Antibody response to the human stress protein BiP in rheumatoid arthritis


Objectives. The human stress protein BiP (immunoglobulin binding protein) has been implicated in the pathogenesis of rheumatoid arthritis (RA) since BiP was found to stimulate synovial T-cell proliferation and anti-BiP antibodies are present in the sera of RA patients. The aim of this study was the development of a rapid and reproducible enzyme-linked immunosorbent assay (ELISA) to determine the specificity and sensitivity of anti-BiP antibodies in RA.

Methods. An ELISA was developed that detected antibodies to BiP. The prevalence of anti-BiP antibodies was determined in sera from patients with early and established RA, sera antedating the onset of RA and sera from patients with other inflammatory and autoimmune diseases and healthy controls.

Results. We have confirmed the increased prevalence of antibodies to BiP in the sera of a large cohort of patients with established RA (specificity 71% and sensitivity 73%) and early RA (specificity 65% and sensitivity 66%). In pre-disease sera, median 2.5 yr (interquartile range 1.1–4.7) before symptoms of joint disease, the sensitivity for anti-BiP antibodies was 45% and the specificity was 65% for the development of RA.

Conclusion. Antibodies to BiP are found in the sera of patients with RA and in sera antedating the onset of RA.

KEY WORDS: Rheumatoid arthritis, Heat shock protein, Antibodies, ELISA.
serum and synovial fluid from a number of patients with RA were also examined.

Materials and methods

Production of BiP

BiP was purified from an E. coli expression system as described previously [15].

Serum collection

Ninety-six RA sera [18] from the Guy’s Hospital rheumatology serum bank were used, with a 2.8:1 female to male ratio. The mean age was 58.2 yr with a range from 24 to 100 yr. The control groups consisted of 51 ‘other inflammatory joint disease’ (OIJD) patients [psoriatic arthritis n = 16, giant cell arteritis n = 1, systemic lupus erythematosus (SLE) n = 2, polymyalgia rheumatica n = 11, ankylosing spondylitis n = 8, palindromic arthritis n = 2, seronegative arthritis n = 2, undifferentiated arthritis n = 1, polyarthritis n = 1, reactive arthritis n = 2, scleroderma n = 1, mixed connective tissue disease n = 1], Behçet’s disease n = 1, primary Sjögren’s syndrome n = 1, inflammatory muscle disease n = 1] and 45 normal controls. The mean age of the OIJD group was 55.2 years and the range 34–100, the female to male ratio was 1.6:1. The mean age of the normal group was 51.8 years with a range of 28–79 and the female to male ratio was 1.8:1.

Appropriate ethical committee approval was granted for the taking and storing of serum from patients included in this study. Patient consent was obtained before the collection of blood samples (Guy’s Research Ethics Committee no 01/05/01).

Sera from pre-diagnosis RA and early RA

Eighty-three individuals fulfilling the classification criteria for RA and who had given blood samples before presenting with any symptoms or signs of joint disease were identified in the Northern Sweden Health and Disease Study Cohort and Maternity Cohort of Northern Sweden. Samples from 382 different control individuals (326 females and 56 males) and 83 cases (69 females and 14 males) were identified for the study. The mean age of the pre-patients when sampled was 48 yr, range 20–67 yr, and of the controls 49 yr, range 19–69 yr, respectively. At their first visit at the Early Arthritis Clinic when RA was diagnosed blood was sampled in 67 (55 females and 12 males) of the original 83 pre-patients. Anti-BiP levels were determined blind and the code broken at the end of the study. Comparisons were made between those individuals that went on to develop RA (83) and the remainder of the samples (382).

Synovial fluid

Heparinized synovial fluid from RA patients was received from the Guy’s Rheumatology Clinic, centrifuged at 2000 rpm for 10 min to remove cells and was stored at −20 °C until used.

Anti-BiP ELISA

ELISA plates (Imunosorp, Nunc) were coated with 10 μg/ml human recombinant BiP in 0.1 M NaHCO₃, pH 8.2. Non-specific binding was blocked by the addition of 10% goat serum (Sigma). 100 μl of 1/100 diluted serum was added and the plate incubated overnight at 4 °C. Detection antibody (goat anti-human IgG-horse radish peroxidase (Sigma) diluted 1/20 000 in PBS with 1% bovine serum albumin and 0.05% Tween 20) was added to each well. The plates were developed with 3,3’,5,5’-tetramethylbenzidine (TMB) (Sigma). Plates were read at 450 nm. In all cases one-half of each plate was coated with BiP and the other half coated with buffer alone to control for non-specific binding of serum to the plate. A reference serum (from a patient with RA), known to bind highly to BiP, was chosen and incorporated into each assay to allow meaningful comparisons of different plates and between different experiments. Results are expressed as the specific binding to BiP (as optical density (OD) of BiP-coated wells (mean of two wells) minus the corresponding non-specific OD) as a ratio of the BiP specific binding of the reference serum. To control for anti-E. coli contaminants and the effect of the histidine tag, β-galactosidase, produced using the same method as BiP, was used as the coating antigen.

