CLINICAL AND VACCINE IMMUNOLOGY, July 2007, p. 880–885 Vol. 14, No. 7
1556-6811/07/$08.00+0 doi:10.1128/CVI.00132-07

Discrepancy between *Mycobacterium tuberculosis*-Specific Gamma Interferon Release Assays Using Short and Prolonged In Vitro Incubation▽

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Received 23 March 2007/Returned for modification 12 April 2007/Accepted 7 May 2007

The sensitivities of various gamma interferon release assays (IGRAs) for the detection of past latent *Mycobacterium tuberculosis* infection are not known. In this study, we aimed to assess the effects of various IGRA formats and in vitro incubation periods on test outcome. The results of the tuberculin skin test (TST) were compared with those of the QuantiFERON-TB Gold in-tube (QFT-GIT) test, an overnight enzyme-linked immunospot assay (ELISPOT), and a 6-day lymphocyte stimulation test (LST) by using the same *M. tuberculosis*-specific peptides and samples from 27 TST-positive persons with a history of exposure to *M. tuberculosis*, 4 patients cured of tuberculosis (TB), and 9 TST-negative controls. Among the TST-positive persons, the LST was more frequently positive (92%; $P < 0.01$) than either the QFT-GIT test (33%) or ELISPOT (46%). While good agreement was observed between the QFT-GIT test and ELISPOT ($\kappa = 0.71$) and between TST and LST ($\kappa = 0.78$), the agreement between TST or LST, on the one hand, and the QFT-GIT test or ELISPOT, on the other, was poor. These data indicate that the QFT-GIT test and overnight ELISPOT are less sensitive for the detection of past latent TB than the 6-day LST. The observed discrepancies between these IGRAs are most likely related to differences in incubation periods. Whether TST-positive persons with positive LST results but negative QFT-GIT and ELISPOT results are at risk for the development of TB needs to be elucidated before short-incubation IGRAs can be used for the screening of individuals for latent TB before immunosuppressive treatment.

In recent years several immunodiagnostic assays have been developed for the diagnosis of *Mycobacterium tuberculosis* infection. The high specificities of these new assays are their main advantage over the tuberculin skin test (TST), which has been used for the detection of *M. tuberculosis* infections for more than a century. TST is based on a delayed-type hypersensitivity response to purified protein derivative, a rough culture supernatant of *M. tuberculosis*; and false-positive results can occur due to cross-reactive immune responses to homologous proteins in *M. bovis* bacillus Calmette-Guérin (BCG) or environmental mycobacteria. The new gamma interferon (IFN-γ) release assays (IGRAs) have specifically been designed to overcome this problem of cross-reactive immune responses by measuring the immune response to antigens specific to *M. tuberculosis.* The availability of the complete genome sequence of *M. tuberculosis* and BCG led to the identification of several proteins which are specific for *M. tuberculosis* and which are absent from BCG and most environmental mycobacteria. Two such antigens, ESAT-6 (Rv3875) and CFP-10 (Rv3874), were first evaluated in a 6-day lymphocyte stimulation test (LST) and were found to be sensitive as well as specific for the diagnosis of tuberculosis (TB) (1, 21, 27, 32). Subsequently, other IGRAs were developed that differed from the classical LST with respect to the in vitro incubation period, the type of cells cultured (whole blood, frozen or fresh peripheral blood mononuclear cells [PBMCs]), and the way that the IFN-γ response is detected (by enzyme-linked immunosorbent assay [ELISA] or enzyme-linked immunospot assay [ELISPOT]).

The evaluation and comparison of new diagnostic assays for the detection of latent *M. tuberculosis* infections have been hampered by the lack of a "gold standard" and, therefore, the inability to reliably calculate their sensitivities and specificities. Most studies used the level of exposure as a surrogate marker for infection, and discrepancies between TST and IGRAs were mostly attributed to prior BCG vaccination (10, 18, 30). However, data from two of our recent studies indicate that this explanation may not account for all discrepant results, as a substantial group of BCG-unvaccinated persons with TST indurations of ≥15 mm had negative results by commercially available IGRAs, the QuantiFERON-TB Gold in-tube (QFT-GIT) test and/or the T-SPOT. TB test (Oxford Immunotec, Abingdon, United Kingdom) (2, 19).

