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Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis

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ABSTRACT: The aim of the present study was to determine the role of previous non-tuberculous mycobacteria sensitisation in children as a factor of discordant results between tuberculin skin test (TST) and an *in vitro* T-cell based assay (T-SPOT.TB; Oxford Immunotec, Oxford, UK).

We enrolled 21 non-bacille Calmette-Guérin-vaccinated paediatric patients for suspicious of latent tuberculosis infection (LTBI). These patients yielded a positive TST and a negative T-SPOT.TB. Cells were stimulated with *Mycobacterium avium* sensitin (having cross-reaction with *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*) and the presence of reactive T-cells was determined by an *ex vivo* ELISPOT.

From the 21 patients, in 10 cases (47.6%), we obtained a positive ELISPOT result after stimulation with *M. avium* sensitin, in six (28.6%) cases, the result was negative and in the remaining five (23.8%) cases, the result was indeterminate.

In conclusion, previous non-tuberculous mycobacteria sensitisation induces false-positive results in the TST for diagnosing LTBI and the use of γ-interferon tests could avoid unnecessary chemoprophylaxis treatment among a child population.

KEYWORDS: Childhood, ELISPOT, interferon-γ release assays, latent tuberculosis infection, *Mycobacterium avium* sensitin, non-tuberculous mycobacteria

The detection and treatment of active TB is a key strategy in the control of childhood tuberculosis (TB) [1]. Children have a high risk of progression to active TB [2]. Therefore, a rapid and specific diagnosis of latent TB infection (LTBI) is essential in preventing the progression to disease. The tuberculin skin test (TST) attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to the purified protein derivative (PPD) [3]. The biggest drawback of TST is that individuals sensitised by previous exposure to non-tuberculous mycobacteria (NTM) or vaccinated with *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) respond immunologically to PPD. Consequently, unnecessary latent tuberculosis treatments are prescribed.

In vitro assays for measuring T-cell-mediated immune responses have been developed. In these assays, infected individuals are identified by the detection of γ-interferon (IFN-γ) released by the T-cells that are sensitised after being stimulated with the specific *Mycobacterium tuberculosis* (MTB) antigens of region of deletion (RD) 1 (early-secreted antigenic target protein (ESAT)-6 and 10-kD culture filtrate protein (CFP)-10) [4, 5]. Promising results from these diagnostic tests in both adults and children have been published [6–11].

However, there are several discordant results between the IFN-γ tests and the TST [12]. One of the more challenging correct interpretations remains in the instance of positive TST and negative IFN-γ results in non-BCG vaccinated children. In our experience [7], among unvaccinated children with a positive TST, the T-SPOT.TB result was negative in 56.6% of the cases.

The aim of the present study was to determine the role of previous NTM sensitisation in children as a factor of discordant results between TST and the T-SPOT.TB test.

MATERIAL AND METHODS

Patients and inclusion criteria

We retrospectively enrolled a total of 21 paediatric patients, who attended Hospital Universitari Germans Trias i Pujol (Badalona, Spain) or TB Control and Prevention Unit of Barcelona (CAP

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Drassanes, Barcalona, Spain) for suspicion of LTBI. These patients were enrolled for contact tracing studies or for screening of LTBI. Inclusion criteria for this selected population were a positive TST, a negative T-SPOT.TB, non-BCG vaccination and no more than 2 weeks of chemoprophylaxis when blood sampling. None of the children presented lymphadenitis at the time of inclusion. We have also included control groups to validate the methodology and the results: 11 children with both TST and T-SPOT.TB negative results, and six individuals with microbiologically confirmed *M. avium* infection (four lymphadenitis and two respiratory infections). Another additional group of 10 children with both TST- and T-SPOT.TB-positive results was included in order to know the background of *M. tuberculosis* and *M. avium* sensitisation in the population. The main demographic characteristics of the groups included in the study are shown in table 1.

Ethics approval for this study was provided by the corresponding Ethics Committees. We obtained written informed consent from all parents before blood sampling. A detailed questionnaire from all patients was completed to indicate the results of any previous TST, BCG vaccination status, details of any contact with a person diagnosed of active TB, history of prior active TB, LTBI and HIV infection, chest radiography and other medical conditions.

