Mycobacterium tuberculosis Region of Difference (RD) 2 Antigen Rv1985c and RD11 Antigen Rv3425 Have the Promising Potential To Distinguish Patients with Active Tuberculosis from M. bovis BCG-Vaccinated Individuals

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Antigens encoded in the region of difference (RD) of Mycobacterium tuberculosis constitute a potential source of specific immunodiagnostic antigens for distinguishing tuberculosis (TB) infection from BCG vaccination. We evaluated the diagnostic potential of specific T-cell epitopes selected from two immunodominant antigens, Rv1985c and Rv3425, from RD2 and RD11, respectively, on the basis of epitope mapping, in TB patients and BCG-vaccinated healthy individuals. Using a whole-blood gamma interferon release assay, a wide array of epitopes was recognized on both Rv1985c and Rv3425 in TB patients. Those epitopes that could specifically discriminate TB infection from BCG vaccination were carefully selected, and the most promising peptide pools from Rv1985c showed a sensitivity of 53.9% and a specificity of 95.5%. When the novel specific peptides from Rv1985c joined the diagnostic antigens in the Quantiferon-TB Gold In-Tube (QFT-IT) assay, the sensitivity was increased from 86.4% to 96.2%, with no drop in specificity. These results indicate that the peptide pools selected from Rv1985c and Rv3425 have the potential to diagnose TB infection by a method that may be routinely used in clinical laboratories.

It is estimated that about one-third of the world population, 2 billion people, is currently infected with Mycobacterium tuberculosis. Although only 10% of them may develop clinical disease in their lifetime (1), this huge reservoir has the potential to produce extremely high absolute numbers of tuberculosis (TB) cases and causes serious global health threats. Globally, about 9 million new cases and 2 million deaths are reported each year (2). To control the global epidemic, it is urgent that simple and specific diagnostic methods for both active TB and latent infection be developed.

The tuberculin skin test (TST) has been widely used for diagnosis of latent tuberculosis infection (LTBI). Purified protein derivative (PPD), the antigens used in TST, is a crude mixture of M. tuberculosis antigens, and some of these antigens are cross-reactive with M. bovis BCG and other environmental mycobacteria (3). This broad antigenic cross-reactivity of PPD brings about poor specificity. Comparative genomic studies have identified some segments on the genome, so-called regions of difference (RDs), which are present only in M. tuberculosis and not in M. bovis BCG or most nontuberculosis mycobacteria (NTM) (4, 5). So far, 6-kDa early secreted antigenic target (ESAT-6) and culture filtrate protein 10 (CFP-10), located in RD1, stand out from a number of RD antigens for their promising diagnostic potential in T-cell-based gamma interferon (IFN-γ) release assays (IGRAs) (6–8). TB7.7 (Rv2654; encoded by RD11) is another immunodiagnostic antigen used in the QuantiFERON-TB Gold In-Tube (QFT-IT; Cellestis, Carnegie, Australia) test (7–10). Compared with TST, IGRAs show much lower false-positive rates in BCG-vaccinated individuals and a better correlation with risk factors for infection with M. tuberculosis (11). Furthermore, IGRAs have a higher predictive value for progression to active disease (12). However, the sensitivities of the IGRAs are still suboptimal, and the tests have considerable indeterminate results, particularly in patients with severe TB or immunosuppressive status (13–15).

To search for novel antigens with diagnostic potential, we screened several recombinant RD2 and RD11 antigens for their ability to induce an antigen-specific T-cell response and found that RD2 antigen Rv1985c and RD11 antigen Rv3425 have good diagnostic potential (16, 17). Rv1985c is a putative chromosome replication initiation inhibitor protein which was specifically recognized by both cellular and humoral responses from patients with TB (17). Rv3425 is a member of the proline-proline-glutamate (PPE) family and was found to be a promising antigen in the serodiagnosis of TB (18, 19). Further study showed that the Rv3425 protein as well as recombinant BCG expressing Rv3425 could lead to an increase in the T-cell immune response in mice (20, 21). In this study, we mapped the immunodominant epitopes on Rv1985c and Rv3425 in TB patients and BCG-vaccinated healthy controls using a whole-blood IFN-γ release assay. Then, the peptides containing specific epitopes were selected and the diagnostic potential of the peptide cocktails was evaluated and compared to that of the well-defined QFT-IT assay.

