Antibody to *Mycobacterium tuberculosis* 65 kDa heat shock protein in patients with rheumatoid arthritis—A survey of antigen-specific antibody isotypes and subclasses in an endemic area of previous tuberculosis infection

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**Abstract**

**Objective**—To clarify the significance of the humoral immune response triggered by the *Mycobacterium tuberculosis* (*M. tb*) 65 kDa heat shock protein (hsp) in the pathogenesis of rheumatoid arthritis (RA).

**Methods**—*M. tb* 65 kDa hsp-specific IgG, IgA, IgM, and IgG subclass antibodies in serum or synovial fluid (SF) of RA and other disease patients were determined by enzyme linked immunosorbent assay (ELISA).

**Results**—RA patients did not show any characteristic increase in mycobacterial 65 kDa hsp-specific antibodies compared with healthy individuals. In contrast, antigen-specific IgG and IgG2 antibody titres in the serum of RA patients were significantly lower than those of patients with tuberculosis and normal controls. In addition, there was also no significant difference in antibody titre between the serum and SF of RA patients, nor was any significant difference found between the SF of RA and Reiter’s patients.

**Conclusion**—The failure to detect a significant increase in IgG anti-*M. tb* 65 kDa hsp antibodies in RA patients does not exclude the possibility of microbial immunity in the aetiology of RA. Nevertheless, anti-*M. tb* 65 kDa hsp antibodies clearly do not appear to be the disease specific markers for RA and their relatively reduced concentrations may argue against their playing a major role in the disease pathogenesis.

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**Patients and methods**

**SERUM AND SYNOVIAL FLUID SAMPLES**

Serum, synovial fluid (SF), or both, were collected from 30 patients with RA, 15 with Reiter’s disease, and 15 patients with gouty arthritis. Twenty seven patients with pulmonary tuberculosis were also included in this study. Control sera were obtained from 30 healthy subjects who were age matched with RA patients.

**ANTIGENS AND MITOGEN**

*M. tb* colonies were collected from Löwenstein-Jensen slants. The sonicated cell lysate was suspended in water at 4°C to extract the protein and unrequired mycobacterial proteins were salted out with 30% ammonium sulphate. The 65 kDa proteins were further precipitated with 70% ammonium sulphate and concentrated with centricron-50 (Amicon). Use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis and Western blotting demonstrated that more than 95% of the prepared components comprised 65 kDa hsp (data not shown). The *Mycobacterium bovis* BCG recombinant 65 kDa hsp

Rheumatoid arthritis (RA) is believed to be an autoimmune disease associated with certain HLA class II alleles, local infiltration of lymphocytes, and the presence of autoantibodies. Its aetiology remains unknown. However, current evidence from an animal model of Lewis rat adjuvant arthritis and human RA suggests that T cells exhibit reactivity to the 65 kDa heat shock protein (hsp) of *Mycobacterium tuberculosis* (*M. tb*). The humoral immunity to *M. tb* 65 kDa hsp also has been evaluated in RA patients. Increased IgG reaction to *M. tb* 65 kDa hsp was claimed to be characteristic of patients with RA. There is little evidence, however, to suggest that *M. tb* 65 kDa hsp-specific antibodies elicit major autoimmune damage in RA. Furthermore, although antibodies to mycobacterial 65 kDa hsp were found to react with chondrocyte cytoplasmic constituents in RA and synovial lining cell of rat adjuvant arthritis and human RA11 data were insufficient to indicate that these antibodies were related to subsequent immunological damage.

Taiwan was an endemic area of pulmonary tuberculosis infection from 1960 to 1980. *Mycobacterium bovis* BCG vaccination is part of a routine schedule for prophylaxis after birth. The aim of this study was to evaluate the significance of the humoral immune response triggered by *M. tb* 65 kDa hsp in the pathogenesis of RA by comparing healthy individuals, patients with tuberculosis (TB), and other arthritic patients.
protein, a generous gift from Dr Ming-Yen Chung, was also used as an antigen.

