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The Tuberculin Skin Test Increases the Responses Measured by T Cell Interferon-Gamma Release Assays

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Abstract

RUTI is a vaccine consisting of *Mycobacterium tuberculosis* bacilli grown in stress conditions that is fragmented, detoxified and liposomed. RUTI was designed to shorten the treatment of latent tuberculosis infection (LTBI) with isoniazid from 9 months to just 1 month, by additional treatment with two inoculations of RUTI 4 weeks apart. During the validation process for monitoring the immunogenicity of administration of RUTI in a Phase I clinical trial, the question arose whether to introduce the tuberculin skin test (TST) in the screening of non-LTBI volunteers. This study was designed to evaluate the effect of TST on subsequent different T-cell interferon-gamma release assay (TIGRA) responses, using a spectrum of *M. tuberculosis*-related antigens (ESAT-6, CFP-10, 16 kDa, 19 kDa, MPT64, Ag 85B, 38 kDa, hsp65, PPD and BCG). The results showed an increase in post-TST response even in non-LTBI subjects for most antigens tested, as measured both by whole blood assay (WBA) and ELISPOT. Increased ELISPOT response decreased toward pre-TST levels within 1 month whereas the WBA response did not. Taking into account that there is no definitive correlation between TST and TIGRA tests to diagnose LTBI and the feasibility that TST might alter the immune monitoring included in clinical trials, these data suggest that TST determination should be carefully planned to avoid any interference with TIGRA.

Introduction

Over one-third of population already has latent tuberculosis infection (LTBI), which is a huge reservoir for maintaining tuberculosis (TB); as many as 10% of people latently infected will develop the disease [1]. One of the main difficulties in reducing this reservoir is that the current gold-standard treatment against LTBI requires the administration of isoniazid for 9 months [2]. However, this strategy is hardly used because it is associated with problems of logistics and compliance. Only the United States has a strong commitment to promoting this strategy, with a clear policy for elimination of TB [3].

Until recently, the tuberculin skin test (TST) has been the only test available to support LTBI diagnosis. However, this test has many known limitations: difficulties in administration and interpretation of results; need for a second-step visit; final results may be affected by prior BCG vaccination or by non-tuberculous mycobacteria (NTM) infection, and low sensitivity both in children and immunocompromised patients. However, despite not being an excellent gold standard, TST is widely used because of its low cost, its high accessibility, large amount of available historical data and, for a long time, the lack of a better method to detect LTBI.

Tuberculin skin test diagnosis is based on the intradermal inoculation of a protein extract, the purified protein derivative (PPD), also known as tuberculin, to trigger a local delayed-type hypersensitivity response. In people with LTBI, the presentation of this antigen by local macrophages to the regional lymph nodes is assumed to induce the proliferation of specific T lymphocytes, IFN-gamma (IFN-γ) producers that accumulate at the site of inoculation and cause local induration. The base of LTBI diagnosis consists of reading the diameter of this induration [4].

The value of this diameter should be correlated with a specific population; the diameter generally changes according to the BCG vaccination policy and the incidence of NTM infection [5]. A positive result is accepted

with a diameter of ≥5 mm (in BCG non-vaccinated subjects) and ≥10 mm (in BCG-vaccinated subjects) after the inoculation of 2 TU (0.04 μg) of PPD batch RT 23 (Statens Serum Institute), according to the Spanish guidelines [6]. However, slight differences should also be considered based on other criteria, such as the HIV status [7].

The correlation between TST and an '*in vitro*' assay using peripheral blood is controversial. Originally, TST was well correlated with '*in vitro*' lymphoproliferation after PPD stimulation [8]. Moreover, the finding of the ESAT-6 antigenic complex in *M. tuberculosis*, deleted in BCG, led to its use in LTBI diagnosis to avoid cross-reaction with BCG-vaccinated people, although this precaution may be unnecessary for subjects inoculated as infants tested 10 years after vaccination [9]. The possibility of cross-reactivity was the base for developing two marketed standardized methods, using both the detection of IFN-γ forming cells by ELISPOT from peripheral blood mononuclear cells (PBMCs) and the concentration of this cytokine in whole blood (WBA), after stimulation with different ESAT-6 complex antigens [10]. These methods are included in the tests known as TIGRA (T-cell interferon gamma release assays).

