Long-Incubation-Time Gamma Interferon Release Assays in Response to Purified Protein Derivative, ESAT-6, and/or CFP-10 for the Diagnosis of Mycobacterium tuberculosis Infection in Children

K. Schepers,a F. Mouchet,b V. Dirix,a I. De Schutter,c K. Jotzo,b V. Verscheure,a P. Geurts,d M. Singh,e J. P. Van Vooren,f F. Mascata,g
Laboratory of Vaccinology and Mucosal Immunity, Université Libre de Bruxelles (U.L.B.), Brussels, Belgium; Pediatric Department, CHU Saint-Pierre, U.L.B., Brussels, Belgium; Department of Pediatric Pulmonology, Cystic Fibrosis Clinic and Pediatric Infectious Diseases–Universitair Ziekenhuis Brussel (UZ Brussels), Brussels, Belgium; Department of Electrical Engineering and Computer Science & GIGA-R, Systems and Modeling, University of Liège (U.Lg.), Liège, Belgium; Lionex Diagnostics & Therapeutics, Braunschweig, Germany; Immune Deficiencies Treatment Unit, Hôpital Erasme, U.L.B., Brussels, Belgium; Immunobiology Clinic, Hôpital Erasme, U.L.B., Brussels, Belgium

The diagnosis of childhood active tuberculosis (aTB) and latent Mycobacterium tuberculosis (M. tuberculosis) infection (LTBI) remains a challenge, and the replacement of tuberculin skin tests (TST) with commercialized gamma interferon (IFN-γ) release assays (IGRA) is not currently recommended. Two hundred sixty-six children between 1 month and 15 years of age, 214 of whom were at risk of recent M. tuberculosis infection and 51 who were included as controls, were prospectively enrolled in our study. According to the results of a clinical evaluation, TST, chest X ray, and microbiological assessment, each child was classified as noninfected, having LTBI, or having aTB. Long-incubation-time purified protein derivative (PPD), ESAT-6, and CFP-10 IGRA were performed and evaluated for their accuracy in correctly classifying the children. Whereas both TST and PPD IGRA were suboptimal for detecting aTB, combining the CFP-10 IGRA with a TST or with a PPD IGRA allowed us to detect all the children with aTB with a specificity of 96% for children who were positive for the CFP-10 IGRA. Moreover, the combination of the CFP-10 IGRA and PPD IGRA detected 96% of children who were eventually classified as having LTBI, but a strong IFN-γ response to CFP-10 (defined as >500 pg/ml) was highly suggestive of aTB, at least among the children who were <3 years old. The use of long-incubation-time CFP-10 IGRA and PPD IGRA should help clinicians to quickly identify aTB or LTBI in young children.

The diagnosis of childhood tuberculosis (TB) remains a real challenge and public health problem, as young children who are infected with Mycobacterium tuberculosis are more prone to developing disseminated disease. Active TB disease is paucibacillary in children, with the bacteria being identified in roughly 50% of cases (1). Children are diagnosed with either confirmed active TB (aTB) if their clinical specimens are positive for M. tuberculosis cultures, or highly probable aTB if they fulfill the criteria of: (i) known contact with an adult index case, (ii) positive tuberculin skin test (TST), and (iii) compatible clinical and/or radiological presentation (2). Unfortunately, in vivo TST has a poor specificity in children sensitized by exposure to nontuberculous mycobacteria (NTM) or who have been vaccinated with Mycobacterium bovis bacillus Calmette-Guérin (BCG) (3). Furthermore, TST does not distinguish between children with aTB from those with a latent form of the infection (latent M. tuberculosis infection [LTBI]).

Other immunodiagnostic tests have been developed that are based on the in vitro secretion of gamma interferon (IFN-γ) by blood cells stimulated with peptides encoded by genomic segments of M. tuberculosis, named the regions of difference (RDs), which are absent from all BCG strains and from most NTM (4). Two such tests are commercialized, the QuantiFERON-TB gold in-tube (QFT-GIT) (Qiagen, Venlo, Netherlands), which measures the concentrations of IFN-γ released in response to peptides from RD-1 and RD-11, and the T-SPOT.TB (Oxford Immunotec, United Kingdom), which counts the number of IFN-γ-secreting cells in response to peptides from RD-1. The major advantage of these tests compared to TST is their specificities. They have been extensively used in adult populations who have been screened for latent M. tuberculosis infection (5). However, as with the TST, these tests are not specific for aTB or for LTBI, and they do not differentiate between infection and disease (6, 7). In addition, despite a sensitivity of 80% in adults with aTB, the negative predictive value is not high enough to recommend its use to reject a diagnosis of aTB (8). The utility of these tests in adults is therefore restricted to the diagnosis of LTBI, but their sensitivities for the diagnosis of remote LTBI were recently challenged (9–13). As the antigens used in the commercialized gamma interferon release assays (IGRA) are poorly expressed by M. tuberculosis during its latency, corresponding antigen-specific T cells in the blood are mainly memory T lymphocytes that are not optimally detected by short-term assays (10, 14). Commercialized IGRA were initially evaluated in adults, with reports in children being scarce for several years. As children who develop TB in countries with low TB prevalence have a high probability of suffering from primary tuberculosis and therefore showing a primary immune response, results may be quite different from those obtained from adults in the same setting who generally suffer from reactivation of TB. The
accuracy of the commercialized IGRA should therefore be better in children than in adults, as they are most often evaluated during the decay of a primary infection and they do not present with remote latent TB at least in their first years of life. Therefore, the infecting mycobacteria probably most often express antigens from the RD-1 of their genome. Meta-analyses of pediatric studies and reviews were recently published (15–17). The authors concluded that the available data do not indicate a clear benefit of the IGRA over the TST in terms of diagnostic accuracy for *M. tuberculosis* infection in children.

