PREDOMINANCE OF IgM ANTI-U1RNP ANTIBODIES IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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SUMMARY

Anti-U1RNP antibodies occur in patients with mixed connective tissue disease (MCTD), systemic sclerosis (SSc), systemic lupus erythematosus (SLE) and other ill-defined connective tissue diseases. To associate the isotypes of anti-U1RNP antibodies with the diagnosis of the disease, namely SLE or MCTD, sequential sera of patients positive for anti-U1RNP antibodies by counterimmuonelectrophoresis (CIE) (32 with SLE, 35 with MCTD) were tested for IgG and IgM anti-U1RNP antibodies by enzyme-linked immunosorbent assay (ELISA) using affinity-purified U1snRNP complexes. Results from ELISA were confirmed by RNA precipitation. IgG RNA precipitation of HeLa cellular extracts was performed using the bulk of the IgG fraction removed from each serum after binding to protein A-Sepharose beads. IgM RNA precipitation was carried out on the IgM fraction of the serum bound to protein A-Sepharose-rabbit anti-human IgM immune complexes. RNAs were electrophoresed in 10.5% acrylamide-7 M urea gels and detected with the silver stain. ELISA showed that all sera were positive to IgG anti-U1RNP, while 12 of the 35 MCTD and 21 of the 32 SLE patients possessed IgM anti-U1RNP (P < 0.025). IgM anti-U1RNP reactivity was found during the follow-up in 20% of 44 sera from 17 MCTD patients and 68% of 112 sera from 23 SLE patients (P < 0.0001). IgG from all the sera precipitated U1RNPs. Eight of the MCTD sera also precipitated U2RNPs and 14 of the SLE sera U2 and/or U4/U6, U5 RNPs. IgM from MCTD sera did not precipitate URNPs, while IgM from SLE sera precipitated predominantly U1RNPs. These data suggest that IgM anti-U1RNP antibodies occur predominantly in patients with SLE. The occurrence of IgG anti-U1RNP without IgM is more frequent in MCTD.

KEY WORDS: Anti-U1RNP, Autoantibodies, Isotypes, SLE, MCTD.

The term 'mixed connective tissue disease' (MCTD) [1] was introduced to describe patients with a combination of features, such as non-erosive arthritis indicative of systemic lupus erythematosus (SLE) [2], Raynauds' phenomenon, puffy hands, sclerodactyly, oesophageal hypomotility indicative of systemic sclerosis (SSc) [3] and muscle involvement indicative of myositis [4]. The sera of these patients recognized a nuclear antigen extracted in isotonic buffers [5] which was found to be a physical complex of two antigens: one sensitive to ribonuclease and trypsin, designated 'nuclear RNA-protein' (nRNP) and the other resistant, designated Sm [6]. The sera of MCTD patients reacted exclusively against the nRNP antigen and, therefore, the anti-nRNP antibodies were considered as a distinct serological feature associated with MCTD [7]. Progress in molecular biology revealed that both nRNP and Sm are complexes of small nuclear RNAs with proteins, in other words small nuclear ribonucleoproteins (snRNPs), and that anti-nRNP antibodies recognize only components unique to U1snRNP, while anti-Sm antibodies recognize common proteins to U1, U2, U4/U6, U5 snRNPs [8–10]. The U1snRNP specific proteins are a 70 kDa polypeptide as well as polypeptides A (34 kDa) and C (22 kDa) which in complex with U1RNP form the U1RNP antigen. The Sm proteins recognized by anti-Sm antibodies correspond to B' (29 kDa), B (28 kDa), D (16 kDa) and occasionally to the peptides E (12 kDa), F (11 kDa) and G (9 kDa) [10–12]. In terms of small linear epitopes, a considerable cross-reactivity exists between peptides B'/B, A and C of the Sm and U1RNP antigens [13]. Antibodies to the 70 kDa polypeptide are mainly of the IgG class, while antibodies to the A polypeptide are of the IgM class [14]. IgG anti-70 kDa and IgM anti-B'/B have been associated with arthralgias and Raynaud's phenomenon [15]. In a recent report, IgM anti-A and anti-D polypeptides were associated with IgM anti-ds DNA in the sera of patients with SLE [16]. However, the previous reports were based on studies using Western blotting and therefore the polypeptides were denatured. In this report, an effort was undertaken to detect the isotypes of antibodies against snRNPs by using the polypeptides of the Sm and U1RNP antigens in their natural configuration in complexes with the respective RNAs, and to associate these isotypes with the disease status.

