Cellular Immune responses during latent tuberculosis : immunodiagnosis and correlates of protection

Leyten, E.M.S.

Chapter 6

Discrepancy between *Mycobacterium tuberculosis*-specific interferon-γ release assays using short versus prolonged *in vitro* incubation

Eliane M.S. Leyten[1,#], Sandra M Arend[1], Corine Prins[1], Frank G. J. Cobelens[3], Tom H.M. Ottenhoff[1,2], Jaap T. van Dissel[1]

Department of Infectious Diseases[1] and Department of Immunohematology and Blood Transfusion[2], Leiden University Medical Center, Leiden. KNCV tuberculosis Foundation, The Hague[3], The Netherlands # Present address: Department of internal medicine, Medisch Centrum Haaglanden, Lijnbaan 32, 2512 VA, the Hague, the Netherlands

ABSTRACT

The sensitivity of various interferon-gamma release assays (IGRA) for detection of past latent *Mycobacterium tuberculosis* infection is not known. In this study, we aimed to assess the effect of varying IGRA formats and *in vitro* incubation periods on test outcome.

The tuberculin skin test (TST) was compared with QuantiFERON®-TB-Gold in-tube (QFT-GIT), with an overnight-ELISPOT and with a 6-day lymphocyte stimulation test (LST), using the same *M. tuberculosis*-specific peptides, in 27 TST-positive person with a history of exposure to *M. tuberculosis*, 4 cured TB patients and 9 TST-negative controls.

Among TST-positive persons the LST was more frequently positive (92%; *P*<.01) than either QFT-GIT (33%) or ELISPOT (46%). While good agreement was observed between QFT-GIT and ELISPOT (κ=0.71) and between TST and LST (κ=0.78), agreement between TST or LST on the one hand and QFT-GIT or ELISPOT on the other was poor.

These data indicate that QFT-GIT and overnight-ELISPOT are less sensitive for the detection of past latent TB as compared to 6-day-LST. The observed discrepancies between these IGRA are most likely related to differences in incubation period. Whether TST-positive persons with positive LST, but negative QFT-GIT and ELISPOT results, are at risk to develop TB needs to be elucidated before short-incubation IGRA can be used for screening of latent TB in individuals before immunosuppressive treatment.

INTRODUCTION

In recent years several immunodiagnostic assays have been developed for the diagnosis of *Mycobacterium tuberculosis* infection. A high specificity is the main advantage of these new assays over the tuberculin skin test (TST), which has been used for the detection of *M. tuberculosis* infections for over a century. The TST is based on a delayed-type hypersensitivity response to purified protein derivative (PPD), 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 interferon-gamma (IFN-γ) release assays (IGRA) have been specifically 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 are absent in 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 TB disease (1, 21, 27, 32). Subsequently, other IGRA were developed that differed from the classical LST with respect to *in vitro* incubation period, type of cells cultured (whole blood, frozen or fresh peripheral blood mononuclear cells (PBMC)), and the way to detect the IFN-γ response (by ELISA or enzyme-linked immunospot assay (ELISPOT)).

Evaluation and comparison of new diagnostic assays for detection of latent *M. tuberculosis* infections has been hampered by the lack of a gold standard and therefore the inability to reliably calculate sensitivity and specificity. Most studies used the level of exposure as a surrogate marker for infection, and discrepancies between the TST and IGRA 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 of ≥ 15 mm had negative results in the commercially available IGRA, QuantiFERON-TB Gold in-tube (QFT-GIT) and/or T-SPOT™.*TB* (Oxford Immunotec, Oxford, UK) (2, 19).

In the present study we further evaluated the latter observation by comparing the performance of two short-incubation IGRA, QFT-GIT and an in-house ELISPOT, with a "classic" 6-day LST and the TST for the diagnosis of latent *M. tuberculosis* infection. As we aimed to assess the effect of varying IGRA formats and *in vitro* incubation periods on test outcome, the same *M. tuberculosis*-specific peptides were used in all three IFN-γ release assays.

