Increased serum anti-mycobacterial antibody titers in rheumatoid arthritis patients

Is there any specific antigenic target?

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ABSTRACT

Objectives: To investigate the presence of immunoreactivity against mycobacterial antigens in the sera of patients with rheumatoid arthritis (RA) and to detect the target of the immune reaction.

Methods: This study was carried out on 60 patients with RA, and 25 patients with no joint diseases in the laboratory of Clinical Microbiology Department of Ankara University Medical Faculty, Ankara, Turkey between July 2003 to January 2004. Secreted and cellular antigens of *Mycobacterium tuberculosis (M. tuberculosis)* H37Rv and *Mycobacterium bovis (M. bovis)* were isolated and purified by high performance liquid chromatography to antigenic fractions. The immunoreactivity of patient and control sera against these antigens were determined by enzyme-linked immunosorbent assay (ELISA).

Results: Immunoreactivity against mycobacterial antigens in RA patients were significantly higher than controls. Significant difference between patients and controls has been determined with *M. bovis* Bacillus Calmette Guerin (BCG) culture fluid and sonicate antigens, but not with *M. tuberculosis* H37Rv. This suggests that the antigen triggering immune response in patients with RA may belong to or mainly expressed on *M. bovis* BCG. The ELISA results showed significant difference between RA patients and controls with all antigenic fractions.

Conclusion: Presence of increased immunoreactivity against mycobacterial antigens in the sera of patients with RA was detected. When statistical analyses was considered, we cannot put forward any antigenic fraction alone as the one responsible for the increased reactivity.

Saudi Med J 2007; Vol. 28 (5): 717-722

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Received 31st October 2006. Accepted 31st December 2006.

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Genetic, autoimmune, infectious and non-infectious environmental factors have been proposed to be involved in the pathogenesis of inflammatory joint diseases.[1] In the multifactorial etiology of rheumatic diseases, infectious agents are regarded as the major environmental factors that may cause inflammatory arthritides in genetically susceptible hosts.[2]

Rheumatoid arthritis (RA) is an immunological disorder of unknown etiology characterized with chronic, recurrent polyarthritis. It is characterized by synovitis, largely of the peripheral joints, erosion of articular cartilage and subarticular bone, and ultimately ankylosis.[3] Immune system is generally considered to be central to the pathogenesis of RA, so the disease is often classified as autoimmune. The immune system's function is the recognition of antigens, so it is natural to ask which are the critical antigens that drive the pathologic immune response in RA. There are 2 main categories of antigens to be considered; autoantigens [type II collagen, proteoglycans, chondrocyte antigens, heat shock proteins (hsp), and immunoglobulins) and foreign antigens (bacterial antigens, viral antigens, superantigens].[4] Many immunologists still believe that RA may be an autoimmune disease triggered by structural mimicry between antigens in the environment and self antigens of the individual. Cartilage proteoglycan was suggested to be the self-antigen responsible for this effect.[5] Despite much research effort, so far it has not been possible to determine any autoantigen responsible for the pathogenesis of RA alone.

The possibility of a relationship with mycobacterial infections, especially *Mycobacterium tuberculosis (M. tuberculosis)*, have been proposed in the etiology of RA, and several recent data suggested a role for mycobacteria in these diseases.[6-8] In the past, RA-like symptoms were reported to occur in certain tuberculosis patients; arthritic symptoms have been described in some patients receiving repeated Bacillus Calmette Guerin (BCG) immunotherapy for cancer.[6] A change in the pattern of glycosylation of IgG (agalactosyl IgG) found in RA was also found in tuberculosis patients. The *M. tuberculosis* protein antigens have been detected in synovial fluid of RA patients. The cross reaction between mycobacterial antigens and host synovial antigens was shown.[7] Significant higher levels of IgG and IgA antibodies to 65 kDa protein of *M. tuberculosis* have been found in RA patients compared with controls. In genetically susceptible rats, injection of *M. tuberculosis* can induce autoimmune arthritis. A 65 kDa protein from *M. tuberculosis* has been shown to have a proliferative response on T cells from Lewis rats with adjuvant-induced arthritis.[8]

