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IDENTIFICATION OF AUTOANTIBODIES TO THE I PROTEIN OF THE HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN COMPLEX IN PATIENTS WITH SYSTEMIC SCLEROSIS

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Objective. **To assess the presence of autoantibodies to the I protein (polypyrimidine-tract binding protein) of the heterogeneous nuclear RNPs (hnRNP) in different connective tissue diseases. Antibodies to other hnRNP proteins (A1, A2, and B) have been previously found in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and mixed connective tissue disease (MCTD).**

Methods. **Sera from 101 patients with various connective tissue diseases and 25 normal controls were investigated by enzyme-linked immunosorbent assay and immunoblotting, for their reactivity to highly purified recombinant hnRNP I. Moreover, reactivity to cellular hnRNP I protein was investigated by immunoblotting using a partially purified preparation of hnRNP proteins (including A1, A2, B, and I), and by indirect immunofluorescence. For the analysis of the fluorescence pattern, affinity-purified antibodies to hnRNP I, obtained from a selected patient, were tested on HEp-2 cells.**

Results. **By immunoblotting, antibodies reacting to recombinant hnRNP I were found in 22 of 40 patients with systemic sclerosis (SSc), 3 of 32 with RA, 0 of 23 with SLE, and 0 of 6 with MCTD. Antibodies to recombinant hnRNP I were more frequently found in patients with pre-SSc or limited SSc (15 of 24) than in those with intermediate or diffuse SSc (7 of 16). In indirect immunofluorescence studies, affinity-purified anti–hnRNP I autoantibodies gave a diffuse nucleoplasmic staining. Using an hnRNP preparation from nuclear extracts, anti–hnRNP I reactivity was detectable in SSc sera, while it was not detectable in RA, SLE, and MCTD sera reacting with hnRNP A/B proteins.**

Conclusion. **Human autoimmune sera show distinct patterns of anti-hnRNP reactivity, i.e., anti-A/B in SLE and RA sera, and anti-I in SSc sera. This suggests that A/B proteins and the I protein may be involved in different dynamic hnRNP complexes that elicit different autoimmune responses. From a clinical perspective, anti–hnRNP I antibodies are frequently associated with pre-SSc features, suggesting an early appearance of these antibodies during the course of the disease.**

RNA (messenger, heterogeneous nuclear, small nuclear, and ribosomal) exist within the cell in association with specific proteins to form RNP complexes (mRNP, hnRNP, snRNP, and ribosomes). It is commonly accepted that the protein component of these complexes is essential to modulate different aspects characterizing the life of the cognate RNA, such as maturation, stability, and function (1). In the last 2 decades a growing number of autoantibodies to RNA-associated proteins have been identified in connective tissue diseases. Most of the described autoantibodies are directed to proteins belonging to the snRNP complexes or to ribosomes. These autoantibodies represent valuable tools for the diagnosis and prognosis of autoimmune rheumatic diseases (2–4).

However, very few studies have addressed the existence of autoantibodies directed to proteins of the mRNP and hnRNP complexes, in spite of their relative abundance and key role in the posttranscriptional control of the mRNA and pre-mRNA fate. This scarcity of data probably derives from the technical difficulties in purifying well-defined protein–hnRNA or –mRNA com-

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plexes. For the same reason, only recently has the structure and function of these complexes begun to be analyzed, particularly with regard to the hnRNP particles (1,5).

About 20 major hnRNP proteins have been identified in mammalian cells as bona fide hnRNP proteins, and they have been given designations ranging from A1 (34 kd) to U (120 kd) (1). The complementary DNA (cDNA) for most of them have been isolated and sequenced. Some of these proteins (hnRNPs A, B, and C) belong to the family of proteins containing the so-called RNA binding domain. Others (hnRNPs I and L) are characterized by protein motifs somewhat related to the RNA binding domain (6). The remaining hnRNP proteins possess unique sequence features. In any case, the definition of the protein component of the hnRNP complexes, the development of biochemical procedures suitable for their purification, and the expression of single-protein species in prokaryotic systems have made it possible to investigate the presence of autoantibodies that are directed toward the hnRNP proteins, although it is not yet possible to analyze their presence in intact hnRNP complexes.

Antibodies to the hnRNP proteins, first reported by Fritzler et al (7), have been detected and characterized in several studies of systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), and rheumatoid arthritis (RA) (8–12). In all of these studies, only the reactivity toward the hnRNP A/B proteins was analyzed. These proteins (A1, A2, B1, and B2) are highly similar in sequence and are therefore immunologically related (1).

