Selected RD1 Peptides for Active Tuberculosis Diagnosis: Comparison of a Gamma Interferon Whole-Blood Enzyme-Linked Immunosorbent Assay and an Enzyme-Linked Immunospot Assay

Delia Goletti, Donatella Vincenti, Stefania Carrara, Ornella Butera, Federica Bizzoni, Giuliana Bernardini, Massimo Amicosante, and Enrico Girardi

Second Division, Health Department of the National Institute for Infectious Diseases (INMI) Lazzaro Spallanzani, Translational Research Unit, INMI, Department of Internal Medicine, University of Tor Vergata, and Epidemiology Department, INMI, Rome, and Division of Infectious Diseases, Ospedale Belcolle, Viterbo, Italy

Received 23 June 2005/Returned for modification 2 August 2005/Accepted 25 August 2005

We recently set up a gamma interferon (IFN-γ) enzyme-linked immunospot assay (ELISPOT), using selected early secreted antigenic target 6 (ESAT-6) peptides, that appears specific for active tuberculosis (A-TB). However, ELISPOT is difficult to automate. Thus, the objective of this study was to determine if the same selected peptides may be used in a technique more suitable for routine work in clinical laboratories, such as whole-blood enzyme-linked immunosorbent assay (WBE). For this purpose, 27 patients with A-TB and 41 control patients were enrolled. Our WBE, using the already described selected peptides from ESAT-6 plus three new ones from culture filtrate protein 10, was performed, and data were compared with those obtained by ELISPOT. Using our selected peptides, IFN-γ production, evaluated by both WBE and ELISPOT, was significantly higher in patients with A-TB than in controls (P < 0.0001). Statistical analysis showed a good correlation between the results obtained by WBE and ELISPOT (r = 0.80, P < 0.001). To substantiate our data, we compared our WBE results with those obtained by QuantiFERON-TB Gold, a whole-blood assay based on region of difference 1 (RD1) overlapping peptides approved for TB infection diagnosis. We observed a slightly higher sensitivity with QuantiFERON-TB Gold than with our WBE (89% versus 81%); however, our test provided a better specificity result (90% versus 68%). In conclusion, results obtained by WBE based on selected RD1 peptides significantly correlate with those generated by ELISPOT. Moreover, our assay appears more specific for A-TB diagnosis than QuantiFERON-TB Gold, and thus it may represent a complementary tool for A-TB diagnosis for routine use in clinical laboratories.
overlapping peptides spanning the whole proteins and employ
enzyme-linked immunosorbent and enzyme-linked immuno-
spot assays (ELISA and ELISPOT), respectively. These over-
lapping peptides are 15 amino acids long (16) or have a vari-
able length of 16 to 26 amino acids (10, 17). Although these
commercial assays provide an accurate diagnosis of M. tuber-
culosis infection, they do not discriminate between A-TB and
LTBI. Thus, further clinical workup is required to rule out
A-TB after a positive response to these tests.

In this context, we have recently set up an IFN-γ ELISPOT
based on multiepitopic peptides from the ESAT-6 protein,
selected by quantitative implemented human leukocyte anti-
gen (HLA) peptide-binding motif analysis. The sensitivity of
this assay for A-TB is 74% (29). More importantly, the positive
response fell below the detection level after 3 months of
anti-TB therapy in those responding to treatment (7). None of
the control patients, including those who were TST positive,
responded to the selected peptides (29). This test has the
potential of being a very useful assay to detect A-TB; however,
ELISPOT is difficult to automate because it requires many
laboratory steps, such as the separation of cells from blood and
the counting of cells and spots. For these reasons, ELISPOT is
not suitable for routine use in diagnostic laboratories.

