

## Cytokines of the Th1 and Th2 type in sera of rheumatoid arthritis patients; correlations with anti-Hsp40 immune response and diagnostic markers

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Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease which affects approximately 1% of the population worldwide. Recent research on the role of heat shock proteins (Hsps) in RA development indicates that they may have pro- or anti-inflammatory effect, most probably *via* modulating cytokine secretion. We investigated type Th1 (INF $\gamma$ , TNF $\alpha$ , IL-2) and type Th2 (IL-10, IL-6, IL-4) cytokine levels in sera of RA patients and healthy controls, using flow cytometric bead array assay, and searched for correlations between the cytokine levels and serum antibodies against bacterial (DnaJ) and human (Hdj1, Hdj2 and Hdj3) Hsp40 proteins, as well as clinical and laboratory parameters. The levels of all cytokines studied were significantly increased in RA patients; the highest increase relative to healthy controls (7-fold) was observed for IL-6 and its levels correlated positively with the antibodies directed to DnaJ and to the C-terminal domain of Hdj2, and with diagnostic parameters (DAS 28, Steinbrocker RTG criteria, ARA/7, ESR, TEN, SW and GH). INF $\gamma$  levels correlated negatively with DAS 28, ESR, TEN and SW. No correlations were found for TNF $\alpha$ , IL-2 or IL-4. Our results support the hypothesis of Hsp40 involvement in RA as well as indicate that IL-6 serum level is a good marker of the RA activity.

**Keywords:** cytokines, heat shock proteins, Hsp40, rheumatoid arthritis

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### INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease with autoimmune etiology (reviewed by Klareskog *et al.*, 2009). RA affects approx. 1% of the population worldwide, with the female/male ratio of 2.5/1 (Lee & Weinblatt, 2001). RA is characterized by persistent and progressive inflammation of peripheral joints, leading to destruction of cartilage and bones (Verhoef, 2001).

Detailed pathogenesis of RA is unknown, however there is strong evidence that immune components, especially T (CD4+) and B cells are involved in the disease progression (Fournier, 2005). Several functional subtypes of CD4+ T cells, including Th1, Th2 and regulatory T cells — Treg, have been described (Skapenko *et al.*, 2005; Shahrara *et al.*, 2008; Bryl *et al.*, 2009). Many reports suggest that RA is mediated by activated pro-inflammato-

ry Th1 cells (Schulze-Koops & Kalden, 2001; Verhoef, 2001) which are characterized mainly by secretion of the IL-2, INF $\gamma$  and TNF $\alpha$  cytokines promoting cellular immune response, whereas Th2 cells produce the IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines which stimulate humoral antibody-mediated immune response (Raziuddin *et al.*, 1998). It has been suggested that rheumatoid inflammation is mediated by the dominance of activated Th1 cells producing pro-inflammatory cytokines over the Th2 cells (Verhoef, 2001; Schulze-Koops & Kalden, 2001).

Heat shock proteins (Hsps) are a family of evolutionarily conserved proteins whose expression at times of cellular stress, including infection and chronic inflammation, is markedly elevated (Zlacka *et al.*, 2006; reviewed by van Eden *et al.*, 2007). Based on their molecular weight, Hsps are classified into protein families, the major being the Hsp100, Hsp70, Hsp90, Hsp60, Hsp40, and small Hsps. Their structure conserved from bacteria to man and high immunogenicity make them attractive targets for investigation in the area of autoimmunity, with Hsp60 and Hsp70 being most extensively studied (reviewed in van Eden *et al.*, 2005; 2007; Wieten *et al.*, 2007). In contrast, the research on Hsp40 involvement in autoimmune diseases, including RA, has been less extensive, despite Hsp40 being probably the largest Hsp family in humans, with at least 50 members (reviewed in Kampinga *et al.*, 2009). *Escherichia coli* DnaJ is one of the immunogenic bacterial Hsp proteins of the Hsp40 family, of which the Hdj1, Hdj2 and Hdj3 proteins are the best characterized human members (Terada & Mori, 2000). The Hsp40 proteins comprise a strongly conserved N-terminal domain and a poorly conserved C-terminal region (reviewed in Cheetham & Caplan, 1998).

