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Anti-Ro52 antibodies frequently co-occur with anti-Jo-1 antibodies in sera from patients with idiopathic inflammatory myopathy

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SUMMARY

We analysed 112 idiopathic inflammatory myopathy (IIM) sera for the presence of anti-Ro, anti-La and anti-histidyl-tRNA synthetase (Jo-1) autoantibodies, and subsequently mapped B cell epitopes on the Ro52 protein recognized by anti-Ro52$^+$ IIM sera. Sera were characterized by immunoblotting, ELISA and RNA precipitation. Both anti-Ro60 and anti-La activity was found in 4% of IIM sera. Anti-Ro52 antibodies were present in 20% of IIM sera. However, in anti-Jo-1$^+$ IIM sera (21%), the frequency of the anti-Ro52 antibodies was found to be much higher (58%). No cross-reactivity between anti-Ro52 and anti-Jo-1 antibodies could be detected in these sera. To learn more about the nature of anti-Ro52 antibodies occurring in IIM sera, we analysed the major epitopes of the Ro52 protein targeted by anti-Ro52$^+$ IIM sera by immunoprecipitation of *in vitro* translated Ro52 deletion mutants. The major epitope was mapped in the region bordered by amino acids 126 and 252. This part of the protein includes a long α-helical region which contains two potential coiled-coil domains as well as a leucine zipper motif. Although no difference in Ro52 epitope recognition between anti-Jo-1$^+$ and anti-Jo-1$^-$IIM sera could be observed, our results suggest that the autoimmune response against Ro52 and Jo-1 in IIM patients is coupled.

Keywords myositis autoantibodies Ro (SS-A) Jo-1 Ro ribonucleoprotein complex

INTRODUCTION

Idiopathic inflammatory myopathies (IIM), the most common forms of which are polymyositis (PM) and dermatomyositis (DM), are defined by chronic inflammation of many groups of muscles. They are a rare but increasingly recognized, diverse group of diseases with a variety of clinical presentations, immunologic abnormalities, and courses. Autoimmune responses to nuclear and cytoplasmic autoantigens are found in about 60–80% of these patients [1]. Some of them are shared with other autoimmune diseases, and some are unique to IIM.

Myositis-specific autoantibodies (MSA) have proved clinically useful in helping to predict signs and symptoms of the disease [2–4]. The most common MSA, seen in about a third of the patients, are directed to aminoacyl-tRNA synthetases, enzymes which catalyse the attachment of a particular amino acid to its cognate tRNA [5]. The occurrence of antibodies to five of the 20 aminoacyl-tRNA synthetases has been described [6–9], but anti-histidyl-tRNA synthetase (anti-Jo-1) is more common than all of the other anti-synthetases combined. A second group of MSA is directed to other cytoplasmic antigens, such as anti-signal recognition particle (SRP) [10]. SRP is a ribonucleoprotein complex involved in the translocation of newly synthesized proteins into the endoplasmic reticulum. An MSA directed to a nuclear antigen is anti-Mi-2, which is seen almost exclusively in patients with DM [11]. The Mi-2 antigen contains at least seven proteins. A protein subunit of 240 kD is the main antigen that reacts with all anti-Mi-2 sera and is a presumed helicase involved in transcriptional activation [12].

Sera from myositis patients also contain autoantibodies which are present in other autoimmune diseases. An example of such autoantibodies are those reactive with Ro ribonucleoprotein particles (Ro RNPs), which primarily occur in diseases like systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). Such autoantibodies are generally referred to as anti-Ro or anti-SSA antibodies. Ro RNPs consist of one of four hY RNA (hY1, hY3, hY4 or hY5) molecules complexed with several proteins. The RNAs are transcribed by RNA polymerase III and vary in length from 84 to 112 nucleotides [13]. The are characterized by a conserved stem structure formed by extensive base-pairing between the highly conserved 5′ and 3′ ends. The protein part of a Ro RNP consists of at least three different proteins: the La protein, involved in the correct and efficient termination of RNA polymerase III transcription [14,15], and the 52- and 60-kD Ro proteins (Ro52 and Ro60). The La protein binds to the oligo-uridylate stretch at the 3′ end of the RNAs, while Ro60 interacts

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with the conserved part of the stem structure [16,17]. The association of Ro52 with the Ro RNP particles remains controversial [18–21], and has been analysed in a number of studies using either monospecific autoimmune sera or affinity-purified antibodies. In most of these studies anti-Ro52 antibodies were able to immunoprecipitate hY RNAs [22–24], indicating that the Ro52 protein is part of at least some Ro RNPs. However, co-precipitation of hY RNAs has also been explained as the result of cross-reactivity of Ro52 antibodies with the Ro60 protein [25].

In this study, we characterized 112 IIM sera for their reactivity with the myositis-specific autoantigen Jo-1 as well as antigenic components of the Ro RNP complex. Using sensitive detection techniques based upon recombinant autoantigens, we found a high frequency of anti-Ro52 antibodies, especially in anti-Jo-1$^+$ sera. To learn more about the nature of anti-Ro52 antibodies occuring in IIM sera, we analysed the major epitopes on the Ro52 protein targeted by these autoantibodies. The major epitope region was mapped in the central part of the Ro52 protein.

