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Interferon-Gamma Release Assay Improves the Diagnosis of Tuberculosis in Children

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Background: Interferon-γ release assays (IGRAs) have been recently developed for the diagnosis of tuberculosis (TB) infection. The aim of the present study was to evaluate the performance of an enzyme-linked immunosorbent assay (ELISA)-based IGRA for detecting TB in children.
Methods: A prospective study in 336 children at risk for TB infection was carried out. All children were tested with tuberculin skin test (TST) and a commercial ELISA-based IGRA [QuantiFERON-TB Gold *In-Tube* (Cellestis)].
Results: TST were positive in 58 of 336 (17.3%) and IGRA in 60 of 336 (17.9%) children. Two (0.6%) IGRA results were indeterminate. The overall agreement between the 2 tests was intermediate (86.2%, κ = 0.533). IGRA was positive in 15 of 16 (93.8%) children with active pulmonary TB. The discordant pattern IGRA−/TST+ was significantly associated with Bacille Calmette-Guérin (BCG) vaccination. Among IGRA+ children (excluding cases of TB disease), TST− were significantly younger than TST+ children.
Conclusions: The good agreement between positive IGRA and active TB disease suggests a good sensitivity of IGRA. Discrepancies between IGRA and TST can be a result of higher specificity of IGRA that is not influenced by previous BCG vaccination. IGRA may be more sensitive in children younger than 48 months.

Key Words: children, diagnosis, interferon-γ release assay, tuberculosis, tuberculin skin test

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The diagnosis of tuberculosis (TB) infection in children is particularly challenging. To date, the gold standard for the diagnosis of TB disease is the detection of *Mycobacterium tuberculosis*.[1] However, because of the paucibacillary nature of the disease in children, smears from sputum or from gastric aspirates are positive in less than 15% of children diagnosed with TB and a positive culture is achieved only in less than 40% of cases.[2,3] Until recently the tuberculin skin test (TST) has been the only test available for the diagnosis of latent tuberculosis infection (LTBI). The main drawback of the TST is its poor specificity in individuals sensitized by prior exposure to nontuberculous mycobacteria (NTM) or by having been vaccinated with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG).[4,5,6] Additionally, TST sensitivity may be low in young children and in individuals with depressed immunity, malnutrition, or advanced TB.[5,7]

New diagnostic blood tests, based on the detection of interferon-gamma (IFN-γ) released by specifically sensitized T cells, have been recently developed. They detect in vitro immune response to *M. tuberculosis* by measuring with enzyme-linked immunosorbent assay (ELISA) or with immunospot assay (ELISpot) the IFN-γ released from T cells in response to mycobacterial antigens.[2,8,9] The specific peptide antigens are encoded by genomic segments of *M. tuberculosis* which are absent from all BCG strains and from most NTM.[2,8]

Two commercial ELISA-based IFN-γ release assays (IGRAs), QuantiFERON-TB Gold (QFT-G, Cellestis) and the newer in-tube format (QFT-IT, Cellestis), have been both approved in Europe and by the United States Food and Drug Administration. A guideline from the Centers for Disease Control and Prevention recommends the use of QFT-G in adults in all circumstances in which the TST is currently used.[10] The United Kingdom National Institute for Health and Clinical Excellence (NICE) TB guidelines recommend a 2-step strategy for LTBI diagnosis with an initial TST, followed by an IFN-γ test in TST positive subjects or in those with less reliable TST testing.[11] Most of the studies in adults comparing IGRAs (using 2 or 3 specific antigens) and TST have shown that IGRA is more specific for detecting LTBI and at least as sensitive as TST for detecting TB disease.[8,9,12–16] Fewer studies have shown a lower sensitivity.[14,17]

The newer in-tube format of the ELISA-based IGRA is a promising and simple to perform test, but it has not been currently approved in children because the published pediatric studies are in a limited number of subjects and provide contradictory results.[18–23]

The aim of this work was to evaluate the performance of an ELISA-based IGRA in children at risk for TB infection by comparing results with TST and exploring possible discordances.

