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A mode of error: Immunoglobulin binding protein (a subset of anti-citrullinated proteins) can cause false positive tuberculosis test results in rheumatoid arthritis

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ABSTRACT

Citrullinated Immunoglobulin Binding Protein (BiP) is a newly described autoimmune target in rheumatoid arthritis (RA), one of many cyclic citrullinated peptides(CCP or ACPA). BiP is over-expressed in RA patients causing T cell expansion and increased *interferon* levels during incubation for the QuantiFERON-Gold tuberculosis test (QFT-G TB). The QFT-G TB has never been validated where *interferon* is increased by underlying disease, as for example RA.

Of ACPA-positive RA patients ($n = 126$), we found a 13% false-positive TB test rate by QFT-G TB. Despite subsequent biologic therapy for 3 years of all 126 RA patients, none showed evidence of TB without INH. Most of the false-positive RA patients after treatment with biologic therapy reverted to a negative QFT-G test. False TB tests correlated with ACPA level ($p < 0.02$).

Three healthy women without arthritis or TB exposure had negative QFT-G TB. *In vitro*, all three tested positive every time for TB correlating to the dose of BiP or anti-BiP added, at 2 ug/ml, 5 ug/ml, 10 ug/ml, and 20 ug/ml.

BiP naturally found in the majority of ACPA-positive RA patients can result in a false positive QFT-G TB. Subsequent undertreatment of RA, if biologic therapy is withheld, and overtreatment of presumed latent TB may harm patients.

Introduction

The interferon-gamma release assays (IGRA) were developed about 20 years ago to screen for exposure to tuberculosis (TB) especially to screen patients with human immunodeficiency virus who had low resistance to TB. The interferon tests for TB were therefore specifically designed for immunosuppressed patients with low CD4 and low induction of interferon. These IGRA tests do not test for TB directly but measure interferon release as a surrogate marker. Actually proving TB infection involves obtaining sputum or biopsy materials to culture tuberculosis which can be difficult and subsequent culture to prove growth of tuberculosis can take months to complete. In the past decade, interferon-gamma release assays were approved, and these tests require one to two weeks for results due to complicated processing at two different laboratory facilities; three different plasma tubes are incubated for 16–24 h at the test site, then the specimens are transported to a central facility equipped for measurement of interferon release. (See supplement 1.) Alternatively, the more traditional tuberculin skin test using purified protein derivative (PPD) testing for TB requires 3 days with minimal personnel training and no equipment. Mantoux PPD tests exemplify classic type IV delayed type hypersensitivity, with no interferon involved for screening TB.

Background/Purpose: Rheumatoid arthritis (RA) is an autoimmune systemic disease that can spontaneously release interferon as well as other immune cytokines [1]. RA develops synovial disease in up to 68 diarthrodial joints, and also a systemic disease stimulating cytokines in the bone marrow, lung, pleural and pericardial tissue, blood vessels, skin, eye, salivary glands, central and peripheral neural tissues. Autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein (ACPA) together have a sensitivity for the diagnosis of RA of up to 85%. Though specificity for RA with RF only is 40%, specificity using serum ACPA rises to 98% [2]. The trigger to initiate symptoms with RA is unknown, but the fact that ACPA is present years before symptoms, has led to speculation that antigen targets of ACPA play important roles in the pathogenesis of RA [3].

