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Original article

Antibodies to the endoplasmic reticulum-resident chaperones calnexin, BiP and Grp94 in patients with rheumatoid arthritis and systemic lupus erythematosus

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Abstract

Objectives. To investigate the presence of autoantibodies against mammalian chaperones of the endoplasmic reticulum (ER) in patients with RA and other immune-mediated diseases.

Methods. Sera from healthy donors, from early RA patients with two follow-up samples, patients with SLE, SSc and IBD were collected and analysed for anti-ER chaperone antibodies. Detection of serum IgG antibodies against immunoglobulin heavy chain binding protein (BiP), glucose-regulated protein 94 (Grp94) and calnexin was carried out using ELISA. The specificity of sera positive for individual ER chaperones was confirmed by immunoblotting. Statistical analysis was performed using Welch's *t*-test, Mann–Whitney U-test, partial correlation and Pearson's correlation.

Results. In patients with RA and SLE, autoantibody titres against BiP, Grp94 and calnexin were significantly higher than those in healthy controls. These autoantibodies were detectable in patients with early RA and titres remained stable for at least 6–12 months. Also several SSc and IBD patients exhibited autoantibodies against these ER chaperones; however, titres and frequencies were lower than in RA or SLE patients. Furthermore, anti-calnexin antibodies correlated significantly with the presence of BiP and Grp94 autoantibodies in patients with RA and SLE.

Conclusion. Calnexin and Grp94 were identified as novel autoantigens in RA and calnexin in SLE. Since calnexin, Grp94 and BiP are ER-resident proteins of eukaryotic cells, our data suggest that autoantibody generation against ER chaperones is independent of initial exposure to the corresponding bacterial chaperones; rather, ER chaperones may represent genuine autoantigens.

Key words: Rheumatoid arthritis, Systemic lupus erythematosus, Chaperone, Heat shock protein, Autoantibodies, ELISA, Calnexin, Grp94, BiP.

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Introduction

RA is a systemic disorder of largely unknown origin characterized by chronic inflammation of synovial joints, leading to progressive erosions of cartilage and bone. The prevalence of RA is ~1% of the world's population with women being three times more often affected than men [1]. Serologically, the majority of RA patients are characterized by the presence of RF and antibodies to cyclic citrullinated peptides (anti-CCPs). RF displays a low specificity for RA, since RF is also detectable in sera of patients with other autoimmune diseases, with bacterial

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infections as well as in healthy donors. In contrast, anti-CCP antibodies are highly specific for RA. Also other autoantibody specificities were described in RA including antibodies to keratin (anti-keratin) [2], heterogeneous nuclear RNP A2/B1 (anti-hnRNP-A2/-B1 or anti-RA33) [3] and collagen type II [4–6]. In addition, autoantibodies to HSPs, mainly of the 60- and 70-kDa family, have been described in patients with RA [7, 8], but they were also found in sera of patients with other autoimmune diseases such as SLE [9], multiple sclerosis [10, 11], IBD [12] and Behçet's disease [13]. HSP family members and mainly those acting as molecular chaperones have been functionally implicated in immunological dysregulation [14]. Chaperones prevent irreversible aggregation of proteins under physiological and stress conditions as they selectively recognize and bind non-native proteins [15]. In addition, endoplasmic reticulum (ER)-resident chaperones, such as immunoglobulin heavy chain binding protein (BiP), glucose-regulated protein 94 (Grp94) and calnexin, are involved in antigen recognition and presentation, because they take part not only in folding and assembly of early intermediates of MHC Class I and II molecules, but also in MHC peptide loading [16, 17]. Furthermore, HSPs have been implicated in immune responses against pathogens and infectious diseases [18]. It has been postulated that HSPs provide a basis for autoimmunity in chronic inflammatory diseases, because they are one of the most conserved protein families [19]. Bacterial infections might therefore induce an immune response against prokaryotic HSPs and may lead to cross-reactivity of T cells and the production of autoantibodies; however, the exact mechanisms remain to be elucidated.

In the present study, we investigated the presence of autoantibodies against three major ER chaperones, namely calnexin, BiP and Grp94, in sera of patients with RA, SLE, SSc and IBD. The specificities of antibodies to BiP and calnexin were confirmed by western blotting. Patients with RA and SLE displayed significantly higher antibody titres against BiP, Grp94 and calnexin compared with healthy donors.

Patients, materials and methods

Study population and clinical evaluation

Sera from 155 RA patients [60% female, mean (S.D.) age 58 (14) years] with average disease duration of 6 months since the onset of first symptoms were collected in 20 collaborating rheumatological centres within the German Network for Competence in Rheumatology. The interval between each visit was at least 3 months and on average 6 months. All patients fulfilled at least four of the 1987 revised classification criteria for RA [20] at study inclusion, referred to as Visit 1. Almost 70% of RA patients were either carriers of one HLA-DR1 or one HLA-DR4 allele. Sera of 70 sex-matched healthy donors (55% female) served as control cohort. A cohort of 60 SLE patients and 22 SSc patients from the Department of Internal Medicine 3, University of Erlangen-Nuremberg, were randomly selected irrespective of stage or severity of the disease. Patients fulfilled the ACR classification criteria for SLE [21, 22] or for early SSc [23], respectively. The diagnoses of IBD were made according to the Lennard-Jones criteria [24]. The study was approved by the ethical committee of the Friedrich-Alexander-University Erlangen-Nuremberg, and informed consent was obtained from all individuals before entering the study.

