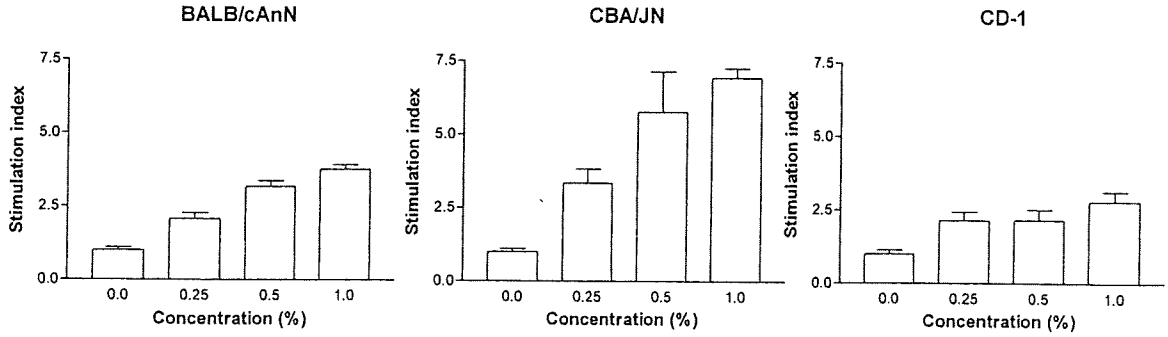

Fig. 1. Dose responses in the modified local lymph node assay with *p*-benzoquinone.
*Data are represented as the mean ± standard error.

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

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

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

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

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

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

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

Acknowledgments

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

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

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

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

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

INTRODUCTION

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

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

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Received 4 July 2003
Revised 26 August 2003
Accepted 3 September 2003

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

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

EXPERIMENTAL

Chemicals and reagents

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

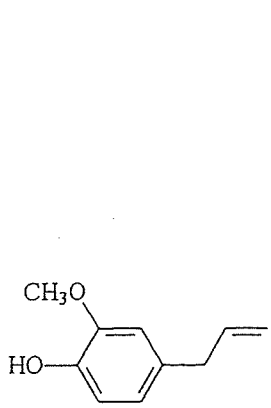
Animals

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

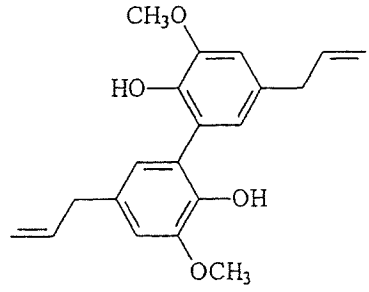
Experimental designs

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

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

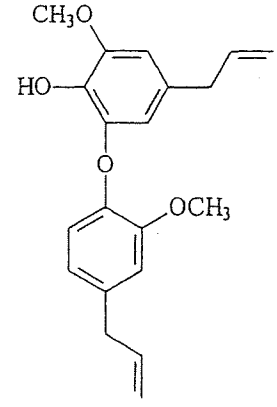


Eugenol



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

- Eugenol dimer 1 -



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

- Eugenol dimer 2 -

Figure 1. Chemical structures of eugenol and its dimers used in this study.

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

Statistical analysis

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

Measurement of EC_3 values

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

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

RESULTS

Guinea pig maximization test

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

Non-radioisotopic LLNA

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

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

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

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

[a] Classified according to the criteria of Magnusson and Kligman (1969).

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

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

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

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

DISCUSSION

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

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

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

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Assessment of statistic analysis in non-radioisotopic local lymph node assay (non-RI-LLNA) with α-hexylcinnamic aldehyde as an example

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Received 31 December 2002; accepted 29 May 2003

Abstract

The murine local lymph node assay (LLNA) is used for the identification of chemicals that have the potential to cause skin sensitization. However, it requires specific facility and handling procedures to accommodate a radioisotopic (RI) endpoint. We have developed non-radioisotopic (non-RI) endpoint of LLNA based on BrdU incorporation to avoid a use of RI. Although this alternative method appears viable in principle, it is somewhat less sensitive than the standard assay. In this study, we report investigations to determine the use of statistical analysis to improve the sensitivity of a non-RI LLNA procedure with α-hexylcinnamic aldehyde (HCA) in two separate experiments. Consequently, the alternative non-RI method required HCA concentrations of greater than 25% to elicit a positive response based on the criterion for classification as a skin sensitizer in the standard LLNA. Nevertheless, dose responses to HCA in the alternative method were consistent in both experiments and we examined whether the use of an endpoint based upon the statistical significance of induced changes in LNC turnover, rather than an SI of 3 or greater, might provide for additional sensitivity. The results reported here demonstrate that with HCA at least significant responses were, in each of two experiments, recorded following exposure of mice to 25% of HCA. These data suggest that this approach may be more satisfactory—at least when BrdU incorporation is measured. However, this modification of the LLNA is rather less sensitive than the standard method if employing statistical endpoint. Taken together the data reported here suggest that a modified LLNA in which BrdU is used in place of radioisotope incorporation shows some promise, but that in its present form, even with the use of a statistical endpoint, lacks some of the sensitivity of the standard method. The challenge is to develop strategies for further refinement of this approach.

Keywords: Local lymph node assay; Sensitivity; Non-radioisotopic; Statistical analysis; α-Hexylcinnamic aldehyde

1. Introduction

The murine local lymph node assay (LLNA) is a validated method for determining the sensitizing potential of chemicals in which activity is mea-

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0300-483X/03/$ - see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/S0300-483X(03)00255-5

sured as a function of induced proliferative responses in draining lymph nodes (Dean et al., 2001; Gerberick et al., 2000; Haneke et al., 2001; Sailstad et al., 2001). The standard LLNA method that employs radioisotope incorporation to measure lymph node cell (LNC) proliferation has been shown to be robust and reliable However, the use of radioisotopes requires some special containment and we have, therefore, explored an alternative strategy. We have previously developed a non-radioisotopic (non-RI) endpoint for the LLNA using 5-bromo-2′-deoxyuridine (BrdU) incorporation (Takeyoshi et al., 2001). Although this alternative method is useful in situations where there is a need to avoid the use of radioisotopes, this alternative endpoint appeared not to be as sensitive as the standard LLNA.

