Novel 68 kDa autoantigen detected by rheumatoid arthritis specific antibodies

S Bläß, C Specker, H-J Lakomek, E M Schneider, M Schwochau

Abstract

Objective—To improve the understanding of the pathogenesis of rheumatoid arthritis (RA) by identifying novel, disease specific autoantibodies.

Methods—Total protein preparations from synovial membranes were separated electrophoretically and immunoblotted. Sera from RA patients were screened for predominant immunoreactions by blotting. A 68 kDa antigen target of the most predominant reaction was detected and further characterised.

Results—The dominant immunoreaction in most of the RA sera tested was with a 68 kDa antigen. The antigen is probably ubiquitously expressed. It has an isoelectric point of 5.1, is O-glycosylated, and is located in the endoplasmic reticulum, the cytoplasm, or both. Antibodies to the 68 kDa autoantigen were present in 64% of 167 RA patients tested, and could also be detected in seronegative RA patients, but were present in only 1% of 98 patients with other rheumatic diseases. They could not be detected in 55 healthy controls.

Conclusions—Because of its high sensitivity (64%) and specificity (99%), the anti-68 kDa autoantibody not only provides another valuable parameter for diagnosis, but also represents an antibody that may be involved in the pathological mechanisms leading to RA. This hypothesis can be tested by investigating if 68 kDa specific T cells are present in RA patients.

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Institute for Genetics,
University of
Düsseldorf,
Düsseldorf, Germany
S Bläß
M Schwochau

Department of
Rheumatology
C Specker
H-J Lakomek

Institute of Blood
Coagulation and
Transfusion Medicine
E M Schneider

Correspondence to:
S Bläß,
Inst. für Genetik,
Heinrich-Heine-Universität
Düsseldorf,
Universitätsstr. 1,
D-40225 Düsseldorf,
Germany.

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Rheumatoid arthritis (RA) is the most frequent systemic rheumatic disease and is characterised by chronic inflammation of synovial membranes, usually followed by irreversible degradation of joint cartilage and bone. The aetiology and pathogenesis of RA are not fully understood, which renders impossible any therapy directed against the cause. Despite commonly applied clinical criteria, early diagnosis of RA often remains difficult, leading to delays in suitable treatment. Recent studies revealing marked progress within the first years of the disease stress the importance of early diagnosis and improved diagnostic confidence^{1 2} to facilitate early commencement of disease modifying therapy.

With respect to diagnosis, the rheumatoid factor (RF) is the only serological parameter

included in the American Rheumatism Association (ARA) criteria for RA:³ a seropositive type of RA is distinguished from a seronegative type on the basis of the presence or absence of IgM RF. RF can be detected in approximately 70% of RA patients, but also in up to 19% of other systemic rheumatic diseases; it even occurs in apparently healthy individuals, so that the RF assay must be adjusted to recognise only up to 5% of these subjects as positive.^{4 5} Additional RA specific autoantibodies have been described, among which is an anti-33 kDa antibody present in 36% of RA patients.⁶ Further serological parameters, especially disease specific autoantibodies, could permit earlier diagnosis of RA, and with greater confidence. Furthermore, the identification of an RA specific autoantigen might help in understanding the pathological mechanisms leading to the disease. Were such an antigen to be known and characterised, the associated T cell immune response could be analysed and probably specifically modified.

In the present study, sera from patients documented in a data bank comprising approximately 2000 patients with various rheumatic diagnoses were screened for RA specific immunoreactions. Total protein preparations of synovium were analysed because synovium is the tissue affected most during the course of RA. We identified, purified and further characterised a previously undescribed autoantigen that was the target of antibodies occurring specifically in RA patients.

Patients and methods

PATIENTS

Patients attending the Rheumatology Unit of the University of Düsseldorf were diagnosed as having RA (according to the ARA criteria³), systemic lupus erythematosus (SLE) (ARA criteria⁷), systemic sclerosis (SSc) (preliminary criteria for the classification of systemic sclerosis⁸), overlap syndrome (clinical signs of at least two systemic rheumatic diseases present), ankylosing spondylitis (AS) (New York criteria⁹), psoriatic arthritis (typical asymmetric peripheral arthritis with destructive and osteoproliferative joint changes, psoriatic skin lesions and lack of rheumatoid factor or subcutaneous nodules), reactive arthritis (Aho¹⁰), morphea (localised sclerosis without the serology and organ manifestation of SSc), and osteoarthritis (OA) (Altman *et al*¹¹).

