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LETTERS

Indeterminate results of a tuberculosis-specific interferon-γ release assay in immunocompromised patients

To the Editors:

Immunocompromised patients with various causes and degrees of immunodeficiencies, such as stem cell and solid organ transplant recipients, patients with autoimmune diseases, patients with chronic renal failure or HIV-positive patients, are at increased risk of progression from latent *Mycobacterium tuberculosis* infection to active disease. Therefore, screening for latent tuberculosis and preventive treatment is recommended in this patient population. Tuberculosis-specific interferon (IFN)-γ release assays (TIGRAs) lacking cross-reactivity with *Mycobacterium bovis* bacille Calmette-Guérin have been introduced into the routine diagnosis of latent tuberculosis infection (LTBI) in the last few years as a more specific alternative to tuberculin skin test (TST). More recent results implicate that QuantiFERON®-TB Gold in tube (QFT-G-IT; Cellestis, Carnegie, Australia) may better predict progression from latent to active disease compared with TST [1, 2].

TIGRAs using stimulation of T-cells with phytohemagglutinin (PHA) as a positive control for identification of false negatives and the classification of indeterminate results may be a better alternative for the prediction of LTBI in immunocompromised patients where IFN-γ release may be affected by immunosuppression. The growing list of data existing on reliability of TIGRAs in immunocompromised patients show that the prevalence of indeterminate results may vary depending on the degree of immunosuppression and the TIGRA test used [3]. In addition, PHA and recall antigens use different IFN-γ secretion pathways [4], which may be differentially affected by immunosuppressive conditions. Thus, an in-depth analysis of factors influencing PHA-associated IFN-γ secretion is important for the assessment of TIGRAs in immunocompromised patients. Since disease group dependent and independent risk factors of indeterminate results in immunocompromised patients have not been evaluated prospectively using the third generation QFT-G-IT test, we tested QFT-G-IT in patients with diverse conditions of immunosuppression to determine the rate of and identify risk factors for indeterminate results.

After approval of the ethical committee of the University of Freiburg (Freiburg, Germany), outpatient or hospitalised immunocompromised patients and 104 healthy students and healthcare workers (controls) were included prospectively (October 2006 to October 2007) in a single centre in Germany. Patients (aged >18 yrs, mean age 64.5 yrs) had either undergone organ transplantation or stem cell transplantation (39 allogeneous, four autologous), had an autoimmune disease (39% rheumatoid arthritis, 12% systemic lupus erythematosus (SLE), 5% chronic inflammatory bowel disease, 44% other autoimmune diseases), had been receiving immunosuppressive therapy for at least 6 months (glucocorticoids equivalent to $\geqslant$10 mg prednisolone, cyclosporin, tacrolimus, methotrexate or mycophenolate), and had primary immunodeficiencies (82% with chronic variable immunodeficiency syndrome) or HIV-infection (mean CD4 count: 435 cells·μL^{-1}; 42 patients with a CD4 count >200 cells·μL^{-1}; 33 with HAART therapy; 33 were male). Of 456 screened patients, nine were excluded for not meeting inclusion criteria or not enough data to define length or dosage of immunosuppressive therapy, two patients (one stem cell transplant and one with autoimmune disease) had active tuberculosis (table 1). Prospectively planned data collection included demographic data, medical history, medication and laboratory values.

QFT-G-IT was performed in one laboratory following manufacturers' instructions after in-house transport at room temperature. After vigorous resuspension of the antigen, test tubes were incubated at 37°C 2–6 h after collection and ELISA was performed after 16–20 h of incubation. Indeterminate results were defined as PHA-associated IFN-γ secretion of <0.5 IU·mL^{-1} or negative control values >8 IU·mL^{-1}. Lymphocyte counts and haemoglobin levels were performed in 68% and 95% of patients, respectively, with a similar percentage in different patient groups. The Chi-square based measure Cramer V was used to determine relationships of categorical variables. For multivariate analysis a backward stepwise logistic regression was performed for variables with more than 25 cases per category testing disease group, sex, age, immunosuppressive medication, blood values (haemoglobin, erythrocyte count and lymphocyte count), and risk factors for immunosuppression (diabetes, smoking, current dialysis). To compare means of numeric variables we used an unpaired t-test.

In our cohort of immunocompromised patients positive QFT-G-IT results were more common than in controls (8.3% (95% CI 5.7–10.7%) *versus* 1.9% (95% CI 2.3–6.8%); p=0.060) and more common in males than in females (10.5% (95% CI 7.2–15.4%) *versus* 5.3% (95% CI 2.7–10%); p=0.056). In a logistic backward regression, a positive QF-GT-IT result was significantly influenced by immigration status and old age (p<0.05) reflecting known risk factors of LTBI in Germany.