MATERIALS AND METHODS

Clinical diagnoses and serum samples

Sequential sera from consecutive patients with autoimmune connective tissue diseases, positive for anti-U1RNP antibodies either alone or associated with other precipitating antibodies by counterimmuno-electrophoresis (CIE) [17], were tested for the presence...
of IgG and IgM anti-U1RNP antibodies by a sensitive and specific enzyme-linked immunosorbent assay (ELISA) [18]. The IgG and IgM reactivities of the sera against U1 and Sm-snRNPs were further characterized by RNA immunoprecipitation [19]. Sera from 32 patients with SLE and 35 with MCTD were tested. The diagnosis of SLE was established according to the 1982 revised criteria of the American Rheumatism Association for the diagnosis of SLE [2]. The diagnosis of MCTD was established when the patients presented anti-U1RNP antibodies by CIE plus three of the following four clinical manifestations: oedema of the hands, synovitis, myositis, Raynaud’s phenomenon and acrosclerosis [20].

Affinity purification of U1RNP from calf thymus extract

Sera positive for antibodies to U1RNP by CIE [17] were further characterized by RNA immunoprecipitation, using HeLa cellular extracts [19]. A serum with reactivity against only U1RNP by RNA precipitation recognizing the 70 kDa specific U1RNP antigen by immunoblotting [21] was chosen for the preparation of an immunoaffinity column for U1RNP purification (Fig. 1). A protein A-Sepharose column (Pharmacia) was used to separate IgG from the monospecific anti-U1RNP serum, according to standard protocols [22]. Briefly, 80 mg of the above IgG were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) at 5 mg IgG/ml of gel according to the manufacturer’s instructions. The remaining active sites were blocked by 0.2 M glycine. Purification of U1RNP was performed as previously described [18]. Briefly, antigen was extracted by homogenizing 100 g of calf thymus in 300 ml of 0.15 M phosphate-buffered saline (PBS) (pH 7.2) and partially purified by ammonium sulphate fractionation. The 30–60% ammonium sulphate fraction of the extract was passed through the column and after extensive washing with PBS (pH 7.2) containing 0.5 M NaCl, the antigen bound to the column was eluted with the 3 M guanidine-HCl (pH 7.2) and dialysed against PBS (pH 7.2).

ELISA

A solid-phase ELISA was developed to detect the antibody class (IgG and IgM) with anti-U1RNP specificity [18]. Antigen [20 µg/ml in PBS (pH 7.2)] was coated onto 96 well polystyrene cuvettes pre-coated with poly-L-lysine (Sigma, Poole, Dorset) (50 µg/ml in PBS). After washing with PBS, the non-specific binding sites were blocked with 10% bovine serum in PBS at 37°C. One hundred microlitres of sera diluted in 10% bovine serum in PBS (1:100 in the case of IgG and 1:100 in the case of IgM) were added in duplicates and incubated for 2 h after washing. Antibody-bound antigen was detected with alkaline phosphatase-conjugated anti-human γ or µ chain specific antisera (SERA-LAB, Sussex). Finally, the plates were washed, substrate solution (p-nitrophenyl phosphate, 1 mg/ml)
in diethanolamine buffer (pH 9.8) was added to each well and the absorbance was read at 405 nm. The washing buffer was PBS containing 0.05% Tween (PBS-Tween). Incubations took place at 37°C. For each ELISA (IgG and IgM), a serum with a strong reactivity, as detected in preliminary experiments, was chosen for the standard curve. The prototype serum for the IgM ELISA was negative for rheumatoid factor (RF) activity. Furthermore, sera positive for RF were tested after absorption of RF by incubating the sera with human heat-aggregated IgG; when IgM ELISA was repeated, IgM anti-U1RNP reactivity was reduced by only 0–10% of the original values. Sera were considered positive when the optical density was higher than the mean value of normals (n = 80) plus 3 S.D.

