Characterisation of nucleolar proteins as autoantigens using human autoimmune sera

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SUMMARY Sera from 271 patients with systemic rheumatic diseases were screened for antinucleolar antibodies using immunofluorescence. Antinucleolar antibodies were found in the sera of 73% of patients with progressive systemic sclerosis, 4% of patients positive for autoantibodies with rheumatoid arthritis, and 8% of patients with rheumatoid arthritis associated with sicca complex, but not in patients with systemic lupus erythematosus, Sjögren's syndrome, undifferentiated connective tissue disease, or in healthy donors. The antinucleolar sera (n=20) were analysed by immunoblotting techniques. In four sera antibodies against nucleolar proteins with molecular weights of 35 kD, 37 kD, 69 kD, 92-93 kD, and 93 kD could be immunodetected. The nucleolar autoantigens were extractable from the nucleoli together with the preribosomal particle fraction and could be solubilised by ribonuclease (RNase) treatment. Their presence in the nucleolus was sensitive to actinomycin D treatment of cells. The 37 kD autoantigen could be identified as nucleolar phosphoprotein B23.

Key words: human antinucleolar antibody, nucleolar autoantigens, systemic sclerosis, rheumatoid arthritis.

Antinuclear antibodies are a characteristic feature of certain autoimmune disorders. They are directed against a varied spectrum of target antigens, deoxy-ribonucleic acid (DNA), ribonucleic acid (RNA), histones, ribonucleoproteins, and other proteins.¹⁻⁴ Autoantibodies reacting with nucleolar components were first reported by Beck⁵ and were frequently found in sera from patients with progressive systemic sclerosis.⁶⁻¹⁰ Detailed information on the number and identity of nucleolar proteins involved is still lacking, however, and one of the first steps towards elucidation of the mechanism by which certain nucleolar components become autoimmunogenic may be the identification of the autoantigens. The present study is aimed at the characterisation of nucleolar proteins reacting with human antinucleolar autoimmune sera obtained from patients with various autoimmune diseases.

Patients and methods

PATIENTS

All patients (n=271) were registered at the Staatliches Rheumakrankenhaus in Baden-Baden. The clinical diagnosis was based on generally accepted criteria: 11 cases with progressive systemic sclerosis (PSS) and 14 cases with systemic lupus erythematosus (SLE) according to the preliminary criteria given by Masi *et al.*¹¹ and Cohen *et al.*¹² 178 cases with rheumatoid arthritis (RA) according to criteria described by Ropes *et al.*¹³ and eight cases with Sjögren's syndrome (SS) as described by Manthorpe *et al.*¹⁴ The autoimmune sera of patients with RA were preselected for positive immunofluorescence of the nucleus or the cytoplasm of HeLa cells, or both. Patients (n=48) with rheumatoid arthritis associated with sicca complex (RA+SS) showed the criteria of RA, of keratoconjunctivitis sicca, and of xerostomia. The diagnosis of undifferentiated connective tissue disease (UCTD) was used when the symptoms did not fully correspond to the criteria of diagnosis cited above (n=12).

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CELLS

HeLa S3 cells and L1210 mouse leukaemia cells were obtained from Flow Laboratories (Bonn, FRG). Cell culture conditions were as previously described.¹⁵

NUCLEOLI

Nucleoli were isolated from L1210 and HeLa suspension cells. The conditions of isolation as described by Muramatsu and Onishi¹⁶ were slightly modified, using a higher concentration of MgCl₂ in the first step (5 mmol/l) and in the sonication step (2 mmol/l). Protein determinations were performed according to the method of Peterson.¹⁷

ELECTROPHORETIC PROCEDURES

Sodium dodecyl sulphate (SDS)-gel electrophoresis and transfer to nitrocellulose sheets (Schleicher and Schuell BA 83) were carried out according to standard procedures.^{18, 19}