Recent studies (Prakken *et al.*, 2002; 2004; Kamphuis *et al.*, 2005; Massa *et al.*, 2007) indicate that recognition of peptides derived from heat shock proteins by the immune system can have an anti-inflammatory effect and down-regulate chronic state of inflammation *via* modulation of cytokine secretion. Recently, it has been demonstrated that in juvenile rheumatoid arthritis (JIA) peptides derived from bacterial and human Hsp40s modulate autoimmune inflammation (Massa *et al.*, 2007).

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**Abbreviations:** ARA, American Rheumatology Association; DAS 28, disease activity score; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; GH, general health; INF, interferon; IL, interleukin; PMBCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; SW, swelling; TEN, tenderness; TNF, tumor necrosis factor

We found previously that sera of RA patients contained significantly elevated levels of anti-DnaJ, anti-Hdj2 and anti-Hdj3 antibodies compared to sera of healthy control individuals (Krzewski *et al.*, 2003; Tukaj *et al.*, 2010). Those results suggested an involvement of Hsp40s in RA development.

In the present study, we investigated type Th1 (INF $\gamma$ , TNF $\alpha$ , IL-2) and type Th2 (IL-10, IL-6, IL-4) cytokine levels in sera of a well characterized group of patients with RA in order to correlate the cytokine levels with anti-Hsp40 humoral response as well as with diagnostic and laboratory parameters of the patients.

## MATERIALS AND METHODS

**Patients.** Rheumatoid arthritis patients were diagnosed according to American Rheumatism Association (ARA) criteria (Arnett *et al.*, 1987). Forty-eight serum samples of RA patients (46 female and 2 male) (age  $46 \pm 16.7$ ; range 20–80); disease duration  $7.9 \pm 8.5$  (range 0.2–32); RTG Steinbrocker's stages: stage I (n=10), stage II (n=10), stage III (n=2) and stage IV (n=14); 50% RF-positive patients; ESR ( $29.63 \pm 24.33$ ; mm/h); DAS 28 ( $3.81 \pm 1.04$ ) from the Pathophysiology Department at the Medical University of Gdańsk and fifty age- and sex-matched healthy volunteers were included in this study. Sera of the RA patients and healthy controls were stored at  $-20^\circ\text{C}$  prior to evaluation of cytokines and anti-Hsp40 antibodies' levels.

**Consent.** The study was approved by the Local Committee for Biomedical Research Ethics at the Medical University of Gdańsk. All subjects were informed of the details of the experiment prior to the taking of a sample of 20 ml peripheral venous blood.

**Chemicals.** Mouse anti-human Fc IgG antibodies coupled with horseradish peroxidase (HRP), the HRP substrate (tetramethylbenzidine) and other chemicals were purchased from Sigma (Poznań, Poland).

**Antigens.** Hsp40 antigens: bacterial DnaJ, human Hdj1, Hdj2, Hdj3, and N-terminal and C-terminal domains of DnaJ (DnaJ $\Delta$ 107-375; DnaJ $\Delta$ 1-199) and Hdj2 (Hdj2 $\Delta$ 106-397; Hdj2 $\Delta$ 1-215) were overproduced in

*E. coli* and purified as described previously (Zylicz *et al.*, 1985; Terada & Mori, 2000; Krzewski *et al.*, 2003).

**Cytokine measurements.** Cytokines (INF $\gamma$ , TNF $\alpha$ , IL-6, IL-4, IL-10 and IL-2) were measured in patients' and controls' sera by a flow cytometric bead array using human Th1/Th2 cytokine kit CBA<sup>TM</sup> (Becton Dickinson, Poland) and a FACScan (Becton Dickinson).