Serum samples

Venous blood was collected from healthy donors and patients with RA into serum tubes (S-Monovette, Sarstedt, Nümbrecht, Germany). To obtain serum, centrifugation was carried out at 2000 *g* for 10 min at 4°C. All serum samples were stored at −20°C.

Purification of HSPs

Canine Grp94 was expressed in *Escherichia coli* and purified as described previously [25]. Recombinant human calnexin was purchased from Stressgen (SPP-865; Biomol, Hamburg, Germany). The expression and purification of murine BiP was carried out according to the protocol of Mayer *et al.* [26].

Detection of anti-ER chaperone antibodies by ELISA

Autoantibodies directed to calnexin, BiP and Grp94 were assessed by ELISA according to the protocol of Schellekens *et al.* [27]. Briefly, Maxisorb polystyrene 96-well plates (Nunc, Wiesbaden, Germany) were coated with 50 μl/well of 1 μg/ml calnexin, BiP and Grp94, respectively, in PBS and incubated overnight at 4°C. To control for non-specific binding of serum one-half of each plate was coated with 1 μg/ml BSA. After four washes with PBS-T (0.05% Tween-20) plates were blocked with 200 μl of 2% BSA in phosphate-buffered saline (PBS; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl pH 7.4) for 1 h. Serum samples diluted 1 : 40 in RIA buffer [10 mM Tris, pH 7.6, 350 mM NaCl, 1% BSA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% SDS] supplemented with 10% rabbit serum (Sigma-Aldrich, Munich, Germany) were subjected in duplicate (100 μl/well) to each well and incubated for 90 min at RT. After five washes, 100 μl of 1 : 5000 diluted (RIA buffer) rabbit anti-human immunoglobulin G–horseradish peroxidase (IgG–HRP) (Dianova, Cat. No. 309-035-082, Hamburg, Germany) was added to each well and incubated for 1 h at room temperature (RT). After a final washing step, antibodies were detected using 100 μl of a 1 mg/ml *O*-phenylenediamine dihydrochloride solution (Sigma-Aldrich, St Louis, MO, USA). The reaction was stopped after 15 min with 1 M HCl. The absorbance at 490 nm was measured using a microplate spectrophotometer (SPECTRA max 190; Molecular Devices, Sunnyvale, CA, USA). To compare different plates, a reference serum from a RA patient that was known to bind the respective ER chaperone, was carried along on each 96-well plate as internal standard. For calculation of relative anti-ER chaperone antibody levels, the mean optical density (OD) of wells coated with BSA as background

value (non-specific binding) were subtracted from the mean OD of each sample. For normalization, all values were finally divided by the mean value obtained for healthy donors.

SDS–PAGE and western blot analyses

Purified calnexin and BiP were loaded at a concentration of 0.5 μg/ml in 1× SDS–PAGE sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 1% 2-mercaptoethanol) to reducing 10% SDS slab gels as described previously [28]. Proteins were transferred to a polyvinyl difluoride membrane (Millipore, Bedford, UK) in a semi-dry apparatus (Serva, Heidelberg, Germany) according to the manufacturer's instructions. The membrane was blocked for 1 h at RT with Tris-buffered saline with Tween-20 (TBS-T) [10 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4] supplemented with 5% milk powder. Subsequently, blots were incubated for 1 h with sera from RA patients (diluted 1 : 200 in RIA buffer containing 5% milk powder), which were found to be either positive or negative for anti-calnexin or anti-BiP antibodies. Anti-calnexin and anti-BiP antibodies (both mouse mAbs; BD Bioscience Pharmingen, San Diego, CA, USA) served as positive controls in a 1 : 1000 dilution. After washing the blots were incubated at RT for 1 h with HRP-conjugated goat anti-mouse IgG or rabbit anti-human IgG–HRP, each diluted 1 : 10 000 in RIA buffer supplemented with 5% milk powder. After a final washing step, proteins were detected using enhanced chemiluminescence (ECL) reagents and Amersham Hyperfilm ECL (Amersham Biosciences, Freiburg, Germany).

Sequence analysis

Protein sequence alignments based on Swiss-Prot information and calculation of sequence identities for canine (P41148) and human (P14625) Grp94 as well as murine (P20029) and human (P11021) BiP were carried out using ClustalW software (Conway Institute, UCD Dublin, Ireland) [29]. For visualization of results the CLC Free Workbench 4 software package (CLC bio, Aarhus, Denmark) was used.