In this study, we evaluated whether a long-term in-house IGRA based on the release of IFN-γ by peripheral blood mononuclear cells (PBMC) stimulated for 4 days with mycobacterial protein antigens would improve the diagnostic accuracy of IGRA for the detection of *M. tuberculosis* in children. We separately tested different mycobacterial antigens to determine if one of them would be sufficient for diagnosis.

**MATERIALS AND METHODS**

**Study subjects and study design.** A prospective multicenter study involving five hospitals was conducted over 5 years and included 266 children between 1 month and 15 years of age. 214 of whom were at risk of recent *M. tuberculosis* infection and 52 of whom were included as controls. All children evaluated for possible aTB disease (having symptoms or signs suggestive of infection) referred because of close contact with recently diagnosed cases of contagious aTB were enrolled, except for those with a congenital or acquired immune deficiency. The absence of consent from legal guardians was also an exclusion criterion. The medical evaluation of these children always included a detailed inquiry of the child’s risk factors for exposure to *M. tuberculosis*, a clinical assessment, TST, chest radiograph, and blood sample for classical biological analysis. Depending on the symptoms, the appropriate biological samples were sent to the laboratory for smear and *M. tuberculosis* culture (samples of sputum, gastric aspirate, and/or bronchoalveolar lavage fluid sample, cerebrospinal fluid sample, tissue biopsy sample), and nucleic acid amplification tests were performed on at least one specimen in the case of suspicion of aTB. For the purposes of this study, an additional blood sample of 2 to 5 ml was collected from each child.

TST were performed by trained nurses according to standard national guidelines using an intradermal injection of 2 units of tuberculin (or the purified protein derivative, PPD RT23; Statens Serum Institute, Copenhagen, Denmark), and a measurement of transverse induration was performed 48 to 72 h later. The cutoff values used for their interpretation were based on recommendations from the Centers for Disease Control and Prevention (CDC), considering the history of TB exposure, individual risk factors, age, and immune status (18). Children in close contact with a smear-positive case were tested again after 8 to 12 weeks in the case of a negative initial result. The chest radiographs were reviewed by an experienced pediatric radiologist.

Based on a consideration of risk factors, clinical assessment, TST, chest X-ray, and the results of routine laboratory tests (microbiological analysis), the pediatricians in charge of the children, who were blinded to the results of long-incubation-time IGRA, retrospectively classified each child into one of three groups which were further analyzed in relation to the results of the IGRA tests:

(i) The aTB group included children with confirmed aTB (*M. tuberculosis* detected in biological samples) or highly probable aTB (known contact with a smear-positive adult, positive TST results, clinical or radiological signs compatible with TB disease, and a response to *M. tuberculosis*-specific treatment on follow-up). A final diagnosis of aTB was confirmed by clinical improvement in these children during treatment, all of whom were followed up for ≥6 months.

(ii) The LTBI group included children without symptoms and radiological signs but who had a history of exposure to an infectious TB index case and a positive TST either at inclusion or at the time of repeat testing.

(iii) The household contact (HC) group included asymptomatic children with a history of exposure to infectious TB and a negative TST at the times of inclusion and at repeated testing.

The control (CTRL) group (n = 52) comprised children evaluated for possible aTB as part of a differential diagnosis but who had a final alternative diagnosis, a negative TST, and a complete recovery in the absence of specific anti-TB treatment.

**Long-term antigen-specific IGRA.** Long-incubation-time IGRA were developed to evaluate the release of IFN-γ by PBMC in response to *in vitro* stimulation with three different protein mycobacterial antigens: PPD, the early secreted antigen-6 (ESAT-6), and the culture filtrate protein-10 (CFP-10). PPD was chosen to compare *in vitro* and *in vivo* responses to tuberculin, whereas ESAT-6 and CFP-10 were chosen because they are both encoded by the RD-1 of the *M. tuberculosis* genome and their peptides are included in the commercialized short-term-incubation IGRA.

PBMC were purified from fresh blood samples and stimulated *in vitro* with the antigens as previously described (19, 20). According to previous standardization, PPD (batch RT49; Statens Serum Institute, Denmark) was used at a concentration of 4 μg/ml, and ESAT-6 and CFP-10 (Lionex, Braunschweig, Germany) were used at concentrations of 5 μg/ml ESAT-6 and CFP-10 are recombinant proteins produced in *Escherichia coli*, and the lipopolysaccharide content of the batches used here was always <0.016 endotoxin units (EU)/μg. In parallel, as the negative control, PBMC were left to culture in the absence of any added antigen and were stimulated with a mitogen, phytohemagglutinin A (PHA) (2 μg/ml; Remel, Lenexa, KS, USA), as the positive control.

IFN-γ concentrations were measured in 96-h cell culture supernatants using sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturers’ instructions (IFN-γ CytoSets ELISA kit; BioSource International, Camarillo, CA, USA). IFN-γ concentrations were calculated using the KC4 software (BRS, Drogenbos, Belgium) by referring to a standard curve generated by serial dilutions of the standard, from 2,500 pg/ml to 10 pg/ml IFN-γ. When detectable, cytokine concentrations obtained under nonstimulated conditions were subtracted from those obtained for the mitogen- or antigen-stimulated cells. The IGRA results were considered indeterminate when the IFN-γ response to PHA was <100 pg/ml. This was the case for 7 children with a median age of 5 months (range, 2 months to 6 years) who all were clinically classified as noninfected. These children were not included in the final statistical analysis of their IGRA results. Due to the limited amount of blood samples in children, all the tests could not be performed on each sample.

