DETECTION OF DNAJ (HEAT SHOCK PROTEIN) AND OTHER MYCOBACTERIAL RELATED ANTIGENS IN RHEUMATOID ARTHRITIS USING ELISA

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SUMMARY: There is strong evidence of an immune response to antigens found in mycobacteria in rheumatoid arthritis (RA) patients. We report here the presence of antigens strongly associated with mycobacteria in serum immune complexes of RA patients. A significant level of mycobacterial antigen was detected in RA patients' serum immune complexes and also in synovial fluids by both polyclonal anti-M. tuberculosis and monoclonal anti-DnaJ antibodies. We have found high levels of mycobacterial antigens using polyclonal antibodies in early stages of RA which however is reduced in late stages of the disease. While reverse situation was noted for DnaJ related antigen, using anti-DnaJ m-Ab, where low levels were detected in early RA they were comparatively high in late RA cases. Possibly in early infection, mycobacteria are involved and subsequently immune system restricts the multiplication of mycobacteria. However, during infection both host and pathogen undergo stress/heat shock resulting in heat shock protein production. The immune response to DnaJ or related antigens of pathogen and other bacteria may cross-react with homologous regions of host protein, and may induce an autoimmune disease.

Key Words: Rheumatoid arthritis, Mycobacterium tuberculosis, Heat-shock protein DnaJ, HLA-DR4, Autoimmune disease, ELISA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, recurrent, systemic inflammatory disease primarily involving joints and which predominantly occurs in HLA DR4 subjects. The cause of the RA is unknown. It is believed to be an immunological disease, possibly with an autoimmune etiology. Like most autoimmune diseases, RA is thought to be caused by an interaction between constitutional and environmental (possibly microbial) factors in genetically susceptible hosts (1,2).

Mycobacterium tuberculosis was found to be a cause of some cases of infective arthritis. This has

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Table 1: Rheumatoid arthritis patients.

	TB patient	Rheumatoid Factor			Race			X-Ray Erosion		
		RF+	RF-	NT *	Asian	Caucasian (Cau.)	Afro-Caribbean	Y	N	NT*
Early (27)	00	17	10	-	03	21	03	08	09	10
Late (68)	03 (Cau.)	54	11	03	06	58	04	56	04	08
Total (95)	03	71	21	03	09	79	07	64	13	18

NT*: Not Tested.

suggested a possible role of *M. Tuberculosis* also in RA (3). It has also been suggested that there are critical *M. tuberculosis* antigens which contain an epitope cross-reactive with self antigen in joint cartilage (4). Recently *M. tuberculosis* antigens have been detected in synovial fluid of RA patients (5). Rheumatoid arthritis patients demonstrated specific T-lymphocytes reactive to a *M. tuberculosis* antigen containing the cross-reactive epitope (6).

An immunological response to bacterial heat shock protein has been implicated in the pathogenesis of arthritis in animals and humans. Autoantibodies to a number of stress proteins have been identified in SLE and Rheumatoid arthritis (7). A role for the 65 kD heat shock protein in the immunopathological process of arthritic disease has been suggested by many workers (8,9). Elevated levels of IgG antibodies to the 65 kD hsp of mycobacteria have been shown to be a characteristic of rheumatoid arthritis patients (10,11).

M. tuberculosis is found to contain the dnaK 70 kD heat shock protein along with two other associated heat shock proteins, GrpE and DnaJ. Albani *et. al.* found that the amino acid sequence of DnaJ of E. coli contains an 11-amino acid run that is homologous to part of the third hypervariable region of human (HLA)DRB*10401 (formally known as HLA Dw4) (12). This raises the possibility that induction of an antibody and/or T cell response to DnaJ, which is a stress protein of many bacteria, might be implicated in an autoimmune process in which DRB 10401 is involved. This haplotype is strongly associated with severe, erosive forms of RA.

We report here the presence of Mycobacterial particularly DnaJ related antigens in serum and synovial fluid immune complexes of rheumatoid arthritis patients. These patients generally have high levels of circulating immune complexes (13,14).