IMMUNODETECTION OF ANTIGENS ON NITROCELLULOSE

After electrophoretic transfer of nucleolar proteins nitrocellulose strips were incubated in saturation buffer (0.01 M inorganic phosphate (Pi)/0.14 M NaCl pH 7.2, 0.5 mM phenylmethanesulphonyl fluoride, 0.5% Tween 20, 10% fetal calf serum) at room temperature. After 30 min incubation human sera diluted 1:100 with saturation buffer were added and the strips further incubated for two hours. The strips were washed several times with washing buffer (0.01 M Pi/0.14 M NaCl pH 7.2, 0.05% Tween 20) and then incubated for two hours with either peroxidase conjugated rabbit IgG specific for human IgG (H+L) (Miles, Frankfurt, FRG) or ¹²⁵I labelled sheep IgG specific for human IgG (H+L) (Amersham Buchler) diluted 1:1000 with saturation buffer or with 0.5 µCi ¹²⁵I labelled IgG/ml saturation buffer respectively. The washing was carried out as before. The peroxidase reaction was developed as described previously.¹⁵ Autoradiography was performed using Kodak-X-Omat film with a Dupont Cronex Quanta III intensifying screen.

AFFINITY PURIFICATION OF AUTOANTIBODIES

Affinity purification of autoantibodies was carried out essentially according to the outline given by Krohne *et al.*²⁰

ENZYMES

RNAse, DNAse I, and proteinase K were obtained from Boehringer (Mannheim, FRG).

IMMUNOFLUORESCENCE

HeLa cells were seeded on sterile glass coverslips

and processed for immunofluorescence after one day's incubation at 37°C.

The cells were washed with 0.01 M Pi/0.14 M NaCl pH 7.2 (phosphate buffered saline, PBS), fixed in a solution of 4% formaldehyde in PBS for 10 min, and made permeable by treatment with methanol at room temperature for 10 min. After thorough washing with PBS the cells were incubated with human autoimmune sera, diluted 1:100 with PBS, and kept at 37°C for 45 min. Thereafter the cells were washed three times with PBS followed by incubation with fluorescein isothiocyanate conjugated rabbit antihuman IgG (H+L) (Miles, Frankfurt, FRG) diluted 1:30 with PBS.

For RNAse or DNAse I treatment the washed cells were made permeable with methanol at room temperature for 10 min. After drying, the coverslips were incubated with RNAse and DNAse I (50 µg/ml each) in PBS/1 mM MgCl₂ at 37°C for 30 min. A control without enzyme was included. After thorough washing with PBS (5×5 min) the cells were fixed with 4% formaldehyde in PBS at room temperature for 10 min followed by further thorough washing with PBS. Thereafter, the cells were incubated with human sera and processed as described above.

ISOLATION OF NUCLEOLAR PROTEIN B23

The isolation of B23 was carried out according to the outlines given by Michalik *et al.*²¹

Results

SERA WITH ANTINUCLEOLAR SPECIFICITY

Sera of patients (n=271) with rheumatic diseases were screened for antinucleolar autoantibodies by indirect immunofluorescence using HeLa monolayer cells fixed with formaldehyde. Most autoimmune sera (228/271) reacted with nuclear or cytoplasmic antigens, or both. Under the given condition 33/48 RA+SS, 4/8 SS, 2/14 SLE, and 4/12 UCTD autoimmune sera were negative in the immunofluorescence test. Autoantibodies against nucleolar components were detected in 20/228 autoimmune sera. Only two of the 20 antinucleolar sera, however, showed a pure nucleolar staining in the immunofluorescence assay. The correlation of antinucleolar antibodies with a distinct diagnosis was as follows: progressive systemic sclerosis (PSS) 8/11 (73%), rheumatoid arthritis associated with sicca complex (RA+SS) 4/48 (8%), autoantibody positive rheumatoid arthritis (RA) 8/178 (4%), Sjögren's syndrome (SS) 0/8, systemic lupus erythematosus (SLE) 0/14, undifferentiated connective tissue disease (UCTD) 0/12, and healthy donors 0/21.

Fig. 1 Immunodetection of nucleolar components (HeLa S3) after SDS-gel electrophoresis and electrophoretic transfer to nitrocellulose. (a) Normal human serum as a control; (b) serum No 1; (c) serum No 2; (d) serum No 3; (e) serum No 4; (c', c'') Immunodetection by affinity purified antibodies derived from serum No 2 specific for (c') 69 kD and (c'') 35 kD components performed in separate experiments. The apparent molecular masses of protein standards are given in kilodaltons.

