Comparison of an *in vitro* Tuberculosis Interferon-γ Assay with Delayed-type Hypersensitivity Testing for Detection of Latent *Mycobacterium tuberculosis:* A Pilot Study in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Recommendations for screening for latent *Mycobacterium tuberculosis* (MTB) infection have been proposed but are not well studied in patients with rheumatoid arthritis (RA). We estimated the prevalence of anergy in RA and evaluated different methods to detect MTB exposure.

Methods. This was a prospective pilot study of 61 patients with RA and 42 healthy controls. Tuberculin skin test (TST) antigen, Candida, and tetanus toxoid were injected intradermally using the Mantoux method. Subjects negative for TST returned for a second-step test. Whole-blood interferon-γ (IFN-γ) release to mycobacterial antigens was evaluated with the first-generation QuantiFeron® test (QIFN).

Results. Cutaneous anergy in patients with RA was not significantly different than healthy controls ($p = 0.154$), and was not affected by disease modifying antirheumatic drugs ($p = 0.270$). In patients with RA, 16.4% had positive TST with 10 mm cutoff vs 11.9% of controls. Using a 5 mm cutoff, 21.3% of patients with RA were positive, and this increased to 29.5% with a second-step TST. QIFN detected MTB exposure in 18% of patients with RA and 19% of controls ($p = 0.897$). However, indeterminate QIFN tests were higher in RA patients (11.5%) compared to controls (2.4%), demonstrating a lower sensitivity to detect latent MTB.

Conclusion. Cutaneous anergy may be less common than previously reported in patients with RA. However, the single-step TST and 10 mm cutoff may fail to detect all cases of latent MTB exposure in RA patients. High rates of indeterminate results in QIFN testing suggest that QIFN should not be employed as an alternative, single-screening test in patients with RA. These pilot results require confirmation in larger studies to determine the optimal screening strategy in RA. (First Release Mar 1 2008; J Rheumatol 2008;35:770–5)

Key Indexing Terms:
TUBERCULOSIS TUBERCULIN TEST SKIN TESTS
DIAGNOSTIC TECHNIQUES RHEUMATOID ARTHRITIS

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Supported in part through grants from Amgen, Inc. and Abbott Immunology, and by a grant from New York State ECRIP and the New York University School of Medicine GCRC NCRR-M01-RR00096. Dr. Greenberg was supported by an Arthritis Foundation/American College of Rheumatology Physician Scientist Development Award. Dr. Reddy was funded through a New York State ECRIP grant, grants from Centocor, and an Amgen Fellowship Award. Dr. Bingham was supported through the Goldstein Young Scholar Award of the New York Chapter Arthritis Foundation and through an Arthritis Foundation Arthritis Investigator Award.

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Accepted for publication December 21, 2007.

The association of *Mycobacterium tuberculosis* (MTB) infection with use of tumor necrosis factor-α (TNF-α) antagonists among patients with rheumatoid arthritis (RA) has been demonstrated in multiple studies[1-3]. While TNF is a central cytokine in the pathogenesis of RA, it is also an essential component of host defense against MTB[4]. In clinical trials of TNF antagonists and in postmarketing experience, cases of reactivation of latent MTB have been seen, often with disseminated presentations and sometimes fatal outcomes[5]. As a result, recommendations have been developed to guide screening of patients with RA to detect latent Tuberculosis (TB) infection before initiation of biologic agents[6-8]. However, clinical evidence to support these recommendations has been limited[6].

To determine whether an individual has been exposed to MTB, most recommendations have relied upon delayed-type hypersensitivity (DTH) skin testing using intradermal administration of 5 Tine units (TU) of tuberculin skin test

(TST) antigen or *M. tuberculosis* purified protein derivative (PPD) and recording induration at 48–72 hours. When negative results are obtained, Spanish Society of Rheumatology guidelines, but not others, advocate a second-step or booster TST 14 days later[6]. Different thresholds for defining positive results have also been suggested. For example, the Spanish guidelines recommend a 5 mm induration threshold for RA patients, whereas, French consensus recommendations recommend a 10 mm threshold[6,7]. Moreover, the British Thoracic Society recommends against TST altogether in patients receiving disease modifying antirheumatic drugs (DMARD), including methotrexate, due to reliability concerns[8].