MATERIALS AND METHODS

Human sera

Sera obtained from 112 Dutch patients with IIM, diagnosed according to the criteria of Bohan *et al*. [26], were analysed by immunoblotting, RNA precipitation and ELISA for the presence of anti-Jo-1, anti-Ro52, anti-Ro60 and anti-La autoantibodies. Other diagnoses were reached as published previously [27].

Expression and purification of recombinant Jo-1 and Ro52

A cDNA encoding the Jo-1 protein was isolated from a human cDNA library by hybridization with an oligonucleotide derived from the published sequence [28]. DNA sequence analysis showed that this cDNA was almost identical to the cDNA published by Raben *et al*. [28]. Merely the ends of the 5′ and 3′ non-coding sequences and the nucleotides at positions 357, 648 and 795 of our cDNA (first coding nucleotide is numbered 1), which are all thymidines in our cDNA, differed from the published sequence. To express Jo-1 in *Escherichia coli* the coding sequence was isolated from the cDNA by a polymerase chain reaction (PCR) approach using primers 5′ CCGCCATGGCAGAGCGTGCGGCGCT 3′ and 5′ GGCAGAT CTGATAGTTTGTTCAGTTCAGCAG 3′, introducing simultaneously a *Nco*I site at the 5′ border and a *Bgl*II site at the 3′ border of the coding sequence of Jo-1. These restriction sites were used to insert the coding sequence of Jo-1 into *Nco*I-*Bam*HI digested expression vector pET-3d. After transformation of *E. coli* B121(DE3)pLysS with this construct, Jo-1 was expressed by diluting 5 ml of an overnight culture in 500 ml L-broth medium. When the culture reached an OD_{600} of 1·0, Jo-1 expression was induced with 1 mM isopropyl-β-D-galactopyranoside. After 3 h at 37°C, bacteria were harvested and resuspended in 10 ml PBS/0·5 mM PMSF, 10 mM $MgCl_2$ and 50 μg/ml DNase I. Cells were lysed by three cycles of freezing and thawing. Lysozyme (1 mg) was added in 4 ml 50% (w/v) sucrose, 10 mM Tris–HCl pH 8·0, 1 mM EDTA and 100 mM NaCl, followed by an incubation for 30 min on ice. Subsequently, 200 μl of Nonidet P-40 (NP-40) and 2 ml of 0·5 M EDTA pH 7·5 were added, followed by an incubation on ice for 15 min. After adding 10 ml of 10% Zwittergent detergent 3-14 (Calbiochem, La Jolla, CA), the cell extract was sonicated three times for 30 s and layered onto a 10 ml 40% (w/v) sucrose solution in PBS. After centrifugation for 30 min at 10 000 ***g***, the pellet was solubilized in 2·5 ml of 8 M urea.

Expression and purification of the Ro52 protein, as well as Ro60 and of the La protein, were performed as previously described [21,29].

ELISA

ELISA using the recombinant antigens Ro60, Ro52, La and Jo-1 was performed as follows. The solutions of bacterially expressed purified antigens were diluted 20 000-fold with coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9·6) and polystyrene microtitre plates were coated overnight at 4°C with the resulting solution. The remaining protein binding sites were blocked with 0·1% bovine serum albumin (BSA) in coating buffer for 2 h at room temperature. Sera from patients with IIM were analysed at a 1 : 200 dilution in buffer A (10 mM Tris pH 7·6, 150 mM NaCl, 0·3% BSA, 1% Triton X-100, 0·1% SDS, 0·5% sodium deoxycholate) containing 10% normal rabbit serum.

To detect bound antibodies, horseradish peroxidase-conjugated rabbit antibodies against human IgA, IgG, IgM (Dakopatts; P212) at a dilution of 1 : 1000 in RIA buffer were used.

Ro52 deletion mutants

The cDNA clone encoding Ro52, subcloned into pGEM-3Zf(+), was previously described [30]. C-terminally truncated mutants were obtained by linearization of the Ro52 cDNA clone with various restriction enzymes, as described by Peek *et al*. [21]. Also the N-terminally truncated mutants ΔN125 (encoding aa 125–292), ΔN136 (encoding aa 136–475) and ΔN208 (encoding aa 208–475) were described previously [21]. In addition, two independently isolated human Ro52 DNA clones, which represent splicing variants of Ro52 mRNAs ([31], unpublished data) were used. One of these lacks the exon 4 sequence leading to the absence of amino acids 169–245 (referred to as Ro52Δ168–246). The second variant cDNA lacks exon 6 which leads to a frame-shift at the exon 5-exon 7 boundary (Ro52ΔC252).

In vitro *transcription and translation*

In vitro transcription and translation were mainly performed as described [21,32]. The Ro52 deletion mutant Δ168–246 was linearized with restriction enzyme FspI; cDNA encoding deletion mutant ΔC252 was linearized with restriction enzyme *Hind*III. Translation products were analysed by 15% SDS–PAGE. The Ro52 mutant ΔC69 was also analysed by Tricine-SDS–PAGE as described by Schägger & von Jagow [33]. After electrophoresis, gels were fixed in 5% methanol/7·5% acetic acid, treated with Amplify (Amersham, Den Bosch, The Netherlands), dried and autoradiographed at −70°C.