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TABLE 1 Demographic characteristics of total study population

| Study group and characteristic | Screening set | Validation set |
|--------------------------------|---------------|---------------|
|                               | TB | LTBI | HC | No. of subjects | 26 | 22 |
| Mean (range) age (yr)          | 46 (20–75) | 35 (21–40) | 10/12 |
| No. (%) BCG vaccinated         | 21 (80.1) | 19 (86.3) | |

**Materials and Methods**

**Subjects and study design.** For the initial screening of the specific T-cell epitope on Rv1985c and Rv3425, a total of 128 HIV-negative Chinese subjects were recruited into three groups: 42 active TB patients (TB group), 30 individuals presumably with latent tuberculosis infection (LTBI group), and 56 healthy controls (HC group). The demographic characteristics of the study populations are described in Table 1. In the TB group, 42 patients with active TB were recruited from Zhejiang and Zhejiang Pulmonary Hospitals, of which 35 patients were diagnosed with pulmonary TB, 5 were diagnosed with tuberculous pleuritis, and 2 were diagnosed with tuberculous lymphadenitis. Twenty-two of 42 active cases were positive both by culture of *M. tuberculosis* from sputum and by acid-fast bacillus (AFB) smear microscopy, 13 were positive only by culture of *M. tuberculosis*, 2 were positive only by AFB smear microscopy, and 2 were negative by smear and culture results were diagnosed with pulmonary TB on the basis of positive histopathological findings, together with a history of close contact with a TB patient, clinical manifestations, and chest radiography. To minimize the effect of anti-TB treatment on the T-cell response, only patients on standard anti-TB therapy for <3 weeks were included in the study. Among 42 patients, 27 were tested prior to initiation of the therapy, 4 had received therapy for <7 days, 7 had received therapy for 8 to 14 days, and 4 had received therapy for 15 to 21 days. The subjects with LTBI were defined as close TB contacts who had lived with the included TB patients for months before enrollment and had a positive result by the T-SPOT TB or QFT-IT assay. None of the LTBI subjects had clinical symptoms or an abnormal chest X-ray. The HC group consisted of 56 healthy students at Fudan University in China. All of the volunteers underwent QFT-IT tests and were questioned about their history of exposure to TB, and only healthy individuals with no evidence of active TB, no contact with TB patients, and negative QFT-IT test results were enrolled.

**For the next evaluation on the diagnostic potential of the selected peptide pools, another 26 TB patients and 22 healthy controls were subsequently recruited on the basis of the same inclusion criteria and were termed the validation set. Nineteen of 26 active cases were positive both by culture of *M. tuberculosis* from sputum and by AFB smear microscopy, and 7 were positive only by AFB smear microscopy. Among 26 patients, 18 were tested prior to initiation of the therapy; 8 had received therapy for <7 days.**

All of the subjects were age 18 years or over and had given their informed consent before blood withdrawal. This study was approved by the Ethics Committee from Huashan Hospital, Fudan University.

**Rv1985c- and Rv3425-derived peptides.** Thirty-one overlapping peptides (25-mers) spanning the complete primary sequence of Rv1985c and Rv3425 were designed and purchased (Sangon, Shanghai). Each peptide was 25 amino acids (aa) long and overlapped its adjacent peptide by 10 aa (Fig. 1). The identity of each peptide was confirmed by mass spectrometry, and the purity (>90%) was checked by high-pressure liquid chromatography. The stock solution of the peptides was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml, and the peptides were further diluted to a working concentration in RPMI 1640 tissue culture medium (Life Technology).

**Whole-blood IFN-γ release assay.** For detecting the T-cell response to the peptide mixtures and 31 individual peptides of Rv1985c and Rv3425, the 1:10-diluted whole-blood assay was performed as described by Weir et al. (22) to measure the T-cell response to the antigens. Briefly, whole blood was collected via venipuncture and drawn into heparinized Vacutainer tubes (Becton, Dickinson). Within 8 h, whole blood was diluted 1:10 with RPMI 1640 tissue culture medium supplemented with 40 μg/ml streptomycin, 40 U/ml penicillin, and 0.04 mM glutamine (Life Technologies Laboratory, Paisley, United Kingdom) and cultured in 96-well tissue culture plates in a total volume of 200 μl with 2.5 μg/ml of phytohemagglutinin (PHA), 5 μg/ml of PPD (RT23; Statens Serum Institute, Copenhagen, Denmark), or 10 μg/ml of the individual tested peptide. After incubation at 37°C for 5 days, serum from cultured cells was collected and stored at −20°C for testing.