In autoimmune disease, there is a breakdown of tolerance mechanisms, resulting in log-rhythmic escalation of autoimmune activity (including spontaneously released interferon). In RA specifically, ACPA

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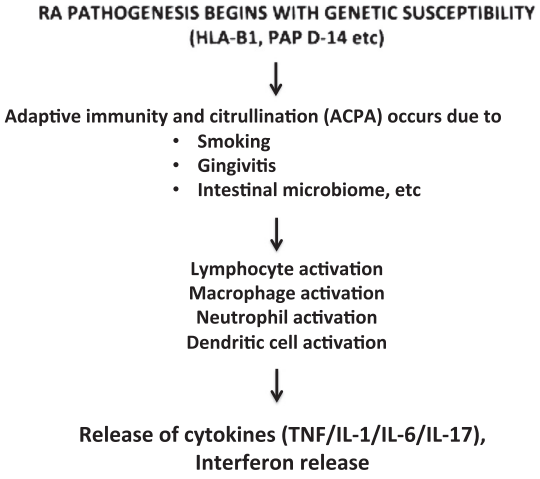

Fig. 1. Pathogenesis of rheumatoid arthritis and subsequent secretion of spontaneous interferon.

are produced in response to citrullination of native proteins. Although citrullination is a physiologic normal response to inflammation, in RA there are very high levels of BiP citrullination; triggers identified to induce citrullination include cigarettes, gum disease, and intestinal bacteria Prevotella copri [4,5,6]. Therefore, the current causal hypothesis for RA is a genetic predisposition (such as HLA-DRB1), followed by an inflammatory condition inducing citrullination (raising ACPA), promoting activated T cells, B cells, and antigen presenting cells. The activated immune cell proliferation results in cytokine and *interferon release*. (Fig. 1)

Active research into the pathogenesis of RA concerns antibody reactivity against antigens that are modified posttranslationally to contain citrulline rather than arginine. Recently, polymorphisms in PAD14 gene, which codes for protein citrullination have been shown to be associated with RA susceptibility [7]. Immunoglobulin binding protein (BiP) contains 27 arginine residues that can be replaced by citrulline [8].

Citrullinated BiP is a newly described subgroup of anti-cyclic citrullinated peptide (ACPA). Citrullinated BiP in both serum and synovial fluid is over-expressed in RA patients and correlates with local lab measurements of ACPA [8]. Peripheral T cells from RA patients show high levels of proliferation in response to BiP, whereas healthy donor T cells do not [8]. T Cells from RA patients have a strong proliferation response to BiP and this response correlates significantly with clinical measures of RA disease activity . In response to BiP, RA peripheral blood mononuclear cells release tumor necrosis factor, IL-6, IL-17 and *interferon-gamma* [9].In one study, in response to BiP, RA synovial T cells produced *interferon-gamma* at >5000 pg/ml [10]. Depletion of CD8 cells further increases cytokines, including *interferon-gamma*. Many of the biologic therapies in use today change the composition of immune cell subsets in RA [11]. This makes interpreting interferon testing in RA extremely complicated since subsets of immune cells vary both with RA disease and with biologic therapy. There are no "control" values for interferon validated in RA. Depending on the immune cell types found in an individual RA patient, stimulation of *interferon-gamma* is widely variable. Also antibody to BiP (anti-BiP) are frequent in RA and higher than in the normal population [10]. Use of IGRA testing in autoimmune diseases where interferon is easily stimulated should be critically suspect and if possible avoided as a screening test for TB.

We postulated that one way to manipulate QFT-G TB testing would be to vary *in vitro* serum BiP and anti-BiP (both naturally found in elevated levels in RA). Either BiP or anti-BiP would cause T cell expansion and increase interferon during incubation of the QuantiFERON-Gold tuberculosis in-tube test (QFT-G TB). Since the test is incubated for 16–24 h, the autoantigen BiP found in RA sera could spontaneously produce interferon due to auto-immune activity, and thus result in a false positive TB test, regardless of the TB antigen in the tube. The interferon spontaneously produced in a untreated active RA patient may be extremely high, then resolve as biologic immune treatment controls the RA autoimmunity (see the follow up data from interferon tests in Fig. 2.) Certainly, after RA patients are treated with biologic immune-modulators, the reproducibility of interferon-gamma levels would be even more unpredictable . Although part of our hypothesis, the effects of biologic immune therapy on QFT-G TB was not tested by specific biologic therapies due to small numbers of subjects in this study, beyond showing that biologic treatment caused the QFT-G TB tests in many cases to revert to normal.