Recently, Schöövinck et al. (23) demonstrated that a sensi-
tive IFN-γ WBE using intact ESAT-6/CFP-10 (RD1) proteins
matched the performance of the ELISPOT. We therefore eval-
uated the performance of our selected RD1 peptides in the
identification of A-TB using a WBE and compared the results
with those obtained by the ELISPOT. The RD1 peptides used
in our test included two that were previously described, se-
lected from ESAT-6 (7, 29), and three that were newly selected
from CFP-10. To substantiate our data, the results obtained
by the two assays were compared with those obtained with
QuantiFERON-TB Gold, a WBE approved for in vitro im-
munological diagnosis of TB infection, based on the use of
RD1 overlapping peptides (10, 19).

MATERIALS AND METHODS

Patient population and study design. Patients that were admitted to the
infectious disease and respiratory disease wards at the National Institute
for Infectious Diseases (INMI) L. Spallanzani in Rome, Italy, between April
2004 and November 2004 were evaluated for enrollment. Subjects were included in
the study if they tested human immunodeficiency virus negative and were not
undergoing cortisone therapy. None of them were undergoing anti-TB therapy at
the time of enrollment.

Enrolled patients underwent clinical and microbiological examinations to con-
firm or exclude the diagnosis of TB. Briefly, sequential respiratory samples (three
expectorated or two induced sputa) were collected over the first 7 days following
hospital admission. Acid-fast bacillus smear (Ziehl-Neelsen) and culture (on
Lowenstein-Jensen and BACTEC 460 media [BD Biosciences Division, Sparks,
Md.] and Middlebrook 7H10/7H11 media) were performed with each specimen.
Chest X rays were done for each patient. Patients underwent TST administered
by the Mantoux method, and those with an induration of ≥10 mm were classified
as TST positive (1, 11, 15).

Enrolled patients were classified as “A-TB” if the diagnosis was confirmed by a
positive M. tuberculosis culture of biological specimens. For controls, we se-
lected patients with diseases other than A-TB who had come to a resolution of
clinical symptoms after antibiotic therapy not involving M. tuberculosis-active
drugs and whose sputum culture for M. tuberculosis was negative.

Within 7 days of admission, two blood samples, one in EDTA (for ELISPOT)
and one in heparin (for WBE), were drawn from all enrolled individuals. Our
WBE and ELISPOT were performed in parallel with a QuantiFERON-TB Gold
assay. Clinicians were blinded to the results of in vitro assays, and laboratory
personnel were blinded to the status of the patient.

The study was approved by the ethics committee at our institution, and all
enrolled patients provided informed written consent.

In vitro assays for TB infection. (i) WBE and ELISPOT based on selected RD1
peptides. The selection of HLA class II-restricted epitopes of ESAT-6 and CFP-10 M. tuberculosis proteins was performed by quantitative implemented
HLA peptide-binding motif analysis as previously described for ESAT-6 (6, 7, 26,
29). Peptides were synthesized as free amino acid termini using 9-fluorenylme-
thyloxy carbonyl chemistry (ABI, Bergamo, Italy). Lyophilized peptides were di-
luted in dimethyl sulfoxide at stock concentrations of 10 mg/ml for each peptide
and stored at −80°C. RD1 peptides used in this study are described in Table 1.
Each selected peptide (Table 1) contained one or more epitopes able to bind at

<table>
<thead>
<tr>
<th>Protein</th>
<th>Position</th>
<th>Sequence</th>
<th>DR serological specificities covereda</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESAT-6</td>
<td>6–28</td>
<td>WNFAGIEAAASAOGNVTSHSL</td>
<td>1, 3, 4, 8, 11(5), 13(6), 52, 53</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>66–78</td>
<td>NALONLARTISEA</td>
<td>3, 8, 11(5), 13(6), 15(2), 52</td>
</tr>
<tr>
<td>CFP-10</td>
<td>18–31</td>
<td>FERISGDLKTQIDO</td>
<td>3, 5, 11(5), 52</td>
</tr>
<tr>
<td>CFP-10</td>
<td>43–70</td>
<td>GOWRGAAGTAAAQAVRFQEANQKQKE</td>
<td>1, 3, 4, 7, 8, 11(5), 13(6), 15(2), 52</td>
</tr>
<tr>
<td>CFP-10</td>
<td>74–86</td>
<td>TNIROAGVQYSSRA</td>
<td>3, 4, 7, 8, 11(5), 12(5), 13(6), 15(2)</td>
</tr>
</tbody>
</table>

a HLA class II alleles putatively able to recognize the selected ESAT-6 and CFP-10 peptides are listed according to serological specificity, i.e., the group of alleles
recognized by the same allo-antisera used for low-resolution HLA typing. Where necessary, the serological HLA class II subgroup to which the HLA-DR allele that
is putatively able to recognize the peptide belongs is shown in parentheses.

