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ORIGINAL ARTICLE

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Characterization of anticytoplasmic antibodies in patients with systemic autoimmune diseases

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Abstract We characterized the cytoplasmic staining patterns identified by indirect immunofluorescence (IF) using human epithelial (HEp-2) cells as substrates, and identified autoantigens using enzyme-linked immunosorbent assay (ELISA) and cognate RNA immunoprecipitation techniques in cytoplasmic antibody-positive sera (CA(+)) in patients with systemic autoimmune diseases. Twenty-three sera (3.7%) of 630 patients were found to have a cytoplasmic staining pattern by IF on HEp-2 cells. The fine-pattern IF specificities were as follows: 12 diffuse fine speckled; 7 coarse granular filamentous speckled; 2 diffuse coarse speckled; 1 condensed large speckled; 1 cytoskeletal. No relationship was found between the staining patterns and the diseases. Anti-SS-A antibodies and antimitochondrial (M2) antibodies were detected by ELISA in 6 and 4 sera, respectively, and antismooth muscle antibody was detected by IF in 1 serum. In RNA immunoprecipitation assays, 6, 11, 3, and 1 patients had antibodies that recognized aminoacyltransfer RNA (tRNA) synthetases (including 2 EJ, 2 PL-7, 1 PL-12, and 1 unidentified tRNA-related), SS-A, ribosomes, and SRP, respectively. Moreover, several other autoantigens were detected by Western blotting using human epithelial (HEp-2) cell lysates. This study suggests that autoantibodies against tRNA synthetases, SS-A, ribosomes, mitochondria, and other autoantigens are present in CA(+) sera from patients with a variety of systemic autoimmune diseases.

Key words Autoantibody · Cytoplasmic antibody (CA) · Human epithelial (HEp-2) cells · Systemic autoimmune disease · Transfer RNA (tRNA)

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Introduction

Autoantibodies are detected in sera derived from patients with a variety of systemic autoimmune diseases, and we have used these autoantibodies as diagnostic tools.[1] Antinuclear antibody (ANA) detection is the most popular examination with systemic autoimmune diseases, and is usually assayed by indirect immunofluorescence (IF) of human epithelial (HEp-2) cells.[2]

Many autoantigens are present in nuclei, but we rarely detect cytoplasmic staining patterns on HEp-2 cells in these diseases. The cytoplasmic antibody (CA) has been reported in sera from patients with systemic lupus erythematosus (SLE) over the past four decades.[3–7] CA was also deteced in sera from patients with diseases other than autoimmune diseases, including malignant melanoma, bronchial asthma, and granulocytic ehrlichiosis.[8–10] However, the identity of autoantigens detected by CA are poorly understood. In this study, we examined by IF the CA in sera derived from Japanese patients with a variety of systemic autoimmune diseases. We also investigated the autoantigens targeted by CA by enzyme-linked immunosorbent assay (ELISA) and RNA immunoprecipitation assay.

Patients and methods

Patients

The sera of 630 patients, who had been referred to the Nagasaki University Hospital or Nagasaki Kita Hospital for

Rheumatic Diseases, were screened by each serology service for ANA by indirect IF using HEp-2 cells. The patients' diagnoses were as follows: 246 rheumatoid arthritis (RA); 111 systemic lupus erythematosus (SLE); 127 primary Sjögren's syndrome (pSjS); 26 polymyositis/dermatomyositis (PM/DM); 12 systemic sclerosis (SS); 32 mixed connective tissue disease (MCTD); 36 Beçhet's disease, and 40 other systemic autoimmune diseases. These diagnoses were confirmed by specialists in rheumatic diseases in our laboratory according to the criteria of the American College of Rheumatology.

Immunofluorescence

The sera were diluted 1:80 (dilutions were decided by our preliminary experiments to eliminate the nonspecific staining.) in phosphate-buffered saline (PBS) and incubated on HEp-2-coated slides (MBL, Nagoya, Japan) for 30 min at room temperature (RT). The slides were then washed in PBS and incubated with fluorescein isothiocyanate (FITC) conjugated polyvalent goat antihuman immunoglobulin (MBL) for 30 min at RT. The cytoplasmic IF patterns were observed using a fluorescence microscope (BX50, Olympus, Osaka, Japan). Antismooth muscle antibody was detected by an IF kit (SRL, Tachikawa, Japan), which used fixed rat kidney and stomach tissues affixed to the slide.

