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Original Article

A Quantitative In Vitro Assay for Detecting Biological Activity of Endotoxin Using Rabbit Peripheral Blood

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SUMMARY: The pyrogen test or the endotoxin test has been playing a crucial role in detecting endotoxin in parenteral drugs. The current test methods, however, have disadvantages such as requiring a relatively high number of animals or an inadequacy in direct evaluation of in vivo activity. We made an attempt to establish a new in vitro assay method that can overcome the shortcomings of the current assay methods. We standardized the system of tumor necrosis factor-alpha (TNF-α) induction from the peripheral blood of rabbits for assaying endotoxin activity. This in vitro assay showed a linear dose-response regression between 0.1 and 5.0 endotoxin units per milliliter of endotoxin and a definite homogeneity of variance by logarithmically transforming the endotoxin and TNF-α concentrations in the reaction mixtures at 5 h of incubation at 37°C. The assay showed a definite correlation with the pyrogen test but not with the endotoxin test when endotoxins from various bacteria were tested.

INTRODUCTION

Lipopolysaccharide (LPS) of gram-negative bacteria, also referred to as endotoxin, is known to perform various biological activities that cause harmful physical effects on humans even in very small amounts (1,2). Contamination of endotoxin has been, therefore, a serious threat to the safety of parenteral drugs. The rabbit pyrogen test (3) has been effectively applied for testing for endotoxin contamination.

The bacterial endotoxin test which was first developed by Levin and Bang (4,5) is based on the highly sensitive clotting of amebocyte lysate of the horseshoe crab (LAL) by endotoxin. The test was markedly improved, thereafter, to allow its practical application to a wide range of parenteral drugs including biological products. Particularly, removal or suppression of the reactivity of LAL reagents to (1-3)-β-D-glucan (6) remarkably proceeded the wide application of the test in Japan by allowing it to avoid the possible confusion caused by reaction with (1-3)-β-D-glucan or other non-pyrogenic substances.

The endotoxin test was approved in the early 1980s in the United States (7) as a replacement of the pyrogen test for evaluating the end product of parenteral human drugs and this approval has been followed by other nations (8,9). The pyrogen test for interferon injections and some blood products was replaced by the endotoxin test in Japan in 1993 (10). Application of the endotoxin test to antibiotic products started in 1995 and was followed by its expanded application in 1998 (11). In the course of implementing the endotoxin test for biological products, special care was taken to set the allowance limits so as to take the in vivo synergistic effect between endotoxin and the products into account for an effective regulation of pyrogenicity, because some products such as interferon-β showed marked enhancement of endotoxin pyrogenicity in rabbits (12). Furthermore, in spite of its excellent sensitivity and accuracy, the endotoxin test shows an inconsistency with the pyrogen test in cases in which pyrogenic response to endotoxin was enhanced synergistically by a product (12). Therefore the endotoxin test has an obviously limited efficacy in regulating the biological activity of endotoxin in such products.

In the present study, we attempted to utilize the system of tumor necrosis factor-alpha (TNF-α) induction (13,14) from peripheral blood of rabbits for assaying endotoxin activity. The results of standardization and evaluation of the assay method are presented in comparison with the endotoxin test.

MATERIALS AND METHODS

LPS: Japanese national reference standard endotoxin (RSE) extracted from *Escherichia coli* UKT-B strain (13,000 EU/vial) (15), USP reference standard endotoxin (EC-6) derived from *E. coli* O113 strain (10,000 EU/vial) and commercial LPSs; *E. coli* O111:B4, *E. coli* O55:B5, *E. coli* 127:B8, *Salmonella typhosa* 0901 (Difco Laboratories, Detroit, Mich., USA), *S. abortus* equi, *Pseudomonas aeruginosa* Serotype 10, *Klebsiella pneumoniae* and *Vibrio cholerae* Serotype Inaba 569B (Sigma Chemical Co., St. Louis, Mo., USA) strains extracted by the Westphal's phenol water method were used. The commercial LPSs were suspended in pyrogen-free distilled water for injection (Otsuka Pharmaceutical Co., Ltd., Tokyo) at a concentration of 1.0 mg/ml and kept at 4°C until use without detectable change in LAL activating activity (LAL activity).

