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Autoantibodies against constituents of nuclear pore complexes in patients with primary biliary cirrhosis and autoimmune hepatitis

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Sera obtained from patients with autoimmune liver disease were screened in indirect immunofluorescence microscopy for the presence of autoantibodies. Patients' sera, which strongly stained nuclei (ANA) with peripheral accentuation, were used for further experiments to define the corresponding antigen(s). Nuclei and nuclear subfractions were isolated from HeLaS$_3$ cells and used as antigen source. Immunoblotting experiments were performed after separation of nuclear proteins by one- and two-dimensional polyacrylamide gel electrophoresis. Some ANA positive sera recognized the nuclear protein with molecular mass of approximately 200 kDa. Further analysis revealed that the patients' sera reacted with gp210, an integral protein of the nuclear pores. The incidence and clinical significance of these antibodies is discussed.

The nuclear envelope, separating the nucleus from the cytoplasm consists of the nuclear membranes, the nuclear pore complexes and the nuclear lamina (for review see [1]). Although these structures are interconnected, they are morphologically and functionally distinct and the constitutive components are not randomly distributed among them. Recently it has been found that proteins of the nuclear envelope are antigen targets in autoimmune liver diseases [2]. Thus, autoantibodies against nuclear lamins were identified in a variety of patients. They are most frequently found in patients with autoimmune hepatitis [3]. More recently antibodies directed against gp210, an integral protein of the nuclear pore complex have been described in human pathology, specifically in primary biliary cirrhosis [4, 5]. However, it is unclear whether these antibodies are restricted to this disease or may also occur in autoimmune hepatitis.

METHODS

Patients. Sera were obtained from 63 patients with autoimmune liver disease and 20 healthy blood donors. Thirty three sera were from patients with primary biliary cirrhosis; 30 sera were collected from patients with autoimmune hepatitis. In addition 20 sera from scleroderma patients were tested.

Indirect immunofluorescence microscopy. Antibodies to nuclear components were detected by indirect immunofluorescence micro-

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[1]Abbreviations: BSA, bovine serum albumin; PBC, primary biliary cirrhosis; PBS, phosphate-buffered saline; NPC-LF, nuclear pore complex-lamina fraction.

scopy using standard cell lines and rodent tissue preparations.

Isolation of nuclei and nuclear pore complex-lamina fraction. To avoid proteolytic degradation of proteins, all isolation steps were performed in the presence of protease inhibitors at 4°C.

The nuclei were isolated as described previously [3]. Briefly, HeLa cells were swollen for 10 min in hypotonic medium and centrifuged. The pellet was suspended again in hypotonic buffer and homogenized after addition of Nonidet P-40 and sodium deoxycholate to a final concentration of 1% or 0.5%, respectively. Then nuclei were pelleted through a sucrose cushion.

The nuclear pore complex-lamina fraction was further prepared from isolated nuclei as described by Dwyer & Blobel [6]. In short, the isolated nuclei were digested with DNase I at pH 8.5, then redigested with both DNase I and pancreatic RNase at neutral pH and extracted in two consecutive steps with high salt buffer and with buffer containing non-ionic detergents. This yielded an insoluble pellet defined as the nuclear pore complex-lamina fraction. After treatment of the pellet with buffer containing a high concentration of monovalent cations and non-ionic detergents, proteins of the nuclear pores became soluble.

Labeling of nuclear pore proteins. Solubilized proteins of the nuclear pore complexes were radioactively labeled using ^{125}I (DuPont-New England Nuclear; Boston, MA) and Iodo-beads from Pierce. Free iodide was eliminated from the incubation assay by gel exclusion chromatography.

Purification of gp210 protein. The gp210 protein was purified by affinity chromatography on lentil lectin-Sepharose. Nuclear pore complexes were solubilized at protein concentration of about 2 mg/ml in high salt buffer (500 mM NaCl, 20 mM Hepes, pH 7.35, 1 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) containing 2% Triton X-100 for 30 min at 4°C. The extract was then centrifuged at 30000 × *g* for 30 min and the supernatant was loaded on preequilibrated lentil lectin-Sepharose. The column was then washed with 20 volumes of high salt buffer containing 0.1% Triton X-100. The gp210 protein was eluted with high salt buffer containing 1 M α-mannose and 0.1% Triton X-100.

Immunoprecipitation. ^{125}I-Labeled nuclear pore proteins, 300 µl, were incubated with 10 µl of patient's serum for 2 h at room temperature. Then 50 µl of washed protein G-Sepharose was added and the incubation was carried on for 2 h at room temperature. Afterwards supernatant was discarded and protein G-Sepharose was washed 4 times with PBS containing Triton X-100 and Nonidet P-40. Immunocomplexes were eluted with glycine buffer or SDS-sample buffer [7] and were analysed.

Electrophoretic separation of proteins. Proteins of nuclear subfractions or purified proteins were separated by one-dimensional and two-dimensional polyacrylamide gel electrophoresis (PAGE). One dimensional-PAGE was performed on 10% SDS slab gels as described by Laemmli [7]. Two-dimensional PAGE was performed according to O'Farrell [8] with some alterations as described in detail previously [9].

