus, together with the reproduction and developmental processes, the immune system is considered to be a major target of the endocrine disrupting chemicals (EDCs) (Degen and Bolt, 2000; Hutchinson et al., 2000). Many reports have shown that the exogenous steroid hormone suppresses the immune functions in mice. Therefore, the effects of hormonally active chemicals or EDCs on animal and human health have been generally considered as immunosuppressive. We have a supportive data that

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thymic atrophy and its functional loss due to aging in adult mice is significantly restored by gonadectomy (Utsuyama and Hirokawa, 1989). However, recent reports indicate that estrogen is required for the normal development of thymus (Erlandsson et al., 2001; Yellayi et al., 2000). Here, we selected diethylstilbestrol (DES), a potent synthetic estrogen as a model compound, which once had been prescribed to pregnant women to ameliorate problems during pregnancy for a certain period of time and caused adverse effects on the female off springs known as 'DES daughter syndrome' (Wingard and Turiel, 1988). In the present paper, we examined the low dose effects of DES on the immune system in young and aged mice of both sexes to elucidate the complexity of the interactions between EDCs and immune system.

2. Materials and methods

2.1. Mice

C57BL/6 male and female mice were purchased from SLC (Shizuoka, Japan) and maintained in a SPF colony of Tokyo Metropolitan Institute of Gerontology. In this study, young mice were 3 months of age and old one were 18 months of age. Five mice were used for each group.

2.2. Experimental protocols

Two experiments were carried out to see the effect of DES (Sigma Chemical, St. Louis, USA) on the immune system of young and old, and male and female C57BL/6 mice.

Experiment 1 was designed to confirm the acute effects of high doses of DES on the immune system. The dose of 3 mg/kg BW (H1) and 15 mg/kg BW (H2), dissolved in corn oil was intraperitoneally (i.p.) injected into young, male and female mice, once a day for 5 days. Control mice were injected with 0.2 ml of corn oil alone. To old mice, the 3 mg/kg BW (H1) of DES was given i.p. in the similar manner. Two days after the last injection, mice were sacrificed and used for the experiment. DES injected per mouse for 5 days was 1500-2025 μg in H2 dose young group, 300–405 μg in H1 young group and 450–585 μg in H1 old group.

Experiment 2 was designed to observe the low dose effects of DES on the immune system. Mice were fed, ad libitum, powdered diet containing 2 different doses of DES. The powdered diet (NFM-1, Oriental Yeast, Japan) was mixed with DES at 0.4 μg/kg (L1) or 4.0 μg/kg (L2), respectively. Control groups were fed NFM-1 alone. Mice were fed the diet for 2 months and used for the experiment. Diet consumption per cage (5 mice) was monitored twice a week and total amount of diet per mouse was calculated. Table 2 shows total amount of diet (g) and DES (μg) estimated for each mouse of different groups during 2 months.

2.3. Flow cytometry

Cell suspension was prepared from spleen and thymus. The percentage of lymphocyte subpopulations was assessed by FACScan (Becton Dickinson, Mountain View, CA). Lymphocytes were first gated with FSC and SSC, and then percentage of each marker positive cells was measured. Monoclonal antibodies employed were anti-CD3, anti-Thy1.2, anti-NK1.1, anti-CD4, anti-CD8, anti-CD44, anti CD45RB and anti-B220 (Pharmingen, San Diego, CA).

2.4. Anti-SRBC antibody response

Mice were injected intraperitoneally (i.p.) with 0.5 ml of 2% sheep red blood cells (SRBC) in saline and sacrificed 4.5 days later. The spleen cells suspensions were prepared and used for the assessment of direct plaque forming cells (PFC) as described previously (Utsuyama and Hirokawa, 1989).

2.5. NK activity

A fixed number of ^{51}Cr-labeled YAC-1 target cell ($4x10^4$) was mixed with either 0.5, 1, 2 or 4 x 10^6 spleen cells in a total volume of 0.2 ml in microplate with round bottomed wells. The plate was incubated for 5h, centrifuged and the radioactivity of the supernatant was counted by a