MATERIALS AND METHODS

The presence of mycobacterial antigens and DnaJ related antigens in the serum and synovial fluid immune complexes of rheumatoid arthritis patients were studied by ELISA using rabbit anti-*M. tuberculosis* H_{37}Rv IgG and anti-DnaJ m-Ab.

Patients

The study was carried out on ninety five patients with rheumatoid arthritis determined using the American Rheumatism Association criteria (15). These were patients attending clinics at Dudley Road Hospital, Birmingham, and Walsgrave Hospital, Coventry.

The patients entering this trial were stratified according to RF seropositivity, race and bone erosion and according to whether the disease was in an early or late onset category (Table 1). Most subjects were RF-positive Caucasians with bone erosion; about two-thirds had late onset disease. Three of these (Caucasians) had concurrent tuberculosis.

Table 2: Normal and disease controls.

Groups	Total Number
Healthy controls	70
Disease controls (other than RA)	29
Systemic Lupus Erythematosus (SLE)	40

Table 3: Antigen-capture ELISA for mycobacterial antigens in serum immune complexes of rheumatoid arthritis patients and controls. Ranges, means and statistical analysis of data.

Antibody	No	Range	Mean ± Standard Deviation	Standard Error	p value		
Polyclonal (Rb. anti-*M. Tuberculosis*)					HC	DC	SLE
Healthy cont.	70	0.021-0.907	0.1434 ± 0.1590	0.0190	-	0.089	<0.01
Disease cont.	29	0.000-0.201	0.0634 ± 0.0700	0.0130	0.089	-	0.014
SLE	40	0.000-2.0	0.2336 ± 0.5154	0.0815	<0.01	>0.014	-
Early RA	27	0.046-1.595	0.5618 ± 0.5688	0.1094	<0.0001	<0.0001	<0.00
Late RA	68	0.008->2.0	0.5238 ± 0.5722	0.0693	<0.0001	<0.0001	<0.00
Total RA	95	0.008->2.0	0.5428 ± 0.5410	0.0656	<0.0001	<0.0001	<0.00

HC : Healthy control
DC : Disease control
SLE : Systemic Lupus Erythmatosus

Normal and disease controls

Ten healthy Laboratory staff (in-house controls with no history of rheumatoid arthritis) and sixty samples from the Regional Blood Transfusion Centre (external controls, presumed negative) were also included in the study to compare with the patients' results. Twenty nine patients' serum samples, other than rheumatoid arthritis and forty SLE patients' samples were also studied as a disease control group (Table 2).

Antibodies

The anti-*M. tuberculosis* H_{37}Rv, raised in a rabbit against *M. tuberculosis* H_{37}Rv, was gifted by Professor Talwar. Antibodies were purified and conjugated with HRP in our Laboratory. Anti-DnaJ m-Ab was developed against *M. tuberculosis* recombinant DnaJ molecule in our Laboratory and was found to cross-react with DnaJ of other bacteria (data not shown).