Statistical analyses

Comparison of means of anti-ER chaperone autoantibody titres between healthy donors and patients with RA and SLE were computed using Welch's test coefficients because variances were not assumed to be equal according to Levene's *F*-test; moreover, both groups had $n > 30$ implying Gaussian distribution of sample means according to the central limit theorem. Means of anti-ER chaperone antibody titres for SSc and IBD patients as well as for the subgroups of men and women had to be analysed for Gaussian distribution of sample means via Lilliefors-corrected Kolmogorov–Smirnov test as the individual groups now comprised only 22 subjects or fewer. If Gaussian distribution of sample means could be assumed, Welch's *t*-test was used; otherwise Mann–Whitney U-test had to be applied.

Partial correlation of antibody titres was performed to test whether high values of calnexin correspond to high titres of BiP or Grp94 in patients with RA, SLE and healthy donors, while controlling for possible mediator variables such as age and gender. For RA patients, retest reliability of antibody titres from at least two visits was also analysed by bivariate Pearson correlation for calnexin, BiP and Grp94 titres over time. Statistical analysis was done using Predictive Analytics Software (PASW) Version 17.0.2 (SPSS Inc., Chicago, IL, USA). $P \leqslant 0.05$ was considered to be statistically significant.

Results

Canine Grp94 and murine BiP share high similarity with their human orthologues

First, we investigated whether our assay system, using recombinantly expressed canine Grp94, murine BiP and human calnexin, was suitable to detect autoantibodies in human sera. Highly purified human proteins are currently not available, thus we used canine Grp94 and murine BiP. According to multiple sequence alignments, canine and human Grp94 are 97.9% identical and 99.4% similar to each other, and the identity and similarity between murine and human BiP is 98.5 and 99.1%, respectively (Fig. 1A and B).

Due to this high-sequence conservation, it was very likely that human serum antibodies would recognize the mammalian orthologues in a respective ELISA system. In an explorative ELISA analysis, we detected antibodies to BiP and calnexin in patients with RA. To ensure the reliability of our testing system, it was necessary to determine whether autoantibodies in RA sera recognize BiP and calnexin specifically. Therefore, highly purified calnexin and BiP were loaded onto SDS–PAGE and sera that had been tested either positive or negative for anti-calnexin (Fig. 2A) or anti-BiP antibodies (Fig. 2B) by ELISA were applied to the blotted membrane. Incubation with negative serum did not result in a signal, whereas with positive serum specific bands were detected for calnexin and BiP. Specific mAbs to calnexin and BiP served as positive controls.

Autoantibodies to BiP, Grp94 and calnexin are elevated in sera of patients with RA and SLE

After assessing the suitability of our antigens and validating the specificity of our ELISA method by western blot, analyses of autoantibodies against the ER chaperones BiP, Grp94 and calnexin in sera of patients with various autoimmune and inflammatory diseases such as RA, SLE, SSc and IBD and in healthy donors was carried out. As summarized in Table 1 and Fig. 3, we found increased levels of autoantibodies of the IgG isotype for all three ER chaperones in patients with RA and SLE as compared with healthy donors. In contrast, similar anti-ER chaperone autoantibody titres were found for patients with IBD and healthy donors. SSc patients displayed significantly increased concentrations of anti-Grp94 autoantibodies. After Bonferroni correction, all antibody titres mentioned

Fig. 1 Protein sequence alignments. Protein sequence alignments for murine (P20029) and human (P11021) BiP (**A**) and canine (P41148) and human (P14625) Grp94 (**B**) are illustrated using CLC Free Workbench software. Sequence identity between murine and human BiP was 98.5 and 97.9%, respectively, for canine Grp94 and its human orthologue.