Methods: ACPA-positive active RA patients (n = 126) meeting the ACR-EULAR 2010 Classification Criteria for RA were tested with QFT-G TB (sourced Cellestis) prior to initiating biologic therapy [12]. Active RA disease were defined as patients with a minimum of 6 tender and 6 swollen joints, and a dermal joint temperature of over 97 F [13]. RA patients were allowed MTX at 15–25 mg/week along with folic acid 1 mg/day but no other traditional disease modifying conventional therapy. No use of tobacco was permitted. All patients had full TB evaluation at baseline and annually for three years with medical and travel history, physical examination, standard hematology and chemistry laboratory testing, chest PA and lateral radiographs, PPD and control skin testing (The IGRA test QFT-G was drawn before the Mantoux PPD skin test was performed.) [14,18] RA patients had ACPA, RF,

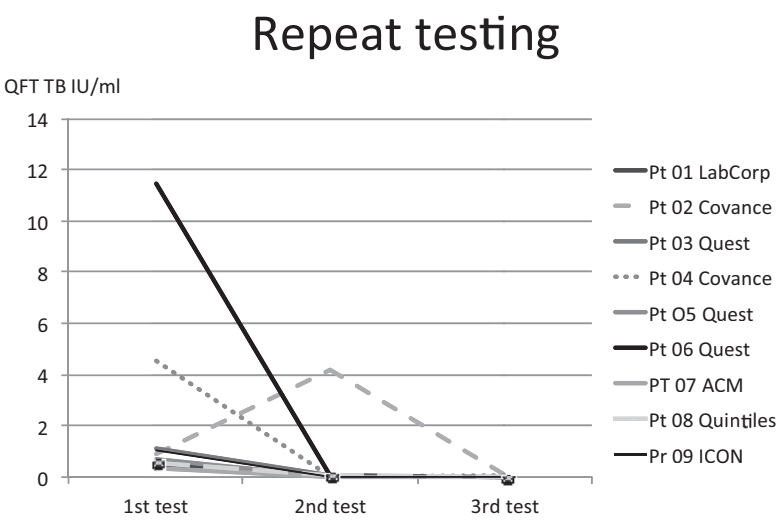

Fig. 2. QFT-G test was repeated in 9 positive ACPA (CCP) RA patients with negative repeat testing. The second test was performed at 1 year after biologic treatment began, and the third test was repeated at 2 years. No patient received INH therapy.

Table 1
Baseline disease characteristics of ACPA (CCP) positive RA subjects: these 16 patients produced elevated levels of IFN which in turn led to false positive QFT-G TB tests.

Age yrs	Yrs of RA	CCP (ACPA)	CCP3 (ACPA)	RF (IU)	DASesr	DAScrp	Jt Temp (F)	IFN (IU/ml)
56 (11.8)	12 (8.8)	148 (288)	663 (848)	62 (73)	4.8 (1.2)	6.2 (1.3)	97.2 (0.1)	1.57* (2.0)

Results listed are average values (SD). Joint temperature is dermal skin measurement over left wrist. [13] ACPA values are reported in mg/L. INF referes to interferon levels measured by QFT_G TB in test tube.
* $p < 0.02$ correlation between CCP and IFN level, and $p < 0.02$ for CCP3 and IFN level.

Westergren esr, CRP, DAS 28, wrist temperature, tender and swollen joint counts at baseline (Table 1). All QFT-G results are reported as described in the label for the test (supplement 2; that is a positive result is recorded if the TB antigen tube interferon value minus the Nil tube interferon value is ≥ 0.35 IU/mL and ≥25% of the Nil tube value). No RA patients were taking corticosteroid medication. Nine of the 126 ACPA positive RA patients with positive QFT-G baseline tests did have incidental repeat QFT-G tests, but this was not the major objective of the original protocol. Although part of our hypothesis included the assumption that biologic immune therapies will also manipulate interferon production (therefore interfere with IGRA testing), specific testing for this part of the hypothesis was not further explored beyond documenting reversion of QFT-G TB to normal in those patients with repeat testing (Fig. 2).