For stimulation with the selected peptide pools from Rv1985c and Rv3425, a 500-μl aliquot of undiluted whole blood was dispensed into the wells of a 48-well tissue culture plate. PHA (2.5 μg/ml) and saline were added as a positive control and a negative control, respectively. Peptide cocktails from ESAT-6, including ESAT-6 from residues 1 to 20 (ESAT-6_1–20), ESAT-6_21–40, ESAT-6_41–60, and ESAT-6_61–80, were selected according to some other research (23, 24) and tested as controls to evaluate the diagnostic performance of the selected peptide pools. On the basis of a dose-response curve ranging from 0 to 20 μg/ml, the working concentration of the peptide mixtures was determined at the concentration with the strongest response, which was 1 μg/ml per peptide. The plate was incubated for 20 h at 37°C. Plasma was collected after centrifugation and stored at −20°C for IFN-γ quantification. All the antigens in our study were tested in triplicate.

**Determination of IFN-γ release.** The IFN-γ levels in supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available agents (Biologend), as per the manufacturer’s instructions. The detection limit of the assay was 1 pg/ml. An antigen- or peptide-specific response was shown as the change in levels (level of IFN-γ release in the antigen-stimulated blood minus that in the unstimulated blood). A positive response to peptide mixtures or individual peptides in 1:10-diluted whole blood was determined when the value was >40 pg/ml, which was the mean IFN-γ release value in unstimulated wells plus 2 standard deviations (20 ± 10 pg/ml). The cutoff value of the peptide cocktails was determined by a receiver operating characteristic curve constructed on the basis of our data, estimated at various sensitivities and specificities, and determined at the maximum Youden’s index (YI), i.e., sensitivity + specificity − 1 (25).

**QFT-IT assay.** The QFT-IT assay was performed according to the manufacturer’s instructions. In total, 1 ml of whole blood was drawn into three QFT-IT assay tubes coated with saline (nil control), *M. tuberculosis*-specific antigens ESAT-6, CFP-10, and TB7.7 (TB antigen), or PHA (mitogen control), respectively, and incubated at 37°C for 24 h. After centrifugation, plasma was harvested from each tube for IFN-γ quantification. IFN-γ was measured by ELISA according to the manufacturer’s instructions (Cellestis). The results were generated using the QFT-IT assay analysis software (version 2.50). The result of IFN-γ release was presented as IU/ml.

**HLA-DR typing.** A PCR-based sequence-specific primer (SSP) method was used for HLA typing. In brief, DNA was isolated from the whole blood of all subjects using a QIAamp system. An HLA-DR low-resolution kit (Invitrogen) was used to type for the DRB1, DRB3, DRB4, and DRB5 alleles. Amplification of DNA and analysis by gel electrophoresis were carried out as specified by the manufacturer. Serologically defined HLA-DR specificities were determined from the genotypes by following the guidelines provided by Invitrogen.

**TABLE 1** Demographic characteristics of total study population

| Study group and characteristic | Screening set | Validation set |
|--------------------------------|---------------|---------------|
|                               | TB | LTBI | HC | No. of subjects | 26 | 22 |
| Mean (range) age (yr)          | 46 (20–75) | 35 (21–40) | 10/12 |
| No. (%) BCG vaccinated         | 21 (80.1) | 19 (86.3) | |
Statistical analysis. The positive rates in the different groups were compared using Pearson’s chi-square test or Fisher’s exact test, and the difference between paired proportions was tested by McNemar’s test. The level of IFN-γ release in the different groups was analyzed by Kruskal-Wallis one-way analysis with Dunn’s posttest. The correlations of the peptide responses were analyzed with a two-tailed nonparametric correlation test (Spearman). SPSS (version 14) and Prism (version 5.0) software (GraphPad Software, San Diego, CA) were used for the analysis.