Here we report investigations to determine the use of statistical analysis to improve the sensitivity of a non-RI LLNA procedure based on BrdU incorporation. α-Hexylcinnamic aldehyde (HCA) a contact allergen that has been shown previously to elicit stable responses in the standard LLNA (Dearman et al., 1998, 2001) was used as the test chemical.

2. Materials and methods

2.1. Chemicals

HCA (HCA, Lot No. 0116AQ; Aldrich Chemical Company Inc., Milwaukee, WI, USA) was dissolved in acetone:olive oil (AOO; 4:1). 5-Bromo-2′-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg/ml.

2.2. Animals

Female CBA/JN mice were obtained from Charles River Japan Ltd. (Kanagawa, Japan). The mice were housed in animal rooms maintained at a temperature of 23±2 °C and a relative humidity of 55±15%. The rooms were ventilated at a frequency of 10–15 cycles/h, and lighted artificially for 12-h daily. The animals were allowed to free access to laboratory diet (MF, Oriental yeast Co., Tokyo, Japan) and tap water.

2.3. Experimental design

Mice were randomly allocated to five or four groups (four mice/group). In experiment 1, a 25 μl volume of the HCA preparation at concentration of either 0% (AOO), 3.125, 6.25, 12.5 or 25% in AOO was applied to the dorsum of both ears daily for 3 consecutive days. A single intraperitoneal injection (5 mg/mouse per injection) of BrdU was given on day 4. On day 5, the auricular lymph nodes were removed, weighed, and stored at −20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure the level of BrdU incorporation. In experiment 2, HCA preparations with concentrations of 0% (AOO), 12.5, 25 and 50% were used in the sameway.

2.4. ELISA for BrdU incorporation

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

2.5. Statistical analyses

The means and standard errors (S.E.) were calculated for the labeling index obtained by ELISA for each treatment group. The stimulation index (SI) relative to the concurrent AOO-treated vehicle control value was then calculated. The BrdU labeling index was analyzed simultaneously using the Bartlett test for homogeneity of variance. If the variances were homogeneous at a level of 5% significance, a one-way analysis of variance (one way-ANOVA) was performed. If the one way ANOVA produced a significant difference, the difference between the control group and each of the experimental groups was analyzed using the Dunnett test. If the variances were not homogeneous, the Kruskal–Wallis test was employed. If this test produced a significant difference, the difference between the control group and each of the experimental groups was analyzed using the non-parametric Dunnett test (Bruning and Kintz, 1997). The estimated concentration of a chemical required to induce a SI of three relative to vehicle-treated controls (EC3 value) was derived by linear interpolation as described previously (Basketter et al., 2000). The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and one immediately below, the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the following equation:

$$EC3 = c + [(3 - d)/(b - d)](a - c)$$

3. Results

HCA has been reported to induce stable responses in the standard LLNA (Dearman et al., 2001). To evaluate the utility of various statistical methods for enhancing the sensitivity of the non-RI LLNA two independent experiments were performed. HCA was tested at application concentrations ranging from 0 to 50% in each of the two experiments. In both cases clear dose responses were observed (Fig. 1). In the context of BrdU incorporation it is apparent that concentrations of HCA in excess of 25% were required to provoke a SI of 3 or greater; the criterion for a positive response in the standard LLNA (Kimber and Basketter, 1992; Dearman et al., 1999). However, if the statistical significance of allergen-induced changes in LNC turnover relative to vehicle control values was measured instead then a different picture emerged. A significant treatment related increase in BrdU incorporation was recorded with 25% HCA in Experiment 1, and with 25 and 50% HCA in Experiment 2 (Table 1).

4. Discussion

The standard LLNA is used for the identification of chemicals that have the potential to cause skin sensitization (Kimber et al., 1994; Loveless et al., 1996). The standard LLNA using radioisotopes is a robust and reliable method for hazard identification and has been evaluated fully and validated formally for this purpose. However, it requires specific facility and handling procedures to accommodate a RI endpoint. For this reason there has been some interest in exploring whether other, non-RI endpoints could be used as a read-

Table 1
Results of non-RI LLNA with HCA in two separate experiments

Group	*N*	Mean	S.E.	SI	Probability (%)
Experiment 1					
0(AOO)	4	0.068	0.002		
3.125%	4	0.079	0.009	1.16	91.20
6.25%	4	0.108	0.024	1.59	40.81
12.5%	4	0.127	0.011	1.87	6.64
25%	4	0.165*	0.034	2.42	2.14
Experiment 2					
0(AOO)	4	0.120	0.012		
12.5%	4	0.190	0.036	1.58	37.14
25%	4	0.289*	0.030	2.40	1.17
50%	4	0.436*	0.049	3.63	0.01

N, number of animals; S.E., standard error; SI, stimulation index. Significantly different from the concurrent vehicle control (parametric or non-parametric Dunnett test, $P < 0.05$).

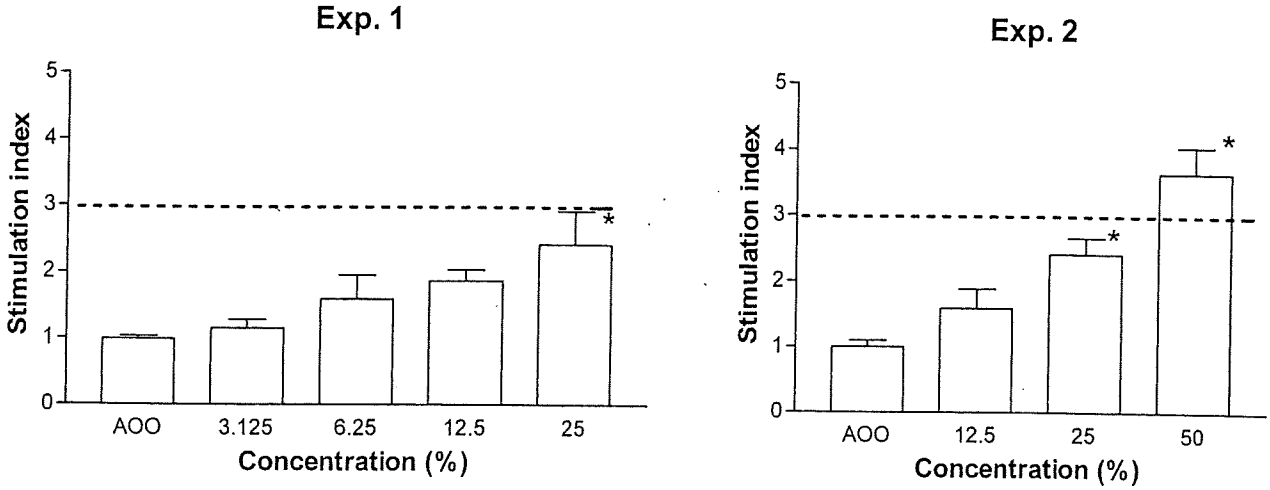

Fig. 1. Dose responses in non-RI LLNA with HCA. * Significantly different from the concurrent vehicle control (parametric or non-parametric Dunnett test, $P < 0.05$).