**Immunoblotting**

This method was used to test the antigenicity of our antigen preparation. Affinity-purified U1 snRNP complexes, expected to contain Sm/U1RNP antigen, were run on a 12% polyacrylamide slab gel [23] and the proteins were transferred to nitrocellulose membranes using Western blotting [21]. Strips cut from the nitrocellulose reacted with 1:100 diluted serum and the binding bands were labelled by horseradish peroxidase (HRPO) anti-human IgG (Sigma) in a dilution 1:1000 to detect IgG anti-Sm/U1RNP reactivity. Five per cent non-fat milk in Tris-buffered saline (TBS)–TWEEN 20 was used as a blocking agent, as well as for the dilution of the sera. Incubation of the nitrocellulose strips of the sera was performed for 4 h and incubation with anti-human IgG–HRPO conjugates for 1.5 h. A 4-chloro-1-naphthol (Sigma) substrate was finally added to detect binding of the conjugate.

**RNA precipitation assays using IgG, IgM autoantibody fractions**

To discriminate between IgG and IgM autoantibody reactivity in patients' sera, the sensitive and accurate RNA precipitation assay was applied to Ig fractions highly enriched for either IgG or IgM antibody classes. This was accomplished by making use of the differential binding ability of the different Ig classes for protein A molecules. As is well established, human IgG molecules (with the exception of the IgG3 subclass) bind strongly to protein A, in contrast to the weak binding exhibited by IgM and IgA antibody classes [22].

Initially, the bulk of the IgG molecules were removed from the serum by incubating 10 μl of serum with 3 mg protein A–Sepharose beads (Pharmacia, Sweden) in NET-2 buffer [150 mM NaCl, 10 mM Tris–HCl (pH 7.5), 0.05% NP-40] for 1.5 h with gentle rocking at 4°C. Following a brief spin and saving of the protein A–IgG complexes in the pellet (fraction a), the supernatant of the serum was applied to a second cycle of incubation to largely deplete the remaining IgG molecules (fraction b). The supernatant of the second cycle was then allowed to react, in a third cycle of incubation, with the complex of protein A–rabbit anti-human IgM, prepared by pre-incubating 3 mg of protein A with 10 μl of rabbit anti-human IgM (fraction c) (13.8 mg/ml; Dako Co., Denmark). The beads of protein A/antibody complexes from the three cycles of incubation were washed three times in NET-2 buffer and applied to RNA precipitation assays using HeLa total cellular extracts as previously described [19]. An aliquot of each immunoprecipitate tested in RNA precipitation was saved for testing the presence of IgG or IgM on a Western blot using rabbit anti-human IgM alkaline phosphatase-conjugated antisera. This experiment revealed that fraction (c) contained only IgM, but not IgG (data not shown). All immune pellets obtained were then phenol extracted and the purified RNA molecules were resolved on 7% urea–10% polyacrylamide gels. RNA on the gel was visualized by silver staining.

**Statistical analysis**

Contingency tables were used where indicated.

**RESULTS**

Diagnoses, serological findings and evolution of patients

There were 32 patients with SLE and 35 with MCTD. The majority of the SLE patients fulfilled four of the American Rheumatism Association (ARA) SLE criteria (22 patients), seven fulfilled five, two patients six and one patient eight of the criteria. The patients with SLE developed full-blown disease (four or more of the ARA criteria) 1–15 yr after the onset of the first symptom compatible with the disease. None of the MCTD patients fulfilled criteria for SLE. Six of the 32 patients also fulfilled criteria for MCTD; three of them presented synovitis, oedema of the hands and Raynaud's phenomenon, two presented synovitis, myositis and Raynaud's phenomenon, and one patient synovitis, oedema of the hands and myositis. Autoantibodies to Ro (SSA), La (SSB) and Sm detected by CIE, RF and anti-dsDNA antibodies in both groups are presented in Table I.

<table>
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<tr>
<th>IgG and IgM isotypes of anti-U1RNP detected by ELISA</th>
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| Since purified U1snRNP fraction contained both the U1snRNP (70 kDa, A and C polypeptides) as well as the Sm antigens (B/B' and D polypeptides), which are common to all U1snRNPs (U1, U2, U4/U6, U5), ELISA cannot discriminate between antibodies of anti-U1RNP and anti-Sm specificities. To avoid confusion, we shall refer to the affinity-purified antibody preparation as "anti-Sm."