Immunoprecipitation of in vitro *translated proteins*

Protein A-agarose beads were incubated with 1 μl antiserum by head over head rotation at room temperature for 1 h in IPP_{500} (10 mM Tris–HCl pH 8·0, 500 mM NaCl, 0·1% NP-40) and, after washing, incubated with a radiolabelled *in vitro* translated protein in IPP_{150} (10 mM Tris–HCl pH 8·0, 150 mM NaCl, 0·1% NP-40) for 1 h at room temperature. Subsequently, beads were washed three times with IPP_{150} and resuspended in SDS sample buffer. Precipitated proteins were analysed by 15% SDS–PAGE or Tricine-SDS–PAGE.

Immunoprecipitation of RoRNPs

Protein A-agarose beads were incubated with 1 μl patient serum or 50 μl 2G10 MoAb (Euro-Diagnostica bv, Arnhem, The Netherlands) for at least 1 h in IPP_{500} followed by washing twice with

IPP_{500} and once with IPP_{150}. The coated beads were incubated with HeLa cell extract (from 10^5 cells) in IPP_{150} by rotation for 2 h at 4°C. After washing three times with IPP_{150}, RNA was isolated by phenol/chloroform extraction and ethanol precipitation. RNA was visualized by pCp-endlabelling [34] and analysed by fractionation on a 6% polyacrylamide-8 M urea gel.

RESULTS

Autoantibody profiles of IIM/Jo-1 sera

Sea from patients with IIM ($n = 112$) were tested for anti-Jo-1, anti-Ro60, anti-Ro52 and anti-La activity by immunoblotting, ELISA and RNA precipitation. The results, summarized in Table 1, show that in the ELISA, in which purified recombinant Jo-1 was used, anti-Jo-1 activity was found in 21% of IIM sera. Strikingly, anti-Ro52 antibodies were also found to occur in a high percentage (20%) of these IIM sera. For PM (70 sera) and DM (35 sera) separately, the prevalence of these autoantibodies was similar to that of the total population of IIM sera. In anti-Jo-1$^+$ IIM sera, however, the frequency of the anti-Ro52 antibodies was 58%. Anti-Ro60 and anti-La antibody frequencies were relatively low (approx. 4%) and did not differ markedly between the different patient groups.

The high incidence of anti-Ro52 activity in anti-Jo-1$^+$ sera might have been caused by cross-reactivity of anti-Jo-1 antibodies with Ro52. To investigate this possibility, we tested whether reactivity of the IIM sera with the recombinant Ro52 protein could be inhibited by recombinant Jo-1 protein. Using up to a 1000-fold molar excess of Jo-1 with regard to the amount of Ro52 used for coating the ELISA plate, no inhibition of anti-Ro52 activity was observed, while anti-Jo-1 activity was completely inhibited by addition of only a 62-fold molar excess of recombinant Jo-1 protein (Fig. 1). Alternatively, when antibodies were eluted from the Ro52 band or Jo-1 band on the immunoblot [35], and subsequently incubated with a second HeLa cell immunoblot, only the antigen from which the antibodies were eluted (Ro52 or Jo-1, respectively) was stained (L Meheus, Innogenetics, Gent, Belgium, personal communication).

Table 1. Presence of anti-Jo-1, anti-Ro60, anti-Ro52 and anti-La antibodies in idiopathic inflammatory myopathy (IIM) sera

	IIM, % ($n = 112$)	PM, % ($n = 70$)	DM, % ($n = 35$)	Jo-1$^+$, % ($n = 24$)
IB				
Jo-1	21	24	17	100
Ro60	3	1	6	4
La	4	3	6	8
ELISA				
Jo-1	21	24	17	100
Ro60	4	4	6	4
Ro52	20	19	17	58
La	4	3	6	8
RNA prec.*				
tRNA	28	29	26	100
Y RNA	15	13	23	25
Other†	24	24	29	29

* RNA precipitation pattern using a HeLa cell extract.
† Other RNAs: U1 snRNA, 5S rRNA, 5·8S rRNA.

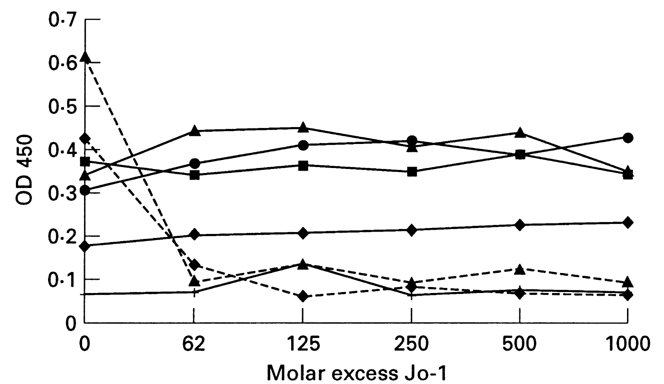

Fig. 1. Competition with recombinant Jo-1 protein for reactivity of idiopathic inflammatory myopathy (IIM) sera with Ro52 or Jo-1. Reactivity of IIM autoantibodies with Ro52 was measured by ELISA after adding increasing amounts of recombinant Jo-1. Up to a 1000-fold molar excess of recombinant Jo-1 with regard to the amount of Ro52 or Jo-1 used for coating the ELISA plates was used. ——, Ro52 ELISA; - - -, Jo-1 ELISA. M83 (●), IIM$^+$/anti-Jo-1$^+$; J102 (■), IIM$^+$/anti-Jo-1$^+$; K160 (▲), IIM$^+$/anti-Jo-1$^+$; H94 (◆), IIM$^+$/anti-Jo-1$^+$ NHS (+).

