ASSAY IN MICE FOR MINUTE AMOUNTS OF ENDOTOXIN BY PRE-ADMINISTRATION OF LEAD ACETATE

HIROSHI FUJIWARA and KAZUO KURATSUKA

*Department of General Biologics Control, National Institute
of Health, Kamiosaki, Shinagawa-ku, Tokyo 141*

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SUMMARY: The enhancing effect of pre-administration of lead acetate on the body-weight decrease due to endotoxin was studied in mice for the purpose of developing a method for determination of minute amounts of endotoxin. By adoption of the body-weight ratio on the first day after endotoxin administration (BWR_1) as a response, the linear regression lines of the dose-response were demonstrated over a wide range of log doses of several different endotoxin preparations tested. By use of these linear regression equations experimentally obtained, the minimum detectable dose was calculated. The minimum detectable dose was defined as a dose of an endotoxin preparation producing a mean body-weight response statistically distinguishable from that of the control. The doses obtained were mostly in nanogram order. The magnitude of the enhancement brought about by the lead treatment was estimated at about 40 to 100 times.

INTRODUCTION

The test for endotoxin contamination constitutes one of the important procedures for the quality control of biological products, blood products, parenteral drugs and medical devices. Recently, more and more items require assurance of freedom from endotoxin contamination; e.g., some vaccines (Mark et al., 1978), erythropoietin (Fumarola, 1977; Braun et al., 1979), cell tissue (Rickles et al., 1979), culture media (Martin et al., 1978), such medical instruments as catheters (Kundsins and Walter, 1980) etc. Since human beings are among the most sensitive species to endotoxin, the test for the safety of those products in respect to endotoxin should be as sensitive as possible. Rabbits are also sensitive to the toxic effects of endotoxin and the rabbit pyrogen test has been widely authorized and adopted in pharmacopoeia and minimum requirements in many countries (Ministry of Health and Welfare, 1973). However, the pyrogen test in rabbit is fairly troublesome for routine use, especially for the quantitative purposes. The limulus lysate test (Levin and Bang, 1964), which has recently become familiar in various fields, remains to overcome its drawback in the quantitative determination, though in sensitivity it seems to exceed any other method and its manipulation is simple. Therefore, it would be much convenient

if a method for detection or quantitation of endotoxin, so simple as to be useful in routine work and as sensitive as the rabbit pyrogen test, was available.

Selye *et al.* (1966) reported that the lethality of the rat due to endotoxin was greatly enhanced by the iv administration of lead acetate. Since then, similar experimental results have been obtained in other animals than rats; e.g., mice (Seyberth *et al.*, 1972; Terao and Katsuno, 1978), chickens (Truscott, 1969) and subhuman primate (Holper *et al.*, 1973).

We noticed that the body-weight decrease in mice occurred with a much smaller dose than a lethal dose of endotoxin and started the study to establish a new assay method for small amounts of endotoxin by incorporating the enhancing effect of lead acetate into the determination of the body-weight decreasing activity instead of the lethal activity of endotoxin. As mice are relatively easy to handle, it is expected to develop a method with a sensitivity comparable to or rather higher than that of the rabbit pyrogen test and being convenient for routine use. Our experimental data obtained in the past with an *E. coli* endotoxin preparation (Kuratsuka and Fujiwara, 1972; Fujiwara and Kuratsuka, 1976, 1978, 1980) suggested a possibility of detecting endotoxin in the nanogram order by pre-administration of lead acetate.

In this paper, the sensitivity of this method for detection of small amounts of endotoxin will be presented with several endotoxin preparations.

