Prevalence of anti-beta-2 microglobulin autoantibodies in sera of rheumatoid arthritis patients with extra-articular manifestations

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SUMMARY The frequency and concentration of specific factors binding β_2 microglobulin were investigated in sera and synovial fluids of patients and in sera of normal controls. High anti- β_2 m activity was detected in the sera of adult RA patients, particularly in those of with extra-articular disease. Similarly, anti- β_2 m was present in the synovial fluids of RA but not of osteoarthritis patients. Both the binding of anti- β_2 m activity to the Sepharose staphylococcal protein A and its elution position in the second 'IgG' peak after Sephadex G-200 gel filtration suggest the antibody nature of the activity. The possibility of differences not only in titre but also in the specificity of heterologous and homologous anti- β_2 m antibodies are discussed.

Soluble beta-2 microglobulin (β_2 m), a shed membrane protein subunit of human HLA antigens, can be found in relatively low concentration in biological fluids of healthy persons.¹ An increased serum β_2 m level has been detected in malignant disease² and in infectious diseases.³ High β_2 m serum concentration is also characteristic of systemic lupus erythematosus (SLE) patients even with normal renal functions,⁴ the joint effusions of rheumatoid arthritis (RA) patients,⁵ and the saliva of patients with Sjögren's disease.⁶

Furthermore serum autoantibodies specific for β_2 m have been observed in SLE patients,⁷ representing a considerable part of the lymphocytotoxic human antibodies reactive against surface antigens.⁸

We previously reported our preliminary observations on the appearance of specific autoantibodies for β_2 m (partly coisolated with rheumatoid factors) in sera and synovial fluids (SF) of RA patients.⁹ Moreover, IgG complexes containing β_2 m from some sera and SFs of SLE and RA patients were detected and analysed.^{10 11}

We report here that anti- β_2 m autoantibodies, reactive with staphylococcal protein A Sepharose (SPRA), can be frequently detected in sera of RA patients and especially in those with extra-articular symptoms.

Materials and methods

Blood was obtained from 104 patients with 'definite' or 'classical' RA according to American Rheumatism Association criteria. This group of patients consisted of 70 females and 34 males; the median age was 47 years, with a range from 32 to 71 years. Thirty-five of the RA patients showed 1 or more extra-articular signs of the disease, that is, subcutaneous nodules, vasculitic lesions of the skin, pleuropulmonary complications, episcleritis, or keratoconjunctivitis sicca. Blood samples of 30 patients with juvenile rheumatoid arthritis (JRA) were also investigated (median age 14 years, range from 5 to 35 years). Eight JRA patients could be classified as having a systemic onset of the disease whose symptoms were mainly fever, rash, iritis, and myocardial involvement. Synovial fluids were obtained from 26 RA patients and from 24 patients with osteoarthritis (OA). The control group included 39 healthy females and 28 males ranging from 15 to 70 years of age.

Antihuman β_2 m rabbit antibody. Monospecific rabbit antibodies against human β_2 m was raised and kindly gifted by Dr P. A. Peterson (Uppsala)¹² and compared with monospecific rabbit antihuman β_2 m purchased from Dako Immunoglobulin Ltd.

β_2 m binding assay by Farr's radioimmune technique. 0.6 ng purified β_2 m labelled with ¹²⁵I (tracer

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in Phadebas- β_2 microtest, Pharmacia, Uppsala) of specific activity: 93 $\mu\text{Ci}/\mu\text{g}$ in 50 μl of 0.1 M borate buffer, pH 8.2, was mixed in polystyrene tubes with 50 μl sample and 100 μl of borate buffer supplemented with 1% Tween 20 and 1% bovine serum albumin. After 1 h incubation at 37°C and 16 h at 4°C, 200 μl of saturated ammonium sulphate was added to each tube. After 20 min at room temperature tubes were centrifuged at 1500 g for 10 min and 200 μl of the supernatant was removed. Total radioactivity (T) of the 0.6 ng $\beta_2\text{m}$ - ^{125}I added to the tubes and of the supernatant (S) was measured. Bound activity was calculated according to the formula: binding % = $\frac{T-2S}{T} \times 100$. Background

values after adding buffer alone were subtracted from the test values. For synovial fluids from RA patients effusions from OA patients served as controls.

As a standard curve for the Farr's radioimmune assay 0.6 ng labelled $\beta_2\text{m}$ in 50 μl buffer was mixed with 150 μl dilutions of both batches of heterologous antihuman $\beta_2\text{m}$ (Fig. 1).