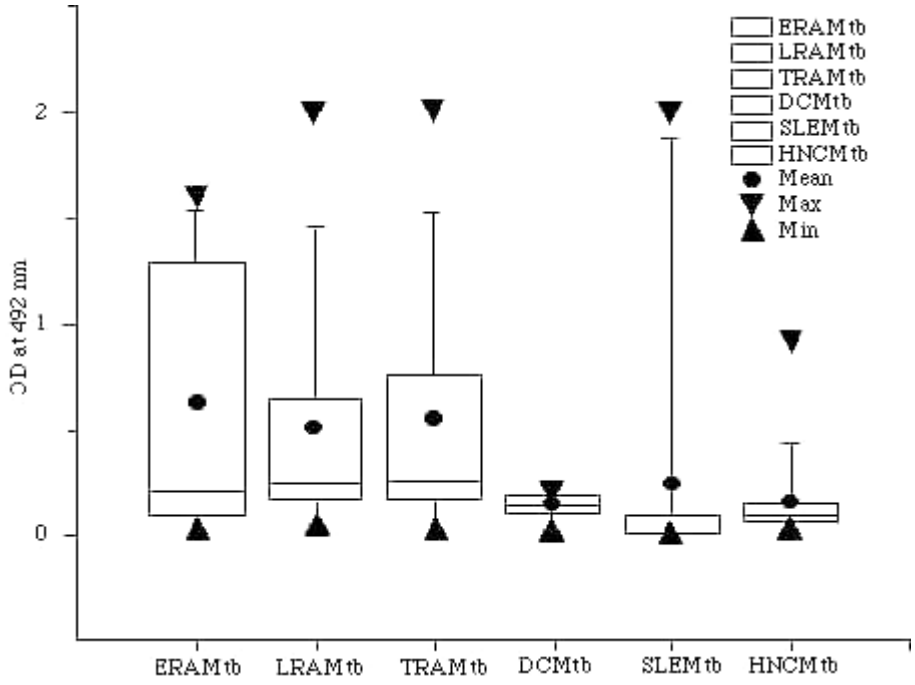
Preparation of immune complexes

The serum immune complexes were precipitated with 2% (w/v) polyethyleneglycol 6000 (PEG) overnight and washed in PEG buffer at 4°C. They are dissolved in veronal buffer. Synovial fluids were similarly treated with PEG to precipitate the immune complexes.

ELISA protocol for detection of mycobacterial antigens in the immune complexes

96 well plates (Nunc Gibco) were coated with 100 μ l/well of 5 μ g/ml of DEAE purified rabbit anti-*M. tuberculosis* H_{37}Rv IgG in coating buffer pH 9.6 and incubated overnight at 4°C. Excess antibodies were washed off with washing buffer. The dissolved immune complexes were mixed with heat-aggregated human IgG (prepared by heating human purified IgG 1 mg/ml at 63°C for 16-20 minutes) to block any rheumatoid factor (RF) activity and 100 μ l were added in duplicate to the antibody-coated plates. The wells were incubated at 37°C for one hour and then washed with washing buffer. 100 μ l of the rabbit anti-*M. tuberculosis* H_{37}Rv-IgG. HRP conjugate (1:4000) in diluting buffer with heat-aggregated human IgG was added, to sandwich the antigen onto the plates, and incubated at 37°C for one hour. Plates were then washed with washing buffer.The presence of bound antibody-conjugate was detected by adding 100 μ l of orthophenylene diamine (OPD) (10 mg OPD/25 ml of the subsrate buffer pH 5.0 with 25 μ l of 30% H_2O_2) to each well. The reaction was allowed to proceed at 37°C for 30 minutes in the dark. The reaction was stopped by adding 50 μ l of 20% (v/v) H_2SO_4 to each well. The plates were read on an ELISA reader(Labsystem Multiskan® MCC) at 492 nm.

Figure 1: Distribution of Mycobacterial antigens in serum immune complexes of rheumatoid arthritis patients and controls.



ERA=Early rheumatoid arthritis
LRA=Late rheumatoid arthritis
Mtb=*M. tuberculosis* antigens
TRA=Total rheumatoid arthritis
DC=Disease controls (other than RA)
SLE=Systemic lupus erythmatosus
HNC=Healthy normal controls

In Figure 1, the single or upper box represents the distribution of OD values in which 50-75% of the subjects lie. The lower box is the 25-50% distribution.

Detection of DnaJ related antigens in serum and synovial immune complexes

For detection of DnaJ related antigens in the serum and synovial immune complexes, 96 well plates (Nunc: Gibco) were coated with

25 μ l of the immune complexes in coating buffer pH 9.6 and incubated overnight at 4°C. Plates were washed with washing buffer to remove unadsorbed antigens. 100 μ l of 5 μ g/ml of purified anti-DnaJ m-Ab and 10 μ g/ml of purified human heat-aggregated IgG was added to each well and incubated at 37°C for one hour, and then, after washing, sheep anti-mouse IgG HRP (1:8000), unreactive with human immunoglobulins, in diluting buffer was applied. After washing, 100 μ l of OPD substrate was added and incubated at 37°C for 30 minutes. The reaction was stopped by adding 50 μ l of 20% (v/v) H_2SO_4 to each well. The plates were read on an ELISA reader (Labsystem Multiskan® MCC) at 492 nm.

