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Original

Influence of endotoxin in blood collection tubes on the results of the tuberculosis diagnosis test QuantiFERON-TB Gold

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Abstract

QuantiFERON®-TB Gold (QFT-G) is an in vitro interferon (IFN)-γ release assay for the specific diagnosis of *Mycobacterium tuberculosis* (TB) infection. Unfortunately, QFT-G is susceptible to false-positive results. To investigate the influence of endotoxin contamination on QFT-G test results, we measured the endotoxin content in blood collection tubes using an extremely sensitive endotoxin-specific limulus test: the turbidimetric kinetic assay. We found that endotoxin content varied between the 5 examined lots. In recently recalled TB antigen tubes, 40 pg of endotoxin per tube was found. When polymyxin B (PMB) was added to the three types of tubes before blood collection, IFN-γ production was significantly diminished in the QFT-G assay. In two of the 16 subjects, the results changed from "positive" to "negative" or from "positive" to "conditionally positive." In one subject, the result changed from "conditionally positive" to "negative." Very small amounts of lipopolysaccharide, 1 pg/ml or less, significantly induced the production of IFN-γ in whole blood cultures of samples obtained from 3 out of 7 people, and production was inhibited by PMB. We propose that the endotoxin content in blood collection tubes should be less than 1 pg (0.007 endotoxin units; EU)/ml.

Key words : *QuantiFERON, tuberculosis, interferon-γ release assay, endotoxin contamination, false-positive reaction*

I. Introduction

A new in vitro test, QuantiFERON®-TB Gold (QFT-G; Cellestis Ltd., Carnegie, Victoria, Australia), has been used as an aid for the diagnosis of *Mycobacterium tuberculosis* (TB) infection[1-5]. In this test, heparinized whole blood from a sensitized person is incubated with mixtures of synthetic *M. tuberculosis* peptides for 24 h, and interferon (IFN)-γ production in the supernatant (plasma) is detected using an enzyme-linked immunosorbent assay (ELISA). Three proteins present in *M. tuberculosis*, ESAT-6, CFP-10, and TB 7.7, are used as stimulating antigens. These proteins are absent from all Bacille Calmette–Guérin (BCG) vaccine strains and from commonly encountered nontuberculous mycobacteria, with the exception of *M. kansasii, M. szulgai*, and *M. marinum*. Thus, QFT-G is potentially more specific for *M.*

tuberculosis than the classical tuberculin skin reaction test. This is since the skin reaction test uses a tuberculin-purified protein derivative, which is found in BCG vaccine strains and many environmental mycobacteria, as the antigen.

However, QFT-G is reportedly susceptible to false-positive results through as-yet unidentified mechanisms[6]. On the other hand, Sekiya et al. reported the endotoxin contamination in blood collection tubes used for the QFT-G[7]. Endotoxin is a cell wall constituent of gram-negative bacilli consisting of a lipopolysaccharide (LPS), which induces a wide variety of biological activities such as lethality to animals, pyrogenicity, shock-inducing activity, and induction of cytokines and IFN by binding to Toll-like receptor-4 on inflammatory cells[8,9]. In this regard, we have reported a range of possible artifacts that can results from endotoxin contamination in collection tubes[10].

Recently, the QFT-G test was recalled because of customer complaints reporting a higher-than-expected rate of QFT-positive results. The manufacturer reported that the abnormality had likely resulted from contamination of the QFT TB antigen tubes with a low level of endotoxin[11].

In this study, we examined the endotoxin content in blood collection tubes from 5 lots and investigated the influence of endotoxin contamination on the test results. Based on our findings, we propose an acceptable endotoxin level for blood collection tubes.

II. Materials and Methods

1. Materials

1) Distilled water, physiological saline, and phosphate buffered saline(PBS): Endotoxin-free distilled water or physiological saline for injection (Otsuka Pharmaceutical, Naruto, Japan) was used after confirming that gelation did not occur even when the sample was incubated for the maximum measurement time (200 min, approximately 0.05 pg/ml or lower) using the turbidimetric kinetic assay method described below. Endotoxin-free PBS was also used. PBS was autoclaved at 121°C for 90 min in order to remove endotoxin.

2) Endotoxin-free tips: Endotoxin-free tips (BioClean tips; Wako Pure Chemical Industries, Ltd, Osaka, Japan.) were used to handle the samples.

3) Limulus amebocyte lysate (LAL) reagent: A lyophilized LAL from American horseshoe crab (Endotoxin Single Test Wako; Wako Pure Chemical Industries) that was designed specifically for endotoxin detection by the addition of an excess amount of β-D-glucan was used[12].