Interestingly, little is known about the interaction between TST and the subsequent use of TIGRA, although there seems to be a neutral effect since some national guidelines have proposed their combined use in a serial manner: first TST and then TIGRA to confirm positive cases [11, 12].

RUTI is a therapeutic vaccine designed to reduce the chemotherapy treatment of latent tuberculosis infection (LTBI) from 9 months to just 1 month [13–15]. During the validation process for monitoring the immunogenicity of administration of RUTI in a Phase I clinical trial the question arose whether to introduce the tuberculin skin test (TST) in the screening of non-LTBI volunteers, as used in previous TB vaccine clinical trials [16, 17]. Our aim was to try to establish the effects of TST on the baseline immune response before the administration of RUTI. With this purpose, we assessed the effect of TST on subsequent TIGRA responses to different *M. tuberculosis* antigens and stimuli, monitoring the evolution of responses during a 4-week follow-up period.

The data provided demonstrate that TST effectively increases the TIGRA responses *in vitro*, thus highlighting that this test is not neutral. An important implication of this finding is that the use of TST must be carefully considered during the development of new immunotherapeutic assays against LTBI.

Material and methods

Subjects. Every year, a TST is performed on everyone working at our Unit as part of TB infection control. During this year's TB test control, a total of nine people from our investigation team were recruited as volunteers for this study. Five of them were known to be latently TB infected according to the Spanish guidelines [6], whereas the remaining volunteers were not. Table 1 shows the clinical characteristics of each volunteer. Oral and written informed consent was obtained from all study subjects.

TST. The TST was performed by inoculating intradermally 2 TU of PPD RT-23 (Statens Serum Institute, Copenhagen, Denmark) in the volar side of the forearm. Results were read at 72 h by a trained physician of the hospital, following standard procedures. A positive result was defined as a diameter greater than 5 mm, and 10 mm in volunteers previously vaccinated with BCG [5].

Table 1 Clinical characteristics of each volunteer regarding *Mycobacterium* infection.

Volunteer	Age	BCG status	Latent tuberculosis infection	Tuberculin skin test (mm) at week 0	Clinical features and prophylactic therapy
Group I					
V1	28	−	−	0	
V2	29	−	−	0	
V3	29	−	−	0	NTM infected
V4	24	−	−	0	
Group II					
V5	40	−	+	0	9-month prophylactic treatment finished 9 years ago
V6	35	−	+	0	6 month prophylactic treatment finished 6 years ago
Group III					
V7	32	−	+	20	6-month prophylactic treatment finished 11 months before the study began.
V8	37	+	+	30	Accidentally exposed. Not prophylactically treated (because the volunteer had >35 years old and was pregnant at the time of diagnosis)
V9	29	−	+	60	Accidentally exposed. At the 8th of 9-month prophylactic treatment when the study began.

Blood collection for TIGRA. A total of 20 ml of whole blood was collected in three tubes: one 8-ml Cell Preparation Tube with Sodium (Citrate BD Vacutainer® CPT™, Plymouth, UK) to obtain fresh Peripheral Blood Mononuclear Cells (PBMCs), and two 6-ml sodium heparinized tubes (BD Vacutainer), on day 0 (w0), prior to TST, and at weeks one (w1), two (w2) and four (w4) post-test.

Stimuli. *Mycobacterium tuberculosis* antigens (ESAT-6, CFP-10, 16 kDa, 19 kDa, 38 kDa, Ag85B and hsp65), and PPD batch RT-50 (SSI, DK) were used as stimuli, at final concentration of 10 μg/ml for both TIGRA. PHA (Sigma-Aldrich, St Louis, MO, USA) was used as positive control.