In the present study we further evaluated the latter observation by comparing the performances of two short-incubation IGRAs, the QFT-GIT test and an in-house ELISPOT, with those of a "classic" 6-day LST and TST for the diagnosis of latent *M. tuberculosis* infection. As we aimed to assess the

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▽ Published ahead of print on 16 May 2007.

TABLE 1. Characteristics of study subjects

Characteristic	No. of subjects	Age (yr)		No. (%) of subjects with:		TST induration (mm)[a]		
		Mean	Range	BCG vaccination	INH Treatment	Mean	SE	Range
Cured of TB	4	51	36–63	0 (0)		21.5	3.9	18–27
TST+[b]	27	48	23–61	8 (30)	8 (30)	16.3	4.8	8–29
Contact investigation	14	51	39–60	2 (14)	4 (29)	16.1	3.3	10–24
Screening	13	45	23–61	6 (46)	4 (31)	15.5	6.8	8–29
TST negative	9	40	28–58	0 (6.6)		0		

[a] TST result during the study.
[b] TST+, persons with a documented TST induration of ≥10 mm in the past.

effects of various IGRA formats and in vitro incubation periods on test outcome, the same *M. tuberculosis*-specific peptides were used in all three IGRAs.

MATERIALS AND METHODS

Study subjects. In order to evaluate the effects of various characteristics on the performances of the assays, we aimed to include a heterogeneous group of persons with presumed recent or more remotely acquired latent TB infection, on the basis of documented TST conversion during contact investigations or screening of high-risk groups. Individuals who were known to have human immunodeficiency virus infection or who had received treatment with immunosuppressive drugs were not eligible for inclusion in the study. The subjects had been included in another study that compared the performance of QFT-GIT test with that of TST on the day of TST administration and on the day of TST reading (19). The present study included subjects whose PBMCs had been collected on the day of TST administration.

Study design. Participants underwent a TST on the day of blood sampling. Prior to TST, 2 ml of blood for the QFT-GIT test and 36 ml of heparinized blood were obtained. The PBMCs were isolated and stored in liquid nitrogen until use. The following data were collected by questionnaire: demographic data, medical history, BCG vaccination status, exposure to *M. tuberculosis*, the date and the results of a previous TST(s), and previous isoniazid (INH) prophylaxis or TB treatment. The study protocol (protocol P04-183) was approved by the Institutional Review Board of the Leiden University Medical Center. Oral and written informed consent was obtained from all study subjects.

TST. TST was performed by experienced personnel by standard procedures. In brief, 0.1 ml (2 tuberculin units) of purified protein derivative (RT23; Statens Serum Institute, Copenhagen, Denmark) was injected intradermally into the dorsal side of the left forearm. The transverse induration at the TST site was measured after 72 h.

QFT-GIT test. Blood samples were collected in two special tubes for the QFT-GIT test (Cellestis Ltd., Carnegie, Victoria, Australia). The in-tube version consisted of two heparinized 1-ml tubes, one coated with *M. tuberculosis*-specific peptides ESAT-6, CFP-10, and TB7.7 (Rv2654 [only peptide 4]) and one coated without antigen for use as a negative control. The tubes were incubated for 24 h at 37°C, followed by centrifugation and cold storage until they were tested as specified by the manufacturer. The concentration of IFN-γ in plasma was measured by using the commercial QFT-GIT ELISA. The test result was determined as negative or positive (a positive result was a concentration of ≥0.35 IU/ml) by using the software of the manufacturer.

ELISPOT for single-cell IFN-γ release. The IFN-γ-ELISPOT was performed as described previously (3). In short, frozen PBMCs were thawed and cultured at 2.5×10^5 per well in 200 μl of complete medium in 96-well ELISPOT plates; the plates were incubated for 18 h with pools of peptides spanning the complete sequences of ESAT-6, CFP-10, and TB7.7 (peptide 4) and a pool containing all peptides of the three antigens together, each at 10 μg/ml/peptide. Tests were performed in triplicate. ELISPOT plates were analyzed on a high-resolution image analyzer (Bioreader pro3000, Bio-Sys, GmbH, Germany). For analysis, the mean number of spot-forming cells (SFCs) per well was calculated from triplicate values for each antigen, and the mean number of SFCs for the negative control wells was subtracted. A positive test result was predefined as ≥5 SFCs per well and at least twice the background value. The ELISPOT result was determined as positive in case the response to one or more of the *M. tuberculosis*-specific peptide pools was positive.