4) LPS: *Escherichia coli* (*E. coli*) O111: B4-derived LPS (Sigma-Aldrich, St. Louis, MO) was suspended in PBS to a concentration of 1 μg/ml, dispensed in small volumes, and stored frozen at −80°C until use. The suspension was diluted immediately before use.

5) Polymyxin B (PMB): PMB was purchased from Sigma-Aldrich and was dissolved in PBS or physiological saline.

6) Anti-factor C monoclonal antibody: The monoclonal antibody (clone No. T8617A; Institute of Immunology Co., Ltd., Tokyo, Japan) was obtained using splenic cells from mice administered with a complex of purified factor C and LPS (*E. coli* O111: B4)[13].

7) Endotoxin extracting solution: The solution (Wako Pure Chemical Industries)

Table 1. Criteria for interpretation of QFT-3G results[15)]

Positive Control (M)*	QFT value (A)**	Assessment	Explanation
Any	≧ 0.35***	Positive	Suspect TB infection
≧ 0.5	≧ 0.1 and <0.35	Conditionally positive	Overall assessment considering degree of risk of infection
≧ 0.5	<0.1	Negative	Not infected with TB
<0.5	<0.35	Indeterminate	Cannot be interpreted, possible immune deficiency

*Positive Control (M): (mitogen control−nil control)
**QFT value (A): (TB antigen−nil control)
***IU/ml

contains human serum albumin (HSA), which can remove endotoxin that has adhered to glass or plastic walls[14)].

2. Methods

1) QFT-G assay: The assay was performed according to the manufacturer's instructions. This assay requires three collection tubes: nil (physiological saline) control, TB antigen, and mitogen control. An anti-coagulant, heparin, had been dried onto the inner wall of the collection tubes. TB antigens and mitogen (phytohemagglutinin) were also dried in the TB antigen tube and mitogen control tube, respectively. Briefly, 1 ml of blood was added to each of the 3 collection tubes and mixed thoroughly. Then, these tubes were incubated for 20 h at 37°C. The tubes were centrifuged at 3,000 rpm for 15 min, and the supernatant (plasma) was stored at −20°C until use. For this experiment, volunteers' blood was used following their informed consent.

IFN-γ content of the supernatant was measured by ELISA as follows. Anti IFN-γ monoclonal antibody conjugated with horseradish peroxidase was added to each well of a 96-well microplate, then 50 μl of the supernatant (plasma) or serially diluted standard IFN-γ was transferred into wells that had been previously coated with anti-IFN-γ monoclonal antibody. The plate was incubated for 2 h at room temperature and then washed six times with washing buffer. Then, a substrate solution (3,3′,5,5′-tetramethylbenzidine) was added to each well. After color generation, H_2SO_4 was added to stop the reaction, and the optical density (OD) of each well was measured using a microplate reader set at 450 nm. IFN-γ content (interferon unit; IU/ml) was calculated using a standard curve plotted according to the OD values of serially diluted standard preparations. Interpretation criteria and explanations for the diagnosis are shown in Table 1[15)].

2) Limulus test: We used the turbidimetric kinetic assay method using an endotoxin-specific LAL reagent (Wako Pure Chemical Industries)[12,16,17)]. The LAL reagent was dissolved with 200 μl of the sample and the test tube was placed in the measurement

Table 2a. Endotoxin contents in the three blood collection tubes from different lots

Product no. (expiry date)	Tubes		
	Nil control (lot. no.)	TB antigen (lot. no.)	Mitogen control (lot. no.)
TH-001 (2010-10)	1.1 ± 0.1 (A090707A)	0.4 ± 0.1 (A0907078)	45.4 ± 13.4 (A0907079)
T-005 (2010-02)	0.1 ± 0.1 (A091105C)	1.5 ± 0.1 (A091105A)	69.8 ± 13.0 (A091105B)
T-025 (2013-06)	15.1 ± 1.3 (A120302A)	3.4 ± 0.9 (A1203026)	1939.8 ± 341.2 (A1203028)

pg/tube, mean ± SD (n=4)

Table 2b. Endotoxin contents in the three tubes from recently sold QFT-G kits

Product no. (expiry date)	Tubes		
	Nil control (lot. no.)	TB antigen (lot. no.)	Mitogen control (lot. no.)
TH-026* # (2014-01)	0.5 ± 0.1 (A1210008)	40.1 ± 1.4 (A1210004)	3397.7 ± 110.4 (A1210006)
TH-028** (2014-07)	0.67 ± 0.2 (A1304012)	4.5 ± 0.1 (A1304011)	12670 ± 766.0 (A1304016)

pg/tube, mean ± SD (*n=3, **n=5); # This product was recalled in April 2013.