The antigens used were provided directly by Lionex Diagnostics & Therapeutics GmbH (Braunschweig, Germany). Purity was always >95%, as measured by SDS-Page/densitometry and western blotting. Endotoxin content was always below the manufacturer' specifications.

A limited amount of available CFP-10 restricted the assays with this antigen to ELISPOT (not WBA): as the antigen amount needed for ELISPOT assays is less than the required for the WB stimulation (because of the final volume to be stimulated), only the ELISPOT assay could be performed.

The characteristics of all the antigens used are detailed in Table 2.

ELISPOT assay. A total of 250,000 fresh PBMCs were cultured per well (in a T-SPOT TB plate, T-SPOT TB; Oxford Immunotech, Oxfordshire, UK) in 150 μl of medium alone or medium plus stimulus. Cells were incubated at 37 °C in 5% CO_2 for 16 h; the ELISPOT assay was performed the day after, following the recommendations of the T-SPOT manufacturer. The results are represented by Spot Forming Units per 1×10^6 cultured PBMCs (Fig. 1) and calculated subtracting the mean number of spots in the medium and cells alone-control well from the mean counts of spots in wells-containing antigens, PPD or PHA. This method was used to obtain the specific responses and to avoid general immune activation.

Whole blood assay (WBA). The whole heparinized blood of each volunteer was cultured alone and with stimuli (1 ml of whole blood per well). It was not possible to stimulate whole blood with CFP-10, due to unavailability of sufficient antigen. The plates were incubated at 37 °C overnight and centrifuged at 600 *g* for 15 min the day after, to harvest about 200–300 μl of plasma from each well. The plasma samples were frozen at −20 °C for 5 weeks, after which they were analysed all at once for human IFN-γ using the marketed WBA-CMI ELISA (Cellestis Ltd., Carnegie, Australia), following the manufacturer's instructions. IFN-γ produced in non-stimulated wells was subtracted from each corresponding antigen-stimulated well, to obtain the specific responses and to avoid general immune activation.

The results are expressed as absolute IFN-γ values (IU/ml) (Fig. 2), as determined from a standard curve run on each plate, taking into account the sample dilution used (if required).

Statistical analysis. To determine if there was a linear relationship between the results of the two assays (ELISPOT and WBA), a linear regression test was used. A P-value <0.05 was considered statistically significant. Otherwise, the small sample size only allowed us to describe the results and no attempt at further statistical analysis was made.

Results

Non-LTBI, TST negative group (Group 1)

The results of the ELISPOT assay (Fig. 1) showed that no subjects responded to ESAT-6 or CFP-10 at w0. Response to ESAT-6 increased in all of them 1 week post-TST (up to 5–60-fold). A post-TST increase was also observed when stimulating with PPD and BCG (an increase of up to 20, and 5–50-fold for PPD and BCG, respectively), but not in Volunteer 3 (V3). When stimulating with the other *M. tuberculosis* antigens, the results showed a generalized increase at 1 week after TST, ranging from 5-fold to 80-fold, depending on the volunteer and the antigen used.

All volunteers experienced an increase in their IFN-γ response to most antigens, measured by the WBA

Table 2 Characteristics of the antigens used.