Taken together, these results indicate that autoantibodies directed to the Ro52 protein are frequently found in IIM and that, at least in the sera used in this study, they often co-occur with anti-Jo-1 antibodies.

Determination of Ro52 epitope(s) recognized by IIM sera

To characterize the nature of anti-Ro52 antibodies occurring in IIM sera, we analysed anti-Ro52 activity in 19 anti-Ro52$^+$ sera by analysing the major epitope regions of the Ro52 protein recognized. Ten of these sera contained anti-Jo-1 activity (group I, Table 2), whereas the other nine sera were not reactive with Jo-1 (group II). In addition, four sera which were anti-Jo-1$^+$ and anti-Ro52$^+$ in ELISA but not diagnosed as IIM were used (group III). An SLE control group of sera containing anti-Ro52 but not anti-Jo-1 antibodies was included as well (group IV). The 23 IIM and anti-Jo-1$^+$ sera were assayed for their ability to immunoprecipitate *in vitro* translated mutants of Ro52. A schematic representation of the Ro52 mutants used is shown in Fig. 2.

The results of representative immunoprecipitations of Ro52 mutants with a subset of the anti-Ro52 sera are shown in Fig. 3. Precipitation efficiencies were determined by comparing the amount of precipitated protein with the amount of input protein and the results obtained are summarized in Table 2. No reductions in reactivity were detectable when the C-terminal 223 amino acids of Ro52 were lacking (ΔC252) (Fig. 3c). However, when an additional 26 amino acids were removed (ΔC226), 59% of the sera showed a significant reduction or even loss of reactivity. With the N-terminally truncated mutants of Ro52 we observed that 91% of the IIM$^+$ and anti-Jo-1$^+$ sera were strongly reactive with mutant (ΔN125) which lacks the N-terminal 124 amino acids and the C-terminal 183 amino acids. However, when 11 additional amino acids were deleted from the N-terminal end (ΔN136), the reactivity of most of the sera decreased significantly (Fig. 3b). Only 26% of IIM$^+$ and anti-Jo-1$^+$ sera were able to precipitate this mutant efficiently. When another 72 amino acids were deleted from the N-terminal end (ΔN208), hardly any or no reactivity was left. The exon 4 skipped Ro52 variant Ro52Δ168–246 was recognized by only three of the 17 sera tested (Fig. 3c). Taken together, the

Table 2. Reactivity of idiopathic inflammatory myopathy (IIM) patient sera with deletion mutants of Ro52

			Reactivity‡												
Group*	Patient	Diagnosis†	wt	ΔC363	ΔC292	ΔC252	ΔC226	ΔC216	ΔC196	ΔC125	ΔC69	ΔN125	ΔN136	ΔN208	Δ168–246
I	B61	PM	+++	+++	+++	+++	−	−	−	−	−	++	+	−	−
	B387	PM	+++	+++	+++	+++	++	−	−	−	ND	+++	+	−	+
	G20	DM	++	++	++	++	−	−	−	−	ND	+	+	−	−
	H94	PM	+++	+++	+++	+++	−	−	−	−	−	+++	+	−	−
	J102	PM	+++	+++	+++	+++	+	+	+	+	+	+++	+	−	+
	K56	PM	+++	+++	+++	+++	−	−	−	−	ND	+++	+++	−	−
	K160	DM	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	ND
	K225	PM	+++	+++	+++	+++	ND	++	++	++	ND	++	+	−	−
	M83	PM	+++	+++	+++	+++	+++	+++	+++	+++	ND	+++	+++	−	−
	F21	PM	+++	+++	+++	+++	+	+	+	−	−	+	+	−	−
II	B275	PM	+++	+++	+++	+++	++	+	+	+	+	+++	++	−	−
	B372	DM	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	−	+
	D188	DM	+++	+++	+++	+++	+	+	+	+	+	+++	+	−	−
	S174	PM	+++	+++	+++	+++	++	++	++	++	+	+++	++	−	ND
	T16	PM	+++	+++	+++	+++	−	−	−	−	−	+++	+	−	−
	T109	PM	+++	+++	+++	+++	+	+	+	±	−	+++	+	±	−
	F6	PM	+++	+++	+++	+++	+	+	+	+	−	+++	+	−	−
	F17	PM	+++	+++	+++	+++	+++	+++	+++	++	+	+++	+	±	ND
	F18	PM	+++	+++	+++	+++	+++	+++	+++	+++	−	+++	+	±	ND
III	H150	RP	+++	+++	+++	+++	−	−	−	−	ND	++	++	−	−
	L65	UCTD	+++	+++	+++	+++	+++	+++	+++	+++	ND	+++	+	±	ND
	S246	RA	++	++	++	++	−	−	−	−	ND	++	+	−	−
	29	SLE/sSS	+++	ND	+++	+++	−	−	ND	ND	ND	+++	+	−	±
IV	106	SLE	+++	ND	+++	+++	++	++	ND	ND	ND	+++	+	−	+
	251	SLE	+++	ND	+++	+++	−	−	ND	ND	ND	+++	+	−	−
	292	SLE	+++	ND	+++	+++	−	−	ND	ND	ND	+++	+	−	−
	S191	SLE	+++	ND	+++	+++	−	−	ND	ND	ND	+++	+	−	−
	S206	SLE	+++	ND	+++	+++	+	+	ND	ND	ND	+++	+	−	±

* Sera were divided into four groups: I, IIM/anti-Jo-1^{+}; II, IIM/anti-Jo-1^{-}; III, not diagnosed IIM/anti-Jo-1^{+}; IV, SLE/anti-Jo-1^{-}.

