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Assessment of pyrogenic response of lipoteichoic acid by the monocyte activation test and the rabbit pyrogen test

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ABSTRACT

Lipoteichoic acid (LTA) is a non-endotoxin pyrogen of a great importance in the pathogenesis of sepsis. The Rabbit Pyrogen Test (RPT) is able to detect all types of pyrogens but involves the use of animals. The Bacterial Endotoxin Test (BET) cannot fully replace the RPT because it only detects endotoxins. The Monocyte Activation Test (MAT) is sensitive to all types of pyrogens and it is based on the same biological mechanism that is responsible for the fever reaction in humans. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has recommended its use for other pyrogens than endotoxin because its equivalence to RPT can be demonstrated. The aim of this study was to evaluate the pyrogenic responses of the RPT and MAT that was induced by LTA. Different LTA concentrations were assayed by the MAT in parallel to the RPT. The results showed that the MAT was more sensitive than the RPT, demonstrating that the MAT detected LTA. This result may contribute to the acceptance of this test by the Brazilian regulatory agencies as a replacement for the animals used in the RPT.

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1. Introduction

Pyrogens are chemically heterogeneous fever-inducing substances that are derived from dead or viable Gram-negative (endotoxin or lipopolysaccharide – LPS) and Gram-positive bacteria (lipoteichoic acid – LTA), viruses or fungi (zymosan). Therefore, these substances can be found even in sterile environments such as in injectables and medical devices (Schindler et al., 2009; ICCVAM, 2008; Hasiwa et al., 2013). Although the endotoxin has been well characterized and has been found to be a contaminant in injectable products for human and veterinary use (Montag et al., 2007; Akers, 2011), there are other neglected pyrogens that represent important major public health problems (Williams, 2007; Schindler et al., 2009). These substances are called “Non-Endotoxin” pyrogens (NEPs, considering the ALT and peptidoglycan) (Montag et al., 2007).

There are three methods for detecting pyrogenic contamination: the Rabbit Pyrogen Test (RPT), the Bacterial Endotoxin Test (BET), and the Monocyte Activation Test (MAT).

The RPT is the most recommended test for ensuring the absence of the pyrogenic contamination in injectable products. This test is based on the measurement of the rectal temperature after injection of products because the rabbit's response has the same pattern as the human fever (Williams, 2007; Hochstein et al., 1990; Pinto et al., 2010). The test interpretation is based on the number of rabbits with a temperature rise $\geq$0.5 °C (fever) and the sum of the temperature rises of all rabbits that were tested (Farmacopeia Brasileira, 2010b; United States Pharmacopeia, 2014b). Although the method detected all types of endotoxins and NEPs, it had limitations in addition to the ethical issue of the animal use (ICCVAM, 2008).

The BET (also known as *Limulus* Amebocyte Lysate – LAL test) is based on the reaction of the hemolymph of the horseshoe crab (*Limulus polyphemus*) after contact with LPS. The BET is susceptible to interference from certain types of products (e.g., biological, products with high protein and lipid levels, glucans), and it is ineffective in detecting NEPs (Spreitzer et al., 2002; Schindler et al., 2003; Schindler et al., 2009; Farmacopeia Brasileira, 2010a; United States Pharmacopeia, 2014a).

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The MAT is based on the activation of human monocytes by pyrogens and on the quantification of cytokine release (IL-1β, IL-6 and TNF) by the Enzyme-Linked Immunosorbent Assay (ELISA) (Hartung and Wendel, 1996; Hoffmann et al., 2005; European Pharmacopoeia, 2014).

Even though the MAT detects all types of pyrogens, there is an urgent need to provide studies that establish of the correlation between *in vivo* and *in vitro* responses for NEPs, such as LTA (ICCVAM, 2008; Rockel and Hartung, 2012).

Morath et al. (2001) developed the first purified biologically active and endotoxin-free LTA from *Staphylococcus aureus*. This development made it possible to use LTA as an appropriate reference material for testing NEPs as one of the main non-endotoxin stimulus of cytokine production in humans (Rockel and Hartung, 2012).