ELISA

Autoantibodies to SS-A (Ro), Jo-1 (histidyl-tRNA synthetase), and M2 (mitochondria M2 antigen) were measured using specific ELISAs (SS-A and Jo-1 ELISA kits from SRL; M2 ELISA kit from MBL) according to the manufacturer's instructions. The detection limit for each assay was 10.0 U/ml, 10.0 U/ml, and 7.0 U/ml for SS-A, Jo-1, and M2, respectively.

RNA immunoprecipitation assay

Immunoprecipitation of RNA was performed as described previously.[11–13] Briefly, HeLa cells ($\sim 2 \times 10^8$) were resuspended in 10 ml NET-2 buffer (50 mM Tris HCl, 150 mM NaCl, 0.05% Nonidet P40, pH 7.4), sonicated, and then centrifuged. The cell supernatant was used as an antigen source. Two milligrams of protein A-Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) was suspended in 500 μl IPP buffer (10 mM Tris HCl, 500 mM NaCl, 0.1% Nonidet P40, pH 8.0) and incubated with 10 μl of patient serum for 12 h at 4°C with end-over-end rotation. After washing the IgG-coated beads, cell supernatant (300 μl) was added and incubated for 2 h at 4°C. The protein A-Sepharose beads were washed five times with 500 μl NET-2 and resuspended in 300 μl of NET-2. RNAs from the immunoprecipitates were extracted with 300 μl phenol/chloroform/isoamyl alcohol (50:50:1) containing 0.1% hydroxyquinoline, precipitated with ethanol, resolved in a 10% polyacrylamide gel containing 7 M urea, and detected by silver staining (Bio-Rad, Hercules, CA, USA). To prepare deproteinized RNA as antigen, RNA was extracted from HeLa cell supernatants with phenol/chloroform/isoamyl alcohol as described previously.[13]

Western blotting

HEp-2 cells were lysed in 1% NP40 buffer including proteinase inhibitor (1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml antipain, 3 μg/ml chymostatin, and 100 μg/ml phenylmethylsulfonyl fluoride), followed by centrifugation at 15 000 r.p.m. for 15 min at 4°C, and the supernatants were frozen at −20°C. Protein concentration was quantified using the Bio-Rad (Melville, NY, USA) protein assay kit. An identical amount of protein for each lysate (10 μg/well) was subjected to 5%–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, Pagel, Atto, Tokyo, Japan). Proteins were transferred to a polyvinyliden difluoride (PVDF) filter (Atto), and the filter was blocked for 1 h using 5% nonfat dried milk in PBS containing 0.1% Tween 20 (PBS-T) at RT, and incubated for 1 h at RT in the presence of each sera diluted 1:50 in PBS using a multiscreen blotter (Postblot, Atto). The filter was washed with PBS-T and incubated with a 1:5000 dilution of goat antihuman IgG coupled to horseradish peroxidase (MBL). The enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA) was used for detection.

Results

Immunofluorescence patterns on HEp-2 cells in CA(+) sera

Of 630 sera tested, 23 sera (3.7%) were found to have a cytoplasmic staining pattern by IF. Figure 1a–g shows the six staining patterns that were identified on HEp-2 cells. Of the IF patterns, there were 12 diffuse fine speckled, 7 coarse granular filamentous speckled, 2 diffuse coarse speckled, 1 condensed large speckled, and 1 cytoskeletal (Table 1). Systematic chart review identified an association with the following diseases: 7 PM/DM (4 DM, 3 PM), 8 RA, 4 pSjS, 2 overlap syndrome, 1 SLE, and 1 aortitis. No relationship was seen between the staining patterns and the diseases.

Detection of anti-SS-A, anti-M2, and anti-Jo-1 by ELISA and antismooth muscle by IF in CA(+) sera

We then examined the three representative cytoplasmic autoantibodies against SS-A, M2, and Jo-1 using ELISA. Anti-SS-A antibodies and antimitochondrial (M2) antibodies were detected in 6 and 4 sera, respectively, while anti-Jo-1 antibodies were not detected in any of 23 CA(+) sera (Table 2). Moreover, antismooth muscle antibodies (ASMAs) were detected in 1 serum sample by IF using rat kidney and stomach tissues.