The 'Düsseldorf Rheumaregister', a database comprising records of some 2000 patients

with different rheumatic diseases, provided data for calculation of sensitivity and specificity of the rheumatoid factor.

RHEUMATOID FACTOR ASSAY

RF values were determined by laser nephelometry applying the LN-latex-RF reagent obtained from the Behringwerke AG, Marburg, FRG.

BIOLOGICAL MATERIAL

Synovial membrane was obtained from RA patients attending our rheumatology unit. The synovium was biopsy material resulting from joint replacements or synovectomies (Prof Dr K-P Schultz, Orthopädische Klinik der Universität Düsseldorf) and was obtained with the patients' permission. It was pulverised in liquid nitrogen. An average of 200 µg of total protein was isolated from 1 g of synovial membrane applying the procedure described below. With HeLa cells or lymphocytes, about 400 µg of protein was typically obtained from 1 g of material.

GENERAL PROCEDURE FOR TOTAL PROTEIN PREPARATION

In order to obtain reproducible isolation of undegraded 68 kDa antigen, we found it essential to proceed *immediately* to the lysis of our cell suspensions or pulverised tissue in buffer G (6 mol/l urea, 5% 2-mercaptoethanol, 1% gardol, 0.1 mol/l NaCl, 10 mmol/l Tris/HCl, pH 7.2). The material was homogenised in a tight fitting homogeniser (Dounce) and lysed subsequently for 15 minutes at 60°C. Proteins were separated from nucleic acids and polysaccharides by centrifugation in a caesium chloride (CsCl) solution with a final density of about 1.6 g/ml for one hour at 30 000 rpm (Beckman SW40, about 120 000 g). The resulting float (proteinaceous material accumulated at the top of the centrifugation tube, above the supernatant) contained mostly peptides, with a few very dense glycoproteins, and could be stored without degradation at temperatures less than -15°C for any length of time. For good and reproducible electrophoretic separations of the isolated antigens, it was necessary to remove most of the gardol and CsCl. Floats were therefore dissolved in buffer N (similar to buffer G, but containing 1% Nouidet P40 (NP40) instead of gardol) and, to avoid degradation, dialysed against 25 mmol/l Tris/HCl at 10°C as quickly as possible (30 minutes, twice). The short time scale was achieved by dialysis in a floppy bag measuring no more than 2 mm high when lying on a flat support. Finally, dialysed proteins were precipitated with ethanol.

IMMUNOBLOTTING

Proteins were separated electrophoretically in discontinuous linear pore gradient gels (10–20% polyacrylamide, 2 mm thick) in the presence of 4 mol/l urea and 0.1% sodium

dodecyl sulphate (SDS). Gels were blotted semi-dry to nitrocellulose sheets; transfer results were visualised by staining with Ponceau S. The nitrocellulose sheets were then cut into strips and incubated with human sera diluted 1:50 in phosphate buffered saline (PBS) after blocking with 0.1% bovine serum albumin (BSA) in the presence of 1% NP40. Immuno-reactions were visualised either with a fluorescein isothiocyanate (FITC) conjugated anti-human Ig (from sheep) (MF01, Wellcome, England) under UV light, or by a biotinylated anti-human Ig, a streptavidin peroxidase complex, and diaminobenzidine (Amersham). The FITC conjugated antibody was used preferentially because it demonstrated fewest side reactions.

We screened 167 sera from RA patients, 98 sera of patients with other rheumatic diseases, and 55 sera from apparently healthy control subjects. Patient and control sera were tested for the anti-68 kDa antibody at the same time with the same antigen preparation, although we had shown the technique to give reproducible results when patients were tested at different time intervals and with different antigen preparations. All results were read by an observer blinded to the diagnosis.

AFFINITY PURIFICATION OF ANTIBODIES

Except for visualisation, blots were treated like immunoblots. The lane containing the antibody-antigen complex was cut out from a blot. The antibodies were eluted from the membrane bound antigen by rocking for 15 minutes in 3 mol/l potassium thiocyanate and 0.1% BSA, and subsequently centrifuged for 40 minutes at 5000 g in centricon micro-concentrators (Amicon), for desalting and concentration. The affinity purified antibody solution was stored at 8°C.