Table 1
Weight of body and organs in Experiment 1

Groups		BW (g)	Adrenals (mg)	Gonads (mg)	Thymus (mg)	Spleen (mg)	Liver (g)
Young	-C	24.3±0.4	5.1±0.2	165.5±7.9	45.1±3.4	87.5±2.4	1.38±0.03
	-H1	27.0±0.9*	6.3±0.4*	169.9±6.4	22.4±1.6*	124.1±7.0*	1.90±0.07*
	-H2	25.8±1.1	6.7±0.6*	187.5±4.4*	18.3±1.1*	104.6±5.3	1.92±0.09*
Young	♀-C	20.7±0.2	7.9±0.6	9.7±0.9	42.8±6.5	93.4±1.5	1.08±0.02
	♀-H1	22.4±0.3*	9.8±0.6*	17.0±1.0*	14.5±1.5*	121.4±5.2*	1.64±0.07*
	♀-H2	21.7±0.4	9.3±0.3*	15.8±0.4*	12.0±0.8*	120.7±4.8*	1.92±0.07*
Old	-C	35.8±1.3	5.4±0.9	165.2±7.5	8.8±1.0	127.3±20.2	2.18±0.05
	-H1	38.6±0.1	4.9±0.9	153.0±13.1	6.8±0.9	153.3±23.4	1.80±0.12*
Old	♀-C	29.6±1.9	7.0±0.3	6.5±0.9	21.4±4.4	171.4±40.4	1.86±0.10
	♀-H1	30.0±1.5	8.5±0.6	13.2±2.8*	9.1±1.4*	169.0±56.0	2.23±0.03*

C, control; H1, 3mg/kg(BW); H2, 15mg/kg(BW). Asterisk (*) indicates statistically significant difference ($P < 0.05$), as compared with control.

gamma-counter (ARC-380, Aloka, Japan), as previously described (Utsuyama et al., 2001).

2.6. Mitogenic response

Assays were performed in microplates (Falcon 2072) as previously described (Utsuyama and Hirokawa, 1989). Briefly, 5 x 10^5 cells in 0.2 ml RPMI 1640 supplemented with 5% FBS, and Streptomycin (0.1 mgU/ml) and Penicillin (10U/ml) were stimulated with optimal concentration of phytohemagglutinin (PHA, 0.5 μg/well, Wellcome Reagent Ltd., England), Concanavalin A (Con A, 0.5 μg /well, Sigma, St. Louis, Mo.), E coli lipopolysaccharide (LPS, 1 μg/ml, Sigma, St. Louis, Mo.) or anti-CD3 mAb (145-2C11; hereafter referred to as anti-CD3; 0.5 μg /ml; Pharmingen, San Diego, CA). The plates were then placed in 5% CO_2 incubator for 66 h. Four hours before harvesting, [^{3}H]thymidine (9.25KBq in 10 μl) was added into each well and processed for β scintillation counting (MicroBeta 1450, Pharmacia Biotech, Turku, Finland).

2.7. RT-PCR

Total RNA of tissues or cells was extracted by using an RNA-extracting kit (Isogen; Nippon Gene, Kanazawa, Japan) and used for RT-PCR. Expression of mRNAs of estrogen receptor α and β was determined by RT-PCR method. GAPDH was set for control of each PCR. Reverse transcription (RT) reaction was run for 60 min at 42 °C using MMLV reverse transcriptase (Takara, Japan) and random hexanucleotide primers (GIBCO, Japan).

The primer designs for PCR were as follows;

ERα; 5'-CCTGCCGCTTCACCAAGTGCC
5'-CAGGCCAGCTGCCCGTGCC
ERβ; 5'-TCTGCAGTGATTATGCATCTGGG
5'-CTTCGTAAGGGACATCATCATGG

The conditions used for PCR were 5 min denaturation at 95 °C, then 35 cycles including 40 s at 94 °C, 1 min at 60 °C, 1 min at 72 °C.

2.8. Cell preparation

Thymocyte subsets (DN; $CD4^-CD8^-$, DP; $CD4^+CD8^+$, 4SP; $CD4^+CD8^-$, 8SP; $CD4^-CD8^+$), T cells ($Thy1.2^+$) and B cells ($B220^+$) were sorted out by FACS Vantage (Becton Dickinson, Mountain View, CA).

2.9. Statistics

Student's *t*-test was employed to test the levels of significance between different experimental groups.