The bacterial endotoxin test: The suspensions of LPSs were serially diluted with pyrogen-free distilled water at fourfold intervals. A 50 μl-volume each of appropriate dilutions was mixed with an equal volume of an endotoxin-specific LAL reagent (Endospecy, Seikagaku Corp., Tokyo) whose reactivity to (1-3)-β-D-glucan was removed. The rate of color development was measured using a specially equipped microplate reader (Well Reader SK601, Seikagaku Corp.) (16). The endotoxin content of the samples was calculated according to the parallel line assay method using logarithmically transformed dose and color-development rate, and was expressed

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as endotoxin units per milliliter (EU/ml).

In vitro assay on TNF-α induction using rabbit peripheral blood: Heparinized (Heparin, Hoechst Marion Roussel, Tokyo) blood from female rabbits of the Japanese white strain (Kitayama Labes Co., Ltd., Nagano or Japan Laboratory Animals, Inc., Tokyo) was used for the TNF-α induction assay within 2 h after bleeding. An appropriate dilution of samples in pyrogen-free saline for injection (0.1 ml) (Otsuka Pharmaceutical Co., Ltd., Tokyo) and 0.15 ml of the heparinized rabbit blood were gently mixed in a pyrogen-free centrifuge tube containing 0.75 ml of pyrogen-free saline. The mixture in duplicate was incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for the desired length of time. Supernatants were isolated from the mixtures by centrifuging at 500 × g for 2 min and stored frozen at −30°C until use. The supernatants were diluted serially at fivefold intervals with distilled water containing 25% of blocking reagent (Block Ace, Dainippon Pharmaceutical Co., Ltd., Osaka) and TNF-α concentration of the dilutions measured by an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed using anti-rabbit TNF-α goat immuno-globulin G (IgG), biotin labeled anti-rabbit TNF-α goat IgG (Research Diagnostics Inc., Flanders, N. J., USA) and horseradish peroxidase conjugated streptavidin (Chemicon International, Inc., Temecula, Calif., USA) in wells of a 96-well micro-plate (Narge Nunc International K.K., Tokyo). TNF-α concentration in the supernatants was calculated according to the parallel line assay method referring to the standard rabbit TNF-α (Research Diagnostics Inc.) using logarithmically transformed doses and absorbance values to express the results as nanograms per milliliter (ng/ml).

Rabbit pyrogen test: Female rabbits of the Japanese white strain weighing approximately 3 kg were housed in cages separately in an air-conditioned animal room. Three animals were allocated for each treatment. They were intravenously injected with 1 ml/kg of a dilution of test samples. Rectal temperature of the animals was monitored for 3 h using an electric thermometer (Scanner Unit X115 with High Accurate Data Logger K730, TECHNOL SEVEN, Kanagawa). The pyrogenicity of a test sample was calculated using the average maximum rise in rectal temperature of the three rabbits during 3 h (ΔT) after sample injection.

Statistical analysis: Analysis of the parallel line assays was carried out according to Finney's method (17). Significance and validity tests were made at a level of $P = 0.05$. The pyrogenicity or TNF-α induction index of each LPS was calculated according to the parallel line assay method referring to the activity of RSE which was assumed as 100.

RESULTS

The kinetics of TNF-α release from rabbit blood stimulated with 0.1 ml-volume each of 5.0 and 1.25 EU/ml of RSE at 37°C was monitored up to 24 h. The blood treated with saline in parallel was also monitored as the control.

While the saline treatment did not stimulate TNF-α release, RSE showed a marked effect as shown in Fig. 1. TNF-α concentration in the supernatant of the blood samples stimulated with RSE started to increase at approximately 1 h and reached a maximum about 4 or 6 h. No significant change was noted, thereafter, up to 8 h of incubation. TNF-α concentration of the RSE groups seemed to have slightly declined by 10 h, then stayed at the same level without changing until 24 h of incubation. Accordingly, 5 h of incubation was chosen for the treatment for TNF-α induction, hereafter.

