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

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Chapter 3

Detection of recent infection with *Mycobacterium tuberculosis* after accidental laboratory exposure, using ELISPOT with *M. tuberculosis-specific peptides*

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ABSTRACT

Improved diagnosis and treatment of recently acquired, latent *Mycobacterium tuberculosis* infection would reduce the number of clinically manifest tuberculosis (TB) cases and therewith contribute to a better control of TB. In this study, we evaluated the diagnostic potential of ELISPOT using *M. tuberculosis*-specific peptide pools of ESAT-6, CFP-10, TB37.6 and TB7.7 for detection of recent infection with *M. tuberculosis* after accidental exposure of personnel in a microbiological laboratory. The tuberculin skin test (TST) was of limited value, as five of the nine exposed individuals were BCG vaccinated. Based on ELISPOT, two laboratory technicians were found to respond strongly to ESAT-6 and CFP-10, precisely those with the highest level of exposure during the accident and in one of them a TST conversion was observed. Interestingly, the peptides 2-6 of TB37.6 were solely recognized by these two recently exposed individuals and not by controls with a history of TST conversion or cured TB who did responded to ESAT-6 and CFP-10. Moreover, follow-up with ELISPOT one year after the accident revealed that responses to TB37.6 had become undetectable, while responses to ESAT-6, CFP-10 remained unchanged. This suggests that a positive ELISPOT response to TB37.6 peptides could be a marker for recent infection. The present findings on an accidental exposure to *M. tuberculosis* in a microbiology laboratory illustrates the value of an IFN-γ-ELISPOT based on multiple *M. tuberculosis*-specific peptides for the detection of recent latent infection, in particular in a setting with a high level of BCG vaccination and possible previous exposure to mycobacteria.

INTRODUCTION

Contact tracing and treatment of individuals who are infected with *Mycobacterium tuberculosis* but have not yet developed clinically manifest lung tuberculosis (TB) is of utmost importance for the control of TB, as most cases of contagious TB arise from this reservoir. However, there is no gold standard for the diagnosis of latent *M. tuberculosis* infection. Since its discovery, the tuberculin skin test (TST) has for almost a century remained the only available method to determine whether an individual is infected with *M. tuberculosis* after exposure to a patient with pulmonary TB. The closest proxy to proof of infection consists of an observed conversion of the TST from negative to positive.

Contact tracing is complicated by several factors that limit the interpretation of the TST. Firstly, prior vaccination with *M. bovis* bacillus Calmette-Guérin (BCG) often induces a positive TST, especially in case of recent BCG vaccination or when repeated skin testing was done (14, 28, 29). Secondly, false positive TST reactions can be caused by cross-reactivity to non-tuberculous mycobacteria. A third factor is that a positive TST result does not discriminate between recent *M. tuberculosis* infection or infection which was acquired in the past, while the risk of developing clinically manifest TB disease is highest in the first two years after infection and rapidly declines to a very low risk thereafter. As a consequence, the potential benefit of treatment of immunocompetent individuals with latent TB infection is highest in case of recent infection.

Recently, a novel method for more reliable detection of infection with *M. tuberculosis* has come in scope. Since the deciphering of the complete genome of *M. tuberculosis* (18), it has become possible to identify genomic regions of difference that are present in *M. tuberculosis* but absent from BCG and most environmental mycobacterial species. One of those was RD1, which was found to encode two potent T cell antigens, ESAT-6 (Rv3875) and CFP-10 (Rv3874) (3, 4, 6, 34). Immunodiagnostic assays based on these two *M. tuberculosis*-specific secreted antigens were found to be sensitive as well as specific for the detection of latent tuberculosis infection, at least in regions with a low prevalence of TB (12). Other studies, using ESAT-6 and CFP-10 in an ELISPOT assay, suggest that this method could even be more sensitive than skin testing for detection of latent *M. tuberculosis* infection (21, 25).

Although ESAT-6 and CFP-10 are potent T cell antigens that are recognized by genetically heterogeneous populations (36), the sensitivity of such immunodiagnostic assays could be further improved by adding other *M. tuberculosis* specific antigens or peptide pools. This was indicated by a recent study, demonstrating that T cell responses to three newly identified *M. tuberculosis*-specific peptide pools combined with ESAT-6 and CFP-10 resulted in a sensitivity of 90 % for detection of *M. tuberculosis* infection with a specificity of 95% (11). Of these, the antigens TB37.6 and TB7.7 were of special interest. TB37.6 (Rv3873, PPE68), a PPE protein encoded by the RD1 region, is predominantly

found in the membrane and cell wall fraction of *M. tuberculosis* (19, 32). The PPE gene family is unique to *M. tuberculosis*, with few homologues in *M. leprae, M. marinum, M. bovis* and other species. PPE genes are hypothesized to be a source of antigenic variability and in this regard they could constitute interesting antigens for immunodiagnosis. Despite being encoded by RD1, TB37.6 was recognized by BCG vaccinated individuals and BCG vaccinated cattle (11, 17, 27, 32). This was explained by cross-reactivity to conserved epitopes within the PPE family, because homologous genes of the PPE family are found throughout the genomes of *M. tuberculosis* and many of these genes are also present in BCG (32). Several peptides of TB37.6, however, were highly *M. tuberculosis* specific, not being recognized by BCG-vaccinated persons (11).