MATERIALS AND METHODS

Endotoxin: The endotoxin preparation mainly used was *Escherichia coli* LPS (lipopolysaccharide) (Control No. 613122, Difco Co., Detroit, MI) prepared by Westphal's method from *E. coli* O 111 B4 cells and designated as *E. coli* LPS (W). All but one preparations listed in Table II were purchased from Difco Co.; *E. coli* LPS (Y) was supplied by Yoshikawa (Yoshikawa and Fukumura, 1966). Each preparation was dissolved in sterilized distilled water (1.0 mg/ml) to make a stock endotoxin solution, which was autoclaved for 20 min at 120 C. From the stock solution, an endotoxin solution of an appropriate concentration was prepared by adding sterilized distilled water.

Lead acetate: Lead acetate (Lot No. 5283, Wako Junyaku Co., Osaka) was dissolved in sterilized distilled water (100 mg/ml); the solution was boiled for 15 min in a water bath. The solution was prepared just prior to use. From our previous studies (Kuratsuka and Fujiwara, 1974), used was a constant dose of 83 μ g/mouse of lead acetate, which enhanced the body-weight decrease due to endotoxin but had no effect by itself.

Diluent: Sterilized pyrogen-free distilled water was used for dilution of endotoxin and lead acetate solutions (Fujiwara and Kuratsuka, 1980). It was also used for lead acetate treatment of (Table II) and for endotoxin administration of control mice (Fig. 3, Table III).

Equipments: All glasswares including syringes, hypodermic needles, etc. were heated for 30 min at 250 C to eliminate activities of possibly contaminating

pyrogens.

Mice: Four-week-old female mice were purchased and weighed every morning for about 7 days. Those showing normal increase in body weight were randomly allotted to the materials and doses.

Body-weight-decreasing activity of endotoxin: As a metameter of body-weight-decreasing activity of endotoxin, body-weight ratio on the first day (BWR_1) was adopted and calculated in each mouse by the following formula:

$$BWR_1 = \frac{B_1}{A} \times 100$$

where A and B_1 mean the body weight (g) before and on the first day after endotoxin administration, respectively. The assumption that BWR_1 normally distributes is based on empirical evidence.

Administration of lead acetate and endotoxin: Lead acetate was given an hour before endotoxin administration. Both were administered intraperitoneally in volumes of 0.25 ml (Fujiwara and Kuratsuka, 1978).

Statistical analysis: Regression analysis and Finney's parallel line assay (1964) were carried out according to Kurokawa et al. (1978). Since the homoscedasticity of variances in each experiment was not denied by the Bartlett's or the Hartley's method, confidence intervals were calculated with the common variances for each experiment. The probability level was fixed at 5% and the confidence coefficient 95%.

RESULTS

Dose-response Regression Lines Including Minute Amounts of Endotoxin

Five doses of serial 10-fold dilutions of *E. coli* LPS (W), ranging from 0.0025 μ g to 25 μ g per mouse, were administered into each group of seven or eight lead-treated mice. A linear log dose-response line covered a wide range of doses of the preparation (Fig. 1). No death of mice was demonstrated even with the dose of 25 μ g per mouse. The minimum dose constituting the regression line was 0.0025 μ g per mouse. The LD_{50} of this preparation was approximately 500 μ g per mouse (unpublished data).

Subsequently, to determine whether the regression line including a dose as low as 0.0025 μ g per mouse can be regarded as linear, the following experiment was carried out. Eight doses of serial twofold dilutions of *E. coli* LPS (W) (0.0025 μ g to 0.313 μ g/mouse) were administered into eight groups of seven lead-treated mice (Fig. 2). No significant deviation from linearity was demonstrated (Table I).

Minimum Detectable Dose of Endotoxin by Pre-administration of Lead Acetate

In the previous experiment, we used 0.0025 μ g of *E. coli* LPS (W) as the

Fig. 1. Dose-response regression line in serial 10-fold dilutions of endotoxin in mice pre-administered with lead acetate.

