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In Vitro Bioassay of Endotoxin Using Fluorescein as a pH Indicator in a Macrophage Cell Culture System

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Based on the biological activity of endotoxin, we propose a possible new method for detecting endotoxin using a pH-indication system of macrophage culture media. After RAW 264.7 macrophage cells were treated with lipopolysaccharide (LPS), the addition of fluorescein to the LPS-treated media reproductively reduced its absorption and emission spectra (it was a dose-dependent reduction). The advantages of this LPS-detection method were compared with the *Limulus* Amebocyte Lysate (LAL) test by using purified bacterial LPS (*Salmonella minnessota, Escherichia coli,* and *Pseudomonas aeruginosa*). Additionally, the absorption and fluorescence intensity of fluorescein, following treatment of RAW 264.7 cells with a high concentration of *Staphylococcus aureus* (Gram-positive, lysed bacteria), could not generally be detected by the LAL test, but they were found to be reduced, in a dose-response relationship, with this new system. The macrophage culture system-method might be a good supplement to the LAL assay for detection of LPS, Gram-negative and Gram-positive bacteria.

Key Words: Lipopolysaccharide, macrophage, fluorescein, *Limulus* Amebocyte Lysate test, *Staphylococcus aureus,* pH

INTRODUCTION

The outer membrane glycolipid component of Gram-negative bacteria is known as lipopolysaccharide (LPS) or endotoxin. LPS acts as a potent stimulus to a variety of cells, and it results in the enhanced expression of cytokines, adhesive proteins and pro-inflammatory molecules.[1] The most widely accepted test for endotoxin is the pyrogen test performed in rabbits, but both ethical and economic considerations call for this test to be replaced by *in vitro* methods. The most widely used *in vitro* alternative is the *Limulus* amoebocyte lysate (LAL) test: however, this test does not exactly parallel *in vivo* pyrogenic activity. The LAL assay is the currently favored method for the detection of endotoxin because of its high sensitivity.[2] It measures small amounts of the major pyrogen, endotoxin (C pathway), and it also measures 1,3-β-glucans (G pathway) of fungi, which are much less pyrogenic,[3] but this test does not react to pyrogenic substances from Gram-positive bacteria. There are several problems associated with this method, and especially when it is applied to biological samples. The same as in other biological assays, the reaction of the LAL assay also differs depending on the chemical and physiochemical structure of the endotoxin.[4]

Macrophages are regarded as functional analogues of *Limulus* amoebocytes, and macrophages are exquisitely sensitive to endotoxin. Therefore, the use of macrophages or other related cell lines as endotoxin indicators has been proposed. The suggested processes that can be used as indicators for endotoxin have included interleukin-1,[5] TNF or interleukin-6 generation,[6] NO production or

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pteridine formation[7] and the enhancement of procoagulatory activity.[8] Such systems that utilize the measurement of cytokine levels have suffered from a high degree of variability (distinct LPS sensitivity) over the range of cell lines. In an effort to avoid these disadvantages, we proposed here a method for detecting endotoxin using a pH-indication system of macrophage culture media that is based on the biological activity of endotoxin. Endotoxin stimulates macrophages and cause their induction of vacuoles (endosomes, lysosomes, etc.), that are kept acidic (pH≅6) via ATP-driven H^+ pumps that are driven by the immune response of the macrophages. A similar or identical vacuolar H^+ ATPase is thought to acidify all endocytic and exocytic organelles, including phagosomes, lysosomes, selected compartments of the Golgi apparatus and many transport and secretary vesicles.[9,10] Therefore, the falling pH of the culture media isolated from endotoxin-stimulated macrophages could dramatically reduce the fluorescence intensity of fluorescein, which can be used as a pH indicator. Fluorescein and many of its derivatives exhibit multiple, pH-dependent, ionic equilibrium. Both the phenol and carboxylic acid functional groups of fluorescein are almost totally ionized in aqueous solutions above pH 9. Acidification of the fluorescein dianion first protonates the phenol (pKa≅6.4) to yield the fluorescein monoanion, and then it induces the carboxylic acid (pKa≅5) to produce the 3 neutral species of fluorescein (Fig. 1).[11]

In this study, we compared the sensitivity and range of detecting endotoxin of Gram-positive and Gram-negative bacteria in a macrophage culture detection system that used fluorescein as a pH-indicator with the standard LAL test.