Antigen	*Mycobacterium tuberculosis* origin	RD1/others	Recombinant/natural	Proteins/peptides	Manufacturer
PPD	Yes	Others	Natural	Proteins	SSI
ESAT-6 (Rv3875)	Yes	RD-1	Recombinant	Proteins	Lionex
CFP-10 (Rv3874)	Yes	RD-1	Recombinant	Proteins	Lionex
16 kDa (Rv 2031c)	Yes	Others	Recombinant	Proteins	Lionex
19 kDa (Rv3763)	Yes	Others	Recombinant	Proteins	Lionex
Ag85B (Rv1886c)	Yes	Others	Recombinant	Proteins	Lionex
38 kDa (Rv 0934)	Yes	Others	Recombinant	Proteins	Lionex
Hsp65 (Rv 0440)	Yes	Others	Recombinant	Proteins	Lionex
BCG	No	Others	Natural		SSI

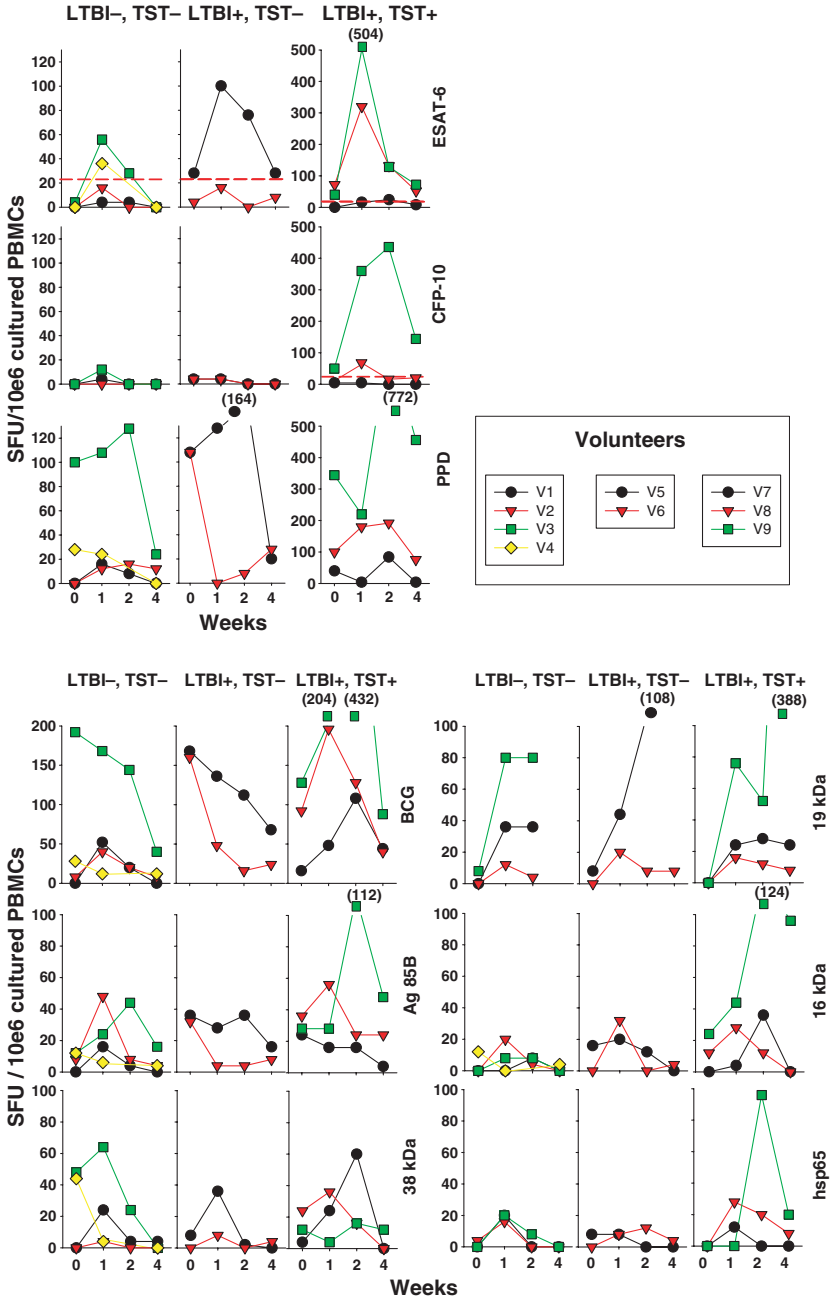

Figure 1 ELISPOT assay results from each volunteer represented by spot forming units per 10^6 cultured PBMCs stimulated overnight with ESAT-6, CFP-10, PPD, BCG, Ag85B, 38, 19, 16 kDa, and hsp65 *Mycobacterium tuberculosis* antigens. The dotted line in ESAT-6 and CFP-10 is for guidance and represents the threshold recommended by the marketed kit.