TB 7.7 (Rv2654) is encoded by the RD 11 region, a phage-inserted region (phiRv2). This is a particularly interesting region for diagnostic purposes because it is highly specific for *M. tuberculosis*; it is not only absent from all tested BCG strains but also from environmental mycobacteria (9, 13, 18). TB7.7 was recently found to contain many human T cell epitopes and was well recognized by the majority of TB patients, while none of the BCG vaccinated individuals responded to this antigen (1, 11). Moreover, TB7.7 was able to induce *M. tuberculosis*-specific skin-test responses in guinea pigs (1).

In a microbiological laboratory several technicians were exposed to *M. tuberculosis* after an accident during which a culture tube was spilled outside of a containment cabinet. As individuals working in a diagnostic microbiological laboratory are often BCG vaccinated and have been at risk for exposure to *M. tuberculosis* in the past, the TST can be expected to be of limited value for the detection of recent *M. tuberculosis* infection. Therefore we evaluated responses to ESAT-6, CFP-10, TB37.6 and TB7.7 in ELISPOT for the detection of recent latent *M. tuberculosis* infection in this setting.

MATERIALS AND METHODES

Study setting

In a microbiological laboratory in the Netherlands, a technician (referred to as A) accidentally dropped a glass tube containing a culture of *M. tuberculosis* on solid Löwenstein-Jensen medium on the floor. The sputum sample had been incubated for 3 to 4 weeks and many *M. tuberculosis* colonies were visible. The glass tube was dropped from a height of about 1.5 m (five feet). The tube broke and pieces of glass and culture medium with *M. tuberculosis* colonies were spilled on the floor of the hall (Fig. 1). Technician A threw a paper cloth soaked in chlorine solution (1000 parts per million) on top of the spills. Subsequently, he cleaned up everything with a colleague (B). They wore gloves but did not wear protective masks. In the mean time another laboratory technician (C) was in the wardrobe (Fig. 1), situated down the hall from where the incident happened. A third

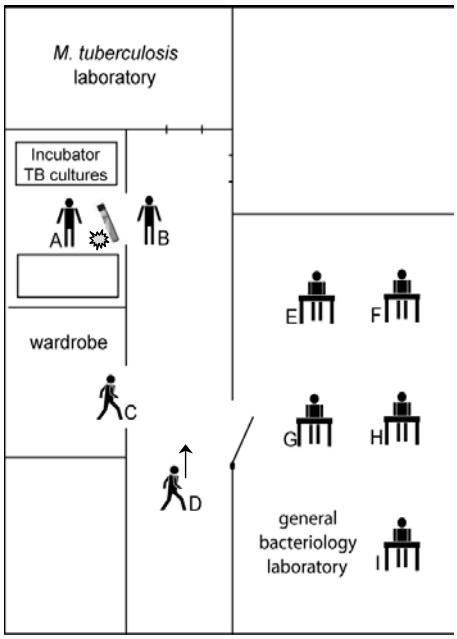

Figure 1. Schematic drawing of the microbiological laboratory where the accident with the TB culture took place.

The position of each technician (A-I), at the time of the accident, is indicated in the floor plan as well as the place where the *M. tuberculosis* culture on solid Löwenstein-Jensen medium was dropped (✵).

colleague (D) inspected the area where the tube was dropped shortly after everything was removed and cleaned with chlorine. Diagonally across from the area where the TB culture was dropped, a laboratory was situated where 5 other technicians (E to I) were at work (Fig. 1). At the time of the accident, the door to this laboratory was open and windows were open on both sides of the laboratory building.

Three months after the accident, all 4 non-BCG vaccinated individuals were screened with a TST while chest radiographs were made of the 5 BCG-vaccinated, exposed technicians. One month later, a TST was also offered to all BCG-vaccinated study subjects. In addition, blood was drawn from 19 laboratory technicians who volunteered to participate in this study, for an ex-vivo ELISPOT assay. One year after the first blood sampling, thus 16 month after the accident, a second blood sample was drawn from 8 colleagues who had been present during the accident, and the ELISPOT assays were repeated.

Study subjects

The study included 9 laboratory technicians (Table 2; A-I) who had been present in the laboratory at the day and time of the accident, as described above (Fig. 1).

In addition, 10 other laboratory technicians (colleagues) who were not present the day of the accident were included (Table 2; lab1–lab10). This group served as a control group with a comparable level of background exposure to *M. tuberculosis.* Three of these technicians were TST negative (lab1-3) and 5 were TST positive (lab4-8) of whom one (lab7) had previously been treated with isoniazide (INH). The remaining two had been successfully treated for limited pulmonary tuberculosis in the past (lab9 in 1970, lab10 in 1994), which was diagnosed during routine check-up before clinical symptoms became apparent. In this group, 5 of the 10 persons were BCG vaccinated.

A second control group was added including 6 well defined *M. tuberculosis* infected individuals. Three persons, TB1, TB2 and TB3 (Table 2), were successfully treated for TB disease respectively 10, 3 and 2 years before this study. In these individuals, the possibility of re-exposure to *M. tuberculosis* is considered to be very small. The 3 TST positive individuals, TST1, TST2 and TST3 (Table 2) had had documented TST conversion after contact with a case of smear positive pulmonary TB, respectively 5, 6 and 10 years before blood sampling. All 3 had been treated with INH. Frozen cells from these individuals were available from a previous study (4). These subjects were chosen because of known positive responses to ESAT-6 and/or CFP-10 in a 6-day lymphocyte stimulation assay.