LTA molecules are attached to the cytoplasmic membrane of Gram-positive bacteria from *S. aureus* via a glycolipid anchor (Fig. 1A). The LTA backbone consists of the repetitive glycerol phosphate units and D-alanyl ester or α-N-acetylglucosamine (Fig. 1B) (Morath et al., 2001, 2002a, 2002b; Draing et al., 2008; Xia et al., 2010).

Keeping the final objective of absolute replacement of this process, the goal of this study was to evaluate the pyrogenic response of LTA in parallel with *in vivo/in vitro* studies between the cryopreserved human whole blood – IL-1β in the monocyte activation test and the rabbit pyrogen test.

2. Animals, materials and methods

2.1. Samples

LPS from the *Escherichia coli* serotype O55:B5 (Sigma–Aldrich, Saint Louis, Missouri, USA) was used as a positive control and LTA from *S. aureus* (Sigma–Aldrich, USA) was used as the NEP stimulus. The 0.9% NaCl pyrogen-free solution (Sanobiol, Brazil) was used as a negative control.

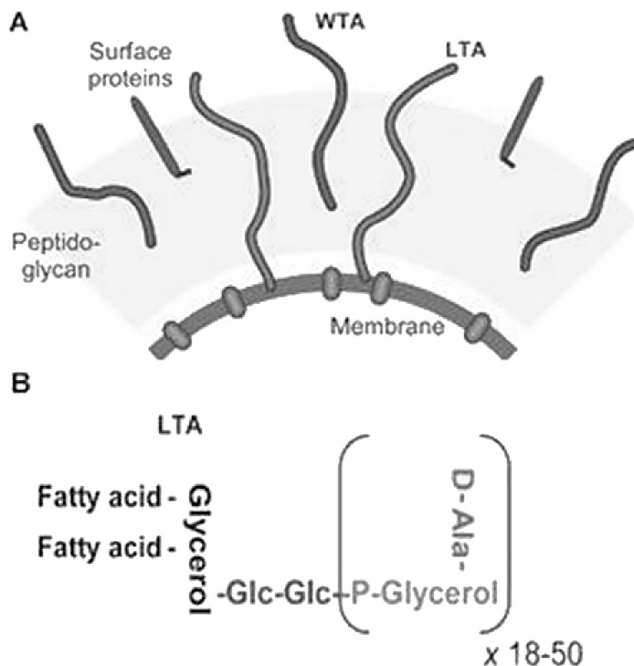

Fig. 1. Schematic localization in the cell envelope (A) and structure (B) of *S. aureus* lipoteichoic acid (LTA). Wall teichoic acids (WTA); P, phosphate; D-Ala, D-alanine; Glc, Glucose (adapted from Xia et al., 2010).

To exclude the endotoxin contamination of the LTA solutions (<0.1 EU/mL) as well as to determine the potency of the LPS solutions, the Endpoint chromogenic *Limulus* Amebocyte Lysate assay (LONZA, Walkersville, MD, USA) was used. The endotoxin standard kit is traceable to the international Reference Standard Endotoxin (RSE).

Concentrations of LPS (0.125; 0.25; 0.5; 1.0; 2.5; 5.0 EU/mL or 0.025; 0.05; 0.1; 0.2; 0.5; 1.0 ng/mL) and LTA (100; 1000; 10,000; 50,000; 75,000; 100,000; 125,000; 250,000; 500,000 ng/mL) were prepared in the 0.9% NaCl pyrogen-free solution.

2.2. Dose-response curve of LTA in rabbits

Healthy adult New Zealand rabbits weighing not less than 1.5 kg were used and individually kept in cages in ventilated racks (20 ± 2 °C). The animals received autoclaved hay as environmental enrichment. The rabbits were supplied by the Laboratory Animal Breeding Center (CECAL/Fiocruz, Brazil). Experiments were performed in accordance with the approved institutional protocols, under License Number LW 44/14 from the Oswaldo Cruz Foundation (Fiocruz) Ethics Committee on Animal Use (CEUA) and followed the recommendations described in the Brazilian Pharmacopeia (BrPh), which is identical to the United States Pharmacopeia (USP).