(Fig. 2). This response varied: for example ESAT-6 had 3–12-fold increase and Ag85B had 1-100-fold increase. The increase in IFN-γ was lower when stimulating with PPD, and there was no increase with BCG.

Previous LTBI, TST negative group (Group 2)

Both volunteers showed a post-TST increase measured by ELISPOT (up to 5–80-fold) when stimulating with ESAT-6, 38 kDa, 19 kDa and 16 kDa, and V5 also with PPD (2-fold increase). Oddly, the response decreased when stimulating with BCG and Ag85B. The WBA analysis showed an increased IFN-γ response (2- to 8-fold) in both volunteers, but a poor response with BCG. However, the response of Volunteer 5 (V5) was always higher than that of Volunteer 6 (V6) when assessed by both techniques.

LTBI, TST positive group (Group 3)

In this group, the volunteers showed an increase when stimulated with any of the antigens tested and assessed by both techniques at w1 and w2. This increase was up to 20-fold and 400-fold in ELISPOT (ESAT-6 and

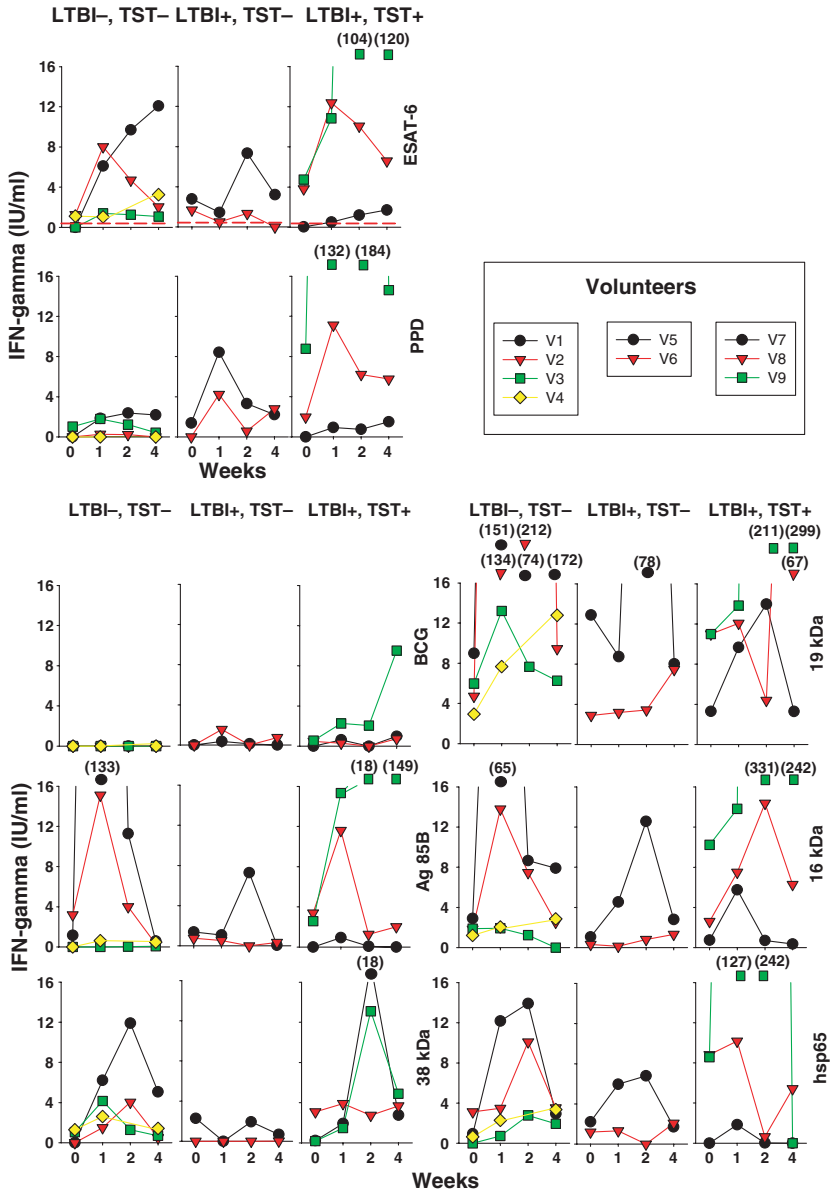

Figure 2 IFN-γ response in the Whole Blood Assay from each volunteer after overnight stimulation of whole blood with ESAT-6, PPD, BCG, Ag85B and 38, 19 and 16 kDa and hsp65 *Mycobacterium tuberculosis* antigens. The dotted line in ESAT-6 is for guidance and represents the threshold recommended by the marketed kit. The results are expressed as IFN-γ IU/ml.