RESULTS

T-cell response to peptide mixtures of Rv1985c and Rv3425. The T-cell recognition of Rv1985c and Rv3425 was first evaluated by measuring and comparing the IFN-γ release levels after stimulation by the peptide mixtures from Rv1985c and Rv3425 using 1:10-diluted whole blood and an incubation time of 5 days. The results are summarized in Table 2. There was no significant difference in the positive response rate between the TB and LTBI groups. After combining the responses for the TB and LTBI groups, Rv1985c and Rv3425, respectively, identified 66.7% (44/66) and 60.9% (28/46) of the subjects with TB infection. PPD was recognized by T cells from 92.5% and 84.6% of the subjects in the TB and LTBI groups, respectively, and also recognized by T cells from 76.8% of the subjects in the HC group.

On the other hand, although recognition of Rv1985c and Rv3425 was not entirely specifically related to TB infection, as

| Peptide No. | aa sequence | Last aa |
|-------------|-------------|---------|
| 1           | MVDPQLDGPQLAALAAAVVEGSFD | 25 |
| 2           | AVDELGFAAERLHVTPAVSQ | 40 |
| 3           | HVTSPAVSQRKSLQQQQVQVLVR | 55 |
| 4           | VQQGQLVRLRCPWRAQTAGIPLR | 70 |
| 5           | ATTAGPILLRAATTTLESEALAE | 85 |
| 6           | ALLESEAEAMGNASLKRTRITIA | 100 |
| 7           | SLKRTRITIAVADSMTWFSAVFD | 115 |
| 8           | MATWFSAVFDGLDVLLDVREDDQ | 130 |
| 9           | LLQVREIQDSRALLRESGMAVAG | 145 |
| 10          | LREGVGAVYTERNPVPGCHVPL | 160 |
| 11          | IPVGRVHPILGEMRYLPVSPRFPVQ | 175 |
| 12          | LPVSPRFVQRHLDGFTAAAKA | 190 |
| 13          | GFTAAAKAPSLAWDDGLQDML | 205 |
| 14          | NRDQGLQLMVRKAFRAIRFPH | 220 |
| 15          | RRAITRTPTHFVPTTEGFAARAGL | 235 |
| 16          | GFTAAARGLWGMFPEKLASSPLA | 250 |
| 17          | PKEKLASSPLADGSFVRCDHLDVP | 265 |
| 18          | RVCDDHLDVPLYQCGWKLDSPIAR | 280 |
| 19          | WKLDSPIARITDORVAAAGLYRG | 295 |
| 20          | RAAASGLYRGQQRRRPG | 303 |

FIG 1 Amino acid sequences of 31 synthetic overlapping peptides covering the entire sequences of Rv1985c and Rv3425. Amino acid sequences are shown in single-letter code from the N terminus to the C terminus.

### TABLE 2 Antigen-induced IFN-γ release by whole blood from TB, LTBI, and HC groups in response to peptide mixtures of Rv1985c and Rv3425

| Antigen | TB group (n = 40) | LTBI group (n = 26) | HC group (n = 56) |
|---------|------------------|---------------------|------------------|
|         | No. positive/total (%) | Median IFN-γ release (pg/ml) (25th–75th percentile) | No. positive/total (%) | Median IFN-γ release (pg/ml) (25th–75th percentile) | No. positive/total (%) | Median IFN-γ release (pg/ml) (25th–75th percentile) |
| PPD     | 37/40 (92.5) | 183.5 (3.0–1,859.0) | 22/26 (84.6) | 330.7 (116.0–2,094) | 43/56 (76.8) | 398.1 (230.8–472.2) |
| Rv1985c | 28/40 (70.0) | 58.1 (27.3–167.0) | 16/26 (61.5) | 47.1 (27.4–131.5) | 20/56 (35.7) | 62.4 (8.4–109.7) |
| Rv3425  | 18/28 (64.3) | 66.6 (25.0–268.2) | 10/18 (55.6) | 62.4 (16.3–336.9) | 20/56 (35.7) | 22.7 (8.4–109.7) |

a Using a cutoff of >40 pg/ml IFN-γ.

b Data are in pg/ml.

c Statistically significantly higher IFN-γ release than the HC group by two-tailed Mann Whitney test, \( P < 0.001 \).

d Statistically significantly higher IFN-γ release than the HC group by two-tailed Mann-Whitney test, \( P < 0.01 \).
both antigens harbored peptides which could induce IFN-γ release in the HC group, the strength of the IFN-γ response was significantly lower in the HC group than the TB or LTBI group (Table 2). Given that there was no significant difference between the TB and LTBI groups in either the positive rate or the stimulated release of IFN-γ, we took these two groups to be the TB infection group throughout the following experiments.