MATERIALS AND METHODS

Study Subjects

Consecutive children (younger than 16 years) at risk for TB infection, who had referred to the Department of Pediatrics, University of Florence, Italy, were prospectively enrolled between July 1, 2005 and December 31, 2006. Patients were recruited from the infectious disease outpatient clinic (including a clinic dedicated to screening immigrants and internationally adopted children) and from inpatients admitted with symptoms suggestive of TB disease. The children eligible for the study were those belonging to the following 3 categories: (a) children with clinical suspicion of TB disease; (b) children in close contact with recently diagnosed cases of contagious TB disease; (c) internationally adopted or recently immigrated children coming from countries with a high prevalence of TB. Immigration within the last 2 years was considered as period of recent immigration. Children with congenital or acquired immunodeficiency disorders (based on their medical history, clin-

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ical examination, and/or laboratory tests) were excluded from the study.

Study Design

Information regarding socio-demographic data, prior TB exposure, and medical history was obtained from each child's parents or from medical documents. Children were considered to have been vaccinated with BCG if there was a clear documentation and/or a BCG scar was present. In the absence of documentation or a BCG scar, the vaccination status was considered unknown.

All children underwent clinical evaluation, venepuncture for IGRA, as part of routine blood examination, and TST. The blood sample for IGRA was taken before the TST administration, to avoid potential booster effect. Chest radiography was performed in all symptomatic children with a positive TST, and in all contacts aged less than 5 years.[24] Children with suspected pulmonary TB had 3 sputa or early morning gastric aspirate samples collected for *M. tuberculosis* detection (by means of microscopy, polymerase chain reaction, and culture).

All results were recorded in the study database. The study was approved by local Ethics Committee and all parents of the enrolled patients gave written informed consent.

Methods

TST was administered, according to the Mantoux method, by injecting intradermally 5 tuberculin units (in 0.1 mL) of purified protein derivative (Biocine Test-PPD, Chiron, Siena, Italy) into the volar surface of the forearm. The TST result was read by experienced infectious disease specialists at 72 hours by measuring the transverse diameter of the skin induration at the site of the PPD application with a flexible ruler. Following the American Academy of Pediatrics guidelines,[25] a positive TST was defined as an induration size $\geq$5 mm for children in close contact with known or suspected contagious case of TB disease or for children suspected to have TB disease (based on clinical evidence and/or chest radiograph) and $\geq$10 mm for children born in countries with a high prevalence of TB and recently immigrated.

TB disease diagnosis was assigned to any child with *M. tuberculosis* culture or detected by microscopy or molecular methods from sputum or gastric aspirate. For culture, both solid and liquid media (Löwenstein-Jensen and BACTEC 460TB, BD Biosciences Division, Sparks, MD) were used. For children it is often difficult to obtain a positive result from the above investigations.[2,3] As a consequence, the TB disease diagnosis was also assigned to any child with clinical and radiologic evidence of active TB, and with either a history of exposure to an infectious case or a positive TST.[2,26] In the absence of a recognized standard, LTBI diagnosis was assigned to any child with a positive TST and no clinical, bacteriologic, or radiographic evidence of active TB.[7] All asymptomatic children with a negative TST were defined as uninfected.

QFT-IT (Cellestis Limited, Carnegie, Victoria, Australia) was performed in the immunology laboratory according to the manufacturer's instructions.[27] Laboratory personnel were blinded to the status of the patient and TST result. QFT-IT was supplied with 3 heparinized blood collection tubes precoated with 3 TB-specific antigens (ESAT 6, CFP-10, and TB7.7 [p4]), a mitogen (phytohemagglutinin), and a negative control. The cutoff for a positive test, indicating likely *M. tuberculosis* infection, was 0.35 IU/mL of IFN-γ for the TB specific antigen-stimulated plasma sample above the amount of IFN-γ in the negative control sample. A positive response to the TB-specific antigens without a response to the mitogen was considered valid with positive result. If there was no detectable IFN-γ response to the mitogen and the TB specific antigens or if the IFN-γ level in the negative control was too high, the test was deemed "indeterminate." Starting from January 2006, quantitative data of IFN-γ response to TB-specific antigen and to mitogen were also recorded.