out for the LLNA. To this end, we have explored the utility of BrdU (Takeyoshi et al., 2001). Several other approaches to find non-RI endpoints for determining a proliferative response in LN have been reported such as the histochemical detection of BrdU (Boussiquet-Leroux et al., 1995), the measurement of interleukin 2 production by LNC (Hatao et al., 1995), flow cytometric analysis of B cell marker on LNC (Gerberick et al., 2002) and flow cytometric analysis of cell division as a function of carboxyfluoresein succinimidyl ether (CFSE) incorporation in LNC (Humphreys et al., 2003). Among them, B cell marker based analysis was found to elicit 1.93 of SI with 50% HCA using CBA/J or CBA/Ca mice, while EC3 of HCA with CFSE based cell division assay in BALB/c mice was estimated as around 5% which close to the EC3 value obtainable in the standard LLNA. In this study, EC3 of HCA was estimated as 37.2% using CBA/JN mice. SIs of 50% HCA for BALB/c and CBA mice were reported to be 10.9 and 9.4, respectively, (Woolhiser et al., 2000). These suggest that non-RI LLNA based on BrdU incorporation was apparently less sensitive than standard LLNA based on radioactive thymidine incorporation. We have no data with regard to the strain-related difference in sensitivity to BrdU. To establish the high sensitive non-RI endpoint, it is necessary to investigate the difference in the strain-related responsiveness to BrdU.

In these investigations, the alternative non-RI method required HCA concentrations of greater than 25% to elicit a positive response based on the criterion for classification as a skin sensitizer in the standard LLNA (the ability at one or more test concentrations to provoke a 3-fold or greater increase in LNC proliferation compared with concurrent vehicle controls; Kimber and Basketter, 1992). Nevertheless, dose responses to HCA in the alternative method were consistent in both experiments and we examined whether the use of an endpoint based upon the statistical significance of induced changes in LNC turnover, rather than an SI of 3 or greater, might provide for additional sensitivity. The results in a series of experiments with HCA clearly support the previous recommendation that an equivocal result would suggest to conduct a repeat test if possible using higher application concentrations (Kimber and Basketter, 1992), and a statistical analysis would also provide effective information to make decision whether the test chemical is a sensitizer or non-sensitizer. The results reported here demonstrate that with HCA at least significant responses were, in each of the two experiments, recorded following exposure of mice to 25% of the chemical. These data suggest that this approach may be more satisfactory—at least when BrdU incorporation is measured. However, even when such a statistical approach is employed, it is clear that this modification of the LLNA is rather less sensitive than the standard

method. It has been reported previously that when employing the standard version of the LLNA positive responses to HCA are observed at application concentrations lower than those required here. Thus, it has been calculated when using the standard assay that between 7 and 12.2% of HCA is necessary to provoke a 3-fold increase in LNC proliferative activity compared with vehicle controls (Dearman et al., 2001). Taken together the data reported here suggest that a modified LLNA in which BrdU is used in place of radioisotope incorporation shows some promise, but that in its present form, even with the use of a statistical endpoint, lacks some of the sensitivity of the standard method. The challenge is to develop strategies for further refinement of this approach.

Acknowledgements

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

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Arch Toxicol (2003) 77: 274–279
DOI 10.1007/s00204-003-0437-z

MOLECULAR TOXICOLOGY

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Development of a high-performance reporter plasmid for detection of chemicals with androgenic activity

Received: 18 June 2002 / Accepted: 5 December 2002 / Published online: 20 February 2003

Abstract A number of chemicals are present in the environment, and some synthetic chemicals may disrupt endocrine function of wild animals and humans. An effective procedure to screen chemicals for endocrine modulating activity has been needed to ensure the safety of chemicals, and the reporter gene assay technique may provide a powerful tool for screening endocrine-disrupting chemicals. We have developed a high-performance reporter plasmid that can trigger high androgen-dependent induction with high selectivity by using mouse mammary tumor virus (MMTV) androgen-responsive elements and a partial fragment of the rat α_{2u}-globulin promoter region. This new type plasmid can induce higher transcriptional activation than a commercial PGV-P-based construct bearing the SV40 promoter fragment, and the basal induction level of this plasmid is much lower than that of the PGV-P-based construct. Moreover, only androgen derivatives could selectively induce a high response in the reporter gene assay with the new reporter plasmid. This new type of reporter plasmid, ARE-AUG-*Luc*+, should be of value in endocrine research and in screening to identify endocrine-modulating chemicals.

Keywords Androgen · α_{2u}-Globulin · Promoter · Endocrine · Reporter gene

Introduction

A number of chemicals are present in the environment, and some synthetic chemicals may disrupt the endocrine function of wild animals and humans. To ensure the safety of chemicals, an effective procedure to screen chemicals for endocrine-modulating activity has been needed by regulatory agencies in several countries, such as the United States Environment Protection Agency (US EPA) and Japan (EDSTAC 1998; OECD 2001). The US EPA developed a chemical screening and testing program consisting of a tiered system to evaluate the endocrine-disrupting effects of chemicals (Earl-Gray 1998). In this program, the hormone receptor-mediated reporter gene assay system is proposed for pre-screening and for the Tier 1 screening battery. The reporter gene assay technique has been used as a tool to investigate gene function, especially to test for enhancer or promoter activity of the regulatory sequences of various genes (Boffelli et al. 1999; Zhang and Teng 2000). Most natural hormones and endocrine-modulating chemicals exert their effects through the transcriptional activation of hormone-responsive genes. Thus, the reporter gene assay technique may be suitable for detecting hormonal activity of chemicals because it has been used to detect enhancers and promoter activity of genes. The reporter gene assay system may also provide a powerful tool to screen for endocrine-disrupting chemicals (Takeyoshi et al. 2002; Yamasaki et al. 2002).