Conflicting evidence has been presented concerning impaired DTH response in RA patients due to the disease itself and the use of DMARD[9-13]. If RA patients have impaired cutaneous DTH responses, then skin testing may result in false-negative TST to detect latent MTB, placing patients at increased risk. Recently, an alternative screening test for MTB exposure has been developed, QuantiFeron®, which evaluates interferon-γ (IFN-γ) release from whole blood in response to incubation with mycobacterial antigens[14,15]. The Centers for Disease Control now include whole-blood IFN assays as an alternative screening strategy to skin testing[15,16]. These guidelines, however, emphasize that first- and second-generation whole-blood IFN assays have not been adequately studied in patients with impaired immune systems.

In light of the conflicting data and recommendations for RA patients regarding MTB screening, we conducted a pilot study with 2 fundamental objectives: first, to estimate the prevalence of cutaneous anergy in RA patients, and second, to evaluate different methods of testing for latent MTB exposure for RA patients including the prevalence of positive TST screening in patients using different induration thresholds for TST, and to evaluate booster responses with a second-step TST.

MATERIALS AND METHODS

Our study was conducted with informed consent under from NYU School of Medicine Institutional Board of Research Associates.

We first evaluated the performance of an anergy panel consisting of 3 antigens for DTH testing in a cohort of control subjects (n = 42) consisting of healthy volunteers prior to evaluating the RA patient cohort (n = 61). Patients with RA met the American College of Rheumatology (ACR) diagnostic criteria[17]. Inclusion criteria for all participants were age between 18 and 65 years with no known history of TB or positive PPD/TST. Exclusion criteria consisted of use of corticosteroids at doses equivalent to prednisone 15 mg daily for more than 2 weeks in the previous 3 months, as well as concurrent use of azathioprine, cyclophosphamide, 6-mercaptopurine, cyclosporine, or any chemotherapeutic agent. We also excluded individuals with other conditions known to depress cell-mediated immunity. Individuals with a PPD test performed within the past 3 months or tetanus booster within 5 years were excluded.

Subjects completed a sociodemographic and TB screening questionnaire to identify individuals with prior Bacillus Calmette-Guerin (BCG) vaccine exposure and other risk factors for TB exposure. DTH skin testing was performed in all controls and RA patients by intradermal injection using a tuberculin syringe with a 27/28-gauge needle. For skin tests, 0.1 ml of a 1:10 dilution in normal saline of tetanus toxoid (Aventis Pasteur), 0.1 ml of Candida skin test antigen (Candin®, Allermed), and 0.1 ml tuberculin skin test (Tubersol®, Aventis Pasteur) were used. 0.1 ml of each antigen was planted on the flexor surface of the forearm according to the Mantoux method. Participants returned after 48 to 72 h for measurement of orthogonal diameters of induration. In healthy controls, 2 study nurses independently assessed indurations in blinded fashion and with high concordance of measurements (kappa 0.94) and little interobserver variability. In RA subjects only one study nurse recorded results.

Subjects having a mean orthogonal diameter < 2 mm for all 3 antigens were classified as anergic according to published guidelines[18]. Hypoergy was defined as having > 2 but ≤ 5 mm reactions. Patients with a negative TST (< 10 mm for healthy volunteers, < 5 mm for RA patients) were asked to return 14 days later (± 3 days) for a second-step intradermal placement of 0.1 ml TST on the contralateral forearm, and readings were performed 48 to 72 h later. We defined 2 TST thresholds of 10 mm and 5 mm, based on the various threshold definitions of existing national guidelines[6,7].

One tube of 10 ml heparinized whole blood was drawn for the IFN assay before PPD testing. The first-generation QuantiFeron® (QIFN) whole-blood IFN assay (Cellestis) available at the time of the study to detect MTB was performed and interpreted according to the manufacturer's instructions. All assays were run in duplicate and represented the average of 2 determinations. Assays with discrepancies were rerun for validation, and all indeterminate results were repeated to ensure accuracy.

Statistical analysis. Means and proportions were calculated and compared to evaluate baseline differences in sociodemographic variables and MTB risk factors. Kappa scores and Pearson correlations were used to assess interrater and test-retest reliability, respectively. Rates of anergy, hypoergy, and presence of positive PPD test were compared between RA patients and controls using chi-square analysis.