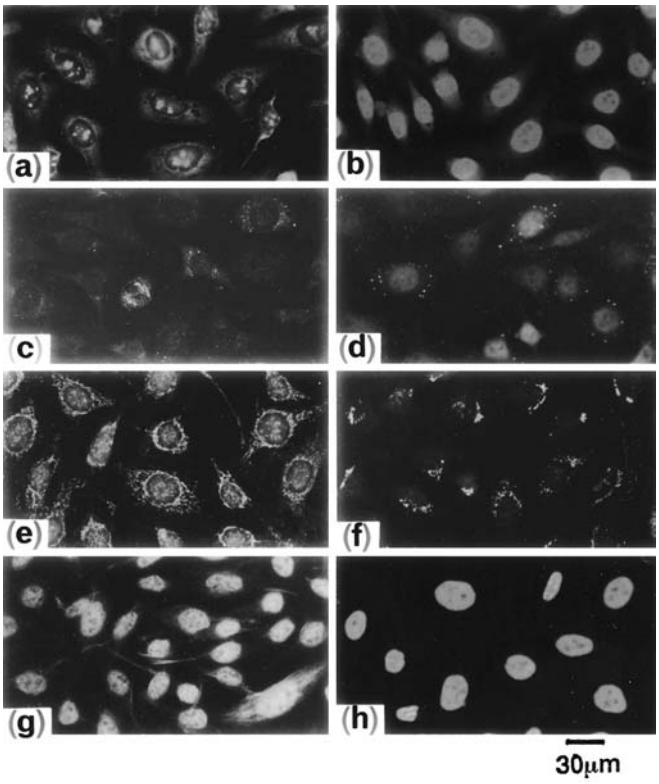

Fig. 1. Cytoplasmic fluorescence patterns on HEp-2 cells. **a** Diffuse fine speckled pattern (patient No. 2, antiribosomal Ab). **b** Diffuse fine speckled pattern (patient No. 15). **c** Diffuse coarse speckled pattern (patient No. 7). **d** Diffuse coarse speckled pattern (patient No. 22). **e** Coarse granular filamentous speckled pattern (patient No. 5, antimitochondrial Ab). **f** Condensed large speckled pattern (patient No. 17, antigolgi Ab). **g** Cytoskeletal pattern (patient No. 19). **h** Cytoplasmic negative pattern (antinuclear antibody (ANA) only). *Bar* 30 μm

RNA immunoprecipitation of HeLa cell extracts in CA(+) sera

As many autoantigens bind RNA in cytoplasm, and because we failed to detect Jo-1 reactivity in the majority of patients, RNA immunoprecipitation assays were performed using 23 CA(+) sera. In this assay, 6, 11, 3, and 1 patients precipitated antibodies that recognized RNAs associated with aminoacyl-transfer RNA (tRNA) synthetases, SS-A, ribosomes, and signal recognition particle (SRP), respectively (Fig. 2, Table 2). RNA immunoprecipitation assays were more sensitive than ELISA in detecting anti-SS-A autoantibodies (Table 2).

Table 1. Cytoplasmic immunofluorescence patterns and diagnosis

Pattern	Total No.	Diagnosis	No.
Diffuse fine speckles	12	DM	3
		RA	3
		PM	2
		Overlap	2
		pSjS	1
		SLE	1
Coarse granular Filamentous speckles	7	RA	3
		pSjS	2
		PM	1
		Aortitis	1
Diffuse coarse speckles	2	RA	1
		pSjS	1
Condensed large speckles	1	DM	1
Cytoskeletal	1	RA	1

DM, dermatomyositis; RA, rheumatoid arthritis; PM, polymyositis; Overlap, overlap syndrome; pSjS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus

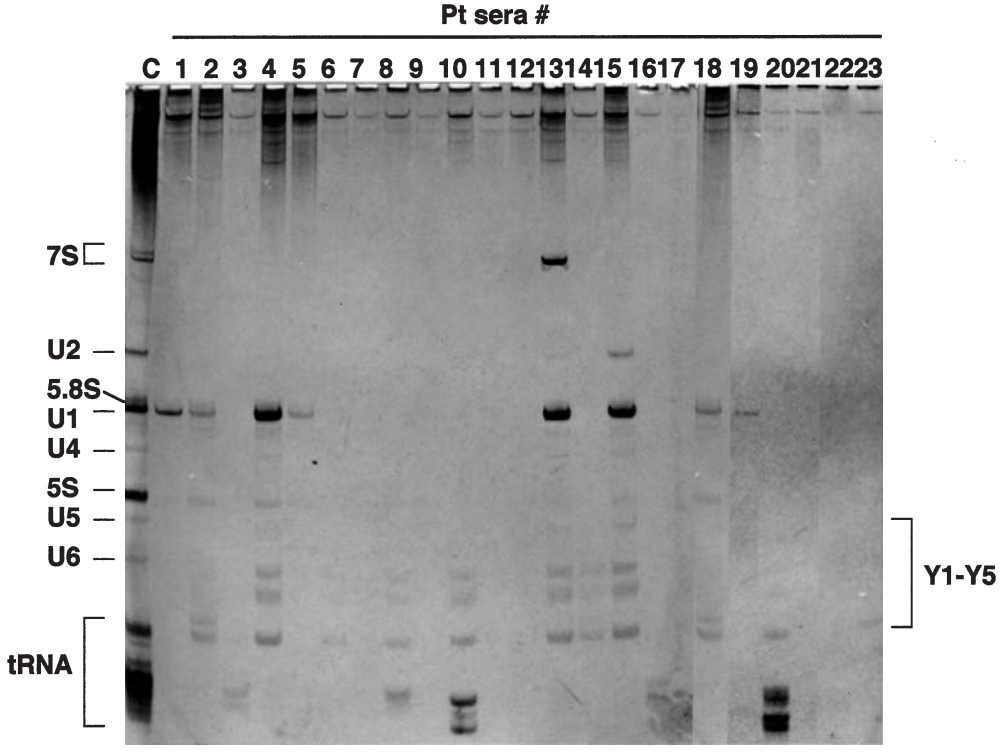

Fig. 2. RNA immunoprecipitation of HeLa cell extracts. CA(+) patient serum samples were incubated with HeLa cell extracts. Precipitated RNA was extracted with phenol, fractionated on polyacrylamide gels, and detected by silver staining. The major RNA species present in total cell extract are shown on the left

Table 2. Summary of autoantibodies using enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation of RNA in patients with CA(+) sera

No.	Disease/sex	Age	ELISA			IF ASMA	Immunoprecipitation of RNA
			SSA	Jo-1	M2		
11	PM	42/F	−	−	+	−	−
16	PM	40/F	−	−	−	−	PL-7
20	PM (+MRA)	58/M	−	−	−	−	PL-12, SSA
3	DM	48/F	−	−	−	−	Unknown (anti-tRNA Ab-related)
8	DM	44/F	−	−	−	−	PL-7, SSA
10	DM	37/F	+	−	−	−	EJ, SSA
17	DM	56/F	−	−	−	−	EJ, SSA
4	Overlap	69/F	−	−	−	−	U1RNP, SSA, ribosome
12	Overlap	54/F	−	−	−	−	−
6	RA	55/F	+	−	+	−	SSA
19	RA	54/M	−	−	−	−	U1RNP
22	RA	71/F	−	−	−	+	−
2	RA (+SjS)	56/F	−	−	−	−	SSB, ribosome
13	RA (+SjS)	38/F	+	−	−	−	SRP, U1RNP, SSA
23	RA (+SjS)	39/F	+	−	+	−	SSA
5	RA (MRA)	64/F	−	−	+	−	Ribosome
21	RA (Felty)	39/F	−	−	−	−	−
7	pSjS	74/M	−	−	−	−	−
14	pSjS	48/F	+	−	−	−	SSA
15	pSjS	70/F	−	−	−	−	U1RNP, Sm, SSA
18	pSjS	69/M	+	−	−	−	SSA, SSB
1	SLE	56/F	−	−	−	−	U1RNP
9	Aortitis	54/F	−	−	−	−	−

IF, indirect immunofluorescence; ASMA, antismooth muscle antibody; MRA, malignant rheumatoid arthritis; SRP, signal recognition particle

Confirmation of tRNA antibodies

We then determined which tRNA were immunoprecipitated by individual serum samples (Fig. 2). Several different patterns of immunoprecipitated tRNAs were identified, including 2 EJ (glycyl-tRNA synthetase), 2 PL-7 (threonyl-tRNA synthetase), 1 PL-12 (alanyl-tRNA synthetase), and 1 unidentified tRNA-related protein, in comparison to the control sera (Fig. 3).