**ELISA assay.** Levels of the anti-Hsp40 antibodies (IgG) in the sera of the RA patients and healthy controls were assayed by an indirect ELISA test performed as described before (Krzewski *et al.*, 2003). Briefly, ELISA plates were coated with 50  $\mu\text{l}$  of each Hsp40 antigen (40  $\mu\text{g}/\text{ml}$ ) and the sera (diluted 1:500) were added to the wells. Subsequently, mouse anti-human Fc IgG antibodies coupled with horseradish peroxidase were added (1:2000). After reaction with the HRP substrate tetramethylbenzidine, absorbance was measured at 450 nm using an ELISA plate reader (Asys Hitech GmbH, Austria). Assays were performed in triplicate.

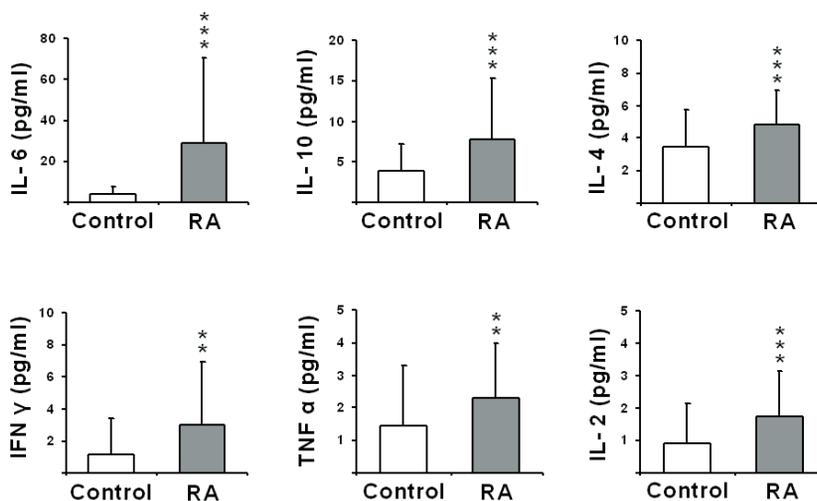
**Statistical analysis.** Statistical analyses were performed using the SPSS 12.0 for Windows program (SPSS Inc, Chicago, IL, USA). The distribution normality was analyzed using the Shapiro-Wilk test. Serum cytokine levels were analyzed using Student's *t*-test for unpaired samples. Serum levels of anti-Hsp40 antibodies were analyzed using U Mann Whitney test. Spearman's rank correlation test was used for multi-parametric data with normal (serum cytokine levels), non-normal (serum anti-Hsp40 levels) and diagnostic and laboratory parameters. *P* values less than 0.05 were considered significant.

## RESULTS

### Rheumatoid arthritis patients have elevated serum levels of the Th1 and Th2 cytokines

Levels of six cytokines representing the Th1 (INF $\gamma$ , TNF $\alpha$ , IL-2) and Th2 (IL-10, IL-6, IL-4) subpopulations were evaluated in the sera of the RA patients (n=48) and healthy control volunteers (n=50) using cytometric bead array (CBA) technique.

We found that the concentrations of all cytokines tested were significantly higher in the sera of RA patients compared to healthy controls (Fig. 1). The RA/healthy ratio varied from approx. 1.4 for IL-4 to about 7.0 for IL-6. INF $\gamma$  in the sera of RA patients was significantly, approx. 2.5-fold, higher ( $P=0.003$ ) than in the controls. Interestingly, measurable levels of the cytokines were not found in all RA or healthy sera. Thus, measurable serum levels of INF $\gamma$  were observed in 24/48 RA patients and in 12/50 of the controls, TNF $\alpha$  in 35/48 sera of RA patients and in 22/50 of the controls, IL-2 in 32/48 RA patients and in 18/50 of the controls, IL-10 in 46/48 RA patients' sera and in 36/50 sera of the controls, and IL-4 were observed in 45/48 RA patients and in 41/50 of the controls. IL-6