† The diagnoses of the patients are indicated: PM, polymyositis; DM, dermatomyositis; RP, Raynaud's phenomenon; UCTD, undifferentiated connective tissue disease; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; sSS, secondary Sjögren's syndrome.

‡ Reactivities with the various deletion mutants is indicated by: +++, strong reactivity; ++, moderate reactivity; +, weak reactivity; ±, hardly above background; −, no reactivity; ND, not determined.

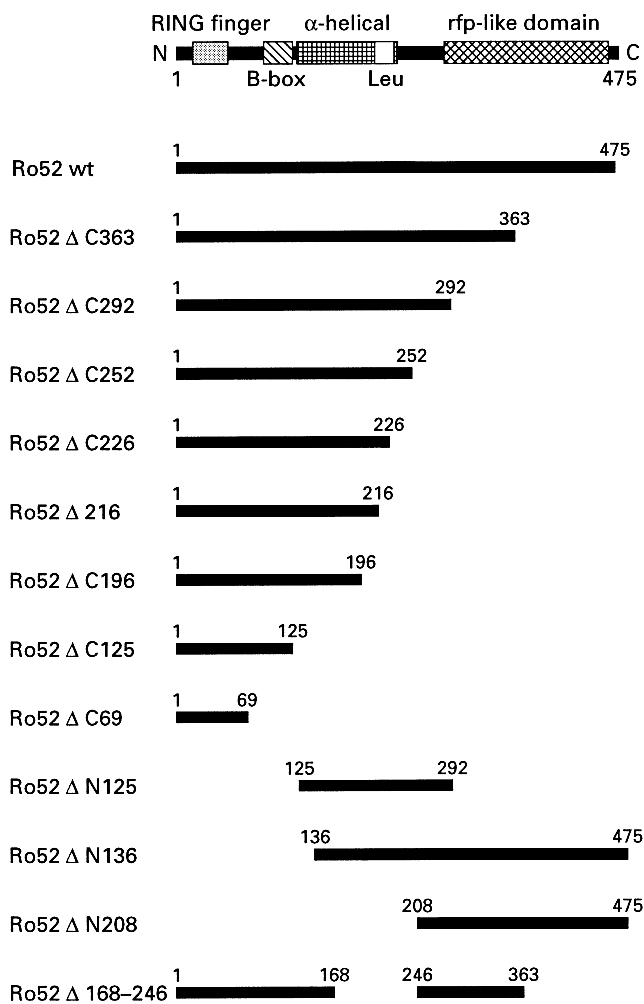

Fig. 2. Deletion mutants of Ro52 used for epitope mapping. C-terminally truncated mutants were obtained by linearization of the Ro52 cDNA with various restriction enzymes. N-terminal deletion mutants (ΔN125, ΔN136 and ΔN208) were obtained by mutagenesis and subcloning of the cDNA. In addition, two different human Ro52 cDNA clones, which represent splicing variants of Ro52, were used. One of these lacks the exon 4 sequence leading to the absence of amino acids 169–245 (Δ168–246). The second variant cDNA lacks exon 6 which leads to a frame-shift at the exon 5–exon 7 boundary (ΔC252).

precipitation data for the C-terminal, N-terminal and internal deletion mutants of Ro52 indicate that the anti-Ro52 reactivity of IIM sera is heterogeneous, but that the major epitope region is located between amino acid 125 and amino acid 252. No significant differences with respect to Ro52 epitopes were observed between anti-Jo-1$^+$ and anti-Jo-1$^-$ IIM sera and between IIM sera and the additional anti-Jo-1$^+$ sera of group III.

Comparison of anti-Ro52 activity of IIM sera with that of SLE sera
Božič *et al.* [30] have previously shown that the residues in Ro52, located between amino acids 216 and 292, are essential for recognition by anti-Ro52 antibodies found in SLE sera. A deletion in this region leads to a strong reduction in reactivity with 70% of SLE sera. In contrast, anti-Ro52 antibodies of patients with SS appear to display a more heterogeneous epitope recognition pattern because they recognize multiple epitope regions located between amino acids 55 and 292 [30]. These results indicate that the Ro52 epitope profile of antibodies produced by SLE patients is often different from that of SS patients. The present results suggest that the major epitope region recognized by IIM antibodies is similar to that recognized by SLE antibodies. To investigate this further the major epitope region recognized by SLE sera (group IV) was mapped in more detail. The Ro52 mutants ΔC292, ΔC252, ΔC226, ΔC216, ΔN125, ΔN136, ΔN208 and Δ168–246 were precipitated with antibodies contained in five SLE sera. As is shown in Table 2, all of these SLE sera precipitated ΔC252 efficiently and all sera showed a decrease in reactivity with mutant ΔC226. Also the precipitation pattern of N-terminal deletion mutants ΔN125, Δ N136 and ΔN208 showed a striking similarity with that of the analysed IIM sera, as is seen by comparing the precipitation results obtained with sera from groups I, II and III with those of group IV (Table 2). Our results indicate that the most important epitope region of Ro52 for reactivity with IIM and SLE autoantibodies is indistinguishable and located between amino acids 125 and 252.