Six-day LST. Frozen PBMCs were thawed and cultured (1.5×10^5/well) in complete medium in triplicate wells of 96-well round-bottomed microtiter plates at 37°C with 5% CO_2, as described previously (1), in the absence or the presence of peptides (the same pools and concentrations used for ELISPOT; see above). On day 6, the supernatants were harvested and the IFN-γ concentration was measured in duplicate by ELISA (U-CyTech, Utrecht, The Netherlands). The mean IFN-γ concentration in the unstimulated wells was subtracted from the mean concentration in the stimulated wells. The LST result was determined as positive in case the response to one or more of the *M. tuberculosis*-specific peptide pools was positive (IFN-γ concentration, ≥100 pg/ml).

Statistical analysis. The percentage of overall agreement between assays was calculated, and a Cohen's kappa value was used to assess the level of agreement. The results of the IGRAs were compared by using McNemar's test. IFN-γ responses were compared by the Mann-Whitney U test. A P value of <0.05 was considered statistically significant. SPSS 12.0 for Windows was used for the statistical analysis.

RESULTS

Study subjects. Forty healthy Dutch individuals participated in this study (Table 1). These included 27 persons with a documented TST induration of ≥10 mm (TST positive [TST+]), 4 patient cured of TB, and 9 TST-negative controls. Of the 27 TST+ individuals, 14 had a positive TST result after a known exposure to a case of smear-positive pulmonary TB, whereas another 13 were found to be TST+ during routine screening because of a profession-related increased risk of exposure to TB patients. The mean interval between a known exposure to *M. tuberculosis* and blood sampling was 5.5 years (standard deviation, 8.9 years; median, 3.8 years; range, 0.5 to 45 years). Only eight TST+ persons had been given INH prophylaxis. Also, only eight persons had previously been vaccinated with BCG. The four subjects who had been successfully treated for active TB had received their therapy 1.5 to 50 years before enrollment in the study.

For seven persons, insufficient numbers of PBMCs were available to perform both ELISPOT and LST. Therefore, ELISPOT was not done with three TST+ persons, whereas LST could not be performed with another three TST+ persons and one of the patients with TB.

Comparison of TST and IGRAs. TST was positive for all the treated TB patients; three had strongly positive QFT-GIT test and ELISPOT results, whereas in one individual who had had an active TB infection 48 years earlier, both the QFT-GIT test and ELISPOT were negative. All TB patients for whom assays were performed were positive by the LST, including the individual with negative results by both the QFT-GIT test and ELISPOT.

Among the TST+ persons with known exposure to *M. tuberculosis*, the TST induration during the study was ≥10 mm (mean induration, 16 mm) for all except two persons, who had TST indurations of 8 and 9 mm, respectively (Table 1). The

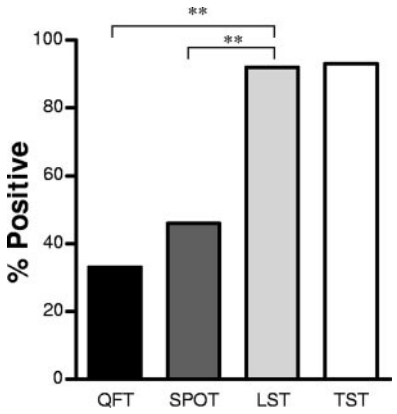

FIG. 1. Comparison of three IGRAs and TST for detection of latent *M. tuberculosis* infection. TST and the QFT-GIT test (QFT) were performed with samples from 27 TST+ persons with known exposure to *M. tuberculosis.* ELISPOT (SPOT) and a 6-day LST were done with samples from 24 TST+ persons by using the same *M. tuberculosis*-specific peptides (ESAT-6, CFP-10, and TB7.7) used for QFT-GIT test. Bars indicate the percentage of positive test results. The cutoffs for positive results were an IFN-γ concentration of 0.35 IU/ml for the QFT-GIT test, 5 SFC/well above the background for ELISPOT, an IFN-γ concentration of 100 pg/ml for LST, and ≥10 mm of induration for TST. **, $P < 0.01$.

QFT-GIT assay result was positive for 9 (33%) of these 27 TST+ individuals. The ELISPOT result was positive for 11 (46%) of the 24 TST+ persons assayed. By contrast, the 6-day LST was positive for 22 of the 24 (92%) individuals (Fig. 1).

