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Morphological and Biochemical Analysis of Anti-Nuclear Matrix Protein Antibodies in Human Sera

Autoimmune sera have been used in the diagnosis of autoimmune diseases as well as the analysis of nuclear substructures. In an attempt to study the biological characteristics of the nuclear matrix, we screened human sera using immunofluorescent staining and immunoblot. We detected antibodies against nuclear matrix (NM), a remnant nonchromatin protein compartment after the treatment of detergent, salt and nuclease, in 212 out of 284 tested sera (74.6%) by immunoblot. Peptides with molecular weights of 70 kDa, 50 kDa and 25 kDa were detected in the order of frequency. Clinical informations of 198 out of 212 cases were available and went as follows: 38 cases were autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis; 132 non-autoimmune and non-neoplastic diseases; 16 neoplastic diseases and 12 cases unclassified. The immunofluorescent staining intensity by anti-nuclear matrix protein (NMP) antibodies decreased variably, but fibrillogranular, speckled and nucleolar immunolocalization patterns were retained after in situ fractionation. Ku70 and La protein were detected by anti-NMP antibodies. Immunolocalization by anti-NMP antibodies indicates that the NMPs constitute a variety of characteristic nuclear substructures and may serve as autoantigens in diverse human diseases. In addition, the presence of Ku70 and La protein as NMPs suggests that the NM can be functionally active in association with DNA or RNA.

Key Words : *Antibodies, anti-nuclear; Nuclear matrix; Nuclear protein; Autoimmune diseases*

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INTRODUCTION

Autoimmune diseases are characterized by various kinds of autoimmune antibodies against cellular proteins and nucleic acids. Autoantigens are either extracellular or intracellular: the one includes cell surface glycoprotein of the cadherin family in pemphigus vulgaris (1) and the other consists of various nuclear proteins. Human autoantibodies have aided the clinician in the differential diagnosis of many autoimmune diseases, since high titers of anti-nuclear antibodies (ANAs) are produced in systemic lupus erythematosus (SLE), scleroderma, Sjögren's syndrome, and mixed connective tissue disease (2). ANAs are also detected, although with lower frequency in other disease conditions, including paraneoplastic neurologic syndromes (3), and cancers of various types (4). In addition, ANAs have been used extensively to isolate cDNA clones encoding target autoantigens, and to study the molecular structure and function of some of those autoantigens. They have contributed to elucidation of nuclear substructures and understanding of important cellular processes such as pre-mRNA splicing, DNA replication and mitosis (5-7).

The nuclear matrix (NM) is a remnant structure after the treatment of detergent, high ionic strength salt and DNase, and consists of about 200 proteins, only some of which are characterized (8). NM is associated with DNA replication (9), RNA transcription (10) and may serve structurally as the "track" for the transport of macromolecules. Therefore, the NM is regarded as a dynamic structural system for genomic functions (11-13).

In this study, to elucidate the structure and function of the NM, we performed immunofluorescent staining and immunoblot analysis with human sera against NMPs. The results indicate that NMPs form a variety of nuclear substructures and may serve as autoantigens in diverse human diseases.

MATERIALS AND METHODS

Human sera and antibody

Five hundred sixteen sera from patients with autoim-

mune diseases and other diseases were included in this study. The sera were immediately frozen in a liquid nitrogen tank until use. For immunofluorescent staining and immunoblot analysis, aliquots of the sera were diluted at 1:200 and sodium azide was added to the final concentration of 0.02%. Monoclonal antibody (mAb) NMB1 against human Ku70 was used for immunoprecipitation (14).