Detection of autoantigens in HEp-2 cell lysates by Western blotting

Since ELISA and immunoprecipitation of RNA failed to identify the antigens recognized by four of the sera (Nos. 7, 9, 12, and 21 in Table 2), we further examined whether autoantibodies were present that could recognize denatured autoantigens derived from the cytoplasm of HEp-2 cells. Figure 4 shows that several autoantigens (pp28, pp30 in No. 7, pp24, pp28 in No. 9, pp29 in No. 12, and pp28, pp85 in No. 21) were detected in all four sera, suggesting that at least one of these antigens represents the antigen(s) seen in HEp-2 cells by IF (see Fig. 1, Table 1).

Discussion

Our studies showed that 23 of 630 patients (3.7%) with systemic autoimmune diseases were found to have a CA detected by IF. This percentage was nearly identical to that noted in previous reports.[6,7] Further examination demonstrated that autoantibodies against aminoacyl-tRNA synthetases, SS-A, ribosomes, mitochondria, and another autoantigens are present in CA(+) sera from these patients.

We identified five types of cytoplasmic pattern on HEp-2 cells, but no relationship was found between the staining patterns and the diseases. Sera show a classical mitochindrial staining pattern in HEp-2 cells by IF, although the same sera included another autoantibody specificity, i.e., SS-A (in sera Nos. 6 and 23) or ribosome (in sera No. 5) determined by ELISA or RNA immunoprecipitation assay (see Table 2). Moreover, sera No. 17 demonstrated a golgi staining pattern (see Fig. 1f), whereas RNA immunoprecipitation assays detected EJ-specific antibody (see Fig. 2) which should not be present in golgi. This suggests that more than one autoantibody specificity is contained in CA(+) sera, and that there is an imprecise correlation between IF and other assays such as immunoprecipitation, Western blotting, and ELISA. No relationship was found between the staining patterns and diseases, or between the staining patterns and autoantigens. As a higher percentage of CA(+) sera was reported in bronchial asthma (20%), malignant melanoma (17%), and granulocytic ehrlichiosis (8%) compared with that in control sera,[8–10] these diseases must also be considered in the differential diagnosis.

Autoantibodies directed against aminoacyl-tRNA synthetases can be found in serum derived from approximately 25%–35% of patients with the chronic, inflammatory muscle disorders, including PM and DM.[14] Table 2 shows that autoantibodies directed against aminoacyl-tRNA syn-