We focused our analysis on the reliability and risk factors of disease group dependent and independent indeterminate results in immunocompromised patients (table 1). As expected, the overall rate of indeterminate result in immunocompromised

TABLE 1 Characteristics of patients and controls and QuantiFERON®–TB Gold in tube results in relation to categorical and continuous variables

	Total	Determinate	Indeterminate	Univariate		Multivariate
				OR (95% CI)	p-value	p-value
Categorical variables: controls						
Female	62; 60 (50–69)	61; 98 (95–100)	1; 1.6 (0–7.3)	0	>0.05	>0.05
Male	42; 40 (31–50)	42; 100 (98–100)	(0–6.7)			
Categorical variables: patients						
Male	258; 58 (53–62)	226; 88 (84–92)	26; 10 (6.4–14)	**0.6 (0.4–0.9): male**	**<0.05**	**>0.05**
Female	189; 42 (38–47)	157; 83 (78–88)	32; 17 (12–23)			
Disease group						
Organ transplant	233; 52 (48–57)	216; 93 (89–96)	17; 7.3 (4–11)	**0.4 (0.2–0.8)**	**<0.05**	
Male	157; 67 (61–72)	147; 94 (88–97)	10; 6.4 (3–11)			
Female	76; 33 (27–39)	69; 90 (82–96)	7; 9.8 (4–18)			
Autoimmune diseases	76; 18 (14–22)	60; 79 (70–88)	16; 21 (12–30)	**2.6 (1.4–5.0)**	**<0.05**	**0.073**
Male	22; 29 (20–40)	22; 100	0; 0			
Female	54; 71 (60–80)	38; 70 (57–80)	16; 30 (19–43)			
Primary immunodeficiency	45; 10.1 (7.3–13)	41; 91 (83–99)	4; 9 (1–18)	0.8 (0.3–2.3)	>0.05	
Male	21; 47 (32–60)	20; 96 (76–100)	1; 4.2 (0–24)			
Female	24; 53 (39–67)	21; 87 (68–96)	3; 13 (3–31)			
Stem cell transplant	42; 9.4 (6.7–12)	23; 55 (40–70)	19; 45 (30–60)	**8.1 (3.0–22)**	**<0.05**	**<0.05**
Male	25; 60 (44–73)	11; 44 (27–63)	14; 56 (37–73)			
Female	17; 40 (27–56)	12; 70 (47–87)	5; 30 (13–53)			
HIV patients	51; 11.4 (8.5–14)	49; 96 (91–100)	2; 3.9 (0–10)	**2.3 (1.5–4.2)**	**<0.05**	
Male	33; 65 (51–76)	32; 97 (83–100)	1; 3 (0–17)			
Female	18; 35 (24–49)	17; 94 (72–100)	1; 6 (0–28)			
Medication						
Steroids	230; 51 (47–56)	195; 85 (80–89)	35; 15.2 (11–20)		>0.05	>0.05
Cyclosporin	147; 33 (29–37)	127; 86 (81–92)	20; 13.6 (8–19)		>0.05	>0.05
Tacrolimus	95; 21 (17–25)	85; 90 (84–96)	10; 10.5 (4–17)		>0.05	>0.05
Methotrexate	25; 6 (4–8)	22; 88 (75–100)	3; 12 (0–25)		>0.05	>0.05
Mycophenolate	173; 39 (34–43)	158; 91 (87–96)	15; 8.7 (4–13)	**0.8 (0.7–1.0)**	**<0.05**	>0.05
Risk factors						
Diabetes	31; 7 (5–10)	28; 90 (80–100)	3; 9.7 (0–20)		>0.05	>0.05
Smoking	28; 6 (4–9)	26; 93 (83–100)	2; 7.1 (0–17)		>0.05	>0.05
Having had dialysis	206; 46 (42–51)	190; 92 (89–96)	16; 7.8 (4–11)		>0.05	>0.05
Continuos variables: patients						
Age yrs	447; 52.4±13.8	389; 52.1±13.7	58; 53.8±14.2		>0.05	>0.05
Erythrocyte count $10^6 \cdot \mu L^{-1}$	431; 4.4±0.7	374; 4.5±0.7	57; 3.9±0.7		**<0.05**	**<0.05**
Leukocyte count $1000 \cdot \mu L^{-1}$	435; 6.9±3.4	378; 7±3.4	57; 6.4±3.4		>0.05	>0.05
Lymphocyte count μL	305; 1614±836	266; 1691±817	39; 1085±771		**<0.05**	**<0.05**
CD3 count μL^{-1}	64; 1114±637	56; 1205±614	8; 481±421		**<0.05**	**<0.05**
CD4 count μL^{-1}	78; 462±300	68; 483±289	10; 313±350		>0.05	>0.05
CD8 count μL^{-1}	74; 585±452	64; 656±446	10; 134±90		**<0.05**	**<0.05**
Length of dialysis months	196; 56±44	182; 55.1±43	14; 68.5±46		>0.05	>0.05
Length of illness months	413; 91±90	360; 94±88	53; 78±102		>0.05	>0.05