RESULTS

Mycobacterial related antigens in serum immune complexes of RA patients

The immune complexes of all patients and controls were tested for the presence of mycobacterial antigen by antigen-capture ELISA using a polyclonal antibody to *M. tuberculosis* H_{37}Rv. The results showed in Figure 1 give the distribution of the patients according to OD in the test, the totals being further are broken down according to early and late onset disease. A value above the mean of the normals ±2SD was considered as positive.Using polyclonal antibodies about 70% of rheumatoid arthritis patients were found to possess mycobacterial related antigens in their serum immune complexes. The concentration of the antigens was

Table 4: Mycobacterial or related DnaJ antigens / epitopes in the serum immune complexes of rheumatoid arthritis patients. Ranges, means and statistical analysis of data.

Antibody	No	Range	Mean ± Standard Deviation	Standard Error	p value		
AB_7 Monoclonal (anti-43 kD DnaJ anti-*M. Tuberculosis*)					HC	DC	SLE
Healthy cont.	70	0.000-0.193	0.0124 ± 0.0313	0.0045	-	<0.001	>0.00
Disease cont.	29	0.000-0.175	0.0617 ± 0.057	0.01164	<0.001	-	<0.05
SLE	40	0.000-0.200	0.0301 ± 0.0348	0.0055	>0.001	<0.05	-
Early RA	27	0.000-1.715	0.2517 ± 0.3379	0.0796	<0.0001	<0.001	0.00
Late RA	68	0.018-1.145	0.3781 ± 0.3062	0.0503	<0.0001	<0.0001	<0.00
Total RA	95	0.106-0.713	0.1908 ± 0.1196	0.0180	<0.0001	<0.0001	<0.00

HC : Healthy control
DC : Disease control
SLE : Systemic Lupus Erythmatosus

found to be slightly higher in early rheumatoid arthritis patients than late rheumatoid arthritis patients.

A significant difference was found between the healthy normals and the rheumatoid arthritis patients. Similar differences were also noted between the disease control group and the RA patients (Table 3).

Antigens were detected in a few cases with other diseases and in normal controls, indicating that these individuals might have been exposed to *M. tuberculosis* or other mycobacteria (Figure 1).

Significant levels of mycobacterial antigens have been detected in >70% RA patients' serum immune complexes in an ELISA using rabbit polyclonal anti-*M. tuberculosis* H_{37}Rv IgG. Recently Lan and Wu (5) have also reported *M. Tuberculosis* antigens in the synovial fluid of RA patients. These results indicate that *M. tuberculosis* may be one of the causative agent of RA.