MATERIALS AND METHODS

Preparation of test materials and treatment on cells

All the purified LPSs that originated from *Escherichia coli* (*E. coli*), *Salmonella minnesota* (*S. minnesota*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) respectively, were purchased from Sigma (St. Louis, MO, USA). The stock solution of LPS of each strain was 10^5 ng/ml (pH 7.2) of Dulbecco's Modified Eagle's Medium (DMEM, without phenol red, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco). The stock solution of *S. aureus* was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and it was 10^7 colony forming units (CFU) ml^{-1} scale in DMEM (without phenol red, pH 7.2) with 10% FBS. This bacterial suspension was lysed by ultrasonication (Misonix Inc., Farmingdale, NY, USA) to obtain the crude bacterial extract, including the bacterial wall component. The macrophage used in this study was the RAW 264.7 macrophage (mouse macrophage cell line). It was obtained from ATCC and cultured at 37℃ in a humidified atmosphere (5% CO_2/95% air) in DMEM containing 10% FBS. For reacting the prepared LPS or sonicated bacterial extractions, the suspension of RAW 264.7 in DMEM without phenol red and with 10% FBS was plated at 4×10^5 cells per well on a 24 well-plate, and the macrophages were allowed to attach for 24 hrs. The stock solution of

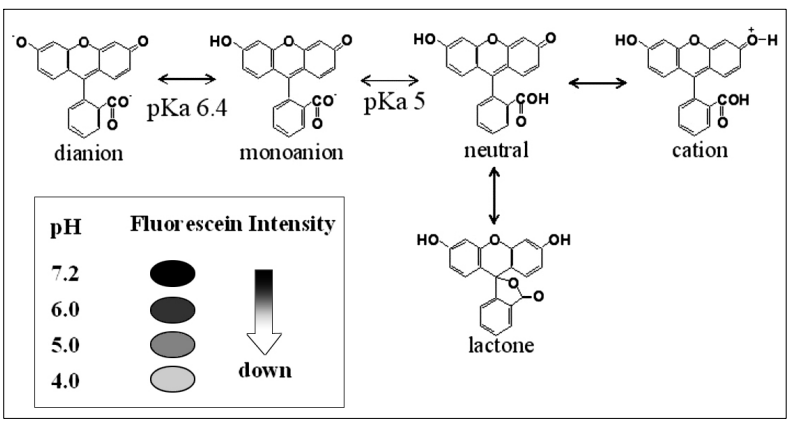

Fig. 1. Fluorescein and many of its derivatives exhibit multiple, pH-dependent ionic equilibria. Lowering the pH of the culture media isolated from endotoxin- stimulated macrophages could dramatically reduce the fluorescence intensity of fluorescein as a pH indicator.

four kinds of purified LPS and the sonicated bacterial solution were prepared to the starting concentration of 250 ng/ml and 10^7 CFU/ml scale, respectively, in DMEM without phenol red (pH 7.2) with 10% FBS. After washing the well-plates with the attached macrophages, the starting concentration of each solution was serially diluted to one fifth of the previous concentration (whose volume was 2 ml in each well of the 6 well-plate), until the final concentration was 0.08 ng/ml and 10 CFU/ml scale, respectively. For the activation of the RAW 264.7 macrophages with the testing materials, the reaction media was incubated in 5% CO_2 at 37°C for 25 hrs.

For the activation of the RAW 264.7 macrophages with the prepared LPS, the LPS-treated cells were incubated in the same conditions and at the same pH as the cultured media that was being evaluated.

Detection of pH-indication in reaction media

After incubation, the reaction media was transferred to a microcentrifuge tube and the supernatant was acquired by centrifuging at 1000 rpm in order to determinate the pH of reaction media without the cells and testing material debris. To determine the absorption of fluorescein as a pH-indication, fluorescein was added to the reaction media to final concentration of 25 M, and the fluorescein intensity, as related to the pH of the reaction media, was detected at a wavelength of 490 nm with an ELISA reader. To determine the emission of fluorescein, it was also added to the media to a final concentration of 10 M, and its intensity was detected by a luminescence spectrophotometer LS50 (PerkinElmer, Bucks, U.K) at wavelengths of 430 nm for excitation and 630 nm for emission.

LAL test

Endotoxin in the testing materials was quantified by the kinetic turbidimetric collection mode of the LAL test by using an EL× 808 incubating microplate reader (Bio-Tek Instruments Inc, Vermont, USA; wavelength filter 340).[12,13] A endotoxin unit (EU) is a standardized amount of endotoxin based on its reactivity in the LAL test (the LAL reactivity of 0.1 ng of US Pharmacopeia (USP) Reference Standard Endotoxin).