**Figure 1.** Serum levels of Th1 (INF $\gamma$ , TNF $\alpha$ , IL-2) and Th2 (IL-10, IL-6, IL-4) cytokines of rheumatoid arthritis patients and healthy controls. Bars present mean  $\pm$  S.D. (standard deviation) values. Student's *t*-test was used for statistical analysis \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

**Table 1. Levels of antibodies against Hsp40 proteins and their domains in sera of RA patients and healthy controls**

Serum antibody levels	RA patients	Control subjects	U Mann Whitney test results
anti-DnaJ	1.34±0.29	0.88±0.22	<i>P</i> <0.001
anti-N-DnaJ	0.31±0.14	0.22±0.09	<i>P</i> <0.01
anti-C-DnaJ	0.13±0.09	0.11±0.11	<i>P</i> <0.05
anti-Hdj1	0.54±0.16	0.59±0.27	n.s.
anti-Hdj2	2.52±0.11	1.95±0.38	<i>P</i> <0.001
anti-N-Hdj2	0.54±0.09	0.4±0.12	<i>P</i> <0.001
anti-C-Hdj2	0.6±0.28	0.51±0.16	n.s.
anti-Hdj3	1.08±0.15	0.91±0.09	<i>P</i> <0.001

Antibodies (IgG) against *E. coli* DnaJ, human Hdj1, Hdj2, Hdj3, and N- and C-terminal domains of DnaJ and Hdj2 were measured by ELISA, as described previously (Krzewski *et al.*, 2003). The values are expressed as mean (±S.D.) of A 450/620. To assay significance of the difference between the RA and control antibody levels U Mann Whitney test was used. n.s., not significant.

was detected in all enrolled RA patients' sera (48/48) and in 33/50 of the controls.

The undetectable levels of cytokines were randomly distributed among the patients and no correlations regarding such low levels of cytokines and disease progression could be found.

#### Correlations between the levels of serum cytokines, anti-Hsp40 response and diagnostic markers

We assayed antibodies (IgG) directed to *E. coli* DnaJ (Hsp40), to its human homologs (Hdj1, Hdj2, Hdj3) and to the N- and C-terminal domains of DnaJ and Hdj2 in sera of RA patients and healthy control individuals using

the ELISA method. We found increased levels of the response against DnaJ, Hdj2 and Hdj3 while there was no significant increase in the anti-Hdj1 response in RA patients. The RA patients had higher response to both DnaJ domains and also to the N-terminal domain of Hdj2 compared to healthy controls (Table 1).

We searched for correlations between the levels of cytokines in the sera of the RA patients and the levels of anti-Hsp40 antibodies, as well as the diagnostic parameters. We found positive correlations between the levels of IL-6 and the antibodies directed to DnaJ protein, and to the C-terminal domain of Hdj2. A negative correlation between the IL-10 levels and the anti-Hdj1 response was observed (Table 2). We found that the levels of

**Table 2. Correlations of serum cytokine concentrations with levels of anti-Hsp40 antibodies, and with the clinical and diagnostic parameters of RA patients**

Antibodies	Correlations with serum cytokines					
	IFN $\gamma$	TNF $\alpha$	IL-2	IL-10	IL-6	IL-4
Anti-DnaJ	n.s.	n.s.	n.s.	n.s.	0.289 <i>P</i> =0.046	n.s.
Anti-Hdj1	n.s.	n.s.	n.s.	-0.312 <i>P</i> =0.031	n.s.	n.s.
Anti-C-Hdj2	n.s.	n.s.	n.s.	n.s.	0.310 <i>P</i> =0.032	n.s.
Parameters						
DAS 28	-0.394 <i>P</i> =0.010	n.s.	n.s.	n.s.	0.534 <i>P</i> =0.000	n.s.
Steinbrocker	n.s.	n.s.	n.s.	0.392 <i>P</i> =0.018	0.581 <i>P</i> =0.000	n.s.
ARA/7	n.s.	n.s.	n.s.	n.s.	0.388 <i>P</i> =0.011	n.s.
ESR	-0.331 <i>P</i> =0.032	n.s.	n.s.	n.s.	0.454 <i>P</i> =0.003	n.s.
TEN	-0.331 <i>P</i> =0.032	n.s.	n.s.	n.s.	0.309 <i>P</i> =0.046	n.s.
SW	-0.313 <i>P</i> =0.044	n.s.	n.s.	n.s.	0.406 <i>P</i> =0.008	n.s.
GH	n.s.	n.s.	n.s.	n.s.	0.354 <i>P</i> =0.022	n.s.
Age	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RF	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Duration	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Spearman's rank correlation test was used for statistical analysis. *P* values less than 0.05 were considered significant. n.s., not significant.