Staphylococcal protein A immunosorbent assay for detection of anti- $\beta_2\text{m}$ autoantibodies. 50 μl of the samples were diluted with 100 μl of 0.05 M Tris-HCl buffer, pH 7.8, supplemented with 0.1 M NaCl and incubated for 1 hour at room temperature with a Sepharose Protein A suspension (Pharmacia) of a concentration of 3×10^4 particles/ml. The

Fig. 1 Binding of beta-2-microglobulin by rabbit IgG specific for 0.001–100 μg human $\beta_2\text{m}$ from individual rabbit antiserum — and Dako anti- $\beta_2\text{m}$ ---. Mean $\pm$ SEM is indicated (4 experiments).

suspensions were washed 3 times with Tris-HCl-NaCl buffer. 0.25 ng $\beta_2\text{m}$ - ^{125}I was added in 20 μl buffer to each tube. After intensive shaking at room temperature for 2 h the suspensions were again washed 3 times in excess buffer and radioactivity absorbed to Sepharose protein A was measured. Background values were subtracted and the results were expressed as a percentage of total radioactivity added to the tubes.

Determination of immune complex (IC) concentrations by polyethylene glycol (PEG) precipitation. A modification¹³ of the method of Creighton *et al.*¹⁴ was used. Fresh samples were diluted 1:50 with 3% PEG 6000 (Merck, Darmstadt, Germany) in 0.1 M borate buffer and incubated for 16 h at 4°C. After centrifugation at 3000 g for 20 min, the precipitates were washed twice in 3% PEG and resuspended to the original sample volume in phosphate buffered saline at pH 7.4. The protein content of the IC-enriched fractions was determined by the method of Lowry *et al.*¹⁵ A standard curve using dilutions of human IgG was plotted.

Determination of rheumatoid factor (RF) titre. RF was measured by differential agglutination of sheep red blood cells sensitised with specific rabbit IgG and by latex agglutination tests.¹⁶

Gel filtration on Sephadex G-200. 0.6 ml samples of 5 sera and 3 SFs were fractionated on a Sephadex G-200 column measuring 1.5 $\times$ 80 cm, equilibrated with 0.1 M borate buffer at pH 8.2, and eluted at 10 ml/h with the same buffer. The fractions of the 3 major peaks were collected, pooled, concentrated to the original volume by PEG 20,000, and dialysed against borate buffer. $\beta_2\text{m}$ binding activity of the fractions was determined as described above.

Results

The results are summarised in Fig. 2. It can be seen that in comparison with the healthy controls significantly higher binding activity was found in some sera of RA patients. In RA patients with extra-articular symptoms the mean of the binding values was especially high (33.71 ± 1.5), compared with the patients without extra-articular manifestations ($t = 4.58$, $P < 0.0001$).

A binding percentage exceeding the mean binding percentage of controls by 2 standard deviations was seen in 10 patients with RA, in 9 of whom extra-articular disease was present.

There was no significant difference between $\beta_2\text{m}$ -binding values of JRA patients and controls even in cases with systemic onset of the disease.

Fig. 2 Binding percentages for β_2m of sera and synovial fluids (SF) from normal controls, rheumatoid arthritis (RA) patients, and juvenile rheumatoid arthritis (JRA) patients. Extra-articular manifestations: $\odot$ = subcutaneous nodules, $\oplus$ = pleuropulmonary signs, $+$ = vasculitic skin lesions, $\circ$ = keratoconjunctivitis sicca. The symbol $\times$ represents values of JRA patients with systemic onset of the disease. Means $\pm$ SD are indicated in all groups. The dotted line shows 2 SD above the mean control level. P values (Student's t test) are calculated by comparing binding percentages of sera from patients with RA or JRA and those of normal controls. SFs from RA patients are compared with effusions from OA patients.

A significantly higher level of β_2m -specific binding factor was found in SFs from RA patients compared with those of OA patients. Higher β_2m binding activity was not found in SFs from 4 RA patients with extra-articular complications compared with joint disease alone (data not shown).

Relatively high β_2m -binding activities were seen mainly in RA patients with pleuropulmonary lesions and nodules. Less striking increases were detected among patients with keratoconjunctivitis sicca syndrome.

Rheumatoid factor titres and immune complexes were also investigated in the sera of RA patients. β_2m binding was not significantly related to RF positivity or with immune complexes (Table 1).

To elucidate the immunochemical nature of the β_2m -binding activity precipitable by 50% saturated

Table 2 Reaction of labelled β_2 microglobulin on staphylococcal protein A immunosorbent precoated with serum dilutions from samples revealing high or low β_2m binding activities

β_2m binding percentages range	n	Percent binding of β_2m - ^{125}I to SprA ¹	Positivity ²	P ³
7.35-10.5	11	0.51 $\pm$ 0.05	2/11	<0.0003
27.51-48.72	13	5.70 $\pm$ 0.17	12/13	

¹For details see Materials and Methods. Mean $\pm$ SEM is indicated.

²Over 2% of the total radioactivity added to tubes.

³ χ^2 test with Yates's correction is used. $\chi^2 = 38.8$.

ammonium sulphate, binding to Sepharose staphylococcal protein A was measured. On the basis of previous Farr's technique 13 serum samples with high and 11 with low β_2m binding percentages were selected for this assay. A significant difference (χ^2 38.8, $P < 0.0003$) was observed between these 2 groups of serum, suggesting that IgG was responsible for the β_2m binding (Table 2).