TNF-α induction by 0.1 ml-volume each of serial fourfold dilutions of RSE from 20 to 0.0781 EU/ml was assayed repeatedly using blood samples from a total of more than 15 rabbits. The measurement of each dose was performed in duplicate in each assay as described in Materials and Methods. Homogeneity of variance is essential for applying the parallel line assay method, and its achievement might be hampered when magnitude of variance would be affected by the magnitude of response. The effect of magnitude of response on variance was evaluated by a correlation analysis using the duplicated data of TNF-α concentration obtained in the repeated assays. A significant correlation was seen between the mean and variance of TNF-α concentrations as shown in Fig. 2a. However, logarithmic transformation of the TNF-α concentrations diminished the correlation as shown in Fig. 2b.

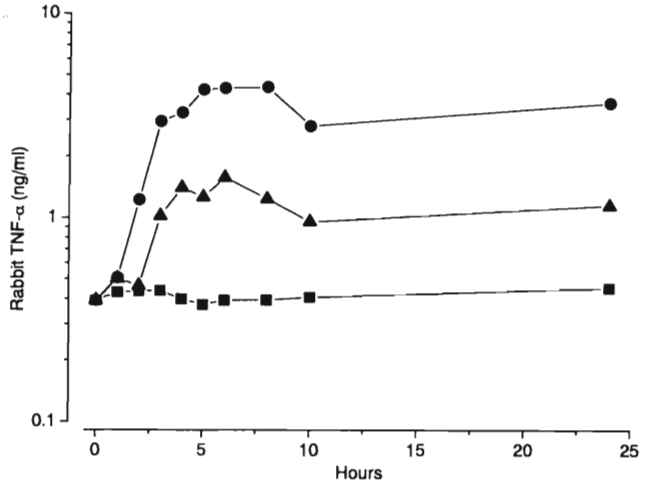

Fig. 1. Kinetics of TNF-α release from rabbit peripheral blood after stimulating with RSE. TNF-α concentration in the supernatant of the blood samples was monitored up to 24 h after stimulation with 5.0 EU/ml (●), 1.25 EU/ml (▲) of RSE or Saline (■) at 37°C. The assay was carried out twice independently using blood samples from different rabbits. Each point represents the mean of two independent assays.

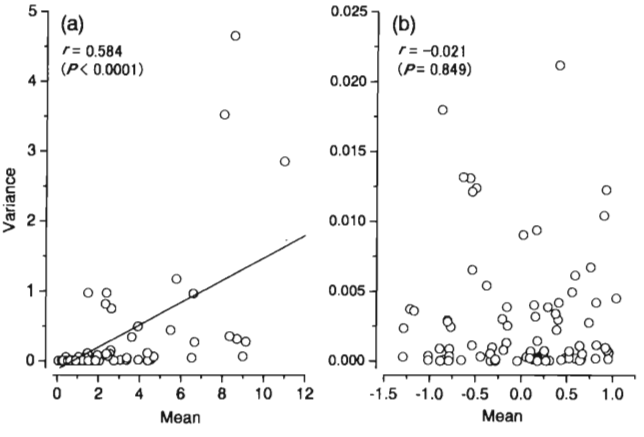

Fig. 2. Correlation between mean and variance of arithmetic or logarithmic TNF-α concentrations. Homogeneity of variance is essential for applying the parallel line assay method. The homogeneity of variance will not be attained when there is a correlation between magnitudes of response and variance. A correlation was seen between mean and variance of arithmetic TNF-α concentrations (a) but it disappeared by the logarithmic transformation (b).

TNF-α-inducing activity of EC-6 in a test sample was assayed relatively to that of RSE in five repeated experiments, and a typical example of the measurement results is depicted in Fig. 3a, b.