19 kDa, respectively) and more than 25-fold in WBA (ESAT-6, Ag85B, 19 kDa and 16 kDa). Response in Volunteer 7 (V7) was small when compared with Volunteers 8 and 9 (V8, V9), who were infected more recently.

The CFP-10 response was only measured by ELISPOT. The results showed that response was generally really poor in both groups 1 and 2, but with individual exceptions: V8 increased from 0 to 70 SFU/10^6, and V9 increased by up to 20-fold at w1.

Correlation between ELISPOT and WBA

The correlation (R) between the two assays was statistically significant ($P < 0.001$) when the samples were stimulated with 16 kDa antigen, PPD or hsp65 ($R > 0.7$), as well as when 19 kDa or 38 kDa antigens were used ($R = 0.59$ and 0.36, respectively). No significant correlation was found when the samples were stimulated with the other antigens. All the results are shown in Fig. 3.

Discussion

The aim of this study was to determine whether TST might affect the monitoring of the immune response triggered by a TB vaccine and, in this particular case, by the use of the therapeutic vaccine RUTI against LTBI. It is noteworthy that the verification of the influence of new marketed tests to confirm LTBI diagnosis was not an objective of this study: although the methodologies for

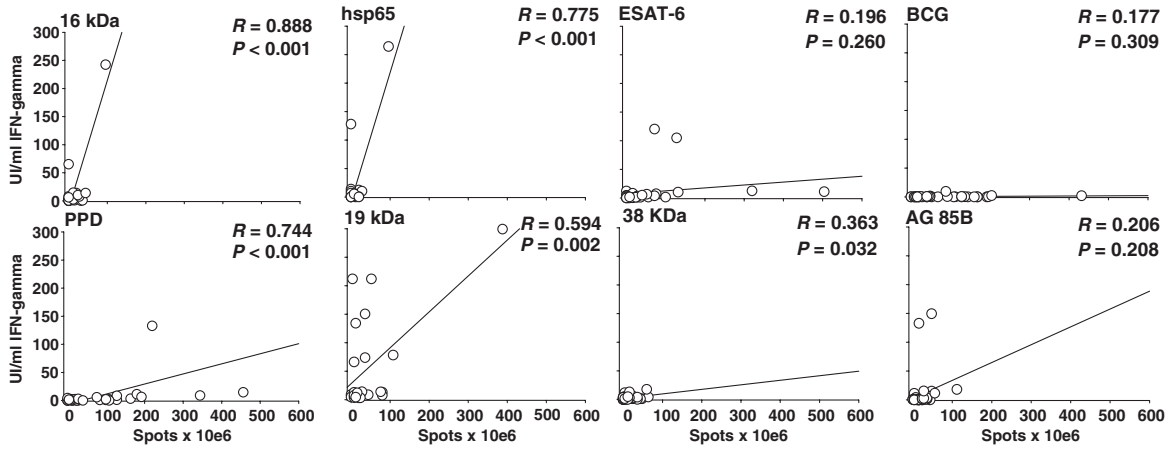

Figure 3 Correlation between the whole blood assay and the ELISPOT results. The results are expressed by each antigen, which was used to stimulate the samples. A linear regression test was used to perform the statistical analysis; the R and P of each analysis are shown.