In situ fractionation on cultured cells

This assay was carried out on cultured cells as described by Staufenbiel and Deppert (15). HeLa cells or HEp-2 cells were maintained in DMEM with 10% FCS in 5% CO_2 incubator at 37℃ They were grown on coverslips to 80% confluency and were washed with ice-cold Kern Matrix (KM) buffer twice. They were extracted with KM buffer containing 0.5% Triton X-100, 1 mM EGTA and 5 mM dithiothreitol (DTT). They were then treated with i) RNase-free DNase I (100 μg/mL in KM buffer) for 2 hr at room temperature, ii) 0.25 M $(NH_4)_2SO_4$ in KM buffer containing 0.5% Triton X-100, 1 mM EGTA and 5 mM DTT for 5 min at room temperature and iii) 2.0 M NaCl in KM buffer for 30 min on ice. Extracted cells were washed with KM buffer, and stainable DNA was checked with fluorescence microscopy. When any stainable nuclear DNA remained, DNase I treatment was repeated for another 1 hr.

Nuclear matrix preparation

The nuclear matrix preparation procedure has been previously described (14). Briefly, cultured cells were centrifuged at 1,000 *g* for 10 min and the harvested cells were resuspended, homogenized in buffer A (10 mM Tris-HCl pH 7.0/10 mM NaCl/1.5 mM $MgCl_2$/1.2 mM PMSF/0.1% Triton X-100) with a teflon pestle on ice for 10 min and spun down at 700 *g*. The pellet was resuspended in buffer B (50 mM Tris-HCl pH 7.5/25 mM KCl/10 mM $MgCl_2$/50 mM EDTA/1.2 mM PMSF), homogenized in a loose fitting glass homogenizer and spun down at 10,000 *g* for 10 min. The pellet (crude nuclear fraction) was extracted in buffer C (10 mM Tris-HCl pH 8.0/140 mM NaCl/L mM $MgCl_2$/L mM EGTA/1.2 mM PMSF) on ice for 45 min, sonicated once for 10 sec at 45 W and centrifuged at 10,000 *g* for 10 min. The resulting pellet (soluble nuclear fraction) was resuspended in 1 mM $NaPO_4$ pH 7.0, sonicated in 10 × 10 sec pulses at 45 W and centrifuged at 10,000 *g* for 10 min. The resulting pellet was residual nuclear protein fraction. The pellet was resuspended in buffer D (10 mM Pipes pH 7.0/50 mM NaCl/300 mM sucrose/3 mM $MgCl_2$/L mM EGTA/1.2 mM PMSF/2 mM vanadylribonucleoside/0.5% Triton X-100/100 μg/mL DNase I) for 45 min at 25℃ and centrifuged at 1,000 *g* for 10 minutes at 4℃. The pellet was resuspended in buffer E (10 mM Pipes pH 7.0/100 mM NaCl/300 mM sucrose/3 mM $MgCl_2$/L mM EGTA/1.2 mM PMSF/2 mM vanadylribonucleoside/0.5% Triton X-100/250 mM $(NH_4)_2SO_2$), stirred in cold room for 10 min and centrifuged at 1,000 *g* for 10 min at 4℃. The pellet was resuspended in buffer F (10 mM Pipes pH 7.0/100 mM NaCl/300 mM sucrose/3 mM $MgCl_2$/1 mM EGTA/1.2 mM PMSF/2 mM vanadylribonucleoside/0.5% Triton X-100), and 4 M stock solution was added slowly until 2 M NaCl was reached at room temperature for 10 min and centrifuged. The resulting pellet was the NM.

Immunofluorescence microscopy

Cultured cells were fixed at room temperature for 10 min with cold methanol, and dipped several times in cold acetone. Nuclear antigen localization was determined after incubating cells with sera at 1:200 dilution for 1 hr at room temperature and then with FITC-conjugated anti-human antibody for 1 hr. Cells were stained for DNA with 1.0 mg/mL of bisbenzimide (Hoechst 33258: Sigma, St. Louis, U.S.A.) in PBS for 10 min and mounted with Fluoromount G (Fisher Scientific, Pittsburgh, USA). Fluorescence images were analyzed using an Olympus Vanox-X fluorescence microscope.