Each concentration was administered intravenously (1 mL/kg body weight) into the marginal ear vein. After the injection, the rectal temperatures of the animals were measured with 30 min intervals with a PyroMon™ system (ELLAB, Hillrod, Denmark, model APT 96, version 2.9.5) for a period of 3 h. For each rabbit, the response was defined as the difference between the basal temperature (obtained prior to injection) and the maximum temperature (recorded after the LTA injection).

Fever was considered when the individual variation of the temperature rise was equal to or higher than 0.5 °C according to the BrPh, USP and Hochstein et al. (1990).

2.3. Monocyte activation test using cryopreserved whole blood

2.3.1. Cryopreservation of whole blood

A volume of 10 mL of fresh human blood was collected by venipuncture from each of four healthy volunteer donors in sterile heparinized tubes (Labor IMPORT). Prior to the collection of the blood the consent forms were signed and questionnaires were completed to eliminate possible factors of exclusion (e.g., infections, inflammatory reactions, etc.). This project is licensed by the Research Ethics Committee of Fiocruz (CEP 368/07).

The cryopreservation procedure was performed within a 4 h period after collection of the blood. The steps of the procedure followed the protocol of Schindler et al. (2006). Endotoxin-free Sörensen Buffer (Acila AG, Mörfelden-Walldorf, Germany), with a pH 6.8 was mixed with 20% v/v endotoxin-free Dimethylsulfoxide (DMSO) (Wak-Chemie Medical GmbH, Steinbach, Germany). The pool of blood from the volunteers was aliquoted in pre-cooled cryotubes (Eppendorf, Hamburg, Germany) and frozen at −80 °C. This pooled cryo-preserved blood was used for all experiments.

2.3.2. Whole blood incubation

Human whole blood incubations were performed as described previously in the European Pharmacopoeia (2010). The pool of blood was thawed at 37 °C for 15 min. Then, 100 μL of LPS and LTA were incubated with 200 μL of the cryopreserved blood and 900 μL of the saline solution in the sterile and non-pyrogenic 1.5–2 mL microtubes (Eppendorf, Hamburg, Germany). The mixture was then left in an incubator (37 °C, 5% CO_2) overnight. After an incubation period of 16 h, the material was immediately analyzed, frozen, or

stored at −20 °C until the cytokine measurement.

The dose–response curve was obtained with the same LTA concentrations that were used in the *in vivo* studies.

The threshold pyrogenic dose was given at 5.0 EU/kg, which corresponds to 1.0 ng of LPS/kg (Hochstein et al., 1990). The results were expressed as pyrogenic (P) and non-pyrogenic (NP).

2.3.3. Measurement of IL-1β by the ELISA method

The determination of the cytokine release was performed by the commercial ELISA kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's information using the VersaMax reader (Molecular Device, software SoftMax® Pro5).

2.3.4. Expression of the equivalent endotoxin units

The pyrogenic response of the preparations was estimated quantitatively by constructing a concentration–response curve of the LPS standard (0.125, 0.25, 0.5, 1.0, 5.0 EU/mL) versus the value of Optical Density (OD) of the release of IL-1β by ELISA of LTA. Thus, the LTA results were expressed in Endotoxin-Equivalent Units per mL − EEU/mL (Montag et al., 2007; ICCVAM, 2008). The data were expressed in term of means ± SE.

2.4. Statistical analysis

Data were transformed as a log (x + 1) and a log (y + 1) to include the zero value (negative control − saline) in the statistical analyses.

The goal was to demonstrate the significance of the difference between the dose–response curves of the doses range below (no fever) the positive threshold of fever and above (fever response). The linear regression of the two dose ranges was calculated by the coefficient of correlation (r) and coefficient of determination (r^2). Then, it was tested whether the slope of the curve (b) showed a statistically significant difference from zero by applying the F test and by calculating the p value. When p was >0.05, the slope was considered to be not statistically significantly different from zero. When p was <0.05, the slope was considered to be statistically significant different from zero.