Statistical analysis

The detection limit is calculated as the minimum concentration of a substance that would diminish the fluorescein absorption and fluorescence less than the mean of the control. The S.D., mean and S.D. of the control were calculated on the basis of all experiments.

RESULTS

Standardization of fluorescein pH-indication

The pH-indicative range of fluorescein was preferentially examined by using a certified pH-standardized culture media [DMEM containing 10% FBS without phenol red; the pH was adjusted to the range of 4.0-7.0 using 1 N HCl]. To determine the absorption of fluorescein as a pH-indicator, fluorescein was added to the cultured media to a final concentration of 25μM, and the absorbance of fluorescein, as related to the media pH, was detected at 490 nm. In addition, the fluorescein emission data, following the addition of fluorescein to the media to a final concentration of 10μM, were determined by luminescence spectrophotometer. Fig. 2A reveals that the absorption of fluorescein effectively decreased from 0.43 (pH 7.0) to 0.12 (pH 4.0) as the media pH was reduced. Fig. 2B shows that the emission intensity of fluorescein significantly decreased from 500.7 at pH 7.5 to 145.6 at pH 4.0, as the media's pH was reduced.

Comparison of the sensitivity to detect endotoxin in the macrophage culture system using fluorescein pH indication with that of the LAL test

The detection limit of the endotoxin (the purified LPS that originated from *E. coli, S. minnesota* and *P. aeruginosa*) in the macrophage culture system, using fluorescein as a pH-indicator, was compared with the LAL test. The stock solution of each strain was 10^5 ng LPS/ml of DMEM (pH 7.2 and without phenol red) supplemented with 10%

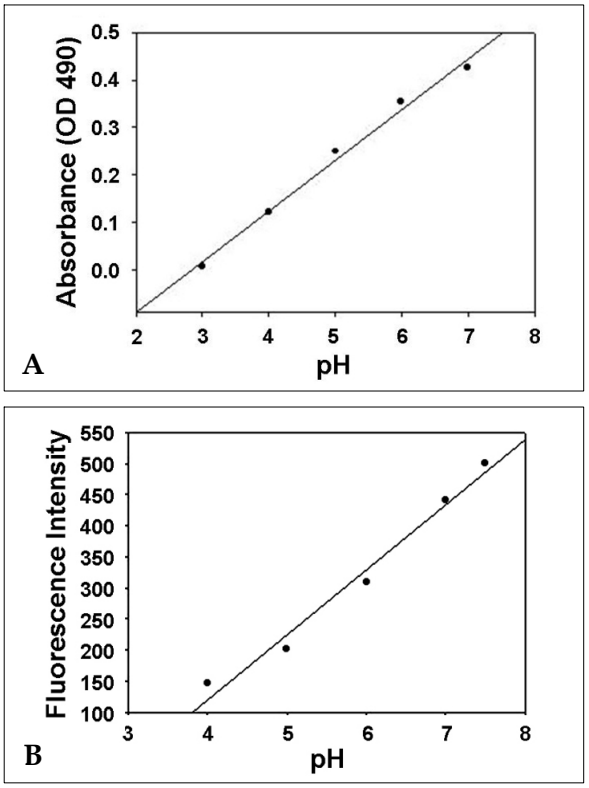

Fig. 2. The pH-indicative range of fluorescein was examined, in terms of both absorption and emission, by a certified pH-standardized culture media (DMEM containing 10% FBS without phenol red; pH 4.0 to 7.0). (A) The absorption of fluorescein decreased with the reduced pH, from 0.43 at pH 7.0 to 0.12 at pH 4.0. (B) The emission intensity of fluorescein decreased significantly as the pH was reduced, from 500.7 at pH 7.5 to 145.6 at pH 4.0.