From all individuals, a blood sample (45 ml) was drawn after a written informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were isolated and stored in liquid nitrogen until used for the assays. The study protocol (p207/99) was approved by the Institutional Review Board of the Leiden University Medical Center.

Mycobacterial antigens and peptides

For the present study, peptides of 20 amino-acids (aa) long, with a 10-aa overlap spanning the complete sequence of CFP-10 and ESAT-6 were synthesized as previously described (7). The overlapping 18-mers peptides from the two proteins TB 7.7 (Rv2654) (5- or 6-aa overlap) and TB37.6 (Rv3873) (8-aa overlap) were synthesized by standard solid-phase methods at Schafer-N, Copenhagen, Denmark (Figure 2). The peptides were purified by reverse phase high-pressure liquid chromatography. Purified peptides were lyophilized and stored dry until reconstitution in phosphate-buffered saline. For TB7.7, a peptide pool was used spanning the complete sequence of the protein (peptide 1-6). For TB37.6, the peptide pool consisted only of peptides 2-6, corresponding to aa 13-70. Recombinant ESAT-6 (batch F800) and CFP-10 (batch 02-III) were expressed in *E. coli* and purified as described elsewhere (10). Purified protein derivative (PPD) RT49, recombinant antigens and the peptide pools of TB37.6 and TB7.7 were made available by the Statens Serum Institute (Copenhagen, Denmark).

TABLE 1. DIAGNOSTIC ANTIGENS AND PEPTIDES

RD [a] region	Rv code	Antigen	Antigenic stimulus	Sequence	Concentration
-	-	PPD	PPD (RT 49 SSI)	-	5 μg/ml
RD1	Rv3875	ESAT-6	peptide pool (p1-p9)	complete sequence	10 μg/ml/peptide
	Rv3874	CFP-10	peptide pool (p1-p9)	complete sequence	10 μg/ml/peptide
	Rv3873	TB37.6	peptide pool (p2-p6)	amino-acid 13-70	10 μg/ml/peptide
RD11	Rv2654	TB7.7	peptide pool (p1-p6)	complete sequence	10 μg/ml/peptide

a RD; Regions of Difference that are present in *M. tuberculosis* but absent from BCG and most environmental mycobacterial species.

ELISPOT assay for single-cell interferon (IFN)-γ release

For ELISPOT, 96-well filtration plates, multiscreen™-HA, sterile plate (MAHAS4510 Millipore, Bedford, MA) were precoated with 5 μg/ml anti–IFN-γ monoclonal antibody (MAb) 1-D1K (Mabtech, Stockholm, Sweden), washed 6 times with Iscoves Modified Dulbecco's Medium (IMDM) with 10% fetal calf serum, and blocked for 2 h with IMDM supplemented with 10% pooled human AB serum, penicillin at 100 U/ml, and streptomycin at 100 μg/ml (complete medium). PBMC at 2.5 x 10^5 per well were cultured in 200 μl of complete medium, in ELISPOT plates with antigen (PPD, rESAT-6, rCFP-10; at 5 μg/ml each) or peptide pools (ESAT-6, CFP-10, TB7.7 and TB37.6 each at 10 μg/ml/peptide) and incubated for 18 hours at 37°C in humidified air containing 5% CO_2. As a positive control, phytohemagglutinin was used at a final concentration of 5 μg/ml and culture medium without antigen served as negative control. Tests were performed at least in triplicate. The ELISPOT plates were developed as previously described (8) and analyzed on a high resolution image analyzer (Bioreader pro 3000, Bio-Sys, GmbH, Germany). For analysis, the mean number of spot-forming cells (SFC) per well from triplicate values for each antigen was calculated and transformed to number of SFC per million PBMC. In order to obtain an antigen-specific response, the mean number of SFC of the negative control wells (background value) was subtracted from the mean number of SFC in the wells containing antigen. A positive test result was predefined as at least 20 SFC per million PBMC (after subtraction of the background value) and at least twice the background value.

Lymphocyte stimulation assay

PBMC (1.5 x 10^5/well) were cultured in complete medium in triplicate in 96-wells round-bottom microtiter plates at 37 °C, 5% CO_2, in the absence or presence of antigen (PPD 5 μg/ml or peptide pools of ESAT-6, CFP-10 each at 10 μg/ml/peptide). At day 6, supernatants were harvested (60 μl/well, pooled per triplicate). IFN-γ concentration

in the supernatants was measure by enzyme-linked immunosorbent assay (ELISA) (U-CyTech, Utrecht, The Netherlands). ELISA samples were tested in duplicate. The mean IFN-γ concentration in unstimulated wells was subtracted from the mean concentration in stimulated wells. A positive response was defined as an IFN-γ level of ≥ 130 pg/ml (3 times the standard deviation of the concentration in unstimulated wells).

Tuberculin skin testing

In all exposed laboratory technicians, including those with a BCG vaccination, a TST was done by trained personnel according to standard procedures. In brief, 0.1 ml (2 Tuberculin Units) of PPD (RT23; Statens Serum Institut, Copenhagen, Denmark) was injected intradermally into the dorsal side of the forearm. Transverse induration at the TST site was measured after 72 hours by experienced personnel.