Herein we report the first evidence of human autoantibodies directed to the hnRNP I, a 60-kd protein that has been proven to be the same as the recently described polypyrimidine-tract binding protein (13). The hnRNP I protein has some distinct features: 1) it binds hnRNA preferentially to the polypyrimidine tract near the 3′ end of introns, but lacks the canonical consensus sequences of the RNA binding domains found in hnRNPs A, B, and C as well as in several snRNP proteins; and 2) it seems to localize diffusely in nucleoplasm, similar to other hnRNP proteins, but is also concentrated in an as-yet-unidentified perinucleolar structure (1,6).

Autoantibodies reacting with hnRNP I are present in sera from patients with various connective tissue diseases. However, their occurrence is different from that of anti–hnRNP A/B because anti–hnRNP I are associated with the clinical features of systemic sclerosis (SSc).

PATIENTS AND METHODS

Patients. This study was carried out using sera from 101 patients with autoimmune connective tissue diseases and 25 healthy control subjects. For each disease group, all sera collected during the 3 months preceding this study were included. Sera were collected for routine diagnostic procedures, i.e., tests for antinuclear antibodies on HEp-2 cells and for antibodies to extractable antigens by counterimmunoelectrophoresis, and were then stored at −80°C. A complete clinical history was obtained for each patient by retrospective chart review.

Twenty-nine patients who had SSc fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) preliminary criteria for the classification of SSc (14) and were grouped in different clinical subsets according to the extent of skin involvement, i.e., limited SSc, intermediate SSc, or diffuse SSc (15). Eleven additional patients had Raynaud's phenomenon, defined as cold-induced blanching and cyanosis of the fingers, and had either anticentromere antibodies or scleroderma-like nailfold capillary abnormalities (16) or both. These patients did not fulfill the ACR criteria for SSc, but were regarded as having a limited SSc or a pre-SSc condition, as previously described (17–20). In the present study, these patients were classified as having pre-SSc; most of them had cutaneous teleangectasias and/or esophageal dysmotility, and 2 had primary pulmonary hypertension. Six other patients had Raynaud's phenomenon and fulfilled the criteria for MCTD proposed by Alarcon-Segovia and Cardiel (22). Finally, 32 patients had RA and 23 had SLE according to the ACR criteria (23,24).

Expression and purification of the recombinant antigen. Polymerase chain reaction was used to tailor the hnRNP I cDNA for expression in *Escherichia coli,* using the expression system pRC23 (25). The human hnRNP I cDNA (kindly provided by C. Morandi [6]) was amplified with N-terminal (5′-AGAATGAATTCCATGGACGGCATTGTCCCA-3′) and C-terminal (5′-AATCAAGCTTATGbpAAGTTGTCGC-AGGGGG-3′) oligonucleotides. The amplified product was digested with *Eco* RI and *Hind* III and subcloned into the corresponding sites of pRC23. After *E coli* transformation (25), the sequence of the recombinant plasmid was verified by the dideoxy-chain termination method (Sequenase kit; United States Biochemical, Cleveland, OH).

Cell growth and induction of the protein were performed as previously described (26). The washed frozen cells from a 2-liter culture were resuspended in 30 ml of buffer A (20 m*M* HEPES, 0.1*M* KCl, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, 0.4 m*M* phenylmethylsulfonyl fluoride, pH 7.9) and lysed by sonication. The suspension was centrifuged at 20,000*g* for 20 minutes at 4°C and the supernatant fraction was loaded on a 50-ml DEAE-cellulose column (2.5 × 10 cm) connected in tandem with a 5-ml agarose–poly U column (AGPOLY U type 6, 1.0 × 6.5 cm; Pharmacia, Brussels, Belgium). Both columns were equilibrated and run with buffer A.

The DEAE column was disconnected and the agarose–poly U column was then washed with 25 ml of buffer A. After subsequent washing with 25 ml each of buffer A containing either 200, 300, 500, or 750 m*M* KCl, hnRNP I was eluted from the column with 15 ml of buffer A containing 1*M* KCl. The eluate was concentrated by ultrafiltration (Centricon-10; Amicon, Beverly, CA) as described by the manufacturer.