Protein antigens of *M. tuberculosis* are known to have an important role in inducing infection. For this reason, great effort has been placed on the characterization of these antigens and immune response against them. With the studies carried out to characterize the antigenic proteins of mycobacteria, several prominent protein antigens involved in antibody and T cell responses to mycobacteria have been identified as members of highly conserved heat shock protein families.[9,10]

The detection of the association between immunodominant antigens of mycobacteria and autoimmunity has revealed an elevated interest on the potential role of these bacteria in the development of inflammatory joint diseases. The cross reactivity between mycobacterial and host's own stress proteins has been considered as a possible mechanism of the disease.[11] Unfortunately, the slow growth and the difficulties of the isolation of mycobacteria makes it hard to clarify their role in the pathogenesis of the diseases.

In this study, we investigated the presence of immunoreactivity against mycobacterial antigens in the sera of patients with RA and we compared the antibody levels in these patients with control patients who have no history of joint disease. The second aim of this study was to determine the target of the immune reaction detected in patients with RA. For this reason *M. tuberculosis* H37Rv and *M. bovis* BCG strains were cultured in liquid Sauton medium and their secreted and cellular antigens were isolated, purified with high performance liquid chromatography (HPLC) to different antigenic fractions and the immunoreactivity of patient and control sera with these antigens were determined with enzyme-linked immunosorbent assay (ELISA) plates coated with antigenic fractions obtained with HPLC. Especially the difference of immune reaction of patients from controls and the target of this reactivity was investigated.

Methods. This study was carried out in the laboratory of Microbiology and Clinical Microbiology Department of Ankara University Medical Faculty, Ankara, Turkey between July 2003 and January 2004. It was carried out on 60 patients (49 females and 11 males, mean age 53 ± 10.9 years) with RA, as defined by the 1987 revised American Rheumatism Association criteria,[12] attending the outpatient clinics of Ankara Physical Medicine and Rehabilitation Center and Rheumatoid Arthritis Outpatient Clinics of Ankara University Medicine Faculty Ibn-i Sina Hospital and 25 patients (13 females and 12 males, mean age 38 ± 9.4 years) with no joint diseases. The patients in the control group were all C-reactive protein and rheumatoid factor negative. Forty-five of the 60 RA patients and all of controls were immunized with BCG vaccine at least 5 years ago, and none of the individuals included in this study reported tuberculosis infection in their medical history. Informed consent was obtained from all patients, and the study procedures were performed in accordance with the principles of the declaration of Helsinki.[13] Sera were stored at -70°C until use.

Mycobacterium tuberculosis H37Rv and *M. bovis* BCG strains were supplied by Tuberculosis Research Department of Refik Saydam Hygiene Center, Ankara. The strains were cultured on Löwenstein-Jensen medium first, then subcultured to 500 ml Sauton liquid medium and incubated for 8 weeks at 37°C.

Culture fluid (cf) was collected with a pipette carefully not to damage the membrane formed on the surface of the medium, and centrifuged at 3500 rpm for 30 minutes. Filtered with paper filters (Whatman No: 1) to purify from big particles, and then filtered with 0.22 µm pore sized membrane filters and distributed to sterile screw capped tubes.

After the collection of bacterial culture from the surface of the medium the bacteria were firstly washed 3 times with phosphate buffered saline (PBS). After the

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last washing they were suspended in PBS and stored at +4°C until sonification. Before sonification bacteria were freezed to -85°C for 30 minutes and after thawed 60 milligrams (wet weight) of bacilli per milliliter was sonified for 20 min with a Branson Sonic Power with an 80-W effect under cooling on ice. The sonic extract was centrifuged at 3500 rpm for 20 minutes at +4°C and supernatant was collected in sterile screw capped tubes.

Protein concentrations of culture fluid and sonicates were determined with Folin-Lowry method[14] and total protein concentrations were calculated. The samples were lyophilizated and stored at +4°C until use.