IL-6, IL-10 and IFN $\gamma$  correlated with diagnostic parameters such as RTG Steinbrocker criteria used to describe progression of radiological changes, DAS 28 index corresponding to activity of RA disease, and four parameters such as ESR, GH, TEN and SW, used to establish the DAS 28 index (Table 2).

## DISCUSSION

In this study we investigated type Th1 (IFN $\gamma$ , TNF $\alpha$ , IL-2) and type Th2 (IL-10, IL-6, IL-4) cytokine levels in the sera of a well characterized group of patients with RA (Fig. 1) and searched for correlations between the cytokine levels and serum antibodies against Hsp40 proteins as well as the clinical and laboratory parameters (Table 2).

Of the cytokines tested, we observed the most dramatic differences between the RA and control groups for IL-6. The mean serum concentration of IL-6 was 7-fold higher in RA patients compared to healthy controls (Fig. 1). This is in good agreement with the well established role of this cytokine in the pathogenesis of RA; increased levels of IL-6 have been found in synovial fluid (Houssiau *et al.*, 1988) and in sera (Lacki *et al.*, 1995; 1997). It is postulated that IL-6 stimulates secretion of IL-17 by Th17 cells; this in turn enhances production of cartilage-destructive enzymes and expression of molecules participating in bone destruction (reviewed by Klareskog *et al.*, 2009).

We found increased levels of antibodies to the Hsp40s of bacterial (DnaJ) and human (Hdj2, Hdj3) origin (Table 1) in sera of the RA patients. The findings concerning DnaJ are in agreement with the early work of Albani *et al.* (1995) and with our results obtained for a different group of patients (UK) (Krzewski *et al.*, 2003). The results concerning human Hsp40s are in agreement with our earlier observation (Tukaj *et al.*, 2010). Furthermore, we observed a positive correlation between the levels of IL-6 and the response to DnaJ and to the C-terminal domain of Hdj2 in RA patients (Table 2). These findings together support the hypothesis of Hsp40s involvement in RA as proposed in early work of Albani *et al.* (1995). It is tempting to speculate that DnaJ induces the increase in humoral anti-DnaJ response and stimulation of Th2 cells to produce IL-6. This hypothesis is in agreement with our results showing that the presence of DnaJ drastically induced secretion of IL-6 by peripheral blood mononuclear cells (PMBCs) *in vitro* (Tukaj *et al.*, 2010). Considering the increased levels of both anti-DnaJ antibodies and IL-6 one should bear in mind that bacterial infection is one of the discussed possible causes of RA and that elevated levels of IL-6 have been found in peripheral blood in a variety of diseases, including bacterial infections (de Benedetti *et al.*, 1994).

IL-6 correlated positively also with diagnostic parameters such as: disease activity score (DAS 28), Steinbrocker RTG criteria, criteria for diagnosis of RA (ARA/7), and the four parameters for establishing the DAS 28 Index (erythrocyte sedimentation rate — ESR, tenderness — TEN, swelling — SW and general health — GH) (Table 2). These findings confirm that the IL-6 serum level is a very good clinical marker of the RA activity.