All control subjects had a negative TST result. Among these subjects, the QFT-GIT test, LST, and ELISPOT results were also negative, with the exception of a positive ELISPOT result for one individual. In one other individual the LST result could not be interpreted due to a high background value.

Thus, a significantly higher percentage of TST+ individuals tested positive by LST than by the other assays with blood, the QFT-GIT test and ELISPOT ($P < 0.01$). No significant differences were observed between the QFT-GIT test and ELISPOT (Fig. 1). In an analysis that excluded BCG-vaccinated individuals, a significantly higher proportion of individuals were similarly positive by LST (15/17) than by either the QFT-GIT test (7/19; $P < 0.01$) or ELISPOT (9/16; $P = 0.03$). The same conclusion held true when those given INH prophylaxis were excluded from analysis ($P < 0.01$) (Table 2).

In summary, the results of all three IGRAs were concordantly positive for only 43% of TST+ persons. Thus, a positive LST result was accompanied by a negative QFT-GIT test or ELISPOT result for almost half (47%) of the TST+ persons.

TABLE 2. Effect of INH treatment on results of assays for diagnosis of latent *M. tuberculosis* infection[a]

INH treatment	No. of subjects	No. of subjects positive by the following test/total no. tested (%)			
		TST	QFT-GIT	ELISPOT[b]	LST
No	19	17/19 (95)	8/19 (42)	8/17 (47)	16/17 (94)
Yes	8	8/8 (100)	1/8 (12.5)	3/7 (43)	6/7 (86)

[a] In 27 persons with a documented positive TST result after a known exposure to TB.
[b] Of 24 subjects blood samples were available for ELISPOT and LST.

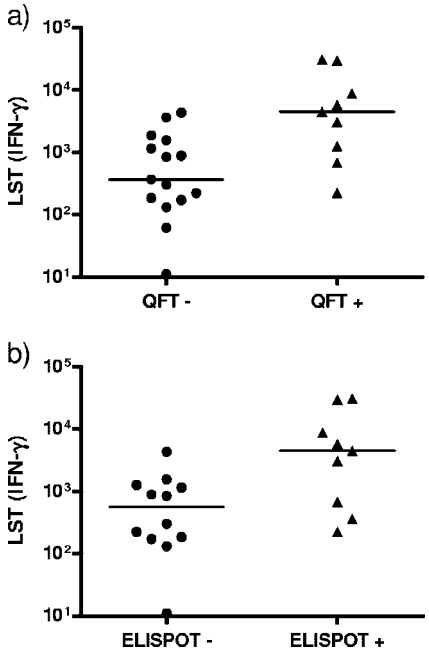

FIG. 2. LST responses in persons with negative versus positive results by the QFT-GIT test and ELISPOT. A 6-day LST was performed with pools of peptides of ESAT-6, CFP-10, and TB7.7. The LST responses are indicated as the highest level of production (in pg/ml) of IFN-γ to one of the *M. tuberculosis*-specific peptide pools. (a) LST responses in QFT-GIT test-negative (QFT-GIT−) and QFT-GIT test-positive (QFT-GIT+) persons (cutoff, IFN-γ ≥ 0.35 IU/ml); (b) LST responses in ELISPOT-negative (ELISPOT−) and ELISPOT-positive (ELISPOT+) persons (cutoff, 5 SFCs/well above the background). Lines indicate the median level of IFN-γ production.

To investigate whether the discrepancy between the LST results and the QFT-GIT test and ELISPOT results could be due to arbitrary differences in the IFN-γ cutoff levels, the LST responses were plotted for persons with negative and positive QFT-GIT test or ELISPOT results (Fig. 2). In this respect, the LST responses are depicted as the highest level of production of IFN-γ in response to one of the *M. tuberculosis*-specific peptide pools. Although the median LST response appeared to be higher in the group positive by the QFT-GIT test or ELISPOT than in the group negative by these assays, there was a large overlap between the two groups, with a substantial number of persons negative by the QFT-GIT test and ELISPOT still having high responses by LST (Fig. 2).