Active RA patients who had negative ACPA testing ($n = 64$) meeting 2010 ACR-EULAR classification criteria for RA, were similarly tested and evaluated as described above for the ACPA-positive RA ($n = 126$) subjects prior to adding biologic therapy.

Three healthy middle-aged female *normal* patients with no arthritis were tested with QFT-G TB test. Their serum was tested *in vitro* with BiP added, with 2 ug/ml, 5 ug/ml, 10 ug/ml, and 20 ug/ml, which are levels seen in the serum of patients with RA (sourced BiP Novus Biologicals.) Sera from these normal patients was also tested with anti-BiP antibody added at 5 ug/ml and 10 ug/ml (sourced BiP Novus Biologicals.) This is reported in Fig. 3.

All patients were examined and tests performed at Desert Medical Advances, Palm Desert, CA. An Institutional Review Board/Independent Ethics Committee-approved the protocol and all subjects signed consent prior to any procedures. Possible correlation between positive QFT-G TB tests and ACPA levels was analyzed by 2 way T test. Descriptive statistics were also performed on the software program, Graphpad Prism 2015. This study is a proof of concept study and no further statistical analysis beyond descriptive statistics were designed.

Results: Of 126 sequential active ACPA-positive RA patients, 16 (13%) tested positive for TB by QFT-G TB, despite no known risk factors for TB (no travel, low endemic rural area, no exposure history), negative chest radiograph, negative PPD with positive candida control, normal blood testing, and no symptoms or signs of TB on clinical examination. Of these 16 RA patients, all had very high levels of ACPA and had active inflammatory disease (mean DAS-esr 6.2). Baseline demographics and disease characteristics for these 16 RA patients are presented in Table 1. There were no differences noted at baseline to mark those RA patients with a false positive QFT-G *versus* those RA patients who did not cause a false alert; although those with the false positive TB interferon testing did have higher levels of CCP. In consultation with an Infectious Disease specialist, all 16 patients received biologic therapy and received no INH prophylaxis. The biologic treatment chosen for the 16 RA patients included anti-TNF ($n = 11$), JAK inhibitor ($n = 3$), anti-IL-17 antibody ($n = 1$), and anti-CD20 antibody ($n = 1$). In follow up after 24–36 months (mean 33 months), none of the 16 RA patients developed signs or symptoms of TB. In 9 RA patients, the ACPA level fell and the QFT-G TB reverted to negative, reflecting control of RA inflammatory disease with biologic therapy (Fig. 2). Mean QFT-G interferon levels in test tubes were 1.57 IU for TB, 0.18 IU for nil, and >10 IU consistently in every titer result for the mitogen tube. Positive TB status was defined by the lab as only positive or negative with a simplified cut-off value using TB tube titer >0.35 IU (this is contrary to the package circular for the test which involves negative, indeterminate, and positive results. See supplement 2 for a description of multiple modes for error in the technique performed by the lab for