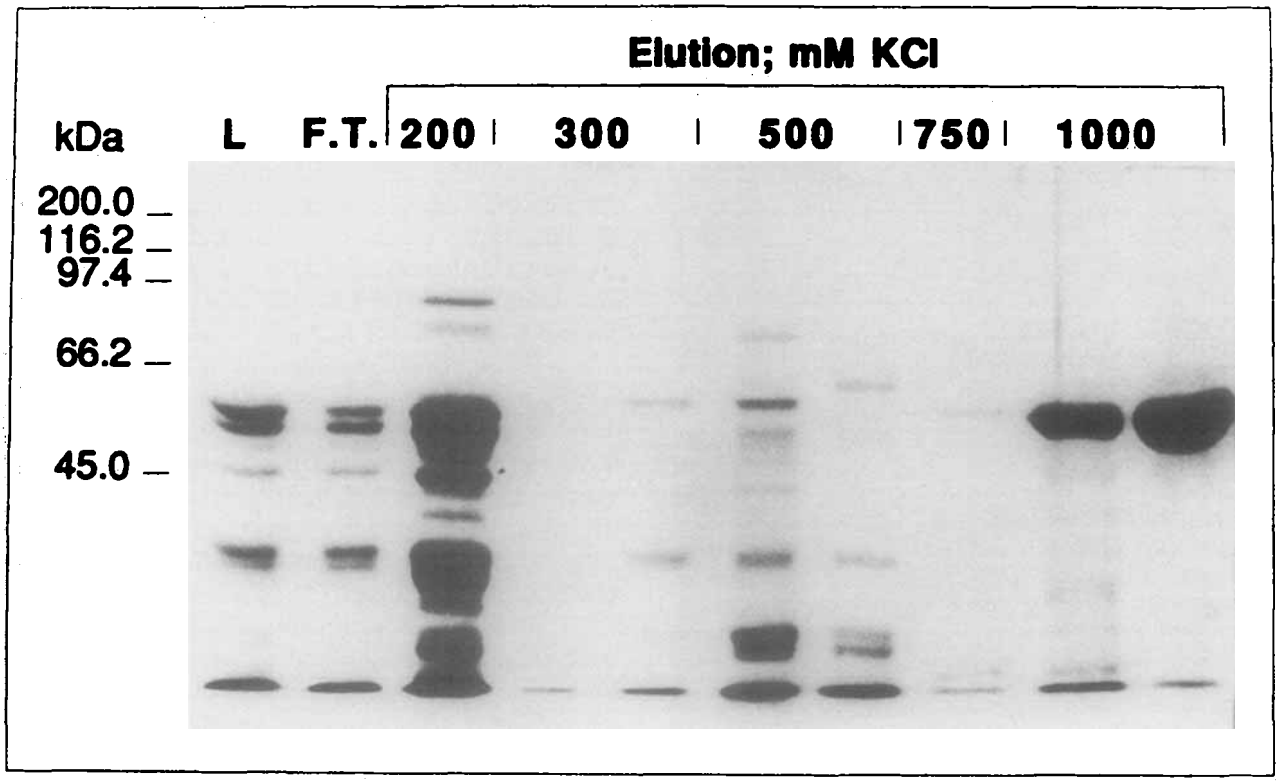

Figure 1. Agarose–poly U chromatography. The material passing through the DEAE-cellulose column (L) interacted with the agarose–poly U column, and heterogeneous nuclear RNP I (hnRNP I) protein bound quantitatively to the column. The flowthrough fraction of agarose–poly U column (F.T.) corresponds to 100 m*M* KCl, and bound proteins were eluted with KCl as indicated. The samples were run on a 12.5% polyacrylamide gel and dye stained with Coomassie blue. Molecular masses in kilodaltons are given on the left.

Purification of hnRNP proteins from nuclear extracts. The hnRNP proteins were purified from 5 gm of exponentially grown HeLa cells, according to the method described by Pinol-Roma et al (27). The purified hnRNP proteins were stored at −80°C in 50% glycerol. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transfer to nitrocellulose, and immunologic detection with antibodies were performed as previously described (28), except that all incubations were done in 5% skim milk (Difco, Detroit, MI). All the sera tested were used at a 1:100 dilution and revealed with peroxidase-conjugated rabbit antibodies to human IgG (Dako, Milan, Italy).

A rabbit antiserum directed against a subset of bovine hnRNP proteins was used as the positive control. This rabbit antiserum was specific for hnRNPs A1, A2, B1, B2, and I, as previously described (6,29). Anti-hnRNP antibody concentration in rabbit serum is very high, and binding to purified recombinant hnRNP I was still detectable by enzyme-linked immunosorbent assay (ELISA) at a final dilution of 1:100,000.