Although only eight TST+ individuals had received INH prophylaxis, we performed a subgroup analysis to evaluate the effect of such treatment on the IGRA results (Table 2). Although the group size became small, it appeared that the proportion of the INH-treated individuals with a positive QFT-GIT test result was lower (12.5%) than the proportion not treated with INH, despite the finding that 86% had a positive LST result ($P = 0.06$). The results of the other assays, i.e., TST, ELISPOT, and LST, were not significantly affected by prior INH treatment.

Agreement between TST and three IGRAs. Next, the overall agreement between the various IGRAs and TST was calculated

TABLE 3. Agreement between results of assays for diagnosis of latent *M. tuberculosis* infection[a]

Assay	Agreement with:					
	TST		QFT-GIT		ELISPOT	
	%[b]	κ[c]	%	κ	%	κ
QFT	58	0.28			86	0.71
QFT (BCG-unvaccinated subjects)	59	0.30				
ELISPOT	65	0.35	86	0.71		
ELISPOT (BCG-unvaccinated subjects)	69	0.40				
LST	91	0.78	60	0.31	59	0.27
LST (BCG-unvaccinated subjects)	93	0.84				

[a] For TST, $n = 40$; for QFT-GIT, $n = 40$; for ELISPOT (performed ex vivo), $n = 37$; for LST, $n = 36$. The in vitro assays were done with the peptides of ESAT-6, CFP-10, and TB7.7.

[b] Number of persons with a concordant positive or negative result divided by the total number tested.

[c] Level of agreement measured by Cohen's kappa (κ).

(Table 3). The level of agreement between the outcomes of the QFT-GIT test and TST was low, and this lack of similarity was not affected if BCG-vaccinated individuals were excluded from the analysis. The findings for the QFT-GIT test compared with those of TST did not differ from those when the outcome of ELISPOT was taken into account. By contrast, the outcomes of the 6-day LST showed excellent agreement (91%; $\kappa = 0.78$) with those of TST. In all, the level of agreement between the outcome of the QFT-GIT test and that of ELISPOT was high (86%, $\kappa = 0.71$), but the outcomes of these short-incubation IGRAs agreed with that of the 6-day LST for only 60% ($\kappa = 0.31$) and 59% ($\kappa = 0.27$) of the cases, respectively. Of note, adjustment of the cutoff levels of the QFT-GIT test and ELISPOT did not improve the level of agreement (data not shown).

DISCUSSION

In this study we compared the sensitivities of three *M. tuberculosis*-specific IGRAs for the diagnosis of latent TB and found a remarkable discrepancy between the outcomes of the two short-incubation IGRAs, i.e., the QFT-GIT test and ELISPOT, on the one hand, and LST with a prolonged, 6-day incubation and TST, on the other. Among TST+ individuals known to have been exposed to *M. tuberculosis* in the past, LST was positive significantly more often than either the QFT-GIT test or ELISPOT. Our findings indicate that that the short-incubation assays have limited sensitivities for the detection of past infection.

We performed all IGRAs using identical *M. tuberculosis*-specific peptides and repeated a TST at the same time. Furthermore, we chose to study a diverse group of persons documented to be positive by TST after exposure to *M. tuberculosis.* Some of these individuals were known to have been exposed to TB decades ago, and others were known to have been exposed more recently. Also, only a few persons had received prophylactic treatment for latent TB. The main limitation of this pilot study is the relatively small number of study subjects. Although a significant difference in sensitivity between LST and both ELISPOT and the QFT-GIT test could be observed, the study size was too small to correlate the observed discrepancy to factors such as the time that had elapsed since the *M. tuberculosis* infection had been acquired.

