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Short Communication

Levels of anti-cytokine antibodies may be elevated in patients with pulmonary disease associated with non-tuberculous mycobacteria

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ABSTRACT

Pulmonary disease due to non-tuberculous mycobacteria (NTM) is caused by several species (particularly *Mycobacterium avium*, *Mycobacterium intracellulare*) that are abundant in the environment. Th1 cytokines such as interferon (IFN)-γ are important in the control of mycobacteria, but *in vitro* production of IFN-γ is not deficient in adult patients with pulmonary NTM disease. Antibodies reactive with IFN-γ have been described in patients with disseminated NTM disease, but it is not clear whether they are common in pulmonary disease. Here we show that patients with pulmonary NTM have a higher level of anti-IFN-γ and anti-GM-CSF antibodies than healthy controls, although some controls also have high levels. Levels of anti-IFN-γ antibodies did not correlate with levels of total immunoglobulin. Longitudinal studies are required to determine whether anti-cytokine autoantibodies are consequence rather than a cause of pulmonary NTM disease.

1. Introduction

Pulmonary disease due to non-tuberculous mycobacteria (NTM) is caused by several species that are abundant in the environment. *Mycobacterium avium* and *Mycobacterium intracellulare* (known collectively as MAC) are the most common in Australia, but other slow growing mycobacteria (SGM) also cause pulmonary disease. These include *Mycobacterium kansasii* and *Mycobacterium lentiflavum*. Diagnoses of pulmonary disease due to NTM have increased worldwide and are more common in post-menopausal women. The most common presentation is nodular bronchiectasis [1,2]. Mechanisms underlying susceptibility are unknown, so pulmonary NTM disease presents as an important medical challenge.

Healthy individuals control infections by intracellular bacteria via Th1 immune responses involving interferon-gamma (IFN-γ). This has led to the speculation that pulmonary NTM disease reflects defects in IFN-γ pathways [3]. However, genetic defects in these pathways associate with disseminated NTM disease (rather than pulmonary NTM) and usually affect children [3].

Recently, anti-cytokine autoantibodies have been proposed as a predisposing factor. Depletion of bioavailable IFN-γ *in vivo* by autoantibodies against Th1 cytokines such as IFN-γ has been associated with other granulomatous conditions such as sarcoidosis, listeriosis and histoplasmosis [4]. Anti-IFN-γ immunoglobulin (Ig)-G autoantibodies were found in patients with disseminated mycobacterial and other bacterial infections [5,6]. Six East Asian women with disseminated NTM disease displayed anti-IFN-γ autoantibodies that were able to block the binding of human IFN-γ to its receptors, thereby inhibiting downstream events in the pathway, specifically IFN-γ dependent phosphorylation of STAT-1, TNF-α and IL-12 [7]. Two more recent reports associating anti-IFN-γ antibodies with disseminated NTM were also from Asian cohorts [8,9].

In healthy controls, low levels of anti-granulocyte–macrophage colony-stimulating-factor (GM-CSF) autoantibodies may neutralise free GM-CSF and regulate GM-CSF mediated inflammation and autoimmunity. However, high levels of anti-GM-CSF autoantibodies have been associated with pulmonary alveolar proteinosis and may limit alveolar macrophage functions. Levels of anti-GM-CSF were inversely related to the basal function of neutrophils *in vivo* (phagocytic capacity) and *in vitro* (assessed through

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chemotaxis) [10]. Anti-GM-CSF autoantibodies are occasionally seen in NTM disease [3].

In this study we have examined the levels of anti-IFN-γ and anti-GM-CSF autoantibodies in plasma samples from patients with NTM disease and healthy controls. Patients with the rapid-growing *Mycobacterium abscessus* [11] were considered separately, as this species causes a range of disease syndromes distinct from those associated with SGM. Data are compared with levels of total IgG to differentiate a specific defect in the immune system from systemic B-cell activation.

2. Materials and methods

2.1. Participants

Adults (over 18 years of age) with pulmonary disease associated with SGM [$n = 135$, age = 70 (42–98) years] were enrolled into the collaborative study through the clinics of Royal Perth Hospital (Western Australia), and the Prince Charles Hospital and Greenslopes Hospital (Queensland). NTM disease was diagnosed using guidelines of the American Thoracic Society [12]. Patients had disease due to slow growing environmental mycobacteria; *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium kansasii* and/or *Mycobacterium lentiflavum*. Patients [$n = 12$, age = 71 (48–86) years] with the rapid-growing *Mycobacterium abscessus* were retained as a separate group. Exclusion criteria included alcohol excess, current smoking, cystic fibrosis, HIV infection or use of immunosuppressive medications. No patients had been treated with exogenous IFN-γ or GM-CSF. All female patients were post-menopausal. Controls comprised offspring of some Western Australian patients [$n = 13$, age = 43 (15–75) years] and healthy individuals from the general West Australian population [$n = 140$, age = 59 (32–84) years]. Patients with SGM comprised 95 females, 32 males and 8 patients where gender was not recorded. This study was approved by the Ethics Review Boards of participating institutions, and informed consent was given by the individuals and/or their guardians.