Data are presented as n; % (95% confidence interval) or n; mean±SD, unless otherwise stated. Significant differences in univariate and multivariate analysis are shown in bold. QuantiFERON®–TB Gold in tube is manufactured by Cellestis, Carnegie, Australia.

patients was significantly higher (13%, 95% CI 9.9–16.1%) than in the control group (0.9%, 95% CI 0–2.75%; p<0,001), and similar to previously published results. However, further comparability is limited due to the use of the second generation QFT-Gold test or due to differences in patient cohorts [3, 5].

Overall indeterminate results differed significantly depending on the disease group. Patients with autoimmune diseases had a significantly higher percentage of indeterminate results than organ transplant patients (p<0.05), which may be due to a previously described decreased IFN-γ response to different

antigens in patients with rheumatoid arthritis [6]. In a subgroup of patients with SLE, seven out of 12 patients had an indeterminate result (univariate $p<0.001$) which may be related to an impaired *in vitro* response to PHA in patients with active SLE [7]. The overall indeterminate result rate was 9.6% if results of stem cell transplant patients were omitted. The high indeterminate result rate in stem cell transplant patients is not surprising as *in vitro* a prolonged impairment of IFN-γ production has been shown in patients with autologous stem cell transplantation [8], and most of our patients have been studied within their first year after transplantation. Thus, the use of TIGRA prior to immunosuppressive therapy is especially important in this patient group, not only because of the high percentage of indeterminate results, but since the donor immune system will be studied after stem cell transplantation.

Indeterminate result rates were higher in females than males (univariate $p<0.05$; not significant in a multivariate analysis comprising medication, haemoglobin and lymphocyte count), a finding that has not been reported in other studies evaluating TIGRAs in immunocompromised patients. The sex-difference was most pronounced in patients with autoimmune diseases (29% in females *versus* 0% in males; $p<0.05$), and it was also significant in multivariate analysis. A nonsignificant sex influence favouring indeterminate results in female over male patients was also found in other patient groups. Several *in vitro* results (in young females, females and female deer) showed a lower IFN-γ response towards PHA stimulation or towards a multi-test cellular-mediated immunity skin test [9]. Therefore, it may well be that TSTs are more frequently false negative in females than in males and that data based on the skin test showing that females are less likely to have latent infection with *M. tuberculosis* must be interpreted more cautiously. Future contact tracing studies combining skin tests and TIGRAs should address this issue prospectively.

Low haemoglobin levels, as well as a low lymphocyte count, that were not correlated ($p=0.179$) correlated significantly with low IFN-γ secretion in univariate analysis ($p<0.001$). In a backward logistic regression analysis a low haemoglobin level as well as a low lymphocyte count were significant factors for explaining variance in QFT-G-IT results ($p<0.001$, Nagelkerke $R=0.29$). In a linear regression model with the same independent variables and the dependent variable ''PHA-induced IFN-γ secretion'', both lymphocyte count and haemoglobin level explained IFN-γ secretion variance significantly ($p<0.001$, $R^2=0.164$) (table 1). A correlation between lymphocyte count and IFN-γ response is expected and is similar to the results published by KOBASHI *et al.* [5]. The reason for the surprising and previously unreported finding of a correlation between low erythrocyte count or haemoglobin level and indeterminate results in QFT-G-IT is unclear and not well explained by the influence of red blood cells on T-cell function, or of erythropoietin on T-lymphocytes and pro-inflammatory cytokine response [10]. If anaemia can be expected, QFT-G-IT tests should be used in immunocompromised patients before its development. Further studies are needed to evaluate the effect of low erythrocyte count/haemoglobin levels on the PHA-associated IFN-γ response in TIGRAs.

In a subgroup of 67 patients (HIV patients and patients with primary immunodeficiencies) where a differential lymphocyte count was available, a strong positive correlation of both CD3+ T-lymphocytes and CD3+CD8+ with the IFN-γ response was found. Similar to previous reports of indeterminate results in patients with HIV infections, in our study no indeterminate results occurred in HIV patients with a CD4-count >200 cells·μL^{-1}. However, indeterminate results did not correlate with CD3+CD4+-T-lymphocytes in this specific subgroup of patients possibly due to the low number of patients, which was a limitation of our study. In this selective patient sub-cohort a low CD8 count was the best predictor of low IFN-γ response after PHA stimulation.