TST

Two intradermal tuberculin units of PPD RT23 Tween 80 (Statens Serum Institut, Copenhagen, Denmark) were used to perform TST. The tuberculin was administered using Mantoux method, and the size of the induration was interpreted after 48–72 h by trained personnel. In this study, TST indurations $\geqslant$5 mm were classified as positive [13].

Detection of T-cell sensitised against MTB specific antigens

Peripheral blood mononuclear cells (PBMCs) were stimulated with ESAT-6 and CFP-10 antigens individually. The presence of reactive antigen-specific T-cells was revealed by ELISPOT (T-SPOT.TB; Oxford Immunotec, Oxford, UK). The test was performed in accordance with the manufacturer's instructions. Unstimulated cells were washed with RPMI medium (Invitrogen, Auckland, New Zealand) and resuspended in freeze medium (80% RPMI and 20% free bovine serum (PAA Laboratories GmbH, Pasching, Austria)), adding dropwise 10% DMSO (Merck, Darmstadt, Germany) and frozen at -80°C.

TABLE 1 Demographic characteristics of patients studied

Variable	Study group	Control groups		
		TST and T-SPOT.TB negatives	TST and T-SPOT.TB positives	Microbiologically confirmed *M. avium* infection
Subjects	21	11	10	6
Sex				
Male	10 (47.6)	3 (27.3)	6 (60)	4 (66.7)
Female	11 (52.4)	8 (72.7)	4 (40)	2 (33.3)
Age yrs	8.81±4.03	11.55±4.52	10±3.02	17.5±20.92
BCG vaccinated				
Yes	0 (0)	5 (45.5)	3 (30)	0 (0)
No	21 (100)	6 (54.5)	7 (70)	6 (100)
Immunosupression				
Yes	0 (0)	0 (0)	0 (0)	0 (0)
No	21 (100)	11 (100)	10 (100)	6 (100)
Birth country				
Immigrants from countries with high prevalence of TB infection	5 (23.8)	6 (54.5)	3(30)	0 (0)
Residents in a non-epidemic TB country	16 (76.2)	5 (45.5)	7 (70)	6 (100)
Origin				
Contact tracing studies	6 (28.6)	8 (72.7)	10 (100)	
Screening of LTBI at school	15 (71.4)	3 (27.3)	0 (0)	
T-SPOT.TB				
Positive	0	0	10	2[#]
Negative	21	21	0	3
Indeterminate	0	0	0	1
***Ex vivo* ELISPOT *M. avium* sensitin stimulation**				
Positive	10	0	5	4
Negative	6	11	3	1[¶]
Indeterminate	5	0	2	1

Data are presented as n, n (%) or mean±SD. TST: tuberculin skin test; *M. avium*: *Mycobacterium avium*; BCG: bacilli Calmette-Guérin; TB: tuberculosis; LTBI: latent TB infection. [#]: in one case, active TB was documented 8 yrs before; [¶]: *M. avium* infection was reported 2 yrs before.

Detection of T-cell sensitised against NTM sensitin

The stimulation of the T-cells was performed using *M. avium* sensitin (Statens Serum Institute, Copenhagen, Denmark). The manufacturer informed that this sensitin has cross reaction with *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. In order to perform *ex vivo* ELISPOT, stimulating with *M. avium* sensitin, cells were thawed and re-suspended in 10 mL of RPMI medium. Finally, cells were washed, re-suspended in AIM-V medium (Invitrogen, Auckland, New Zealand) and stimulated with medium alone (as nil control), phytohaemagglutinin (as positive control) and *M. avium* sensitin at a concentration of 10 $\mu g \cdot mL^{-1}$. Plates were incubated for 16–20 h at 37°C with 5% CO_2. Following incubation, wells were washed with PBS and incubated for 1 h at 2°C with a monoclonal antibody to IFN-γ conjugated to alkaline phosphatase. The presence of reactive antigen-specific T-cells was revealed as a spot in the well.

Interpretation of the results

Spots were scored using an automated ELISPOT plate reader (Lector AID Elispots; Autoimmun Diagnostiks GmbH, Germany). All readings were also manually verified. The results of the assays were expressed as ESAT-6, CFP-10 and *M. avium* sensitin specific responder cells per million PBMCs. Test wells were scored as positive if the number of responder cells per million PBMCs minus their number in the control negative was >24. The result of the assay was considered indeterminate if the number of positive control cells per million PBMCs was <80, and the response to both of the antigen panels was negative.