IMMUNOBLOTTING WITH ANTINUCLEOLAR SERA

In order to identify individual nucleolar autoantigens only the 20 antinucleolar autoimmune sera selected by immunofluorescence were further characterised by immunoblotting experiments. The individual components of isolated HeLa S3 nucleoli were separated by SDS-gel electrophoresis and transferred to nitrocellulose sheets. Distinct immunoreactive nucleolar components could be detected only with 4/20 sera.

The immunoreactive components had molecular weights of 35 kD and 37 kD reacting with serum No 1 (RA+SS, Fig. 1b), 35 kD and 69 kD reacting with serum No 2 (PSS, Fig. 1c), 92–93 kD reacting with serum No 3 (RA + SS, Fig. 1d), and 93 kD reacting with serum No 4 (RA, Fig. 1e). The non-identity of the 93 kD component reacting with serum No 4 and the 93 kD component of the double band resulting from the reaction with serum No 3 (Fig. 1d) was indicated by the differences in the immunofluorescence pattern obtained after treatment of HeLa cells with actinomycin D (Figs 3g and h).

AFFINITY PURIFICATION OF AUTOANTIBODIES

Monospecific autoantibodies are required for immunolocalisation of individual nucleolar components by the immunofluorescence assay since autoantibodies present against other components might interfere with the immunofluorescence pattern of a distinct component. Affinity purification of monospecific autoantibodies was performed with nitrocellulose strips carrying the individual autoantigens. These were cut out from nitrocellulose sheets after Western transfer of nucleoli separated by SDS-gel electrophoresis.

The affinity purified antibodies were used in immunofluorescence studies to confirm the nucleolar localisation of corresponding autoantigens.

A comparison of the fluorescence patterns obtained with the autoimmune sera and the pattern obtained with affinity purified antibodies is shown in Fig. 2. The affinity purified antibodies showed an almost exclusively bright nucleolar fluorescence (Figs 2e–i). Interfering fluorescence of the nucleus and the cytoplasm disappeared from the sera as shown in Figs 2a and e and Figs 2d and i. In addition, the affinity purified antibodies were also used to decide whether the 35 kD and 37 kD components (serum No 1) or the 35 kD and 69 kD components (serum No 2) showed any cross reactivity. The nucleolar components 35 kD and 37 kD immunoreactive with serum No 1 (Fig. 1b) showed strong cross reactivity, indicating that the 35 kD component may be a degradation product of the 37 kD component since the 35 kD affinity purified antibody also reacted with the 37 kD component and the 37 kD affinity purified antibody with the 35 kD component in the immunoblotting experiment. The components 35 and 69 kD immunoreactive with serum No 2, however, did not show any cross reactivity in the immunoblotting experiment after affinity purification (Figs 1c' and c'') but yielded similar fluorescence patterns probably due to similar distributions in the nucleoli. This 35 kD component (serum No 2) showed no cross reaction with the 35 kD/37 kD components specified by serum No 1. Affinity purification of antibodies to only one of the two nucleolar components (92–93 kD) immunoreactive with serum No 3 could not be achieved because of the small distance between the components separated by SDS-gel electrophoresis. Cross reactivity of these two components, therefore, cannot be excluded.

ENZYME SENSITIVITY OF AUTOANTIGENS

The chemical nature of autoantigens was established by treatment with various enzymes. Isolated nucleoli were treated with DNase I (1.7 mg/ml).

Fig. 2 Immunofluorescence patterns obtained in HeLa S3 monolayer cells with the antinucleolar autoimmune sera. (a) Serum No 1; (b) serum No 2; (c) serum No 3; (d) serum No 4; and with the corresponding affinity purified antibodies against the nucleolar components (e) 37 kD serum No 1; (f) 69 kD serum No 2; (g) 35 kD serum No 2; (h) 92–93 kD serum No 3; and (i) 93 kD serum No 4. Bar represents 10 μ m.

Fig. 3 Immunofluorescence patterns obtained with antinucleolar autoimmune sera in HeLa S3 monolayer cells pretreated with (a-d) RNase or preincubated with (e-h) actinomycin D. (a, e) Serum No 1; (b, f) serum No 2; (c, g) serum No 3; and (d, h) serum No 4. Bar represents 10 μ m.

