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APLICABILITAT CLÍNICA DE LES TÈCNIQUES DE DETECCIÓ *IN VITRO* DE L'INTERFERÓ-GAMMA EN LA INFECCIÓ I LA MALALTIA TUBERCULOSA

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cannot be extrapolated to countries with moderate or low TB prevalence. Indeed, a decreased T-cell response against ESAT-6 stimulation was reported in household contact children exposed to *Mycobacterium africanum* (responsible for up to half of TB cases in western Africa), rather than that observed in children exposed to *M. tuberculosis* [89].

Regarding the specificity of tests in NTM infection, Detjen *et al.* analyzed 23 children with bacteriological NTM lymphadenitis and 22 with other nonmycobacterial respiratory tract infections, and reported a specificity for the T-SPOT.TB and QFN-G-IT tests of 100% [90]. Although the specificity of IFN-γ-based tests is excellent, frequent discordant results with the TST have been described [72,73,76,77,83,84,91–93]. Among 511 TST-positive children screened for LTBI at school (14–15 years old) in Norway [84], only 44 (9%) had a confirmed positive QFN-G result. In another study conducted in Barcelona (Spain) among children who were not BCG-vaccinated, QFN-G-IT was negative in 60.4% of children with a positive TST, and T-SPOT.TB assay in 56.6% of cases [76]. In fact, a priority research area is to understand discordant TST and IFN-γ-based test results, including the role of NTMs [11].

With regard to indeterminate results, Ferrara *et al.* noted that QFN-G obtained in children under 5 years gave a higher proportion of indeterminate results than the T-SPOT.TB (32 vs 0%) [12]. A higher ratio of QFN-G-indeterminate results (17%) was also obtained by Connell *et al.* [72]. However, in a later study, Connell *et al.* reported a higher proportion of indeterminate results by the T-SPOT.TB than the QFN-G-IT assay [73]. The available results seem to indicate that indeterminate results using QFN-G are age-dependent, being that IFN-γ is released in significantly lower quantities in response to phytohemaglutinin in young children [72,92]. By contrast, T-SPOT.TB, except in the first weeks of life [81], seems not to be age-dependent [12]. Nevertheless, Nicol *et al.* [93] reported a decrease in the number of children below the age of 1 year who scored positive T-SPOT.TB, whereas the TST results were unaffected by age. On the other hand, the QFN-G-IT test seems to offer a lower number of indeterminate results than the QFN-G test [75,76]. Indeed, recently, Bergamini *et al.*, in a retrospective study involving 496 children, reported that indeterminate results were associated with younger age (<4 years of age) for both QFN-G and QFN-G-IT, but not for T-SPOT.TB [94]. Lewinsohn *et al.*, using an in-house IFN-γ-based test based on ELISA detection, found that young household contact children (<2 years old) produced IFN-γ responses comparable to adults [85]. Liebeschuetz *et al.* reported that ELISPOT was less affected by HIV co-infection, malnutrition or age under 3 years old, than the TST [22]. Probably one of the main reasons that can explain the indeterminate results is the impossibility of isolating a high number of T cells in children if less of 4 ml of blood is collected.

One unresolved issue with IFN-γ-based tests is reversions and conversions observed in contact patients [95,96]. Hill *et al.* conducted a longitudinal study in the Gambia comparing ELISPOT results with those of the TST [96]. They included 740 contacts of 177 TB patients, all contacts being at least 15 years old. 3 and 18 months after the moment of recruitment, ELISPOT was repeated in contact patients. 3 months after recruitment ELISPOT reversion was detected in 40% (54 out of 134) of contacts with a previous positive ELISPOT, and 18 months after recruitment reversion occurred in 36% (28 out of 78). On the contrary, the ELISPOT conversion rate was 27% (20 out of 75) at 18 months. Intriguingly, the rate of TST reversion was lower than in the ELISPOT, and the rate of TST conversion at 18 months was higher (50%). One plausible explanation is that reversion might reflect the clearance of bacilli from the organism [95], or might reflect the transition into latency [96]. In addition, the different rate of conversion between the ELISPOT and TST may also be due to a combination of factors: increased TST sensitivity, a different interval between initial exposure and test conversion, boosting the effect of TST results, and early ELISPOT reversion [96]. Therefore, more studies are needed to determine the biological variability of IFN-γ responses over time in the absence of TB exposure, and also to define the exact criteria for ELISPOT reversion and conversion. Further research is required for better understanding the meaning of these reversions and conversions [97]. All these facts should be considered when preparing IFN-γ-based test guidelines.