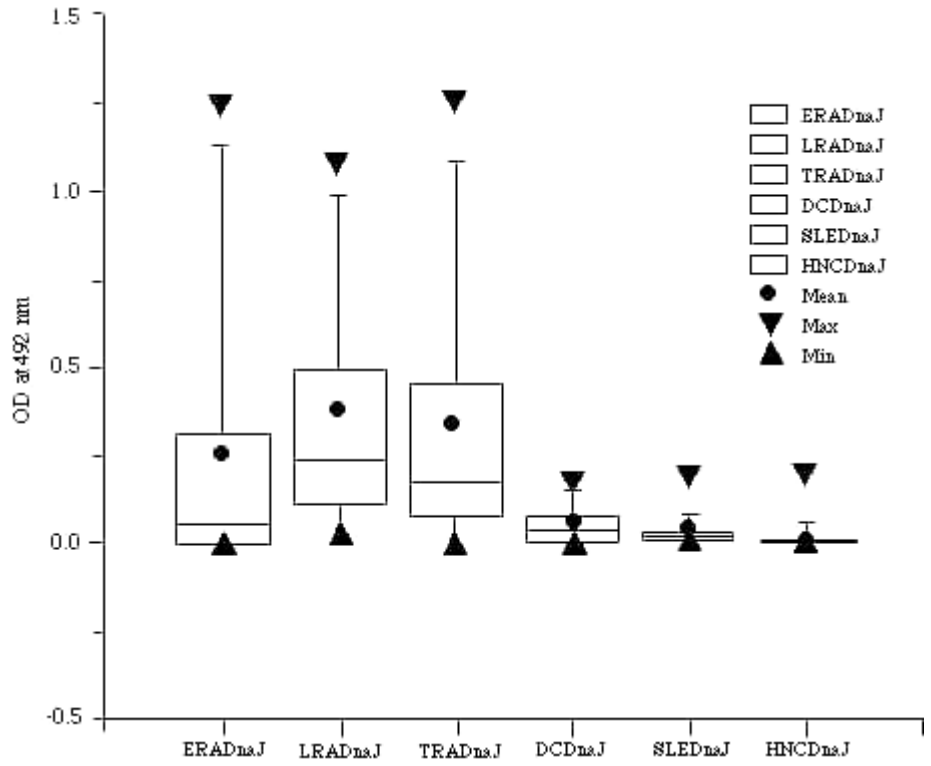
Several antigens found in *M. tuberculosis* are not restricted to this species. Many antigens of mycobacteria are widely shared throughout the genus and some more widely throughout the microbial world. Antiserum raised to *M. Tuberculosis* can also crossreact with other species. Thus, the presence of antigens associated with mycobacteria found in serum immune complexes of RA patients cannot be assigned definitively to *M. tuberculosis* by using polyclonal anti-*M. tuberculosis*. However, our data support the view that mycobacterial, or other bacteria with common antigens, may be involved in RA (3,8,16,17). There is substantial evidence of an immune response to antigens found in mycobacteria in RA patients.

Early RA patients show higher levels of mycobacterial or related antigens in their serum immune complexes. It is possible that clinical or subclinical mycobacterial or other bacterial infection induces an antibody response, which in turn reduces antigen levels at a later stage of the disease. Other forms of immune response may also restrict the multiplication of mycobacteria.

DnaJ related antigens in serum immune complexes of RA patients

The immune complexes of RA patients, diseases

Figure 2: Presence of DnaJ related antigens or its epitopes in serume immune complexes of Rheumatoid arthritis patients.



ERA=Early rheumatoid arthritis LRA=Late rheumatoid arthritis TRA=Total rheumatoid arthritis
DC=Disease controls (other than RA) SLE=Systemic lupus erythmatosus HNC=Healthy normal controls
DnaJ=(heat-shock protein)

In Figure 2, the single or upper box represents the distribution of OD values in which 50-75% of the subjects lie. The lower box is the 25-50% distribution.

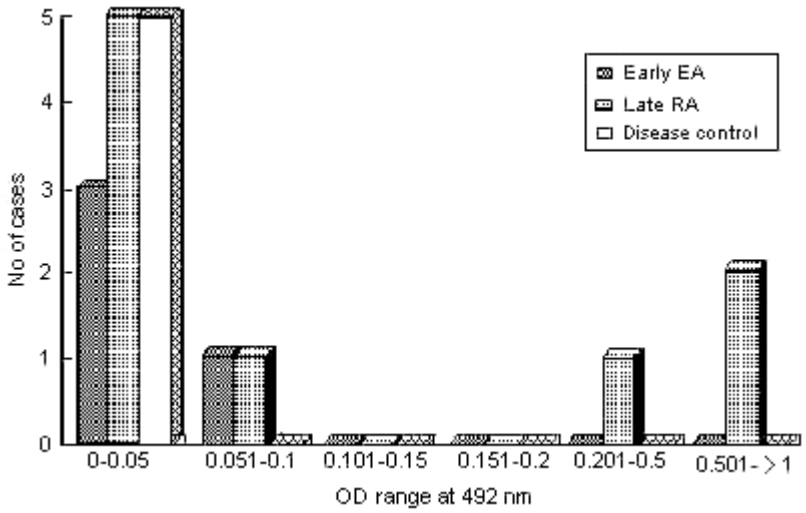
and healthy controls were also tested for the presence of DnaJ related antigen by ELISA using the anti-DnaJ (AB_7) monoclonal antibody. The results shown in Figure 2 give the distribution of the patients according to OD in the test, the totals being further broken down according to early and late onset disease. A value above the mean of normals ±2SD was considered as positive.