RESULTS

Demographic characteristics of the RA and healthy control cohorts are summarized in Table 1. Among the 61 patients with RA, 80.3% were rheumatoid factor-positive. RA patients had a mean (SD) disease duration of 11.2 (8.8) years, with 14.5 (8.8) tender and 12.8 (7.8) swollen joints. Thirty-four percent of the RA patients had never been prescribed DMARD and were classified as DMARD-naive. Prednisone was currently prescribed for 34.4% of patients with a mean dose of 6.8 mg. Methotrexate was prescribed for 49.2% and TNF antagonists were prescribed in 21.3%.

Results of individual antigen DTH responses. In the healthy controls, mean (SD) DTH skin induration for Candida was 8.53 (7.40) mm, for tetanus 7.12 (7.42) mm, and for PPD-1

Table 1. Baseline sociodemographic and *M. tuberculosis*-related risk factors in rheumatoid arthritis (RA) and healthy control participants.

	RA Cohort, n = 61	Healthy Controls, n = 42	p
Age, yrs, mean, SD	49.5 ± 11.1	35.0 ± 8.4	< 0.001
Sex, % female	83.6	76.2	0.448
Caucasian, %	60.0	43.2	0.164
Born outside US, %	77.0	36.6	< 0.001
BCG-positive, %	27.8	23.3	0.781

BCG: Bacillus Calmette-Guerin.

2.41 (6.39) mm. In the RA cohort, mean (SD) DTH induration for Candida was 11.51 (8.10) mm, for tetanus 4.36 (6.92) mm, and for PPD-1 3.08 (6.27) mm. Mean indurations did not differ between controls and RA patients (for Candida p = 0.11, tetanus p = 0.07, PPD-1 p = 0.60). No patient developed skin necrosis or required systemic steroid treatment for skin reactions.

Anergy results based on DTH testing. Using a panel of 3 antigens (Candida, tetanus, and single-step PPD), the proportion of patients with cutaneous anergy (DTH reactions < 2 mm for all 3 antigens) was assessed (Figure 1). In controls, 6 (14.3%) of 42 individuals were anergic, and none met the criteria for cutaneous hypoergy. In the RA cohort, 3 (4.9%) of the 61 individuals were anergic, and none was hypoergic. There was no statistical difference (p = 0.270) between rates of anergy between DMARD-naive and DMARD-treated patients with RA.

PPD DTH thresholds. In the control cohort, for the first PPD test (PPD-1), 5 (11.9%) of 42 individuals were classified as positive using the 10 mm orthogonal diameter cutoff (Figure 2). Lowering the threshold to define a positive to 5 mm did not change the number (5/42) of patients who would be classified as TST-positive (11.9%) among the controls. Of the 37 healthy controls with negative TST tests, 24 returned for a second-step TST (PPD-2). None of these achieved a booster response, using a 5 mm cutoff for PPD-2.

In contrast, both the threshold and PPD-2 booster increased the numbers of RA patients who would be classified as TST-positive. In the RA cohort, 10/61 (16.4%) were PPD-positive using the 10 mm cutoff, but 13/61 (21.3%) were PPD-positive using the 5 mm cutoff. A total of 33/61 (69%) RA patients returned for a second PPD test. For PPD-2, 5 (15%) additional RA patients were classified as having a positive TST result using the 5 mm cutoff and 4 (12.1%) using a 10 mm cutoff. The proportion of patients with a positive TST using the 5 mm cutoff and the second-step PPD increased, compared to the proportion who were TST-positive using only a single-step PPD and a 10 mm cutoff.

In vitro detection of latent TB. At the time our study was conducted, the first-generation QuantiFeron® (QIFN) test was the only commercially available assay for evaluating latent TB. Later generations of this test have reported further increases in specificity (e.g., the QuantiFeron-TB Gold), and similar assays are now available from other vendors (e.g., the T-SPOT.TB)[16,19]. Results of the QIFN test are summarized in Table 2. Eighteen percent of RA patients and 19% of controls had positive MTB results detected by the QIFN test, and roughly 50% of individuals in each group had negative results, with no differences between cases and controls. There was a strong trend approaching significance (p = 0.059) toward RA patients having higher rates of indeterminate test results (false-negatives) and also a trend for more controls with Myco-positive non-TB (false-positive) results (p = 0.112). In addition, we observed 2 RA patients who were QIFN-positive but failed to trigger a positive TST using either the 10 mm or 5 mm threshold.