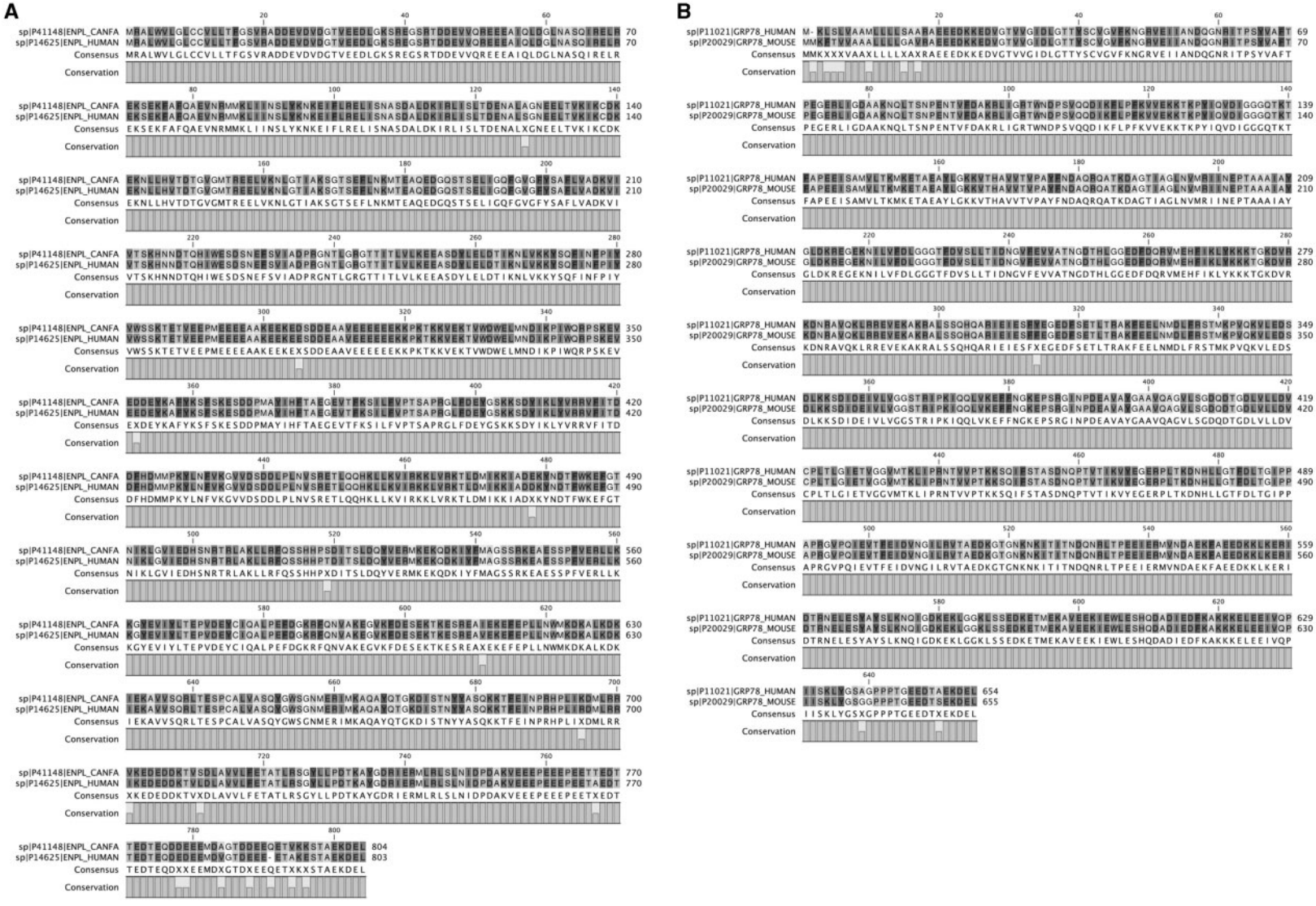


FIG. 2 Specific recognition of calnexin (**A**) and BiP (**B**) by antibodies that are contained in sera of RA patients. Western blotting of purified calnexin (A) and BiP (B) was carried out. Sera, previously identified by ELISA to contain either high or no anti-calnexin or anti-BiP autoantibody titres were selected and termed as positive and negative sera, respecitvely. mAbs to calnexin and BiP served as internal positive controls.

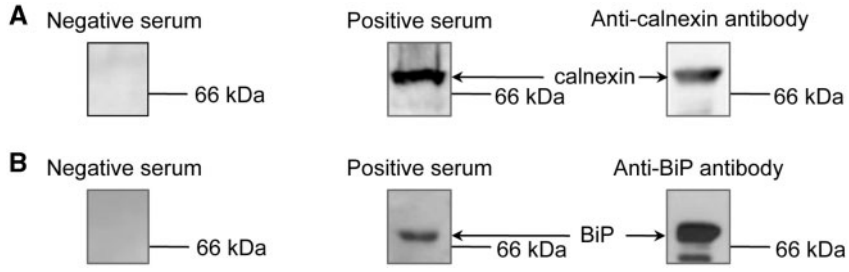


TABLE 1 Statistical analysis of antibodies to calnexin, BiP and Grp94 detected in patients with RA, SLE, SSc and IBD compared with healthy donors

Autoantibodies against	*n*	Mean (S.D.), AU	95% CI	*P*-value
Calnexin				
NHD	63	1.01 (0.81)	0.81, 1.22	
RA Visit 1	119	1.83 (1.73)	1.51, 2.14	<0.001*
SLE	60	3.04 (3.98)	2.01, 4.07	<0.001*
SSc	22	1.07 (1.00)	0.62, 1.51	NS**
IBD	14	0.88 (0.66)	0.50, 1.26	NS*
BiP				
NHD	70	1.22 (1.27)	0.92, 1.52	
RA Visit 1	131	2.95 (3.73)	2.31, 3.40	<0.001*
SLE	60	2.45 (1.60)	2.04, 2.87	<0.001*
SSc	22	1.32 (0.70)	1.01, 1.63	NS*
IBD	14	1.63 (1.27)	0.89, 2.36	NS**
Grp94				
NHD	70	1.07 (1.00)	0.83, 1.31	
RA Visit 1	131	1.77 (1.82)	1.45, 2.08	0.001*
SLE	60	2.43 (3.68)	1.48, 3.38	0.007*
SSc	22	2.34 (2.75)	1.13, 3.56	0.04**
IBD	14	0.74 (1.11)	0.10, 1.38	NS**

P-values for RA, SLE, SSc and IBD patients were calculated in comparison with NHD. **P*-values were calculated using Welch *t*-test. ***P*-values were calculated using Mann–Whitney U-test. AU: arbitrary units; NHD: normal healthy donor; NS: not significant.

above remained significantly different when compared with healthy controls apart from Grp94 titres in SSc.