We report here the establishment of a high-performance reporter plasmid that can trigger high androgen-dependent induction with high selectivity by using mouse mammary tumor virus (MMTV) androgen-responsive elements and a partial fragment of the rat α_{2u}-globulin promoter.

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Materials and methods

Test chemicals

The chemicals used in this study are listed in Table 1. All chemicals were dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) at concentrations of 10 mM, and the solutions were serially diluted in the same solvent at a common ratio of 1:10 with an automated pipetting device (Biomek 2000; Beckman Coulter Company, Tokyo, Japan) to prepare stock solutions with concentrations of 1 mM, 100 μM, 10 μM, 1 μM, 100 nM and 10 nM.

Table 1 Test chemicals and results of reporter gene assay using a new reporter plasmid, ARE-AUG200-*Luc*+

Category	Test chemical			Transcriptional activity	
	Name	CAS No.	Manufacturer	PC10(M)	PC50(M)
Natural and synthetic estrogen derivatives	17β-Estradiol	50-28-2	Wako	–	–
	17α-Estradiol	57-91-0	Wako	–	–
	Estrone	53-16-7	Wako	–	–
	Estriol	50-27-1	Wako	–	–
	Ethynyl estradiol	57-63-6	Aldrich	3.55E−06	–
3- or 5- Derivatives of estradiol	Estradiol valerate	979-32-8	Sigma	–	–
	β-Estradiol 3-benzoate	50-50-0	Wako	–	–
	β-Estradiol 3-benzoate 17-*n*-butyrate	63042-18-2	Sigma	–	–
	β-Estradiol 17-acetate	1743-60-8	Sigma	–	–
	Estriol 3-methyl ether	1474-53-9	Sigma	–	–
	Estrone 3-methyl ether	1624-62-0	Sigma	–	–
	Estradiol dipropionate	113-38-2	Sigma	–	–
	Mestranol	72-33-3	Wako	–	–
	β-Estradiol 17-cypionate	313-06-4	Sigma	–	–
	β-Estradiol 3-carboxymethyl ether	41164-36-7	Sigma	–	–
	β-Estradiol 17-hemisuccinate	7698-93-3	Sigma	–	–
	β-Estradiol 17-enanthate	4956-37-0	Sigma	–	–
	17α-Ethynylestradiol 3-cyclopentyl ether	152-43-2	Sigma	–	–
	Estrone acetate	901-93-9	Sigma	–	–
	Estradiol 3-carboxymethyl ether	69260-14-6	Sigma	–	–
	β-Estradiol 17-propionate	3758-34-7	Sigma	–	–
	Estriol 3-benzyl ether	18650-87-8	Sigma	–	–
16 or 17- Stereoisomers/derivatives of estradiol	17-Epiestriol	1228-72-4	Sigma	–	–
	Estradiol 16,17-diacetate	805-26-5	Sigma	–	–
	Estriol triacetate	2284-32-4	Sigma	–	–
	16α-Hydroxyestrone	566-76-7	Sigma	–	–
	16-Ketoestradiol	566-75-6	Sigma	–	–
1- or 3- Derivatives of estradiol	2-Hydroxyestradiol	362-05-0	Sigma	–	–
	4-Hydroxyestradiol	5976-61-4	Sigma	–	–
	2-methoxy-β-estradiol	362-07-2	Aldrich	–	–
	2-Hydroxyestriol	1232-80-0	Sigma	–	–
	3-Deoxyestrone	53-45-2	Sigma	3.29E−07	–
	4-Hydroxyestrone	3131-23-5	Sigma	–	–
	2-Methoxyestrone	362-08-3	Sigma	–	–
Other estradiol derivatives	6β-Hydroxyestradiol-17β	547-81-9	Sigma	–	–
	6α-Hydroxyestradiol	1229-24-9	Sigma	–	–
	6-Ketoestradiol	571-92-6	Sigma	–	–
	6-Dehydroestrone	2208-12-0	Sigma	–	–
	6-Ketoestradiol 6-(*o*-carboxymetyl)oxime	35048-47-6	Sigma	–	–
	6-Ketoestrone	1476-34-2	Sigma	–	–
	Equilin	474-86-2	Sigma	–	–
Androgen derivatives	Testosterone	58-22-0	Wako	1.40E−09	1.24E−08
	Methyltestosterone	58-18-4	Wako	2.00E−11	1.71E−10
	4,5α-Dihydrotestosterone	521-18-6	Wako	2.98E−10	2.93E−09
	Norethynodrel	68-23-5	Sigma	2.52E−09	2.13E0−7
	Norethindrone	68-22-4	Sigma	7.57E−10	3.57E−09
	4-Androstene-3,17-dion	63-05-8	Sigma	3.97E−09	2.86E−08
	Levonorogestrel	797-63-7	Sigma	3.19E−11	4.15E−10
	Testosterone enanthate	315-37-7	Wako	2.22E−08	5.55E−07
Other steroids	Androsterone	53-41-8	Wako	1.18E−07	–
	5α-Androstane	438-22-2	Sigma	–	–
	5α-Androstan-3β-ol	1224-92-6	Sigma	–	–
	Progesterone	57-83-0	Sigma	7.05E−09	–
	Corticosterone	50-22-6	Sigma	–	–
	5α-Androstane-3β,17β-diol	571-20-0	Sigma	6.70E−06	–
	Dehydroisoandrosterone	53-43-0	Wako	–	–
Non-steroids	*p-sec*-Butylphenol	99-71-8	TCI	–	–
	p-t-Butylphenol	98-54-4	TCI	–	–
	Nonylphenol	84852-15-3	TCI	–	–
	Bisphenol A	80-05-7	TCI	–	–
	Diethylstilbestrol	56-53-1	Wako	–	–

Cells

A *Cercopithecus aethiops* kidney cell line (CV-1, ATCC No. CCL-70) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and supplemented with 10% dextran-coated-charcoal (DCC)-treated fetal bovine serum (FCS; Gibco BRL, Rockville, MD, USA) at 37°C in a humid atmosphere containing 5% CO_2 (Miller et al. 2000). The cells were passaged every 3–5 days at 60–80% confluence.