To further investigate the indeterminate results (mitogen-nil < 1.5), we evaluated both nil and mitogen responses of the RA patients and controls. The nil response indicates baseline IFN production by cells incubated with saline. The

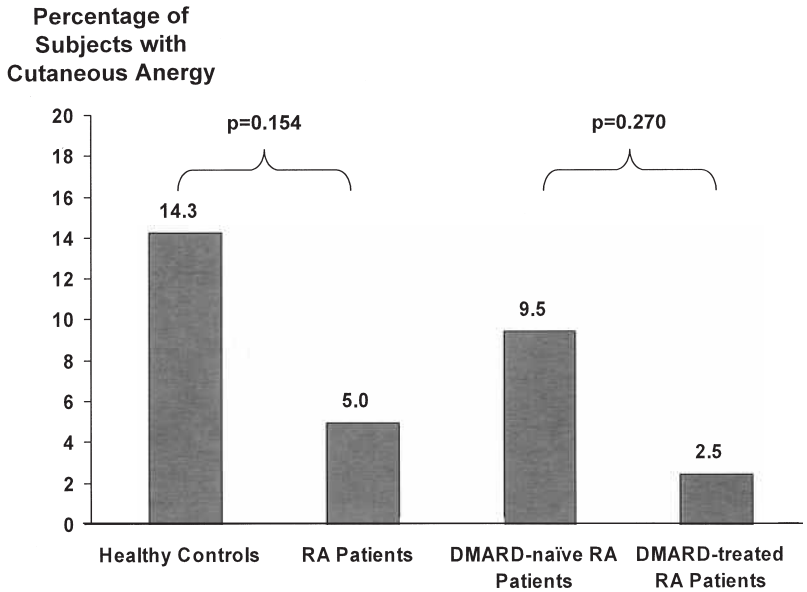

Figure 1. Individuals (%) with anergic cutaneous delayed-type hypersensitivity responses. Anergy was defined using a panel of 3 individual test antigens placed intradermally. Presence of anergy was defined as no individual skin test result demonstrating > 0.2 mm induration in the orthogonal diameter. DMARD: disease modifying antirheumatic drug.

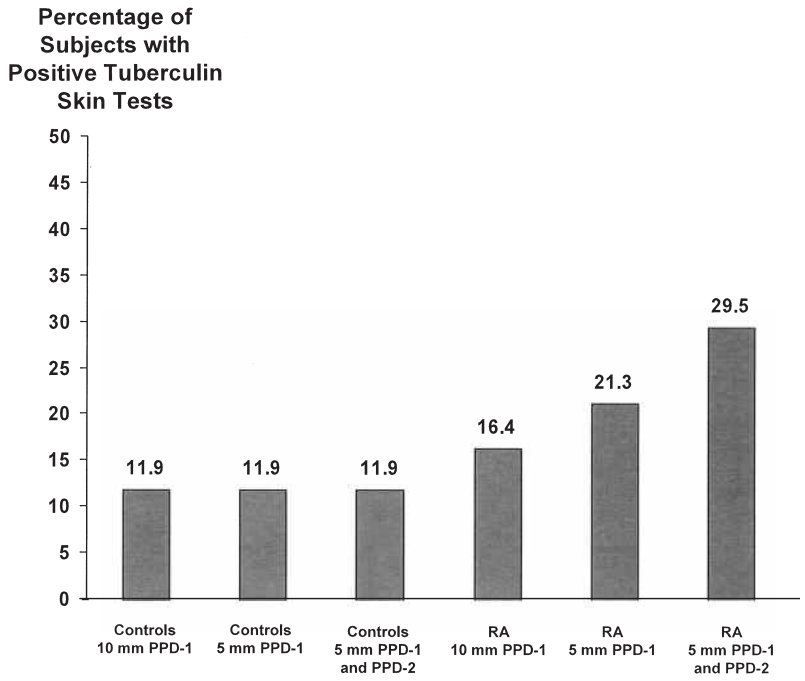

Figure 2. Individuals (%) with positive tuberculin skin tests using different thresholds to define positives, and comparing first-step (PPD-1) and second-step (PPD-2) tests.