port of a Toxinometer ET-5500 (Wako Pure Chemical Industries, Ltd.). The transmittance at the start was set at 100%, and the gelation time was defined as the reaction time required for the percent transmittance of the reaction mixture to be reduced to 92% as a result of gel formation. We then calculated the amount of endotoxin in the sample based on a standard curve generated using standard LPS derived from *E. coli* O111: B4 (Wako Chemical Industries). One endotoxin unit (EU) of the standard LPS is equivalent to 0.127 ng of LPS (i.e., 1 pg LPS to 0.007 EU). Plasma endotoxin levels were measured by the turbidimetric kinetic assay using pretreated plasma with the 10-fold dillution and heating method to inactivate interfering factors for the test[17]. The minimum detection limit of this plasma endotoxin assay was about 0.4 pg/ml.

3) Statistical analysis: All the results are expressed as the mean±SD. The statistical significance of differences between the two groups was determined using Student's t-test. A value of $p<0.05$ was considered statistically significant.

III. Results

1. Endotoxin contents in three types of blood collection tubes

The three types of blood collection tubes (nil control, TB antigen, and mitogen control) were randomly selected from a packed box. One milliliter of water was added to the tube and was mixed vigorously; the quantity of endotoxin in the water was then measured using the limulus test. Based on a preliminary linearity test using serially diluted samples, the presence of inhibitory activity in an undiluted sample for the limulus test was suggested. Thus, the endotoxin content was measured in samples diluted 10-fold. In three products (Table 2a), endotoxin levels of 1–15 pg and 0.4–3.4 pg were detected in the nil control tubes and the TB antigen tubes, respectively. In the mitogen control tubes, endotoxin levels of 45–1900 pg were detected. Use of the endotoxin extracting solution did not significantly increase endotoxin contents in the solutions in comparison to contents of the tubes subjected to water extraction (data not shown). The incubation time, i.e., 30 min or overnight at room temperature, did not influence the endotoxin contents (data not shown). We performed inhibition experiments using PMB and an anti-factor C monoclonal antibody to confirm that the limulus activity was caused by endotoxin contamination. The limulus activity found in the nil control and TB antigen tubes of the three products was completely inhibited after the addition of PMB (endotoxin$<$0.03 pg/ml) or an anti-factor C monoclonal antibody (endotoxin$<$0.03 pg/ml). Activity in the mitogen control tube of a product from Lot No. T-005 was reduced to less than 10 pg/ml by addition of PMB or an anti-factor C monoclonal antibody.

The endotoxin levels in the tubes of recent lots are shown in Table 2b. The amount (40.1 pg) in the TB antigen tube of Lot No. A1210004 was higher than that of previous lots. Conversely, the endotoxin content of a new lot (No. A1304011) was lower than that of the previous lot.

2. Inhibition experiment involving the addition of PMB to the blood collection tubes

In order to investigate the effect of the contaminated endotoxin on IFN-γ production, endotoxin-neutralizing antibiotic PMB was added to the three types of collection tubes before the addition of blood. Blood from 16 volunteers and PMB were added to the blood collection tubes of Lot No. T-005, and the IFN-γ production levels were determined. This treatment significantly inhibited the IFN-γ production in (mitogen control) – (nil control) among 16 specimens (Fig. 1a) and showed a tendency to reduce the production in (TB antigen) – (nil control) (Fig. 1b). Considering the QFT-G results we obtained, it is clear that in the (TB antigen) – (nil control) values for specimens 7 (S7), 6 (S6), and 14 (S14), the results changed from "positive" (1.32 IU/ml) to "negative" (0 IU/ml), from "conditionally positive" (0.15 IU/ml) to "negative" (0 IU/ml), and from "positive" (0.35 IU/ml) to "conditionally positive" (0.32 IU/ml), respectively (Table 1). In S10, PMB treatment engendered a remarkable reduction in IFN-γ production, although this did not change the final classification of the sample.