However, this approach does not ensure that there is not a statistically significant difference between the two coefficients of correlation. Thus, we used the Fischer's t test for comparing the two independent correlation coefficients (r) by transforming the values of r to Z, as described in Kenny (1986). When p was <0.05, it meant that there was a statistically significant difference between the two correlation coefficients.

3. Results

3.1. Dose-response curve of LTA obtained by the rabbit pyrogen test

To evaluate the correlation between the LTA cytokine release, we compared the *in vivo*/*in vitro* responses. Rabbits were stimulated with increasing concentrations of LTA from *S. aureus*. The results showed that the fever response started at the 75,000 ng/mL dose (Fig. 2). Comparing the responses for the range of the NP doses (negative control dose was up to 50,000 ng/mL), the F value was determined to be 0.1862 and p = 2.917. This showed that the slope was not significantly different from zero (the total absence of slope). Pyrogenic doses for the range from 75,000 ng/mL up to 500,000 ng/mL presented an F value of 63.98 and p = 0.0041. This showed that the slope presented a statistically significant difference from zero. Comparing the coefficients of correlation by Fischer's t test, the obtained result was Z = 5.06 (p < 0.05). This result showed that there was a statistically significant difference between the two correlation coefficients. This result showed that the response to 50,000 ng/mL from the NP dose was significantly different from the response to 75,000 ng/mL from the P dose (Fig. 2).

3.2. Concentration–response curve of LTA in monocyte activation test

The results of the standard curve of LPS demonstrated that there were differences in the release of IL-1β depending on the concentration (Fig. 3). These differences in release were statistically demonstrated by the coefficient of Determination $R^2 = 0.9360$, where F = 73.18 (p < 0.001) demonstrated that the slope was significantly different from zero. It was also demonstrated that there was a statistically significant linear relationship between the concentrations and the responses (p = 0.3333).

Furthermore, the results of the LTA concentrations showed a pyrogenicity in the range from 50,000 ng/mL up to 500,000 ng/mL (Fig. 4). The difference in the release of IL-1β was dependent on the concentration of LTA. This was statistically demonstrated by the coefficient $R^2 = 0.9284$, where F = 103.7 (p < 0.0001) demonstrated that the slope was significantly different from zero (p = 0.1905).

3.3. Comparison of results obtained using LTA in RPT and MAT

When compared to the RPT, the MAT responded to LTA at the dose of 50,000 ng/mL. This can be explained by the fact that MAT is more sensitive than the RPT. To show the equivalence to endotoxin, the results were expressed in EEU (Table 1).

4. Discussion

Based on the need to provide studies on the application of the MAT for non-endotoxin pyrogens, a study was conducted to compare the equivalence of the obtained results from this method with those obtained in the RPT using a pyrogen-free saline solution (NaCl 0.9%) that was intentionally “spiked” with a different concentrations of LTA.

ICCVAM (2008) recommends that “optimally, a study that includes three-way parallel testing, with the *in vitro* assays being compared to the RPT and the bacterial endotoxin test, should be conducted to comprehensively evaluating the relevance and comparative performance of five methods by the MAT. These studies may be conducted with historical data from the RPT supplied that the same substances (i.e., the same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill the existing data gaps”. Therefore, there is an urgent need for the application of an alternative methods that allow the establishment of the correlation of responses between the *in vivo* and *in vitro* methods (ICCVAM, 2008).

In this study, the results allowed to demonstrate the difference between the pyrogenic (≥75,000 ng/mL) and the non-pyrogenic doses (≤50,000 ng/mL) of LTA in rabbits. We consider these data highly relevant because this correlation had not been previously established.

The RPT is still used in some cases where *in vitro* test cannot be applied. It has the disadvantage of being an expensive method because it involves the structure for maintaining an animal facility, it is time consuming, and it involves physiological variations of the animals (Williams, 2007; ICCVAM, 2008).