Using anti-DnaJ m-Ab more than 50% of rheumatoid arthritis patients was found to possess the DnaJ antigen in their serum immune complexes. The concentration of the antigen was found to be slightly higher in late rheumatoid arthritis patients than in early.

A significant difference was found between the healthy normals and the rheumatoid arthritis patients. Similar differences were also noted between the other disease control group and the RA patients (Table 4).

High levels of DnaJ, related antigens or epitopes on other antigens, have been detected in the serum immune complexes of RA patients. DnaJ is a heat shock conserved protein present in a wide range of bacteria. The anti-mycobacterial DnaJ m-Ab was found to cross-react with the DnaJ of a wide range of Gram negative bacteria (data not shown). Therefore the presence of DnaJ or related antigens in the serum immune complexes of RA patients cannot be taken to prove the

Figure 3: Mycobacterial related antigens in synovial fluids detected by ELISA using rabbit anti-*M. tuberculosis*.



involvement of mycobacteria specifically in the aetiology of RA.

High levels of DnaJ related antigens were seen in serum immune complexes by using anti-DnaJ m-Ab in late RA. There are reports suggesting a possible role of some hsps in the survival of mycobacteria (18-21). Surviving bacteria could continuously boost the immune response particularly to hsps, which have been implicated in the pathogenesis of arthritis in animals and humans (7,12).

Mycobacterial antigens (DnaJ) related antigens in synovial fluid of RA patients

Mycobacterial-related antigens were also detected in synovial fluid samples of rheumatoid arthritis patients using polyclonal rabbit-anti-*M. Tuberculosis* H_{37} Rv IgG. More than one-third of synovial fluid samples of the late rheumatoid arthritis patients was found to possess high levels of mycobacterial related antigens. This elevated level was not seen in the early rheumatoid arthritis patients or in the disease control group (Figure 3).

The DnaJ antigen was also studied in some rheumatoid arthritis patients' synovial fluid immune complexes. Slightly elevated levels of this antigen were detected in the late rheumatoid arthritis serum immune complexes as shown in Figure 4.

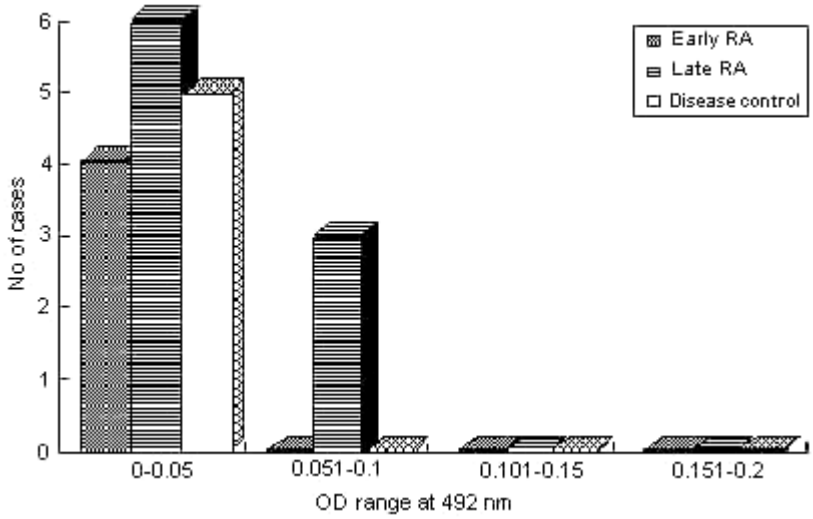
DISCUSSION

Recent studies implicate a putative role for mycobacteria and other microorganisms as well as autoantigens cross-reactive with these microbes in the aetiology and autoimmunity of arthritis. In our study, the presence of antigens strongly associated with mycobacteria was observed in the serum immune complexes of RA patients detected by antigen capture ELISA. Significant differences in antigen levels were noted between normal and patients' sera using polyclonal antibodies to *M. tuberculosis* H_{37}Rv.