Statistical Analysis

Age, TST size, and IFN-γ values were expressed as median and interquartile range of months, millimeters, and IU/mL, respectively. The kappa (κ) statistic was used to assess the agreement between 2 tests. Differences in frequencies were evaluated by the Fisher exact test. The nonparametric Mann-Whitney *U* test was used to compare the medians. Linear regression analysis was used when appropriate. Because the QFT-IT ELISA cannot accurately measure IFN-γ values $>$10 IU/mL, values $>$10 IU/mL were treated as 10 IU/mL in all the analyses.[28] Data were analyzed using SPSS 11.5 (SPSS, Chicago, IL). Significance was defined by $P \leq 0.05$.

RESULTS

Three hundred-thirty six children were enrolled in the study. The median age was 54.0 (31.2–81.7) months. Their demographic characteristics and the reasons for testing are summarized in Table 1. Table 2 shows the BCG vaccination status, the TST size, and the

TABLE 1. Socio-Demographic and Clinical Characteristics of Study Population

	Total n (%)
<48 mo	152 (45.2)
Male	199 (59.2)
Region of origin	
Italy	11 (3.3)
Sub-Saharian Africa	54 (16.1)
North-Africa	9 (2.7)
Asia	43 (12.8)
Eastern-Europe	139 (41.4)
Latin-America	80 (23.8)
Reason for testing	
Screening	
Recently immigrated	13 (3.9)
Internationally adopted	276 (82.1)
Contact	
Immigrants	29 (8.6)
Italians	9 (2.7)
Clinical suspicion of active pulmonary TB	
Immigrants	7 (2.1)
Adopted	0
Italians	2 (0.6)

TABLE 2. BCG Vaccination Status, TST Size, and IGRA Result According to Diagnosis

	Uninfected n = 276 n (%)	Latent TB n = 44 n (%)	TB Disease n = 16 n (%)	Total n = 336 n (%)
BCG vaccination				
Yes	152 (55.1)	20 (45.5)	1 (6.3)	173 (51.5)
No	23 (8.3)	8 (18.2)	3 (18.8)	34 (10.1)
Unknown	101 (36.6)	16 (36.4)	12 (75.0)	129 (38.4)
TST induration diameter (mm)				
<5	253 (91.7)	0	2 (12.5)	255 (75.9)
≥5 and <10	23 (8.3)	2 (4.5)	2 (12.5)	27 (8.0)
≥10 and <15	0	20 (45.5)	5 (31.3)	25 (7.4)
≥15	0	22 (50.0)	7 (43.8)	29 (8.6)
IGRA result				
Negative	251 (90.9)	22 (50)	1 (6.2)	274 (81.5)
Positive	23 (8.3)	22 (50)	15 (93.8)	60 (17.9)
Indeterminate	2 (0.7)	0	0	2 (0.6)

IGRA results according to diagnosis. The median time between the vaccination and the tests performing was 43 (22.0–65.0) months.

The diagnosis of active TB disease was made in 16 (4.8%) children. All these children presented with pulmonary disease. Nine (56.3%) of them had symptoms compatible with TB (eg, persistent cough, fever, night sweats, or weight loss), 4 (25.0%) had been in close contact with an infected adult, and 3 (18.8%) were diagnosed, after screening examinations for children coming from regions, with high prevalence of TB. Fourteen (87.5%) of the 16 patients diagnosed with TB disease gave a positive TST. Fifteen children underwent gastric aspirate or sputum examination for *M. tuberculosis*. This investigation was not performed in 1 patient who arrived to our attention while already on treatment. Six (40%) children had *M. tuberculosis* detected from the culture. Microscopy and polymerase chain reaction were positive in 3 (20%) and in 4 (26.6%) children, respectively. Forty-four (13.1%) children were diagnosed with LTBI. The age of children with LTBI and the age of those with TB disease were not significantly different. Two hundred seventy-six (82.1%) children were defined as uninfected.