Mycobacterium tuberculosis H37Rv and *M. bovis* BCG culture filtrates (H37Rv-cf and BCG-cf respectively), *M. bovis* BCG sonicates (BCG-son) were purified to antigenic fractions by HPLC (Hewlett Packard series 1100) with a gel filtration colon (TSK-GEL G3000 SW, separation range: 10.000 – 500.000 Dalton) according to their molecular weights. Mobile phase used was 30 mM Tris-HCL + 0.1 M NaCl, pH 7.5. The flow rate was 3 ml/minute, UV detector was arranged to 280 nm and the antigenic fractions were collected with 30 second intervals (in 1.5 ml volumes). The same chromatographic conditions were carried out for all samples. Lyophilized BCG-cf, BCG-son and H37Rv-cf were diluted with sterile distilled water to get a protein concentration of 2 mg/ml. The samples were filtrated with 0.22 µm pore sized membrane filters after centrifugation for 10 minutes at 12000 rpm and then applied to HPLC with 1 ml volume. Protein concentrations of considerable peaks detected on the chromatograms were calculated with Folin-Lowry method.

All antigenic proteins were prepared in carbonate-bicarbonate buffer solution (0.05 M; pH 9.6). The H37Rv-cf, BCG-cf, H37Rv-son and BCG-son were coated onto 96 well flat-bottomed microtiter plates (Greiner) at 50 µg/ml concentration in carbonate-bicarbonate buffer solution (0.05 M; pH 9.6). On the second step ELISAs, the antigenic fractions obtained with HPLC were coated at 10 µg/ml concentration and incubated overnight at +4°C. Excess antigen was washed off twice with washing buffer (0.1 M phosphate-buffered saline with 0.05 % Tween 20(PBS-T); pH 7.4), then post-coated with 100 µl per well 5% bovine serum albumin (BSA) in PBS-T at 37°C for 1 hour, followed by a further 4 washes. One hundred microliters of the test and control sera were added in 1/100 dilution in PBS-T, and the plates incubated at 37°C for 1 hour. Four further washes were followed by 100 µl/well horse radish peroxidase-conjugated anti-human IgG (SIGMA) diluted 1/2000 in PBS-T with 0.5% BSA, at 37°C for 30 minutes. After this incubation period plates were washed again 4 times and then 100 µl/well 3,3',5,5'-Tetramethyl-benzidine liquid substrate (SIGMA) was added onto each well. After 25-30 minutes color development was stopped with 50 µl/well 3M H_2SO_4 and assessed at 450 nm, using an ELISA reader (Spectra max plus 384).

To determine the molecular weight (MW) of antigenic fractions used in this study a molecular weight standard consisting of 212 kDa, 122 kDa, 83 kDa, 52 kDa, 35 kDa, 28 kDa, 20 kDa and 7.2 kDa proteins was applied to HPLC. The relationship between MW of the protein and its Ve (The volume that a particular protein elutes from a column is called the elution volume) is given by the following equation;[15] $Ve = \alpha \ln (MW) + \beta$.

In this equation α and β are constants obtained by the elution volume of proteins with known sizes (α=-0.515 and β=7.231). So the formula we used was:

$$Ve = 7.231 - 0.515 \ln (MW).$$

Comparison in groups were made with Friedman test and comparisons between groups were made with Kruskal Wallis Test. When significant difference was detected with these tests Scheffe multiple comparison test was used to determine where the difference is between.[16,17]

Results. Patients with RA showed significant higher optical absorbance (OpA) values with BCG-cf (p<0.01) and BCG-son (p<0.05) when compared with controls, however, OpA values with Rv-cf and Rv-son showed no difference between the 2 groups (p>0.05) (**Figure 1**).