Immunoblots and ELISA. One microgram of recombinant hnRNP I was mixed in 62.5 m*M* Tris HCl, pH 6.8, containing 5% 2-mercaptoethanol, 2% SDS, and 0.1% bromphenol blue, heated at 100°C for 5 minutes, and then centrifuged. The supernatant was loaded on a 10% SDS-PAGE column and run at 30 mA for 4 hours. The gel was blotted onto a nitrocellulose sheet that was cut into strips, washed, and processed as previously described (28). Human sera were tested at a 1:500 dilution, and peroxidase-conjugated antibodies to human IgG were used as a secondary probe.

Patient sera, reacting with recombinant hnRNP I, were also studied by ELISA at 1:100 and 1:1,000 dilution. ELISA was performed in triplicate, as previously described, for recombinant A1 protein (11) using peroxidase-conjugated antibodies to human IgG (Dako). The optical density (OD) was measured at 492 nm using a Titertek multiscan reader (Flow, Opera, Italy). The cut-off value was established as 3 SD above the mean for normal controls (8 controls for each 96-well plate). Arbitrary units were obtained according to the formula patient OD value/cut-off value. To rule out nonspecific binding, all sera were also tested on uncoated wells.

Affinity purification of anti–hnRNP I antibodies and indirect immunofluorescence. Affinity purification of anti–hnRNP I antibodies was performed essentially as previously described (30). Briefly, 200 μg of purified recombinant hnRNP I protein was loaded onto the entire length of a 10% SDS-PAGE. After the gel had been run, proteins were transferred to a nitrocellulose filter and stained with Ponceau. A nitrocellulose strip corresponding to the hnRNP I was cut, incubated for 30 minutes in blocking buffer composed of Tris buffered saline (TBS; 10 m*M* Tris HCl, pH 7.4, 0.9% NaCl) rendered in

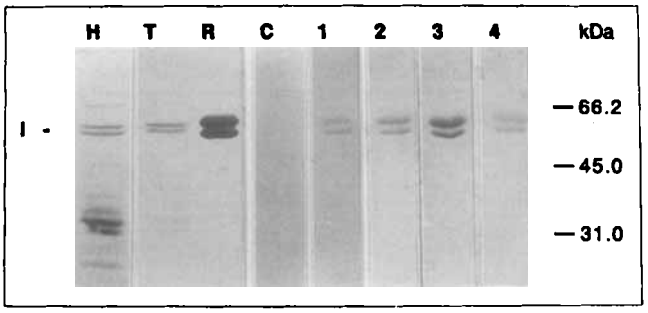

Figure 2. Reactivity of anti-hnRNP rabbit antibodies and human systemic sclerosis (SSc) sera (1:500 dilution) to recombinant hnRNP I protein. Four different human pre-SSc and SSc sera (lanes 1–4), 1 normal human serum (lane C), and rabbit antibodies (1:10,000) (lane R) were utilized in immunoblots to stain 1 μg of recombinant hnRNP I protein. For comparison, staining of total cell extracts (10 mg of wet cell pellets) from HeLa cells (lane H) and NT2/D1 teratocarcinoma cells (lane T) with the same rabbit antibodies is shown. The position of the cellular hnRNP I protein is indicated on the left. The doublet detectable in lanes R–4 corresponds to hnRNP I and its major degradation product, which originated during purification. In contrast, the 2 bands present in total cell extract (lanes H and T) probably represent hnRNP I isoforms (see ref. 6). Molecular masses in kilodaltons are given on the right. See Figure 1 for other definitions.

2% skim milk, and then incubated overnight at 4°C with 300 μl of patient serum at 1:4 dilution with gentle shaking in an Eppendorf tube.

The serum remaining after this incubation showed no recognition of hnRNP I either in the ELISA or in the immunoblotting studies. This serum was kept for further immunofluorescence analysis (depleted serum). After rinsing the filter twice in TBS, the antibodies were eluted in 500 μl of elution buffer (0.2*M* glycine, pH 2.8, 1 m*M* EGTA) and immediately neutralized by adding 50 μl of 1*M* Tris base. As expected, affinity-purified antibodies showed a strong reactivity for hnRNP I by immunoblotting.

Indirect immunofluorescence was carried out, as described (31), on methanol-fixed HEp-2 cells, using total serum and anti–hnRNP I–depleted serum at 1:20 dilution, and on affinity-purified antibodies, as obtained by the above-described procedure, without further dilution. Fluorescein isothiocyanate–conjugated rabbit anti-human IgG antibodies (Dakopatts, Copenhagen, Denmark) were used as a secondary antibody.