RSE and the resultant TNF-α concentrations showed a straight linear dose-response relationship from 0.0781 up to 1.25 EU/ml of RSE as seen in Fig. 3a. EC-6 measured together with RSE also showed a straight linear dose-response but with a much smaller regression coefficient, therefore suggesting the applicability of the method of analysis for slope ratio assays. However, logarithmic transformation of endotoxin and TNF-α concentrations was found to give parallel dose-response regression lines of RSE and EC-6 as seen in Fig. 3b and Table 1. A straight linear dose-response regression line for RSE was attained from 0.0781 to 5.0 EU/ml by means of logarithmic transformation. Consequently, hereafter in the present study, the TNF-α-inducing activity of a test sample was determined by referring to RSE activity by means of the parallel line assay method using the logarithmically transformed dose and TNF-α concentrations.

Figure 4 shows the results of five repeated measurements of the TNF-α-inducing activity of EC-6. The level of TNF-α induced by RSE or EC-6 varied to some extent in different experiments. The slope of the dose-response regression also showed considerable variation in different experiments. However, so far as the activity of EC-6 was calculated relatively

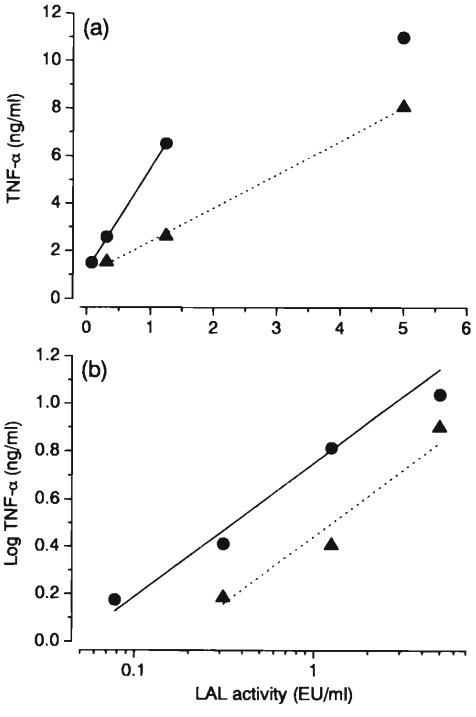

Fig. 3. Comparison of arithmetic and logarithmic dose-responses of TNF-α induction by endotoxins. TNF-α-inducing activity of EC-6 (▲) was measured as a test sample referring to the activity of RSE (●). Each point shows the mean value of duplicated measurements in an experiment. Arithmetic concentrations (a) of endotoxin and TNF-α showed a linear dose-response relationship up to a TNF-α concentration of around 8 ng/ml, suggesting the applicability of the slope ratio analysis. Logarithmic transformation (b) of endotoxin and TNF-α concentrations was found to allow application of the parallel line assay method.

Table 1. Analysis of variance table

Factor	Sum of square	df[1]	Mean square	F[2]
Preparation	0.0433	1	0.0433	7.427
Regression	1.3963	1	1.3963	239.325[3]
Parallelism	0.0097	1	0.0097	1.668
Linearity	0.0367	3	0.0122	2.098
Between doses	1.4861	6	0.2477	42.495[3]
Error	0.0408	7	0.0058	
Total	1.5269	13		

Analysis on the data depicted in Figure 3b.
[1]df: degrees of freedom.
[2]F: *F value*.
[3]Significant at $P < 0.05$.

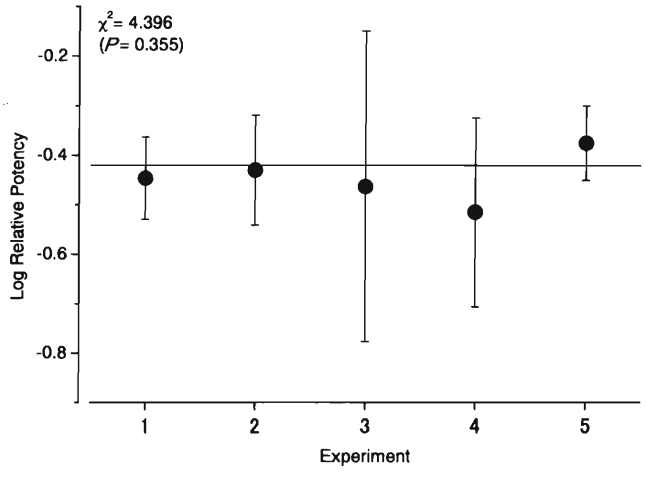

Fig. 4. Reproducibility of the TNF-α induction assay. Weighted mean of logarithmic potency of TNF-α-inducing activity of a test sample was calculated relatively to that of RSE. The vertical bars show 95% confidence intervals. χ^2 test showed homogeneity of the relative potency values, suggesting the assay's strong reproducibility.

to that of RSE in each experiment, the TNF-α induction assay was shown to be highly reproducible as seen in Fig. 4, showing no significant deviation of the result of each measurement from the weighted mean.