Results

Prevalence of anti-BiP antibodies in RA, other inflammatory joint diseases and healthy controls

Figure 1a shows antibody levels to BiP of 96 RA patients and 96 controls from the Guy’s Hospital serum bank. There is a significant difference (P = 0.001, Mann–Whitney U-test) between the binding of the RA sera (mean = 0.44, s.d. ± 0.28) and the control population (mean = 0.29 ± 0.21). When the control data are separated into OIJD and healthy controls, RA sera were significantly different from both the OIJD (P = 0.01) and the normal healthy control sera (P = 0.001). Moreover there is a significant difference between the RA group and other arthritides (reactive arthritis, psoriatic arthritis and osteoarthritis) (mean = 0.44 ± 0.28 vs 0.28 ± 0.19; P < 0.001, Student’s t-test). To confirm these findings, 60 RA sera were obtained from AGT (Athens, Greece). The results were determined blind, with occasional duplicated samples. The Greek sera showed no significant difference from the Guy’s sera (Greek mean 0.48 ± 0.36; Guy’s mean 0.44 ± 0.28; P = 0.7, Student’s t-test). Moreover the repeat samples showed less than 5% variation (data not shown).

The receiver operating characteristics curve was generated by calculating the specificity and sensitivity of the assay at a range of cut-off values. At a cut-off value set at the upper 95% confidence interval (95% CI) of the control data, then the specificity of the assay for RA is 71.0% and the sensitivity 73.0%.

In 14 RA and 11 OIJD synovial fluid samples tested (Fig. 1b), the anti-BiP antibody levels in RA synovial fluid were significantly higher than in OIJD (P = 0.04). The levels of anti-BiP antibody were, however, similar in the RA paired serum and synovial fluid (data not shown). By contrast, there was a significant decrease in the concentration of anti-BiP antibodies in the OIJD synovial fluid compared with their matched serum.

Anti-BiP levels in pre-diagnosis RA sera

In the 98 samples from 83 individuals pre-dating the onset of RA (pre-patients) the prevalence of anti-BiP antibodies was 45% compared with 35% in controls (χ² = 3.405, P > 0.05). When the samples from the pre-patients were stratified for time, samples collected more than 1.5 yr before symptoms showed a sensitivity for anti-BiP antibodies of 40% and a specificity of 70%. In pre-patient samples taken less than 1.5 yr before symptoms the sensitivity for anti-BiP antibodies was 55% and the specificity 56%. In patients with early RA the prevalence of anti-BiP antibodies was significantly increased, 66% (44/67), compared with controls (χ² = 22.72, P < 0.001). The frequency of anti-BiP antibodies increased significantly (χ² for trend = 9.00, P < 0.01) during the antedating time (calculated on divided intervals) the closer to the time of disease onset (Fig. 2). Upon developing anti-BiP antibodies, all individuals but five remained positive and the
We have confirmed, by means of a specific ELISA, previously published data [15], using Western blotting, that levels of antibodies to BiP are raised in the sera of patients with RA. Our original observation was confirmed by Blass et al. [17] using BiP purified from a mammalian cell source. These authors suggested that glycosylation of BiP was necessary for antibody binding. Our data, however, show that this is not necessary since the BiP used in our ELISA is produced in an *E. coli* expression system and is not glycosylated. A direct comparison, however, of glycosylated and non-glycosylated BiP needs to be performed to resolve this issue. To this end, experiments in collaboration with Professor Burmester are being discussed. We have shown an increase in the levels of anti-BiP antibodies in a cohort of established RA patients at Guy’s Hospital. There is a significant increase above the levels found in the sera of control groups, either normal healthy controls or patients with other inflammatory joint diseases. Moreover, this level of antibody was mirrored in a cohort of RA sera from patients with established disease in Athens, suggesting that the presence of anti-BiP antibodies is not confined to racial groups or geographical areas but, rather, is a manifestation of the disease itself. Our data suggest that antibodies to BiP, as measured by this method, have sensitivity for established RA of 73% and a specificity of 71% and for early RA of 66 and 65%, respectively. The paper by Blass et al. [17] gave a sensitivity value of 68% for antibodies to BiP, and a specificity of 96%.