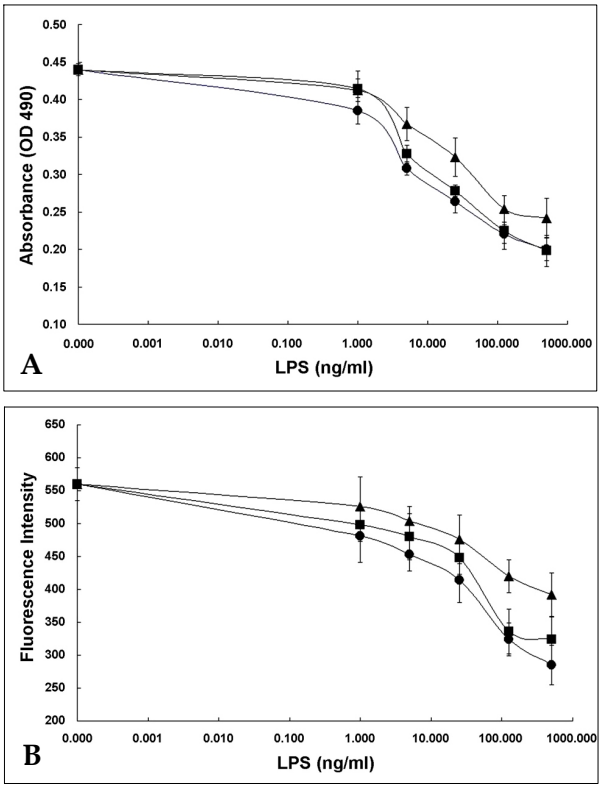

Fig. 3. Dose dependent diminution of fluorescein absorption (A) and fluorescence (B) after LPS treatment [the LPS originated from three kinds of Gram-negative bacteria, *S. minnesota* (●), *E. coli* (■), and *P. aeruginosa* (▲)]. To determine the absorption of fluorescein as a pH-indicator, fluorescein was added to the reaction media to a final concentration of 25 M, and the absorbance, as related to the pH of the reaction media, was detected at 490 nm by an ELISA reader. To determine the fluorescein emission, fluorescein was also added to the media to a final concentration of 10 M, and its intensity was detected by a luminescence spectrophotometer at 430 nm of excitation and 630 nm of emission.

FBS. The detection limit of this study was 1 ng/ml LPS of *E. coli, S. minnesota*, and *P. aeruginosa* in the case of both the fluorescein absorption and fluorescence assay (Fig. 5). The absorption and fluorescence intensity of fluorescein in the control media without LPS was 0.44 ± 0.008 and 560 ± 20 (mean ± S.E.M.), respectively. Fig. 3A establishes that the absorption of fluorescein in the cultured media was significantly reduced with the increasing LPS concentration (on the whole scale from 1 to 500 ng/ml) of *E. coli, S. minnesota*, and *P. aeruginosa*. At a 1 ng/ml LPS concentration of *S. minnesota*, the absorption of fluorescein decreased from 0.44 of the non-treated control to 0.385, and at 500 ng/ml, it decreased to 0.2. The fluorescence data also demonstrated the dose (LPS)-dependent decrease of fluorescence intensity of fluorescein in cultured media in all cases of *E. coli, S. minnesota*, and *P. aeruginosa* (Fig. 3B). In the case of *S. minnesota*, the emission of fluorescein was reduced from 560 for the non-treated control to 481 at 1 ng/ml of LPS, and it was reduced to 285 at 500 ng/ml. The results in LAL test also had a similar pattern to those results of our methods (Fig. 4). Among the bacteria species examined, the endotoxin unit (EU, the LAL reactivity of 0.1 ng of Reference Standard Endotoxin) of *S. minnesota* was detected from 0.49 of the control to 1.85 at 1 ng/ml of LPS and to 6982 at 500 ng/ml, which was more sensitive than that of *E. coli* and *P. aeruginosa*.

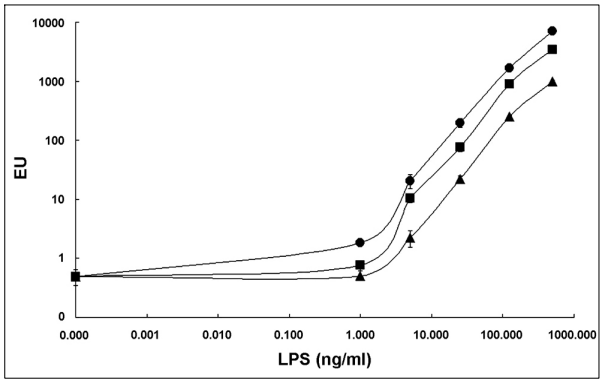

Fig. 4. LAL test for detection of endotoxin unit (EU) in accordance with LPS concentration. A serial dilution of 3 different LPSs [*S. minnesota* (●), *E. coli* (■), and *P. aeruginosa* (▲)] was assayed using the kinetic turbidimetric method.