**Ethical statement.** Ethical approval for the study was provided by the central ethics committee of Hôpital Erasme and by the respective ethical committees of the different hospitals where children were included (Centre Hospitalier Universitaire [CHU] Saint Pierre, Universitair Ziekenhuis Brussel, Hôpital Universitaire Des Enfants Reine Fabiola [HUDERF], and CHU Charleroi). Written informed consent was obtained from the legal guardians of all study participants.

**Statistical analyses.** All children with complete clinical data and at least one available IGRA result were included in the final analysis. Indeterminate results were excluded from the final statistical analysis. Differences between the groups were assessed by the nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparison tests or by the nonparametric Mann-Whitney U test when appropriate. Receiver operating characteristic (ROC) curves and the areas under the curves (AUC) were established for each mycobacterial antigen. Statistical analysis was performed with the GraphPad Prism software version 4.0b (GraphPad Software, San Diego, CA, USA).

**RESULTS**

**Patient characteristics.** The main demographic characteristics of the children included in the study are shown in Table 1. Among the 266 included children, 135 children were identified as being...
infected with *M. Tuberculosis*, whereas the other 111 children were considered to be noninfected. The group of infected children comprised 63 with aTB and 92 (35%) considered to be LTBI. The group of uninfected children comprised those referred for having close contact with an index case of aTB and who were considered to be noninfected HC (*n*/H11005 59) and children who were evaluated for possible aTB but were considered after evaluation to be not infected with *M. Tuberculosis* (CTRL, *n*/H11005 52). As the children belonging to these two last groups (HC and CTRL) were all uninfected, they were further considered to be one group referred to as noninfected (NI) children.

The median age of all children was 5.07 years (range, 0.08 to 15.51 years), but the LTBI children were older than children from other groups (*P*/H11021 0.0001). Sixty-two percent of the children were born in Belgium, but 80% of those had an ethnic origin from an area that is highly endemic for TB. Fifty-nine percent of the children had a known TB contact, and this was a family member in 70% of the cases. Seventy children (26%) had been vaccinated with BCG. Whereas most cases of aTB were pulmonary TB (PTB) (86%), 9 children (14%) had extrapulmonary aTB (EPTB): 4 cases of miliary TB and 1 case each of cervical adenitis, peritoneal infection, pericarditis, and spondylodiscitis.

### TABLE 1 Characteristics of the study population (*n* = 266)

| Subject characteristics                  | Data by group (*n*): | Noninfected (111) | LTBI (92) | aTB (63) |
|-----------------------------------------|----------------------|-------------------|-----------|----------|
| Age (median [range] yr)                 |                      | 3.41 (0.08–12.35) | 7.66 (0.88–14.72) | 3.94 (0.48–15.51) |
| % of children <5 yr age                 |                      | 63                | 24        | 62       |
| Gender (no. of males/no. of females)    |                      | 50/61             | 50/42     | 34/29    |
| Ethnicity (no./total no. [%] of children)|                      |                   |           |          |
| Caucasian                               |                      | 34/111 (31)       | 30/92 (33) | 12/63 (19) |
| North African                           |                      | 40/111 (36)       | 23/92 (25) | 24/63 (38) |
| Sub-Saharan African                     |                      | 24/111 (22)       | 26/92 (28) | 23/63 (37) |
| Other                                   |                      | 13/111 (11)       | 13/92 (14) | 4/63 (6)  |
| History of BCG vaccine (no./total no. [%]) |                      |                   |           |          |
| Vaccinated                              |                      | 17/111 (15)       | 36/92 (39) | 17/63 (27) |
| None                                    |                      | 93/111 (84)       | 50/92 (54) | 46/63 (73) |
| Unknown                                 |                      | 1/111 (1)         | 6/92 (7)  | 0        |
| Contact with a smear-positive adult (no./total no. [%]) | | | | |
| Yes                                     |                      | 59/111 (53)       | 58/92 (63) | 41/63 (65) |
| Unknown                                 |                      | 6/111             | 34/92     | 22/63    |
| TST induration size (no./total no. [%]) |                      |                   |           |          |
| 0–4 mm                                  |                      | 111/111 (100)     | 0/92 (0)  | 4/63 (6)  |
| 5–9 mm                                  |                      | 6/92 (6.5)        | 2/63 (3)  |           |
| 10–14 mm                                |                      | 19/92 (21)        | 12/63 (19) |          |
| ≥15 mm                                  |                      | 61/92 (66)        | 44/63 (70) |          |
| Unknown                                 |                      | 6/92 (6.5)        | 1/63 (2)  |           |
| Country of birth (no./total no. [%])    |                      |                   |           |          |
| Belgium                                 |                      | 68/111 (61)       | 52/92 (56) | 44/63 (70) |
| Other                                   |                      | 8/111 (7%)        | 19/92 (21%) | 14/63 (22%) |
| Unknown                                 |                      | 35/111 (32)       | 21/92 (23) | 5/63 (8)  |
| Positive bacteriology (no./total no. [%]) |                      |                   |           |          |
| Pulmonary                               |                      | 0                 | 0         | 28/63 (44) |
| Extrapulmonary                          |                      | NA                | NA        | 54/63 (86) |
| Unknown                                 |                      | NA                | NA        | 9/63 (14) |
| Positive IGRA results (no./total no. [%]) |                      |                   |           |          |
| PPD-IGRA                                |                      | 8/102 (8)         | 83/89 (93) | 55/57 (96) |
| ESAT-6 IGRA                             |                      | 6/98 (6)          | 44/78 (56) | 37/52 (71) |
| CFP-10 IGRA                             |                      | 4/93 (4)          | 47/64 (73) | 38/46 (83) |