Bakir *et al.*, in a study conducted in Turkey involving more than 900 children and adolescents with recent household TB exposure, found that among the 381 contacts with positive ELISPOT, 11 developed active TB, and among the 550 with positive TST, 12 developed active TB [98]. They concluded that a positive ELISPOT result predicted the development of active-TB as well as the TST.

In children, IFN-γ-based tests have demonstrated to be useful in diagnosing LTBI, showing association between positive ELISPOT results with exposure to *M. tuberculosis* and not being affected by BCG vaccination. They could also be a useful tool for helping in the diagnosis of active TB. It is necessary to cautiously interpret a positive TST in screening studies, as the high ratio of discordant results with IFN-γ-based tests is probably due to NTM infection.

Diagnosing active TB

Some studies [12,13,23,30,34,99] and case reports [63,74,100–102] referring to the utility of IFN-γ-based tests in diagnosing active TB in immunocompromised patients have been reported. Nowadays, IFN-γ-based tests can be used as a complementary tool in the diagnosis of active TB, being helpful in areas where the prevalence of LTBI infection is very low. In addition, these tests can also be of use in diagnosing extrapulmonary TB, where approximately half of the cases have not been microbiologically diagnosed, and which often require complex and invasive diagnostic tools. Finally, tests could also be useful for pulmonary smear-negative patients. However, IFN-γ-based assays, as they are currently designed, cannot distinguish between active and latent TB infection.

Nevertheless, a novel approach has been proposed for diagnosing active TB. It is based on applying ELISPOT in samples collected directly from the site of infection. The recruitment of specific T cells during active TB and how antigen-specific cells clonally expand and migrate to the site of infection have been described [103].

Indeed, the utility of the ELISPOT in diagnosing active TB in immunocompromised patients using samples different from blood has already been reported. Richeldi *et al.* described how

an ELISPOT based on RD1 antigens on a pleural effusion sample accelerated the diagnosis of disseminated TB in a leukemic patient with persistent fever and a negative TST [104]. In this case, the ELISPOT on blood was positive 5 weeks before the TST became positive; and the ELISPOT on the pleural fluid sample was also positive 1.5 weeks before the mycobacteria isolated on the peripheral blood was identified as *M. tuberculosis*. Lange *et al.* also described the diagnosis of active TB in a patient receiving anti-TNF-α therapy by means of T-SPOT.TB on a pleural effusion sample [105]. The TST was negative (0 mm), the smear of sputum and bronchoalveolar lavage (BAL) fluid was also negative, and only the sputum culture and the pleural biopsy study (histopathology and nucleic acid-amplification technique) confirmed active TB. However, the sputum culture result was delayed between 7 and 35 days, and the nucleic acid-amplification technique on pleural biopsy was performed retrospectively.

By contrast, Baba *et al.* designed a study for validating the QFN-G assay on pleural fluid samples, in a group of HIV-infected patients with confirmed TB, probable TB and non-TB pleuritis [106]. The authors found that the QFN-G assay obtained 52% of indeterminate results, mainly caused by the high background observed in the negative control of both TB groups.

The detection of reactive T cells against the *M. tuberculosis*-specific antigens in BAL fluid by ELISPOT has been also described as compatible with the diagnosis of pulmonary active TB [107]. Regarding the experience in immunocompromised patients, its utility has been described in a patient receiving immunosuppressive therapies for a mixed connective tissue disease [108]. The patient had a negative TST, and smear and nucleic acid-amplification techniques were also negative in both the sputum and BAL fluid. The treatment was initiated, based on the ELISPOT result obtained from BAL fluid, 18 days before *M. tuberculosis* was isolated in the sputum culture.