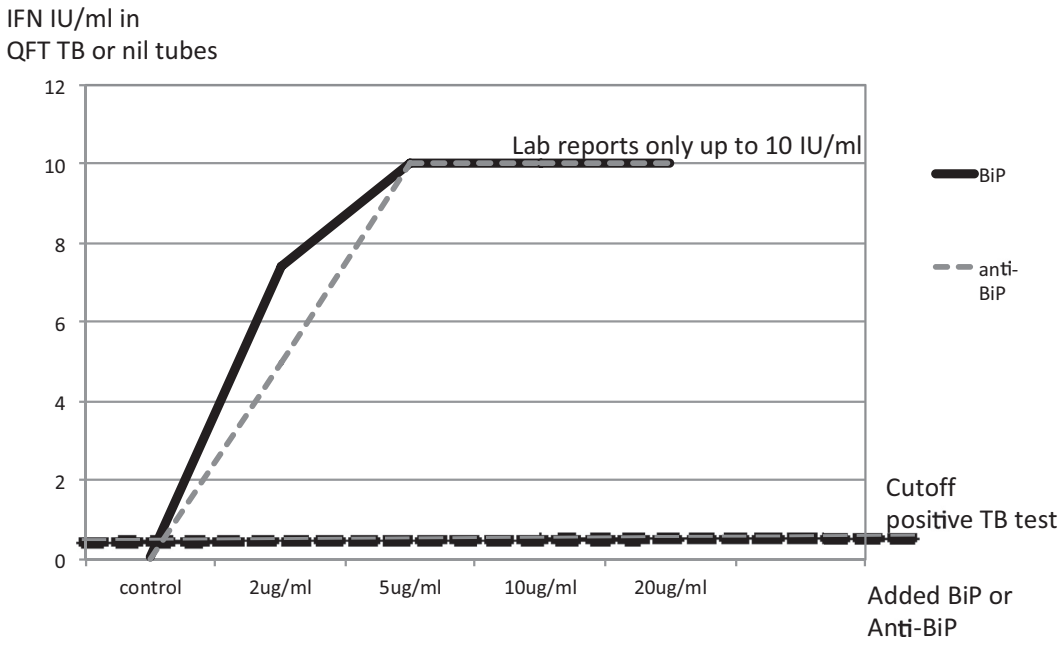

Fig. 3. Adding BiP or anti-BiP to test tubes in healthy *normal* patients resulted in very positive IFN levels (both in the TB test tube and the nil tube).

QFT-G).

Of the sequential 64 active RA patients negative for ACPA, during evaluation for biologic therapy, none had a positive QFT-G TB test. This negative ACPA cohort was not different at baseline from the positive cohort ($n = 126$). Both cohorts met the same entry criteria.

Three *normal* healthy women with no arthritis or TB exposure had negative QFT-G TB tests at baseline. These three subjects tested positive every time for TB by QFT-G correlating to the dose of BiP added *in vitro*, at concentrations of 2 ug/ml, 5 ug/ml, 10 ug/ml, and 20 ug/ml (Fig. 3). The QFT-G test was also positive in all three healthy normal patients when anti-BiP antibody was added *in vitro* to the sera at 5 ug/ml and 10 ug/ml (Fig. 3).

Discussion

The 13% RA patients described in this proof of concept study were all negative for TB by clinical criteria and 3 year follow up. We believe that the QFT-G test is not testing for TB but for interferon background levels spontaneously produced in some RA patients. We believe our results are consistent with the hypothesis that the QFT-G test is following BiP levels. Since ACPA (BiP) does generally fall with effective RA treatment, the fluctuation in the QFT-G test is consistent with that hypothesis. Also we have shown that sero- negative RA (those without BiP) do not have curious QFT-G tests. Many investigators have documented ACPA correlates with RA disease; further we have referenced studies showing very high levels of interferon background release in RA. We believe our hypothesis is sound based on the 13% false tests (RA patients on biologic therapy never developed TB over 3 years), QFT-G test results fluctuated downward with treatment of RA, and sero- negative RA did not have false positive tests. The RA patients with false QFT-G tests also had positive interferon in the Nil tube (0.18 IU/mL, sd 0.23, CI 0.13). Lastly, as proof of concept, we added very small amounts of BiP or anti-Bip to normal sera and could reproducibly cause false positive QFT-G tests. We believe these four findings are consistent with our hypothesis that BiP levels can result in false positive QFT-G tests.