2.2. ELISA methods

Half-well microplates were coated with 50 μL/well recombinant human IFN-γ (0.5 μg/mL; ORF Genetics, Iceland) or GM-CSF (0.5 μg/mL; Aurora Biosystems, Germany] overnight at 4 °C, washed 3 times with 0.05% Tween-20 in phosphate buffer saline (PBS), blocked with 100 μL/well 5% bovine serum albumin in PBS for 1 h and washed. A patient sample with high levels of IFN-γ and GM-CSF autoantibodies was used as a standard and given an arbitrary concentration of 1000 Units/mL. Plasma samples (diluted 1/100 and 1/500) were added (50 μL/well) for 2 h, plates were washed 4 times and reacted with 50 μL/well anti-human IgG horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) for 2 h. After washing, tetramethylbenzidine (TMB, Sigma–Aldrich) substrate dissolved in phosphate/citrate buffer was added (50 μL/well) for 10 min, the reaction was stopped with 25 μL 2 M H_2SO_4 and read at 450 nm. Total IgG was quantified in plates coated with goat anti-human IgG [0.5 μg/mL; Invitrogen, Australia] using with same protocol and greater plasma dilutions (1/18,000 and 1/54,000). The standard was purified human IgG [2-fold serial dilutions from 0.5 μg/mL; Sigma–Aldrich].

2.3. Statistical analysis

All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA). Non-parametric tests Mann–Whitney tests were used to compare results between NTM patients, family members and healthy controls. Spearman's non-parametric correlation tests were used to calculate correlation coefficients. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

The level of anti-IFN-γ autoantibodies was 1.3-fold higher in SGM patients than healthy controls ($p = 0.0016$; Fig. 1A), whilst levels were not elevated in the family members or *Mycobacterium abscessus* patients. Although NTM disease is more common in post-menopausal females [1,13], the level of anti-IFN-γ autoantibodies was similar in male and female patients ($p = 0.726$). Levels were not significantly affected by donor age in patients ($r = 0.17$, $p = 0.06$) or controls ($r = -0.17$, $p = 0.09$).

The level of anti-GM-CSF autoantibodies was also higher in SGM patients than healthy controls ($p = 0.003$) and patients with *Mycobacterium abscessus* ($p = 0.0006$; Fig. 1B). The level of anti-IFN-γ did not correlate with anti-GM-CSF autoantibodies in SGM patients ($r = -0.01$, $p = 0.91$; Fig. 1F) or healthy controls ($r = 0.11$, $p = 0.18$). Levels of anti-GM-CSF antibodies were not associated with age.

The level of total IgG was also higher in SGM patients than healthy controls ($p = 0.003$; Fig. 1C), whilst levels were not elevated in the family members or *Mycobacterium abscessus* patients. The level of total IgG did not correlate significantly with anti-IFN-γ autoantibodies in SGM patients ($r = 0.11$, $p = 0.21$; Fig. 1D) or patients with *Mycobacterium abscessus* ($r = 0.37$, $p = 0.24$) but there was a weak correlation in healthy controls ($r = 0.25$, $p = 0.003$). The level of total IgG correlated weakly with anti-GM-CSF autoantibodies in SGM patients ($r = 0.29$, $p = 0.001$; Fig. 1E) and healthy controls ($r = 0.28$, $p = 0.001$), but not in patients with *Mycobacterium abscessus* ($r = 0.08$, $p = 0.80$).

4. Discussion

Levels of autoantibodies to IFN-γ and GM-CSF, and total IgG, were higher in patients with pulmonary NTM than healthy controls. Increased levels of autoantibodies to IFN-γ have been described in smaller studies of Asian patients with disseminated NTM disease [14,15]. Patients in our study were mostly Caucasian and all had pulmonary disease.

Anti-IFN-γ autoantibodies were detectable in healthy Asian controls [7] and in controls in our study. One can surmise that these have no biological activity as they were not associated with any disease syndrome, though controls were not tested for subclinical infections [16,7]. Here the median levels of each antibody differed by only 1.3-fold between SGM patients and controls, and some healthy donors had high levels of autoantibody (Fig. 1A). These small differences and the wide individual variation between individuals in each group are equally compatible with anti-cytokine autoantibodies being a consequence of chronic NTM (or other) infections, rather than a predisposing factor. It may be relevant that levels of anti-IFN-γ autoantibodies were not higher in female patients or older patients – i.e. they do not provide a simple explanation for the greater susceptibility of older females evident from the makeup of this cohort and from the literature.

If levels of anti-IFN-γ autoantibodies were increased by the inflammatory environment created by NTM disease, then levels of anti-GM-CSF autoantibodies and total IgG (reflecting B-cell hyperactivity) would also be increased. Increases were evident in our cohort (Fig. 1B and C) but there was no correlation between levels of autoantibodies to IFN-γ and total IgG in patients with SGM, so the role of B-cell activation in the elevation of anti-IFN-γ autoantibodies may be minor.