Table 2. Results of whole-blood interferon-γ release in response to mycobacterial antigens using the QuantiFeron® test system compared to results of tuberculin skin tests. Data are percentages.

	RA Patients, N = 61	Healthy Controls, N = 42	p
QuantiFeron Results, %			
MTB+	18.0 (n = 11)	19.0 (n = 8)	0.897
MTB–	55.7 (n = 34)	47.6 (n = 20)	0.422
Indeterminate	11.5 (n = 7)	2.4 (n = 1)	0.059
Myco+ non-TB	13.1 (n = 8)	26.2 (n = 11)	0.112

mitogen response reflects generation of IFN in response to mitogen, as an indirect determination of potential T cell activation. No differences (p values all > 0.3) were observed between RA patients and controls in nil control responses (2.0 vs 2.0 IU/ml, respectively) or between DMARD-naive and DMARD-treated RA patients (1.6 vs 2.2 IU/ml, respectively). Mitogen responses were also similar between RA patients and controls (15.4 vs 16.6 IU/ml, respectively; p = 0.73); however, mitogen responses were significantly lower among the DMARD-naïve compared to the DMARD-treated RA patients (7.8 vs 17.7 IU/ml, respectively; p = 0.01).

DISCUSSION

The objective of our pilot study was to investigate currently utilized screening strategies for MTB exposure in a cohort of patients with RA. Because of reports of impaired DTH responses and cutaneous anergy in RA patients[9-13], we assessed cutaneous anergy in patients compared to healthy controls. The prevalence of cutaneous anergy in both DMARD-naïve and DMARD-treated RA patients was low, and was not significantly different than in healthy controls. Our data indicate that TST screening in RA patients, whether they are receiving DMARD or not, may represent a highly sensitive screening test, particularly using a 5 mm cutoff and a second-step TST. In contrast, we observed that a whole-blood IFN assay was indeterminate in 11.5% of RA patients, particularly among DMARD-naïve patients, who exhibited an attenuated mitogen response compared to patients treated with DMARD. These results, although preliminary, should alert clinicians and researchers to the challenges associated with interpreting the results of a single diagnostic screening test for evidence of MTB exposure in patients with RA.

Further, in this New York City cohort, we detected an unexpectedly high proportion of RA patients with positive tuberculin skin tests, considering that patients with known positive PPD were excluded from participation. The cohort we studied represents an urban population, with a large proportion of immigrants, and coming from an area in which the prevalence of MTB is significant[20]. The high prevalence of possible latent MTB emphasizes the importance of screening prior to use of TNF inhibitor.

Our results show that using a 5 mm cutoff for TST screening and a second-step PPD may increase the sensitivity of detecting latent MTB exposure among patients with

RA. Although the absence of a "gold-standard" of MTB exposure makes it difficult to derive definitive conclusions, the second-step PPD did not increase the number of positive results in healthy controls, suggesting that the booster phenomenon in RA patients may be important. Although concerns have been raised about impaired T-lymphocyte function and anergy in RA[9-13], we found the prevalence of cutaneous anergy in both DMARD-naïve and DMARD-treated patients with RA was low (< 10%) and was not significantly different than in healthy controls, consistent with the findings of Pope, *et al*, who reported that the prevalence of cutaneous anergy was 0% but that 27.3% of RA patients exhibited a "hypoergic" or depressed DTH response[9]. In contrast, multiple studies have observed rates of cutaneous anergy ranging from 24% to 50% in patients with RA[10-13].

One explanation for the conflict of our results with those of studies reporting higher rates of cutaneous anergy is that previous studies used the Multitest CMI® multipuncture system, whereas we used individual intradermal administration of skin test antigens and the Mantoux method. The Multitest was a panel of 7 antigens and a control preincorporated on an implantation device; however, that product was withdrawn from the market before initiation of this study. Validation studies of Multitest CMI® compared to the Mantoux method for DTH reactions have been limited[21,22]. Indeed, the Multitest CMI® has not been validated in patients with RA or other autoimmune disorders. A more recent study of HIV patients, directly comparing the results of the Multitest CMI® with DTH responses, reported substantial discrepancies using these methods[22]. Thus, the discrepancy between our findings and earlier reports may be explained on the basis of the diagnostic technique employed.