When comparisons in groups were made, we determined that control group showed significant lower OpA values with BCG-cf (p<0.001) when compared with BCG-son, Rv-cf and Rv-son while there were no significant difference between BCG-son, Rv-cf and Rv-son (p>0.05) (**Figure 2**). The OpA values of patients with

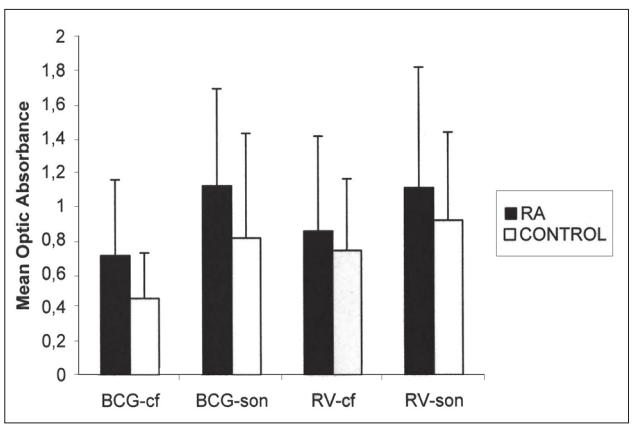

Figure 1 - Comparison of OpA values of patient and control groups with BCG-cf, BCG-son, Rv-cf, and Rv-son (comparison between groups). OpA - optical absorbance, RA - rheumatoid arthritis, BCG - bacillus Calmette Guerin, cf - culture fluid son - sonicates, RV - *Mycobacterium Tuberculosis* H37Rv

RA were significantly higher with BCG-son and Rv-son when compared with BCG-cf and Rv-cf (p<0.001; p<0.01 versus p<0.001; p<0.01) while there were no significant difference between BCG-son and Rv-son. In addition, their OpA with Rv-cf was significantly higher than BCG-cf (p<0.001). Briefly, OpA values of patients with RA were significantly higher with sonicate antigens (**Figure 2**). Comparisons between groups showed that discrimination of patients from controls were easier with antigenic proteins of BCG.

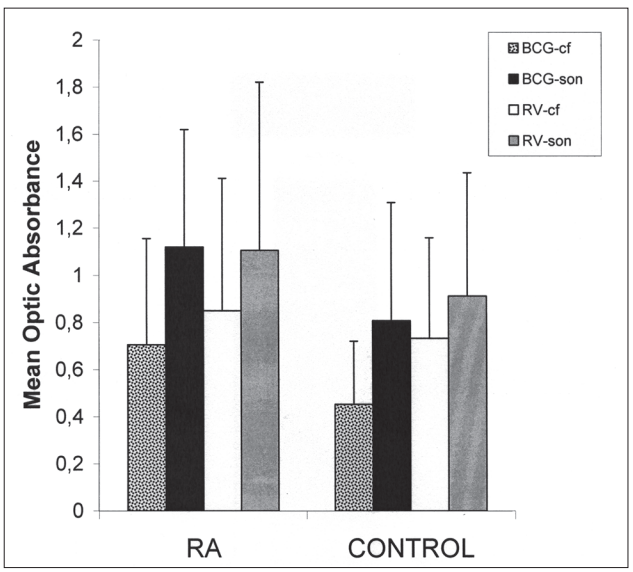

Figure 2 - Comparison of OpA values with BCG-cf, BCG-son, Rv-cf and Rv-son in each patient group (comparison in groups). OpA - optical absorbance, RA - rheumatoid arthritis, BCG - bacillus Calmette Guerin, cf - culture fluid son - sonicates, RV - *Mycobacterium Tuberculosis* H37Rv.

On the chromatograms of BCG-cf and Rv-cf there were 2 different peaks seen at 22 and 24 minutes, which were not detected on the chromatogram of BCG-son. Other peaks of BCG-cf and Rv-cf were common with BCG-son. So we studied with these fractions besides fractions detected on the chromatogram of BCG-son.

Mean absorbance plus 2 SD of control group were considered as the cut-off value and sera of RA patients with higher values were used on the second step of this study to determine from which antigenic fraction the higher optic absorbance values are arising from. For this purpose, we carried out a serial of ELISA assays with the antigenic fractions obtained by HPLC with these RA patients and control group. After the ELISA assays OpA values of each group with the antigenic fractions used were compared with each other. With all antigens tested, except BCG-son #78 (p>0.05), there was significant difference between RA patients and controls (p<0.001) (**Figure 3**).