DISCUSSION

The Limulus lysate assay is currently being widely used in most situations that are concerned with the quantification of endotoxins because of the assay's high sensitivity. This test has become even more sensitive in recent years, and this is due to a replacement of the classical clotting assay by turbidimetric and colorimetric test formats and to the determination of endpoints by kinetic assays, as well as other additional improvements. However, this assay is not specific for all kinds of endotoxin, and it is drastically modified by the presence of inhibitors or activators of the enzymes included in the cascade reaction of the *Limulus* assay.[14,15] In addition, endotoxin undergoes chemical enzymatic degradation or modification in the presence of certain host factors, such as lipoprotein.[16,17] In most LAL formats, the highly pyrogenic endotoxin and the much less pyrogenic 1,3-β-D-glucans induce a similar LAL reaction.[3] Previous reports have emphasized the variability of the LAL test, particularly when using the test to analyze complex biological specimens. Urbaschek et al.[18] reported that the LAL test with FCS was hampered by factors that interfered with the LAL reaction. For example, Eperon et al.[19] tested various FCS lots with two distinct formats of the LAL test (WAKO and Haemachem), and with the cell culture test. Some FCS lots assayed with two LAL test formats were positive on one, but not on both types of tests. One single FCS lot that was positive on both LAL assays induced TNF generation in monocytoid cells. In light of these previous findings, the determination of endotoxin by the *Limulus* test, as in other biological assays, does not always reflect the true amount of endotoxin.

In the current study, we have created an *in vitro* alternative (the macrophage culture system using fluorescein as the pH-indicator) to the LAL test, and we compared the sensitivity and range for detecting endotoxin of Gram-positive and Gram-negative bacteria in a system of macrophage culture by using fluorescein as a pH-indicator with the standard LAL test. The sensitivity to detect endotoxin in the macrophage culture system using fluorescein as pH-indicator for both the absorption and emission wavelength was compared with LAL test. Fig. 3 demonstrated that the absorption and emission of fluorescein in the reaction media were significantly reduced in accordance with an increasing concentration of LPS that originated from *E. coli, S. minnesota*, and *P. aeruginosa*. The results of the LAL test also demonstrated that the EU value was highly dependent on the dose of LPS (Fig. 4). With a high concentration of LPS, both the macrophage culture-method and LAL test showed similar patterns for detecting the purified bacterial LPS, but the correlation between the detection method and LPS concentration was less significant than the absorption at minute concentrations below the unit ng/ml scale (Fig. 5). In the LAL test, there was a good linear relationship between dose and response with a small coefficient of intra-assay variation at high concentrations. However, for example, *P. aeruginosa* demonstrated a insensitive dose-response relationship in the range from 0.04 to 1.0 ng LPS/ml (Fig. 5C). One of the most potent LPSs on the LAL test was LPS from *S. minnesota,* which was consistent with the results of the macrophage culture system. It is quite within the realms of possibility that for different endotoxins, the LAL test is less sensitive than the macrophage culture system for the detection of minute concentrations of LPS that originate from different bacteria.

In the current LAL study, the *S. aureus* bacteria that were grown in pyrogen free media and had no LPS contamination were not detected in a