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MATERIALS AND METHODS

Study subjects

In order to evaluate various characteristics on the performance of the assays, we aimed to include a heterogeneous group of persons with presumed recent, or more remotely acquired, latent TB infection, as based on documented TST conversion during contact investigations or screening of high-risk groups. Individuals with a known HIV infection or treatment with immunosuppressive drugs were not eligible for inclusion. The subjects had been included in another study comparing performance of QFT-GIT on the day of TST administration and on the day of TST reading (19). The present study included subjects of whom PBMC had been collected on the day of TST administration.

Study design

Participants underwent a TST on the day of blood sampling. Prior to the TST, 2 mL of blood for QFT-GIT and 36mL of heparinized blood were obtained. PBMC 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*, date and results of previous TST(s), and use of isoniazid (INH) prophylaxis or TB treatment. The study 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.

Tuberculin skin test

The TST was performed by experienced personnel according to standard procedures. In brief, 0.1 mL (2 TU) of purified protein derivative (PPD, RT23; Statens Serum Institute, Copenhagen, Denmark) was injected intradermally into the dorsal side of the left forearm. Transverse induration at the TST site was measured after 72 hours.

Quantiferon-TB gold in-tube

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

Enzyme-linked immunospot assay for single-cell IFN-γ release

The IFN-γ-ELISPOT was performed as described (3). In short, frozen PBMC were thawed and cultured at 2.5×10^5 per well, in 200 µl of complete medium in 96-well ELISPOT plates and incubated for 18 hours with peptide pools spanning the complete sequence of ESAT-6, CFP-10 and TB7.7 (4) and a pool containing all peptides of the 3 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 (SFC) per well from triplicate values was calculated for each antigen and the mean number of SFC of the negative control wells was subtracted. A positive test result was predefined as ≥ 5 SFC 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 lymphocyte stimulation test (LST)

Frozen PBMC were thawed and cultured (1.5×10^5/well) in complete medium in triplicate wells in 96-well round-bottomed microtiter plates at 37°C and 5% CO_2 as previously described (1), in the absence or presence of peptides (the same pools and concentrations as used for ELISPOT; see above). At day 6, supernatants were harvested and IFN-γ concentration was measured in duplicate by ELISA (U-CyTech, Utrecht, The Netherlands). The mean IFN-γ concentration in unstimulated wells was subtracted from the mean concentration in 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 ≥ 100 pg/mL).

Statistical analysis

The percentage of overall agreement between assays was calculated and a Cohen's Kappa was used to assess the level of agreement. Results of IGRA were compared using a McNemar's test. IFN-γ responses were compared using a Mann-Whitney u test. A *P*-value <.05 was considered statistically significant. For the statistical analysis SPSS 12.0 for Windows was used.

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+), 4 cured TB patients and 9 TST-negative (TST-) controls. Of the 27 TST+ individuals, 14 had a positive TST

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Table 1. CHARACTERISTICS OF STUDY SUBJECTS [a]

	No.	Age		BCG		INH		TST [b]		
		mean	range	No.	(%)	No.	(%)	mean	SE	range
Cured TB	4	51	36-63	0	(0)	-	-	21.5	3.9	18-27
TST+ [c]	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 –	9	40	28-58	0	(6.6)	-	-	0	-	-

a TST, tuberculin skin test; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin vaccination; TB, tuberculosis
b TST result during the study.
c TST+, persons with a documented TST of ≥ 10 mm in the past.

after a known exposure to a case of smear-positive pulmonary TB, whereas another 13 were found to be TST positive during routine screening because of 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 year (±SD 8.9), median 3.8 year (range: 0.5-45 year). Only 8 TST+ persons had been given INH-prophylaxis. Also, only 8 were previously vaccinated with BCG. The 4 subjects who had been successfully treated for active TB had received their therapy 1.5 to 50 years before study enrolment.