Autoimmune diseases involve a complex set of interactions, including multiple classes of T cells, B cells, neutrophils, macrophages, plasma cells, autoantibodies, cytokines and interferons. Patients with autoimmune disease are in no way similar to the normal population in terms of immune background nor interferon release. This is due both to the underlying autoimmune disease and the immune-biologic therapies in general use in this patient population. Use of a test for latent TB relying on interferon levels in autoimmune patients is not reasonable without extensive testing to evaluate reproducibility of IGRA at baseline, on steroids, or after each of many biologic therapies.

Shoda's group has published data showing that citrullinated BiP is a strong autoantigen for T and B cells [8,15]. Data has documented specifically strong immune responses in RA to mycobacterial antigens. Furthermore, this positive immune response to mycobacterial antigens clearly correlated with serum anti-BiP antibody titers [15]. Going further, RA mononuclear cells response to BiP is markedly decreased by anti-HLA-DR blocking antibodies as well as by depletion of CD-25 (Treg) cells [16]. This reiterates the prior studies mentioned before that variation in immune subsets such as the number of CD4 and CD8 cells [10] influence QFG-T test results. The variables in immune subsets are further influenced by immune-modulating therapies with different pathways used to treat RA patients [11].

In our study, BiP or anti-BiP antibody added to the sample of any *normal* healthy patient did yield false positive QFT-G tests, at microgram levels. This suggests again that BiP is one mode of action causing error in the interferon results of QFT-G tests.

BiP and anti-BiP antibody is naturally found in higher levels in the majority of RA patients. We found that the highest CCP patients also had the highest QFT-G TB interferon levels ($p < 0.02$). In the clinical setting, a false positive QFT-G test in RA may lead to with-holding the appropriate biologic treatment in an active RA patient. Conversely, the false positive QFT-G test in an individual RA patient may lead to overtreatment of a presumed disease not present, if INH or other TB drugs are added unnecessarily. Both responses affect patient care.

There has also been no specific quality control testing of QFT-G in auto-immune disease. BiP has been found in the sera of up to 30% of SLE patients, and T cell proliferation and cytokine release including interferon is found in SLE as well, although the responses are lower in SLE than in RA [17]. Our clinic documented one false positive QFT-G test in our SLE population (the result reverted to negative after treatment of the SLE patient with sifulilumab; data available upon request to the corresponding author). In our clinic, there have been no positive QFT-G tests in psoriatic arthritis ($n = 86$), ankylosing spondylitis ($n = 13$) or inflammatory bowel disease ($n = 7$). (Data available from corresponding author upon request). Elevated levels of BiP and anti-BiP have not been documented in these sero-negative diseases. However, there is no reason to believe that ACPA is the only pathway to alter interferon levels and using interferon as a latent TB test in any autoimmune disease should be critically analyzed.

In the US where the incident risk of TB is low, false-positive TB testing is the more likely test result [14,18,19,20]. Using QFT-G screening in a low prevalence region refutes Baynesian analysis. Rather than test autoimmune patients for latent TB in the US, a region of low TB prevalence, another approach would be to evaluate patients prior to biologic immune therapy only for active TB. Physical exam, history of TB exposure, signs and symptoms of TB should be a minimum evaluation. Further evaluation with a chest radiograph or PPD testing can be added dependent on circumstances (for example, an employee in a hospital). Direct evaluation of TB if indicated can be done now with pharyngeal nasal washings sent for TB RNA polymerase chain reaction analysis and results are available within 24 h [21]. Alternatively, newer gas sensor technologies give same day results, with sensitivity and specificity for TB of 94% (Oxford Immunotec) [22]. If testing for latent TB is considered, use of the traditional PPD type 4 delayed type hypersensitivity test may be a more rational choice [23]. A version of PPD that does not cross react with BCG has been patented (Rutgers). Certainly advances in diagnosis of latent TB will come in the future, and there is no necessity to use interferon release as a surrogate measure going forward.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jctube.2017.08.004.

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