RESULTS

Tuberculin skin testing and chest radiographs

A TST was offered to all 9 laboratory technicians who were potentially exposed to *M. tuberculosis* during the accident. Two of the 5 previously BCG vaccinated technicians agreed to have a TST done and all 4 BCG unvaccinated persons had a TST done (Table 2). A TST conversion was observed in one of the BCG unvaccinated technicians; the TST was 25 mm, while previous yearly routine TSTs had been negative (0 mm) (Table 2; B). Thus this person, who helped cleaning up, was most likely infected with *M. tuberculosis* during the accident. Technician A, who accidentally dropped the *M. tuberculosis* culture had a TST of 20 mm (Table 2; A). No previous TSTs were done as he was BCG vaccinated in the past (> 20 years ago).

Chest radiographs of all 5 BCG vaccinated individuals and of the person with the TST conversion were normal, excluding active pulmonary TB disease. However, the possibility of latent *M. tuberculosis* infection due to the accidental exposure could not be excluded in the BCG vaccinated technicians. Therefore, blood was drawn from exposed and unexposed personnel of the microbiological laboratory to perform an IFN-γ ELISPOT assay using PPD, ESAT-6, CFP-10, TB7.7 and TB37.6 as antigenic stimuli (Table 1).

ESAT-6 and CFP-10 specific T cells

The individual results are shown in Table 2. High numbers of ESAT-6 and CFP-10 specific T cells were detected in the two laboratory technicians (A and B) who had been most closely involved in the accident and had a positive TST. No significant numbers of ESAT-6 or CFP-10 specific T cells were observed in subject C, who was in the proximity of the accident, or subject D, who inspected the area of the accident just after the cleaning with

chlorine was completed.. In agreement, their TSTs were negative. None of the other tested subjects (E-I) were positive, but for one individual the ELISPOT results were inconclusive due to high background values (median 96 SFC/million PBMC).

As a control group, 10 colleagues who had not been present during the accident with *M. tuberculosis* were tested. The 3 technicians with a negative TST also had negative in vitro responses to PPD as well as to all 4 peptide pools (lab1-lab3 in Table 2). Of the 5 TST positive technicians (lab4-lab8), only one (lab6) responded to PPD and ESAT-6 in the ELISPOT. Of note, the two technicians who were treated for limited pulmonary TB respectively 33 and 8 years ago (lab9 and lab10) did not recognize any of the 4 *M. tuberculosis* specific peptide pools in an overnight ELISPOT assay.

Because the ELISPOT is a short-term culture for detection of effector T cell responses, a 6 day lymphocyte stimulation assay was done in 16 subjects (A-I, lab1-3 and lab7-10) in order to evaluate whether results would be different after prolonged incubation allowing more time for memory T cells to respond. With this 6 day assay, 4 additional laboratory technicians (F,G, H and lab7) could be identified who recognized ESAT-6 (IFN-γ of 506, 395, 157 and 245 pg/ml respectively), although IFN-γ responses were less strong than those found in technician A and B (IFN-γ of 6438 and 16801 pg/ml). In the two colleagues (lab9 and lab10) with a history of limited pulmonary TB, no responses to ESAT-6 or CFP-10 were found in the 6 day culture, which was in accordance with the ELISPOT results (data not shown).

The second control group consisted of individuals with a well documented *M. tuberculosis* infection (Table 2; TST1-3 and TB1-3). All 3 TST converters and 2 of the 3 TB patients responded to ESAT-6 and CFP-10 in an overnight IFN-γ ELISPOT. For one of the cured TB patients, who suffered from TB disease 10 years ago, ESAT-6 and CFP-10 specific T cells were just below the cut-off level for a positive response. In a 6 day culture, PBMC that had been obtained during the same venapuncture from this person responded positively to these two antigens with the production of significant amounts of IFN-γ.

Responses to recombinant ESAT-6 and CFP-10 proteins were very similar to those to the peptide pools spanning the complete sequence of these antigens (data not shown). This is in accordance with an earlier study (7).

TB37.6 and TB7.7 specific T cells

Stimulation with the *M. tuberculosis* specific peptide pool of TB37.6 resulted in a significant number of IFN-γ producing T cells using PBMC from subjects A and B (52 and 53 SFC/million PBMC respectively; Table 2). These two individuals were most directly exposed, were or had become TST positive and also recognized ESAT-6 and CFP-10. Interestingly, none of the other 9 TST positive individuals responded to TB37.6. Three of these individuals (Table 2; TST1-3), who had had a TST conversion 5, 6 and 10 years ago for which they were treated with INH, did respond strongly to ESAT-6 and CFP-10.

TABLE 2. CLINICAL INFORMATION AND IFN-γ ELISPOT RESULTS OF STUDY SUBJECTS [a,b]