When compared with the other antigenic fractions, higher levels of mean optic density were detected with BCG-son #28, #31, #36, #41, #60 and #62 with both RA patients and normal controls. In addition, in RA patients, we detected significant higher OpA levels with Rv-cf #45, but not in the control sera.

Calculated MW values of the antigenic fractions used in ELISA assays according to the equation Ve = 7.231 – 0.515 ln (MW) were 174 for BCG-son #28; 141 for BCG-son #31; 99 for BCG-son #36; 69 for BCG-son #41; 18 for BCG-son #60; 16 for BCG-son #62; 12 for BCG-son #66; 8 for BCG-son #71; 6 for BCG-son #75; 5 for BCG-son #78; 52 for Rv-cf #45 and 37 for Rv-cf #50. After the analysis, 83.3% (R^2=0.833) of the variation in Ve was determined to be explained with MW.

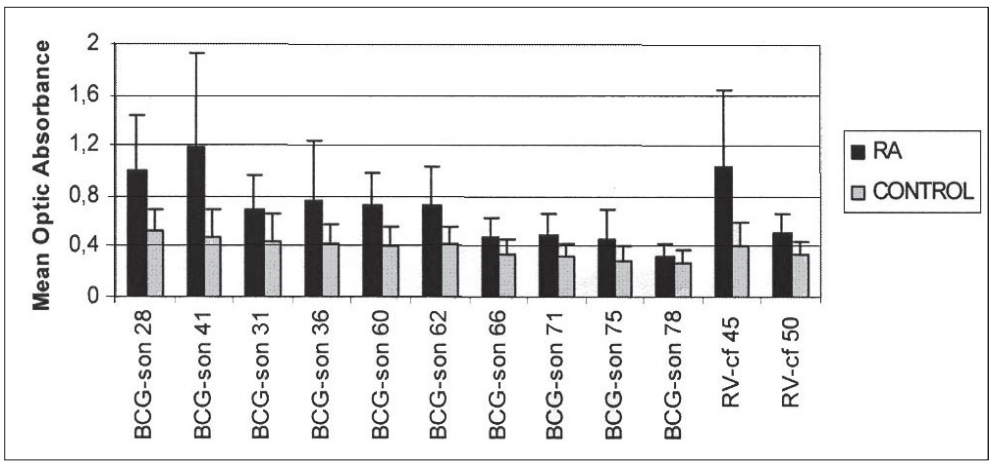

Figure 3 - Comparison of OpA values of RA patients and controls with the antigenic fractions obtained by chromatography (comparison between groups). OpA - optical absorbance, RA - rheumatoid arthritis, BCG - bacillus Calmette Guerin, cf - culture fluid son - sonicates, RV - *Mycobacterium Tuberculosis* H37Rv.

Discussion. Several evidences have been obtained in many studies suggesting a role for mycobacteria in the pathogenesis of RA. However, since attempts to isolate mycobacteria from joints of RA patients have failed in the past, their role in the etiopathogenesis of these diseases have not yet been clarified. Despite these findings, the possibility of *Mycobacterium* involvement in RA cannot be completely ruled out. Tsoulfa et al[18] demonstrated significantly raised IgA and IgG antibody levels to the mycobacterial 65 kDa HSP in the sera of patients with RA. In another study, the investigators attempted to determine whether this phenomenon is specific for RA, and whether it is seen only with the mycobacterial homologue of this particular HSP gene family. They therefore, screened antibody levels to the mycobacterial and *Escherichia coli (E. coli)* HSP65, and the mycobacterial, *E. coli*, and human HSP70, in the sera from RA patients, patients with systemic lupus erythematosus, tuberculosis and Crohn's disease, and control donors. The RA sera showed the greatest increase in IgA binding to the mycobacterial HSP65, but no increase in IgA binding to the *E. coli* homologue. The RA sera showed increased IgG binding to the mycobacterial HSP65, but no increase to the *E. coli* homologue. Thus, they suggested that elevated IgG antibody levels to the mycobacterial HSP65 shows some disease specificity.[3]