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Autoantibodies in patients with MCTD and SLE</th>
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<tr>
<td>Available serum samples</td>
<td>MCTD (n = 35)</td>
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<tr>
<td>Anti-DNA</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Anti-Ro</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Anti-La</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-Sm</td>
<td>2 (6)</td>
</tr>
<tr>
<td>RF</td>
<td>11 (31)</td>
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U1snRNP fraction applied in ELISA assays as the snRNP antigen. Nonetheless, since, as shown in Table I, only 6% of MCTD sera and 19% of SLE sera also contained anti-Sm antibodies, we assumed that ELISA data reflected by and large the specificity against U1snRNP components. ELISA was used to screen the sera of the patients for IgG and IgM anti-U1snRNP reactivity against affinity-purified U1snRNP components. In a first experiment, the oldest serum samples from each patient that were available in our laboratory were tested. Twelve out of the 35 MCTD patients were positive for IgM anti-U1snRNP antibodies vs 21 of the 32 SLE patients ($P < 0.025$) (Fig. 2). On the contrary, all the patients were positive for IgG anti-U1snRNP antibodies.

Sera from patients with SLE and MCTD which had been collected during their follow-ups were withdrawn for testing with an order of one sample every 9 months during their regular visits to the out-patient clinic. A total of 44 sera from 17 MCTD patients and 112 sera from 23 SLE patients were collected. The IgG and IgM reactivity to U1snRNP antigen was detected in these sera by ELISA. Nine of the 44 MCTD sera (20%) vs 77 of 112 SLE sera (68%) were positive for IgM antibodies to U1RNP ($P < 0.0001$). In fact, temporary increases in IgM anti-U1snRNP reactivity above the upper limit of normals occurred in four patients with MCTD. On the contrary, a persistent increase in IgM anti-U1snRNP reactivity above control levels occurred in 18 of the 23 SLE patients. Figures 3 and 4 represent the IgM anti-U1RNP activity in a group of eight SLE and MCTD patients. These patients were collected as being representative of each group in terms of the following: (a) the SLE patients did not present MCTD related features other than synovitis, which occurred in three of them; the MCTD patients presented a stable clinical feature for years non-evolving to SLE or scleroderma; (b) the patients of both groups had a long follow-up; (c) from the initial patients’ sera, those with highest IgM anti-U1snRNP activity were used; (d) sequential serum samples were available in our serum bank.

As is shown, five of the eight MCTD patients are initially negative for IgM anti-U1snRNP antibodies and tend to be negative thereafter, with temporary increases in the IgM anti-U1snRNP titre above normal values. On the other hand, five sera of SLE patients are persistently positive for IgM anti-U1RNP antibodies, three were initially negative and became positive over a period of 2–3 yr.

The RF activity of the sera does not interfere with their IgM anti-U1RNP activity

Fifteen of the 23 patients positive for RF were also positive for IgM anti-U1snRNP antibodies. In addition, 18 of 44 patients negative for RF were

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**Fig. 2.**—The absorbance values of anti-U1RNP sera of SLE and MCTD patients as well as normal human sera tested for IgG(G) or IgM(M) anti-U1RNP antibodies.
also positive for IgM anti-U1snRNPs. By using contingency tables, no statistically significant association between the above variables was observed ($\chi^2 = 3.57, P > 0.05$). To further strengthen the above observation, we eliminated serum RF by absorption with human heat-aggregated IgG as RF activity was not further detectable and the ELISA was repeated. The absorbance values were decreased from 0 to 10% of the initial values in all the sera.

**Characterization of the IgM anti-U1RNP reactivity by RNA precipitation**

Although ELISA helped us to show the presence, in high frequency, of IgM anti-snRNP reactivity in sera...
of SLE when compared to MCTD patients, it could not provide information on the specific antigenic component (Sm vs U1snRNP) involved. Therefore, sera from SLE and MCTD were further characterized regarding their IgM, IgG reactivity by RNA precipitation of HeLa cellular extracts. For each serum, three immunoprecipitates were analysed with respect to UsnRNA content. Immunoprecipitates (a) and (b) referred to the snRNA species bound by the bulk and the residual IgG class, respectively. On the other hand, immunoprecipitate (c) should contain snRNP species recognized solely by IgM class bind to the protein A–rabbit anti-human IgM complex. All the sera were positive for IgG anti-U1RNPs, as was shown by IgG RNA immunoprecipitation. However, eight of the MCTD sera also precipitated U2RNPs, while 14 of the SLE sera also precipitated U2RNPs and eight of them in addition precipitated U4/U6, U5 RNPs. Figure 5 indicates the reactivity of each of the above samples (obtained from five MCTD and three SLE sera) against snRNPs by RNA immunoprecipitation. As is shown, four out of the five MCTD sera have IgG anti-U1RNP specificity in the absence of any detectable IgM reactivity. Serum no. 3 was the only one with a very weak anti-Sm specificity. This serum also precipitates U1RNP by IgM antibodies; while the rest of the sera are negative for IgM anti-U1RNPs. The results obtained by ELISA showed that of the above five sera this serum was weakly positive for IgM anti-snRNP reactivity. Two out of the three SLE sera shown in Fig. 5 are positive for IgM anti-snRNPs, and these are the sera which also have anti-Sm specificity, as is clear by the presence of U2, U4/U6, U5 RNPs which are precipitated by their IgG antibodies. The one SLE serum (no. 7) with IgG anti-U1RNP alone does not contain any detectable specificity of the IgM class.