In the literature, we did not find articles that related the fever response in rabbits for LTA. However, there is only one publication that comparatively evaluated the MAT with the human and rabbit blood for IL-1β and IL-8 (Schindler et al., 2003). This article showed that only the human blood released IL-1β, while the rabbit blood

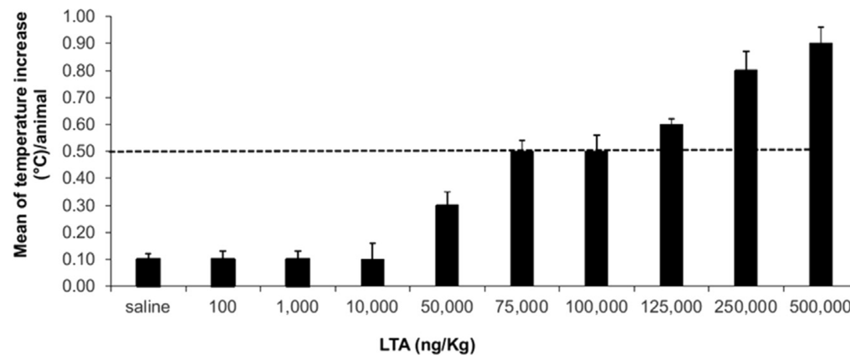

Fig. 2. Dose-response curve of *S. aureus* Lipoteichoic Acid by the RPT. Results are expressed as the mean of temperature increase (°C)/animal ± standard error. Animals received doses of 100; 1000 and 10,000 (n = 3); 50,000; 75,000; 100,000; 125,000; 250,000 and 500,000 ng/mL, and control group (n = 8) in the marginal ear vein. The dashed line represents the cut off temperature of 0.5 °C, corresponding to a fever.

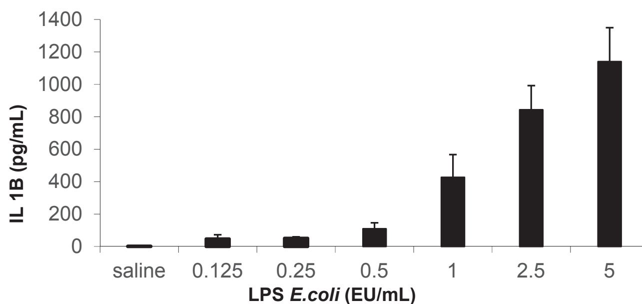

Fig. 3. IL-1β release from different concentrations of LPS from *E. coli* O55:B5 in cryopreserved blood. Blood pooling the cryopreserved samples was used. There was a difference in the release of IL-1β concentration dependent LPS. Means ± SE of four independent experiments in duplicate.

Table 1
Results presented by the Rabbit Pyrogen Test and Monocyte Activation Test to cryopreserved blood/IL-1β for evaluation of lipoteichoic acid of *S. aureus*.

LTA (ng/mL)	Rabbit pyrogen test (mean ΔT) (°C)*	MAT (IL-1β) EEU/mL
100	0.1 ± 0.03[NP]	0.0[NP]
1000	0.1 ± 0.03[NP]	0.6[NP]
10,000	0.1 ± 0.06[NP]	2.26[NP]
50,000	0.3 ± 0.05[NP]	5.41[P]
75,000	0.5 ± 0.04[P]	5.84[P]
100,000	0.5 ± 0.06[P]	6.27[P]
125,000	0.6 ± 0.02[P]	7.33[P]
250,000	0.8 ± 0.07[P]	10.79[P]
500,000	0.9 ± 0.06[P]	13.69[P]

NP = Non-Pyrogenic, P = Pyrogenic, *mean ± SE, LTA = Lipoteichoic acid, MAT = Monocyte activation test, EEU = Endotoxin Equivalent Units.

showed a lower reactivity than the human blood for the IL-8. Our results showed that the fever response for LTA in rabbit was comparable to the MAT. Complementary to Schindler's findings, we demonstrated that MAT was more sensitive than the RPT.