MEASUREMENT OF ANTIBODY BINDING TO M. TB 65 KDA HSP
Antigen-specific antibodies of the IgG, IgA, and IgM isotypes and IgG subclasses in serum, SF, or both, were measured by enzyme linked immunosorbent assay (ELISA) using alkaline phosphatase-conjugated mouse anti-human Ig1, IgG2, IgG4, IgG, IgA, and IgM antibodies, at 1/500 dilution. The reaction was stopped after 30 minutes and the amount of bound enzyme assayed; absorbance values were read at 405 nm in an ELISA reader (Metertech, Σ960). Absorbance was calculated as the mean of triplicate well readings. In each experiment, 65 kDa antigen-specific monoclonal antibody was used as positive control. The optic density (OD) ratio was calculated as OD of the test samples divided by OD of a standard normal serum used in each plate. The control solution (phosphate buffered saline) never gave a value greater than 0-01. The standard deviations of the triplicate wells were all less than 10%.

In order to verify the validity of using OD values of IgG1, IgG2, and IgG4 to compare the antibody titres of tested samples, the OD values of alkaline phosphatase conjugated IgG1, IgG2, and IgG4 antibodies were plotted against a serial titration of known IgG1, IgG2 and IgG4 immunoglobulin concentrations (0·03–30 μg/ml).

In addition, the IgG2/IgG4 ratio was calculated for all groups of patients, to evaluate the humoral immune regulation associated with this antigen.

COMPARISON OF M. TB 65 KDA HSP-SPECIFIC ANTIBODIES IN THE SF AND SERUM OF RA PATIENTS
Because migration of antigen-specific lymphocytes into the synovial cavity has been suggested to be related to local inflammation in RA,13 we determined the isotypes and subclasses of the antigen-specific antibodies in the SF of RA patients.

COMPARISON OF M. TB 65 KDA HSP-SPECIFIC ANTIBODIES IN SF OF RA AND REITER’S PATIENTS
To verify the specificity of antibody production in the synovial cavity of RA patients, the anti-M. tb 65 kDa antibodies in the SF of Reiter’s disease patients were also determined.

STATISTICAL ANALYSIS
Statistical differences were sought by non-parametric group comparisons (Mann-Whitney U rank two tail test) processed and plotted with JMP2.0.5 software (SAS Institute, Inc.).

Results

M. TB 65 KDA HSP-SPECIFIC ANTIBODY ISOTYPES IN THE SERUM OF RA, OTHER DISEASE PATIENTS AND HEALTHY SUBJECTS
The mean concentration of IgG antibodies to the M. tb 65 kDa hsp was significantly greater than that of IgA and IgM isotypes in the serum of all tested individuals (fig 1A), and that in RA patients was significantly less than that in control subjects (p = 0·041) and TB patients (p = 0·032), but there was no significant difference of IgG binding to M. tb 65 kDa hsp in patients with gouty arthritis, Reiter’s disease or TB compared with control subjects. The concentration of IgA antibody in TB patients was greater than that of RA patients (p < 0·035) (fig 1B). Concentrations of IgM antibodies to M. tb 65 kDa hsp did not differ significantly between any groups (fig 1C).

IGG SUBCLASSES OF ANTI-M. TB 65 KDA HSP ANTIBODY IN THE SERUM OF PATIENTS AND HEALTHY SUBJECTS
The plot of immunoglobulin optical densities against known concentrations revealed a high correlation coefficient among the IgG subclasses (fig 2A).

Concentrations of antigen-specific IgG1 and IgG4 subclasses were much smaller than those of IgG2 in all groups of patients and normal controls (fig 2B). The mean concentration of IgG2 in RA patients was smaller than that in
patients with gouty arthritis or TB, and in normal subjects, but differences were significant only between RA and TB patients (p = 0.036) and RA patients and normal controls (p = 0.048).

The antigen-specific IgG2:IgG4 ratio in the sera of RA patients was significantly less than that of TB patients (p = 0.014), as was that of patients with Reiter’s disease (p = 0.021). In contrast, the IgG2:IgG4 ratios in TB patients were not significantly different from those of normal individuals or GA patients. The IgG2:IgG4 ratio in Reiter’s patients was not significantly different from that of RA patients. Figure 2C shows the IgG2:IgG4 ratios in the groups studied.

COMPARISON OF M. TB 65 KDA HSP-SPECIFIC ANTIBODIES IN SF AND SERUM OF RA PATIENTS
No significant difference in occurrence of antigen-specific Ig isotypes, IgG subclasses, or IgG2:IgG4 ratio was found between the SF and serum of these patients (fig 3A).