Anti-Ro RNP reactivity in IIM sera
The IIM sera which were used in this study represent an as yet unrecognized class of anti-Ro52 antibody-containing sera. The reason for this is probably that anti-Ro52 reactivity cannot be detected by standard immunodiffusion or counterimmunoelectrophoresis techniques, which are routinely applied in a first screening for autoantibodies in patient sera, due to the fact that anti-Ro52 autoantibodies do not detectably cross-react with the antigen from bovine or rabbit tissues [24]. Anti-Ro reactivity detected in SLE or SS sera by these standard techniques probably represents anti-Ro60 reactivity. This suggests that most anti-Ro52$^+$ IIM sera are devoid of anti-Ro60 reactivity, which would be in clear contrast to SLE sera, in which anti-Ro52 and anti-Ro60 antibodies almost invariably co-occur [24].

Because the association of Ro52 with Ro RNP particles is still a matter of debate [19–21], we analysed the RNA precipitation pattern of IIM sera (Table 1). Fifteen percent of the sera were able to immunoprecipitate hY RNAs, whereas in only 4% of the sera could anti-Ro60 activity and in 4% could anti-La activity be detected (ELISA data, Table 1). However, 20% of sera were reactive with Ro52, which might suggest that at least a subset of hY RNAs can be immunoprecipitated via Ro52 antibodies. In addition, 25% of anti-Jo-1$^+$ IIM sera are able to immunoprecipitate hY RNAs, which might be related to the more frequent presence of anti-Ro52 activity (58%) in this group of sera.

Furthermore, we tested some IIM/Jo-1 sera, in which, by all techniques available to us, no anti-Ro60 and anti-La reactivity could be detected (Table 1), for the ability to precipitate Ro RNPs from a HeLa cell extract. As positive controls serum S67 (anti Ro/La serum) and MoAb 2G10 (anti-Ro60) were used. Co-precipitation of the Ro RNP associated Y RNAs was monitored by RNA extraction followed by ^{32}P-pCp-labelling. As shown in Fig. 4, hY RNAs were immunoprecipitated by IIM sera K56 and K225. As expected, all IIM/Jo-1 sera analysed were able to precipitate histidyl-tRNA. Because neither anti-Ro60 nor anti-La activity could be detected in these sera, it is likely that recognition of the Ro RNP particle occurs via the Ro52 protein.

DISCUSSION

In this study, 112 IIM sera were analysed for their reactivity with several autoantigens. Reactivity with Jo-1, La/SS-B and the