TABLE 2. Epidemiological and demographic characteristics of
enrolled subjectsa

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value for group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active TB</td>
</tr>
<tr>
<td></td>
<td>(microbiological</td>
</tr>
<tr>
<td></td>
<td>diagnosis)</td>
</tr>
<tr>
<td>Total no. of patients</td>
<td>27</td>
</tr>
<tr>
<td>Mean age (yr) ± SE</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>7</td>
</tr>
<tr>
<td>Males</td>
<td>20</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>Western Europe</td>
<td>5</td>
</tr>
<tr>
<td>Non-Western Europe</td>
<td>22</td>
</tr>
<tr>
<td>Diseases other than TB</td>
<td></td>
</tr>
<tr>
<td>Bacterial pneumonia</td>
<td>8</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>5</td>
</tr>
<tr>
<td>Fever of unknown origin</td>
<td>2</td>
</tr>
<tr>
<td>Bacterial abscess</td>
<td>1</td>
</tr>
<tr>
<td>Bacterial pleuritis</td>
<td>1</td>
</tr>
<tr>
<td>Pulmonary carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Pseudotumor</td>
<td>1</td>
</tr>
</tbody>
</table>

a Abbreviations: TB, tuberculosis; TST, tuberculin skin test; SE, standard error; BCG, bacillus Calmette et Guerin.
least (i) four different HLA-DR, (ii) two different HLA-DP, and (iii) two different HLA-DQ specificities with a putative binding ability of 80% of the maximum binding for any allele belonging to an HLA class II serological specificity. Together, the designed peptide epitopes were able to cover more than 90% of the HLA class II haplotypes present in different human populations (data not shown). Sequence homology searches of all known protein sequences confirmed that the sequences of the selected peptides were uniquely restricted to M. tuberculosis complex proteins.

Samples were analyzed in parallel by WBE and ELISPOT. For the 40-hour ELISPOT, the PBMC were treated as previously described (29). For WBE, 0.5 ml per well of heparinized blood was seeded in a 48-well plate and treated with selected RD1 peptides and phytohemagglutinin (Sigma, St. Louis, MO) at the same concentration used in ELISPOT (29). Samples were then incubated for 24 h at 37°C in the presence of 5% CO2. IFN-γ levels in culture supernatants were assessed by a commercially available kit (QuantiFERON-CMI kit; Cellestis Limited, Carnegie, Victoria, Australia). Cutoff values were determined by constructing a receiver operator characteristic (ROC) curve by means of LABROC-1 software. The cutoff values that we used in the ELISPOT, expressed as spot-forming cells (SFC)/million PBMC, were 60 for phytohemagglutinin and 34 for selected RD1 peptides, and the cutoff value for all stimuli in WBE experiments was 0.6 IU/ml.