Our data confirmed a significantly increased antibody response to BiP in patients with RA and to Grp94 in SLE patients, as reported previously [30–32]. Our observation of significantly increased Grp94 and calnexin autoantibody titres in patients with RA and calnexin in patients with SLE is novel (Fig. 3B and C and Table 1).

Furthermore, statistical analysis using Pearson correlation for retest reliability of individual anti-ER chaperone autoantibody titres at different timepoints (Visits 1–3) revealed that autoantibodies to all three ER chaperones remained constant at each follow-up visit without major changes (Fig. 4). The Pearson correlation coefficients were highly significant for autoantibodies to BiP ($r = 0.80$; $P < 0.001$) and Grp94 ($r = 0.86$; $P < 0.001$) in two visits and for calnexin over three visits ($r = 0.92$; $P < 0.001$). Hence, we conclude that autoantibody titres against BiP, Grp94 and calnexin remain stable over a period of at least 6–12 months. Furthermore, the autoantibody response against BiP, Grp94 and calnexin is a rather early event in RA, since the first serum samples (Visit 1) of RA patients were obtained as early as 6 months after disease onset.

High mutual correlation of autoantibodies against ER chaperones

As autoantibody titres against calnexin, BiP and Grp94 were found to be significantly increased in patients with RA and SLE, we analysed the co-existence of these

Fig. 3 Detection of autoantibodies to BiP (**A**), Grp94 (**B**) and calnexin (**C**) in sera of patients with RA, SLE, SSc and IBD compared with healthy donors. Autoantibodies to mammalian BiP, Grp94 and calnexin in sera of RA patients with consecutive visits (Visit 1: $n = 131$; Visit 2: $n = 81$; Visit 3: $n = 68$), SLE patients ($n = 60$), SSc patients ($n = 22$), patients with IBD ($n = 14$) and healthy controls ($n = 70$) were analysed by ELISA. For detection of antibodies to IgG-type murine BiP, canine Grp94 and human calnexin wells was coated and ELISAs were carried out as has been described in detail in the 'Patients, materials and methods' section. Data points represent the percentage of the mean of the healthy donors. Statistical analysis was performed using Welch's *t*-test for RA and SLE patients and Mann–Whitney U-test for SSc and IBD patients if Gaussian distribution could not be assumed.

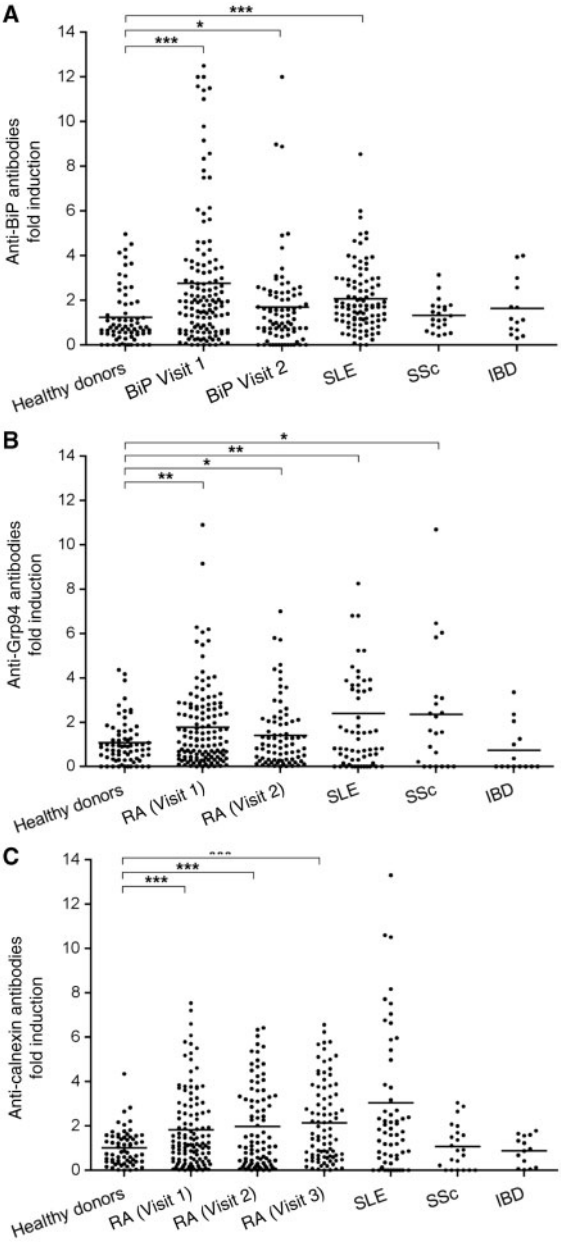
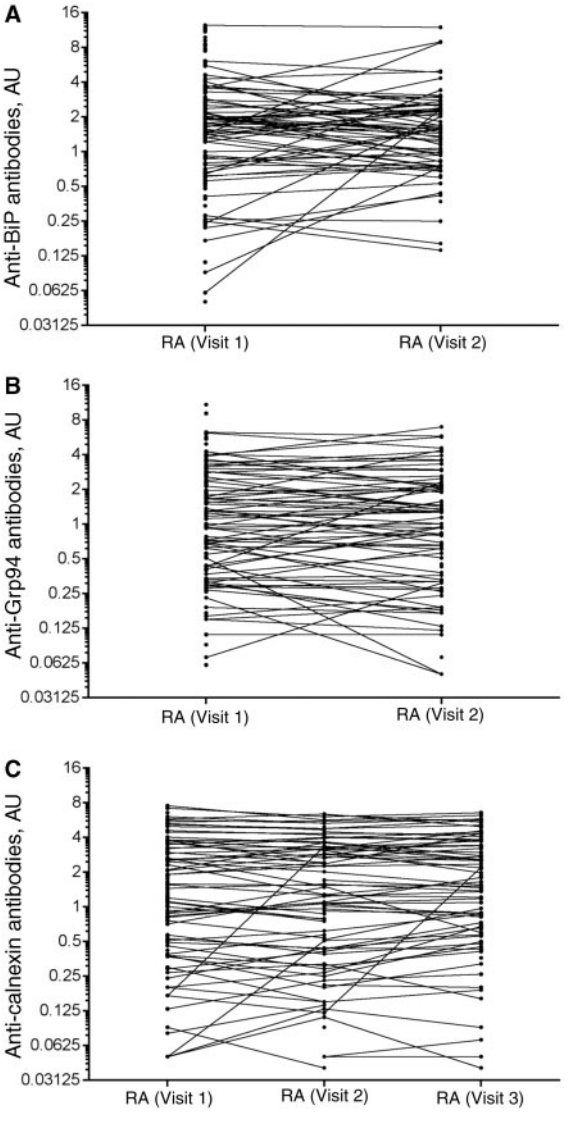