Plasmid construction and transient transfection assay

The full open reading frame of human androgen receptor (AR) coding cDNA (Genbank Accession No. M20132) was cloned into the pcDNA3.1 mammalian expression vector (Invitrogen Corp., Groningen, Netherlands) to make AR/pcDNA3.1. Rat α_{2u}-globulin promoter fragment (Genbank Accession No. U28152) was cloned by using the TaKaRa LA PCR in vitro cloning Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions with gene-specific and adapter-specific primers from rat genomic DNA. An oligonucleotide containing three copies of the mouse mammary tumor virus (MMTV) androgen-responsive element (ARE), 5′-TGAGGTACCA AGCTAGAACA GCATGTTCTG ATCAAGCTAG AACAGCATGT TCTGATCAAG CTAGAACAGC ATGTTCTGAT CGAGCTCTTG-3′, was synthesized and concatenated in the upstream region of the partial rat α_{2u}-globulin promoter fragments. This concatenated fragment was cloned into the multiple cloning site of PGV-B vector (PicaGene basic vector; Toyo Ink MFG Co. Ltd., Tokyo, Japan) to create reporter vector ARE-AUG-*Luc*+. PGV-P vector (PicaGene promoter vector; Toyo Ink MFG Co. Ltd.) bearing SV40 promoter, which was also employed to evaluate promoter performance.

CV-1 cells grown in 90-mm dishes to 60–80% confluence were washed twice with phosphate-buffered saline (PBS). The cells were then transiently transfected with both 2 μg AR/pcDNA3.1, designed to express human AR, and 4 μg ARE-AUG-*Luc*+, designed to detect ARE-mediated transcriptional activation, with 12 μl LipofectAMINE (Gibco BRL) and 60 μl PLUS reagent (Gibco BRL) according to the manufacturer's protocol. After overnight incubation, the cells were trypsinized, resuspended in EMEM without phenol red containing 10% DCC-treated fetal bovine serum, and plated on a flat-bottomed microplate (Corning Coster Corp., Cambridge, MA, USA) at a density of 10^4 cells/well. Each test chemical, diluted in DMSO, was added to the wells at final concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}–10^{-5} M), in quadruplicate. Positive control wells ($n=6$) treated with a natural ligand (10 nM dihydrotestosterone, DHT), and negative control wells ($n=6$) treated with DMSO alone were also prepared on every assay plate. After adding the chemicals to induce the reporter gene product, the assay plates were incubated for 24 h. After washing three times with PBS the cells were lysed with cell culture lysis reagent (CCLR; Promega Corp., Madison, WI, USA). Luciferase activity was measured with the commercial luciferase assay reagent (Promega Corp.) and luminometer (LUMIstar; BMG, Durham, NC, USA) as the integrated value over 5 s. The luminescence signal data were processed and the average and standard deviation for the negative control wells were calculated. The integrated value for each test well was divided by the average integrated value of the negative control well to obtain individual relative transcriptional activity. The average transcriptional activity was then calculated for each concentration of the test chemical. The PC50 and PC10 values were calculated to evaluate the AR agonist potency of each chemical. These PC values were defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response (Takeyoshi et al. 2002; Yamasaki et al. 2002). Description of these parameters are provided in Fig. 1.

Results

We prepared three types of plasmids, ARE-AUG150-*Luc*+, ARE-AUG200-*Luc*+, and ARE-AUG250-*Luc*+, concatenated with different lengths of rat α_{2u}-globulin promoter fragment (Fig. 2). PC50 values calculated for each plasmid were 2.85×10^{-9} M (ARE-PGV), 2.34×10^{-9} M (ARE-AUG100-*Luc*+), 2.93×10^{-9} M (ARE-AUG150-*Luc*+) and 3.21×10^{-9} M (ARE-AUG200-*Luc*+). The values (PC50) were identical for each of the plasmids used in the assay system. However, since ARE-AUG200-*Luc*+ showed the highest induction level for dihydrotestosterone (Fig. 3), suggesting that ARE-AUG200-*Luc*+ had a suitable promoter length for detection of androgen receptor-mediated transcriptional activation, we used ARE-AUG200-*Luc*+ as the standard plasmid to test chemicals for androgenic activity. We confirmed antagonist detectability with these plasmids when cyproterone acetate was used as antagonist against 0.5 nM of DHT (data are not shown).

In the experiment with the positive control substance, 10 nM DHT, the androgen-inducible transcriptional activation level of the plasmid was higher than that of the commercial PGV-P-based construct bearing the SV40 promoter fragment, and the basal induction level of the plasmid was much lower than that of the PGV-P-based construct (Fig. 4).

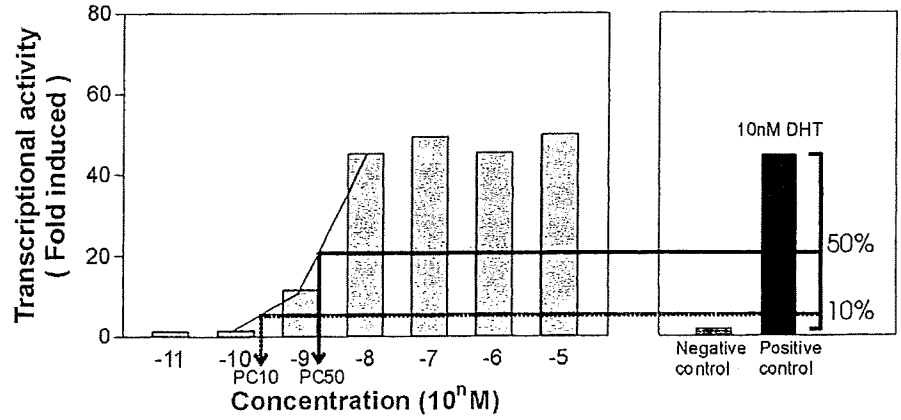

Fig. 1 Schema for the estimation of PC50 and PC10 values for evaluation of the hormonal activity of chemicals (*DHT* dihydrotestosterone)