The agreement between the QFT-GIT test and ELISPOT was high, but the outcomes of these assays showed poor agreement with those of both TST and LST, assays whose results were highly concordant. About half the TST+ individuals had negative results by both the QFT-GIT test and ELISPOT, while most were positive by the 6-day LST. Of note, all three assays also measured the levels of IFN-γ production in response to the peptides of ESAT-6, CFP-10, and TB7.7, antigens that were found to be highly specific for *M. tuberculosis* (4, 24), when they were used to test a 6-day cell culture (1, 21, 27). Among the participants negative by the QFT-GIT test and ELISPOT, high levels of IFN-γ could be produced by LST, indicating that the observed discrepancy was not simply explained by differences in the levels of detection of IFN-γ. A plausible explanation for the difference in sensitivity would be the differences in the in vitro incubation periods for the QFT-GIT test and ELISPOT, on the one hand, and that for LST, on the other. We hypothesize that after 24 h incubation only circulating effector memory T cells have had sufficient time to produce IFN-γ, while central memory T cells first started producing IFN-γ after a more prolonged incubation. In individuals who have been infected with *M. tuberculosis* in the past, the number of circulating effector cells could be low, causing negative results in a short-incubation assay but positive responses after a prolonged incubation. In accordance with this line of thought are findings from a recent study of hepatitis C virus showing that short-term ELISPOT responses were not influenced by depletion of lymphotropic chemokine receptor 7-positive T cells, representing memory cells, while the depletion of these memory cells did decrease the antigen-specific responses after prolonged culture (13). Our findings suggest that prolonged incubation of the IGRAs, such as a 6-day LST, might be the most sensitive method for screening for latent *M. tuberculosis* infection in persons with an increased risk of the development of a reactivation of TB, such as those eligible for transplantation or treatment with tumor necrosis factor alpha antagonists (17). A recently published case of pulmonary TB in a liver transplant patient with a negative QFT-GIT test result before transplantation illustrates that the results of the QFT-GIT test must be interpreted with caution in this setting (8).

Although only a limited number of study subjects had been treated with INH, the data suggest that the QFT-GIT test results were more affected by prior INH treatment than were those of ELISPOT, LST, or TST. Three previous studies indicated there is a trend toward decreased ELISPOT responses at the end of treatment for latent TB (6, 11, 31). In Indian health care workers, the QFT-GIT test result remained positive after INH treatment, but these individuals continued to be exposed to cases of pulmonary TB (23). Further studies are needed to evaluate the kinetics of different IGRAs during treatment. Although INH treatment could have a differential effect on the results of IGRAs with different test formats, the observed discrepancy between IGRAs with a short incubation compared with those with a prolonged incubation remained when INH-treated individuals were excluded from analysis.

There is a lack of knowledge on the performance of IGRAs with a short incubation period compared to those of IGRAs

with a more prolonged incubation period in relation to the detection of *M. tuberculosis* infection. One study that compared the overnight ELISPOT and the 6-day LST reported that ELISPOT performs slightly better (28). While that finding is in contrast to our findings, the study included patients with active TB, while we studied TST+ persons with exposure to *M. tuberculosis* more remote in time. In another study, the overall agreement between ELISPOT and a 3-day-incubation whole-blood IGRA was good (29), but the 3 days of incubation for the IGRA could have been too short to reliably detect a memory response. In accordance with our data are the observations of negative responses to a panel of RD1 peptides in an overnight ELISPOT and positive responses in a cultured ELISPOT in three cured TB patients (14). Several studies compared one short-incubation IGRA with TST for the detection of latent *M. tuberculosis* infection (2, 5, 7, 9, 10, 12, 15, 16, 20, 22, 25, 30), but the levels of agreement between TST and the IGRA varied widely between studies. In line with the hypothesis that a short-incubation IGRA might have a lower sensitivity for the detection of past latent infection are the observations of several other studies (16, 20, 26). In two cross-sectional studies performed in South Africa, approximately one-third of adults with a TST induration of >15 mm had a negative QFT-GIT test result (20, 26) and 38% had a negative T-SPOT. TB test result (26). Another study noticed that in a mostly BCG-vaccinated Korean control population, 51% of the subjects were TST+ and only 4% were QFT-GIT assay positive, while the expected prevalence of *M. tuberculosis* infection was 33% (16).

In conclusion, a major discrepancy was observed between the results of two short-incubation IGRAs (the QFT-GIT test and an in-house ELISPOT) and those of a 6-day LST. This study raises the hypothesis that short-incubation IGRAs mainly detect recent or ongoing infection with *M. tuberculosis*, while prolonged-incubation IGRAs seem to be more sensitive for the diagnosis of past latent infection. Further studies are needed to confirm this hypothesis and evaluate the consequence of this hypothesis for the predictive value for the risk of TB.

ACKNOWLEDGMENTS

This work was partially supported by the European Commission.

We thank all volunteers who participated in this study, many of whom were employees of the KNCV Tuberculosis Foundation, The Hague, and of the Department of Infectious Diseases, Leiden University Medical Center, Leiden. Krista van Meijgaarden is gratefully acknowledged for her general help and useful advice in the laboratory.

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