RESULTS

Expression and purification of hnRNP I. The cDNA for the human hnRNP protein I has been isolated recently by immunologic screening of a human liver cDNA library (6). In order to extend the characterization of autoantibodies directed to proteins of the hnRNP complexes, we induced expression of hnRNP protein I in *E coli*. Recombinant protein was purified to homogeneity by column chromatography using DEAE-cellulose and agarose–poly U matrices. Since most of the protein I (>90%) eluted in the flowthrough, and in the washes (100 m*M* KCl) of, DEAE-cellulose (results not shown), a simple purification scheme was adopted in which, in order to speed up the process of purification, the DEAE-cellulose column was connected in tandem with the agarose–poly U column. This matrix was chosen on the basis of the reported binding preference of protein I to poly U ribopolymers (29). As shown in Figure 1 (intentionally overloaded), the protein eluted, after extensive washes, at 1*M* KCl as a homogeneous protein.

The purified recombinant hnRNP I protein had an apparent molecular mass of 60 kd, as shown in Figure 1. After concentration and upon storage, it resolved into a doublet of bands, both of which were recognized by the rabbit antiserum utilized to isolate the cDNA (Figure 2). The band with a slightly lower molecular mass represents a proteolytic product that originated during the purification procedure.

Reactivity to recombinant hnRNP I. Human sera were tested against purified recombinant hnRNP I by both ELISA and immunoblotting. A screening test was carried out by means of ELISA with a 1:100 dilution of the sera. Sera from 25 normal controls and 101 patients (40 with SSc, 32 with RA, 23 with SLE, and 6 with MCTD) were tested. Higher than normal serum values for hnRNP I reactivity were found in nearly 80% of SSc patients, 20% of RA patients, and 20% of SLE patients, as well as in 2 of 6 MCTD patients (Figure 3). Using a 1:1,000 dilution of the sera, however, only 11 sera (all from SSc patients) gave positive values for hnRNP I reactivity.

To exclude nonspecific binding, all sera were also tested on uncoated wells. No SSc serum, tested in this way, showed OD values higher than the cut-off value. To rule out the possibility that the observed results could be due to a reactivity to bacterial contaminant proteins, albeit undetectable by SDS-PAGE analysis (see Figure 1), we used the human sera in immunoblot experiments. In these experiments, 1 μg of recombinant protein was used and probed with a 1:500 dilution of the sera. No differences were observed in the staining of protein bands between these experiments and those using the purified recombinant protein, thus confirming the data obtained in the previous analysis. Some representative results are shown in Figure 2. A significant reactivity was found in 22 of 40 patients with either SSc or pre-SSc and in only 3 of 32 patients with RA. None of the 28 patients with SLE or MCTD showed detectable anti–hnRNP I reactivity. No reactivity was ever observed with control sera at the dilution utilized.

It is worth noting that all patient sera showing >2

arbitrary units in the screening test by ELISA were also positive for hnRNP I staining by immunoblotting (Figure 3). Anti–hnRNP I antibodies, as detected by immunoblotting, were present in all of the different SSc subsets (Table 1), although they were more frequently found in patients with pre-SSc and limited SSc (15 of 24) than in those with either intermediate or diffuse SSc (7 of 16). No specific association was found with either anticentromere or anti–Scl-70/topoisomerase I or with any other clinical characteristics.

Reactivity to hnRNP proteins purified from nuclear extract. As shown previously, 11 of the analyzed sera had antibodies to recombinant hnRNP protein I, detectable by both immunoblotting and ELISA at 1:1,000 dilution. We then investigated whether the same sera were able to recognize the cellular hnRNP protein.