IFN-γ production (IU/ml) in response to selected RD1 peptides evaluated by WBE was significantly higher in patients with A-TB (5.5 ± 1.3 IU/ml) than in controls (0.95 ± 0.7 IU/ml) \((P < 0.0001)\). This difference was statistically significant independently of the TST status of controls, either positive (1.7 ± 1.3 IU/ml, \(P < 0.0001\) versus A-TB) or negative (0.1 ± 0.03 IU/ml, \(P < 0.0001\) versus A-TB) (Fig. 1A). Similarly, the IFN-γ production in response to the same peptides, evaluated by ELISPOT and expressed as SFC per million PBMC, was significantly higher in patients with A-TB (156.8 ± 26.8 SFC) than in controls (23.8 ± 10 SFC, \(P < 0.0001\)). This difference also was statistically significant independently of the...
TST status of controls, either positive (39.7 ± 19.5 SFC, \( P < 0.0001 \) versus A-TB) or negative (7.2 ± 1.8 SFC, \( P < 0.0001 \) versus A-TB) (Fig. 1B). When the response to selected RD1 peptides obtained by WBE was compared to that found by ELISPOT, the number of SFC per million PBMC counted by ELISPOT correlated significantly with the IU/ml measured by WBE (\( r = 0.80, P < 0.001 \)) (Fig. 1C).

In terms of results scoring, in WBE experiments the response to selected RD1 peptides was scored positive in 22/27 (81%) A-TB patients and in 4/41 (10%, \( P < 0.0001 \)) controls (4/21 among TST-positive individuals, \( P < 0.0001 \); 0/20 among TST-negative individuals, \( P < 0.0001 \)). Thus, in the population studied, the sensitivity of the WBE for A-TB was 81% and the specificity was 90% (Table 3). ELISPOT responses were scored positive in 20/27 (74%) patients with A-TB and in 3/41 (7%) \( P < 0.0001 \) controls (3/21 among TST-positive individuals, \( P < 0.0001 \); 0/20 among TST-negative individuals, \( P < 0.0001 \)). Thus, in the population studied, the sensitivity of the WBE for A-TB was 81% and the specificity was 90% (Table 3). ELISPOT responses were scored positive in 20/27 (74%) patients with A-TB and in 3/41 (7%, \( P < 0.0001 \)) controls (3/21 among TST positives, \( P < 0.0001 \); 0/20 among TST negatives, \( P < 0.0001 \)), with resulting sensitivity and specificity values for A-TB of 74% and 93%, respectively (Table 3). We observed 86.7% concordance of positive and negative results between the two tests (\( k = 0.71; P < 0.001 \)).

WBE and ELISPOT proved to have good internal reproducibility, with less than 10% difference between duplicate samples. The ELISPOT was always run in duplicate, while duplicate samples were assayed for 10 patients with the WBE (data not shown). Moreover, samples from five patients were retested after a week, and again, less than 10% difference among the results obtained on the two different experiment days was found (data not shown).

In conclusion, these data indicate that the response to selected RD1 peptides is associated with A-TB and that WBE results correlate significantly with ELISPOT data.

**Comparison between our WBE for selected RD1 peptides and the QuantiFERON-TB Gold assay.** We next compared the results obtained by our above-described WBE with those obtained by QuantiFERON-TB Gold, a whole-blood assay approved for the diagnosis of TB infection, based on RD1 overlapping peptides (Table 3). In the QuantiFERON-TB Gold assay, IFN-\( \gamma \) production in response to TB antigens was significantly higher in patients with A-TB (10.01 ± 2.04 IU/ml) than in controls (0.84 ± 0.34 IU/ml, \( P < 0.0001 \)), and this difference was statistically significant independently of the TST status of controls, either positive (1.5 ± 0.6 IU/ml, \( P < 0.0001 \) versus A-TB) or negative (0.1 ± 0.04 IU/ml, \( P < 0.0001 \) versus A-TB) (Fig. 2). Responses were scored positive in 24/27 patients with A-TB (89%) and in 13/41 controls (32%, \( P < 0.0001 \); 11/21 among TST positives, \( P = 0.008 \); 2/20 among TST negatives, \( P < 0.0001 \)). Thus, in the population studied, the sensitivity of this assay for A-TB was 89% and the specificity was 68% (Table 3). In a comparison of the results generated by QuantiFERON-TB Gold with those obtained by our WBE, no difference was found in the proportion of positive responses among patients with A-TB (\( P = 0.6 \)), but a significantly higher response by TST-positive controls was observed in the QuantiFERON-TB Gold assay (11/21 versus 4/21, \( P = 0.016 \)).