Polyacrylamide gel electrophoresis and immunoblot

Nuclear matrix proteins of HEp-2 cells were separated by SDS-polyacrylamide (10%) gel electrophoresis (16), using the dual slab minigel system (Bio-Rad Lab, Hercules, U.S.A.) for 2 hr at 100 V. High molecular markers were coelectrophoresed to determine size. Proteins were then transferred to nitrocellulose paper (0.45 μm pore size; Schleicher & Schuell, Dassel, Germany) as described (17), using the mini Trans-Blot cell (Bio-Rad Lab). Nitrocellulose membranes containing transferred proteins were blocked by incubation in 0.5% Tris-BSA for 30 min at room temperature under agitation. Nitrocellulose papers were reacted with sera for 1 hr. Immune complexes were detected by incubating membranes for 1 hr in 1:1,000 horseradish peroxidase-conjugated antihuman immunoglobulin in blocking solution, followed by color development with 5-bromo-4-chloro-3-indolphosphate toluidinium and nitro blue tetrazolium (Pierce, Rockford, IL) as substrates.

Screening of cDNA libraries

A HeLa cell cDNA library (Stratagene, La Jolla, CA) was subjected to immunoscreening with a human serum using modified standard procedures (18). Appropriately diluted bacteria (XL1-Blue MRF strain) were incubated with the lamda phage for 20 min at 37℃ to which 3 mL of top agar prewarmed at 48℃ was added. They were plated on LB

agar plates, and incubated for 3-3½ hr at 42℃. Nitrocellulose membranes saturated with 10 mM IPTG solution were put on the plates to transfer the plaques onto them for 3 hr at 37℃. Nitrocellulose papers were incubated in the blocking solution (3% BSA in TBST solution) overnigh at 4℃ in the human serum for 2 hr at RT, and then in anti-human antibody for 2 hr at RT, sequentially. They were washed three times in washing solution I, II and III for 10 min, each. They were developed in NBT/BCIP solution and dried. The positive plaques were picked up, mixed in SM buffer and chloroform, and stored at 4℃ for sequencing.

RESULTS

Immunofluorescent analysis of autoimmune sera

To detect the presence of anti-nuclear antibodies, over five hundred human sera were screened by indirect immunofluorescent staining on intact HEp-2 cells. We could detect