Mapping of the T-cell epitopes on Rv1985c and Rv3425 in subjects with TB infection. In search of the epitopes on both tested antigens specifically recognized by T cells, we used 1:10-diluted whole blood from the subjects in the TB infection group who responded to Rv1985c and Rv3425 peptide mixtures (44 subjects for Rv1985c and 28 subjects for Rv3425) to test their reactivity against 31 individual peptides covering the complete sequence of the two antigens. The results showed that the epitopes on Rv1985c clustered at the middle (peptide 5 [P5] to P10) and carboxy terminus (P14 to P20) of the molecule (Fig. 2A). Peptide 19 was the most frequently recognized, being recognized in 75.0% (33/44) of the TB subjects, and P6, P7, P9, P14, P15, P19, and P20 were recognized in >50% of subjects. For Rv3425, P21 and P23 were the most frequently recognized peptides, with recognition frequencies of 75.0% and 82.1%, respectively. Those peptides with a recognition frequency of >50% also induced relatively high levels of IFN-γ by specific T cells. There appeared to be a wide array of T-cell epitopes on both Rv1985c and Rv3425. On the other hand, there were no significant differences between the TB and LTBI groups either in the recognition frequency or in the strength of the T-cell response against the peptide (data not shown).

HLA-DR typing of the subjects who responded to at least one of the peptides demonstrated that they represented a heterogeneous group of HLA-DR molecules encoded by HLA-DRB1, -DRB3, -DRB4, and -DRB5 genes, including HLA-DR1, -DR2, -DR3, -DR4, -DR5, -DR6, -DR7, -DR8, -DR9, -D51, -DR52, and -DR53. The result indicated the permissive nature of immunodominant peptides within Rv1985c and Rv3425 in recognition by subjects with TB infection.

Mapping of T-cell epitopes on Rv1985c and Rv3425 in BCG-vaccinated healthy people. To identify specific peptides of the diagnostic antigens, we further characterized the cross-reactivity of the T-cell epitopes on Rv1985c and Rv3425. The T-cell responses to 31 peptides in the HC group (23 subjects for Rv1985c and 20 subjects for Rv3425) were analyzed and compared with those in subjects with TB infection. These results revealed that both Rv1985c and Rv3425 harbored peptides that could induce IFN-γ release in healthy individuals. However, unlike the TB infection group, only a few peptides induced such a response in the HC group (Fig. 2).

In the Rv1985c peptide cocktail, P6, P7, P14, P19, and P20 were recognized by more than 50% of the controls and induced a relatively high level of IFN-γ, and those peptides were scattered throughout the molecule of Rv1985c. For Rv3425, strong recognition of P21, P22, and P23, in the N-terminal part of the mole-
A specific peptide pool of Rv1985c and Rv3425 was found. For some peptides, such as P6, P7, P19, P20, and P23, the intensity of the reaction and the recognition frequency in the HC group were equal to or even higher than those in the TB infection group. When we divided the healthy controls into two groups (those with and those without BCG vaccination), no significant difference either in the positive rate or in the amount of IFN-γ released by antigen-specific T cells was observed between them (data not shown).

According to the data for the HC group, specific peptides without a cross-reactive T-cell response were cautiously selected. Peptides that were recognized by more than two healthy subjects and induced an IFN-γ release of over 400 pg/ml in any control were excluded. Four peptide cocktails were chosen for Rv1985c: Rv1985c pool1 contained P2 to P4, Rv1985c pool2 contained P8 to P10, Rv1985c pool1 contained P11 to P13, and Rv1985c pool4 contained P15 and P16. For Rv3425, the C-terminal region, including P27 to P31, was chosen to make a cocktail, Rv3425 pool (Fig. 2B).