Detection of *M. tuberculosis* antigens or antibodies against these antigens in synovial fluid or synovial tissue has been proposed to be important for the pathogenesis of RA.[19] However, Lydyard et al[6] have investigated the IgG and IgA levels in both synovial fluid and serum of 23 RA patients, and reported that antibody levels were low in the synovial fluid compared with serum. For this reason in our study we preferred to study with serum instead of synovial fluid.

According to the data obtained on the first step of our study, patients with RA seem to have significant higher levels of antibodies to mycobacterial antigens when compared with controls. The increased levels of antibodies in RA patients when compared with controls show correlation with previous studies.[3,18,20]

When the reactivity of sera from patients with RA, and controls with BCG-cf, BCG-son, Rv-cf and Rv-son were assessed, significant difference between patients and controls has been determined with BCG-cf and BCG-son, but not with Rv-cf and Rv-son. This data allowed us to suggest that the antigen triggering the immune response in patients with inflammatory arthritis may belong to *M. bovis* or it may mainly be expressed on this microorganism.

The BCG immunization status of the patients is thought to be a factor, which may have influence on immune reaction of the patients to mycobacterial antigens. The fact that all controls and most of the patients were BCG positive and the OpA values were significantly higher in RA patients makes us think that there was a different immune reaction against the same antigen. If BCG immunization was effective alone then there should be higher OpA values in control patients also. Thus, we consider that other factors influencing the immune reaction against this antigen (genetic, environmental factors, and others) exist.

The ELISA results of the first step of this study showed that patients with RA showed significantly higher OpA values than controls. When comparisons in groups were made, we detected that sera of RA patients and controls showed significant difference between different antigenic fractions.

On the second step of this study, ELISA results showed significant difference between RA patients and controls with all antigenic fractions ($p<0.001$), (not with BCG-son#78). When compared with the other antigenic fractions, higher levels of mean optic density were detected with BCG-son #28, #31, #36, #41, #60 and #62 in both RA patients and normal controls and significant difference has been detected between OpA values of RA and control patients. Even though the mean optic densities obtained with the other antigens were lower, these antigens (not BCG-son #78) also showed significant differences between RA patients and normal controls. For this reason these results do not indicate any antigen to be an ideal candidate responsible of the increased reactivity. In addition, on the first step of the study, we determined that there is no significant difference between patient and normal controls with Rv-cf, but in the second step, we purified this antigen with HPLC and when we studied with each antigenic fraction obtained with HPLC, we have seen that patients with RA showed significant higher mean optic density on ELISA with fraction 45. This might be due to the low level of this fraction in the total antigen of Rv-cf, so further studies must be carried on with *M. tuberculosis* H37Rv culture fluid beside *M. bovis* BCG.

With the analyses of HPLC, which was performed with MW standard to determine the MW of antigen fractions used in ELISA, fraction 41, which showed the higher reactivity with RA patients was determined as a protein of approximately 69 kDa. The method we used to determine the MW was not 100% accurate, so we suggest that this 69 kDa protein may represent mycobacterial 65 kDa heat shock protein. This finding shows correlation with several studies indicating an increased reactivity to mycobacterial 65 kDa heat shock protein.[3,5,18]

In conclusion, presence of increased immuno-reactivity against mycobacterial antigens in the sera of patients with RA support the role of mycobacteria in the

pathogenesis of RA. However, when statistical analyses are considered, we see that we cannot put forward any antigenic fraction alone as the one responsible for the increased reactivity in the sera of patients with inflammatory arthritis. Further studies are needed to clarify their role in the pathogenesis and the underlying target of the immune reaction.

Acknowledgment. *The authors wish to thank Associate Professor Dr. Atilla Elhan for excellent statistical comments on the study.*

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