The relationship between LAL activity and biological activities such as pyrogenicity and the TNF-α induction activity of LPSs of the bacteria listed in Materials and Methods was examined. RSE and other LPSs were diluted to have an equal level of LAL activity, and the pyrogenicity of each of their serial threefold dilutions were assayed using three rabbits to calculate the activity of LPSs relative to the activity of RSE using the parallel line assay method. The TNF-α induction activity of the appropriate serial fourfold dilutions of each LPS was also assayed according to the parallel line assay method. Figures 5a and 5b depict TNF-α-inducing activity and pyrogenicity of the LPSs, respectively.

TNF-α-inducing activity and the pyrogenicity of the LPSs with equivalent LAL activity ranged from 1.5% to 141.7% and from 6.6% to 480.6% of the activities of RSE, respectively. The results suggested that there is no correlation between LAL activity and both of the biological activities of the LPSs so far tested.

On the other hand, when correlation between the pyrogenicity and the TNF-α-inducing activity of the LPSs was analyzed, it was found that there was a fine correlation between these

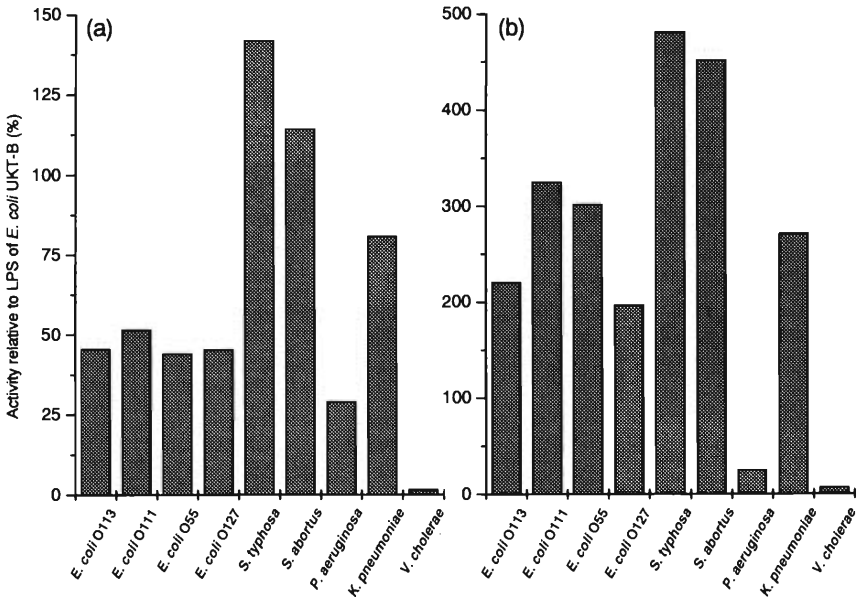

Fig. 5. Relationships between LAL activity and TNF-α-inducing activity or pyrogenicity. TNF-α-inducing activity (a) and pyrogenicity (b) were measured on LPSs of various bacterial strains adjusted to have equivalent LAL activity. In each assay the biological activities of the LPSs were compared with those of RSE which was assumed to be 100%. Both activities showed considerable variation among different LPSs to suggest a discrepancy between these activities and LAL activity.