Diagnostic potential of the selected peptide cocktails. To validate the diagnostic potential of the selected peptide cocktails, we recruited 48 different individuals, consisting of 26 TB patients and 22 QFT-IT assay-negative healthy controls. Undiluted whole blood individually stimulated by the five selected peptide pools was incubated for a shorter time (20 h) to detect antigen-specific IFN-γ release, and the same procedures used for the QFT-IT assay were followed. We also tested a peptide pool from ESAT-6 (ESAT-6 pool) to verify our whole-blood detection system and for comparison with our new selected peptide pools. The peptide pool from ESAT-6 has a sensitivity of 70% and a specificity of 90%, according to other research (23, 24). The related IFN-γ levels and the diagnostic value are listed in Table 3. The QFT-IT assay achieved a sensitivity of 84.6%. Among the peptide cocktails, ESAT-6 pool and Rv1985c pool4 were recognized by 65.4% and 53.9% of TB patients, respectively. They also achieved high specificities of 100% and 95.5%, respectively, for healthy controls. Meanwhile, Rv1985c pool2, Rv1985c pool3, and Rv3425 pool4 achieved moderate sensitivities of 46.2%, 42.3%, and 46.2%, respectively, whereas their specificities were over 95%. However, the diagnostic potential of Rv1985c pool1 turned out to be poor (sensitivity, 23.1%; specificity, 95.5%), which was not consistent with the results from the individual peptides.

In addition to recognition frequency, the strength of the response of IFN-γ to different peptide pools was also compared by the Kruskal-Wallis test. ESAT-6 pool and Rv1985c pool4 showed the strongest response, with no significant difference between them. In contrast, Rv1985c pool1, Rv1985c pool2, Rv1985c pool3, and Rv3425 pool4 gave rise to lower levels of IFN-γ release than ESAT-6 pool and Rv1985c pool4. No significant difference was found among Rv1985c pool1, Rv1985c pool2, Rv1985c pool3, and Rv3425 pool4.

We further combined the promising peptides in such a way that a combination could really improve the sensitivity with the least drop in specificity. The parallel combination strategy was used, in which a positive result was determined when at least one of the tests was positive, and a negative result was defined only when all the tests were negative. For the 26 TB patients and 22 QFT-IT assay-negative healthy controls, the combination of Rv1985c pool4 and ESAT-6 pool could detect 84.6% of TB patients. The use of Rv1985c pool4 and the QFT-IT assay had a sensitivity of 92.3%, and the use Rv1985c pool2 and Rv1985c pool4 with the QFT-IT assay achieved the highest sensitivity of 96.2%. On the other hand, the specificity of all of the combinations was >95% (Table 4).

**DISCUSSION**

Differentiation of *M. tuberculosis* infection in BCG-vaccinated populations may rely on finding pathogen-specific diagnostic antigens other than PPD, which fails to distinguish *M. tuberculosis* from *M. bovis* BCG. The proteins encoded in the regions of differences (RDs) of *M. tuberculosis* have become the most promising candidates as diagnostic antigens. Our previous study demonstrated that recombinant antigens of Rv1985c and Rv3425 had great potential for specific immune-based diagnosis of TB infection in the BCG-vaccinated population (16, 17). In this study, 25-amino-acid-long peptides covering the whole length of the two antigens were first tested for their ability to stimulate IFN-γ release. It was found that mixtures of synthetic overlapping peptides had potency equivalent to that of the whole-protein antigens (26). However, the result was not completely consistent with our previous work, in that the IFN-γ response to peptides of both Rv1985c and Rv3425 was found in BCG-vaccinated healthy controls but was significantly lower than that in the subjects with TB infection.