Immunoblotting. Proteins separated on slab gels were electrophoretically transferred onto nitrocellulose sheets and stained by Ponceau S. The blots were saturated with 3% bovine serum albumin (BSA) in PBS, then exposed to appropriate concentrations of patients' sera, extensively washed and incubated with ^{125}I-labeled Protein A (DuPont-New England Nuclear, Boston, MA). The reactive antigens were detected by autoradiography using X-OMAT S film (Kodak, Rochester, NY).

Lectin binding. The blots were blocked with 3% BSA in PBS and incubated with the affinity purified lectins: *Lens culinaris* and wheat germ agglutinin conjugated with biotin. The lentil lectin is specific for α-D-mannose and wheat germ lectin reacts with *N*-acetylglucosamine. The blots were extensively washed and incubated with the streptavidin-alkaline phosphatase complex. The staining was performed in buffer consisting of 100 mM Tris/HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$ using bromo chloro indolyl phosphate/nitro blue tetrazolium as substrate. In control assay 1 M α-methylmannoside or *N*-acetylglucosamide was added as competitor to the lectin solution.

RESULTS AND DISCUSSION

Some patients' sera gave a punctate staining of the nuclear periphery suggesting the

presence of antibodies to constituents of the nuclear envelope.

To determine reactive antigen(s), the nuclear pore complex-lamina fraction (NPC-LF) was isolated, proteins were electrophoretically separated and used for immunoblotting. Figure 1 shows that several patients' sera stained predominantly a protein at about 200 kDa. Some sera recognized additionally two protein constituents within molecular mass range between 60 kDa and 70 kDa. The position and pattern of the reactive two bands were characteristic for nuclear lamins A and C.

To further characterize the reactive protein band in the high molecular mass region, proteins of the nuclear pores were extracted from NPC-LF and used for both immunoblotting as well as for immunoprecipitation experiments. To facilitate the recognition of immunoprecipitated antigen(s), the proteins of the nuclear pores were previously labeled with ^{125}I. As shown in Fig. 2 three proteins were predominantly ^{125}I-labeled. The analysis of samples after immunoprecipitation using sera from primary biliary cirrhosis patients, revealed a radioactive protein band at 200 kDa, (Fig. 3, lane 3 and 4). A normal serum used as control did not precipitate any protein (Fig. 3, lane 2). These results indicate that the patients' sera are able to recognize both denatured as well as

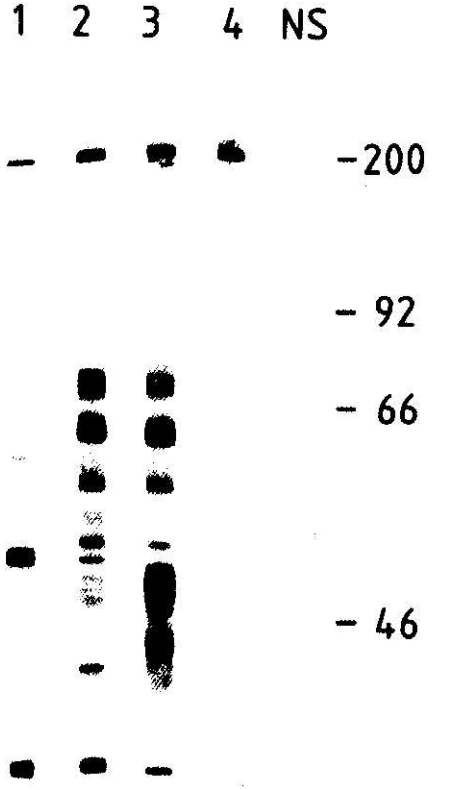

Fig. 1. Immunoblotting using the nuclear pore complex-lamina fraction (NPC-LF) as antigen source.
Proteins were separated on 10% SDS-slab gels. Lanes 1-4: incubation with the sera from the patients with primary biliary cirrhosis; NS: incubation with normal serum. Position of marker proteins is indicated.

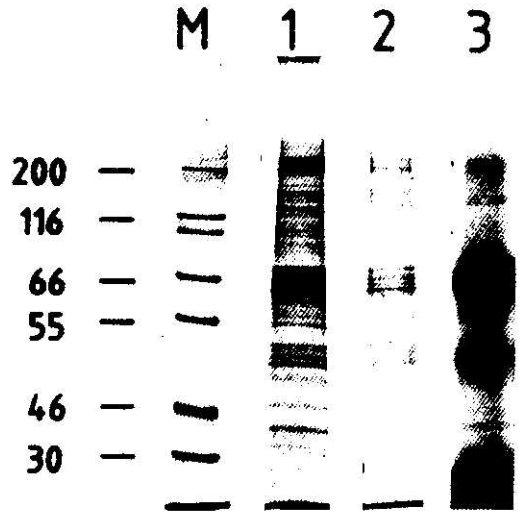

Fig. 2. ^{125}I-Labeling of nuclear pore complex-lamina fraction proteins.
Lane 1: proteins used for labeling; lanes 2–3: ^{125}I-labeled proteins. Lanes 1–2: Coomassie blue staining; lane 3: autoradiography. M, protein markers.