Code[c]	BCG	TST (mm)	TB	Treat-ment	PPD		ESAT-6		CFP-10		TB37.6		TB7.7	
					year 0	year 1	year 0	year 1	year 0	year 1	year 0	year 1	year 0	year 1
A	yes	20	no	yes	**275**	**76**	**71**	**78**	**469**	**190**	**52**	<	7	<
B	no	25	no	yes	**146**	**160**	**370**	**275**	**104**	**148**	**53**	8	5	<
C	yes	3	no	no	5	**98**	<	9	<	<	<	<	0	<
D	no	0	no	no	12	16	6	3	4	1	1	1	0	8
E	yes	-	no	no	**27**	**23**	7	<	7	4	6	0	6	<
F	yes	-	no	no	*	*	*	*	*	*	*	*	*	*
G	yes	>10	no	no		<		5		5		11		<
H	no	0	no	no	5	**42**	<	4	<	<	<	<	<	<
I	no	0	no	no	**46**		<		<		1		2	
Lab 1	no	0	no	no	<		<		<		<		2	
Lab 2	yes	0	no	no	15		<		<		<		<	
Lab 3	yes	0	no	no	2		<		<		<		3	
Lab 4	yes	22	no	no	14		<		<		<		<	
Lab 5	no	>10	no	no	0		<		<		<		<	
Lab 6	un-kown	>10	no	no	**271**		**27**		7		<		19	
Lab 7	no	>10	no	yes	3		<		<		<		<	
Lab 8	no	11	no	no	6		<		<		<		<	
Lab 9	yes	-	cured TB *d*	yes	**22**		<		<		3		<	
Lab 10	yes	11	cured TB *d*	yes	17		<		<		<		<	
TB 1	no	-	cured TB	yes	**144**		18		19		5		12	
TB 2	un-kown	-	cured TB	yes	**52**		**84**		**34**		<		16	
TB 3	no	-	cured TB	yes	**196**		**63**		**104**		<		3	
TST 1	no	>10	no	yes	**325**		**35**		**275**		<		<	
TST 2	no	>10	no	yes	**243**		**77**		**111**		2		**115**	
TST 3	no	>10	no	yes	**181**		**324**		**240**		7		16	

a IFN-γ ELISPOT was done 4 months (year 0) and 16 months (year 1) after the accidental exposure. ELISPOT results are shown in mean spot forming cells (SFC)/10^6 PBMC minus the mean of medium value. Bold numbers indicate a positive response.

b Symbols: <, number of SCF less than in medium; -, not done; „, not interpretable due to high medium values.

c A-I, technicians present during the accident; decreasing exposure level. Lab1-10, technicians not present during the accident. TB 1-3, cured TB patients, treated 10, 3 and 2 years before, TST 1-3, tuberculin skin test converters after a known exposure to TB 5, 6 and 10 years before.

d Limited pulmonary TB diagnosed during routine check-up before clinical symptoms became apparent; lab9 32 years ago and lab10 8 years ago.

Also none of the 5 individuals with a history of TB recognized the peptides of TB37.6 in the ELISPOT assay.

TB7.7 was not recognized by any of the laboratory technicians that were present during the accident nor by any of their colleagues. Among subjects with a history of TB disease, TB7.7 did not induce significant numbers of IFN-γ producing T cells in an overnight ELISPOT assay either. Only in one of the 3 TST converters TB7.7 specific T cells (115 SFC/million PBMC) were found (Table 2; TST 2).

Follow-up of *M. tuberculosis*-specific T cell responses

One year after the first ELISPOT assays were done, another blood sample was obtained from the laboratory technicians who had been present at the time of the accident with *M. tuberculosis* and ELISPOT assays were repeated. The results showed that the number of IFN-γ producing T cells in response to PPD and the peptide pools of ESAT-6, CFP-10 and TB7.7 had remained of the same order of magnitude as one year earlier (Table 2). In contrast, responses to the TB37.6 peptides had become undetectable in subjects A and B, who were most likely infected with *M. tuberculosis* after the laboratory accident, and were treated for 6 month with INH (the isolate was INH sensitive). The INH treatment was finished 7 months before the second blood sampling. None of their colleagues with a positive TST had received INH treatment.

DISCUSSION

This study illustrates the diagnostic potential of ELISPOT using *M. tuberculosis*-specific peptide pools of ESAT-6, CFP-10, TB37.6 and TB7.7 for detection of recent infection with *M. tuberculosis.* Especially when many exposed individuals are BCG vaccinated or already TST positive due to previous exposure to mycobacteria, as was the case in our study setting, where personnel of a microbiological laboratory was accidentally exposed to *M. tuberculosis,* the TST has limited value for detection of recent TB infection. In one of 4 BCG non-vaccinated persons with potential exposure to *M. tuberculosis*, the ELISPOT showed positive responses to ESAT-6 and CFP-10; this was the only person in whom a TST conversion was observed. Among 5 BCG vaccinated person with possible accidental exposure, one responded to ESAT-6 and CFP-10 in ELISPOT. These two ESAT-6/CFP-10 positive persons were precisely those laboratory technicians who had been most closely involved in the accident and would be expected to have had the highest level of exposure. Interestingly, the peptide pool of TB37.6 was only recognized by T cells from these two subjects, while individuals with a history of TST conversion in the past, did also respond to ESAT-6/CFP-10 but did not respond to TB37.6. When the ELISPOT assay was repeated one year later, responses to TB37.6 had become undetectable in the

two persons who were most likely infected with *M. tuberculosis* during the accident, while responses to ESAT-6 and CFP-10 had remained of similar heights. This suggests that the pattern of antigen-specific responses may reflect the phase of infection. Alternatively, a positive ELISPOT response to TB37.6 could reflect an exposure to a high bacterial load and that these responses become negative when the bacterial load decreases during the course of the infection. Either way, a positive ELISPOT response to TB37.6, together with a positive response to ESAT-6/CFP-10, could be a marker for recent exposure and subsequent infection.