In conclusion, different disease groups bear an independent risk of indeterminate results in the QFT-G-IT. It is prudent to perform TIGRAs for the diagnosis of latent tuberculosis as soon as the need for therapeutic immunosuppression is evident. Prospective studies may be able to confirm that low lymphocyte, CD8 T-cell and haemoglobin levels are better predictors of indeterminate QFT results than disease group or immunosuppressive medication and confirm the surprising finding of female sex being a risk factor for indeterminate results.

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Dermcidin identification from exhaled air for lung cancer diagnosis

To the Editors:

Exhaled breath condensate (EBC) analysis is a simple and truly non-intrusive approach to acquire information on understanding airway inflammation and other diseases of the respiratory system, such as tumourigenesis [1–3]. There are several striking advantages to utilising breath testing for screening purposes: 1) it does not influence airway function or cause inflammation [4]; 2) it can be performed repeatedly within short intervals [5]; 3) it is not significantly affected by age, sex or disease status [5]; and 4) it can be considered as a lung-specific analytic approach. Cancer cells have distinct properties from normal cells in that they may synthesise new proteins or change the protein expression levels during tumourigenesis [6]. A lot of soluble components of the lung exist in the epithelial lining fluid of alveoli [7]; therefore, the secreted new synthetic proteins can be digested into peptides under enzymatic processes. Subsequently, the small peptides have the possibility of adding to exhaled breath like other EBC compounds. The exploration of endogenous peptides, created by enzymatic cleavage of proteins in particular cellular environments, can result in relevant biomarker candidates [8, 9]. However, trace amounts of materials in EBC make detection a challenging task. This study was designed to determine the peptidome of EBC and to search for potential biomarkers for lung cancer diagnosis.

The study protocol was approved by the Institutional Review Board of the Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). Patients with histological evidence of primary lung cancer who were admitted to the Division of Pulmonary and Critical Care Medicine (Dept of Internal Medicine, Kaohsiung Medical University Hospital), between January 2008 and August 2008 were enrolled in the study. We enrolled patients with squamous cell carcinoma, adenocarcinoma, small cell carcinoma, pneumonia and chronic obstructive pulmonary disease (COPD), as well as healthy subjects (table 1). The inclusion criteria included patients with newly diagnosed lung cancer before treatment. The diagnosis of lung cancer was confirmed by histological examinations of biopsy and/or cytology specimens obtained during fibreoptic bronchoscopy or with computed tomography-guided transthoracic needle aspiration biopsy. The stage of lung cancer was determined according to the staging system of the American Joint Committee on Cancer TNM classification. The early stage of lung cancer indicated stage I, II or IIIA. The late or advanced stage of lung cancer indicated stage IIIB and stage IV. Informed consent was obtained from each participant before enrolment in the study. In order to participate patients had to be aged >18 yrs. The control group of the study included subjects without lung cancer matched for socioeconomic group and age and who were nonsmokers, smokers and ex-smokers (defined as not having smoked for $\geqslant$1 yr). The controls were healthy or had had pneumonia or COPD during hospitalisation in the Kaohsiung Medical University Hospital (table 1). The exclusion criteria included: 1) patients refused to enter this study; 2) patients with lung cancer but experienced chemotherapy or radiotherapy; 3) patients with pulmonary tuberculosis; 4) patients with unclassified nonsmall cell lung cancer (NSCLC); 5) patients with other solid or haematological malignancy; and 6) patients with lung cancer but who could not open their mouth due to betel chewing or oral diseases.

The number of patients in each category and their ages and disease stages are listed in table 1. EBC collection was performed using an EcoScreen condenser (Jaeger, Wuzburg, Germany). Subjects were asked to breath at a normal frequency and tidal volume for 15 min while wearing a nose-clip. The exhaled air passed through a mouthpiece and a two-way non-rebreathing valve and was then frozen at -20°C. The condensates (>1 mL) were thawed and transferred to 1.5-mL microtubes and immediately stored at -70°C. Bruker Cu magnetic beads (Bruker Daltonics, Leipzig, Germany) were used to purify EBC before mass spectrometer (MS) analysis. For MALDI-TOF (matrix-assisted laser depolarisation/ionisation-time of flight) MS analysis, measurements were performed on a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). For liquid chromatography (LC)/MS analysis, a nano-LC system coupled with Thermo LTQ-FTICR MS was used. Chromatographic separation was achieved by using self-packed C_{18} column at a split flow rate of 300 mL·min^{-1} in a 60-min running cycle. For FTICR MS analysis, the full-scan mass survey was set at m/z 320-1,800 with mass resolution of 100,000 at m/z 400. Singly charged ions were rejected from MS/MS sequencing.

Based on MS/MS analysis and the MASCOT search, ~20 to 100 peptides that were deduced to be from <10 proteins were identified in each EBC sample. A total of 20 types of predicted