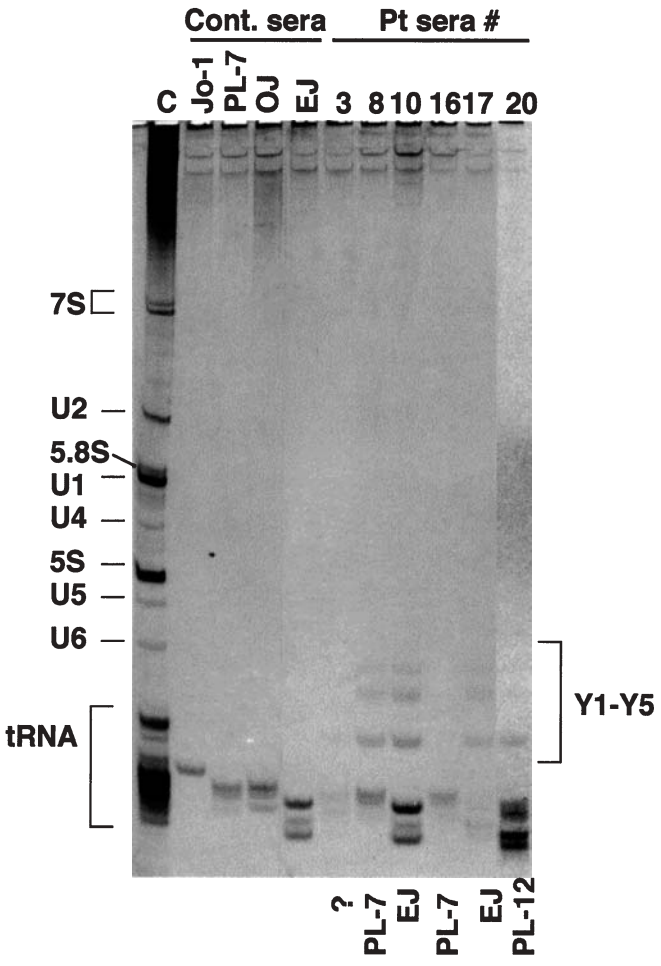

Fig. 3. RNA immunoprecipitation for antisynthetase positive sera compared with control sera with antiaminoacyl-tRNA synthetase antibodies. RNA immunoprecipitation assay was performed in six sera depicting tRNA precipitates (see Fig. 2), and compared with control sera with antiaminoacyl-tRNA synthetase antibodies. Sera used for immunoprecipitation include the antisynthetase sera indicated, including antibodies to Jo-1 (histidyl-tRNA synthetase), PL-7 (threonyl-tRNA synthetase), PL-12 (alanyl-tRNA synthetase), OJ (isoleucyl-tRNA synthetase), and EJ (glycyl-tRNA synthetase)

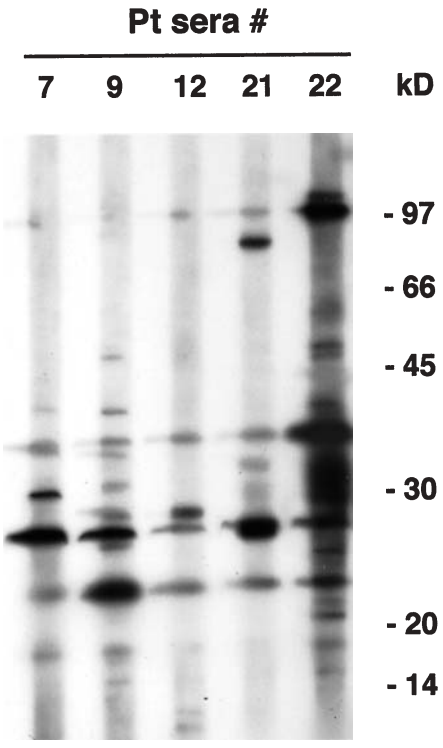

Fig. 4. Detection of autoantigens in HEp-2 cell lysates by Western blotting. HEp-2 cell lysates were subjected to 5%–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred to a polyvinyliden difluoride (PVDF) filter. The filter was incubated in the presence of each sera (using the sera from patients Nos. 7, 9, 12, and 21 in which autoantigens could not be identified by enzyme-linked immunosorbent assay (ELISA) or immunoprecipitation of RNA (see Table 2), and one patient's serum (No. 22) which targeted smooth-muscle antigen). The serum was diluted 1:50 in phosphate-buffered solution (PBS) using a multiscreen blotter after blocking with 5% nonfat dried milk in PBS-T. The filter was washed with PBS-T and incubated with goat antihuman IgG coupled with horseradish peroxidase. The enhanced chemiluminescence system was used for detection. The positions of the molecular weight markers are indicated on the right (in kD)

thetases, including EJ, PL-7, and PL-12, were found in 5 of 23 CA(+) sera (21.7%), and detected only in PM and DM. These results suggest that these autoantibodies are highly specific to PM and DM, which is consistent with a previous report.[15] When CA(+) sera are encountered by clinicians, historical and physical examinations are needed to identify patients with PM/DM (i.e., those having myalgia, muscle weakness, etc.), and to check for elevations of muscle enzymes in sera. Thus, it is very useful for clinicians to identify aminoacyl-tRNA synthetase-specific autoantibodies in CA(+) patients. Moreover, when the autoantigens cannot be detected in CA(+) patients by using ELISA and RNA immunoprecipitation assays, other methods may be developed (e.g., mass spectrometry or protein microarray analysis) to discover novel autoantigens.

Apoptosis is not only a morphologically, but also a biochemically, defined form of cell death that plays a significant role in autoimmunity.[16] Many substrates (autoantigens) are cleaved by proteases during the apoptotic process inside cells, and new epitopes are created that are potentially presented to antigen-presenting cells, such as dendritic cells and macrophages. The antigen-presenting cells are thought effectively to present the autoantigens to T cells, leading to help to autoreactive B cells and autoantibody production. Apoptotic cells may thus be a major source of autoantigens.[17] Recently, a serine proteinase, granzyme B, has been reported to cleave many autoantigens, including cytoplasmic antigens (histidyl tRNA synthetase, SRP-72, etc.).[17,18] Although a small number of cytoplasmic autoantigens are recognized by sera derived from patients with systemic autoimmune diseases compared with the number of nuclear autoantigens, it is possible that our study may identify new insights regarding cytoplasmic autoantigens.

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