3. IFN-γ production triggered by very small amounts of LPS and inhibition by PMB

We then determined whether small

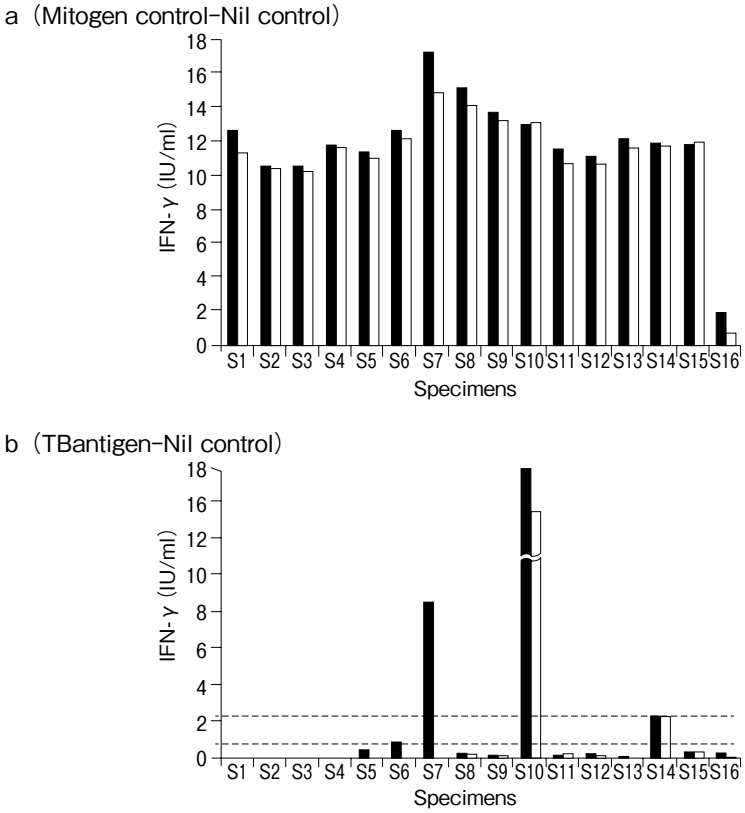

Fig. 1. Effect of PMB on IFN-γ production in the GFT-G assay.
This experiment was performed using tubes from Lot no. T-005. Two hundred microliters of PMB (10 μg/ml) or physiological saline was added, and then the test tube was held at an angle and rotated several times to ensure that the inner wall was washed with the solution to extract the endotoxin. This procedure was also performed to bind the endotoxin and PMB before binding of the endotoxin with leukocytes added subsequently to the tube. Blood specimens (S1-S16) obtained from 16 volunteers (13 males, age 24–63 y; 3 females, age 28–41y) were used in this experiment.
For (mitogen control) – (nil control), the average ± SD IFN-γ concentration in the absence of PMB was 12.06 ± 3.17 IU/ml, whereas that in the presence of PMB was 11.51 ± 3.11 IU/ml among the 16 specimens. PMB significantly reduced IFN-γ production ($p<0.05^*$) (Fig. 1a). For (TB antigen) – (nil control), PMB tended to reduce IFN-γ production (0.678 ± 2.20 vs. 0.506 ± 1.90 IU/ml, $p=0.063^*$) (Fig. 1b). *Student's t-test (paired, one-tailed).

doses of LPS are capable of inducing IFN-γ production in whole blood cultures. In 3 of 7 blood specimens, 0.1 pg/ml of LPS clearly induced the production of IFN-γ, as shown in Figure 2-a, b, c. In contrast, moderate- (Fig. 2-d, e), and low-responders (Fig. 2-f, g) to LPS were also found. In the presence of PMB, IFN-γ production was strongly attenuated.

IV. Discussion

We detected varying amount of endotoxin in the blood collection tubes in 5 examined lots of QFT-G kit (Table 2a, 2b). Endotoxin, i.e. LPS, is found in the environment and induces IFN-γ production in peripheral blood mononuclear cells, including monocytes and T-cells [17]. Therefore, endotoxin contamination