In gel filtration experiments we separated the 3 macromolecular fractions from 3 sera and 3 SFs with high and from 2 sera with low β_2m binding activity. In the 6 positive cases the majority of the binding activity was recovered in the second 'IgG' peak, while only insignificant binding was found in peaks of negative samples. Fig. 3 shows typical elution patterns from sera of RA patients with high (Fig. 3a) and with low (Fig. 3b) β_2m binding activity. Gel filtration of a synovial fluid with high β_2m binding activity is also shown (Fig. 3c).

Table 1 β_2 microglobulin binding factors in sera of RA patients with high and low RF titres and IC levels

	n	β_2m binding percentages (mean $\pm$ SEM)	P
Rheumatoid factor ¹			
1:16-1:64	27	26.46 $\pm$ 0.79	NS
1:128-1:4096	29	27.30 $\pm$ 1.08	
² PEG precipitation			
≤ 200 μ g/ml ³	24	24.99 $\pm$ 0.66	NS
> 200 μ g/ml	27	27.72 $\pm$ 1.65	

¹Waller-Rose indirect agglutination. All patients had positive latex agglutination in their sera.

²3% PEG 6000 precipitation test (see Materials and Methods).

³Limit of positivity in PEG assay (higher than mean of IC level in sera of 80 healthy blood donors increased by two SD).

Fig. 3 β_2 microglobulin binding by fractions obtained after gel filtration on Sephadex G-200 of 2 sera (a, b) and 1 synovial fluid (c) of RA patients. Void volume is marked by broken line.

Discussion

The data reported here confirm our previous observations⁹ on the incidence of anti- β_2 m autoantibodies in the sera and synovial fluids of RA patients and focus our attention especially on those with different extra-articular manifestations (predominantly pulmonary and nodular). Findings in radioimmune Farr's assay clearly show the prevalence of anti β_2 m factors in this subgroup of patients. In sera of patients with SLE a similar frequency of positivity was reported.⁷ We have also investigated serum samples from 30 patients with SLE (unpublished observation) in the same system and found a comparable high mean of binding percentage (30.5 ± 7.0) and the same incidence of positive sera (16.6%).

Furthermore the binding of β_2 m by globulins from SFs of RA patients ($22.5 \pm 11.5\%$) exceeded that of SFs from OA patients ($14.1 \pm 9.2\%$). Interestingly, the absolute levels of the β_2 m binding in SFs were relatively low compared to serum. The basis for this difference requires further analysis but high local accumulation of β_2 m^{5, 17} and β_2 m-containing complexes found in synovial fluid^{10, 11} could also be responsible for a lower level of free anti- β_2 m activity.

The binding activity for β_2 m in different samples demonstrated in our study is attributed to anti- β_2 m

antibodies on the following evidence: (1) the ability of Sepharose staphylococcal protein A to bind the IgG fractions with high β_2 m binding activity; (2) most of the β_2 m binding material was eluted in the IgG peak after chromatography on Sephadex G-200 gel.

Poor attachment of IgG₃ and other immunoglobulin classes to SprA allows the possibility that other immunoglobulin molecules also contribute to the total binding activity found in sera and SFs. The presence of nonantibody components among the β_2 m binding molecules has also been proposed.^{18, 19} The participation of soluble HLA chains in the binding of β_2 m is less probable, as we failed to detect significant binding reactivity in the third peak of Sephadex G-200 gel filtration, the region corresponding to the molecular weight of the free HLA chains.

The nature of the binding activity found in the first macromolecular peak needs further clarification, since β_2 m-IgG complexes in antibody excess and with free binding sites for β_2 m may be also present in this fraction.¹¹

The binding percentages even in human serum samples with relatively high β_2 m binding activity were low compared to that of standard rabbit anti- β_2 m IgG. The binding capacity of $0.1-0.01 \mu\text{g}$ of the heterologous antibody is equivalent to the 1:4 dilutions of the human serum and SF samples. It is attractive to speculate that we measure only a part of the autoantibody response, as autoantibodies with higher avidity are probably rapidly removed by surface β_2 m of different cells. As an alternative interpretation, a difference between the serological specificity of heterologous antibodies produced against purified urinary β_2 m and naturally occurring autoantibodies to β_2 m can be suspected. The stimulus for the formation of β_2 m-specific autoantibodies is still unknown. β_2 m shed from cells or in immune complexes, virus-modified surface β_2 m, β_2 m microaggregates, or altered configuration of cytophylic β_2 m reassociated to the cell membranes might all be autoantigenic.

The immunopathological significance of these autoantibodies in SLE has been convincingly shown by their lymphocytotoxic properties in this disease.⁸ Heterologous antibodies directed to human β_2 m have been reported to interfere with different forms of cellular immune response *in vitro*.^{20, 21} Moreover anti- β_2 m heteroantibodies can evoke a dissociation of HLA- β_2 m complex in the cellular membrane, causing a loss of the alloantigenic configuration of the HLA chains.²²

Autoantibodies might also interfere with different cell functions and provoke the formation of ICs of β_2 m specificity.

Analysing the reactivity of anti- β_2m autoantibodies may help to elucidate the immunopathological features of RA and especially its extra-articular manifestations.

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