Our study is the first to our knowledge to compare these 2 methods of assessing cutaneous anergy in an RA population taking DMARD. On the basis of skin testing, there were no statistically significant differences between patients with RA and controls; however, the results of the IFN-γ assay would suggest an inherent impairment of T cell function in our RA population. Twelve percent of our RA patients (7/61) had an indeterminate result, primarily due to low mitogen responses. The low mitogen responses we observed are consistent with studies indicating that RA patients have impaired cell-mediated immunity[23]. Our results suggest some discrepancy between impaired *ex vivo* mitogen-stimulated mononuclear cells and the skin testing with specific antigen to detect cutaneous anergy.

Further, our results suggest that both the threshold for positive results and the second-step testing may increase the sensitivity to detect possible latent MTB exposure in patients with RA. Although the absence of a gold standard to diagnose latent MTB infection precludes definitive conclusions, our data support the Spanish recommendation of using a 5 mm cutoff for interpreting TST results in RA patients. Whereas 10 of 61 (16.4%) RA patients had a positive TST using the 10 mm cutoff (per some current recommendations), the prevalence of a positive TST increased to 13 in 61 (21%) using a second-step TST. Moreover, our results also support Spanish recommendations for using a second-step TST, detecting an additional 5 cases. In comparison with the 16% prevalence of TST-positive results using the single TST and 10 mm cutoff, the 5 mm cutoff coupled with the second-step TST doubled the detection rate to 30% of patients with RA. Larger studies are required to confirm these findings, although evidence suggesting that RA patients have impaired cutaneous cell-mediated immunity and attenuated TST response supports using the 5 mm threshold and a booster second-step TST[24]. Further research is required to determine the optimal TST screening thresholds for RA patients with impaired immune function.

Our concurrent evaluation of the IFN-γ assay as a second screening technique for RA patients and healthy individuals allowed a comparison of diagnostic strategies. These assays have been validated for healthy individuals at low risk of MTB exposure[25]. In contrast, validation studies in immunocompromised patients such as those with HIV have proven less promising[16-26]. Our findings suggest that the IFN assay may detect additional cases of MTB exposure that would otherwise be missed using TST screening techniques. However, 12% of the RA patients had indeterminate QIFN results, raising concerns about the QIFN assay as a single screening technique in RA patients. Rather, use of a QIFN assay as a screening tool in combination with TST screening for high-risk patients could offer optimal sensitivity for detecting latent MTB.

Our study has several limitations. Sample sizes of both groups were small, which may have limited our ability to detect between-group differences. Patients with RA were older and more likely to be immigrants. In addition, the RA cohort was heterogeneous with respect to disease activity and medication use, each of which may have independent influences on T cell function and cellular immunity. However, our study was representative of the range of drugs used by RA patients in clinical practice, but was not powered to detect differences with different DMARD. Finally, although the proportion of individuals who reported having received BCG vaccinations was similar in both groups, we did not verify this (e.g., ascertaining presence of a BCG scar). BCG vaccination has not been a standard childhood procedure in the US for many years, but this is not the case in all countries. Thus, it is conceivable that some subjects reported being vaccinated when in fact they were not, and others without knowledge of an immunization may have underestimated exposure. Moreover, because the RA patients were older and boosting increases with age (presumably because of more remote exposure), we cannot conclude that boosting in the RA patients was directly related to having the disease.

Our study indicates that the current standard recommendations of TST screening for RA patients at high risk for latent TB exposure may fail to detect a proportion of exposed individuals. In our study, no single method was sufficient in detecting all cases. Our finding that the first-generation QIFN may be less reliable in DMARD-naïve patients with RA due to decreased mitogen responses requires additional evaluation.

The results of our pilot study support the use of TST as a screening method for detecting latent MTB in patients with RA and demonstrate that additional sensitivity may be obtained using a 5 mm threshold and second-step PPD. While the first-generation QIFN assay may improve detection of latent MTB in some patients with RA, it is unlikely to be reliable as a single diagnostic test. Further studies are needed to confirm sensitivity and specificity tradeoffs of using 5 mm thresholds and second-step TST in patients with RA.

ACKNOWLEDGMENT

The authors acknowledge the participation of Vivian Abellana, RN, CCRC, and Eileen McCullagh, RN, CCRC, for performing skin testing, and of Hayf Al-Mussawir for technical assistance with QuantiFeron® assays.

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