In conclusion, these data indicate that in patients with A-TB, responses to selected RD1 peptides detected by WBE correlate with those obtained by QuantiFERON-TB Gold; however, the different results of TST-positive controls in the two assays suggest that our test provides a higher specificity.

**DISCUSSION**

We recently set up an IFN-\( \gamma \) ELISPOT, using selected ESAT-6 peptides, that appears to be specific for A-TB (7, 29). However, ELISPOT is difficult to automate. Thus, the objective of this study was to develop a test to detect A-TB that is suitable for routine use in diagnostic laboratories, such as an ELISA performed with whole blood. By using selected peptides, three of which were new multiepitopic peptides of CFP-10 protein and two of which were already characterized peptides of ESAT-6 protein (7, 29), we found that the response was significantly higher in those with A-TB than in controls by both WBE and ELISPOT. Data obtained by the two tests correlated significantly. To substantiate our data, we compared WBE results with those obtained by QuantiFERON-TB Gold, which is a whole-blood ELISA based on RD1 overlapping peptides obtained by WBE was compared to that found by ELISPOT, the number of SFC per million PBMC counted by ELISPOT correlated significantly with the IU/ml measured by WBE (\( r = 0.80, P < 0.001 \)) (Fig. 1C).

**TABLE 3. Comparison of the different assays to perform immune diagnosis of TB**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Test</th>
<th>Active TB patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of patients positive/total no. (%)</td>
<td>TST positive</td>
</tr>
<tr>
<td>Selected RD1</td>
<td>WBE</td>
<td>22/27 (81)</td>
<td>4/21 (19)</td>
</tr>
<tr>
<td></td>
<td>ELISPOT</td>
<td>20/27 (74)</td>
<td>3/21 (14)</td>
</tr>
<tr>
<td>RD1 overlapping</td>
<td>QuantiFERON-TB-Gold</td>
<td>24/27 (89)</td>
<td>11/21 (52)</td>
</tr>
</tbody>
</table>

* Total, TST-positive and TST-negative control subjects.

FIG. 2. Correlation between WBE and QuantiFERON-TB Gold results. The highest IFN-\( \gamma \)-secreting T-cell response is reported in IU/ml for each individual. Symbols: ○, active TB; ▼, TST-positive controls; △, TST-negative controls.
peptides approved for TB infection diagnosis. Among patients with A-TB, the results obtained by WBE did not differ significantly from those obtained by QuantiFERON-TB Gold. Conversely, a higher proportion of TST-positive controls were scored positive by QuantiFERON-TB Gold than by our WBE.

Using our selected RD1 peptides, we found that IFN-γ production was significantly higher in patients with A-TB than in controls. WBE showed a slightly higher sensitivity for A-TB than ELISPOT (81% versus 71%), although the difference was not statistically significant. The higher sensitivity may likely be due to the fact that blood cells are incubated with the reagents without any prior manipulation. Thus, such a technique, in addition to being easier to automate, may also be more sensitive. Regarding the sensitivity issue, it is important to note that three of those five A-TB patients who did not respond in vitro to the selected peptides in the WBE test were probably in a condition of “M. tuberculosis-specific unresponsiveness,” since they did not respond in assays with QuantiFERON-TB Gold, either. This condition could be a consequence of the intrinsic limits of such immune diagnostic assays, which is in line with data recently published showing only 70% positive responses to RD1 ELISPOT in children with active pulmonary TB (18).