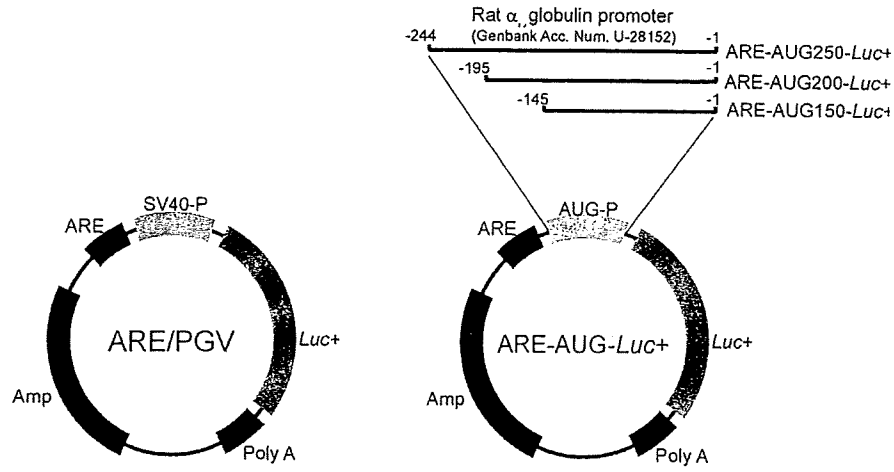

Fig. 2 Structure of hormone-responsive reporter plasmid (ARE-AUG-*Luc*+) used in this study, together with that of a commercially available plasmid (ARE/PGV) *ARE* androgen-responsive elements, *AUG-P* rat α_{2u}-globulin promoter fragment

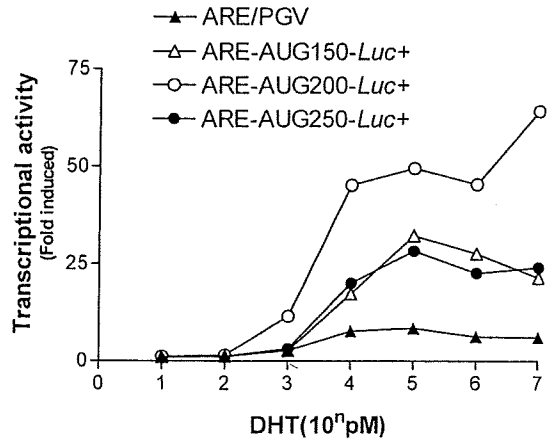

Fig. 3 Transcriptional activity of the androgen-responsive elements (ARE)-mediated reporter gene assay with different types of reporter plasmids. Values are presented as the mean transcriptional activity (of dihydrotestosterone) from quadruplicate assays

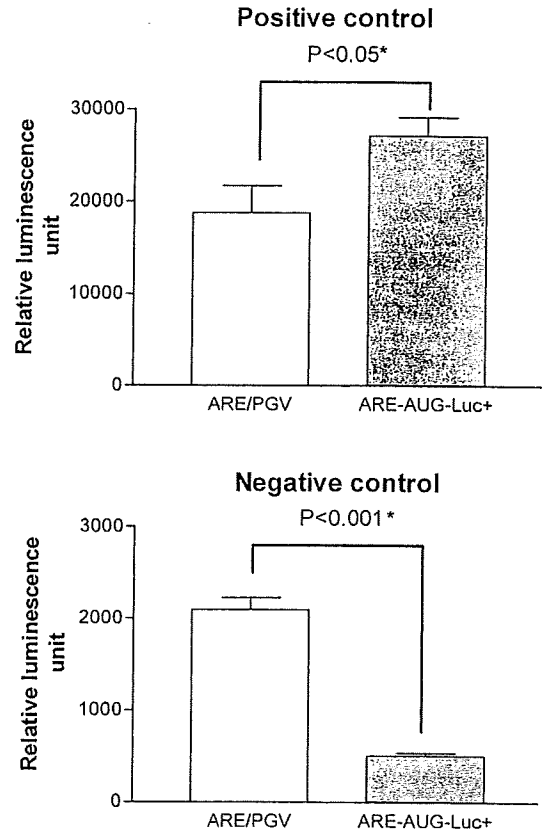

Fig. 4 Comparison of positive and negative control responses in reporter gene assay using ARE-AUG200-*Luc*+ or commercially available plasmid (PGV/ARE). *Data were analysed by Student's *t*-test

The results of a pilot study of 56 steroid derivatives and five non-steroid chemicals are shown in Table 1. PC50 values were calculated only for androgen derivatives, including testosterone, methyltestosterone, 5α-dihydrotestosterone, norethynodrel, norethindrone, 4-androstene-3,17-dione, levonorogestrel and testosterone enanthate (Fig. 5). PC10 values were calculated for ethinyl estradiol, 3-deoxyestrone, androsterone, progesterone, and 5α-androstene-3β,17β-diol, in addition to the compounds for which the PC50 was calculated.

Discussion

Tens of thousands of chemicals are currently used around us and many of them escape into the environment as pollutants. However, since some of the synthetic chemicals that are widely distributed in our environment may mimic estrogens or otherwise disrupt the endocrine system, an effective procedure to screen chemicals for endocrine-modulating activity has been needed by regulatory agencies in several countries, including the USA and Japan, to ensure the safety of chemicals (EDSTAC 1998; OECD 2001). The US EPA developed a chemical screening and testing program consists of a tiered system to evaluate the endocrine-disrupting effects of chemicals (Earl-Gray 1998), and the hormone receptor-mediated reporter gene assay system is proposed for pre-screening and in the Tier 1 screening battery. Since no effective high-throughput screening procedure is available to detect androgen-active compounds, in this study we have developed a new plasmid construct that can be used for the reporter gene assay system to screen chemicals