Our observation that sera of RA patients contained significantly elevated levels of IL-10 compared to healthy controls (Fig. 1) is in agreement with the work of Lacki *et al.* (1997). Recently, a significant increase of IL-10 was found only in RA patients hav-

ing an increased CRP value (Minami *et al.*, 2006). We did not include CRP assay in our study, however, we found no correlation between IL-10 levels and ESR values. A positive correlation for IL-10 and Steinbrocker RTG criteria was observed (Table 2), which is to some extent surprising, since IL-10 is the main anti-inflammatory cytokine and its administration reduced inflammation in animal models of RA (Groux *et al.*, 2003). However, the high IL-10 levels may represent a counteracting mechanism which is insufficient to balance the production of pro-inflammatory cytokines by the Th1 subpopulation. We found that the IL-10 levels were negatively correlated with the anti-Hdj1 humoral response (Table 2). It remains to be tested whether Hdj1 has an inhibitory effect on IL-10 secretion by PMBCs.

We found increased levels of IFN $\gamma$ , which is a pro-inflammatory cytokine with immunoregulatory and anti-tumor activity (Billiau, 1996), playing a central role as a mediator of the signs and symptoms of chronic autoimmune inflammation (Schulze-Koops & Kalden, 2001). IFN $\gamma$  was observed to correlate negatively with the disease activity (DAS 28) as well as with three out of the four criteria used to establish the DAS 28 index (ESR, TEN and SW) (Table 2). Previously, Funauchi *et al.* (1991) found no correlation between IFN $\gamma$  serum levels and the clinical findings — the discrepancy with our results could be due to differences between the groups of patients, including variations in their therapies. The negative correlation between IFN $\gamma$  and activity of the disease is in agreement with observations that patients treated with IFN $\gamma$  showed improvement concerning clinical parameters (Machold *et al.*, 1992), and suggests that IFN $\gamma$  plays an important role in maintaining immune homeostasis in patients with RA. Furthermore, our data suggest that its blood level might be a good marker of the RA activity.

TNF $\alpha$  is postulated to play a crucial role in RA development, together with IL-6 and IL-1, and agents blocking its function are effective drugs in RA therapy (reviewed by Klareskog *et al.*, 2009). Our finding that the RA patients had increased TNF $\alpha$  serum levels is in agreement with the above facts, although we were unable to find any correlation between TNF $\alpha$  and the other parameters tested. Previous assessments showed either no significant increase of TNF $\alpha$  serum levels in RA patients (Fröde *et al.*, 2002) or an increase correlating with systemic inflammation (Altomonte *et al.*, 1992). These discrepancies indicate that TNF $\alpha$  serum level is not a good candidate for the disease marker, probably because of the relatively short half-life of TNF $\alpha$ . However, one should remember about the cascade of cytokines, meaning that TNF $\alpha$  induces IL-6 production, and since IL-6 has a much longer half-life, it is a better marker for practical measurements in patients' sera. The lack of correlation between TNF $\alpha$  levels and the severity of the disease could be also explained by the fact that we assayed TNF $\alpha$  in serum, not in the synovial fluids.

The serum levels of IL-2 and IL-4 were increased in RA patients but we found no correlations with the anti-Hsp40 antibodies or clinical and diagnostic parameters (Table 2). Our results concerning IL-4 levels are consistent with a previous report (Rivas *et al.*, 1995) and suggest that IL-4 does not contribute to the deleterious effects of the disease. In the case of IL-2, an earlier report showed a decrease of its serum levels in RA patients (Altomonte *et al.*, 1992).

Our finding that the levels of the Th2- as well as Th1-type cytokines were increased in RA patients compared to the healthy controls shows that the serum levels of cytokines do not simply reflect the theory of the dominance of the Th1 over the Th2 response in RA, the theory which is based on the research on synovium (Verhoef, 2001; Schulze-Koops & Kalden, 2001).

## CONCLUSION

The existence of a positive correlation between the serum levels of IL-6 and antibodies against bacterial Hsp40 (*E. coli* DnaJ) and C-terminal domain of human Hdj2) in RA patients supports the hypothesis of Hsp40 involvement in RA. The increased levels of both Th1- and Th2-type cytokines show that the serum levels of cytokines do not simply reflect the theory of the dominance of the Th1 over the Th2 response in RA.

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