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**TABLE 3** Diagnostic performance and IFN-γ release of the selected peptide pools from Rv1985c and Rv3425 in TB patients and healthy controls

| Antigens | No. positive/no. | Sensitivity | Median IFN-γ release | Specificity | Median IFN-γ release |
|----------|-----------------|-------------|----------------------|-------------|----------------------|
|           | tested | (%) | (25th–75th percentile) | (%) | (25th–75th percentile) |
| Rv1985c pool1 | 6/26 | 23.1 | 9.0–43.7 | 12/26 | 95.5 | 77.2–99.9 |
| Rv1985c pool2 | 12/26 | 46.2 | 26.6–66.6 | 2/26 | 100.0 | 82.5–100.0 |
| Rv1985c pool3 | 11/26 | 42.3 | 23.4–63.1 | 0/22 | 95.5 | 77.2–99.9 |
| Rv1985c pool4 | 14/26 | 53.9 | 33.4–73.4 | 1/22 | 95.5 | 77.2–99.9 |
| Rv3425 pool | 12/26 | 46.2 | 26.6–66.6 | 2/26 | 100.0 | 82.5–100.0 |

**a** The IFN-γ response stimulated by the selected peptide pools was detected using an undiluted-whole-blood assay and a 20-h incubation time. The cutoff value was determined by receiver operating characteristic curve analysis: Rv1985c pool1, 32.6 pg/ml; Rv1985c pool2, 14.2 pg/ml; Rv1985c pool3, 20.7 pg/ml; Rv1985c pool4, 35.0 pg/ml; Rv3425 pool, 30.8 pg/ml; ESAT-6 pool, 30.5 pg/ml.

**b** Sensitivity was calculated as the percentage of responding patients out of all TB patients tested.

**c** Specificity was calculated as the percentage of negative healthy controls out of all healthy control subjects tested.

**d** Data are in pg/ml.

**f** Statistically significantly lower sensitivity than ESAT-6 pool by McNemar’s test, *P* = 0.05.

**g** Statistically significantly lower IFN-γ release than ESAT-6 pool by two-tailed Mann-Whitney test, *P* < 0.01.

**CI**, confidence interval.
infection (Table 2). This may suggest that a certain degree of cross-reactivity to those epitopes could exist in the BCG-vaccinated subjects. Many factors could contribute to the difference, including the different method used to detect IFN-γ release, the different incubation time, as well as the cutoff value used for a positive response. 

Rv1985c, encoded in RD2, was absent from 8 M. bovis BCG strains analyzed, including the BCG-Danish strain, which is used most frequently for vaccination in China (4). When we divided the healthy controls into two subgroups (those who had received and those who had not received BCG vaccination), there was no significant difference between subgroups in either the positive rate or the amount of IFN-γ release in response to Rv1985c stimulation (data not shown). Thus, it is unlikely that the strong recognition of Rv1985c-derived peptides in BCG-vaccinated controls was due to cross-reactivity with BCG strains. A search of the genome from the publicly available mycobacterial database identified Rv1985c homologues in only some nonpathogenic mycobacteria, including M. smegmatis, M. vanbaalenii, and M. gilvum. Rv3425 is a protein from the large PPE family. Although PPE protein Rv3873 from RD1 was found to have epitopes highly conserved in a large number of PPE family members from various species of mycobacteria (27, 28), those highly conserved epitopes were not found in Rv3425. Moreover, BLAST searches against available mycobacterial databases have shown that the region encoding Rv3425 is absent from all M. bovis BCG strains (4) and all environmental mycobacterial genomes searchable at present. Thus, it was speculated that the antigens shared epitopes with organisms unrelated to M. tuberculosis, which led to the positive response in the HC group. This is in agreement with previous observations on some other diagnostic RD antigens (9, 10, 28).

In the present study, cell-mediated immune responses were monitored by using an in vitro IFN-γ release assay with whole blood. The whole-blood assays are easy to perform and require small quantities of blood, so that this method could be used in a situation in which TB is endemic (29, 30). In our study, two different whole-blood assays were used. For mapping the T-cell epitopes on Rv1985c and Rv3425, we used 1:10-diluted whole blood and an incubation time of 5 days to detect antigen-specific T-cell responses; this assay was first described by Weir et al. (22). The method was proved to be sensitive enough to detect the T-cell recognition of different antigens by measuring cytokines such as IFN-γ (22). More importantly, it allowed us to test the 31 individual peptides and controls in triplicate using a very small quantity of blood, which could easily be obtained from the subjects by venipuncture with few compliance problems. However, when assessing the diagnostic ability of the 5 selected peptide pools, we chose undiluted whole blood and a relatively short incubation time (20 h), the same experimental conditions used for the commercial QFT-IT assay, which made it possible to equally compare our peptide pools with the diagnostic antigens in the QFT-IT assay.

