Original Article

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Relationship between T-SPOT.TB responses and numbers of circulating CD4+ T-cells in HIV infected patients with active tuberculosis

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Summary **This study sought to evaluate the performance of the T-SPOT.TB assay for the diagnosis of active tuberculosis (TB) in human immunodeficiency virus (HIV) infected patients. One hundred confirmed HIV-infected patients with active TB and known T-SPOT.TB and CD4+ T-cell counts were enrolled in this clinical retrospective study. We found that patients with lower CD4+ T-cell counts (11-50 cells/μL) had the lowest T-SPOT.TB positive rates (50%), and patients with higher CD4+ T-cell counts (50-100 cells/μL) had the highest T-SPOT.TB positive rates (75%). However, there were no significant differences between the T-SPOT.TB positive rates of patients with different CD4+ T-cell counts (< 10, 11-50, 51-100 and > 100 cells/μL) (χ^2 = 3.7747, p = 0.287). The patients with positive TB culture results had significantly higher T-SPOT.TB positive rates (78.9%) than patients that were culture-negative (44.3%) (χ^2 = 12.8303, p < 0.001). Other variables, including gender, age, TB disease classification, HIV RNA level, and highly reactive antiretroviral therapy (HAART), had no significant effects on T-SPOT.TB positive rates. The number of spot-forming cells (SFCs) reactive with ESAT-6, CFP-10 and ESAT-6/CFP-10-specific T cells detected by T-SPOT.TB were positively correlated with the number of circulating CD4+ T-cells (r_s = 0.3791, p = 0.0001; r_s = 0.2929, p = 0.0031; r_s = 0.3345, p = 0.0007, respectively). This study suggests that the number of SFCs is strongly related to the degree of immunodeficiency, while the T-SPOT.TB positive rates are less dependent on the level of CD4+ T-cell depletion in HIV infection and active TB.**

Keywords: HIV, active tuberculosis, T-SPOT.TB

1. Introduction

Tuberculosis (TB) is one of the most common infectious diseases in the world. In 2012, there were an estimated 8.6 million people with active TB. Among these, there were 1.1 million (13%) co-infections with HIV (TB-HIV) and 320,000 active TB-HIV deaths according to a report from the World Health Organization (*1*). Patients with advanced immunodeficiency have a greater risk of active TB (*2*).

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Patients with active TB need early diagnosis and prompt treatment. Currently, the gold standard for active TB diagnosis remains the isolation of *Mycobacterium tuberculosis* positive cultures - a procedure that is time-consuming (*3*). Also, it is sometimes difficult to obtain suitable culture specimens other than sputum in HIV-infected patients suffering from extrapulmonary TB (*4,5*). Furthermore, *M. tuberculosis* culturing is often not available in some regions, and a Ziehl-Neelsen stain may take more than 10 days. There is a great need for new methods for diagnosing TB in HIV-infected subjects throughout the world, especially for patients with advanced immunodeficiency. The introduction of an interferon (IFN)-γ release assay (IGRAs) using the immunogenic and specific *M. tuberculosis* antigens early secreted antigenic target (ESAT-6) and culture

filtrate protein 10 kDa (CFP-10) for immunodiagnosis is, therefore, a potential advantage. There are two commercial forms of the IGRAs licensed for use in the developed world, including the T-SPOT.TB (Oxford Immunotec, Abingdon, UK), which is based on the enzyme-linked immunosorbent spot (ELISPOT) assay, and the whole blood-based QuantiFERON-TB Gold in-tube (QFT-IT; Cellestis, Melbourne, Australia), which uses an enzyme-linked immunosorbent assay (ELISA) to detect IFN-γ released into culture supernatants (*6*). The results of QFT-IT are impacted by peripheral CD4+ T-cell counts, as reported by previous studies, whereas similar problems for the T-SPOT.TB assay were not detected in several studies (*7-12*). It is thought by some that the T-SPOT.TB assay is less likely to be affected by low CD4+ T lymphocyte counts, as ELISPOT technology is the most sensitive method for detecting IFN-γ-secreting antigen-specific T cells derived from blood. Therefore, although the magnitude of the mitogen response was slightly lower in low CD4+ T-cell counts, responses were still well above the threshold for the positive control (*13*), suggesting that the T-SPOT.TB assay might be an appropriate test method for HIV-infected individuals with advanced immunodeficiency. However, previous studies suggest that advanced immunodeficiency may affect the performance of the T-SPOT.TB assay (*14-16*). The value of the T-SPOT.TB assay for the diagnosis of active TB in HIV-infected individuals with very low CD4+ T-cell counts has not yet been established.