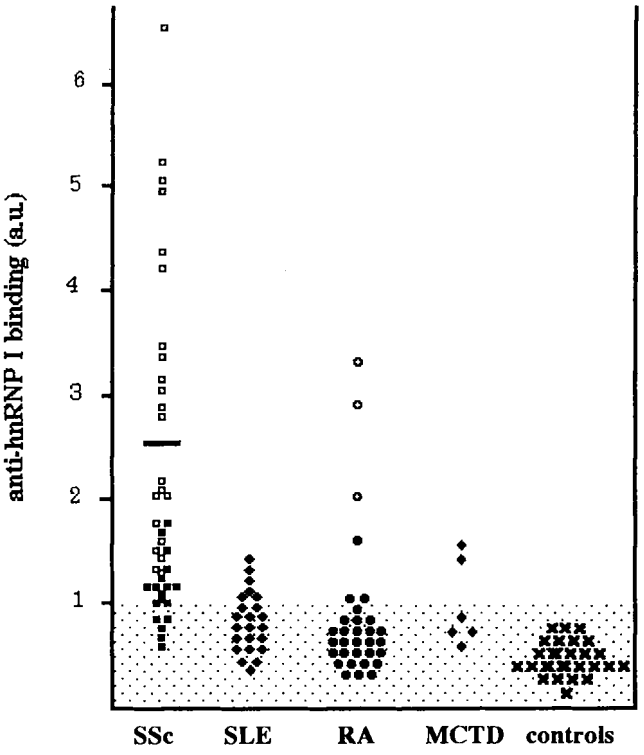

Figure 3. Binding of human sera from patients with different autoimmune diseases to purified recombinant hnRNP I protein, as determined by enzyme-linked immunosorbent assay (screening test at a 1:100 dilution of the sera). Shaded area represents the range of optical density values lower than the cut-off value of 1 arbitrary unit (a.u.). The cut-off value was chosen as 3 SD above the mean for normal control optical density values. Open symbols in the systemic sclerosis (SSc) and rheumatoid arthritis (RA) disease subsets represent sera with antibodies to recombinant hnRNP I that were also detectable by immunoblotting. Horizontal bar in the SSc column represents the mean value. SLE = systemic lupus erythematosus; MCTD = mixed connective tissue disease. See Figure 1 for other definitions.

Table 1. Occurrence of antibodies to recombinant heterogeneous nuclear RNP I (hnRNP I), as detected by immunoblotting, in the different systemic sclerosis (SSc) subsets*

Clinical feature	n	Anti-hnRNP+ (n = 22)	Anti-hnRNP− (n = 18)
Pre-SSc	11	8 (73)	3 (27)
Limited SSc	13	7 (54)	6 (46)
Intermediate/diffuse SSc	16	7 (44)	9 (56)
Anticentromere	17	9 (53)	6 (47)
Anti–Scl-70	8	3 (37)	5 (63)
Anti–SS-A/Ro	2	2 (100)	0
Rheumatoid factor	13	7 (54)	6 (46)

* Values are the number (%) of patients positive for each clinical feature. No reactivity for definite nuclear antigens, other than Scl-70, SS-A/Ro (by counterimmunoelectrophoresis), or centromere (by indirect immunofluorescence using HEp-2 cells), was found in the patients with SSc. Among all 40 patients with SSc, 55% were positive and 45% were negative for anti–hnRNP I.

Toward this aim, hnRNP proteins were chromatographically purified from HeLa cells. As shown in Figure 4 (lane C), this method led to the isolation of a set of hnRNP proteins with molecular masses ranging from 34 kd to 68 kd. The presence, in this preparation, of hnRNP proteins A/B and I was detected by immunostaining with rabbit antibodies specific for this set of polypeptides (the same antibodies utilized for the isolation of both A1- and I-specific cDNAs) (6,26) (Figure 4, lane A). Of note, protein I was underrepresented in our hnRNP preparation; nevertheless, the high-positive sera (detectable by ELISA at a 1:1,000 dilution) were found to bind cellular hnRNP I in immunoblot assay. Neither control sera nor sera with low-positive values (detectable only by ELISA at a 1:100 dilution) were found to react with hnRNP I. All tested RA, SLE, and MCTD sera showed no recognition of hnRNP I. However, as expected on the basis of previously reported data (11), several of these sera were found to recognize other hnRNP proteins, namely A1, A2, B1, and, probably, C. Control sera were consistently negative for all of the hnRNP proteins (Figure 4).

Immunofluorescence studies. All 11 sera with high-titer anti–hnRNP I showed a positive immunofluorescence test result for antinuclear antibodies on HEp-2 cells. Three of these sera had diffuse nuclear fluorescence only, 5 had a centromeric pattern associated with weak diffuse nucleoplasmic staining, and 3 had nucleolar fluorescence associated with a diffuse nucleoplasmic staining. Serum that showed the highest binding in ELISA was selected for affinity purification experiments. Reactivity of this serum to hnRNP I protein purified from HeLa cells is shown in lane 1 of Figure 4. Figure 5 shows the indirect immunofluorescence patterns obtained with total serum (Figure 5A), affinity-