Thus, anti-U1RNP reactivity alone in either MCTD or SLE is of the IgG isotype. On the contrary, anti-U1RNP antibodies when existing together with anti-Sm can be of both the IgG and IgM isotype. Anti-Sm reactivity in SLE can also be of the IgM isotype. The above results are compatible with those obtained by the ELISA.

DISCUSSION

The present report indicates that among patients with precipitating antibodies to snRNP components, the patients with SLE possess anti-snRNP antibodies of the IgM class, while the patients with MCTD possess anti-snRNP (mainly anti-U1RNP) antibodies with predominance of the IgG class. The above information was based on results obtained by ELISA using purified U1RNP antigen as the antigen source. Since the purified antigen also contained Sm antigenic determinants [6, 8, 12, 18], it was important to discriminate the reactivity of the sera attributed to the anti-U1RNP and not to the anti-Sm antibodies which probably exist in some of the sera [18, 24]. RNA precipitation, in agreement with CIE, indicated that the majority of both MCTD and SLE sera precipitated only U1RNAs, and thus they contain antibodies to the U1RNP specific components. This was not surprising since anti-Sm antibodies are rare in Greek [24] and other European populations [25] with SLE. Therefore, the results obtained by ELISA indicated mainly anti-U1RNP reactivity of the sera tested. Although this finding was clear for IgG anti-U1RNP reactivity, the IgM anti-U1RNP reactivity needed substantial proof. To discriminate between IgG and IgM autoantibody to snRNPs in patients' sera, we applied the RNA precipitation assay as an extremely sensitive way of identifying anti-U1RNP and anti-Sm specificities. The RNA precipitation assay confirmed that antibodies of the IgM class detected by ELISA really existed, since these antibodies could precipitate snRNPs in the absence of the IgG fraction. Furthermore, the IgM fraction of the anti-Sm sera recognized predominantly U1RNP, although these sera, by their IgG fraction,
patients also possess IgG anti-UlRNP antibodies considered as a sign of a previous and never renewed cross-reactivity between peptides B'/B and D of the Sm peptides is inevitable. Therefore, small linear epitopes, immunogen for patients with SLE and MCTD, and of certain autoantibodies remain unresolved, it is snRNPs, as well as to dsDNA [28].

In the present report, an effort was made to answer the question of whether the IgM anti-U1RNP reactivity of the sera can also be explained by IgM antibodies recognizing the native forms of the U1RNP related peptides. The ELISA was used as a screening test and revealed a considerable number of SLE patients to be positive for IgM antibodies to U1srNRP components, in contrast to MCTD patients. The RNA precipitation confirmed the IgM reactivity of the sera and showed that this reactivity is turned on against mainly U1RNP. Therefore a continuous, antigen-driven, T- and B-cell activation occurs in SLE, in contrast to MCTD. In this regard, the IgG anti-U1RNP activity observed in MCTD patients. The ELISA was used as a screening test and revealed a considerable number of SLE patients to be positive for IgM antibodies to U1srNRP components, in contrast to MCTD patients. The RNA precipitation confirmed the IgM reactivity of the sera and showed that this reactivity is turned on against mainly U1RNP. Therefore a continuous, antigen-driven, T- and B-cell activation occurs in SLE, in contrast to MCTD. In this regard, the IgG anti-U1RNP activity observed in MCTD patients.

Although many mechanisms for the production of certain autoantibodies remain unresolved, it is becoming clear that intact U1RNP is a major immunogen for patients with SLE and MCTD, and that SLE is a disease where a continuous activation of the immune system with snRNPs operates.

References