Previous studies demonstrated that the antigens used in this study are highly specific for M. tuberculosis and are unlikely to elicit false-positive test results (4, 11, 16, 26, 30, 31, 35). Therefore, positive responses can be regarded as a correlate of infection. However, the sensitivity of ELISPOT for detection of accidental infection has thus far not been reported and, thus, the question can be posed whether the negative ELISPOT results that were observed in our study reliably exclude infection with M. tuberculosis. In previous studies, the sensitivity of an ELISPOT-based assay using specific antigens was reported to be 90 % in HIV+ and up to 100% in HIV-negative patients with clinically manifest TB (16, 35). In contacts of smear-positive pulmonary TB patients, the ELISPOT was not only found to correlate better with degree of exposure than the TST, but even seemed to be more sensitive (21). During the accident that was described in the present study, the technicians were exposed to a number of bacilli that was potentially much higher than the number that is inhaled during exposure to an individual with a natural infection. Up to a certain level of exposure, the strength of immune responses will increase directly with the amount of antigen (2, 22, 23), and accidental infection can therefore be expected to result in an equal if not higher number of antigen-specific T cells. Thus, we think that the persons with negative ELISPOT results were not infected during the accident. During a follow-up period of 3 years, none of them developed active TB disease.

Of the antigens that were used in the present study, ESAT-6 and CFP-10 were used in most previous studies of *M. tuberculosis*-specific immunodiagnostic assays, as was recently reviewed (33). However, it has become clear that T cell responses to these antigens can be found in persons who have been infected with *M. tuberculosis* in the past and may even persist after adequate treatment (4, 34, 39). Therefore, new diagnostic antigens should not only be able to further optimize the sensitivity of immunodiagnostic assays but ideally should also differentiate between recent and remote *M. tuberculosis* infection. The study setting of an accidental exposure to *M. tuberculosis* in a laboratory was particularly suitable to evaluate the value of novel diagnostic antigens for the detection of recent infection and to study the kinetics of immune responses, because the exact moment of exposure was known. This is in contrast to the usual contact investigations in which individuals have been exposed to a person with contagious TB for an extended period of time that can often not be specified.

We evaluated the most promising novel diagnostic antigens identified in recent studies, TB37.6 and TB7.7 (1, 11, 27), of which TB7.7 was recognized poorly. The *M. tuberculosis*-specific peptide pool of TB37.6, however, was found to be of particular interest as our results indicate that it is only recognized by individuals with a recent infection. Previous studies showed that TB37.6 is recognized by 42% of TB patients tested 0-6 months after diagnosis (32) and by 53% of untreated TB patients (27). In seeming contrast to our results, another study demonstrated that remote TST converters can recognize TB37.6, but in that study whole recombinant antigen was tested in a 5 day culture assay (32), while we tested only the *M. tuberculosis*-specific peptides and used a short culture assay, which could explain the difference in observation. Previously, *M. tuberculosis*-specific peptide pool of TB37.6 was only tested in patients with active TB and a small group of recently latently infected individuals, inducing IFN-γ responses in 38-46% of these persons (11). In our study, both accidentally infected persons were treated and responses to TB37.6 had become undetectable in both of them after treatment was completed, but this does not necessarily imply a causal relationship between treatment and the kinetics of the immune response. Two other studies indicate that there might be a sequential appearance of antigen-specific responses after infection with *M. tuberculosis* (37, 38). It has been reported that ELISPOT responses to ESAT-6 and CFP-10 became negative during successful treatment of active TB, while remained positive in those without clinical improvement (15). A prospective follow-up study of recently infected persons with or without treatment for latent TB infection may clarify the natural kinetics of T cell responses to TB37.6 and assess the effect of treatment.

Among the laboratory controls were several persons who had most likely been infected with *M. tuberculosis* in the past, as several of the BCG non-vaccinated persons had a positive TST result and two persons had a history of limited pulmonary TB for which they had received treatment. Yet, a positive response to ESAT-6 was observed in only one of them and a response to the other specific antigens in none. This finding is in seeming contrast with previous studies showing that responses to ESAT-6 and CFP-10 are frequent among TST positive individuals and (cured) TB patients (4, 39). Besides a relatively small study size, there are two possible explanations for this apparent discrepancy. First, it has been reported that ELISPOT responses decrease rapidly during effective treatment (15) and this may have been the case in the technicians who had been treated in the past. Secondly, an overnight-ELISPOT is highly sensitive but mainly detects responses of circulating effector memory T cells as exist during active or recent infection, while a more prolonged cell culture allows detection of responses of central memory T cells as well. In individuals who have been infected with *M. tuberculosis* in the past, the number of circulating antigen-specific effector cells could be low, causing negative results in a short-term assay but positive responses after prolonged culture. In accordance, in some of the persons who were most probably latently infected with *M. tuberculosis* in the

past, we observed positive responses to ESAT-6 and CFP-10 in a 6 day culture, but not in an overnight ELISPOT. Thus, ELISPOT could be the method of choice for detection of recent infection with *M. tuberculosis* while more prolonged cultures may be required when detection of more remote infection is relevant as well. The latter is important in immunocompromised persons or those eligible for immunosuppressive treatment, e.g. such as is used in transplant recipients or TNF-alpha antagonistic drugs (5, 24).

A diagnostic assay that could specifically detect recently acquired latent infection would allow more targeted treatment, because the risk of progression to active TB decreases significantly with time after infection. The present study provides a novel hypothesis in this regard, namely that ELISPOT responses to TB37.6 indicate recent infection. Selection of latently infected individuals would benefit even more if it were possible to identify persons specifically at risk of developing active TB disease. One study found that that recently exposed individuals with high ESAT-6 responses were most likely to progress to active TB (20).