Each point shows the mean BWR₁ value at the indicated endotoxin dose. X in the formula and on the abscissa: $X = \log D - \log 0.00025$, where D is the actual dose (μg/mouse) of endotoxin. The figure in parentheses represents the number of mice per dose. The vertical bar represents the confidence interval of the mean at a 95% probability level. Lead acetate was administered an hour before endotoxin administration.

smallest dose (Figs. 1 and 2). Thereupon, we tried to find the minimum dose, of which mean value of BWR₁ was significantly different from that of the control mice, not injected with endotoxin but treated with lead acetate. Thus, such a dose can be regarded as "the minimum detectable dose" of the preparation.

Each group of seven or 10 lead-treated mice was administered with each of serial twofold dilutions of *E. coli* LPS (W) (0.00063 μg–0.01 μg/mouse) (Fig. 3). Another group of seven or 10 lead-treated mice was injected with distilled water for control. The BWR₁ values and their 95% confidence limits were calculated for each group as shown in Fig. 3. Similar experiments were repeated three times. The difference in the mean of BWR₁ was significant between the group administered with 0.00125 μg of the endotoxin and that with water in Experiment 1 and also between the group administered with 0.0025 μg and that with water in Experiments 2 and 3. Consequently, the minimum detectable dose of *E. coli* LPS (W) seems to have been between 0.00125 μg and 0.0025 μg.

In each experiment, the experimental equation of dose-response line was obtained and the linearity was confirmed. By the equations, the BWR₁ values

Fig. 2. Dose-response regression line in serial twofold dilutions of endotoxin in mice pre-administered with lead acetate. Seven mice were used for one dose. For the points, the vertical lines, lead acetate and log doses, see the legend for Fig. 1.

TABLE I

Analysis of variance for the data presented in Fig. 2

Nature of variation	SS	df	MS	F	F(0.05)
Regression	125.39	1	125.39	28.56	4.04
Linearity	39.75	6	6.63	1.51	2.30
Between doses	165.14	7	23.59	5.37	2.21
Error	210.57	48	4.39		
Total	375.71	55			

corresponding to two doses, 0.00125 μg and 0.0025 μg , were calculated at 97.0 and 96.4 in Experiment 1, 98.6 and 98.1 in Experiment 2 and 99.3 and 98.4 in Experiment 3, respectively. The mean of the six values was 97.97. From these data, the minimum detectable dose of *E. coli* LPS (W) was defined as the dose corresponding to 98 in terms of the BWR₁ value in these experiments.

The Linear Relationship and the Minimum Detectable Doses of Several Endotoxin Preparations

We studied the minimum detectable doses of six endotoxin preparations including *E. coli* LPS (W), being different one another in respect to the bacterial species and the purification processes.

TABLE II
The dose-response lines and minimum detectable doses of several endotoxin preparations

Endotoxin preparation†	Formula of linear dose-response line**		Minimum dose used ($\mu\text{g}/\text{mouse}$)		Minimum detectable dose calculated ($\mu\text{g}/\text{mouse}$)*	
	control group	lead-treated group**	control group	treated group	control group (C)	treated group (T)
<i>S. typhimurium</i> LPS (W)*	$Y = -3.51X + 112.00(5) \ddagger$	$Y = -2.41X + 103.78(8)$	0.244	0.004	2.436	0.063
<i>Sh. flexneri</i> LPS (W)	$Y = -4.92X + 114.27(5)$	$Y = -2.90X + 101.72(8)$	0.244	0.004	0.507	0.005
<i>Ser. marcescens</i> LPS (W)	$Y = -5.26X + 123.66(5)$	$Y = -3.03X + 107.99(7)$	3.906	0.061	18.894	0.495
<i>E. coli</i> LPS (W)	$Y = -4.36X + 110.69(5)$	$Y = -2.91X + 101.57(8)$	0.244	0.004	0.203	0.004
<i>E. coli</i> LPS (B)	$Y = -5.01X + 113.94(5)$	$Y = -2.95X + 101.62(8)$	0.244	0.004	0.380	0.004
<i>E. coli</i> LPS (Y)	$Y = -5.16X + 115.71(5)$	$Y = -3.05X + 102.60(8)$	0.244	0.004	0.676	0.008
Common coefficient	-4.70	-2.88				

† *S.*: *Salmonella* *Sh.*: *Shigella* *Ser.*: *Serratia* *E.*: *Escherichia*

* Method of endotoxin purification: W: Westphal's method B: Boivin's method Y: Yoshikawa's method.