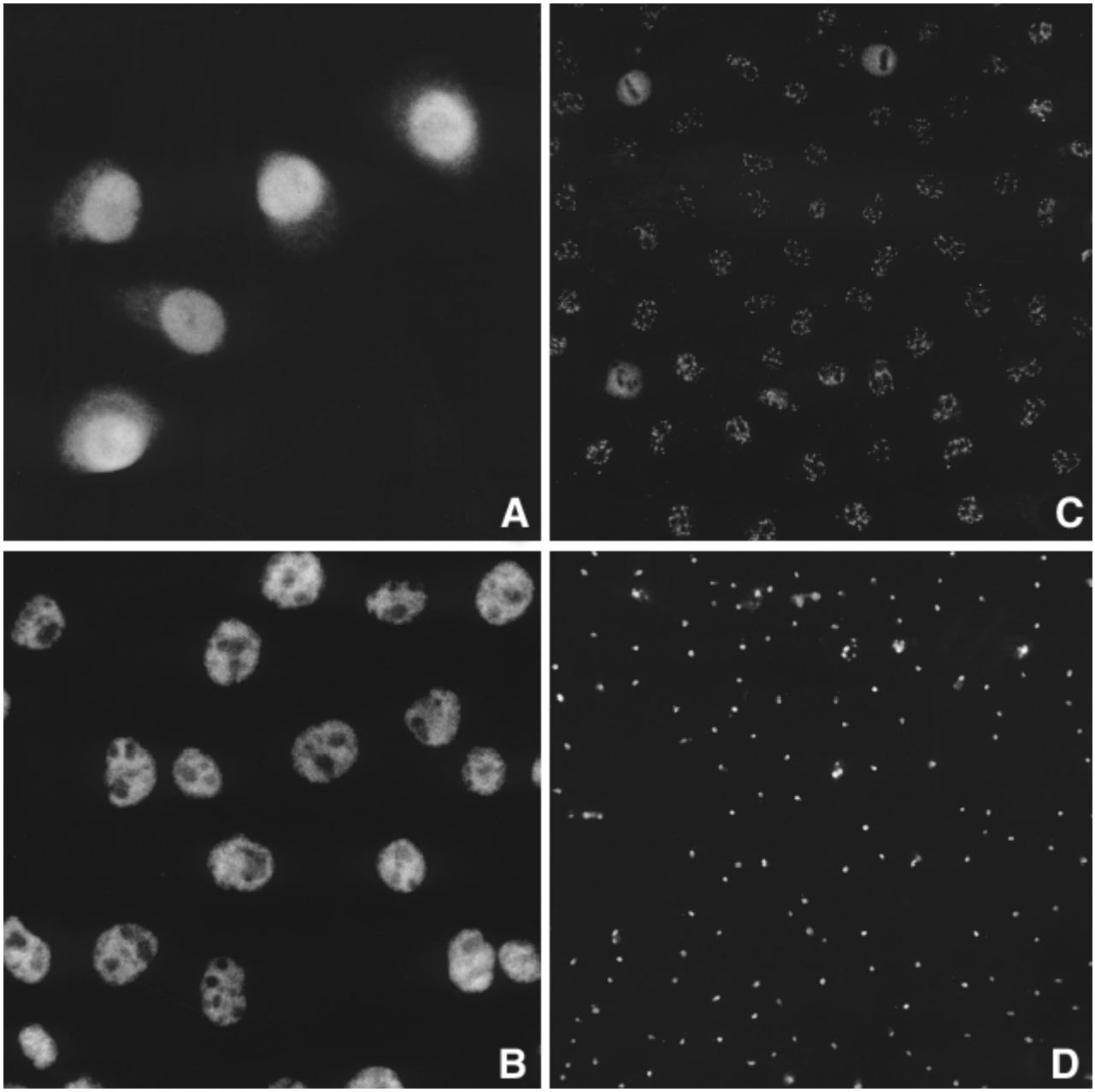

Fig. 1. Immunofluorescent staining patterns by antinuclear antibodies: A, diffuse homogeneous pattern; B, fibrillogranular pattern; C, speckled pattern; D, nucleolar pattern.

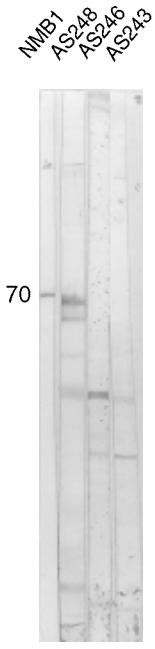

Fig. 2. Representative immunoblot on NMPs of HEp-2 cells by anti-NMP antibody containing sera: one to several peptides from NMPs of HEp-2 cells are detected.

various nuclear staining patterns as follows; diffuse homogeneous (Fig. 1A), fibrillogranular (Fig. 1B), coarse speckled (Fig. 1C), nucleolar (Fig. 1D), centromeric and mixed. A diffuse homogeneous pattern indicated smoothly rubbed nuclear staining. The fibrillogranular pattern consisted of diffusely scattered fine granules and underlying reticular network. Speckles were variably larger than granules.

Immunoblot analysis of anti-NMP antibodies

To investigate the presence and incidence of anti-NMP antibodies in autoimmune sera, we performed Western blotting with nitrocellulose papers on which nuclear matrix proteins of HeLa cells were transferred. We detected one to several bands in 212 among 284 sera, which stained nuclei in variable intensity and patterns. Ninety-one sera recognized only 1 peptide, 53 sera recognized 2 peptides, 22 sera distinguished 3 peptides, and more than 4 bands were recognized by 46 sera. The most frequently identified peptides were 70 kDa, 50 kDa and 25 kDa in the order of frequency (Fig. 2).

Clinical features or diagnosis of 198 out of 212 cases which had anti-NMP antibodies are summarized in Table 1. Thirty eight were autoimmune diseases, including systemic lupus erythematosus (16), rheumatoid arthritis (13), Sjögren's syndrome (2), scleroderma (1), antiphospholipid antibody syndrome (2), primary biliary cirrhosis (1), autoimmune cholangitis (2), and autoimmune hepatitis (1). There were 160 cases of non-autoimmune diseases, among which 132 were nonneoplastic diseases, 12 carcinomas and 4 benign neoplasms. Twelve cases presented nonspecific symptoms such as fever and myalgia.