ISOELECTRIC FOCUS (IEF)

The 68 kDa protein separated in the first dimension in SDS-urea-polyacrylamide gel electrophoresis (PAGE) was cut out, homogenised in a sample buffer containing NP40 (or Tween 20) and run in the second dimension in an IEF. Gels were subsequently incubated in 5% perchloric acid and 25% isopropanol/5% acetic acid for about three days, changing solutions twice a day, and transferred semi-dry to nitrocellulose sheets. Immunoblotting was performed as described above.

CYTOIMMUNOFLUORESCENCE

Cells grown overnight on object slides were denatured in 3.7% formaldehyde and 0.1% NP40. Primary and secondary antibodies were incubated for one hour at room temperature each. The secondary antibody was FITC conjugated.

ANALYSIS OF GLYCOSYLATION

Deglycosylation experiments were performed either with immunoblots or with solvatised

Figure 1 Reaction of RA sera with immunoblots of synovium, lymphocytes and HeLa cells to detect the 68 kDa antigen, using FITC labelled sheep anti-human Ig. Lane 1 was incubated with secondary antibody only, lane 2 with a negative control serum, lanes 3–12 with the same set of RA sera for each tissue. A 68 kDa antigen is specifically detected by RA sera with comparable sensitivities. Further reactions are attributable to the secondary antibody, are present also in healthy controls, or are of much lower sensitivity.

protein material, to establish if the corresponding antibodies detected glycoepitopes within the 68 kDa antigens.

For preferential cleaving of O-linked sugars from polypeptide chains by alkaline β -elimination, dissolved or membrane bound proteins were treated with water or different concentrations of NaOH at 37°C for 16 hours. They were subsequently incubated with a negative control serum or anti-68 kDa positive RA serum. Immunoreactivity was detected using FITC conjugated sheep anti-human Ig.

Enzymes and lectins (O-glycosidase, neuraminidase, N-glycosidase; obtained from Boehringer/Mannheim, FRG) were applied as recommended by the manufacturer.

For sugar competition experiments, the anti-serum was pre-incubated with a 1 mol/l solution of the respective sugar (D-glucosamine or mannopyranoside). Subsequently the blot strip was incubated with this serum-sugar

solution, followed by incubation with secondary antibody.

Results

THE 68 kDa AUTOANTIGEN

Screening of sera from RA patients by immunoblotting revealed that 66% detected a 68 kDa antigen (fig 1). This 68 kDa antigen could not be detected by applying sera of healthy donors or the secondary antibody alone. No differences in protein or immunostaining patterns were evident when different RA or non-RA synovia were applied. Other human tissues (lymphocytes, HeLa cells, liver, spleen, sperm) were investigated, and all contained the 68 kDa antigen specifically reactive with RA sera. Affinity purified antibodies reactive with the 68 kDa synovial antigen also detected a 68 kDa antigen on lymphocyte and HeLa blots (fig 2), suggesting that the synovial 68 kDa protein is identical to that detectable in other tissues, and that the antigen is ubiquitously expressed. Further proof of the identity of the antigen in the various tissues was obtained in two dimensional separation studies in which 68 kDa lanes from SDS-PAGE separations of synovial, lymphocyte, and HeLa proteins were conducted to isoelectric focusing. A protein with an apparent isoelectric point of pH 5.1 was detected by RA sera in all tissues examined (data not shown). The antigen was demonstrated to be located in the endoplasmic reticulum or cytoplasm close to the nucleus, as identified by standard immunofluorescence techniques (fig 3). The antigen was also detected in mouse NS0 cells, but not in insect tissues or cells (data not shown).

Expression of the 68 kDa antigen could not be enhanced applying heat shock.

Deglycosylation experiments in which alkaline β -elimination was applied to HeLa 68 kDa total protein (dissolved or blotted) demonstrated hydroxide concentration dependent abolition of the immunoreactivity. Abolition was complete when a concentration of 10 mmol/l NaOH was used, and partial with 5 mmol/l NaOH (fig 4). No gel shift exceeding 1–2 kDa was observed (data not shown). Incubation of blot strips overnight at 37°C with O-glycosidase (5 mU) and neuraminidase

Figure 2 Cross reactivity of the 68 kDa antigens of synovium, lymphocytes, and HeLa cells. Affinity purified anti-68 kDa antibodies reacted with lymphocyte and HeLa blots, as detected using FITC labelled sheep anti-human Ig. Lane 1 was incubated with a negative control serum, lane 2 with a positive control, and lane 3 with the affinity-purified anti-68 kDa antibody from synovium. A 68 kDa band may be seen on both blots.