In 7 persons there were insufficient numbers of PBMC available to perform both ELISPOT and LST. Therefore, ELISPOT was not done in 3 TST+ persons whereas LST could not be performed in another 3 TST+ persons and in one of the TB patients.

Comparison of TST and IGRA

In the treated TB-patients, the TST was positive in all; 3 had strongly positive QFT-GIT and ELISPOT results whereas in one individual who had suffered an active TB infection 48 years ago, both the QFT-GIT and ELISPOT were negative. All TB patients so assayed were positive in the LST, including the individual with negative results in both QFT-GIT and ELISPOT.

In TST+ persons with known exposure to *M. tuberculosis*, the TST during the study was ≥10 mm (mean value of 16 mm) with the exception of two persons who had a TST of 8 and 9 mm, respectively (Table 1). The QFT-GIT assay was positive in 9 (33%) of these 27 TST+ individuals. The ELISPOT result was positive in 11 (46%) of the 24 TST+ persons assayed. By contrast, the 6-day LST was positive in 22 out of the 24 (92%) individuals (Figure 1).
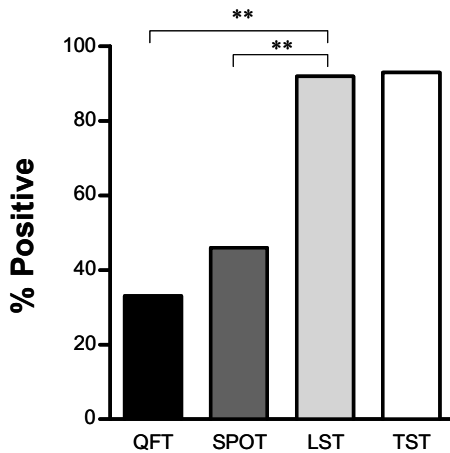
Figure 1. Comparison of three IFN-γ release assays and TST for detection of latent tuberculosis.
Tuberculin skin test (TST) and QuantiFERON TB Gold in-tube (QFT) were performed in 27 TST positive persons with known exposure to *M. tuberculosis.* ELISPOT (SPOT) and a 6-day lymphocyte stimulation test (LST) were done in 24 TST+ persons, using the same *M. tuberculosis*-specific peptides of ESAT-6, CFP-10 and TB7.7 as were used for the QFT-GIT. Bars indicate the percentage of positive test results. Cut-off for a positive result were respectively, IFN-γ of 0.35 IU/mL for QFT-GIT, 5 SFC/well above background for ELISPOT, IFN-γ of 100 pg/mL for LST and ≥ 10 mm induration for TST. **; *P* <.01.

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

Thus, in the LST a significantly higher percentage of TST+ individuals tested positive as compared with the other blood assays QFT-GIT and ELISPOT (*P*<.01). Between QFT-GIT and ELISPOT no significant difference were observed (Figure 1). In an analysis excluding, BCG-vaccinated individuals, similarly the LST was positive in a significantly higher proportion of individuals (15/17), than tested either by QFT-GIT (7/19; *P*<.01) or ELISPOT (9/16; *P*=.03). The same conclusion holds true when those given INH-prophylaxis were excluded from analysis (*P*<.01)(Table 2).

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

To investigate whether the discrepancy between LST and QFT-GIT and ELISPOT could be due to arbitrary differences in IFN-γ cut-off level, LST responses were plotted in persons with a negative versus those with a positive QFT-GIT or ELISPOT (Figure 2).

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Table 2. EFFECT OF ISONIAZID TREATMENT ON DIAGNOSTIC ASSAYS FOR LATENT TUBERCULOSIS [a]

	No.	TST +		QFT-G +		ELISPOT +		LST +	
		No.	(%)	No.	(%)	No. [b]	(%)	No. [b]	(%)
INH[c] treatment:									
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 tuberculin skin test (TST+) after known exposure to TB.
b Of 24 subjects blood samples were available for ELISPOT and LST.
c INH; isoniazid

In this respect, LST responses are depicted as highest IFN-γ production to one of the *M. tuberculosis*-specific peptide pools. Although the median LST response appears to be higher in the QFT-GIT or ELISPOT positive group compared with the assay negative groups, there was a large overlap between the two groups, with a substantial number of QFT-GIT and ELISPOT negative persons still having high responses in LST (Figure 2).