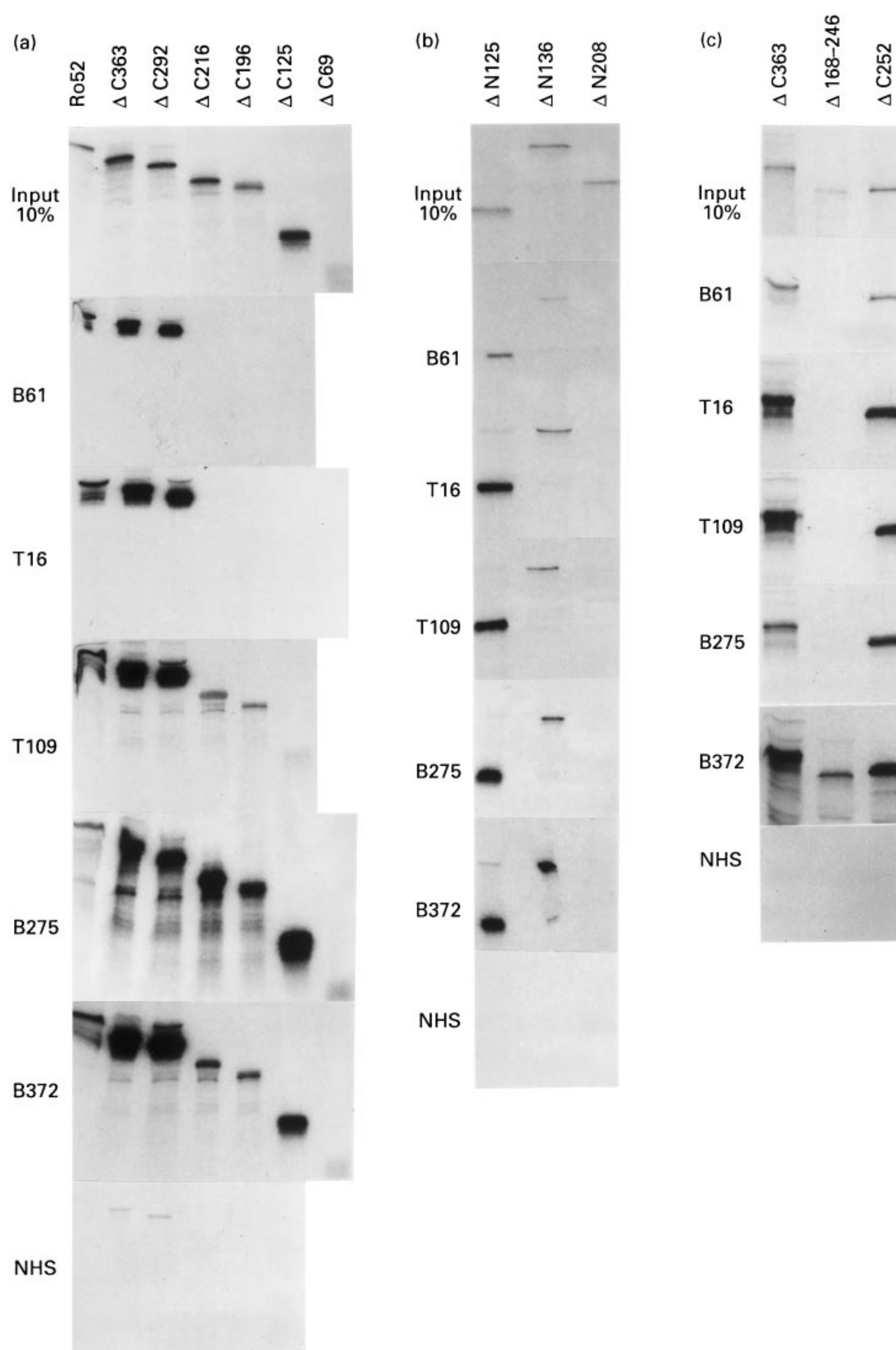

Fig. 3. Immunoprecipitation of deletion mutants of Ro52 by idiopathic inflammatory myopathy (IIM) sera. *In vitro* translated, ^{35}S-methionine-labelled, C-terminally (a) and N-terminally truncated mutants of Ro52 (b) and splicing variants Ro52Δ168–246 and ΔC252 (c) were immunoprecipitated using anti-Ro52^{+} IIM sera. Sera are indicated on the left side of each panel. Precipitated proteins were size-fractionated on a 15% SDS–polyacrylamide gel. The upper panel of each figure shows 10% of the amount of protein used for immunoprecipitation. The lower panels show the results for control precipitations with normal human sera (NHS).

Ro/SS-A proteins was tested by immunoblot and ELISA. Also the RNA precipitation pattern was determined. While only a relatively small percentage of the IIM sera was reactive with Ro60 (4%) and La (4%), 21% of the sera was found to be reactive with Jo-1, and, surprisingly, 20% with the Ro52 protein. In anti-Jo-1^{+} sera, the frequency of anti-Ro52 antibodies was even much higher (58%). This increase in anti-Ro52 activity in Jo-1 sera was not due to cross-reactivity between Jo-1 and Ro52, since (i) no competition for the binding to Ro52 could be detected with up to a 1000-fold molar excess of recombinant Jo-1 protein, and (ii) affinity-purified anti-Ro52 and anti-Jo-1 antibodies did not cross-react with each other. Our results imply that anti-Ro52 antibodies are more

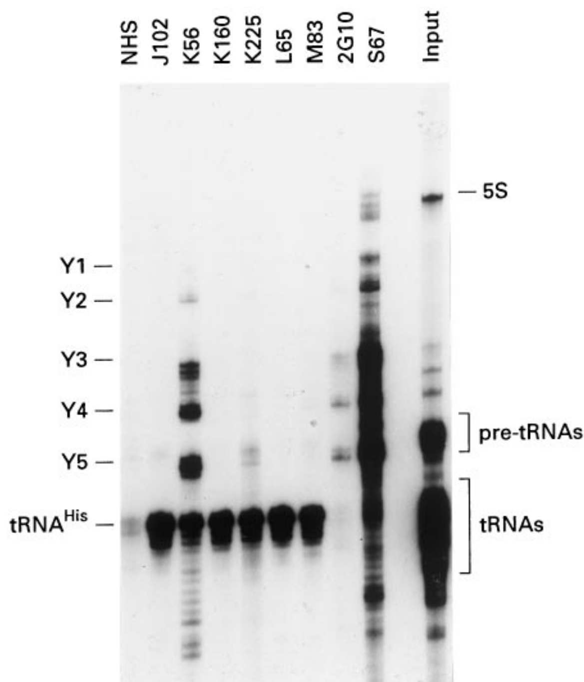

Fig. 4. RNA precipitation by anti-Jo-1$^+$ sera. Immunoprecipitations of RNA from a HeLa cell extract were performed with idiopathic inflammatory myopathy (IIM) anti-Jo-1 sera in which no anti-Ro60 or anti-La reactivity could be detected by counterimmunoelectrophoresis, immunoblotting and ELISA. As a positive control an anti-Ro60 MoAb (2G10) was used as well as an anti-Ro/Anti-La serum S67. Normal human serum (NHS) was included as a negative control. In the input lane RNA from 5% of the cell extract used for immunoprecipitations was loaded. (Co-precipitated) RNAs were visualized by pCp-labelling and denaturing PAGE.

frequent in anti-Jo-1$^+$ than in anti-Jo-1$^-$ (IIM) sera. No significant differences were found between PM and DM sera. Anti-Ro52 antibodies appear to co-occur only with anti-histidyl-tRNA synthetase antibodies (Jo-1), because they were found in the (few) sera that precipitated tRNA but in which no anti-Jo-1 activity was detected.