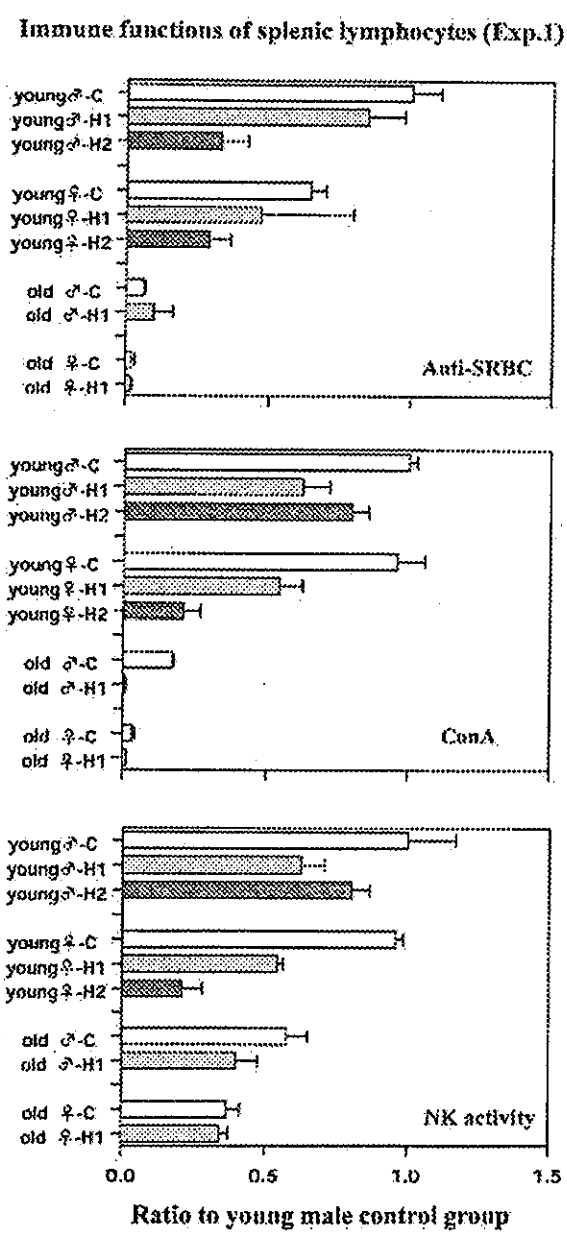

Fig. 1. Immune functions of splenic lymphocytes in mice treated with DES at pharmacological high dose (experiment 1). Anti-SRBC, anti-SRBC antibody response. Con A, proliferative activity of splenic lymphocytes after stimulation with concanavalin A. NK activity, natural killer cell activity against YAC-1 cells. DES dosages are C, control; H1, 3mg/kg(BW); H2, 15mg/kg(BW). Asterisk (*) indicates statistically significant difference ($P < 0.05$), as compared with control. Levels are indicated as ratio to values of young male control group. Each bar and line indicate mean + standard error ($n = 5$).