Figure 3 HeLa cells denatured in 3.7% formaldehyde and 0.1% NP40 reacted with anti-68 kDa antibody, as detected using FITC conjugated secondary antibody. **A:** Phase contrast micrograph. **B:** Fluorescence activation micrograph. Anti-68 kDa antibodies are present in the cytoplasm adjacent to the nucleus or the endoplasmic reticulum. No staining was detected with the control antibody. Horizontal bar represents 1 μ m.

(40 mU) abolished detection with anti-68 kDa positive sera, but incubation with N-glycosidase F (1 U) did not (fig 5). N-Acetyl glucosamine and D-glucosamine competed with the 68 kDa antigen for antibody binding,

Figure 4 Hydroxide concentration dependent abolition of immunoreactivity of the anti-68 kDa antibody to its antigen: deglycosylation applying alkaline β -elimination to HeLa total protein. **A:** Nitrocellulose membrane bound (Western blot) HeLa total protein preparations. Preincubation with: water (lanes 1 + 2); 5 mmol/l NaOH (lanes 5 + 6); 10 mmol/l NaOH (lanes 3 + 4). Incubation with: negative control serum (lanes 1, 3, 5), or anti-68 kDa positive RA serum (lanes 2, 4, 6). Detection with FITC conjugated sheep anti-human Ig. The extra bands at molecular masses less than 68 kDa are patient specific and are not relevant here. **B:** Dissolved HeLa total protein. Preincubation with: water (lanes 5 + 6); 5 mmol/l NaOH (lanes 1 + 2); 10 mmol/l NaOH (lanes 3 + 4). Separation by SDS-PAGE. Incubation with: negative control serum (lanes 1, 3, 5), or anti-68 kDa positive RA serum (lanes 2, 4, 6). Detection with FITC labelled sheep anti-human Ig.

Figure 5 Abolition of 68 kDa immunoreactivity by O-glycosidase and neuraminidase, but not N-glycosidase. HeLa blots incubated with: O-glycosidase + neuraminidase (lanes 1 + 2); N-glycosidase F (lanes 3 + 4); buffer (lanes 5–8). Subsequent incubation with anti-68⁺ RA serum (lanes 1–6) or negative control serum (lanes 7 + 8).

but mannopyranoside did not. Subsequent washing and incubation with serum alone reconstituted the reaction (fig 6). The 68 kDa antigen bound concanavalin A (con A) and could readily be eluted from con A Sepharose columns. Taken together, these data revealed the nature of the 68 kDa protein as an O-glycosylated glycoprotein gp68 with only a few sugar residues.

THE ANTI-68 kDa AUTOANTIBODY

We identified an RA specific anti-68 kDa autoantibody by applying human synovial membrane protein preparations as a homotypic antigen pool. Of the 167 RA sera screened on immunoblots to test the sensitivity of this antibody, 66% were anti-68 kDa positive (fig 7). Antibodies to a 68 kDa antigen with sensitivities (75% and 58%) comparable to that of the synovial 68 kDa antigen were observed in lymphocyte and HeLa total protein preparations (fig 1). The 98 sera of patients with other rheumatic diseases screened on immunoblots to test specificity comprised 26 SLE, 15 SSs, four overlap syndrome, 12 AS, 16 psoriatic arthritis, four reactive arthritis,

Figure 6 Sugar competition experiments. HeLa blots incubated with preincubated sera. Lanes 1–2: Reacted with RA serum pretreated with glucosamine; detection with FITC conjugated secondary antibody. Lane 1: Subsequently incubated with untreated RA serum. Lane 3: Negative control serum incubated with glucosamine. Lanes 4–6: Same situation for incubation with mannopyranoside. Lane 7: Untreated positive control. Lane 8: Untreated negative control.

Figure 7 Sensitivity and specificity of the anti-68 kDa antibody compared with rheumatoid factor. Separate sera were screened, on synovium blots for the anti-68 kDa antibody, and by laser nephelometry for rheumatoid factor. Sensitivities: 66% for the anti-68 kDa antibody (□) and 68% for the RF (■). Specificities: 99% for the anti-68 kDa antibody and 76% for RF.

10 morphea, 10 OA. Only one serum of the rheumatic control group (an SLE patient) and none of 55 apparently healthy controls also screened (fig 7) showed immunoreaction with the 68 kDa antigen. Thus specificity of the anti-68 kDa antibody can be calculated as 99%.