‡ Numbers of doses of serial four-fold dilutions of endotoxin.

** Lead acetate: 83 $\mu\text{g}/\text{mouse}$ (an hour interval between the administrations of lead acetate and endotoxin).

Calculated by the formula substituted with 98 for Y.

** $X = \log D - \log 0.00025$, where D is the dose of endotoxin.

Number of mice per doses of endotoxin was eight.

Fig. 3. The minimum detectable dose of endotoxin by pre-administration with lead acetate. Experiment 1 (●): seven mice were used per dose of endotoxin. Experiments 2 (○) and 3 (×): 10 mice were used per dose of endotoxin. Control mice were administered with distilled water instead of endotoxin. *: Significantly different ($\alpha < 0.05$) from control. For the vertical lines, the points, lead acetate and log doses, see the legend for Fig. 1.

Four to eight doses, including one close to the expected minimum detectable dose (Table II), of serial fourfold dilutions of each preparation were administered into the lead-treated and the water-treated control groups. The formulae of the regression lines are presented in Table II. Among both of six regression coefficients in the lead-treated group and those in the water-treated control group, there was no significant difference. The regression lines in the lead-treated group were significantly flatter than those in the control groups as seen previously (Fujiwara and Kuratsuka, 1980).

As described in the preceding section, the dose corresponding to 98 in BWR_1 value was defined as the minimum detectable dose of *E. coli* LPS (W). On the assumption that this relation is valid for the other endotoxin preparations, 98 was adopted provisionally as one of the response values to calculate the minimum detectable dose of the respective endotoxin preparations in the lead-treated mice. The doses of the six endotoxin preparations thus calculated by substituting 98 for Y in the respective formulae in lead-treated groups are shown in column T in Table II. These values were in the nanogram order in all but one preparation. The value thus obtained with, for instance, *E. coli* LPS (Y), was somewhat larger than the minimum dose used, 0.004 μg per mouse, being within a range of linear regression.

The experiments for obtaining the BWR_1 values for control mice injected with distilled water only (water-control in water-treated mice) were repeated six times. The results are shown in Table III. The mean BWR_1 value (101.4) of the water control in water-treated mice was not different significantly from that

TABLE III

Lower limit of 95% confidential interval in BWR₁ of mice given water

Response (BWR ₁)*	Experiment No.						Total
	1	2	3	4	5	6	
mean	102.9	101.4	99.6	102.1	101.1	100.5	101.4
variance	3.91	3.16	10.27	2.99	11.66	7.73	6.78
n†	12	10	8	10	10	10	60(10)
lower limit**	101.6	100.1	97.5	100.9	98.7	98.8	100.7(99.4)‡

* Body-weight ratio on the first day (See the text).

** Lower limit of 95% confidential interval in BWR₁.

† Number of mice used for statistical analysis.

Distilled water : 0.5 ml/mouse/ip.

‡ Lower limit at n=10.

(99.9) of the three water controls in lead-treated mice shown in Fig. 3. No significant difference was demonstrated between the common variance of water control in water-treated mice (6.89) and that (3.51) of water control in lead-treated mice. In the experiments for the dose-response lines of *E. coli* LPS (W) shown in Table II, the homoscedasticity of variances in water-treated and lead-treated mice was not denied, either. Therefore, it may be justifiable to use the value of 98 in terms of BWR₁ for calculation of the minimum detectable dose also in water-treated mice. The estimates of doses calculated by substituting 98 for Y in the respective formula in water-treated groups are shown in column C in Table II. Then the ratios C/T were calculated as an expression of the magnitude of the enhancement in the body-weight decrease due to endotoxin by lead acetate treatment as used in the present experiment (column C/T in Table II). The values ranged from about 40 to 100.