*TST*, tuberculin skin tests.
TABLE 2 Sensitivities and specificities of the different tests

| Test (cutoff) | Sensitivity (%) for: | Specificity for LTBI (%) |
|--------------|----------------------|--------------------------|
|              | Confirmed            | Probable EP             |
| TST (≥5 mm)  | 93                   | 94                       | 78                       | 100                     | NA* |
| PPD IGRA (≥500 pg/ml) | 96             | 97                       | 100                      | 93                      | 92  |
| ESAT-6 IGRA (≥200 pg/ml) | 71             | 71                       | 67                       | 56                      | 94  |
| CFP-10 IGRA (≥50 pg/ml) | 81             | 85                       | 75                       | 73                      | 96  |

*NA, not applicable.

P < 0.0001), whereas IFN-γ concentrations were not significantly different between children with aTB and LTBI.

To evaluate the sensitivity and specificity of the PPD-IFN-γ release assay (PPD IGRA) for the discrimination of infected from NI children, a ROC curve was constructed. The area under the curve (AUC) was 0.98 (95% confidence interval [CI], 0.96 to 0.99) for aTB and 0.96 (95% CI, 0.93 to 0.99) for LTBI (Fig. 1B), indicating a good global accuracy of the PPD IGRA for discriminating infected from NI children. A cutoff of 500 pg/ml of IFN-γ was selected as the most appropriate for differentiating the groups of infected from noninfected subjects. It provided a sensitivity of 96% for the diagnosis of aTB and of 93% for the identification of LTBI, the specificity being 92% for both groups (Table 2). The specificity was probably affected by the interference of prior BCG vaccination in some children as, whatever the group, BCG-vaccinated children secreted more IFN-γ in response to PPD than nonvaccinated children, with medians (ranges) of 3,973 pg/ml (12 to 120,661 pg/ml) and 477 pg/ml (10 to 81,157 pg/ml), respectively (P < 0.05).

ESAT-6- and CFP-10-IFN-γ release assays. As shown in Fig. 1C, PBMC from the aTB and LTBI children secreted significantly more IFN-γ in response to ESAT-6 than NI children (medians of 1,098 pg/ml and 296 pg/ml compared to 10 pg/ml; P < 0.0001), whereas the IFN-γ concentrations were not significantly different between children with aTB and LTBI. Similar results were obtained with the CFP-10-IFN-γ release assay (CFP-10 IGRA), with median IFN-γ concentrations of 1,067 pg/ml in aTB children, 322 pg/ml in LTBI children, and 10 pg/ml in NI children (P < 0.0001) (Fig. 1E). Again, IFN-γ concentrations were not significantly different between the aTB and LTBI children.

To evaluate the sensitivity and specificity of the ESAT-6- and CFP-10 IGRA to discriminate between infected and NI children, ROC curves were constructed. The AUC values for ESAT-6 were 0.88 (95% CI, 0.81 to 0.94) and 0.82 (95% CI, 0.75 to 0.88) for aTB and LTBI, respectively, and for CFP-10, the AUC values were 0.93 (95% CI, 0.87 to 0.98) and 0.88 (95% CI, 0.81 to 0.94) for aTB and LTBI, respectively (Fig. 1D and F). The cutoffs of 200 pg/ml IFN-γ and of 50 pg/ml IFN-γ for ESAT-6 and CFP-10, respectively, were selected as those providing the best discrimination between infected and noninfected children. Based on this, the sensitivity of the ESAT-6 IGRA was 71% for the diagnosis of aTB and 56% for the diagnosis of LTBI, the specificity being 94% for both groups. The sensitivity of the CFP-10 IGRA was 83% for aTB and 73% for LTBI, with 96% specificity for both groups (Table 2).

Diagnostic accuracy of immune-based tests in children with active TB. Considered individually, both the TST and the PPD IGRA provided an excellent sensitivity for the diagnosis of aTB (94% and 96%, respectively) (Table 2). However, combining these tests afforded a 100% sensitivity for reaching the objective of detecting all the children with aTB; the PPD IGRA was positive for the 4 children who had a negative TST, whereas the TST was positive for 2 PPD IGRA-negative children (Fig. 2A).

Similarly, although the sensitivity of the CFP-10 IGRA was only 83%, combining these results with those of the TST (Fig. 2C) or the PPD IGRA (Fig. 3) provided 100% sensitivity for the diagnosis of aTB, with the CFP-10 IGRA affording a high specificity (96%) for the children who were positive for this test.

In contrast, the sensitivity of the ESAT-6 IGRA remained poor...
(71%), and even when combining these results with the TST (Fig. 2B) or the PPD or CFP-10 IGRA, the sensitivity did not reach 100% (data not shown).

These results were not different when considering confirmed versus probable aTB ($P = 0.71$) (Table 2). In contrast, with the exception of the PPD IGRA, lower sensitivities were obtained for the diagnosis of EPTB than for PTB (with CFP-10 IGRA, 75% for EPTB and 85% for PTB; with TST, 78% for EPTB and 96% for PTB, at a 5-mm cutoff).

These results therefore suggest that combining the PPD IGRA and the CFP-10 IGRA was more efficient for diagnosing aTB in children than the current approach using TST alone, even if, like TST, none of these IGRA were able to differentiate aTB from LTBI.