COMPARISON OF M. TB 65 KDA HSP-SPECIFIC ANTIBODIES IN SF OF RA AND REITER’S PATIENTS
M. tb 65 kDa hsp-specific antibodies were present in SF of Reiter’s disease patients in concentrations which were not significantly different from those in RA patients (fig 3B). The IgG2:IgG4 ratios in SF of RA and Reiter’s patients were not significantly different (fig 3B).

Discussion
Increased concentrations of IgG5-7 and IgA antibodies to the M. tb 65 kDa hsp protein have been found to be characteristic of patients with RA, being nearly three times those in the sera of normal subjects.7 Moreover, in one of these studies, the titres of antibodies in RA patients were significantly greater than those of tuberculosis patients.5 Our current study has not shown any significant increase in these antibodies in RA patients compared with TB patients and healthy individuals. The reason for this discrepancy is unknown, but it may be significant that, in Taiwan, M. bovis BCG vaccination was part of a routine prophylactic schedule after birth; whether BCG vaccination influences the concentration of anti-M. tb 65 kDa hsp antibody is uncertain. Nevertheless, the presence of this antibody in healthy individuals does not support a direct role for anti-M. tb 65 kDa hsp antibodies in the pathogenesis of RA.

The IgG1 fraction of the M. tb 65 kDa hsp-specific antibody was scarce, although 60–65%

Figure 2 Comparison of concentrations of mycobacterial 65 kDa hsp-specific IgG subclasses (IgG1, IgG2, IgG4—1, 2, 4) of antibodies in the serum of patients with rheumatoid arthritis (RA) (n = 30), gouty arthritis (GA) (n = 15), Reiter’s disease (RD) (n = 15), or tuberculosis (TB) (n = 27), and normal controls (C) (n = 30). A: Plot of alkaline phosphatase conjugated IgG1 (●), IgG2 (○), and IgG4 (□) antibodies against known concentrations of IgG1, IgG2 and IgG4 immunoglobulin concentration (0.03–30 μg/ml). B: Subclasses of antibody reactive to the mycobacterial 65 kDa hsp in patients and normal controls. Horizontal lines indicate mean OD ratio. C: Antigen-specific IgG2:IgG4 ratios in patients and healthy controls. Horizontal lines indicate means; diamonds enclose means with 95% confidence intervals.

Figure 3 Comparison of anti-mycobacterial 65 kDa hsp antibody isotypes and IgG subclasses: A—in the synovial fluid (SF) and serum (S) of patients with rheumatoid arthritis (RA) (n = 15); B—in the SF of RA (n = 10) and Reiter’s disease (RD) patients (n = 10). Ratio = IgG2:IgG4 ratio.
of the total IgG pool may be expected to comprise IgG1.14 Furthermore, there was no significant difference between peripheral and synovial anti-M. tb 65 kDa hsp antibody titres in RA patients, nor was any significant difference found between the concentrations in SF of RA and Reiter’s patients. This would appear to indicate no specificity or local synthesis of these antibodies in the synovial cavity of RA patients. The IgG2:IgG4 ratio in TB patients was significantly greater than that of RA and Reiter’s disease patients, mainly because of the significant increase in IgG2 concentrations in TB patients.

Decreased titres and frequencies of anti-M. tb 65 kDa IgG and IgG2 antibodies in RA patients were found in this study, but the reason for this is unknown. Disease activity or drug effects may not be implicated, because polyclonal hypergammaglobulinaemia and increased rheumatoid factor were present in RA patients compared with healthy individuals. In addition, antibody titres appeared not to be associated with disease severity (mild, moderate, or severe degree) in RA patients or with clinical pattern (fibrosis, cavity formation, and miliary dissemination) in TB patients (data not shown). Decreased titres of IgG2 antibodies resulting from the formation of immune complexes between IgG2 anti-M. tb 65 kDa antibodies and the 65 kDa hsp antigen are also unlikely.15

In conclusion, neither high titres nor specific IgG subclass production of anti-M. tb 65 kDa hsp antibodies was observed in RA patients compared with patients with other diseases and healthy controls. Our failure to detect significant increases in anti-M. tb 65 kDa hsp antibodies in RA should not be interpreted as excluding the possibility of microbial immunity in the aetiology of RA, but anti-M. tb 65 kDa hsp antibodies clearly do not appear to be disease specific markers for RA and their relatively lower concentration is evidence against their playing a major role in the disease pathogenesis of RA.

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