DISCUSSION

The validity of the two kinds of the estimates obtained in the present experiment, the minimum detectable dose of endotoxin and the magnitude of enhancement by the lead acetate treatment, depends on the authenticity of the assumptions on which the calculations of the estimate were made.

One of the two assumptions is that the regression line is still linear within a range of doses close to the estimated minimum detectable dose. As the smallest dose used in each experiment was very close to the estimated minimum detectable dose (Table II) and no deviations from linearity of the regression line including the smallest dose was demonstrated, this assumption may be considered valid.

The other assumption is that the value of 98 in terms of BWR₁, adopted as the smallest mean response statistically distinguishable from mean BWR₁ of the control mice, can be valid both in the lead-treated and in the water-treated control mice. This assumption is based on the findings that there was no significant difference in mean BWR₁ or in the variance between the lead-treated

TABLE IV

The minimum detectable doses calculated with 99 and 100 as BWR₁ value in control mice and C/T ratios

Endotoxin preparation	Minimum detectable dose in control mice with BWR ₁ value (μ g/mouse)			
	BWR ₁ =99	C/T*	BWR ₁ =100	C/T*
<i>S. typhimurium</i> LPS (W)	1.264	20.1	0.656	10.4
<i>Sh. flexneri</i> LPS (W)	0.317	63.4	0.199	39.8
<i>Ser. marcescens</i> LPS (W)	12.196	24.6	7.872	15.9
<i>E. coli</i> LPS (W)	0.120	30.0	0.071	17.5
<i>E. coli</i> LPS (B)	0.240	60.0	0.152	38.0
<i>E. coli</i> LPS (Y)	0.433	54.1	0.277	34.6

* Ratio to the minimum detectable dose calculated with 98 in lead-treated mice shown in Table II.
For details see the footnote of Table II.

and the water-treated controls and that the difference in the variance between the lead treated-endotoxin injected mice and the water treated-endotoxin injected mice was not significant, either. The magnitude of the increase in the sensitivity largely depended on this assumption. To find the range of the magnitude, by the minimum detectable doses were recalculated in the same way as that in Table II using the values of 99 and 100 for the water-treated mice instead of the value of 98 and summarized in Table IV. Though the ratios (C/T) became fairly smaller than those in Table II, the magnitude of enhancement is still larger than 10.

The LD₅₀ values in mice of partially purified endotoxin preparations were usually in the milligram order (Berczi et al., 1966), while the mouse body-weight-decreasing doses of similar preparations were found to be in the sub-microgram order (Ishida, 1968). The comparison of the lethal dose with the minimum dose inducing a detectable body-weight decrease in mice could also be made from the data given in Fig. 1, in which no death of mice occurred even by injection of 25 μ g.

The minimum detectable dose of an endotoxin preparation of *Serratia marcescens* was much higher than those of other species. Such a difference might be due to the poor solubility in water or to the low purity of the preparation. Nevertheless, the magnitude of enhancement by the lead treatment was in a similar degree to that of endotoxin preparations of other species.

The specificity of the enhancing effect of lead acetate is an important factor when the test is to be used for detecting possible contamination of endotoxin in a given test sample. Selye et al. (1966) reported that among numerous reticuloendothelial blocking agents tested in rats, none equalled the sensitizing effect of lead acetate on the lethality. It remains still unsolved whether lead acetate affects the body-weight-decreasing activity of substances other than endotoxin. As an approach to the problem, the immunological method might be useful, since anti-endotoxin guinea-pig sera inhibited the body-weight-decreasing

activity of endotoxin in mice (Kuratsuka and Shimazaki, 1973; Kuratsuka *et al.*, 1973).

The six dose-response lines of the endotoxin preparations shown in Table II were parallel one another. This fact suggests that the parallel line assay method may be applicable to precise and reproducible micro-determination of endotoxin.

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