Table 1. Clinical diagnosis of 198 patients with anti-NMP antibodies

Clinical diagnosis	No. of patients
Autoimmune diseases	**38**
Systemic lupus erythematosus	16
Rheumatoid arthritis	13
Sjögren's syndrome	2
Scleroderma	1
Antiphopholipid antibody syndrome	2
Autoimmune liver disease	4
Non-autoimmune non-neoplastic diseases	**129**
Neurologic diseases	30
Renal diseases	23
Liver diseases	19
Cardiovascular diseases	12
Dermatologic diseases	10
Gastrointestinal diseases	7
Endocrinologic diseases	7
Bone and joint diseases	7
Ophthalmologic diseases	4
Lung diseases	4
Gynecologic diseases	3
Infectious diseases	3
Neoplastic diseases	**16**
Carcinomas	12
Benign neoplasms	4
Unclassified	**15**

Immunofluorescent analysis of anti-NMP antibodies

Immunofluorescent staining by anti-NMP antibody positive sera which were tested by immunoblot analysis revealed diffuse fibrillogranular, speckled and nucleolar patterns. Twenty-one representative sera, which reacted with NMPs, were applied on the in situ fractionated HEp-2 cells. The immunofluorescent staining results of the 21 cases are summarized in Table 2. Fifteen sera revealed diffuse fibrillogranular pattern, 4 sera stained speckles, and 2 sera reacted with mainly nucleoli. Staining intensity decreased markedly in 11 cases, which included 9 diffuse fibrillogranular pattern, 1 speckled, and 1 nucleolar pattern. Ten sera retained their

Table 2. Immunofluorescent staining results on in situ frac-tionated HeLa cells

Nuclear staining pattern	No. of cases
Fibrillogranular	15
Speckled	4
Nucleolar	2
Mixed	0
Nuclear staining intensity	
Stationary	10
Decrease	11