In our studies, monoclonal antibodies to DnaJ of *M. tuberculosis* (43 kDa hsp) have been produced, and used to detect DnaJ or a related or cross-reactive antigen by ELISA in the serum immune complexes of RA patients. High levels of DnaJ or related antigens were detected in both serum and synovial fluids immune complexes.

It was interesting to note that high levels of mycobacterial antigens were seen in serum immune

Figure 4: DnaJ or related antigens in synovial fluids detected by ELISA using anti-DnaJ monoclonal antibody.



complexes by using polyclonal antibodies in early RA, which reduces in late RA. The reverse situation was noted for the DnaJ antigen, using the anti-DnaJ m-Ab, where low levels were detected in early RA and comparatively higher levels in late RA cases (Figures 1 and 2).

There are reports suggesting a possible role of some hsps in the survival of mycobacteria (18-21). Surviving bacteria could continuously boost the immune response particularly to hsps, which have been implicated in the pathogenesis of arthritis in animals and humans (7,12). These and other data provide some evidence for the possible involvement of mycobacteria in RA (15,22). Slow bacterial infections and possibly subclinical tuberculosis as well as BCG immunotherapy have been reported to cause reactive arthritis (23).

Susceptibility to RA is associated predominantly with a human histocompatibility antigen (HLA DR4) (2,12,25). Patients carrying the DR4 group antigens with RA have worse disease than those who lack the DR4 antigen. Albani *et. al.* (12) found that the amino acid sequence of DnaJ of E. coli contains an 11-amino acid run that is homologous to part of the third hypervariable region of human HLA-DR4. This sequence data suggests that immunological cross-reactivity might exist between HLA DR4 and DnaJ.

The epitope recognized by the AB_7 anti-DnaJ m-Ab has not been mapped. At this stage, it is not possible to ascertain whether anti-DnaJ m-Ab recognize the same homologous sequence or a different one on the same molecule. Therefore, it is necessary to study the amino acid sequence of the DnaJ recognized by the anti-DnaJ m-Ab.

Albani *et. al.* (12) Worthington *et. al.* (26) and more recently Jawaheer *et. al.* (27) suggested the role of DnaJ hsp in the RA. Worthington *et. al.* (26) measured antibodies to DnaJ by using monoclonal antibodies in a competitive ELISA and detected antibodies only in RA patients. The target epitope includes the so called "shared epitope sequence", a likely target for aberrant recognition via a mechanism of molecular mimicry in RA.

Recently Albani *et. al.* (28) studied the T cell proliferation response to DnaJ in RA and various other chronic inflammatory diseases. They observed that an increased immune reactivity to DnaJ is characteristic of RA and the magnitude of immune response is linked to disease activity.

DnaJ has been detected in a range of Gram negative bacteria by using anti-DnaJ m-Ab (data not shown). This finding of homology between epitopes of DnaJ and some components of susceptible RA patients, raises the possibility that induction of an antibody and/or T cell responses to DnaJ, which is a stress protein of many bacteria, might be implicated in an autoimmune process in which the DRB 10401 haplotype (DR4) is involved (12,24,27). This haplotype is strongly associated with severe, erosive forms of rheumatoid arthritis. The mechanism by which a shared epitope could increase susceptibility to RA is unknown and is likely to be complex.

This finding may suggest that *M. Tuberculosis* or a related bacterial species expressing DnaJ with identical antigenic structure is implicated as a source of antigen in immune complexes in rheumatoid arthritis patients.

RA is considered to be an autoimmune disease and associated primarily with DR4 haplotype and there is sufficient evidence to support the hypothesis of involvement of the QKRRA shared epitope between DnaJ and the third hypervariable region of human HLA-Dw4. However Boki *et. al.* (29) and De Vries (30) do not find this association in Greek and Israeli Jews. These studies indicate that some other factors, probably other hsp or antigens or some infection, could also contribute in the aetiopathogenesis of RA. It is also possible that DnaJ of some unidentified species may have sequence homology with DR1 or other haplotype.

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