Although only 8 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 becomes small, it appeared that among INH-treated individuals the proportion with a positive QFT-GIT was lower (12.5%) than in the group without treatment, despite the finding that 86% had a positive LST result ($P = 0.06$). Results of the other assays, e.g., TST, ELISPOT and LST, were not significantly affected by prior INH-treatment.

Agreement between TST and three IGRA

Next, the overall agreement between various IGRA and the TST was calculated (Table 3). The level of agreement between outcome of QFT-GIT 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 versus TST did not differ from those taking the outcome of the ELISPOT. By contrast, the outcome of the 6-day LST showed excellent agreement (91%; κ=0.78) with those of the TST. In all, agreement between outcome of QFT-GIT and ELISPOT was high (86%, κ=0.71), but outcome of these short-incubation IGRA did agree with that of the 6-day LST in only 60% (κ=0.31) and 59% (κ=0.27), respectively, of the cases. Of note, adjusting cut-off levels of QFT-GIT and ELISPOT did not improve the level of agreement (data not shown).
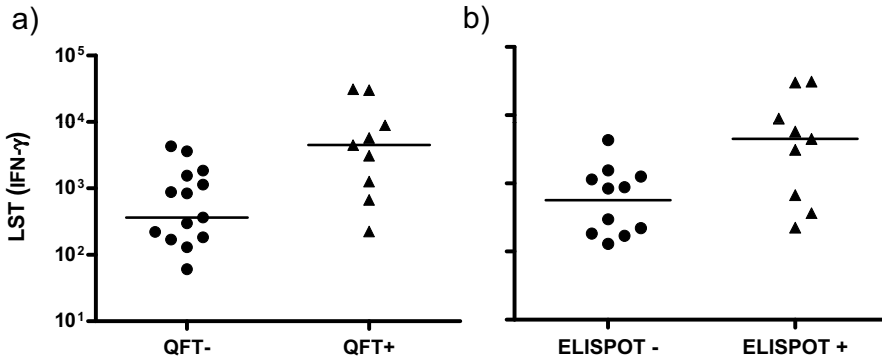
Figure 2. LST responses in persons with negative versus positive results in QFT-GIT and ELISPOT
A 6-day lymphocyte stimulation test (LST) was performed using peptide pools of ESAT-6, CFP-10 and TB7.7. LST responses are indicated as the highest IFN-γ production (in pg/ml) to one of the *M. tuberculosis*-specific peptide pools. a) LST responses in QFT-GIT negative and positive persons (cut-off: IFN-γ ≥ 0.35 IU/mL). b) LST responses in ELISPOT negative and positive persons (cut-off: 5 SFC/well above background). Lines indicate the median IFN-γ production.

DISCUSSION

In this study we compared the sensitivity of three *M. tuberculosis*-specific IGRA for the diagnosis of latent TB and found a remarkable discrepancy between outcome of the two short-incubation IGRA, i.e., QFT-GIT and ELISPOT, on the one hand and the 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 significantly more often positive than either QFT-GIT or ELISPOT. Our findings implicate that the short-incubation assays have limited sensitivity to detect past infection.

We performed all IGRA using identical, *M. tuberculosis*-specific, peptides and repeated a TST at the same time. Further, we chose to study a diverse group of persons with a documented positive TST after exposure to *M. tuberculosis*. Some of these individuals were known to be exposed to TB decades ago, and others more recently. Also, only 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 QFT-GIT could be observed, the study size was too small to correlate the observed discrepancy to factors such as the time elapsed since *M. tuberculosis* infection was acquired.