Fig. 4 Anti-BiP (**A**), anti-Grp94 (**B**) and anti-calnexin (**C**) antibody profiles of patients with RA of consecutive visits. Autoantibodies to BiP, Grp94 and calnexin were analysed by ELISA in sera of RA patients with consecutive visits. Antibody titres in arbitrary units are shown in natural logarithmic scale with linked dots for the same patient of consecutive visits.



autoantibody specificities by partial correlation and controlled these relationships for the influence of age and gender. Table 2 displays a highly significant correlation between antibodies to calnexin and those to BiP and to Grp94 in at least two visits of patients with RA.

Similarly, the presence of autoantibodies against these three ER chaperones correlated with each other in patients with SLE (Table 3). Partial correlation was also performed for healthy donors, although very few healthy donors displayed elevated anti-ER chaperone antibody titres. The appearance of anti-calnexin and anti-Grp94

Table 2 Partial correlation for anti-ER chaperone antibodies in patients with RA controlled for gender and age

	Partial correlation controlled for gender and age		RA					
			Calnexin Visit 1	BiP Visit 1	Grp94 Visit 1	Calnexin Visit 2	BiP Visit 2	Grp94 Visit 2
RA	Calnexin Visit 1	$r_{partial}$						
		P-value						
		n						
	BiP Visit 1	$r_{partial}$	0.213**					
		P-value	0.022					
		n	115					
	Grp94 Visit 1	$r_{partial}$	0.579**	0.548**				
		P-value	0	0				
		n	115	127				
	Calnexin Visit 2	$r_{partial}$	0.914**	0.444**	0.856**			
		P-value	0	0.004	0			
		n	71	79	79			
	BiP Visit 2	$r_{partial}$	0.444**	0.830**	0.519**	0.375**		
		P-value	0.001	0	0	0.001		
		n	70	78	78	78		
	Grp94 Visit 2	$r_{partial}$	0.819**	0.569**	0.894**	0.802**	695**	
		P-value	0	0	0	0	0	
		n	70	78	78	78	78	
	Calnexin Visit 3	$r_{partial}$	0.917**	0.458**	0.791**	0.869**	0.428**	0.807**
		P-value	0	0	0	0	0.001	0
		n	53	61	61	67	61	61

**$P < 0.01$ (two-tailed).

antibodies seems to be synchronized also at lower levels, as the partial correlation was significant (Table 3).

Anti-ER chaperone antibodies did not correlate with serological and clinical parameters of RA diagnosis such as the presence of anti-CCP antibodies, RF, with disease activity measured by 28-joint DAS (DAS-28) or RA susceptibility alleles, namely HLA-DR1 and HLA-DR4 (data not shown).