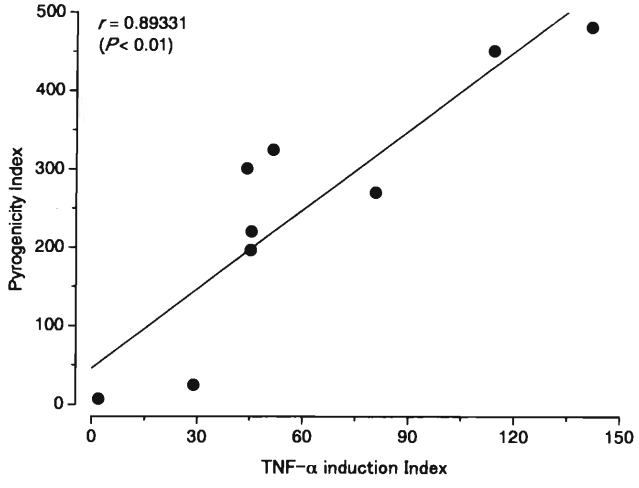

Fig. 6. Correlation between TNF-α-inducing activity and pyrogenicity of LPSs. The biological activities of the LPSs were shown as pyrogenicity index and TNF-α induction index that were calculated referring to RSE assuming its activities to be 100. Each point shows the activity of each LPS relative to that of RSE. A clear correlation was seen between TNF-α -inducing activity and pyrogenicity.

activities, showing a correlation coefficient of 0.893 ($P < 0.01$) as seen in Fig. 6. The in vitro assay method for endotoxin using rabbit blood was suggested to allow an assay that closely reflects the in vivo biological activity of endotoxin.

DISCUSSION

Although the bacterial endotoxin test has been playing a crucial role in regulating endotoxin contamination in parenteral drugs, it has a limited efficacy in evaluating the harmful biological activities of endotoxin contained in drugs in the case where a drug enhanced the biological activity of endotoxin synergistically (12). The pyrogen test would be capable of detecting this synergistic effect and could be effective in regulating not only endotoxin contamination but also overall pyrogenicity including any possible synergism. The pyrogen test also has limitations, however, in its utility due to its insufficient accuracy and its inferior sensitivity as compared to the endotoxin test. Furthermore, it requires a relatively large number of animals. We made an attempt to establish an assay method that requires a minimal number of animals and has levels of sensitivity and accuracy similar to those of the endotoxin test and can even effectively detect the biological activity of endotoxin as can the pyrogen test. TNF-α is known as the earliest detectable inflammatory cytokine released from macrophages or monocytes by stimulation with endotoxin (13,14,18). In the present study, we attempted to utilize the in vitro system of TNF-α induction from peripheral blood of rabbits for detecting the biological activity of endotoxin.

The concentration of TNF-α released in the supernatant of fresh rabbit blood treated with endotoxin was found to reach a peak at around 5 h of incubation at 37°C as seen in Fig. 1. The most sensitive detection of endotoxin activity was found to be possible, therefore, at 5 h of incubation. Although a straight linear dose-response regression could be attained between concentrations of TNF-α and endotoxin to suggest the applicability of the slope ratio analysis method, the range of the concentrations of TNF-α and endotoxin for the straight linear dose-response relationship seemed to be limited without any transformation (Fig. 3a). A logarithmic transformation of the values was shown to be adequate for achieving homogeneity of variance and parallelism of the regression lines so as to allow analysis by the parallel line assay method (Figs. 2b, 3b and Table 1). Thus, the transformed dose and response enabled the assay to have comparable levels of accuracy and sensitivity as those of the endotoxin test (Table 1). The assay method could detect approximately 0.1 EU/ml of endotoxin in a test sample or, therefore, 0.01 EU/ml of that in the reaction mixture and showed a straight linear dose-response relationship up to 5 EU/ml or 0.5 EU/ml of test sample or reaction

mixture, respectively (Fig. 3b).

Relationships of the endotoxin test, the pyrogen test and the in vitro rabbit TNF-α induction test were examined using LPSs derived from various bacterial strains listed in Materials and Methods. It has been widely accepted that results of the pyrogen test and the endotoxin test show reasonable correlation in routine quality control tests (19,20). However, when LPSs from various differing bacterial strains were compared, the results of both tests showed considerable mutual discrepancy as shown in Fig. 5b. A similar discrepancy was also seen between the results of the endotoxin test and the in vitro rabbit TNF-α induction test as shown in Fig. 5a. Although no correlation was found between LAL activity and pyrogenicity or in vitro rabbit TNF-α induction activity, the results of the in vitro rabbit TNF-α induction test was found to correlate well with those of the pyrogen test as shown in Fig. 6.