IGRA was positive in 60 (17.9%) children and negative in 274 (81.5%). Two (0.6%) children had an indeterminate result. All the indeterminate results were found in patients who tested negative to the TST. In 288 (86.2%) of 334 cases there was concurrence of the TST and IGRA results. The overall agreement between the IGRA and the TST (without considering the indeterminate results) was intermediate with a κ value of 0.533. The IGRA was positive in 15 of 16 (93.8%) children diagnosed with TB disease, showing a very good agreement between the result of the test and the diagnosis. Of the 44 children defined as LTBI according to the TST results, 22 (50.0%) had a positive and 22 (50.0%) had a negative IGRA result. Of the 276 remaining children, defined uninfected, 251 (90.9%) were tested negative and 23 (8.3%) were positive. Figure 1 (Supplemental Digital Content 1, http://links.lww.com/A972) shows the results of the IGRA stratified according to the results of the TST. In the LTBI subgroup, a positive IGRA result was significantly associated with larger diameter of the TST (11 [10–15] mm in the children IGRA − vs. 15 [11–20] mm in the IGRA+ [$P = 0.027$]).

To evaluate the performance of the IGRA, possible causes for the discordant results between the 2 tests were analyzed. Nineteen (86.4%) of 22 children with LTBI, as diagnosed by TST, but a negative IGRA were tested because they came from regions with a high prevalence of TB. Among the 28 children with LTBI diagnosed by TST and a known BCG status, those with a negative IGRA were more likely to be vaccinated with BCG, compared with those with a positive IGRA (14/16 [87.5%] vs. 6/12 [50%], $P = 0.04$). These results may indicate higher specificity of the IGRA which is not influenced by previous BCG vaccination. All children diagnosed with LTBI, including the 22 children with discordant pattern IGRA−/TST+, received the preventive treatment for LTBI with isoniazid and/or rifampin.[11,24]

Twenty-four children had a positive IGRA but a negative TST result. Twenty-three (95.8%) of these children were internationally adopted, and one had a close contact of a TB case. One patient was diagnosed with TB disease and the IGRA result was, therefore, in agreement with the diagnosis. Of the 23 children defined as uninfected by TST but with a positive IGRA results, 4 were lost to follow-up. One was a close contact of a TB contagious case and consequently we treated this child for 2 months with isoniazid. Both TST and IGRA, repeated at the end of the preventive treatment, as well as the chest radiograph, were negative. Eighteen children had a TST repeated after 2 months and the result was still negative. A chest radiograph was not performed for this group. At the time we planned the study, there were insufficient data regarding the use of ELISA-based IGRA in children. In the absence of accepted guidelines, we decided not to perform a chest radiograph in asymptomatic children with a negative TST. Their IGRA follow-up is ongoing. During the follow-up none developed active TB disease. Among children with a positive IGRA result (excluding the cases of TB disease), the age of those with a negative TST (37 [20.0–71.0] months) was significantly lower than that of those with a positive TST (90.5 [49.0–134.5] months), ($P = 0.01$).

IFN-γ quantitative data were available for 32 of 60 (53%) patients with a positive IGRA. A significant correlation between IFN-γ levels in response to TB specific antigens and the diameter of TST ($r = +0.48$, $P = 0.005$) was observed (Fig. 2A). No correlation between the age of the children and the IFN-γ values ($r = +0.05$, $P = 0.773$) was found (Fig. 2B). The median of IFN-γ concentrations in children defined as uninfected (1.35 [0.49–2.84])