RESULTS

From the 21 children with positive TST and negative T-SPOT.TB, a positive ELISPOT result after stimulation with *M. avium* sensitin was obtained in 10 (47.6%) cases. In six (28.6%) cases the result was negative and in the remaining five (23.8%) cases the result was indeterminate. The number of responder T-cells after *M. avium* sensitin stimulation was significantly higher than the number of responder T-cells after specific MTB antigens (ESAT-6 and CFP-10) stimulation: p=0.001 and p<0.001, respectively.

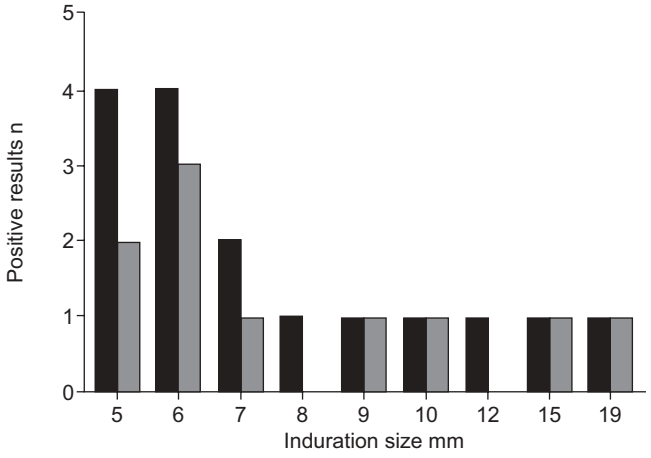

FIGURE 1. Induration size distribution of positive results of the tuberculin skin test (■) and *ex vivo Mycobacterium avium* sensitin ELISPOT (■) among the children with a valid result.

Among the 10 children that obtained a positive result after stimulation with *M. avium* sensitin, five children were aged 6–7 yrs and the other five children were aged 11–16 yrs. Additionally, eight were enrolled during LTBI screening at school and the remaining two, during a contact tracing study. Regarding the induration of the TST, eight of these children were in the range of 5–10 mm, one case was 15 mm and the other case was 19 mm (fig. 1).

In all children with both TST- and T-SPOT.TB-negative results included as controls, negative ELISPOT results after stimulation with *M. avium* sensitin were obtained. There were no significant differences between the number of responder T-cells after stimulation with ESAT-6, CFP-10 and *M. avium* sensitin. The differences in the number of responder T-cells to *M. avium* sensitin between the patients study group and this control group were significant (p=0.004) (fig. 2). In the group of individuals with microbiologically confirmed *M. avium* infection, four out of five cases with valid results, cells