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Table 3. AGREEMENT BETWEEN DIAGNOSTIC ASSAYS FOR LATENT *M. TUBERCULOSIS* INFECTION [a]

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

a TST, tuberculin skin test (n=40); QFT, QuantiFERON-TB Gold in-tube (n= 40); ELISPOT, *ex vivo*
  IFN-γ enzym-linked immunospot assay (n = 37); LST, 6-day lymphocyte stimulation test (n=36).
  In vitro assays were done using peptides of ESAT-6, CFP-10 and TB 7.7.
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).
d BCG, *M. bovis* bacillus Calmette-Guérin vaccine.

The agreement between QFT-GIT and ELISPOT was high, but the outcome of these assays showed poor agreement with both TST and LST, assays that were highly concordant. About half the TST+ individuals had negative results in both QFT-GIT and ELISPOT, while most were positive in the 6-day LST. Of note, all three assays measured the IFN-γ production in response to peptides of ESAT-6, CFP-10 and TB7.7, antigens that were found to be highly specific for *M. tuberculosis* (4, 24), also when tested in a 6 day cell culture (1, 21, 27). Among the QFT-GIT and ELISPOT negative participants, high levels of IFN-γ could be produced in the LST, indicating that the observed discrepancy was not simply explained by differences in detection level of IFN-γ. A plausible explanation for the difference in sensitivity would be the difference *in vitro* incubation period for QFT-GIT and ELISPOT on the one hand and for LST on the other. We hypothesize that after 24 hours 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 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 prolonged incubation. In accordance with this line of thought are findings from a recent study on hepatitis C 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 IGRA, such as a 6-day LST, might be the most sensitive method to screen for latent *M. tuberculosis* infection in persons with an increased risk to develop reactivation TB, such as those eligible for transplantation or treatment with tumour necrosis factor-alpha antagonists (17). A recently published case of pulmonary tuberculosis in a liver transplant patient with a negative QFT-G test before transplantation illustrates that QFT-G has to 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 QFT-GIT results were more affected by prior INH treatment than those of ELISPOT, LST or TST. Three previous studies indicated there is a trend towards decreased ELISPOT responses at the end of treatment for latent TB (6, 11, 31). In Indian health-care workers, QFT-GIT 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 IGRA during treatment. Although INH treatment could have a differential effect on the result of IGRA with different test format, the observed discrepancy between IGRA with a short versus a prolonged incubation remained when INH-treated individuals were excluded from analysis.

There is a lack of knowledge on the performance of short-incubation IGRA compared to IGRA with a more prolonged-incubation period in relation to TB infection. One study, comparing overnight-ELISPOT and 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-positive persons with more remote exposure to *M. tuberculosis*. In another study, overall agreement between ELISPOT and a 3-day-incubation whole-blood IGRA was good (29), but the 3-day IGRA could have been too short to reliably detect a memory response. In accordance with our data is the observation in 3 cured TB patients with negative responses to a panel of RD1 peptides in an overnight-ELISPOT and positive responses in a cultured-ELISPOT (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 level of agreement between TST and IGRA varied widely between studies. In line with the hypothesis that short-incubation IGRA might have lower sensitivity for the detection of past latent infection are the observations of several other studies (16, 20, 26). In two cross-sectional study in South Africa, approximately one third of adults with a TST > 15mm had a negative QFT-GIT (20, 26) and 38% a negative T-spot.*TB* (26). Another study noticed that, in a mostly BCG-vaccinated Korean control population, 51% was TST positive and only 4% were QFT-G positive, while the expected prevalence of *M. tuberculosis* infection was 33% (16).

In conclusion, a major discrepancy was observed between results of two short-incubation IGRA (QFT-GIT and an in-house ELISPOT) and 6-day LST. This study raises

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the hypothesis that short-incubation IGRA mainly detect recent or ongoing infection with *M. tuberculosis*, while prolonged-incubation IGRA seem to be more sensitive for diagnosis of past latent infection. Further studies are needed to confirm this hypothesis and evaluate the consequence for the predictive value for TB risk.

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