The objectives of this retrospective study in HIV-infected individuals were to determine the performance of the T-SPOT.TB assay among active tuberculosis patients whose CD4+ T-cell counts were below 10 and 50 cells/μL. The performance of the T-SPOT.TB assay in patients with CD4+ T-cell counts below 10 cells/μL has not been reported.

2. Materials and Methods

2.1. *Selection of study subjects*

One hundred HIV-infected patients naïve to anti-TB therapies were enrolled from July 2012 to October 2013 at Shanghai Pubic Health Clinical Center in Shanghai, China. The patients diagnosed with confirmed active TB had presented to the clinic with signs and symptoms of TB. Written informed consent was obtained from all participants.

2.2. *Procedures*

All patients had a symptom assessment, a full clinical assessment, and sputum (three samples if productive or at least one sample induced with hypertonic 3% saline if not) was sent for smear microscopy and mycobacterial culturing. Chest radiographs and/or CT scans were performed. As needed, other appropriate specimens were obtained for microscopy and culturing. A fixed number of peripheral blood mononuclear cells from venous blood samples were used to perform the T-SPOT.TB assay according to the manufacturer's instructions using the T-SPOT.TB kit (Oxford Immunotec Ltd., Oxford, UK). Test wells were considered positive if the number of spot-forming cells obtained from the test antigens, either or both of the ESAT-6 or CFP-10-derived peptides, was more than twice that of the negative control, and if they had six or more spots than the negative control. Wherever possible, patients with indeterminate results had another sample tested until a definitive positive or negative result was achieved.

2.3. *Statistical methods*

Chi-squared (χ^2) tests were used for proportional comparisons among different subgroups. The Wilcoxon rank-sum test was used for non-normal data. The Shapiro-Wilk test was used to assess normality of the data. Spearman's rank correlation was used for correlation calculations. All *p*-values were two-sided with $\alpha = 0.05$. All data were analyzed using STATA 10.0 software (StataCorp, College Station, Texas, USA).

3. Results

3.1. *General information of the patients studied*

The average age of the patients was 42.0 ± 13.8 years. Eighty-nine percent of the cohort was male. 45% patients had specimens (sputum, stool, or abdominal cavity effusion) that were smear microscopy positive. For other characteristics see Tables 1 and 2.

3.2. *Analysis of positive and negative T-SPOT.TB performance results*

The sensitivity of the T-SPOT.TB assay was 59% among the 100 patients. Patients with lower CD4+ T-cell counts (11-50 cells/μL) had the lowest T-SPOT.TB positive rates (50%). Patients with higher CD4+ T-cell counts (50-100 cells/μL) had the highest T-SPOT.TB positive rates (75%). There were no significant differences between the T-SPOT.TB positive rates of patients with different CD4+ T-cell counts ($p = 0.287$). We divided the CD4 + T-cell counts into two categories, 1-50 cells/μL and > 50 cells/μL, although there was still no significant association ($p = 0.082$).

The patients with positive TB culture results had significantly higher T-SPOT.TB positive rates (78.9%) than the TB culture-negative group (44.3%) ($p < 0.001$). Other variables, including gender, age, TB disease classification, HIV RNA level, and highly reactive antiretroviral therapy (HAART), had no significant effects on T-SPOT.TB positive rates (Table 2).

Table 1. Group and subgroup of patients with active tuberculosis

Group	Number	Subgroup	Number
Pulmonary TB	59	Ordinary pulmonary TB	57
		Blood disseminated pulmonary TB	2
Extrapulmonary TB	15	Lymph node TB	5
		Tuberculous meningitis	5
		Pancreatic TB	1
		Tuberculous peritonitis TB	1
		Tuberculous pleurisy and spinal TB	1
		Tuberculous meningitis and lymph node TB	1
		Intestinal and abdominal cavity lymph node TB	1
Pulmonary and extrapulmonary TB	26	Pulmonary TB and tuberculous pleurisy	8
		Pulmonary TB and tuberculous meningitis	5
		Pulmonary TB and lymph node TB	4
		Pulmonary and intestinal TB	2
		Pulmonary and laryngeal TB	1
		Pulmonary and mediastinal lymph node TB	1
		Pulmonary TB plus tuberculous meningitis and lymph node TB	1
		Pulmonary TB plus tuberculous pleurisy and lymph node TB	1
		Pulmonary TB plus tuberculous pleurisy and tuberculous peritonitis	1
		Blood disseminated pulmonary TB plus tuberculous pleurisy and lymph node TB	1
		Pulmonary TB, tuberculous pleurisy plus tuberculous peritonitis and lymph node TB	1