Discussion

In this study, we identified the ER chaperones calnexin and Grp94 as novel autoantigens in RA as well as calnexin in SLE. Autoantibodies against Grp94 have been described very recently in SLE patients [30–32]. Patients with RA and SLE had significantly higher titres of autoantibodies against calnexin, BiP and Grp94 compared with healthy donors or with patients with SSc or IBD. The selection of calnexin, BiP and Grp94 as potentially new antigens was based on the following considerations: (i) BiP appears to be involved in the pathogenesis of experimental arthritides, since immunization or tolerance induction with BiP markedly influenced the disease course [31]; (ii) due to their essential chaperone function, HSPs play an important role in proper MHC folding and peptide loading and certain MHC alleles have been associated with RA [33, 34]; and (iii) calnexin, BiP and Grp94 are ER-resident proteins of eukaryotic cells. With respect to the immunogenic mechanism, a bacterial trigger for induction of autoantibody generation against Grp94 or BiP cannot be completely excluded. However, cross-reactivity to prokaryotic HSP and chaperone family members is unlikely. With regard to Grp94 and BiP, bacteria express solely the cytoplasmic representatives HtpG and DnaK that belong to the family of Hsp90 and Hsp70, respectively. The identity between human Grp94 and HtpG from *E. coli* is 32%, whereas human BiP shows 46% identical stretches with DnaK from *E. coli* or *Streptococcus pneumoniae* (data not shown). Likewise, a bacterial infection as initial trigger for autoantibody production against calnexin is extremely unlikely, since calnexin is entirely restricted to eukaryotic cells.

In 1986, Srivastava *et al.* [35] reported induction of an anti-tumour immune response by immunization of mice with tumour-derived Grp94. These experiments created the basis for further investigations on cytosolic and ER chaperones that have been shown to bind antigenic peptides generated within the cells. Chaperones transport and load these peptides to the MHC Class I and II molecules and, therefore, interact physically with MHC I and II molecules [33, 34, 36]. Under yet undefined pathological circumstances, ER chaperones, or fragments of ER chaperones, might be displayed on the cell surface by hijacking the MHC II surface transport machinery. Selective proteolysis, especially for chaperones such as BiP and Hsp60 in peripheral blood mononuclear cells of RA patients has been described, providing a putative mechanism for the generation of neoantigens [37].

The generation of antibodies against calnexin, BiP and Grp94 seems at least to be independent of the presence

TABLE 3 Partial correlation for anti-ER chaperone antibodies in normal healthy donors and in patients with SLE controlled for gender and age

Partial correlation controlled for gender and age	NHD			SLE		
	Calnexin	BiP	Grp94	Calnexin	BiP	Grp94
Calnexin						
$r_{partial}$						
P-value						
n						
BiP						
$r_{partial}$	0.153			0.547**		
P-value	0.251			0.000		
n	57			52		
Grp94						
$r_{partial}$	0.454**	0.280*		0.494**	0.402**	
P-value	0.000	0.025		0.000	0.000	
n	57	63		52	52	

*$P < 0.05$ and **$P < 0.01$ (two-tailed).

of HLA-DR1 and HLA-DR4 alleles, because no correlation between HLA-DR1- or HLA-DR4-positive RA patients and the generation of anti-ER chaperone antibodies was observed. Interestingly, antibodies against ER chaperones were detectable already early during the disease course of RA within the first 3–12 months after onset of symptoms, and their titres remained stable. Hence, autoimmunity to ER chaperones might not be just a consequence of long-lasting tissue destruction in RA. Rather, anti-ER chaperone immune responses could be involved in the pathogenic process. Moreover, antibodies against calnexin, BiP and Grp94 also occur in the absence of RF or anti-CCP antibodies, suggesting that antibodies to these ER chaperones might represent a useful novel diagnostic tool in RA, especially in those lacking RF or anti-CCP antibodies.

Rheumatology key messages

- Sera from patients with RA and SLE contain significantly higher anti-calnexin, anti-BiP and anti-Grp94 antibody titres than healthy donors.
- ER chaperone autoantibody titres mutually correlate in RA and SLE patients.

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References

1 Doan T, Massarotti E. Rheumatoid arthritis: an overview of new and emerging therapies. J Clin Pharmacol 2005;45:751–62.

2 Young BJ, Mallya RK, Leslie RD, Clark CJ, Hamblin TJ. Anti-keratin antibodies in rheumatoid arthritis. Br Med J 1979;2:97–9.

3 Steiner G, Hartmuth K, Skriner K *et al*. Purification and partial sequencing of the nuclear autoantigen RA33 shows that it is indistinguishable from the A2 protein of the heterogeneous nuclear ribonucleoprotein complex. J Clin Invest 1992;90:1061–6.

4 Burkhardt H, Koller T, Engstrom A *et al*. Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse. Arthritis Rheum 2002;46:2339–48.

5 Burkhardt H, Yan T, Broker B *et al*. Antibody binding to a collagen type-II epitope gives rise to an inhibitory peptide for autoreactive T cells. Eur J Immunol 1992;22:1063–7.