RNAse (1.7 mg/ml), or proteinase K (1.7 mg/ml) for 30 min at 37°C. After gel electrophoresis and Western transfer of digested nucleolar components only the nucleoli treated with proteinase K lost their immunoreactivity with the autoimmune sera, indicating that the components recognised by these four sera are proteins. The association of the protein autoantigens with subnucleolar structures containing nucleic acids was investigated with similar techniques using immunofluorescence: HeLa monolayer cells were made permeable with methanol, dried and treated with RNAse (50 µg/ml) or DNase I (50 µg/ml) for 30 min at 37°C. Non-treated controls were included. After washing and fixation the cells were used for indirect immunofluorescence with the antinucleolar sera Nos 1–4. In all samples the nucleolar fluorescence was almost completely lost after RNAse treatment (Figs 3a–d). Controls without RNAse treatment or cells treated with DNase I showed the bright nucleolar staining as in Figs 2a–d, indicating that all nucleolar autoantigens shown in Fig. 1 are associated with RNA fibrils.

ACTINOMYCIN D TREATMENT

In order to obtain information on the association of the identified nucleolar autoantigens with potential nucleolar functions we used pretreatment of cells with actinomycin D which stops elongation of ribosomal transcripts. We exposed HeLa monolayer cells on slides to 1 µg/ml actinomycin D for three hours before processing the slides for immunofluorescence. Nucleoli appeared intact in phase contrast after this treatment. All four antinucleolar sera (Nos 1–4) showed a different fluorescence

pattern. The nucleolar protein autoantigens were now localised at the periphery of the nucleoli or were diffusely distributed over the whole nucleus. The central parts of the nucleoli were free of nucleolar autoantigens (Figs 3e–h). We concluded from these results that the nucleolar autoantigens were either directly or indirectly associated with components of the ribosomal transcription units and were released upon transcription arrest.

AUTOANTIGENS IN NUCLEOLAR SUBFRACTIONS

For the localisation of the nucleolar autoantigens in nucleolar subfractions HeLa cell nucleoli were subsequently extracted as described by Rothblum *et al.*²² This procedure yielded a NaCl/ethylene-diaminetetra-acetate (EDTA) fraction (Fig. 4a), a 10 mM TRIS (trometamol) fraction containing the preribosomal particles (Figs 4b and c) which could be subdivided into a supernatant (Fig. 4b) and a 100 000 g pellet (Fig. 4c), and the fraction of nucleolar chromatin (Fig. 4d). This procedure did not yield a clear cut separation of nucleolar subfractions, but it was useful for obtaining preliminary information. The nucleolar subfractions were characterised by SDS-gel electrophoresis and immunoblotting using the autoimmune sera Nos 1–4 (Figs 4I–IV). All identified nucleolar autoantigens were extractable with the 10 mM TRIS fraction. The 10 mM TRIS fraction was further separated into a pellet containing a preribosomal particle and a soluble supernatant by high speed centrifugation (100 000 g, 16 h). The 69 kD and 35 kD proteins (Fig. 4II, serum No 2) and the 93 kD protein (Fig. 4IV, serum No 4) were predominantly recovered in the

Fig. 4 Immunodetection of fractions obtained by sequential extraction of HeLa S3 nucleoli after SDS-gel electrophoresis and electrophoretic transfer to nitrocellulose using antinucleolar autoimmune sera. (I) serum No 1; (II) serum No 2; (III) serum No 3; and (IV) serum No 4. (a) Extraction with NaCl/EDTA; (b) soluble supernatant and (c) pellet obtained by extraction with 10 mM TRIS; (d) nucleolar chromatin.

Fig. 5 Immunodetection of isolated nucleolar protein B23 after SDS-gel electrophoresis and electrophoretic transfer to nitrocellulose using (a) normal human serum; (b) antinucleolar autoimmune serum No 1; and (c) molecular weight standards given in kilodaltons.

fraction containing the preribosomal particle (Fig. 4c), but smaller portions were still present in the fraction of nucleolar chromatin. The 37 kD protein

(Fig. 4I, serum No 1) was exclusively found in the 10 mM TRIS supernatant, whereas 92–93 kD proteins (Fig. 4III, serum No 3) were predominantly detected in the 10 mM TRIS supernatant and in the fraction of nucleolar chromatin, and to a lesser extent in the fraction containing the preribosomal particle.