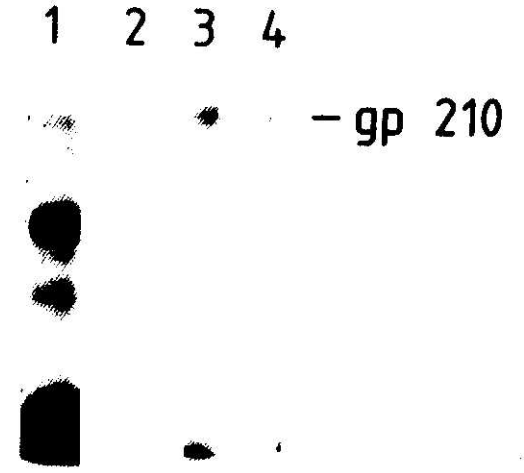

Fig. 3. Immunoprecipitation of ^{125}I-labeled nuclear pore complex-lamina fraction proteins (for details see Materials and Methods) — autoradiography.
Lane 1: sample submitted to immunoprecipitation, lane 2: precipitation using normal serum; lanes 3-4: precipitation using sera from patients with primary biliary cirrhosis.

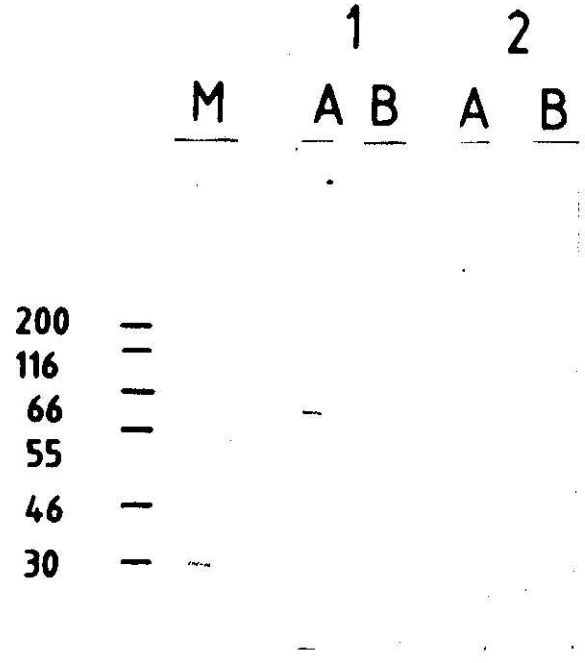

Fig. 4. Identification of gp210 protein using specific lectin.

Nuclear pore complex-lamina fraction proteins separated on 10% SDS gels and transferred onto membrane were incubated with specific lectin (for details see Materials and Methods). 1. Incubation with wheat germ agglutinin lectin protein markers. 2. Incubation with lentil lectin. A, Binding in standard buffer; B, binding in the presence of competing sugar; M, protein markers.

native antigen, and point to high specificity of the autoantibodies.

The molecular mass of the reactive antigen coincided with that characteristic for gp210, an integral nuclear pore complex protein. To prove the identity of the reactive protein band of 200 kDa with gp210, blot strips were probed with lentil lectin, known to have the affinity to mannose substituted glycoproteins. Indeed, the reactive protein band at 200 kDa did bind lentil lectin. The lectin binding was completely abolished after addition of α-methylmannoside to the incubation buffer. Finally, the gp210 glycoprotein purified by affinity chromatography on lentil lectin-Sepharose was used in immunoblotting experiments. The sera reacted strongly with this antigen purified to homogeneity. To check whether the anti-gp210 autoantibodies are restricted to PBC, the sera from patients with autoimmune hepatitis and with scleroderma were also tested. Anti-gp210 antibodies were not detected in scleroderma sera. Of thirty sera from patients with autoimmune hepatitis only two (6%) reacted with gp210 glycoprotein. On the other hand, patients with PBC frequently had autoantibodies against gp210. Nine of thirty three PBC sera (28%) reacted with gp210.

"Mature" gp210 is composed of three main domains. A large amino-terminal domain of 1783 amino acids is located in the perinuclear space. A short 20 amino-acid hydrophobic segment penetrates the nuclear pore membrane and a carboxy-terminal tail domain of 58 amino acids faces the cytoplasm.

It is not known why patients with PBC develop autoantibodies against gp210 and how these autoantibodies are related to the pathogenesis of PBC. The autoantibodies against gp210 glycoprotein of the nuclear pore detected so far only in autoimmune liver disease seem to be characteristic for PBC and could be of particular clinical significance in the diagnosis of primary biliary cirrhosis, especially in cases when the antimitochondrial antibody titer is low or undetectable.

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