Figure 2 Correlations between the TST induration size and the IFN-γ concentrations released in the different long-term IGRA. The induration size (mm) of the TST measured 48 to 72 h after the intradermal injection of 2 U PPD was compared to the IFN-γ concentrations released in 96-h cell culture supernatants from PBMC stimulated in vitro with PPD (A), ESAT-6 (B), or CFP-10 (C). The vertical dotted lines indicate the cutoff values chosen for the different IGRA. The black circles represent children with aTB, whereas the gray circles represent children with LTBI.

Diagnostic accuracy of the immune-based tests in LTBI children. By definition, all children with LTBI had a positive TST. However, the relatively poor specificity of TST is well known to be a consequence of the interference of a previous BCG vaccination or infection with NTM. Among IGRA, the best sensitivity for detecting LTBI was obtained with the PPD IGRA (93%), probably because this test used the same antigen as the TST, but the specificity was therefore suboptimal (92%). The specificities of both the TST and PPD IGRA are affected by the proportion of BCG-vaccinated controls and are therefore quite variable among different countries. This proportion was rather low in our NI population (15%); however, 18% of them had a positive PPD IGRA compared to only 5% of the non-BCG-vaccinated NI children ($P = 0.0012$).

In contrast, an excellent specificity for LTBI diagnosis was provided by the CFP-10 IGRA (96%), but the sensitivity was low (73%). However, combining the CFP-10 IGRA and the PPD IGRA allowed us to diagnose 96% of the LTBI children, with only 4/92 being negative for both tests (Fig. 3). We therefore suggest this combination as optimal to provide a good specificity, at least for the children with a positive CFP-10 IGRA result.

Figure 3 Correlation between the results from the PPD IGRA and the CFP-10 IGRA. IFN-γ concentrations were measured in 96-h cell culture supernatants from PBMC stimulated in vitro with PPD or with CFP-10. The dotted lines indicate the cutoff values chosen for the different IGRA. The black circles represent children with aTB, the gray circles represent children with LTBI, and the open circles represent NI children.

Discrimination between aTB and LTBI in children <3 years old. Whereas the IFN-γ concentrations secreted in response to PPD, ESAT-6, or CFP-10 were not significantly different between children with LTBI and aTB, significant differences appeared when focusing on the results obtained for children <3 years of age. In this specific group, the PBMC from aTB secreted significantly more IFN-γ than those from LTBI in response to the three antigens tested, suggesting that higher IFN-γ concentrations might be more suggestive of a diagnosis of aTB than of LTBI (Fig. 4). PPD-induced IFN-γ concentrations of >5,000 pg/ml (found in 16 children) gave a probability of 94% of having aTB versus 6% of having LTBI, whereas no NI children had such results. For ESAT-6- or CFP-10-induced IFN-γ concentrations, results of >500 pg/ml provided probabilities of having aTB of 83% and 88%, respectively, compared to probabilities of having LTBI of 11% and 12%, respectively. Only 1/18 NI children had such results in response to ESAT-6 and none in response to CFP-10.

In view of the high specificity of the CFP-10 IGRA, we suggest that a high IFN-γ response to CFP-10 can be used as a biomarker of highly probable aTB.

Discussion

Whereas everyone agrees on the difficulty of accurately diagnosing $M.\ tuberculosis$ infection in children, current guidelines do not recommend replacing TST with one of the commercially available IGRA (21, 22). Evidence from the literature does not indicate a
clear benefit for the diagnostic accuracy of the IGRA over the TST (15, 17), especially in children <5 years of age (23), in whom a high proportion of indeterminate IGRA results have been reported (15). IGRA are suggested to be used as a complementary tool to TST only in certain situations (21), but both the CDC and the American Academy of Pediatrics recommend the limited use of IGRA in children <5 years of age (24, 25).

The early diagnosis of aTB in children remains a challenge, and the sensitivity estimates for the different immune-based tests are comparably low, averaging 84% in high-income countries (15). We report here that combining the results from two different long-incubation-time IGRA, in response to CFP-10 and PPD, allowed us to identify all the children who were later diagnosed as having aTB. Both bacteriologically confirmed and highly probable aTB infections were confirmed by these combined IGRA, in contrast to the results reported for the QFT-GIT, which is more sensitive for the detection of bacteriologically proven aTB (26). Furthermore, these long-incubation-time IGRA also provide optimal sensitivities for the diagnosis of EPTB, as all cases were detected by the PPD IGRA compared to only 78% with the in vivo TST. This poor sensitivity of the TST for the diagnosis of EPTB was previously reported (27–29). The results obtained with the combined CFP-10 and PPD IGRA were thus better than those obtained with the TST, which missed 4/63 children with aTB in this cohort. Even if the specificity of the PPD IGRA was limited by possible false-positive results occurring due to previous BCG vaccination or NTM infections, an excellent specificity was nonetheless in the majority of children with a positive CFP-10 IGRA result. The accuracy of these new long-incubation-time IGRA for diagnosing aTB in children therefore appears to be better than the published results using the commercialized IGRA (15); this should therefore allow patients to avoid skin testing, a method that remains difficult to standardize and requires two visits at 48- to 120-h intervals, which sometimes makes compliance difficult. These results were obtained by testing a large cohort of children; however, the study was performed in a country with low TB incidence and with a low BCG vaccination rate. Belgium belongs to the WHO group 3, with an incidence TB rate of 10.3/100,000 in 2010, and children <5 years represent 3.5% of the nationally reported cases (see www.fares.be/documents/Regtbc2010.pdf). However, the study was conducted in Brussels, where the incidence rate is 3 times higher than the national incidence.

In contrast to the diagnostic accuracy of the combined CFP-10 and PPD IGRA for aTB, a combination of the results obtained with the two major antigens included as peptides in the commercial tests, ESAT-6 and CFP-10, provided only 85% sensitivity for the diagnosis of aTB in our study. We thus confirmed the data reported in a pediatric meta-analysis for high-income countries (15), as well as the higher diagnostic value of CFP-10 compared to ESAT-6 for the diagnosis of aTB (30).