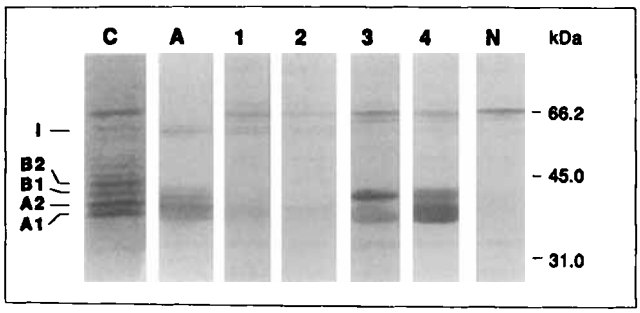

Figure 4. Presence of antibodies against different hnRNP proteins in autoimmune sera. Lane C, hnRNP proteins purified from HeLa cells, analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue. Lane A, Western blot analysis of hnRNP proteins with rabbit antibodies. Lanes 1–N, hnRNP proteins immunostained with sera (1:100 dilution) from different autoimmune patients (1 with pre-systemic sclerosis [SSc], 2 with SSc, 3 with rheumatoid arthritis, and 4 with systemic lupus erythematosus) or from a normal individual (N). The position of relevant hnRNP proteins is indicated on the left. Molecular weight markers are indicated on the right. The band at about 66 kd represents a nonspecific reaction, since normal serum also seems to react with it. The band of approximately 62 kd, detectable in lanes 1 and 3, has not yet been identified, but on the basis of its molecular mass, we may speculate that it corresponds to hnRNP L or M (see ref. 1). See Figure 1 for other definitions.

purified anti–hnRNP I antibodies (Figure 5B), and anti–hnRNP I–depleted serum (Figure 5C) on HEp-2 cells. Depletion of anti–hnRNP I antibodies resulted in a selective disappearance (or intensity reduction) of the diffuse nuclear staining, without affecting centromeric fluorescence. In contrast, as expected, affinity-purified anti–hnRNP I antibodies showed a prevalent diffuse nucleoplasmic staining.

DISCUSSION

This study demonstrates the presence, in human sera, of autoantibodies to the hnRNP I protein, one of the abundant hnRNP proteins in human cells (1). Evidence for the presence of anti–hnRNP I in human sera is based on 1) reactivity to purified recombinant hnRNP I by both immunoblotting and ELISA, 2) specific immunostaining of protein I in hnRNP protein preparation from HeLa cells, 3) selective disappearance of a weak diffuse fluorescent nuclear staining after absorption on purified recombinant hnRNP I protein, and 4) diffuse nucleoplasmic staining with affinity-purified anti–hnRNP I antibodies from a selected positive serum.

These findings extend the as-yet limited analysis of hnRNP-directed autoimmunity. In addition, our results seem to open new perspectives on the study of diagnostic parameters for rheumatic diseases and on the study of the biologic functions of single hnRNP proteins. In this context, it is worth noting that almost all sera that strongly reacted with the A/B proteins did not recognize the I protein (Figure 4). Furthermore, anti–hnRNP I reactivity was mainly associated with features of SSc, whereas SLE, RA, and MCTD patient sera preferentially recognized A/B proteins. Accordingly, significant binding to hnRNP I was found in SSc sera, but not in SLE and RA sera, when the purified recombinant polypeptide was used as the antigen source in immunoblotting studies. Differences in the frequency of positive sera observed between immunoblotting studies and ELISA seemed mainly due to the different serum dilutions used.

Positive results for hnRNP I reactivity were ob-

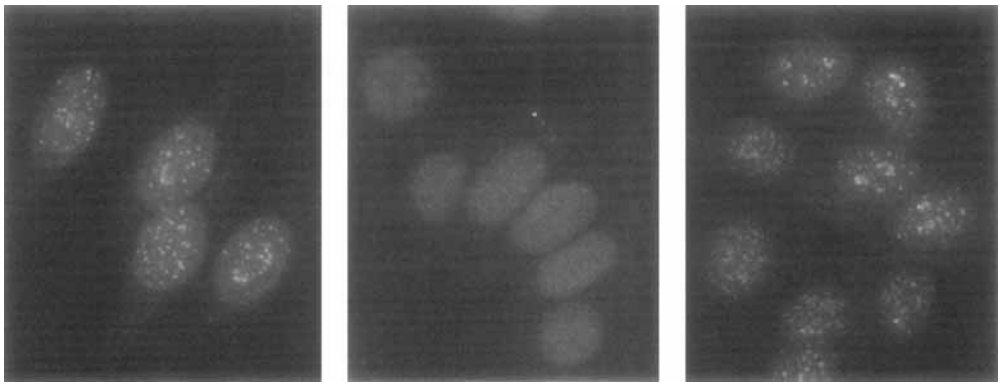

Figure 5. Immunofluorescence staining of HEp-2 cells with the same hnRNP I–positive serum used in lane 1 of Figure 4. The fluorescence patterns obtained with whole serum (**A**), anti–hnRNP I affinity-purified antibodies (**B**), and anti–hnRNP I antibody–depleted serum (**C**) are shown. See Figure 1 for definitions.