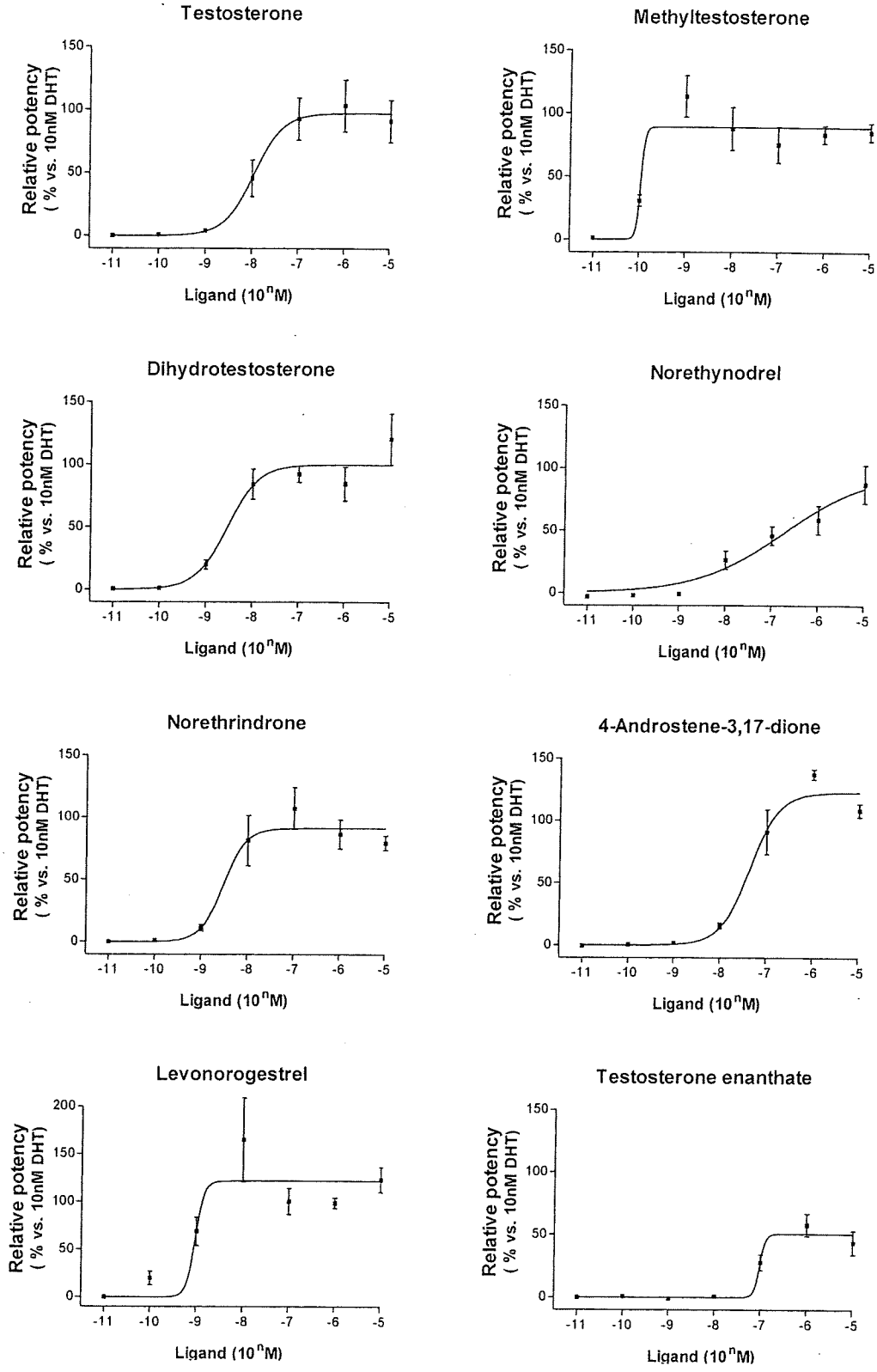

Fig. 5 Induction of luciferase activity by androgen derivatives in the HeLa cell transiently transfected with AR/pcDNA3.1 and ARE-AUG200-*Luc*+. Luciferase activities were expressed as the mean relative potency ±SE, relative to the positive control response induced by 10 nM dihydrotestosterone

for androgenic potential. A partial fragment of the rat α_{2u}-globulin promoter sequence was used as the basal promoter unit. The rat α_{2u}-globulin is known as a hormone-inducible protein, and its biosynthesis is under multi-hormonal regulation (Kulkarni et al. 1985; Kurtz et al. 1976; Roy and Neuhaus 1967; Roy et al. 1987).

The rat α_{2u}-globulin promoter fragment used in this plasmid has no hormone-responsive motifs, but the fragment seems to have properties suitable for constructing reporter plasmids to detect hormonal activity of chemicals. In this study, a new plasmid bearing rat α_{2u}-globulin promoter showed high hormonal induction with remarkably low background induction. A higher assay background induction for negative control with a lower maximum induction for positive control result in less sensitivity of the assay system because it would lead to a narrow dynamic range in the assay system. The new reporter plasmid ARE-AUG-*Luc*+ has excellent properties with regard to the assay background and positive induction levels when compared with a commercial plasmid. Accordingly, a sensitive reporter gene assay system would be established with this new reporter plasmid. Moreover, among 56 test chemicals consisting of several types of steroids, only the androgen derivatives selectively triggered a high response in the reporter gene assay using the new reporter plasmid, ARE-AUG-*Luc*+. Recently several reporter gene systems for detecting endocrine-active chemicals have been described (Gagne et al. 1994; Vinggaad et al. 1999; Balaguer et al. 1999, 2001; Miller et al. 2000) but most of them were intended for detecting estrogenic activity of chemicals, whereas our new plasmid construct is suitable for detecting the androgenic activity of chemicals. Adoption of high-throughput screening for the androgenic and anti-androgenic activity of chemicals will surely lead to safer chemicals, and the reporter gene assay system should provide a powerful tool to screen for endocrine-disrupting chemicals (Takeyoshi et al. 2002; Yamasaki et al. 2002). This new type of reporter plasmid, ARE-AUG-*Luc*+, will be of value in endocrine research and in the screening for endocrine-modulating chemicals.

Acknowledgements We wish to thank Ms. Nobuko Mitoma, Ms. Harumi Hada and Ms. Miyoko Kumagai for their skillful technical assistance

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Reproductive Toxicology 16 (2002) 367–369

Reproductive Toxicology

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Effect of gonadotropin-releasing hormone antagonist on ovarian and uterine weights in immature female rats

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Received 24 January 2002; received in revised form 18 April 2002; accepted 5 May 2002

Abstract

The immature rat uterotrophic assay has been proposed as a screening test method for detecting estrogenic and antiestrogenic chemicals. Although the immature rat uterotrophic assay is advantageous because the test animals are not traumatized by the ovariectomizing process, the effect of endogenous estrogen on ovarian and uterine weight in the immature animals that are used in immature rat uterotrophic assay has not received much attention. In this study, 19-day-old rats were treated with antide, a gonadotropin-releasing hormone antagonist, antide, to block gonadal production of endogenous estrogen. Uterine and ovarian weights of the antide-treated animals were markedly lower than those of control animals. This finding suggests that endogenous gonadal estrogen may already be acting on the uterus and ovaries in immature rats. Blocking endogenous estrogen with a gonadotropin-releasing hormone antagonist may enhance the sensitivity of the immature rat uterotrophic assay; however, the possibility that this protocol may interfere with the ability of the immature rat uterotrophic assay to detect centrally-mediated effects can not be discounted.