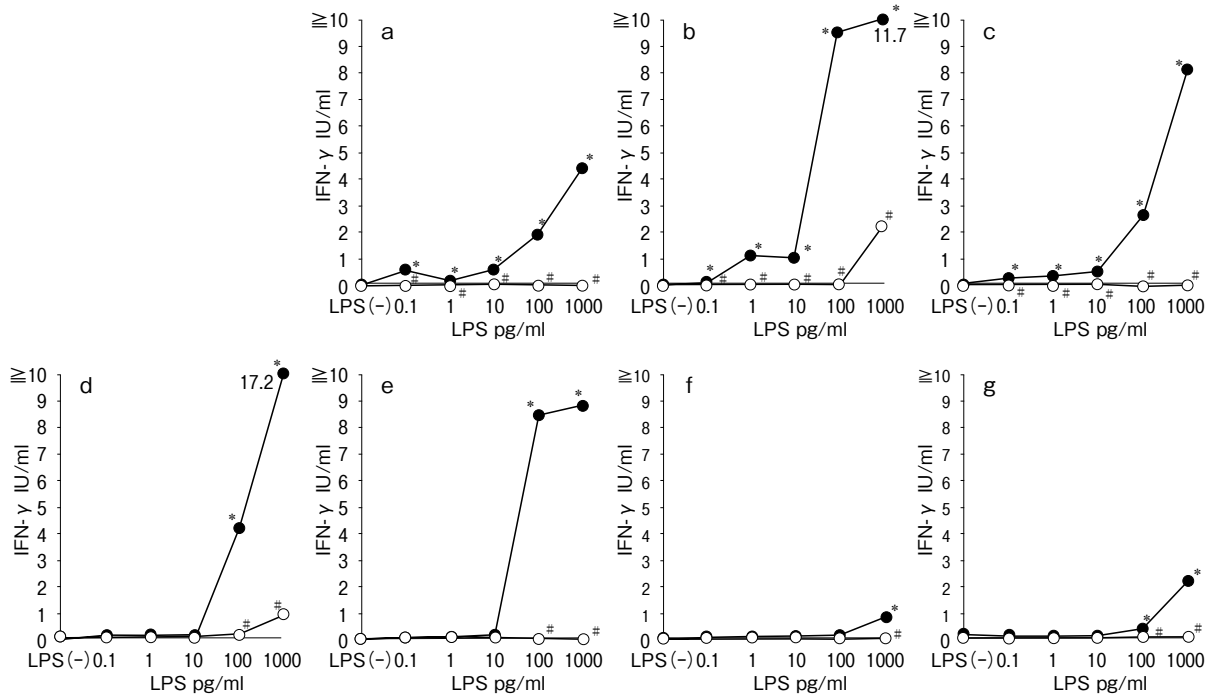

Fig. 2. Quantification of IFN-γ production in response to LPS added to blood samples in the presence or absence of PMB.

One milliliter of heparinized blood obtained from a volunteer was added to a 1.8-ml plastic tube (NUNC 375418, Roskilde, Denmark) and incubated for 20 h at 37℃ in the presence (○) or absence (●) of PMB (10 μg/ml). The supernatant was obtained by centrifugation and assayed for IFN-γ production using the ELISA kit provided with the QFT-G. Each value is the average from 3 assays. The endotoxin content in the plasma was measured using a turbidimetric kinetic assay. The plasma endotoxin content in all the blood samples used in this experiment was lower than the detection limit (0.4 pg/ml). The mean values obtained from the data for 3 wells in the ELISA experiment are shown. The SD values have not been depicted because they are small. *$p<0.05$, against the control value (without LPS); Student's t-test. #$p<0.05$, against the corresponding LPS concentration without PMB; Student's t-test.

in the collection tubes will lead to an artificial result of the QFT-G test. Sekiya et al. [7] first reported that 0.46 EU (65.8 pg) of endotoxin was detected in the TB antigen tubes, and this finding led to the recall of the QFT-G kit. We measured the endotoxin content in three lots of the kit that had been sold after the recall and found that the endotoxin content in the TB antigen tubes was improved to about 1 pg/tube or less (Table 2a). Recently, the problem of endotoxin contamination in the blood collection tubes was raised again and then reported to be improved[11]. In this study we confirmed endotoxin contamination (40.1 pg/tube) in the TB antigen tubes as the problem, and found that the content in the resold tubes had decreased to 4.5 g/tube (Table 2b).

The limulus activity in these tubes was clearly caused by endotoxin contamination, because the activity was inhibited by co-treatment with the endotoxin-neutralizing

antibiotic PMB[19,20] or the limulus cascade reaction inhibitor anti-factor C monoclonal antibody. The turbidimetric kinetic limulus assay, which is now used as a National Health Insurance-covered endotoxin assay, is more sensitive than the previously employed synthetic chromogenic substrate assay[21]. This assessment is based on the quantification of the gelation time in a Toxinometer. This endotoxin assay enables the detection of about 0.04 pg/ml of LPS when the reaction time is extended to 200 min, which is the maximum reaction time of the Toxinometer. In a limulus test, the reagents, test tubes, and pipette tips used to handle the samples and reagents should not be positive for endotoxin, especially when using a highly sensitive endotoxin assay system, and the handling of the reagents and specimens needs to be performed very carefully. The structural requirements for lipid A species in the activation of clotting enzyme from the horseshoe crab were investigated in detail by Takada et al.[22], and a strict structure of lipid A for the activation of the limulus cascade has been elucidated.