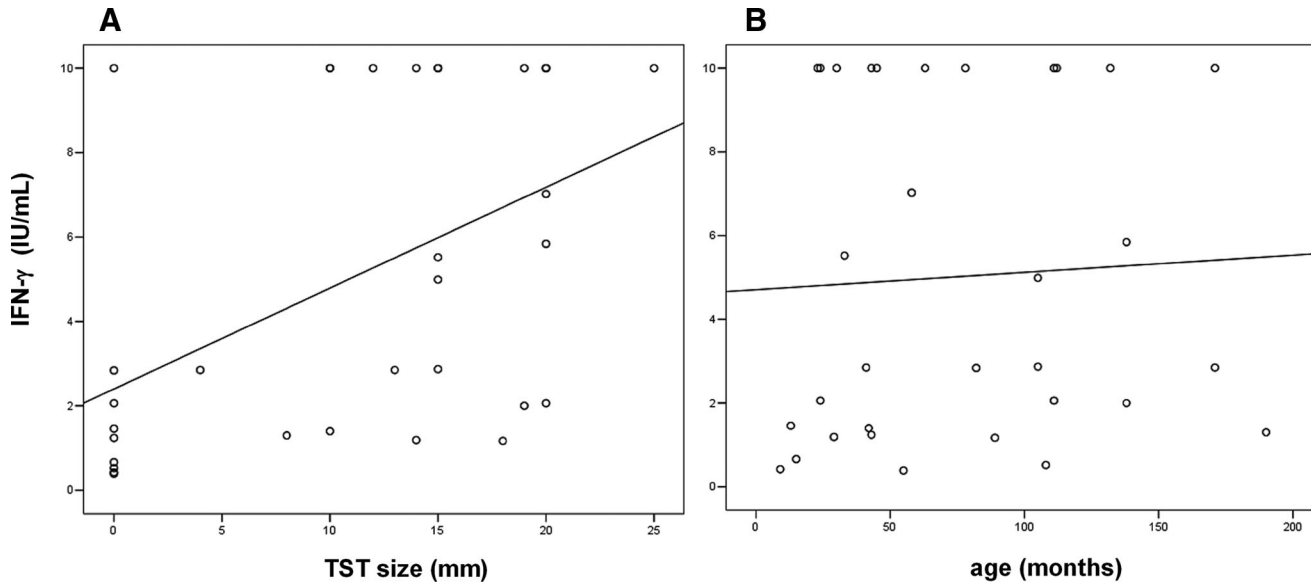

FIGURE 2. IFN-γ values in response to tuberculosis specific antigens according to TST size (A) and age of children (B).

was significantly lower than that of those with LTBI (5.84 [2.13–10]), ($P = 0.008$). No difference in IFN-γ values between the children with LTBI and the children with TB disease (5.84 [2.13–10] vs. 7.02 [2.03–10], $P = 0.948$) was found (Fig. 3, Supplemental Digital Content 2, http://links.lww.com/A974). In children with discordant pattern IGRA+/TST−, the median of IFN-γ levels in response to TB specific antigens was 1.35 (0.56–2.65).

DISCUSSION

To our knowledge, this is the largest study on the reliability of ELISA-based IGRA in children. It is also the first study carried out including international adoptees, which is an increasingly relevant phenomenon within developed countries. The main findings of our study were a very good agreement between positive IGRA and active TB and an intermediate agreement between IGRA and TST. Unlike other published studies, we had very few indeterminate results despite the high number of children younger than 48 months. This is an important aspect to be considered, as other authors indicate the high number of indeterminate results in children as a limiting factor for the use of the test in this population.[13,18]

Half of the children with LTBI had a negative IGRA result. This disagreement can be interpreted as the IGRA being either more specific or less sensitive, or more likely, the TST giving a false positive as suggested by the observation that the children with discordant pattern IGRA−/TST+ were more likely to be vaccinated with BCG. These findings may reflect an higher specificity of IGRA, as already suggested.[12–15] Less than 10% of the children defined uninfected by TST had a positive IGRA. The age of these children was lower than that of those with positive results from both tests. It is known that TST can be falsely negative in children younger than 48 months.[1,5,25] This could explain the difference between the 2 tests and may suggest that the IGRA is more sensitive in young children. Longer follow-up of these children could provide a better understanding of the results and more solid conclusions regarding this issue. It should also be determined whether treating individuals diagnosed with LTBI by the IGRA will result in protection against active disease.