The frequency of anti-BiP antibodies was not significantly different between pre-patients and controls in the samples from northern Sweden. However, our data show an increasing antibody level to BiP as the patient approaches the onset of arthritis. By the time the patient is diagnosed with early RA there is a significant increase in anti-BiP antibodies when compared with controls. These data may provide a potential diagnostic role for anti-BiP antibodies in RA when used in patients with early arthritis.

ELISA used to measure serum antibodies can be affected by a number of factors. Therefore, verification of such an ELISA needs to be performed. One complicating factor which might result in false results is that of non-specific binding of the serum to the plate. To control for this we have calculated our anti-BiP levels by subtracting the non-specific binding (that seen with no antigen, or irrelevant antigen produced in the same expression system as BiP and isolated using the same technique) away from the specific binding, thereby giving a true representation of the anti-BiP levels. Levels of RF, which may affect ELISAs measuring immunoglobulin levels, did not influence anti BiP levels. Correlations between RFs of the IgM, IgG and IgA type and anti-BiP binding showed no link between RF and anti-BiP. Neither does heat aggregated IgG, monomeric IgG or IgM bind in the assay (data not shown).

We also demonstrate anti-BiP antibodies in the synovial fluid of patients with RA at corresponding levels to those seen in the serum. The levels are significantly increased over those seen in control synovial fluid. This would suggest that anti-BiP antibodies are made in both the primary and secondary lymphoid organs as well as the joint. It is of interest to note that IgM RF is

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**Fig. 1.** (a) There is a significant increase in antibody levels to BiP in RA sera (mean = 0.44, s.d. = 0.28) when compared with all controls (mean = 0.29, s.d. = 0.21; *P* = 0.001 Mann–Whitney *U*-test). When the control sera are subdivided there is a significant increase in anti-BiP in RA sera compared with OIJD sera (mean = 0.33, s.d. = 0.20; *P* = 0.01), ‘normal’ sera (mean = 0.24, s.d. = 0.21; *P* = 0.001) and ‘other arthritides’ (mean = 0.28, s.d. = 0.19; *P* < 0.001). Box and whisker plot shows median, 5th, 10th, 25th, 75th, 90th and 95th percentiles of the data set. (b) There is a significant increase in anti-BiP antibody levels in the synovial fluid of RA patients (mean = 0.32, s.d. = 0.33) when compared with OIJD (mean = 0.09, s.d. = 0.11; *P* = 0.04 Mann–Whitney *U*-test). Box and whisker plot shows median, 5th, 10th, 25th, 75th, 90th and 95th percentiles of the data set.

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**Fig. 2.** The titre of anti-BiP antibodies antedating the onset of RA (*n* = 83) in blood samples taken >1.5–16 yr before symptoms (sample 1) and <1.5 yr before symptoms (sample 2) and at the diagnosis of RA (*χ*² for trend = 9.00, *P* < 0.01).
found at a higher concentration in the joint fluid, with the implication that there is local production [19]. This has been confirmed by studies demonstrating the presence of RF producing plasma cells in the RA synovial compartment [20].

The mechanisms that lead to the induction of autoantibodies are still poorly understood, but events during apoptosis may be involved. Rao et al. [21] have proposed that BiP can act as an apoptosis regulator by controlling the cellular response to endoplasmic reticulum stress-induced cell death. They report that BiP can block caspase activation and thereby block caspase-mediated cell death. This is contradicted by the observation that BiP messenger ribonucleic acid is up-regulated during programmed cell death in nerve cells [22], although this could be as consequence of the cell’s attempt to protect against apoptosis. It is of interest to note that Ro, La and BiP are found in the same small surface apoptotic blebs [23]. Furthermore, Kinoshita et al. [24] have reported that mice, when repeatedly immunized with both Ro52 and Ro60 protein, eventually mount an antibody response against BiP, suggesting an interplay between the proteins. However, we were unable to find a correlation between antibodies to BiP and Ro and La in our studies (data not shown).

In summary, we have developed an ELISA that has shown elevated levels of anti-BiP antibodies in RA. The ELISA is robust and gives a specificity of 71% and a sensitivity of 73% for established RA. Antibodies to BiP are identified in samples predating the onset of RA, although with a low specificity. This study did not attempt to correlate anti-BiP levels with either disease severity or duration of established disease, but both of these parameters are under investigation. We suggest that anti-BiP antibodies may be a useful additional autoantibody test in patients with RA.

### Key messages

- Antibodies to the human heat shock protein BiP are elevated in RA.
- Antibodies to BiP are present before diagnosis of RA and increase towards that diagnosis.

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MB-S, VC and GP are shareholders in, and GP is a director of, Immune Regulation Ltd, a biotechnology company investigating the role of heat shock proteins in autoimmune disease, which holds patent rights in BiP for diagnostic tests. The other authors have declared no conflicts of interest.

### References