Anti-Ro antibodies have been described as being rather specific for SS or SLE. In the last decade, however, it became evident that these antibodies are also in low frequencies present in sera from patients with other autoimmune diseases [36,37]. For example, Marguerie and coworkers [37] described that anti-Ro antibodies are present in patients with anti-Jo-1 antibodies. Anti-Ro (isoform unknown) activity could be detected in five out of 19 anti-Jo-1$^+$ sera. We observed anti-Ro60 and anti-Ro52 reactivity in one and 14 sera, respectively, out of 24 Jo-1$^+$ IIM sera. Due to differences in techniques used and related to the fact that it is unclear whether all five anti-Ro$^+$ patients described in the previous study [37] had myositis, it is difficult to compare these data. By using sensitive detection techniques based upon recombinant autoantigens we were able to make a distinction between anti-Ro60 and anti-Ro52 activity. We found a frequency of 4% for anti-Ro60 antibodies in our Dutch cohort of IIM sera and of 4% in anti-Jo-1$^+$ IIM sera. The anti-Ro52 frequency of 20% in the total group of IIM sera was, therefore, unexpectedly high. Even more surprising was the ubiquitous presence of anti-Ro52 activity in anti-Jo-1$^+$ IIM sera (58%), suggesting that the immune response against Jo-1 and Ro52 might be coupled in some way. None of the IIM patients with both anti-Jo-1 and anti-Ro52 activity met the criteria for SS or SLE.

MSA like Jo-1 are of special interest because they are closely linked to clinical manifestations in IIM [36]. Similarly, diagnostic values of autoantibodies to the Ro proteins for SS [38,39], SLE [40], rheumatoid arthritis [41] and neonatal lupus syndrome [42] have been described. To learn more about the nature of the anti-Ro52 antibodies occuring in IIM sera, we analysed the major epitope region of the Ro52 protein targeted. This region was mapped in the middle part of the Ro52 protein between amino acids 125 and 252. This same central part of Ro52 (amino acids 128–234) is predicted to form a long α-helical domain with the potential to form coiled-coils with a leucine zipper motif at its C-terminal end. Leucine zippers were originally described in DNA binding proteins, but later these motifs were shown to be involved in protein–protein interaction and dimer formation as well (reviewed in [43]). When either C-terminally (ΔC226) or N-terminally (ΔN136) amino acids were deleted from the α-helical domain, 59% and 70% of the sera, respectively, precipitated the Ro52 protein less efficiently. In addition, the splicing variant Ro52β (Δ168–246) which lacks a large part of the α-helical domain is not or very inefficiently precipitated by all IIM sera analysed. Our results thus indicate that the major epitope region recognized by these sera corresponds to the putative α-helical domain.

Several studies have addressed the antigenic regions of the Ro52 protein [30,44–50]. The results vary depending on the patient population and detection methods used in these studies, but they have in common that the most important epitopes were found in the central part of the protein, within the α-helical domain. Furthermore, it has been shown that the recognition pattern of the Ro52 protein by several autoimmune sera shows some disease specificity [30,45–47]; the residues located between amino acids 216 and 292 appears to be essential for reactivity with anti-Ro52 antibodies from SLE sera, whereas antibodies of SS patients generally recognize multiple epitopes between amino acids 55 and 292 [30]. Having determined the antigenic regions of the Ro52 protein recognized by SLE sera in more detail (Table 2), we conclude that the major epitope region recognized by antibodies in IIM sera is identical to that recognized by SLE antibodies, i.e. the putative α-helical domain located between amino acids 125 and 252.

Although the frequency of anti-Ro52 antibodies is increased up to 58% in anti-Jo-1$^+$ IIM sera in comparison with 9% of anti-Jo-1$^-$ IIM sera (Table 1), no distinction in Ro52 epitope recognition was detected between these two groups of IIM sera. The reason why such an increase in frequency of anti-Ro52 antibodies is seen remains unclear, but it is tempting to speculate that the autoimmune response against these two, mainly cytoplasmic, autoantigens is coupled in some way.

Because anti-Ro52$^+$ IIM sera represent an as yet unrecognized class of anti-Ro52-containing sera, we tested whether they are able to immunoprecipitate Ro RNP-associated hY RNAs. Although co-precipitation of hY RNAs by anti-Ro52$^+$ sera has been demonstrated [21,24,25], the association of Ro52 with Ro RNPs remains controversial [18,19,25]. We determined the RNA precipitation pattern of several IIM sera in which no anti-Ro60 activity could be detected by the most sensitive techniques available. Two sera (K56 and K225) out of a panel of six IIM sera tested were able to precipitate Ro RNPs. The lack of precipitation of Ro RNPs by the other anti-Ro52 sera might be explained by differences in epitope

recognition, as has been reported before [21]. A more detailed analysis of the major epitope(s) recognized by anti-Ro52 antibodies in IIM sera would be required to conclude whether epitope differences between Ro RNP precipitating and non-precipitating antibodies are present. Co-precipitation of hY RNAs with the IIM sera, however, further supports the idea that at least a subset of Ro52 molecules is associated with at least a subset of Ro RNPs.

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