both types of study may be very similar (e.g. some reagents and timings), the antigens were not the same. Therefore, we would like to clarify that the data we obtained from the TIGRA test must be interpreted carefully and within its own context, highlighting the need to specifically discern about this issue.

The data presented here show that a low antigenic concentration (particularly 0.04 μg of intradermal PPD) may enhance an immune response. At these amounts, an increased immune response in LTBI, TST-positive patients with expected specific T cell concentrations may be expected. Surprisingly, a slight increase in the immune response in non-LTBI, TST-negative patients was also observed. This observation may be related to the fact that TST positiveness is based on the assumption of determined population thresholds after the administration of a specific concentration of PPD [5], which cannot exclude the presence of specific T cells that remain undetected with this specific method.

The 'boosting effect' used in some populations with a low immune response and/or at high risk of LTBI (i.e. old people or health care providers, respectively) is well known. In these cases, the immune response is 'triggered' by repeating the TST 1 week after a negative TST recording. In our observation, those volunteers who were previously TST negative may have shown a similar 'boosting effect', when their peripheral blood was re-challenged with specific antigens at 1 week after the TST, a phenomenon that could be described as '*in vitro* boosting' of TST.

Besides, this boosting effect was also observed in other population-based studies that were not aiming to address this particular issue. Specifically, Mawa *et al.* [18] described this effect in their '*in vitro*' stimulation of diluted whole blood with CFP-10 *M. tuberculosis* antigen for 6 days. A similar effect was also described by Thom *et al.* [19] when measuring IFN-γ release after stimulating the infected whole blood of *Mycobacterium bovis* 1 week after the tuberculin testing.

Despite observing an increase in the immune response to most *M. tuberculosis* antigens used in this trial, there seems to be some differences between the two assays employed (ELISPOT and WBA). First, although the increase was observed in all volunteers with most antigens and by both techniques, the magnitude of increase was different for each subject. The reasons for such differences are unknown and we cannot explain them, appearing unrelated to any clinical issue.

Regarding the follow-up monitoring, the increased response observed by ELISPOT usually dropped at week 3 and returned to pre-stimulation levels by week 4; in contrast, the response observed by WBA still remained high until week 4 in some cases, or decreased more slowly.

Indeed, sometimes volunteers showing a higher increase by ELISPOT were different from those with a higher increase by WBA (see for example Group I, ESAT-6). This observation encouraged us to determine the correlation between the two assays. Using a linear regression test on the results, only in five out of the eight antigens used in both techniques showed a significant linear relationship between them and only in three of them R was >0.7. We attribute this apparent disparity to the fact that even though both techniques measure IFN-γ production, they are different: ELISPOT determines IFN-γ positive cells from a known number of PBMCs, whereas WBA shows IFN-γ release from a definite whole blood volume. In WBA, it is not known how many cells are secreting IFN-γ, whereas in ELISPOT, the number of cells is known, but their contribution to IFN-γ in whole blood is not.

Although the final concentration of the antigens used in the marketed kits (both T-SPOT TB and QTF-TB-Gold) is not disclosed, we decided to show in our figures the threshold recommended as a guidance. In half

of the healthy volunteers, the increase in response to ESAT-6 was high enough to surpass the T-SPOT TB threshold. The response to CFP-10 reached such threshold in no cases, but response was poor save in Group 3. When whole blood was stimulated with ESAT-6, WBA showed positive baseline values in V2 and V4 (1.25 and 1.12 IU/ml, respectively) with regard to the cut-off value recommended by the QTF-TB-Gold kit (the one used in the diagnosis of LTBI). However, the two volunteers were considered to be non-LTBI subjects according to their previous negative TST and their clinical history.