Table 2. General characteristics of the study subjects (n = 100) according to the results of the T-SPOT.TB assay

Variable	Positive T-SPOT.TB	Negative T-SPOT.TB	p-value
Subjects (n)	59	41	-
Gender			
Males	54	35	0.333
Females	5	6	
Age			
≤ 40 years	27	24	0.209
> 40 years	32	17	
TB disease			
Pulmonary	35	24	0.186
Extrapulmonary	6	9	
Pulmonary and extrapulmonary	18	8	
CD4+ T cell counts			
1-10 cells/μL	11 (52.4%)	10 (47.6%)	0.287
11-50 cells/μL	19 (50%)	19 (50%)	
51-100 cells/μL	15 (75%)	5 (25%)	
101-536 cells/μL	13 (61.9%)	8 (38.1%)	
1-50 cells/μL	30 (50.8%)	29 (49.2%)	0.082
51-536 cells/μL	28 (68.3%)	13 (31.7%)	
HIV RNA[a]			
≥40 copies/mL	38	32	0.482
<40 copies/mL	6	3	
HAART treatment			
YES	14 (63.6%)	8 (36.4%)	0.544
NO	44 (56.4%)	34 (43.6%)	
TB culture[aa]			
Positive	30 (78.9%)	8 (21.1%)	< 0.001
Negative	27 (44.3%)	34 (55.7%)	

[a] The HIV RNA of 21 patients was not detected. [aa] The culture results in two patients were other microorganisms.

3.3. *Correlation of CD4+ T-cells with T-SPOT.TB*

The number of spot-forming cells (SFCs) reactive with ESAT-6, CFP-10 and ESAT-6/CFP-10-specific T cells detected by T-SPOT.TB were positively correlated with the number of circulating CD4+ T-cells (Table 3).

4. Discussion

It is more difficult to detect tuberculosis infections in HIV-infected patients than in uninfected individuals due to the TB-associated decline of some immune phenomena that is secondary to the destruction of the immune system (*17*).

Table 3. Correlation of numbers of circulating CD4+ T-cells with numbers of SFCs in response to incubation with ESAT-6 and CFP-10 in the T-SPOT.TB test

Correlation of CD4+ T-cells per μL with	Spearman's rho	*p*-value
T-SPOT.TB ESAT-6 SFCs per million PBMC	0.3791	0.0001
T-SPOT.TB CFP-10 SFCs per million PBMC	0.2929	0.0031
T-SPOT.TB ESAT-6/CFP-10 SFCs per million PBMC	0.3345	0.0007

The T-SPOT.TB assay has been evaluated for the detection of TB in HIV-infected people by many studies, although there were only limited data from a small number of HIV-infected patients with advanced immunodeficiency (*17-19*), and there were few data regarding its performance in active TB patients with HIV and CD4+ T-cell counts below either 10 or 50 cells/μL.

Therefore, we examined the relationship between T-SPOT.TB responses and CD4+ T-cells in HIV infected patients with active tuberculosis. The sensitivity of the T-SPOT.TB assay was 59% in our study. This was greater than the reported sensitivity of the T-SPOT.TB test in HIV-1 infected persons with active TB in the studies of Chen *et al*. (*20*) and Yu *et al*. (*21*) (34.2%, 13/38; 41.3%, 19/46, respectively), poorer than the sensitivities reported in the studies of Clark *et al*. (*19*), Chen *et al*. (*22*) and Ling *et al*. (*23*) (90.3%, 28/30; 77.4%, 493/637; 82%, 80/98, respectively). A similar sensitivity was reported in the studies of Santin *et al*. (*24*), Metcalfe *et al*. (*25*), Oni *et al*. (*26*), Markova *et al*. (*27*), and Jiang *et al*. (*28*) (65%, 202/311; 68%, 177/261; 68%, 58/85; 62%, 8/12; 65.6%, 21/32, respectively). We speculate on the sensitivity of the T-SPOT.TB test in HIV-1 infected persons with active TB is approximately 70% in studies using a large number of samples; different results can be obtained from studies of small numbers of samples.