In the retrieval study from the 'Düsseldorf Rheumaregister', data of 277 RA patients and 893 patients with other rheumatic diseases were analysed for the presence of the rheumatoid factor. Sensitivity was 68% and specificity 76%, taking into account that 5% of the apparently healthy population is also positive (fig 7). The anti-68 kDa antibody is thus of equal sensitivity (66% v 68%) and of even greater specificity (99% v 76%) than the rheumatoid factor (fig 7).

The anti-68 kDa antibody was also detected in seven out of 12 seronegative RA sera. Clinical comparison of 31 seropositive and seronegative RA patients (table) did not show a statistically significant difference between them ($p > 0.05$).

Discussion

In this study a 68 kDa antigen, originally detected in synovium, was identified as an autoantigen specifically recognised by sera from RA patients. A 68 kDa antigen was also detectable in all human tissues investigated, and in mammalian, but not insect, cells. Affinity purified antibodies directed against the 68 kDa antigen of one tissue cross reacted with a 68 kDa antigen of others. The isoelectric

point of the 68 kDa antigen in the various tissues was shown to be 5.1. These data lead us to conclude that the 68 kDa antigen is identical in all tissues investigated and is probably expressed ubiquitously in man, or even in mammals generally. Its subcellular localisation was demonstrated to be the endoplasmic reticulum or the cytoplasm. Procedures cleaving off O-linked sugars abolished immunoreactivity with RA sera. There was no gel shift visible, and N-acetyl glucosamine competed with the 68 kDa antigen for binding to RA specific antibodies. O-glycosylated antigens localised in the endoplasmic reticulum or cytoplasm are not unprecedented.¹²

The physicochemical data of the 68 kDa antigen argue against identity with one of the autoantigens already known to be associated with autoimmune diseases. The 62 kDa human antigen (identical with Epstein-Barr virus antigen-1) is a nuclear antigen,¹³ while the 68 kDa antigen is cytoplasmic or endoplasmic. The molecular mass of RA33¹⁴ is much smaller than the 68 kDa antigen, although 33 kDa could be a degradation product of 68 kDa, which remains to be tested; the same applies for the 50 kDa Sa antigen,¹⁵ which could not be detected in HeLa cells, for example. The 68 kDa antigen was not heat inducible and thus appears not to be one of the hsp70 class of heat shock proteins. In addition, heat shock proteins differ from the 68 kDa antigen in their isoelectric points,¹⁶ as does the 68 kDa antigen of soluble nuclear ribonucleoproteins.¹⁷ In order to identify disease specific autoantibodies, a large number of xenotypic and homotypic antigen pools have been investigated.^{18, 19} It may be speculated that the particular method of protein preparation which we used, together with separation via SDS-PAGE in the presence of urea, enabled us to detect a hitherto unidentified antigen.

The anti-68 kDa autoantibody was present in 66% of RA sera, but in only one control patient (SLE). It was also present in seronegative patients, indicating that this novel antibody is not correlated with RF. The data suggest that this anti-68 kDa antibody could be a promising serological parameter well suited to promote earlier and improved diagnosis of RA when available in a recombinant form. Development of an enzyme linked immunosorbent assay could not only yield higher sensitivities, but would also permit screening of a greater number of patients, to investigate a probable association between disease activity and the presence of the anti-68 kDa antibody.

Existence of a ubiquitously expressed autoantigen fits well with the concept currently under discussion, that major proteins of cell metabolism are target antigens in autoimmune diseases.²⁰ The high RA specificity of the anti-68 kDa antibody also implies a role for autoimmunity against the 68 kDa antigen we describe, in the pathological mechanisms leading to RA. It is now generally accepted that RA is T cell mediated and maintained. Autoreactive T cells specific for the purified

The anti-68 kDa antibody in RA

	Anti-68 kDa	
	Positive (n = 20)	Negative (n = 11)
Disease duration (yr)	7.1	6.4
No of ARA criteria	6.3	5.2
Subcutaneous nodules (%)	58	20
Erythrocyte sedimentation rate (mm/1st h)	51	26
No of radiological changes (0-4)	2.8	1.8

ARA = American Rheumatism Association.

68 kDa antigen were demonstrated in a reasonable number of RA patients and are currently under investigation. Application of the recombinant antigen will further confirm and elucidate our findings.

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