We also examined human (21) and C3H/HeN mouse (22) blood for their utility in the in vitro TNF-α induction assay (data not shown). Human blood was shown to react with endotoxin to release TNF-α. However, its reactivity was affected considerably depending on the source individuals or especially on their immunization state. Blood from those having immunity against influenza virus could not be used for the effective detection of endotoxin activity in influenza vaccines. In addition, this procedure using human blood might raise ethical issues or create possible biohazards. Mouse blood was also found to be inappropriate due to its insufficient sensitivity even though using blood from the mice whose responsiveness of the endotoxin-dependent TNF-α release has been enhanced by BCG-sensitization (23,24). The in vitro TNF-α induction test using rabbit blood was, therefore, suggested to be the most appropriate test method for detecting the biological activity of endotoxin so far.

The in vitro assay method using rabbit peripheral blood was shown to have advantages in comparison to the other test methods as described above. This assay method is able to assay many test samples using only one rabbit and can use the same rabbit repeatedly, differing from the pyrogen test which cannot use rabbits that showed positive response in a previous measurement and which requires three rabbits per sample. LAL reagents for the endotoxin test are also very expensive and the endotoxin test costs at least 5 times the present assay using an ELISA system. The new in vitro assay method using rabbit peripheral blood has an advantage in regarding cost.

However, this assay method still has possible limitations. A rabbit might have immunity to a pathogen that has an antigen common with a vaccine under test. The test might be hampered for acellular pertussis vaccines if the blood was taken from a rabbit with positive immunity against *Bordetella bronchiseptica* which might have an antigen common to a component of acellular pertussis vaccines. It would be therefore important to examine the responsiveness of animal blood by means of an interference test. If TNF-α induction by spiked endotoxin was interfered for such a vaccine product, we need to avoid using such an animal which may have immunity to the organism associated with the product. As the assay method makes use of only TNF-α induction, it may show a discrepancy with the pyrogen test especially when testing a drug which can affect a physiological step that follows TNF-α release. Furthermore, the test may have a limitation in predicting the safety of a drug that is suspected to have a host-specific synergistic effect with endotoxin such as interferon-γ injection which is known to have a significant structural difference (25) from its corresponding rabbit interferon. Although the rabbit in vitro TNF-α induction assay was found to correlate well with the pyrogen test, there was an endotoxin that showed a significant discrepancy in the two tests. The reason for the differing results of the two assays could not be explained in the present study.

It is important for a new method for testing endotoxin contamination to guarantee the same level of safety as that of the pyrogen test. Although the endotoxin test is specific for detecting endotoxin, it cannot detect other pyrogens such as peptidoglycan that constitute the cell walls of both gram-negative and gram-positive bacteria. It might be interesting to examine the possibility of detecting other pyrogens such as peptidoglycan by the present in vitro rabbit TNF-α induction assay method.