In conclusion, ELISPOT using peptide pools of ESAT-6, CFP-10 and TB37.6 allowed specific detection of recent *M. tuberculosis* infection after accidental exposure to *M. tuberculosis,* irrespective of BCG vaccination or TST results. In particular, responses to *M. tuberculosis*-specific peptides of TB37.6 were indicative for a recently acquired latent infection. Clearly, the ideal diagnostic test for detection of latent TB infection is not yet available, but the findings of this study contribute to another step in the right direction.

REFERENCE LIST

1. Aagaard, C., I. Brock, A. Olsen, T. H. Ottenhoff, K. Weldingh, and P. Andersen. 2004. Mapping immune reactivity toward Rv2653 and Rv2654: two novel low-molecular-mass antigens found specifically in the Mycobacterium tuberculosis complex. J. Infect. Dis. 189:812-819.
2. Alter, G., C. M. Tsoukas, D. Rouleau, P. Cote, J. P. Routy, R. P. Sekaly, and N. F. Bernard. 2004. Assessment of longitudinal changes in HIV-specific effector activity in subjects undergoing untreated primary HIV infection. AIDS 18:1979-1989.
3. Andersen, P., M. E. Munk, J. M. Pollock, and T. M. Doherty. 2000. Specific immune-based diagnosis of tuberculosis. Lancet 356:1099-1104.
4. Arend, S. M., P. Andersen, K. E. van Meijgaarden, R. L. Skjot, Y. W. Subronto, J. T. van Dissel, and T. H. Ottenhoff. 2000. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. J. Infect. Dis. 181:1850-1854.
5. Arend, S. M., F. C. Breedveld, and J. T. van Dissel. 2003. TNF-alpha blockade and tuberculosis: better look before you leap. Neth. J. Med. 61:111-119.
6. Arend, S. M., A. C. Engelhard, G. Groot, K. de Boer, P. Andersen, T. H. Ottenhoff, and J. T. van Dissel. 2001. Tuberculin skin testing compared with T cell responses to Mycobacterium tuberculosis-specific and nonspecific antigens for detection of latent infection in persons with recent tuberculosis contact. Clin. Diagn. Lab Immunol. 8:1089-1096.
7. Arend, S. M., A. Geluk, K. E. van Meijgaarden, J. T. van Dissel, M. Theisen, P. Andersen, and T. H. Ottenhoff. 2000. Antigenic equivalence of human T cell responses to Mycobacterium tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. Infect. Immun. 68:3314-3321.
8. Arend, S. M., K. E. van Meijgaarden, K. de Boer, E. C. de Palou, D. van Soolingen, T. H. Ottenhoff, and J. T. van Dissel. 2002. Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with Mycobacterium marinum or M. kansasii. J. Infect. Dis. 186:1797-1807.
9. Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284:1520-1523.
10. Berthet, F. X., P. B. Rasmussen, I. Rosenkrands, P. Andersen, and B. Gicquel. 1998. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 144 (Pt 11):3195-3203.
11. Brock, I., K. Weldingh, E. M. Leyten, S. M. Arend, P. Ravn, and P. Andersen. 2004. Specific T cell epitopes for immunoassay-based diagnosis of Mycobacterium tuberculosis infection. J. Clin. Microbiol. 42:2379-2387.
12. Brock, I., K. Weldingh, T. Lillebaek, F. Follmann, and P. Andersen. 2004. Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. Am. J. Respir. Crit Care Med. 170:65-69.
13. Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L. M. Parsons, A. S. Pym, S. Samper, D. van Soolingen, and S. T. Cole. 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc. Natl. Acad. Sci. U. S. A 99:3684-3689.
14. Bugiani, M., A. Borraccino, E. Migliore, A. Carosso, P. Piccioni, M. Cavallero, E. Caria, G. Salamina, and W. Arossa. 2003. Tuberculin reactivity in adult BCG-vaccinated subjects: a cross-sectional study. Int. J. Tuberc. Lung Dis. 7:320-326.
15. Carrara, S., D. Vincenti, N. Petrosillo, M. Amicosante, E. Girardi, and D. Goletti. 2004. Use of a T cell-based assay for monitoring efficacy of antituberculosis therapy. Clin. Infect. Dis. 38:754-756.
16. Chapman, A. L., M. Munkanta, K. A. Wilkinson, A. A. Pathan, K. Ewer, H. Ayles, W. H. Reece, A. Mwinga, P. Godfrey-Faussett, and A. Lalvani. 2002. Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of Mycobacterium tuberculosis-specific T cells. AIDS 16:2285-2293.
17. Cockle, P. J., S. V. Gordon, A. Lalvani, B. M. Buddle, R. G. Hewinson, and H. M. Vordermeier. 2002. Identification of novel Mycobacterium tuberculosis antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. Infect. Immun. 70:6996-7003.
18. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, and . 1998.

Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393:537-544.
19. Demangel, C., P. Brodin, P. J. Cockle, R. Brosch, L. Majlessi, C. Leclerc, and S. T. Cole. 2004. Cell envelope protein PPE68 contributes to Mycobacterium tuberculosis RD1 immunogenicity independently of a 10-kilodalton culture filtrate protein and ESAT-6. Infect. Immun. 72:2170-2176.
20. Doherty, T. M., A. Demissie, J. Olobo, D. Wolday, S. Britton, T. Eguale, P. Ravn, and P. Andersen. 2002. Immune responses to the Mycobacterium tuberculosis-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. J. Clin. Microbiol. 40:704-706.
21. Ewer, K., J. Deeks, L. Alvarez, G. Bryant, S. Waller, P. Andersen, P. Monk, and A. Lalvani. 2003. Comparison of T cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak. Lancet 361:1168-1173.
22. Godkin, A. J., H. C. Thomas, and P. J. Openshaw. 2002. Evolution of epitope-specific memory CD4(+) T cells after clearance of hepatitis C virus. J. Immunol. 169:2210-2214.
23. Hill, P. C., A. Fox, D. J. Jeffries, D. Jackson-Sillah, M. D. Lugos, P. K. Owiafe, S. A. Donkor, A. S. Hammond, T. Corrah, R. A. Adegbola, K. P. McAdam, and R. H. Brookes. 2005. Quantitative T cell assay reflects infectious load of Mycobacterium tuberculosis in an endemic case contact model. Clin. Infect. Dis. 40:273-278.
24. Keane, J., S. Gershon, R. P. Wise, E. Mirabile-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N. Engl. J. Med. 345:1098-1104.
25. Lalvani, A., A. A. Pathan, H. Durkan, K. A. Wilkinson, A. Whelan, J. J. Deeks, W. H. Reece, M. Latif, G. Pasvol, and A. V. Hill. 2001. Enhanced contact tracing and spatial tracking of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells. Lancet 357:2017-2021.
26. Lalvani, A., A. A. Pathan, H. McShane, R. J. Wilkinson, M. Latif, C. P. Conlon, G. Pasvol, and A. V. Hill. 2001. Rapid detection of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells. Am. J. Respir. Crit Care Med. 163:824-828.
27. Liu, X. Q., D. Dosanjh, H. Varia, K. Ewer, P. Cockle, G. Pasvol, and A. Lalvani. 2004. Evaluation of T cell responses to novel RD1- and RD2-encoded Mycobacterium tuberculosis gene products for specific detection of human tuberculosis infection. Infect. Immun. 72:2574-2581.
28. Menzies, D. 1999. Interpretation of repeated tuberculin tests. Boosting, conversion, and reversion. Am. J. Respir. Crit Care Med. 159:15-21.
29. Menzies, D. 2000. What does tuberculin reactivity after bacille Calmette-Guerin vaccination tell us? Clin. Infect. Dis. 31 Suppl 3:S71-S74.
30. Mori, T., M. Sakatani, F. Yamagishi, T. Takashima, Y. Kawabe, K. Nagao, E. Shigeto, N. Harada, S. Mitarai, M. Okada, K. Suzuki, Y. Inoue, K. Tsuyuguchi, Y. Sasaki, G. H. Mazurek, and I. Tsuyuguchi. 2004. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am. J. Respir. Crit Care Med. 170:59-64.
31. Munk, M. E., S. M. Arend, I. Brock, T. H. Ottenhoff, and P. Andersen. 2001. Use of ESAT-6 and CFP-10 antigens for diagnosis of extrapulmonary tuberculosis. J. Infect. Dis. 183:175-176.
32. Okkels, L. M., I. Brock, F. Follmann, E. M. Agger, S. M. Arend, T. H. Ottenhoff, F. Oftung, I. Rosenkrands, and P. Andersen. 2003. PPE protein (Rv3873) from DNA segment RD1 of Mycobacterium tuberculosis: strong recognition of both specific T cell epitopes and epitopes conserved within the PPE family. Infect. Immun. 71:6116-6123.
33. Pai, M., L. W. Riley, and J. M. Colford, Jr. 2004. Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. Lancet Infect. Dis. 4:761-776.
34. Ravn, P., A. Demissie, T. Eguale, H. Wondwosson, D. Lein, H. A. Amoudy, A. S. Mustafa, A. K. Jensen, A. Holm, I. Rosenkrands, F. Oftung, J. Olobo, F. von Reyn, and P. Andersen. 1999. Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis. J. Infect. Dis. 179:637-645.
35. Scarpellini, P., S. Tasca, L. Galli, A. Beretta, A. Lazzarin, and C. Fortis. 2004. Selected pool of peptides from ESAT-6 and CFP-10 proteins for detection of Mycobacterium tuberculosis infection. J. Clin. Microbiol. 42:3469-3474.
36. Shams, H., P. Klucar, S. E. Weis, A. Lalvani, P. K. Moonan, H. Safi, B. Wizel, K. Ewer, G. T. Nepom, D. M. Lewinsohn, P. Andersen, and P. F. Barnes. 2004. Characterization of a Mycobacterium tuberculosis peptide that is recognized by human CD4+ and CD8+ T cells in the context of multiple HLA alleles. J. Immunol. 173:1966-1977.

37. Shi, L., Y. J. Jung, S. Tyagi, M. L. Gennaro, and R. J. North. 2003. Expression of Th1-mediated immunity in mouse lungs induces a Mycobacterium tuberculosis transcription pattern characteristic of nonreplicating persistence. Proc. Natl. Acad. Sci. U. S. A 100:241-246.
38. Silva, V. M., G. Kanaujia, M. L. Gennaro, and D. Menzies. 2003. Factors associated with humoral response to ESAT-6, 38 kDa and 14 kDa in patients with a spectrum of tuberculosis. Int. J. Tuberc. Lung Dis. 7:478-484.
39. Wu-Hsieh, B. A., C. K. Chen, J. H. Chang, S. Y. Lai, C. H. Wu, W. C. Cheng, P. Andersen, and T. M. Doherty. 2001. Long-lived immune response to early secretory antigenic target 6 in individuals who had recovered from tuberculosis. Clin. Infect. Dis. 33:1336-1340.