Keywords: Gonadotropin-releasing hormone antagonist; Ovarian weight; Uterine weight

1. Introduction

Some synthetic chemicals may disrupt the endocrine function of wild animals and humans. Recently, an international study program sponsored by the Organization for Economic Cooperation and Development (OECD) was initiated to validate the use of the uterotorophic assay as a test method for determining the estrogenic and antiestrogenic potentials of synthetic chemicals [1]. Several studies have validated the use of immature rat uterotrophic assays [2–5]. However, the endocrine status of these immature animals is not stable, and important changes in several hormone levels can occur during the course of these assays [6–9]. The effect of endogenous gonadal estrogen on ovarian and uterine weight in the immature animals that are used in the immature rat uterotrophic assay has not received adequate attention.

We report here the changes in ovarian and uterine weights in immature rats treated with a gonadotropin-releasing hormone antagonist to block endogenous gonadal estrogen.

2. Materials and methods

2.1. Chemicals

Antide, a gonadotropin-releasing hormone antagonist, was obtained from Sigma Chemical Company (St. Louis MO, USA), and dissolved in propylene glycol/distilled water [10,11].

2.2. Animals

Pregnant Crj: male CD (SD) IGS rats (specific pathogen free) were obtained from Charles River Japan, Inc. (Shiga, Japan) on gestation day 14 and permitted to give birth. On postnatal day (PND) 4, litters were culled to eight per dam, and the dams and pups were kept in polycarbonate cages until weaning. Rats were weaned on PND 20 and housed individually in hanging stainless steel wire-mesh cages throughout the study. The animals were given free access to diet (MF, Charles River Japan, Inc.) and water. At the start of each experiment, the rats were assigned to groups using a stratified randomization procedure. The animal room was maintained at a temperature of 23 ± 2 °C and a relative humidity of

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0890-6238/02/$ – see front matter
PII: S0890-6238(02)00043-6

Table 1
Body and organ weight (mean ± S.D.)

	PND (*n* per treatment group)			
	20 (15)	23 (15)	30 (10)	37 (5)
Body weight (g)				
Control	48.3 ± 3.4	60.9 ± 3.3	103.8 ± 7.5	148.6 ± 14.2
Antide	48.6 ± 3.1	61.5 ± 4.3	105.3 ± 8.1	150.3 ± 21.1
Uterine wet weight (mg)				
Control	–	46.1 ± 4.7	90.2 ± 43.4	364.2 ± 149.4
Antide	–	28.7 ± 2.2**	37.0 ± 15.2*	268.3 ± 143.7
Uterine weight (blotted; mg)				
Control	–	44.6 ± 4.5	84.2 ± 34.6	278.0 ± 56.7
Antide	–	27.4 ± 2.1**	35.6 ± 14.6*	202.7 ± 67.2
Ovarian weight (mg)				
Control	–	17.3 ± 2.4	22.2 ± 3.6	51.8 ± 15.1
Antide	–	12.0 ± 1.0**	14.1 ± 3.1**	25.2 ± 9.7*
Pituitary weight (mg)				
Control	–	2.4 ± 0.2	4.9 ± 0.4	8.2 ± 0.9
Antide	–	2.7 ± 0.2	5.0 ± 0.4	7.3 ± 0.8
Thyroid weight (mg)				
Control	–	4.6 ± 1.4	4.8 ± 1.6	7.0 ± 1.6
Antide	–	5.1 ± 1.4	5.3 ± 0.7	7.0 ± 1.5

* $P < 0.05$ compared to control, Student's *t*-test.
** $P < 0.01$ compared to control, Student's *t*-test.

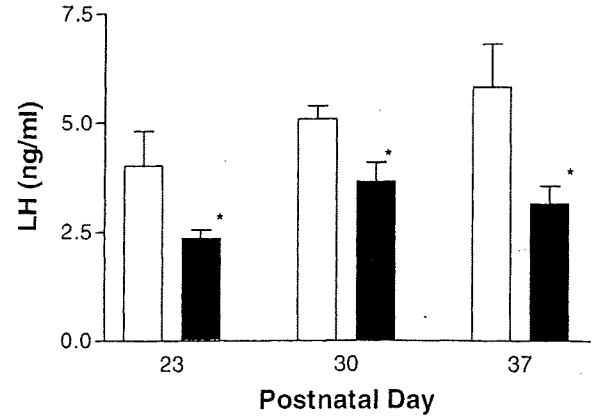
55 ± 10% with 10–15 air changes per hour and artificial light for 12 h daily. Animals were treated according to the principles outlined in the guide for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

2.3. Experimental design

A single subcutaneous injection (10 mg/kg) of antide or vehicle alone was given on PND 19. Administered in this manner, antide was reported to have persistent effects over 2 weeks [10]. The animals were sacrificed by exsanguinations under ether anesthesia on PND 23, PND 30, or PND 37. Animals were then subjected to gross necropsy. The uterus and ovaries were dissected and trimmed of all fascia and fat. The vagina was removed from the uterus at the level of uterine cervix. Uterine and ovarian weights were recorded. Pituitaries and thyroids were dissected and weighed. Blood samples were collected from the abdominal aorta, and the serum was separated by centrifugation. The serum concentration of luteinizing hormone (LH) was determined in animals using an enzyme-linked immunosorbent assay (ELISA).

3. Results

There was no significant difference in body weight change between control and antide-treated animals (Table 1). No toxicologic signs were observed in any of the animals. The wet and blotted uterine weights of antide-treated rats were significantly lower than those of the control animals sacrificed on either PND 23 or PND 30. The ovarian weights of the antide-treated rats were significantly lower than those of control animals sacrificed on PND 23, PND 30, and PND 37 (Table 1). There was no difference in pituitary or thyroid weight on any day of evaluation. Serum LH levels are shown in Fig. 1. The LH levels in the antide-treated animals were significantly lower than those of control animals on PND 23, PND 30, and PND 37.



immature female rats
*: Significantly defferent from the control (p<0.05, Student's *t*-test)

Fig. 1. Effects of antide treatment on serum LH levels in immature female rats. Open bars represent vehicle control treatment and closed bars represent antide treatment. Mean ± S.D. *: Significantly defferent from the control ($p < 0.05$, Student's *t*-test).