The IGRA evaluated here not only allowed us to diagnose all the children classified as having aTB but also permitted us to discriminate between aTB and LTBI in children <3 years of age. Our results indicate that in this subgroup of young children, high IFN-γ concentrations secreted in response to CFP-10 or PPD or ESAT-6 in the long-incubation-time IGRA was highly suggestive of aTB, with very few LTBI children secreting IFN-γ at such elevated concentrations. This is in contrast with most studies that suggest poor sensitivity of the IGRA in young children attributed to impaired IFN-γ production during early childhood, but relevant studies are limited (31). More recently, Critselis et al. also reported a high IFN-γ-producing capacity of infants in response to mycobacterial antigens (32). These very high IFN-γ secretions in young children with aTB might reflect a high mycobacterial burden in patients who were recently infected and without subsequent depression of cellular immune responses (33), in contrast to what is observed in persistent infections in adults (34). This, to our knowledge, is the first report of an IGRA that differentiates aTB and LTBI in children. It is limited to young children, but the risk of rapid and primary progression to aTB when infected is important in this age category, as the risk of aTB is thought to be inversely correlated to age (35). Moreover, these young children often remain paucisymptomatic even while suffering from aTB; hence, the decision to treat them for aTB or LTBI may be quite difficult. We suggest that a rapid therapeutic decision can be made based on the results of the CFP-10 IGRA.

The same PPD and CFP-10 IGRA combination for aTB allowed us to accurately detect LTBI cases. Only 6% of the LTBI cases were negative for the combined CFP-10 and PPD IGRA. As there is no gold standard to diagnose M. tuberculosis-infected children, it remains difficult to conclude whether children with a positive TST and a negative IGRA were really infected with M. tuberculosis. In addition, various cutoffs may be used to define a positive TST result, with the specificity of the test being lower.
when considering a low cutoff level of 5-mm diameter. In this study, if the CFP-10 IGRA was quite specific for *M. tuberculosis* infection, the specificity of the PPD IGRA was only 92%, a fact that may be attributed to a previous BCG vaccination, as those children produce significantly more IFN-γ in response to PPD than unvaccinated children. Caution is thus recommended when interpreting the results of the PPD IGRA. This does not really represent a problem in Belgium (where BCG vaccination is no longer recommended), and only 15% of the NI children in our study were BCG vaccinated. However, only the results from the CFP-10 IGRA provided a higher sensitivity (73%) for the detection of LTBI in children than the results reported in the literature for the QFT-GIT; a recent large prospective study performed on 336 children indicated that only 50% of the children with an LTBI were identified using this test (36).

A limitation of our study is that we cannot directly compare the results of the long-incubation-time IGRA to those obtained with the commercialized IGRA that were not performed here, as they are not recommended in the guidelines for the diagnosis of pediatric *M. tuberculosis* infection. Due to the limited amount of blood available from the child participants, we decided to focus our research on the evaluation of long-incubation-time IGRA characterized by a separate testing of the antigens in contrast to commercialized IGRA, and this allowed us to determine the superior diagnostic accuracy of CFP-10 over ESAT-6 for the diagnosis of *M. tuberculosis* infection in children. The good sensitivity of the CFP-10 IGRA for the detection of LTBI in children that we reported here may be attributed to the 96-h incubation time of the tests compared to a 24-h incubation time for the commercialized IGRA. As CFP-10 is poorly expressed by mycobacteria during latency, the long *in vitro* incubation time of the blood samples with the antigen allows for a better expansion of the antigen-specific T lymphocytes, which are mostly memory T cells (10, 14). Short-incubation-time IGRA are more appropriate for the detection of effector T lymphocytes that represent the majority of the circulating lymphocytes during recent or ongoing *M. tuberculosis* infection (14).

Although these results should be confirmed by other independent and large studies, our data suggest that the two different long-incubation-time IGRA, one in response to CFP-10 and the other in response to PPD, can be used as diagnostic tests for *M. tuberculosis* infection or disease in children living in countries with a low incidence of TB. All the cases of aTB in this study were correctly identified, suggesting that these tests may be used as a supplementary diagnostic tool for the evaluation of active TB disease.

**ACKNOWLEDGMENTS**

This work was supported by the European Commission within the 6th Framework Program (contract no. LSHP-CT-2005-018736). K. Schepers was supported by a fellowship from the Fond Erasme, and F. Mascart was partially supported by a grant from the Fonds National de la Recherche Scientifique (FNRS). These funding agencies had no involvement with this publication.

We thank all parents and children who kindly agreed to participate in this study, all the pediatricians involved in the recruitment procedure (particularly A. Vergison and N. Rangelou), and James Ansty for critical reading of the manuscript.

We declare no conflicts of interest.

**REFERENCES**

1. Starke JR. 2000. Diagnosis of tuberculosis in children. Pediatr. Infect. Dis. J. 19:1095–1096. http://dx.doi.org/10.1097/00006454-200011000-00015.

2. Marais BJ, Gie RP, Hespel AC, Schaaf HS, Lombard C, Enarson DA, Beyers N. 2006. A refined symptom-based approach to diagnose pulmonary tuberculosis in children. Pediatrics 118:e1350–e1359. http://dx.doi.org/10.1542/peds.2006-0519.

3. Farhat M, Greenaway C, Pai M, Menzies D. 2006. False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? Int. J. Tuberc. Lung Dis. 10:1192–1204.

4. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284:1520–1523. http://dx.doi.org/10.1126/science.284.5419.1520.

5. Diel R, Goletti D, Ferrara G, Bethamley G, Girillo D, Kampmann B, Lange C, Losi M, Markova R, Migliori GB, Nienhaus A, Ruhwald M, Wagner D, Zellweger JP, Huitric E, Sandgren A, Maniresso D. 2011. Interferon-γ release assays for the diagnosis of latent *Mycobacterium tuberculosis* infection: a systematic review and meta-analysis. Eur. Respir. J. 37:88–99. http://dx.doi.org/10.1183/09031936.00115110.

6. Menzies D. 2008. Using tests for latent tuberculosis infection to diagnose active tuberculosis: can we eat our cake and have it too? Ann. Intern. Med. 148:388–390. http://dx.doi.org/10.7326/0003-4819-148-5-200803040-00011.

7. Pai M, Menzies D. 2007. Interferon-gamma release assays: what is their role in the diagnosis of active tuberculosis? Clin. Infect. Dis. 44:747–77. http://dx.doi.org/10.1086/509927.

8. Sester M, Sotgiu G, Lange C, Ghiel G, Girardi E, Migliori GB, Bossink A, Dheda K, Diel R, Domenguez J, Lipman M, Nemeth J, Ravn P, Winkler S, Huitric E, Sandgren A, Maniresso D. 2011. Interferon-γ release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur. Respir. J. 37:100–111. http://dx.doi.org/10.1183/09031936.00114810.

9. Hougardy JM, Schepers K, Place S, Drouart A, Lechevin V, Verscheure V, Debrie AS, Doherty TM, Van Vooren JP, Loct C, Mascart F. 2007. Heparin-binding-hemagglutinin-induced IFN-gamma release as a diagnostic tool for latent tuberculosis. PLoS One 2:e2962. http://dx.doi.org/10.1371/journal.pone.0002962.

10. Leyten EM, Arend SM, Prins C, Cobedans FG, Ottenhoff TH, van Dissel JT. 2007. Discrepancy between *Mycobacterium tuberculosis*-specific gamma interferon release assays using short and prolonged in vitro incubation. Clin. Vaccine Immunol. 14:880–885. http://dx.doi.org/10.1128/CVI.00132-07.

11. Goletti D, Butera O, Vanini V, Lauria FN, Lange C, Franken KL, Angeletti G, Ottenhoff TH, Girardi E. 2010. Response to Rv2628 latency antigen associates with cured tuberculosis and remote infection. Eur. Respir. J. 36:135–142. http://dx.doi.org/10.1183/09031936.00140009.

12. Corbière V, Pottier G, Bonkain F, Schepers K, Verscheure V, Lecher S, Doherty TM, Loct C, Mascart F. 2012. Risk stratification of latent tuberculosis defined by combined interferon gamma release assays. PLoS One 7:e43285. http://dx.doi.org/10.1371/journal.pone.0043285.

13. Hinks TS, Dusanjh DP, Innes JA, Pasvol G, Hackforth S, Varia H, Millington KA, Liu XQ, Bekir M, Soysal A, Davidson RN, Gunathesan R, Lalvani A. 2009. Frequencies of region of difference 1 antigen-specific but not purified protein derivative-specific gamma interferon-secreting T cells correlate with the presence of tuberculosis disease but do not distinguish recent from remote latent infections. Infect. Immun. 77:5486–5495. http://dx.doi.org/10.1128/IAI.01436-08.

14. Butera O, Chiacchio T, Carrara S, Casetti R, Vanini V, Meraviglia S, Gugino G, Diel R, Vecchi M, Lauria FN, Marruchella A, Laurenti P, Singh M, Caccamo N, Girardi E, Goletti D. 2009. New tools for detecting latent tuberculosis infection: evaluation of RD1-specific long-term response. BMC Infect. Dis. 9:182. http://dx.doi.org/10.1186/1471-2334-9-182.

15. Mandalakas AM, Detjen AK, Hespel AC, Benedetti A, Menzies D. 2011. Interferon-gamma release assays and childhood tuberculosis: systematic review and meta-analysis. Int. J. Tuberc. Lung Dis. 15:1018–1032. http://dx.doi.org/10.5888/ijtlld.J06.0361.

16. Sun L, Xiao J, Miao Q, Feng WX, Wu XR, Yin QQ, Jiao WW, Shen C, Liu F, Shen D, Shen AD. 2011. Interferon gamma release assay in diagnosis of pediatric tuberculosis: a meta-analysis. FEMS Immunol. Med. Microbiol. 63:165–173. http://dx.doi.org/10.1111/1574-695X.2011.00838.x.

17. Machingaidze S, Witsonge CS, Gonzalez-Angulo Y, Hatherill M, Moyo...
S. Hanekom W, Mahomed H. 2011. The utility of an interferon gamma release assay for diagnosis of latent tuberculosis infection and disease in children: a systematic review and meta-analysis. Pediatr. Infect. Dis. J. 30:694–700. http://dx.doi.org/10.1097/INF.0b013e318214b915.

18. CDC. 2011. Fact sheets: tuberculin skin testing. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/TB/publications/factsheets/testing/skintesting.htm.

19. Masungi C, Temmerman S, Van Vooren JP, Drowart A, Pethe K, Menozzi FD, Locht C, Mascart F. 2002. Differential T and B cell responses against Mycobacterium tuberculosis heparin-binding hemagglutinin in infected healthy individuals and patients with tuberculosis. J. Infect. Dis. 185:513–520. http://dx.doi.org/10.1086/338833.