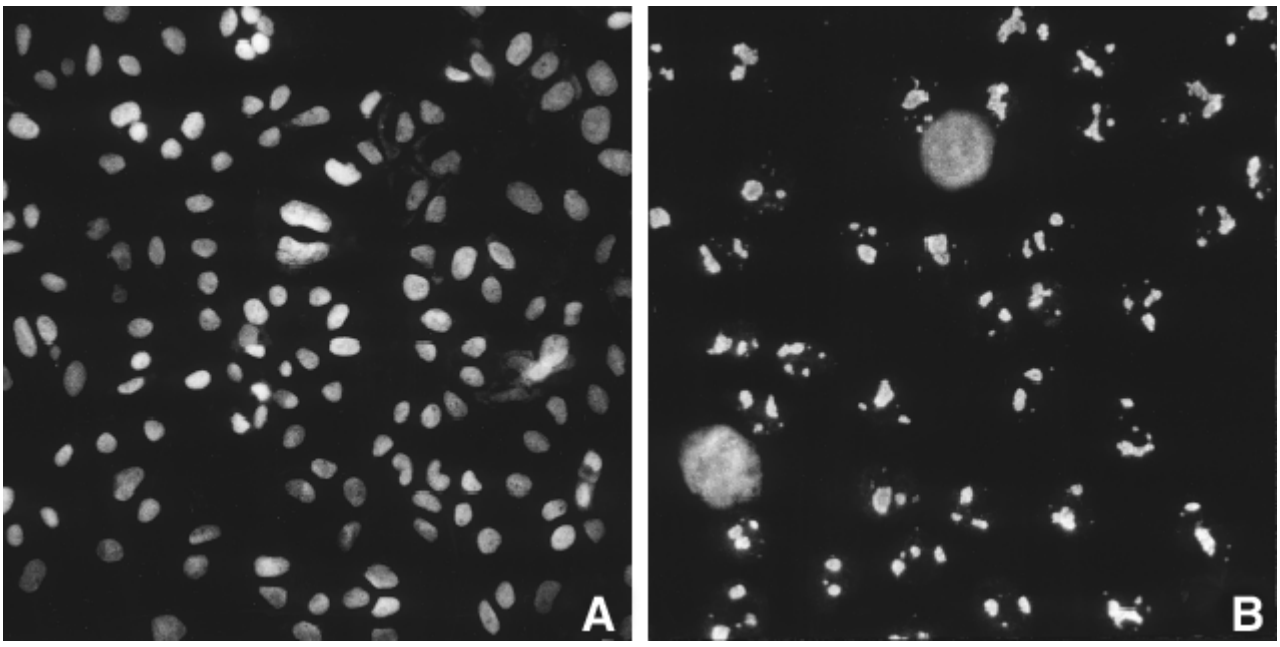

Fig. 3. Immunofluorescent staining patterns by anti-NMP antibodies on in situ fractionated HEp-2 cells: A, diffuse fibrilogranular; B, speckled pattern.

staining intensity and patterns after in situ fractionation. They included 6 sera showing diffuse fibrillogranular pattern (Fig. 3A), 3 sera revealing speckled pattern (Fig. 3B) and 1 serum decorating nucleoli.

Detection of Ku protein in NM

HEp-2 cell nuclear protein was immunoprecipitated by anti-Ku70 antibody, NMB1 and immunoblot was done with NMB1 and anti-NMP human sera which revealed a diffuse fibrillogranular immunofluorescent staining pattern. The peptide of 70 kDa, corresponding Ku70, were consistently identified (Fig. 4).

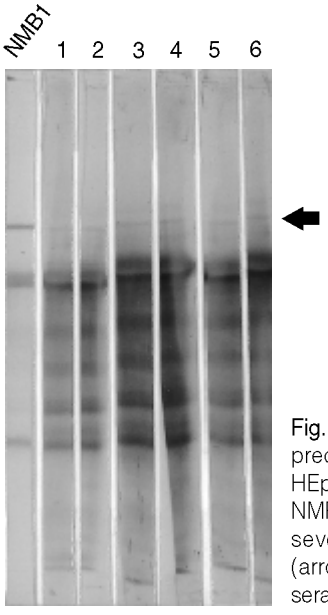

Fig. 4. Immunoblot on immunoprecipitated nuclear proteins of HEp-2 cells by NMB1 with anti-NMP antibody contatining sera: several bands including Ku70 (arrow) are detected by tested sera.

Detection of La protein in NM

We selected a serum which reacted three peptides with molecular weight of 70 kDa, 50 kDa and 45 kDa. The serum stained HEp-2 cell nuclei diffusely and its finely fibrillogranular staining pattern was similar to that by NMB1. But the serum did not stain in situ fractionated HEp-2 cell nuclei. HeLa cDNA library was screened with the serum and La protein gene was identified (Fig. 5) (19).

DISCUSSION

We identified a high incidence of antibodies against both in vitro and in vivo NMP by immunoblot analysis and immunofluorescent staining. We screened 284 ANA positive autoimmune sera and detected anti-NMP antibodies (212 among 284; 74.64%) by immunoblot analysis. The presence of anti-NMP antibodies in autoimmune sera was known to represent a specific type of autoimmune disease and revealed a characteristic staining pattern (20-22). However, we could demonstrate anti-NMP antibodies in various non-autoimmune diseases. ANAs are directed against components of functionally important subnuclear structures (5-7) and rec-

167
CACGAGGTTGAACCGTCTAACAACNNACTTTAATGTAATT

GTGGAAGCATTGAGCAAATCCAAGGCAGAACTCATGGAAA
286
TCAGTGAAGATAAAACTAAAATCAGAAGGTCTCCAAGCAA

Fig. 5. A partial cDNA sequence obtained by immunoscreening with a human serum. Oligonucleotide sequence exactly matched with that of human La cDNA is underlined by solid lines.

ognize highly conserved, conformation-dependent epitopes of the targeted subnuclear structures (23, 24). Thus, diffusely distributed NMP can frequently produce autoantibodies.