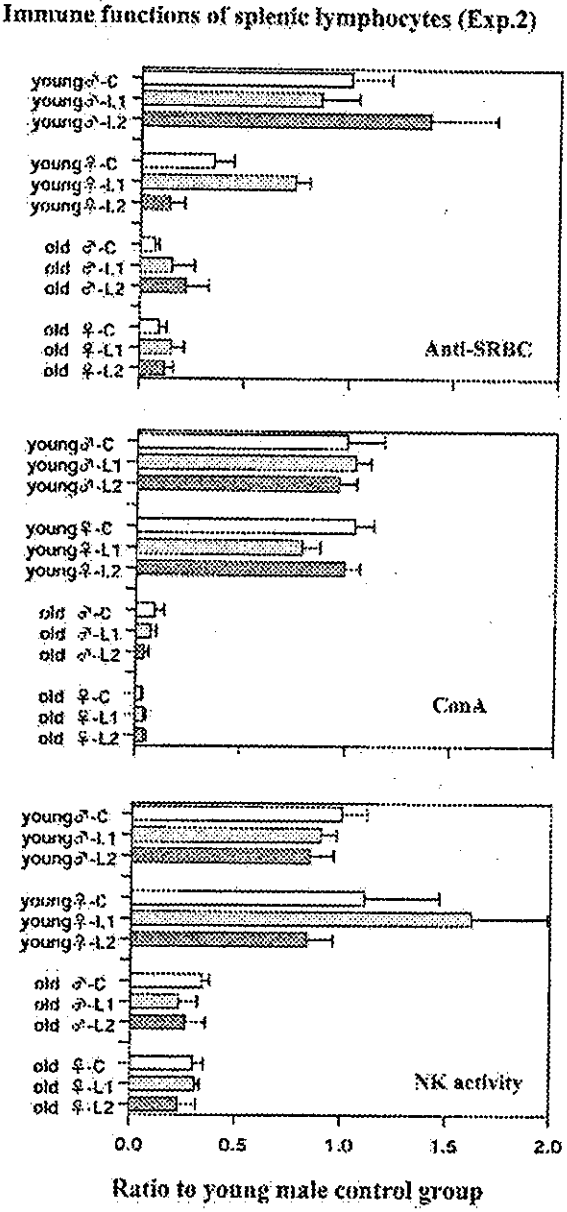

Fig. 2. Immune functions of splenic lymphocytes in mice fed diet containing DES at low dose (experiment 2). Anti-SRBC, anti-SRBC antibody response. Con A, proliferative activity of splenic lymphocytes after stimulation with concanavalin A. NK activity, natural killer cell activity against YAC-1 cells. DES dosages are C, fed control diet alone; L1, fed the diet containing 0.4 μg of DES/Kg of diet; L2, fed the diet containing 4.0 μg of DES/Kg of diet. Total amount of DES estimated for each mouse is shown in Table 2. Asterisk (*) indicates statistically significant difference ($P < 0.05$), as compared with control. Levels are indicated as ratio to values of young male control group. Each bar line indicate mean + standard error (n = 5).

3. Results

3.1. High dose DES effects on the immune system.

Body weight slightly increased in male and female young mice after administration of H1 of DES (Table 1). Weight of adrenal glands significantly increased in young male and female mice of H1and H2 groups. Weight of testes slightly increased at H2 dose, whereas ovaries weight prominently increased. A remarkable increase was observed in the weight of liver in both male and female (Table 1). Thymus atrophy was observed in both young and old, female and male groups in a dose-dependent manner. The thymic cell numbers were in good correlation with the thymus weight and the major decrease in thymocytes was $CD4^+CD8^+$ cells (data not shown). In old male mice, thymic atrophy by DES was monitored in female, but not in male due to severe senile atrophy.

In young mice, a significant increase was observed in the weight of spleen. However, the absolute number of both T cells and B cells did not change significantly. Within T cell subpopulations, $CD4^+CD44^{low}CD45RB^{high}$ (naive T) cells increased in percentage with a concomitant decrease of $CD4^+CD44^{high}CD45RB^{low}$ (memory T) cells (data not shown). In old mice, an increase in spleen weight was observed in male, but not in female. Both T cells and B cells decreased in numbers. Within T cell subpopulations of old mice, naive and memory T cells did not change. Percentage of NK cells significantly decreased in both male and female old mice.

In young mice, anti-SRBC antibody response decreased in both male and female in a dose dependent manner. A marked decrease was also observed in proliferative response of splenic lymphocytes to concanavalin A (Con A) in both male and female by administration of H1 and H2 dose of DES. NK activity did not change significantly. In old mice, proliferative response of splenic lymphocytes to Con A decreased significantly in both male and female. Both anti-SRBC antibody and NK activity that was declined in old mice compared to young mice were not influenced by administration of DES (Fig. 1).

3.2. Low dose of DES on the immune system.

Table 2 shows a total amount of diet and DES given to each mouse during 2 months. The daily intake of DES was estimated as 16–25 ng/mouse for L2 dose group and 1.6–2.4 ng/mouse for L1 dose group. It is calculated as 0.044–0.085 μg/kg bw/day for L1 and 0.461–0.905 μg/kg bw/day according to body weight and diet consumption. Considering that the serum level of estrogens (estrone, estradiol and estriol) in human female at the late pregnancy is approximately 100 ng/ml, the daily intake of DES in this experiment is regarded as within the physiological dose range (Table 2).

The weight of body, kidneys and liver did not change significantly by the treatment in any groups. The weight of thymus was significantly decreased only in L1 of young male group and did not change greatly in other groups (Table 3). When looking at subpopulations of thymocytes, $CD4^+CD8^+$ cells significantly decreased in both

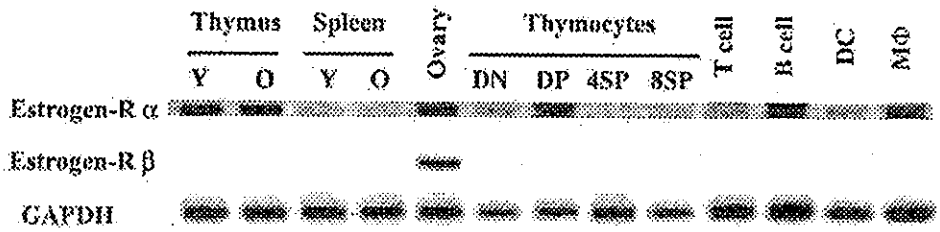

Fig. 3. Expression of estrogen receptor (α and β) in tissues and cells of the immune system. Detection by RT-PCR. Ovarian tissue is used as positive control. Estrogen-R, estrogen receptor; Y, Young; O, Old; DN, $CD4^-CD8^-$; DP, $CD4^+CD8^+$; 4SP, $CD4^+CD8^-$; 8SP, $CD4^-CD8^+$.