Expert commentary

Since the development of IFN-γ-based assays as an *in vitro* alternative method to the TST for the immunodiagnosis of LTBI, promising results in adults and also in children have been published [12,70,72,76,79,80,109–111]. Although IFN-γ-based tests are designed for diagnosing LTBI, they have also been evaluated as an aid in the diagnosis of active TB [109,112–114]. A high specificity of the IFN-γ-based tests has been demonstrated, being that the IFN-γ-based tests were not affected by BCG vaccination or infection by the most common NTM. In addition, in the absence of a gold standard test to determine LTBI, IFN-γ test results have demonstrated to be closer than the TST to the degree of exposure to *M. tuberculosis* [80,100,115]. One of the key questions for its utilization is establishing the capacity of these tests in predicting the development of disease. In studies recently published [98,116,117], positive IFN-γ-based assays predicted development of active TB in adult and children with recent TB contact.

IFN-γ-based tests offer general advantages over the TST [118]: avoidance of cross-reaction with BCG-vaccinated individuals and with NTM infection, logistical convenience (result available in 24 h), avoidance of poorly reproducible measurements, no follow-up visit required and the result remains confidential. Furthermore, an injection of PPD for the TST can boost subsequent TST responses, primarily in NTM-infected or BCG-vaccinated individuals. On the contrary, IFN-γ-based tests could be performed serially without inducing the boosting phenomenon.

According to cumulative evidence, the T-SPOT.TB assay showed a higher number of positive results, and a lower number of indeterminate results than the QFN tests. Given that the number of cells stimulated in the T-SPOT.TB instead of QFN test is standardized to 250,000 per culture, negative and indeterminate results associated with low lymphocyte counts could be reduced.

Nevertheless, although the T-SPOT.TB test seems to be more sensitive than the QFN-G-IT test, some operational aspects have to be noted for the performance of both IFN-γ-based tests (Figure 1). The T-SPOT.TB assay requires same-day processing of specimens, given the early and often inconvenient time limits for sample collection. This is especially important if a courier service is involved. Recently, the T-SPOT.TB manufacturer introduced a complementary reagent to allow the isolation of lymphocytes from whole blood up to 32 h following venopuncture. However, although the manufacturer claims that there is not a significant decrease in peripheral blood mononuclear cell yields or T-cell populations when comparing with whole-blood samples stored for less than 8 h, the procedure has not been extensively assessed and further experience is required.

Furthermore, T-SPOT.TB technical performance makes it difficult to run a large number of samples in a single day. In this sense, the QFN-G-IT test allows more flexible timing for specimen collection and transport owing to the fact that the antigens are already incorporated in the sample collection tube. The QFN-G-IT test also makes it possible to store the samples after stimulation with the antigens and to run batches. However, there are few studies evaluating whether stimulation of T cells with antigens together instead of separately could affect the sensitivity or specificity of the test. In a previous study evaluating LTBI in a rural area of South Africa, the QFN-G-IT test (201/358; 56%) obtained more positive results than the conventional QFN-G test (137/358; 38%) [119]. Furthermore, T-SPOT.TB requires more expertise in the readout of the results than QFN-G-IT, especially when the result is close to the cut-off value.

For immunosuppressed patients, additional advantages are required, such as a low ratio of indeterminate results, a high predictive value and a higher sensitivity than TST for avoiding false-negative results due to anergy.

Indeterminate results were mainly associated with immunosuppression, especially related to immunosuppressant therapy, and in patients with suppressed cellular immunity. Nevertheless, it is important to remark that in immunosuppressed patients with a negative TST result, an indeterminate IFN-γ test result should be considered as very useful clinical information.

The fact that IFN-γ-based tests measure effector T-cell responses rather than memory T cells seems to indicate that IFN-γ-based tests are a good indicator of recent LTBI, allowing the identification of patients with remote LTBI. Although this fact could be useful for selecting candidates to receive prophylaxis in an immunocompetent population, at the moment it is recommended to treat all immunocompromised patients with LTBI (both recent and remote infections).