The nucleus is compartmentalized according to its function, but morphological details of the nuclear substructures are not well known because of the absence of solid membrane boundaries. The NM, an integral nuclear substructures, constitutes the nuclear skeleton as well as serves the field for various nuclear functions. However, there are those who still argue about the in situ structure of NM (25). There are two ways for detecting the structure of NM, monoclonal antibody libraries and antinuclear autoimmune sera. Using immunofluorescent analysis in this study, we can classify nuclear matrix structures as follows; 1) diffuse fibrillogranular, 2) speckled, 3) nucleolar and 4) mixed. The diffuse fibrillogranular pattern was similar to that detected by mAb NMB1 against Ku70, which recognized the nuclear matrix structure, consisting of fine meshwork and overlying fine granules. Speckled pattern in this study may be a part of spliceosomes, some components of which are in the NM. Nucleolar pattern was clearly uncovered after in situ fractionation with detergent, salt and DNase, which means that the nucleolar matrix is a major part of the NM in addition to the central and peripheral NM.

The immunogens inducing autoimmune responses are likely to be aggregates of diverse molecules forming subcellular particles (7-9). Therefore, autoantibodies against such subnuclear particles must be diverse in various autoimmune dieseases. However, only a few NMPs are immunogenic among more than 200 peptides of NM separated on 2 dimensional gel electrophoresis (25). Salden et al. (20) detected anti-NMP antibodies in 10 patients suffering from mixed connective tissue disease (MCTD) and 7 patients with SLE. The MCTD antibodies gave rise to the so-called 'speckled' pattern, excluding the nucleoli which was persistent after the in situ fractionation. In contrast, peripheral nuclear fluorescent pattern by SLE IgG fraction disappeared after DNase I treatment (20). In this study, we identified peptides with molecular weight of 70 kDa, 50 kDa and 25 kDa in the order of frequency. In screening of 21 MCTD sera, antigens of mol. wt 70 kDa, 31 kDa, 23 kDa and 19 kDa were identified in the matrix fraction (22). In contrast, Deng et al. reported 36 kDa and 30 kDa proteins as the most important nuclear matrix antigenic targets of anti-NMP antibodies (21).

The similarity in immunofluorescent staining pattern by mAb NMB1 and some anti-NMP antibodies suggested that the peptide of 70 kDa might be Ku70. We could detect Ku70 by the human sera on immunoprecipitated nuclear proteins by mAb NMB1. Ku protein is diffusely present in both normal and neoplastic human tissues except spermatids (14). However, anti-Ku antibody would not be pathogenic unless Ku protein has been exteriorized and becomes accessible to the antibody. It has been suggested that apoptosis may be involved in the release of potentially immunostimulatory antigens in privileged sites such as the nucleus, which is normally inaccessible to circulating antibodies (26-29).

We did immunofluorescent staining on in situ fractionated nuclei with limited cases of ANAs. The reactivity was stationary, decreased or disappeared. The discrepancy between the immunoblot analysis and immunofluorescent staining results can not be fully explained. But the fractionation procedure could be affected by delicate temperature change and reaction time.

We could select cDNA of La protein with a serum which showed fibrillogranular immunofluorescent pattern and reacted three peptides, including a peptide of 50 kDa from the NMP of HEp-2 cells. These results indicate that La protein (50 kDa) can be a NMP and form a very similar nuclear structure to that by Ku protein. But immunolocalization of La protein by a monoclonal antibody was different from that by the serum we tested (30). Thus, it can be suggested that La protein may be associated with other proteins and form the fibrillogranular nuclear matrix structure.

We could not identify any specific differences in clinical features between the patients with anti-NMP antibodies and those without anti-NMP antibodies. In addition, many patients could not be classified into autoimmune patients. ANAs can be detected in healthy controls in 31.7% at 1:40 dilution (31), therefore the roles of anti-NMP antibodies in

pathogenesis of autoimmune diseases are still undetermined.

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