It is important to know whether very small amounts of endotoxin are capable of influencing IFN-γ production in the blood. Even 0.1 pg/ml of LPS was found to induce the production of IFN in blood samples from 3 of 7 individuals (Fig. 2). IFN production was due to the presence of LPS, since it was completely inhibited by co-treatment with PMB. We found that LPS-induced IFN-γ production was dependent upon the responsiveness of the donor's blood. The donor dependency of the LPS response has been reported by Mattern et al.[23]; approximately 50% of the tested blood samples were responders when monocyte-dependent T lymphocyte proliferation in response to LPS was examined. Therefore, false-positive reactions due to the presence of a small amount of LPS might have occurred in the LPS high-responder individuals.

To investigate the ability of PMB to minimize the false-positive rate, the antibiotic was added to the collection tubes prior to blood collection, and its effect on the results was observed. A significant reduction of IFN-γ production by PMB was observed in the mitogen control tubes (Fig. 1a). The boundary value is only 0.50 IU of the (mitogen control) – (nil control) value (Table 1). A value of less than 0.5 IU is not interpretable due to the low mitogenic response of the T cells, and, thus, the result is regarded as "indeterminate." However, there are likely to be some cases in which the monocyte response of IFN-γ production to endotoxin[18] is intact, even if the T cell response is diminished. In these cases, IFN-γ will be produced by the endotoxin that has contaminated the tubes, thus causing a false-positive test result. The IFN-γ −inducing ability of T lymphocyte mitogens is reportedly enhanced by the presence of LPS[24,25]. Therefore, the production of IFN-γ in mitogen tubes is susceptible to contamination with LPS. We would like to emphasize that endotoxin contamination in the mitogen control tubes is likely responsible for exceeding the critical value of 0.5 IU used to define an "indeterminate" decision as a result of the low response to mitogen.

PMB also diminished IFN-γ production in some blood specimens in TB antigen tubes and the results changed in 3 (S6, S7, and S14) of the 16 blood specimens (Fig. 1b). In this

assay, the boundary value that determines positivity or negativity is only 0.35 IU of the (TB antigen) – (nil control) value. Thus, these results suggest that the contaminating endotoxin may interfere with the results of the QFT-G.

Since endotoxin is strongly pyrogenic, the maximum permissible endotoxin level for injectable drugs is set at 5 EU/kg (2 EU/ml for intrathecal drugs) by the U.S., European, and Japanese Pharmacopoeias[26]. One EU represents an originally ruled value regarding 1 ng of standard LPS preparation. In the endotoxin assay kit of Wako Pure Chemical Industries, 1 pg of *E. coli* O111: B4 LPS is equivalent to 0.007 EU. Thus, 5 EU of LPS is calculated to be 715 pg. This raises the issue of how much LPS contamination is deemed "allowable" in *ex vivo* blood cultures. Based on our current findings, we propose that the endotoxin content in QFT-G tubes should be less than 1 pg/ml (0.007 EU/ml).

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Conflict of Interest: None to declare

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結核診断法クオンティフェロン TB －ゴールド（QFT-G）採血管における エンドトキシン汚染の影響について

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要旨

結核診断法クオンティフェロン TB －ゴールド（QFT-G）は結核抗原と血液の培養によりインターフェロン - γ（IFN- γ）産生を測定し判定をする．ここでは QFT-G の陰性，陽性，結核抗原の採血管のエンドトキシン（ET）汚染について検討した．1) 比濁時間分析法を用いた ET 測定で，リコールされたロットの結核抗原の採血管に約 40pg の ET が検出された．その後のロットでは数 pg であった．T 細胞マイトジェンが含まれる陽性採血管では，調べた 5 ロットいずれも ET は高値で発売順に増加していた．2) それらの採血管に予めポリミキシン B を添加し血液培養し非添加と比較したところ，16 名中 3 名で陽性から陰性，陽性から判定保留，さらに判定保留から陰性へと判定が変わった．3) 7 名の血液に各種濃度の LPS を加え培養したところ，3 名で微量の LPS（0.1-1pg/ml）により IFN- γ が有意に産生され，ポリミキシン B により阻害された．以上から採血管の非常に微量のエンドトキシン汚染が結果の判定に影響することが示唆された．