20. Temmerman S, Pethe K, Parra M, Alonso S, Rouanet C, Pickett T, Drowart A, Debrise AS, Delogu G, Menozzi FD, Sergheraert C, Brennan MJ, Mascart F, Locht C. 2004. Methylation-dependent T cell immunity to Mycobacterium tuberculosis heparin-binding hemagglutinin. Nat. Med. 10:935–941. http://dx.doi.org/10.1038/nm1090.

21. Perez-Velez CM. 2012. Pediatric tuberculosis: new guidelines and recommendations. Curr. Opin. Pediatr. 24:319–328. http://dx.doi.org/10.1097/MOP.0b013e32835357c3.

22. European Centre for Disease Prevention and Control. 2011. Use of interferon-gamma release assays in support of TB diagnosis. European Centre for Disease Prevention and Control, Stockholm, Sweden.

23. Okada K, Mao TE, Mori T, Miura T, Sugiyama T, Yoshiyama T, Mitarai S, Onozaki I, Harada N, Saint S, Kong KS, Chhour YM. 2008. Performance of an interferon-gamma release assay for diagnosing latent tuberculosis infection in children. Epidemiol. Infect. 136c:1179–1187. http://dx.doi.org/10.1017/S0950268807009831.

24. Mazurek GH, Jereb J, Vernon A, LoBue P, Goldberg S, Castro K. 2010. Updated guidelines for using interferon gamma release assays to detect Mycobacterium tuberculosis infection—United States, 2010. MMWR Recomm. Rep. 59(RR5):1–25.

25. Pickering LK, Baker CJ, Kimberlin DW, Long SS. 2009. Red book: 2012 report of the Committee on Infectious Diseases, 28th ed. American Academy of Pediatrics, Elk Grove Village, IL.

26. Kampmann B, Whitaker E, Williams A, Walters S, Gordon A, Martinez-Alier N, Williams B, Crook AM, Hutton AM, Anderson ST. 2009. Interferon-gamma release assays do not identify more children with active tuberculosis than the tuberculin skin test. Eur. Respir. J. 33:1374–1382. http://dx.doi.org/10.1183/09031936.00153408.

27. Munt PW. 1972. Miliary tuberculosis in the chemotherapy era: with a clinical review in 69 American adults. Medicine (Baltimore) 51:139–155. http://dx.doi.org/10.1097/00005792-197203000-00004.

28. Rooney JJ, Jr, Crocco JA, Kramer S, Lyons HA. 1976. Further observations on tuberculin reactions in active tuberculosis. Am. J. Med. 60:517–522. http://dx.doi.org/10.1016/0002-9343(76)90718-X.

29. Yaramij A, Gurkan F, Elevli M, Söker M, Haspolat K, Taş MA. 1998. Central nervous system tuberculosis in children: a review of 214 cases. Pediatrics 102:E49. http://dx.doi.org/10.1542/peds.102.5.e49.

30. Fox A, Jeffries DJ, Hill PC, Hammond AS, Lugos MD, Jackson-Sillah D, Donkor SA, Owiafe PK, McAdam KP, Brooks KE. 2007. ESAT-6 and CFP-10 can be combined to reduce the cost of testing for Mycobacterium tuberculosis infection, but CFP-10 responses associate with active disease. Trans. R. Soc. Trop. Med. Hyg. 101:691–698. http://dx.doi.org/10.1016/j.trstmh.2007.03.001.

31. Nicol MP, Davies MA, Wood K, Hatherill M, Workman I, Hawkridge A, Eley B, Wilkinson KA, Wilkinson RJ, Hanekom WA, Beatty D, Hussey G. 2009. Comparison of T-SPOT.TB assay and tuberculin skin test for the evaluation of young children at high risk for tuberculosis in a community setting. Pediatrics 123:38–43. http://dx.doi.org/10.1542/peds.2008-0611.

32. Critsels E, Amanatidou V, Syridis G, Spyridis NP, Mavrikou M, Papadopoulos NG, Tsolia MN. 2012. The effect of age on whole blood interferon-gamma release assay response among children investigated for latent tuberculosis infection. J. Pediatr. 161:632–638. http://dx.doi.org/10.1016/j.jpeds.2012.04.007.

33. Wilkinson KA, Wilkinson RJ, Pathan A, Ewer K, Prakash M, Klenerman P, Maskell N, Davies R, Pasvol G, Lalvani A. 2005. Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. Clin. Infect. Dis. 40:184–187. http://dx.doi.org/10.1086/426139.

34. Hougardy JM, Place S, Hildebrand M, Drowart A, Debrise AS, Locht C, Mascart F. 2007. Regulatory T cells depress immune responses to protective antigens in active tuberculosis. Am. J. Respir. Crit. Care Med. 176:409–416. http://dx.doi.org/10.1164/rccm.200701-084OC.

35. Marais BJ, Gie RP, Schaaf HS, Hesseling AC, Ohbara CC, Nelson LJ, Enarson DA, Donald PR, Beyers N. 2004. The clinical epidemiology of childhood pulmonary tuberculosis: a critical review of literature from the pre-chemotherapy era. Int. J. Tuberc. Lung Dis. 8:278–285.

36. Bianchi L, Galli L, Moriondo M, Veneruso G, Becciolini L, Azzari C, Chiappini E, de Martino M. 2007. ESAT-6 and heparin-binding hemagglutinin. Nat. Med. 319–328. http://dx.doi.org/10.1038/nm1090.

37. Papadopoulos NG, Tsolia MN. 2012. The effect of age on whole blood interferon-gamma release assay response among children investigated for latent tuberculosis infection. J. Pediatr. 161:632–638. http://dx.doi.org/10.1016/j.jpeds.2012.04.007.