Therefore, although among immunosuppressed patients the percentage of negative TST results is higher, and IFN-γ-based tests obtain more positive results with a higher specificity, the presence of discordant results between TST and IFN-γ-based tests, and the high risk of progression to active TB in this population, it seems prudent to recommend the utilization of IFN-γ-based tests after a negative TST result in order to increase the sensitivity of detecting LTBI cases in severely immunosuppressed patients. According to the data available, there is no risk of TST boosting in the IFN-γ-based test response if these tests are performed after a negative TST [120–124]. The possibility of using IFN-γ-based tests alone to avoid a large number of false-positive TST results should be considered for BCG-vaccinated nonsevere immunocompromised patients.

Although more studies comparing IFN-γ-based tests with the TST are needed to sufficiently expand knowledge, IFN-γ-based tests seem to be, in combination with the TST, a useful method for diagnosing TB infection in immunocompromised patients and children, according to their exact role in the different immunocompromising situations, the degrees of immunosuppression and the specific risks of LTBI for each patient.

Five-year view

In the next 5 years, the evolution of the IFN-γ-based tests in diagnosing TB infection will require advancements in two directions. First, it will be necessary to perform studies with a large number of patients to establish the prognostic value of positive test results in the different populations of immunosuppressed patients, especially in those that undergo an anti-TNF-α therapy.

Guidelines for IFN-γ-based test utilization in each group of immunocompromised patients and in each individual situation will be established. Given that *in vitro* assays depend on the secretion of IFN-γ, which is largely produced by CD4 T cells, more studies will be developed in order to define the CD4 threshold at which the performance of these assays declines, in particular when CD4 T-cell counts are under 50 cells/μl. These studies should explore the necessity and accuracy of new cut offs for diagnosing LTBI in immunosuppressed patients, and for distinguishing between active and latent TB infection.

Furthermore, specific studies will be performed in order to understand the discordant results between IFN-γ-based tests and the TST, especially in the pediatric population where the effect of NTM infection may play a very important role. The knowledge of the meaning of conversion and reversion results will also increase, and the clinical explanation of discordant and indeterminate results between IFN-γ-based tests will be expanded.

Second, technical modifications will be performed on IFN-γ-based tests [125]. The refinement of IFN-γ-based tests will include the exploration of alternative readouts to measure IFN-γ release [125], the utilization of new *M. tuberculosis*-specific antigens [6,126], and the simultaneous measurement of chemokines [127] and interleukins [128]. The next generation of IFN-γ-based tests will significantly enhance diagnostic sensitivity without diminishing specificity, and reduce the number of indeterminate results. In addition, the commercial IFN-γ-based test manufacturers should increase efforts to simplify technology and enhance its applicability in resource-limited settings.

Similarly, the detection of *M. tuberculosis*-specific T cells in nonblood samples using ELISPOT is a promising tool for the diagnosis of active TB in immunocompromised patients with negative smears. The definition of methodological procedures and the establishment of an accurate cut off will also be performed.

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Key issues

- IFN-γ-based tests produce more positive results than the tuberculin skin test (TST) for diagnosing latent TB infection (LTBI) in immunosuppressed patients, but less than in immunocompetent patients.
- Concordance between the TST and IFN-γ-based tests is poor, but IFN-γ-based test results are more likely associated with risk factors for LTBI than the TST.
- T-SPOT.TB shows a higher number of positive results than the QuantiFERON®-TB (QFN) test.
- Available data suggest that the T-SPOT.TB assay is less influenced by immunosuppression status than the QFN test.
- The T-SPOT.TB assay has a lower number of indeterminate results than the QFN test.
- Differences in the performance of both commercial tests should be considered when selecting the adequate IFN-γ-based test for each population and setting.
- In children, IFN-γ-based tests show a higher number of positive results than the TST, and have an especially higher specificity than the TST.
- The role of nontuberculous mycobacteria infection in children requires further studies for determining their effect in discordant results between the TST and IFN-γ-based tests.
- IFN-γ-based tests seem to be, in combination with the TST, a useful method for diagnosing TB infection in severely immunosuppressed patients.

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