In our study, the groups of CD4+ T-cell counts were < 10, 11-50, 51-100 and > 100 cells/μL. The CD4+ T-cell counts in the first two groups were extremely low, especially in the first group. However, there were no significant differences among the T-SPOT. TB positive rates of patients with different CD4+ T-cell counts. The non-significant association between CD4+ T-cell counts and T-SPOT.TB responses might be due to the small number of subjects in each group of CD4+ T-cell counts. We subsequently divided the CD4+ T-cell counts into two categories, 1-50 and > 50 cells/μL. Nevertheless, there was still no statistically significant difference in T-SPOT.TB positive rates. Our results strongly support previous studies that showed T-SPOT.TB positive rates are not correlated with T-cell stratification in patients with HIV-infection and active TB (*20,26*). Chen *et al*. (*20*) divided the CD4+ T-cell counts into two categories, 1-99 and ≥ 100 cells/μL. Oni *et al*. (*26*) divided the CD4+ T-cell counts into four categories, < 100, 100-199, 200-299, and > 300 cells/μL. Neither study found a significant association between CD4+ T-cell counts and T-SPOT.TB responses. Consequently, we demonstrated that T-SPOT.TB positive results were not dependent on CD4+ T-cell counts in HIV-infected patients with active TB.

We found that the number of SFCs reactive with ESAT-6 and CFP-10 positively correlated with the number of circulating CD4+ T-cells. This result differs from those of previous studies (*8,9,20*). Chen *et al*. (*20*) did not find a correlation between CD4+ T-cell counts and the amount of SFCs reactive with ESAT-6 or CFP10 in 35 individuals with active TB and HIV infection. Leidl *et al*. (*9*), using the QFT-IT assay, found that in HIV-infected patients, the concentration of IFN-γ in response to specific M. tuberculosis antigens directly correlated with the number of circulating of CD4+ T-cells. In contrast, SFCs in the ESAT-6 or CFP-10 antigen wells in the T-SPOT.TB assay were not correlated to the numbers of circulating CD4+ T cells (*9*). In another study by Karam *et al*. (*8*), the total number of SFCs reactive to ESAT-6 and CFP10 did not vary with changes in CD4+ T-cell counts. However, the proportion of positive responses to the ELISPOT assay decreased with decreasing CD4+ T-cell counts in HIV-infected patients (*8*). The reasons that these results differ from those in our study might be because our study subjects were all active TB patients with HIV infections.

In our study, the T-SPOT.TB positive results in the group with positive TB cultures was significantly higher than the culture-negative group (78.9% *vs*. 44.3%, respectively). Our result is similar to previous observations that showed 75% T-SPOT.TB positive results in HIV infected TB patients with positive TB cultures, greater than 22% positive results for the culture negative group (*29*). Oni *et al*. (*26*) found 68% T-SPOT.TB positive results in 85 HIV infected TB patients with positive TB cultures. This result was slightly lower than ours. Ribeiro *et al*. (*30*) found the mean ESAT-6 or CFP-10 SFC counts were higher for the positive sputum culture group compared to the culture-negative group, although this difference did not reach statistical significance before patients received anti-TB treatment. The study individuals were composed of 58 patients with pulmonary tuberculosis, of whom 57 were HIV seronegative (*30*). Ling *et al*. (*31*) thought the T-SPOT.TB assay did not have added value beyond clinical data and conventional tests for diagnosis of TB in smear-negative children in a high-burden setting. Although the T-SPOT.TB positive rate

may be lower in HIV infected TB patients with negative TB cultures, TB cultures are time-consuming and the T-SPOT.TB assay is faster.

In conclusion, the SFCs in the T-SPOT.TB assay are strongly related to the degree of immunodeficiency, while the T-SPOT.TB positive rates are less dependent on the level of CD4+ T-cell depletion.

Our study has several limitations. First, we did not explain why the T-SPOT.TB positive results of patients with < 10 CD4+ T-cells/μL were similar to other groups that had greater CD4+ T-cell counts. Second, we collected a limited number (100) of active tuberculosis patients that were co-infected with HIV. Third, most of the individuals were male patients, which may have affected the results.

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