Earlier, the MAT detected the LTA pyrogenic concentration (50,000 ng/mL) corresponding to 5.41 EEU/mL that was determined as the threshold concentration for the *in vitro* test in our study. By analyzing this result, we can infer that the MAT is more sensitive than the test in rabbits. This difference may be related to the variability of response of animals that are intrinsic to the animal itself and/or the environmental factors (Williams, 2007; Hasiwa et al., 2007).

One of the advantages of the MAT is that it does not require the cell isolation procedures, thus it reduces the possible sources of contamination (Daneshian et al., 2009). Another important point is that the MAT has the same biological mechanism that is responsible for the reaction of the pyrogen-induced fever in humans without making the extrapolation between the species (ICCVAM, 2008).

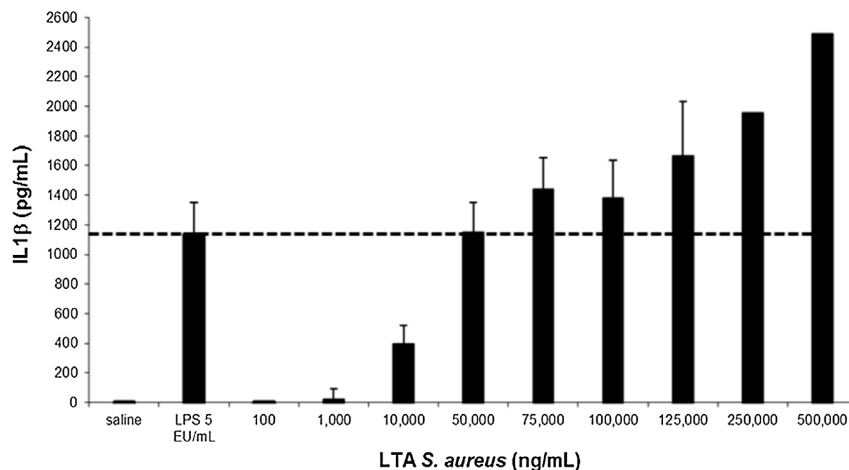

Fig. 4. IL-1β release to different concentrations of LTA from *S. aureus* by ELISA in cryopreserved blood. Blood pooling the cryopreserved samples was used. Mean of four independent experiments in duplicate and standard error. The dashed line represents the cut off in the concentration of 5 EU/mL.

Some studies have discussed that the MAT applicability has not been widely used because this assay needs a large amount of blood as in the case of the pharmaceutical industry. Recently, Wunderlich et al. (2014) showed other mononuclear cell sources for MAT from the bovine blood. They proposed that the use of the bovine assay avoids possible contaminations. Furthermore, the genetic factors and problems that related to the lifestyle of the human donor differed significantly between the donors. Whereas, the animals can be housed under the standardized pathogen-free conditions. However, results showed that bovine blood was less reactive and sensitive compared to the human blood mainly for the evaluated NEPs. Moreover, the use of bovine blood goes against the 3Rs concept and brings back the problem of data extrapolation between the species, as well as the RPT.

This study is intended to fill in the gaps of correlation between the RPT and MAT responses by showing that the MAT detects LTA in smaller amounts than the RPT. Complementary studies should be performed with the use of other NEP in MAT that have to undergo a product specific validation because the LAL does not detect this type of contaminant and the *in vivo* test presents ethical and methodological limitations.

The MAT may become the replacement for the RPT. Thus, the institutions that work with the quality control of products in Brazil, as INCQS, and worldwide will be able to reduce the number of rabbits used (Freitas, 2008; ICCVAM, 2008). The high sensitivity of MAT is an important point of view when it is related to the safety assessment of the injectable products that were subjected to the sanitary surveillance.

5. Conclusions

We conclude that the LTA limit dose, for the 0.5 °C cut-off, which causes fever in animals was 75,000 ng/mL, while the limit concentration for MAT was established at 50,000 ng/mL or 5.41 EEU/mL. These results suggest that the MAT can be a full replacement for the RPT. Complementary studies that compare the two methods should be carried out for other pyrogenic substances and also demonstrate the applicability for other injectable products contaminated by LTA.

Conflicts of interest

The authors declare that they have no conflict of interest.

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