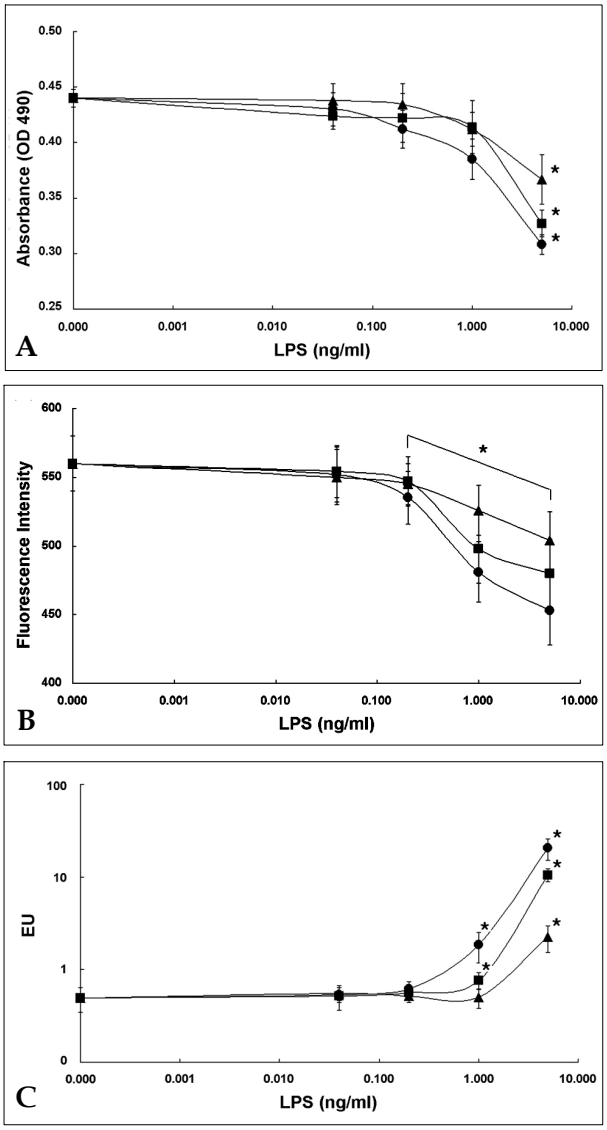

Fig. 5. Comparison of the macrophage culture system-method and the LAL test at minute concentrations of LPS below the unit ng/ml scale [*S. minnesota* (●), *E. coli* (■), and *P. aeruginosa* (▲)]. Absorption (A) and fluorescence intensity (B) of fluorescein in the reaction media and the EU values (C) of 3 different LPSs were detected and compared with each other in the order of magnitude increases for concentration from 0.04 EU to 5 EU. The asterisk indicates statistical significance at $p \leq 0.05$, as compared with untreated controls. Data represent mean values (± S.D.) of triplicate samples per condition for the experimental method.

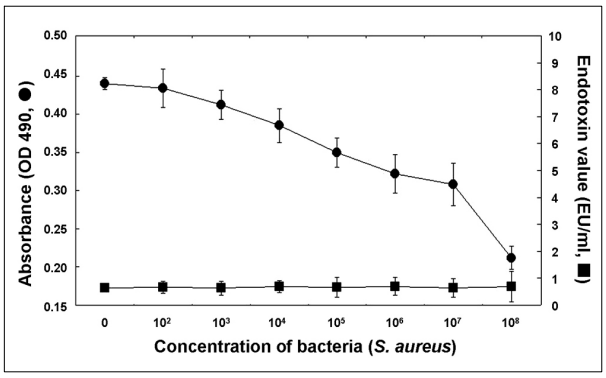

Fig. 6. Comparison of the macrophage culture system and the LAL test, in terms of sensitivity and specificity to sonicated *S. aureus* (Gram-positive bacteria) extracts. Absorption (●) of fluorescein in the reaction media and EU values (■) of the lysed *S. aureus* extracts were detected and compared with each other in the order of magnitude increases for concentrations from 10^2 CFU/ml scale to 10^7 CFU/ml scale (n=4).

suspension of 10^8 bacteria/ml (Fig. 6). For the detection of *S. aureus* by the LAL test, the current test exhibits control EU levels at all bacterial concentrations because Gram-positive bacteria have no LPS in their cellular components. Thus, this result supports the concept that the cell culture test has a higher degree of specificity than the LAL tests for the detection of unknown endotoxins such as bacterial particles that originate from Gram-positive bacteria. Monocytes are thought to be primary target cells for LPS, and they are the main source of inflammatory cytokines that are induced during bacterial infection.[20] Apart from LPS, other components of Gram-negative and Gram-positive bacteria, e.g. muramyl peptide[21] or lipoteichoic acid,[22] are known to interact with monocytes and macrophages and to stimulate cytokine secretion.[23] For example, Moesby et al.[24] reported that the production of IL-6 by Mono Mac 6 (MM6) cells was a reliable indicator for LPS stimulation. Although the MM6 assay showed a dose-response relationship between LPS and IL-6 over a wide concentration range (2.5-1000 pg/ml), in the case of Gram-positive bacteria, a detection limit of approximately 3×10^5 CFU/ml *S. aureus* was obtained, which is comparable to the detection limit of the macrophage-fluorescein bioassay in our study (10^3 CFU/ml). In the current study, *S. aureus*, a Gram-positive bacterium, demonstrated no significant reduction of absorption until the concentration reached 10^2 CFU/ml (lysed bacteria) scale. However, the absorption decreased significantly at higher bacterial concentrations ($> 10^3$ CFU/ml

scale, Fig. 6). A simple *in vitro* assay that is less susceptible to nonspecific interference and has sensitivity to various pyrogens comparable to that of the rabbit pyrogen test or even better would be preferable. The current study might be a good supplement to the LAL test for the detection of LPS, Gram-negative and Gram-positive bacteria.

In conclusion, the results in the present study indicated that the macrophage culture system-method presented in this study demonstrates a significant advantage in terms of its sensitivity and its ability to detect unknown endotoxins such as the bacterial particles that originate from Gram-positive bacteria.

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