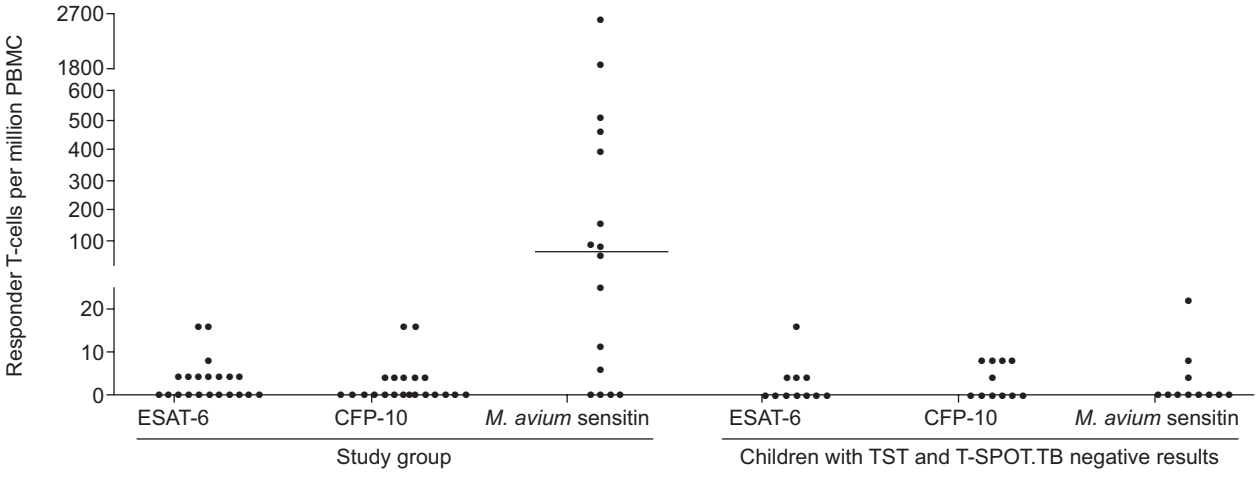

FIGURE 2. Number of responder T-cells enumerated by *ex vivo* ELISPOT after stimulation with the specific *Mycobacterium tuberculosis* antigens (early secretary antigen target (ESAT)-6 and culture filtrate protein (CFP)-10) and *Mycobacterium avium* (*M. avium*) sensitin in the study group and children with tuberculin skin test (TST)- and T-SPOT.TB-negative results. PBMC: peripheral blood mononuclear cells.

sensitised against *M. avium* were detected. The results obtained by the study and all control groups are presented in table 1. The indeterminate results were due to the low number of cells recovered after thawing.

DISCUSSION

Although specificity of IFN-γ tests is excellent because the assay is not affected by BCG vaccination [6–8], frequent discordant results with TST have been described [6, 7, 14]. In fact, it has been recommended for priority research to obtain data to understand discordant TST and IFN-γ tests results, including the role of NTM [12]. To date, the effect of NTM on IFN-γ tests results has been poorly studied. In this sense, we have studied the effect of previous NTM sensitisation to try to give an explanation for the discordant results of positive TST and negative IFN-γ results in non-BCG vaccinated children. Among the 16 children with a valid result, 10 (62.5%) children had a specific response of T-cells after stimulation with *M. avium* sensitin.

It has been described that asymptomatic infections with *M. avium* and other NTM are common [15] and probably acquired in childhood [16–19]. In our area, the estimation of NTM infection in children with a positive TST (5–10 mm) ranged 20–50% [16]. According with our results, using the *ex vivo* ELISPOT, eight (80%) of the 10 children reactive against *M. avium* sensitin had a positive TST between 5 and 10 mm, and nine (90%) of them between 5 and 15 mm. Indeed, in the children control group with TST- and T-SPOT.TB-positive results, the presence of T-cell sensitised against *M. avium* was detected in five out of the eight cases with valid result.

In our study, eight out of 10 children with a positive *M. avium* sensitin T-cell assay from our study group were enrolled from a routine screening of LTBI without known exposure to any active TB patient. Given that NTM infection affects the TST reading, it is in this group of children where IFN-γ tests could be used to confirm the diagnosis in case of a positive TST result.

Regarding the six remaining discordant results without T-cell response after *M. avium* sensitin stimulation, there are three possible explanations. First, a real LTBI not detected by the IFN-γ test. Nevertheless, the sensitivity of the IFN-γ tests is considered to be higher than the TST, or at least at the same level. Secondly, the IFN-γ test enumerates effectors T-cells that have recently been in contact with the antigen, in contrast, TST remains positive a long period after past *M. tuberculosis* infection [20]. However, in children the infection is usually recent. The third explanation is that the positive TST was due to a previous infection by a NTM without *M. avium* sensitin cross-reaction. It was impossible to test more NTM sensitins given that we didn't have more PBMCs stored from these patients.

One limitation of our study is that the skin test reactions to *M. avium* sensitin were not performed at the moment of inclusion of the children; therefore, it was not possible to correlate with the *ex vivo* result. Another limitation is that we have tested a reduced number of children. Nevertheless, despite these limitations, the results obtained are sufficiently consistent to draw some conclusions.

In summary, our results show enough evidence to state that previous NTM sensitisation in children induces false-positive results in the TST for diagnosing LTBI and that the IFN-γ tests could avoid both unnecessary chemoprophylaxis treatment among child populations and consuming resources searching the index case.

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STATEMENT OF INTEREST

A statement of interest for J. Domínguez can be found at www.erj.ersjournals.com/misc/statements.dtl

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