The disagreement between TST and IGRA results could be a consequence of the distinct immunologic mechanisms responsible for positive TST or IGRA tests. TST is a delayed-type-hypersensitivity reaction that measures both effector and memory T cells function, whereas IFN-γ, with its short period of incubation, measures mostly the effector T-cell function. Thus, a positive IGRA result may indicate a more recent or ongoing TB infection, whereas a positive TST result could indicate a more remote TB infection.[14,17]

There is no reliable cutoff that definitely indicates TB infection, although in general practice a TST >15 mm has been considered a reliable cutoff.[6,25] In our study, a positive IGRA in children with LTBI was significantly associated with a larger TST with the median size being 15 mm, although there was an evident overlapping in TST size between the positive and negative IGRA results.

Although the number of cases was small, the significant correlation between TST size and IFN-γ values, as well as the higher IFN-γ concentrations in children with LTBI and TB disease, suggest a possible role of IFN-γ quantitative data for diagnosis of TB infection. However, the absence of difference between the IFN-γ concentrations in LTBI and TB disease indicates that IFN-γ quantitative data cannot be used to distinguish between infection and disease. These data need to be confirmed in further studies.

Evaluating the reliability of IGRA may be difficult, particularly in children. *M. tuberculosis* detection is the gold standard for TB disease diagnosis. Therefore, the sensitivity of a new test should be evaluated using cases of confirmed TB disease. However, *M. tuberculosis* detection is rarely obtained in children, and so a large data set would be needed. In the absence of a gold standard for the diagnosis of LTBI, optimal estimation of sensitivity and specificity is not possible.[9,29]

Our findings confirm those obtained in previous studies in adults and some studies in children.[2,9,13,20,21] The use and assessment of the ELISA-based IGRAs in children is limited at present.[2,9] Similarly to our findings, Connell et al, in 2 subsequent studies, showed a good agreement between positive IGRAs and TB disease, whereas a poor correlation between IGRAs and TST for the diagnosis of LTBI in children was found.[18,23] Ferrara et al studied a population comprising both adults and children claiming a good agreement between QFT-G and TST. However, this agreement was lower in the BCG-vaccinated group and these findings were interpreted as the QFT-G being more specific.[13] Dogra et al showed a substantial concordance between QFT-IT and TST with no impact of BCG on either tests.[19] Nakaoka et al found that the agreement between TST and QFT-IT was inconsistent and suggested that the pattern of disagreement in children at high risk was not random and might reflect a higher sensitivity of the QFT-IT.[20] Detjen et al found that both IGRAs currently available (QFT-IT and T SPOT-TB) have high diagnostic value in bacteriologically confirmed childhood TB. Both tests also allowed to distinguish positive TST results caused by NTM disease, reducing TB overdiagnosis.[21] Recently, Taylor et al evaluated the potential impact of the new National Institute for Health and Clinical Excellence TB guidelines in pediatric TB screening. After the 2-step-strategy, 85% fewer children would have been given LTBI treatment but 2% children with possible TB disease would not have been identified.[22]

A limiting factor in our study was that we could investigate a relatively small number of children that either had been in contact with TB-infected adults or had active TB. On the other hand, the majority of children were internationally adopted and the information regarding medical history, BCG vaccination status, and social data were often missing. In these children the cutoff for a positive TST is generally considered to be ≥10 mm.[25] However, the lack of information regarding their potential close contact with a TB case, as well as their previous tuberculin testing, makes it difficult to define with certainty a positive or negative TST result and consequently to compare the 2 tests. Moreover, we were not able to investigate the role of NTM infections and we could not test any child with no risk of TB infection due to ethical reasons. The IFN-γ quantitative analysis was limited by the fact that the quantitative data were available only for half of the children with positive IGRA.

Our study shows that ELISA-based IGRA can have an important role for the detection of TB in children. Undoubtedly, large and well-designed trials and long-term follow-up studies are needed to confirm the value of the IGRA. However, this test holds the potential to become a useful diagnostic tool for TB control and an important step towards the achievement of the World Health Organization Millennium Development Goals, which target to have halted TB and begun to reverse its incidence by 2015.[30]

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