REFERENCES

1. Sasaki, S. (1979): Biological activity of lipopolysaccharides isolated from bacteria in human periodontal lesions. Bull. Tokyo Dent. Coll., 20, 159-174.
2. Grisman, S. E. and Hornick, R. B. (1969): Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. Proc. Soc. Exp. Biol. Med., 131, 1154-1158.
3. U.S. Pharmacopeia. 24th (2000): Pyrogen test.
4. Levin, J. and Bang, F. B. (1964): The role of endotoxin in the extracellular coagulation of limulus blood. Bull. Johns Hoplins Hosp., 115, 265-274.
5. Levin, J. and Bang, F. B. (1968): Clottable protein in Limulus; its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh., 19, 186-197.
6. Morita, T., Tanaka, S. and Iwanaga, S. (1981): A new (1-3)-β-D-glucan-mediated coagulation pathway found in limulus amebocytes. FEBS Lett., 129, 318-321.
7. U.S. Pharmacopeia. 24th (2000): Bacterial endotoxin test.
8. European Pharmacopoeia. 3rd ed. (2000): supplement.
9. Japanese Pharmacopoeia. 13th ed. (1995): (in Japanese).
10. Ministry of Health and Welfare (1993): Minimum Requirements for Biological Products (in Japanese).
11. Ministry of Health and Welfare (1998): Minimum Requirements for Antibiotic Products of Japan (in Japanese).
12. Kawasaki, H., Moriyama, M. and Tanaka, A. (1987): Augmentation of endotoxin fever by recombinant human beta interferon in rabbits. Infect. Immun., 55, 1121-1125.
13. Mahony, J. R. Jr., Beutler, B. A., Trang, N. L., Vine, W., Ikeda, Y., Kawakami, M. and Cerami, A. (1985): Lipopolysaccharide-treated RAW 264.7 cells produce a mediator that inhibits lipoprotein lipase in 3T3-L1 cells. J. Immunol., 134, 1673-1675.
14. Beutler, B., Greenwald, D., Hulmes, J. D., Chang, M., Pan, Y. C. E., Mathison, J., Ulevitch, R. and Cerami, A. (1985): Identity of tumor necrosis factor and the macrophage-secreted factor cachectin. Nature, 316, 552-554.
15. Murai, T., Nakagawa, Y., Miyawaki, E., Ogawa, Y. and Horiuchi, Y. (1997): The endotoxin reference standard of the National Institute of Health Sciences (the Japanese Pharmacopoeia endotoxin reference standard) (Control 971). Bull. Natl. Inst. Health Sci., 115, 202-203 (in Japanese).
16. Tanaka, S. and Iwanaga, S. (1993): Limulus test for detecting bacterial endotoxins. Methods Enzymol., 223, 358-364.
17. Finney, D. J. (1978): Statistical Methods in Biological Assay. 3rd ed. Charles Griffin Co., Ltd., London.

18. Zee, K. J. V., Deforge, L. E., Fischer, E., Marano, M. A., Kenney, J. S., Remick, D. G., Lowry, S. F. and Moldawer, L. L. (1991): IL-8 in shock, endotoxemia, and after IL-1 administration. J. Immunol., 146, 3478-3482.
19. Naito, S., Fujiwara, H., Asakawa, S., Homma, R., Ishida, S., Chino, F., Tanaka, S., Ohki, M., Tsuchiya, M. and Matsuura, S. (1992): Application of the limulus amebocyte test to measurement of endotoxin in therapeutic human plasma protein fraction. Comparison with the rabbit pyrogen test. J. Pharm. Soc. Jpn., 112, 551-556 (in Japanese).
20. Asakawa, S., Fujiwara, H., Naito, S., Homma, R., Ishida, S., Chino, F., Tsuchiya, M., Matsuura, S., Tanaka, S. and Ohki, M. (1994): Application of the limulus test for practical quality control on endotoxin content in commercial human serum albumin (HSA) products. In comparison with the rabbit pyrogen test. J. Pharm. Soc. Jpn., 114, 888-893 (in Japanese).
21. Foster, S. J., McCormick, L. M., Ntolosi, B. A. and Campbell, D. (1993): Production of TNF-α by LPS-stimulated murine, rat and human blood and its pharmacological modulation. Agents Actions, 38 Special Conference Issue, C77-79.
22. Glode, L. M., Scher, I., Osborne, B. and Rosenstreich, D. L. (1976): Cellular mechanism of endotoxin unresponsiveness in C3H/HeJ mice. J. Immunol., 116, 454-461.
23. Peavy, D. L., Baughn, R. E. and Musher, D. M. (1979): Effects of BCG infection on the susceptibility of mouse macrophages to endotoxin. Infect. Immun., 24, 59-64.
24. Vogel, S. N., Moore, R. N., Sipe, J. D. and Rosenstreich, D. L. (1980): BCG-induced enhancement of endotoxin sensitivity in C3H/HeJ mice. I. In vivo studies. J. Immunol., 124, 2004-2009.
25. Farrar, A. M. and Schreiber, D. R. (1993): The molecular cell biology of interferon-γ and its receptor. Annu. Rev. Immunol., 11, 571-611.