CROSS REACTIVITY OF HUMAN AUTOIMMUNE SERA WITH MOUSE NUCLEOLAR ANTIGENS

Interspecies cross reactivity was studied with the four autoimmune sera specified above using isolated mouse nucleoli. Antibodies to 35 kD, 37 kD, 92–93 kD, and 93 kD proteins showed strong cross reactivity with the corresponding mouse nucleolar proteins of 36 kD, 37 kD, 92–93 kD, and 93 kD respectively, whereas antibodies to the 69 kD protein cross reacted only slightly with the analogous mouse protein of 69 kD.

IDENTIFICATION OF THE 37 kD ANTIGEN AS NUCLEOLAR PHOSPHOPROTEIN B23

Comparison of the nucleolar autoantigens identified in this paper with nucleolar proteins described in the literature indicated a possible identity of the 37 kD autoantigen with nucleolar phosphoprotein B23, which also has a molecular weight of 37 kD. We isolated nucleolar phosphoprotein B23 as described by Michalik *et al.*²¹ The identity of both proteins was verified by gel electrophoresis and immunoblotting of isolated B23 (Fig. 5), by an enzyme linked immunosorbent assay with serum No 1 and by immunofluorescence studies (Fig. 6). After absorption of serum No 1 with isolated B23 (150 µg/ml serum) (Fig. 6b) the ability of staining nucleoli of HeLa cells was completely abolished.

Fig. 6 Immunofluorescence patterns in HeLa S3 monolayer cells obtained with (a) serum No 1 and (b) serum No 1 absorbed with B23 (150 µg/ml serum). Bar represents 10 µm.

Discussion

The investigations presented in this paper characterise at least five nucleolar autoantigens and identify one of them as nucleolar phosphoprotein B23.

All five autoantigens could be identified as proteins and were detected in the fraction containing the preribosomal particles of the nucleoli. The sensitivity of the nucleolar location of the five autoantigens to actinomycin D treatment of cells and the RNase dependent solubility in situ lead us to the conclusion that the described autoantigens are components of the ribonucleoprotein or interact with the nucleolar ribonucleoprotein fibres.

In some earlier studies concerned with nucleolar autoantigens reacting with sera from patients suffering from rheumatic diseases a 4-6 S RNA was identified as the reacting component.⁹ Furthermore, ribonucleoprotein particles containing 7-5 S-A and 7-5 S-B RNA,²³ 7-2 S RNA,^{24, 25} or U3 RNA²⁵ have been described. Only one protein exclusively located in the nucleolus, RNA polymerase I, has been described so far to be immunoreactive with rheumatic autoimmune sera.²⁶ We are now able to identify the 37 kD autoantigen as phosphoprotein B23, a major nucleolar protein.^{21, 27} Further nucleolar autoantigens are represented by the 92-93 kD (serum No 3) and 93 kD (serum No 4) antigens, which appear not to be identical as seen by the differences in the immunofluorescence pattern obtained after treatment of HeLa cells with actinomycin D (Figs 3g and h) and by their different distribution in the 10 mM TRIS fractions of nucleoli (Figs 4III and IV). One of them, however, may be identical with a 94 kD nucleolar protein also associated with ribonucleoprotein fibres.²⁸ To our knowledge nucleolar proteins analogous to our 35 kD and 69 kD nucleolar autoantigens (serum No 2) have not yet been characterised. Our data concerning the correlation with clinical diagnosis and the frequency of expression of antinucleolar antibodies, which was especially high (73%) in patients with PSS, are essentially in accordance with other reports.^{7, 8} It is noteworthy that despite the fact that 8/11 autoimmune sera of patients with PSS showed positive nucleolar immunofluorescence only 1/11 autoimmune sera reacted with nucleolar components after SDS-gel electrophoresis. This may be due to SDS sensitive epitopes or to epitopes present on RNA which do not bind to nitrocellulose under the conditions used. On the basis of these findings it is necessary to include other autoantigens, such as scl-70 and centromere,² into molecular immunodiagnoses of PSS. The identified nucleolar autoantigens represent a promising basis for further investigations of the specific cellular functions of

autoantigens, which might help to elucidate the aetiologies of autoimmune disease.

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