It is well-known that recognition of mycobacterial antigens and peptides by CD4+ T cells in peripheral blood is mostly restricted by the HLA-DR molecule (31, 32). Recognition by an HLA-heterogeneous group of donors is an essential requirement for new antigens to qualify as diagnostic reagents and vaccine candidates. HLA-DR typing of the donors showed that immunodominant epitopes of Rv1985c and Rv3425 were recognized by T cells from individuals with various HLA backgrounds which represented all the frequently expressed HLA-DR molecules. These results were encouraging and suggested that both Rv1985c and Rv3425 have genetically promiscuous epitopes that can be recognized by a high proportion of donors.

Although the QFT-IT assay had a high sensitivity of 84.6% in the present study, which is consistent with other research in a BCG-vaccinated population (11), 15.4% (4/26) of the patients still did not respond to ESAT-6, CFP-10, and TB7.7. Our result suggested that the sensitivity of the QFT-IT assay could be raised when it was used in combination with Rv1985c peptide pools and Rv1985c pool4, just as previous studies did (10, 16, 17). Though the additive effect on sensitivity was not statistically significant, the trend may be more obvious and relevant if tested on a broader scale. It was noticed that when the QFT-IT assay was combined with Rv1985cpool2 and Rv1985cpool4, just as previous studies did (10, 16, 17).

| Table 4: Diagnostic performance of the selected peptide pools in combination with QFT-IT assay |
|---------------------------------------------|
| Antigen combination | TB patients (n = 26) | Healthy controls (n = 22) |
| | No. positive/no. tested | Sensitivity (%) | No. positive/no. tested | Specificity (%) |
| Rv1985cpool2 + ESAT-6pools | 22/26 | 84.6 (65.1–95.6) | 1/22 | 95.5 (77.2–99.9) |
| Rv1985cpool2 + ESAT-6pools | 20/26 | 76.9 (56.4–91.0) | 0/22 | 100.0 (82.5–100.0) |
| Rv3425pool + ESAT-6pools | 19/26 | 73.1 (52.2–88.4) | 1/22 | 95.5 (77.2–99.9) |
| QFT-IT assay | 22/26 | 84.6 (65.1–95.6) | 0/22 | 95.5 (77.2–99.9) |
| Rv1985cpool4 + QFT-IT assay | 24/26 | 92.3 (74.9–99.1) | 1/22 | 95.5 (77.2–99.9) |
| Rv1985cpool4 + QFT-IT assay | 23/26 | 88.5 (69.9–97.6) | 0/22 | 100.0 (82.5–100.0) |
| Rv1985cpool4 + Rv1985cpool4 + QFT-IT assay | 25/26 | 96.2 (80.4–99.9) | 1/22 | 95.5 (77.2–99.9) |

*The IFN-γ response stimulated by the peptide pools was measured using undiluted whole blood and a 20-h incubation time.

*Sensitivity was calculated as the percentage of subjects positive for either test out of all TB patients. Data in parentheses represent 95% confidence intervals.

*Specificity was calculated as the percentage of subjects negative for all tests combined out of all healthy controls. Data in parentheses represent 95% confidence intervals.

*d = only QFT-IT-negative healthy controls were included in this study.
duced as a result of the selection criteria used for the LTBI and HC groups, which were mainly dependent on a positive or a negative QFT-IT assay response. In addition, the prognostic values of the tested peptides could be introduced to predict the possibility of progression to active TB disease (12, 14). Further study is still required to confirm the diagnostic performance of the selected peptides.

In conclusion, this is the first study to map the T-cell epitopes of Rv1985c and Rv3425 recognized by specific T cells in donors with TB infection and healthy controls. The whole-blood IFN-γ release assay suggested that among selected peptide pools containing specific T-cell epitopes, Rv1985c_poo2, Rv1985c_poo4, and Rv3425_poo4 showed great potential for specific diagnosis of TB infection in the BCG-vaccinated population since they could improve the sensitivity when combined with the QFT-IT assay. The result demonstrated that selected peptide pools from Rv1985c and Rv3425 are useful supplements in a diagnostic protein mixture.

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