tained in nearly 80% of SSc patients, 20% of SLE patients, and 20% of RA patients, using ELISA at a 1:100 dilution. At a higher dilution (1:1,000), 28% of SSc sera were found positive, while none of the 61 sera from patients with other autoimmune rheumatic diseases showed positive results. These data suggest that antibodies to hnRNP I can be detected in the majority of sera from patients with SSc, and that high-titer antibodies are almost specific for this condition, whereas SLE and RA sera mainly react with the A/B hnRNP proteins (9–12), suggesting a different pattern of anti-hnRNP reactivity. In particular, the entire set of basic hnRNP core proteins (A/B) seems to constitute a common target of the autoimmune response in RA and SLE patients (Figure 4), as has been recently addressed (10).

Complex structures are common targets of autoimmunity in connective tissue diseases, so that antibodies directed to different proteins of a unique complex can be found in a single patient and in patients with the same disease (2–4). The data presented herein reveal that hnRNP proteins A/B and I are distinct targets of different autoimmune responses. According to the antigen-driven origin of autoantibodies, one may speculate that these proteins may be involved in different dynamic hnRNP complexes. This would not be surprising, since hnRNP protein binding is not random with respect to RNA sequence, and each different hnRNA associates with a unique combination of hnRNP proteins (1). Furthermore, A/B proteins and the I protein are sharply different in their predicted structures and considerable evidence has accumulated to suggest different functions in pre-mRNA processing (1).

Relevant details on the distinct features of hnRNP I have been previously described (6). The hnRNP I protein binds RNA structures that are particularly exposed and is released from hnRNP complexes more readily than most other abundant proteins, which might explain, in part, the selective autoimmune response to hnRNP I. Moreover, immunofluorescence microscopy with monoclonal antibodies has localized hnRNP I to the nucleoplasm of interphase HeLa cells and has shown 1 (occasionally, 2) spots per nucleus, which are always closely apposed to a nucleolus. It seems likely that these regions with a higher concentration of hnRNP I represent sites of transcription of specific hnRNA species (6). This preferential perinucleolar localization is intriguing, since nucleolar or nucleolus-related antigens are a specific target of autoimmune response in patients with SSc and related disorders (32–34).

Another intriguing point is the relationship between hnRNPs and RNA polymerase, since some hnRNP proteins assemble on RNA polymerase II transcripts as soon as they are synthesized (1,35) and autoantibodies to RNA polymerases occur specifically in SSc (36,37). We believe that studies on the association of anti–hnRNP I with different SSc-associated autoantibodies might provide new insights on co-localization and function of the hnRNP I protein.

In our patients, anti–hnRNP I reactivity was associated with a fluorescence pattern characterized by diffuse nucleoplasmic staining. A similar diffuse staining was also observed with affinity-purified anti–hnRNP I antibodies from a selected positive patient serum. The lack of perinuclear spots in our immunofluorescence studies could be explained by the different experimental system used (1,6).

Most autoantibodies found in SSc are known to mark off separate clinical subsets of the disease. For example, anti–Scl-70 (topoisomerase I) are usually associated with diffuse SSc, anticentromere with limited SSc, and antifibrillarin and anti–PM-Scl with SSc/polymyositis overlap disease (17,18,32,38,39). Antibodies to hnRNP I were present in all SSc subsets in our study, but were found more frequently in those patients with pre-SSc and limited SSc. The occurrence of these antibodies in patients with features of pre-SSc suggests an early appearance during the course of the disease, similar to that observed for anticentromere antibodies (17,19). No significant association between anti–hnRNP I and anticentromeric reactivity was found in our study. However, the present series was too small to drawn any definite conclusion.

Further studies on larger series are needed to evaluate the relationship of these antibodies to hnRNP I with clinical and serologic features, as well as to assess the behavior of these antibodies during the course of the disease. In particular, it would be interesting to prospectively evaluate those patients with pre-SSc or with isolated Raynaud's phenomenon to ascertain whether these antibodies would be predictive of an evolution to overt SSc. At present, this can only be suggested by the lack of correlation of these antibodies with Raynaud's phenomenon in other connective tissue disorders such as MCTD.

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