6 Burkhardt H, Sehnert B, Bockermann R, Engstrom A, Kalden JR, Holmdahl R. Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. Eur J Immunol 2005;35:1643–52.

7 Goeb V, Thomas-L'Otellier M, Daveau R *et al*. Candidate autoantigens identified by mass spectrometry in early rheumatoid arthritis are chaperones and citrullinated glycolytic enzymes. Arthritis Res Ther 2009;11:R38.

8 Lydyard PM, Tsoulfa G, Sharif M, Broker B, Smith M, Rook GA. Immunity to heat shock proteins in rheumatoid arthritis. Clin Exp Rheumatol 1990;8(Suppl. 5):69–74.

9 Jarjour WN, Jeffries BD, Davis JS 4th, Welch WJ, Mimura T, Winfield JB. Autoantibodies to human stress proteins. A survey of various rheumatic and other inflammatory diseases. Arthritis Rheum 1991;34:1133–8.

10 Georgopoulos C, McFarland H. Heat shock proteins in multiple sclerosis and other autoimmune diseases. Immunol Today 1993;14:373–5.

11 Salvetti M, Buttinelli C, Ristori G *et al*. T-lymphocyte reactivity to the recombinant mycobacterial 65- and 70-kDa heat shock proteins in multiple sclerosis. J Autoimmun 1992;5:691–702.

12 Stevens TR, Winrow VR, Blake DR, Rampton DS. Circulating antibodies to heat-shock protein 60 in Crohn's disease and ulcerative colitis. Clin Exp Immunol 1992;90: 271–4.

13 Pervin K, Childerstone A, Shinnick T *et al*. T cell epitope expression of mycobacterial and homologous human 65-kilodalton heat shock protein peptides in short term cell lines from patients with Behcet's disease. J Immunol 1993;151:2273–82.

14 Nardai G, Vegh EM, Prohaszka Z, Csermely P. Chaperone-related immune dysfunction: an emergent property of distorted chaperone networks. Trends Immunol 2006;27:74–9.

15 Buchner J. Supervising the fold: functional principles of molecular chaperones. FASEB J 1996;10:10–9.

16 Williams DB, Watts TH. Molecular chaperones in antigen presentation. Curr Opin Immunol 1995;7:77–84.

17 Srivastava PK. Peptide-binding heat shock proteins in the endoplasmic reticulum: role in immune response to cancer and in antigen presentation. Adv Cancer Res 1993;62: 153–77.

18 Zugel U, Kaufmann SH. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clin Microbiol Rev 1999;12:19–39.

19 Theofilopoulos AN. The basis of autoimmunity: part I. Mechanisms of aberrant self-recognition. Immunol Today 1995;16:90–8.

20 Arnett FC, Edworthy SM, Bloch DA *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; 31:315–24.

21 Tan EM, Cohen AS, Fries JF *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.

22 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997;40: 1725.

23 LeRoy EC, Medsger TA Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol 2001;28:1573–6.

24 Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol 1989;170(Suppl. 170):2–6; discussion 16–9.

25 Frey S, Leskovar A, Reinstein J, Buchner J. The ATPase cycle of the endoplasmic chaperone Grp94. J Biol Chem 2007;282:35612–20.

26 Mayer M, Kies U, Kammermeier R, Buchner J. BiP and PDI cooperate in the oxidative folding of antibodies in vitro. J Biol Chem 2000;275:29421–5.

27 Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest 1998;101:273–81.

28 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–5.

29 Chenna R, Sugawara H, Koike T *et al*. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003;31:3497–500.

30 Bodman-Smith MD, Corrigall VM, Berglin E *et al*. Antibody response to the human stress protein BiP in rheumatoid arthritis. Rheumatology 2004;43:1283–7.

31 Corrigall VM, Bodman-Smith MD, Fife MS *et al*. The human endoplasmic reticulum molecular chaperone BiP is an autoantigen for rheumatoid arthritis and prevents the induction of experimental arthritis. J Immunol 2001;166: 1492–8.

32 Boehm J, Orth T, Van Nguyen P, Soling HD. Systemic lupus erythematosus is associated with increased auto-antibody titers against calreticulin and grp94, but calreticulin is not the Ro/SS-A antigen. Eur J Clin Invest 1994;24:248–57.

33 Ishii T, Udono H, Yamano T *et al*. Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. J Immunol 1999;162:1303–9.

34 Li Z, Menoret A, Srivastava P. Roles of heat-shock proteins in antigen presentation and cross-presentation. Curr Opin Immunol 2002;14:45–51.

35 Srivastava PK, DeLeo AB, Old LJ. Tumor rejection antigens of chemically induced sarcomas of inbred mice. Proc Natl Acad Sci USA 1986;83:3407–11.

36 Srivastava PK. New jobs for ancient chaperones. Sci Am 2008;299:50–5.

37 Schulz M, Dotzlaw H, Mikkat S, Eggert M, Neeck G. Proteomic analysis of peripheral blood mononuclear cells: selective protein processing observed in patients with rheumatoid arthritis. J Proteome Res 2007;6:3752–9.