Table 2
Total amount of diet and DES in Experiment 2

Groups		Body Weight (g)	Diet (g/mouse)	DES (μg/mouse)	DES (μg/kg bw/day)
Young	♂-C	29.1±0.8	311	(-)	0
	♂-L1	29.3±1.0	301	0.120	0.083
	♂-L2	28.3±0.5	302	1.208	0.850
Young	♀-C	23.5±0.5	370	(-)	0
	♀-L1	24.0±0.5	372	0.149	0.085
	♀-L2	23.7±0.7	384	1.536	0.905
Old	♂-C	31.1±1.5	255	(-)	0
	♂-L1	34.9±1.5	244	0.098	0.044
	♂-L2	33.5±1.9	247	0.988	0.461
Old	♀-C	32.7±1.0	322	(-)	0
	♀-L1	37.4±1.7	312	0.125	0.060
	♀-L2	35.7±1.8	353	1.410	0.701

Diet: Average amount of diet consumed by a mouse in 2 months period. L1 groups, fed the diet containing 0.4 μg of DES/Kg of diet. L2 groups, fed the diet containing 4.0 μg of DES/Kg of diet.

L1 and L2 groups, regardless of age and sex (Table 3). The testes significantly decreased in the L2 group and the ovaries showed a trend of increase in L2 group. The weight of adrenal gland was did not change greatly. However, it is interesting to note that the change of adrenal weight is inversely related to that of thymus weight in young male group (Table 3).

In young mice, the weight of spleen slightly increased in dose dependent manner but not significantly. In old mice, the weight showed a trend of increase at L1 and decrease at L2, generating inverted U-shaped dose response curves (Table 4).

The complex changes in percentage of T cells, T cell subpopulations and NK cells are shown in Table 4. T cells showed a significant increase in young female (L2) and old male (L2), a trend of increase in young male (L2) and a significant increase in old female (L1). B cells showed a significant decrease in young male (L2) and old male (L2), and a trend of decrease in young female (L2) and old female (L2). NK cells significantly increased in old female (L1 and L2).

Table 3
Weight of body and organs, percentage of $CD4^+CD8^+$ subpopulation of thymocytes in Experiment 2

Groups		BW (g)	Adrenals (mg)	Gonads (mg)	Thymus (mg)	$CD4^+CD8^+$ thymocyte (%)	Liver (g)
Young	♂-C	29.1±0.8	3.7±0.6	199.8±1.6	49.2±2.7	84.0±0.7	1.77±0.07
	♂-L1	29.3±1.0	4.5±0.2	184.6±3.6*	37.0±4.3*	78.8±2.2*	1.77±0.11
	♂-L2	28.3±0.5	3.4±0.8	154.2±13.9*	40.6±4.5	81.0±1.0*	1.55±0.10
Young	♀-C	23.5±0.5	6.9±0.5	15.1±1.6	45.8±3.8	88.8±0.7	1.42±0.05
	♀-L1	24.0±0.5	6.8±0.3	14.6±1.1	57.6±5.0	82.3±2.3*	1.32±0.04
	♀-L2	23.7±0.7	7.2±0.3	17.0±0.8	49.1±3.7	82.9±2.5*	1.48±0.07
Old	♂-C	31.1±1.5	5.5±1.0	173.8±18.7	13.6±3.0	81.5±1.5	2.01±0.08
	♂-L1	34.9±1.5	5.0±0.9	151.6±9.9	14.9±1.6	77.3±6.0	2.29±0.09
	♂-L2	33.5±1.9	5.4±1.1	139.5±7.3	14.1±2.5	79.3±3.4	2.03±0.19
Old	♀-C	31.4±1.0	7.9±0.3	12.9±1.8	17.2±3.3	82.6±1.9	1.96±0.09
	♀-L1	34.8±1.9	8.0±0.8	14.2±2.2	12.0±1.3	78.7±4.3	1.99±0.06
	♀-L2	34.7±1.6	7.6±0.6	18.4±2.3	14.3±2.1	79.4±2.6	2.14±0.09

C, L1 and L2, same as shown in Table 2. Asterisk (*) indicates statistically significant difference ($P < 0.05$), as compared with control.