Volunteer 3 (Group 1) had high baseline values at w0 (ELISPOT: PPD, BCG, 38 kDa; WBA: PPD, 19 kDa and 16 kDa), but was negative for TST, and also had negative values for ESAT-6 (ELISPOT and WBA) and CFP-10 (ELISPOT, WBA was not done). According to the clinical history, the laboratory results and the physician's judgement, marketed T-SPOT and QuantiFeron-TB-Gold-In Tube Method tests were performed to this volunteer 3 months after the conclusion of the study. Both tests were negative, and thus the subject was considered to be NTM infected.

Our report has several limitations, such as the wide differences between the clinical issues of the volunteers, which were difficult to group; however, since volunteers were recruited at random, they may be quite representative of most of the characteristics that could be found in the population of health-care workers. Further, the foremost limitation of this study is its sample size. The small sample size only allowed us to describe the results but, even if no firm conclusion can be drawn because of it, with this paper we mean to raise attention to the need of more and better designed studies and clinical trials. Our results, although limited, suggest the need to explore in more detail the immunology of TB infection and its diagnosis, especially in light of new assays being developed and implemented in clinical practice together with TST, since previously unknown interactions with TST could influence diagnosis algorithms.

Although the data obtained from the studies carried out with TIGRAs to date have been promising – especially those comparing TIGRAs with TST– [20–26], several studies have highlighted the limitations of TIGRAs and have suggested that there is still a long way to go before these assays can be really useful in everyday clinical practice [23, 27–30].

Further studies should be conducted to establish the adequate strategy for diagnosing LTBI in high-risk populations and to determine the role of TIGRAs in that scenario; these studies should be done before making any adjustments to diagnostic practice. Consequently, several guidelines have recently been published in order to include TIGRAs in the diagnosis of LTBI for TB infection control. In December 2005, the US Centers for Disease Control and Prevention (CDC) included in their guidelines that the QuantiFERON TB Gold Test (QFT) assay approved by Food and Drug Administration (FDA) could replace TST in all circumstances in which the TST is currently used [31, 32].

In addition, UK guidelines by The National Institute for Health and Clinical Excellence [11] and Italian Guidelines [12] recommend a two-stage strategy for TST testing followed by a TIGRA to confirm a positive TST result. However, despite doubts on the matter [33], few studies have assessed how TST might affect TIGRA results [34, 35].

Recently, Leyten *et al.* [34] did not find any significant raise in IFN-γ responses on the QuantiFeron TB Gold results, 3 days after performing the TST, but they could not exclude a boosting effect after a longer interval. Although we did not use the antigens included in the marketed kits in our study, we observed an objective effect on TIGRAs that should at least encourage the review of current guidelines suggesting a double-step strategy to diagnose LTBI (in order to determine exactly when to conduct the second step after TST); we would also encourage additional studies to confirm and determine this effect in order to avoid it, especially using the marketed kits. It would also be desirable to further study the '*in vitro* boosting' phenomenon, due to its potential usefulness for detecting LTBI in immunodepressed people; further, whether a double-step strategy might be also used in TST-negative individuals, for confirmation in doubtful cases.

Finally, these data have convinced us to avoid the use of TST for the screening of healthy non-LTBI volunteers, at least in this initial RUTI Phase I clinical trial. The reason for our decision is that monitoring of RUTI responses requires measurement of IFN-γ production in peripheral blood; thus it seems reasonable to suppose that TST screening 1–2 weeks before RUTI inoculation could affect the immune response and hence render the interpretation of dose-response records to RUTI difficult.

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