The fact that ELISPOT and whole-blood assays correlate well has already been described by Schölvink et al., although in a different experimental setting (23). In fact, our WBE differs from those described by others (10, 16–18, 20–23, 30) in terms of the reagents used (selected RD1 peptides, overlapping peptides, and intact proteins), methodology, and the results obtained. It could be argued that the incubation times rather than the reagents used could account for the difference in specificities of our test and others. However, this is not the case, since incubation times varied in different studies from 1 day (our WBE and those described previously [10, 16, 17, 20–22]), 2 days (the ELISPOT described here and those described previously [7, 29]), and 3 days (18, 23, 30). In this report, our WBE differed from the QuantiFERON-TB Gold test only in the use of the selected peptides, and this was the only factor that could account for the difference in terms of assay specificity for active disease (90% versus 68%). At present, the reason(s) for the discrepancy of the responses to selected peptides (our WBE and 7, 29), overlapping peptides (10, 16, 17, 20), and intact proteins (18, 21–23, 30) remains unclear. Studies are ongoing in our laboratory to explain such an interesting phenomenon. A possible explanation for the specificity of the selected RD1 peptide response for A-TB may rely on both the different immunological status of the patients with A-TB and LTBI and the selection of a cutoff by ROC analysis able to maximize the accuracy of the diagnosis of A-TB. Regarding the immune status of the patients, it must be considered that metabolically active and viable bacilli secrete proteins, such as ESAT-6 and CFP-10 (2). Consequently, IFN-γ-producing T cells specific to the selected epitopes are also present at high frequency only during the active replication of mycobacteria. Previous papers have demonstrated that lymphocytes with immediate effector memory function circulate for a limited time, until the antigen has been cleared (20). It can be hypothesized that the frequency of T cells specific to the single epitopes contained in the selected peptides might dramatically decrease during latent infection in the absence of bacterial replication and continuous exposure to M. tuberculosis-secreted antigen.

Thus, in the presence of a small antigenic load, as in patients with LTBI or with A-TB after efficacious treatment, the limited number of effector cells specific to the selected peptides in the peripheral blood could fall below the cutoff, while the number of T cells specific to intact proteins or to the overlapping peptides covering the whole proteins, which contain multiple epitopes, could remain high enough to be detected. Moreover, it is worth noting that our cutoff value was found by ROC analysis and was selectively chosen to maximize the accuracy of the diagnosis of A-TB, whereas that of the commercially available assays was probably chosen to maximize the accuracy of detection of TB infection (10, 16). This different statistical approach may, in part, explain the different results obtained.

Some limitations of the present study need to be mentioned. First, the study was performed with a small population, and thus, the estimates for sensitivity and specificity may be imprecise. Second, the positive in vitro response to RD1 antigens could be related to infection with mycobacteria other than M. tuberculosis, such as M. kansasii, M. szulgai, M. flavescens, M. marinum, and Mycobacterium leprae, because the ESAT-6 gene or a homologue is also present in these mycobacteria (13, 14). Third, we did not assess the role of different HLA backgrounds in response to selected peptides. In fact, although the population evaluated here is heterogeneous, as the patients came from different areas, the fact that subjects sharing the same HLA alleles could not respond to the same HLA-restricted epitopes (4) may represent a limit when using selected peptides. Therefore, to confirm the specificity of our assay, it will be important to evaluate a larger population in future studies, including patients with mycobacterial diseases other than TB, and also to assess the response to peptides in the context of the patients’ HLA class II background.

In conclusion, this whole-blood assay based on selected RD1 peptides may be a potentially useful and easy-to-use tool for TB control. In fact, this assay may facilitate the diagnosis of active TB in the clinical setting. Moreover, in the context of a screening program, our assay using selected RD1 peptides may help to rule out A-TB in individuals with positive results to either the TST or an assay of RD1 overlapping peptides. The simple assay format would be readily adaptable to routine laboratories, even in medically underserved environments where the research application of such whole-blood assays to populations has been shown to be entirely feasible (5).

ACKNOWLEDGMENTS

We are grateful to all patients and nursing staff who took part in this study. We thank C. Nisii, G. M. Fimia, and T. Alonzi for critical review of the paper.

The study was supported by a grant from the Italian Ministry of Health (RF 04.126).

D. Goletti, S. Carrara, D. Vincenti, M. Amicosante, and E. Girardi have a patent pending on a T-cell assay based on selected RD1 peptides (PCT international patent no., PCT/EP2005/050728; priority date, 19 February 2004).

REFERENCES


