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Original Article

Association between anti-U1 ribonucleoprotein antibodies and inflammatory mediators in cerebrospinal fluid of patients with neuropsychiatric systemic lupus erythematosus

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Running head

Anti-U1RNP Abs and inflammatory mediators in CSF

Abstract

Objective: We investigated possible associations between neurotoxic inflammatory mediators (IMs) and anti-U1RNP antibodies (Abs) in cerebrospinal fluid (CSF) of neuropsychiatric systemic lupus erythematosus (NPSLE).

Methods: Serum and CSF anti-U1RNP Abs were detected using an RNA-immunoprecipitation assay and CSF anti-U1RNP Ab levels were measured by ELISA. IFN-α, MCP-1 and IL-8 levels in CSF were determined by quantitative multiplex cytokine analysis. IM levels were compared among anti-U1RNP-positive and anti-U1RNP -negative NPSLE as well as other rheumatic disease controls (controls).

Results: Anti-U1RNP Abs were detected in serum (58%) and in CSF (18%) of 82 NPSLE patients. CSF MCP-1 levels were higher in NPSLE than in controls. CSF IFN-α level was higher in CSF anti-U1RNP Ab-positive than in -negative patients or controls. When limited to serum anti-U1RNP Ab-positive patients, however, levels of all 3 IMs in CSF were higher in CSF anti-U1RNP Ab-positive than in -negative patients. Anti-U1RNP Ab levels in CSF correlated with CSF MCP-1, but not IFN-α and IL-8 levels.

Conclusions: CSF anti-U1RNP Ab positivity is associated with the increased level of CSF IFN-α. MCP-1 levels correlated with CSF anti-U1RNP Ab levels, whereas the increased CSF MCP-1 was not specific to CSF anti-U1RNP Ab-positive NPSLE.

Key Words: anti-U1RNP antibodies, cerebrospinal fluid, IFN-α, MCP-1, neuropsychiatric systemic lupus erythaematosus

Introduction

Systemic lupus erythaematosus (SLE) is a chronic multisystem autoimmune disease, and neuropsychiatric SLE (NPSLE) is a severe life-threatening condition. Although the pathophysiology of NPSLE remains unclear, a particular subset of autoantibodies (auto-Abs) has been shown to be associated with neuronal injury.[1-3] Abs binding to a certain sequence of the *N*-methyl-D-aspartate receptor subunit NR2 (anti-NR2 Abs) cause excitatory synaptic transmissions in central nervous system (CNS).[4] Arinuma et al[5] reported that anti-NR2 Abs in the cerebrospinal fluid (CSF), but not in serum, were involved in diffuse central NPSLE. Okada et al[6] reported that 13 of 14 patients (7 SLE, 5 mixed connective tissue disease [MCTD] or overlap syndrome, 1 undifferentiated CTD, 1 Sjögren's syndrome) with aseptic meningitis attributed to CTD had serum anti-U1 ribonucleoprotein (RNP) Abs. Recently, using an RNA-immunoprecipitation assay (RNA-IPP), we found that anti-U1RNP Abs were detected in CSF; anti-U1RNP Abs in CSF could be a useful biomarker of NP manifestations in SLE.[7]

In addition to auto-Abs, inflammatory mediators (IMs) have been found in CSF of NPSLE patients.[8, 9] Previous reports showed that the levels of IFN-α[10], IFN-γ-inducible protein (IP)-10[11, 12], IL-8[11], monocyte chemotactic protein (MCP)-1[11, 13], fractalkine (CX3CL1)[14], the regulated and normal T-cell expressed and secreted (RANTES/CCL5) chemokine[11] and IFN-γ-induced monokines (MIG/CXCL9)[11] in CSF are significantly higher in NPSLE than in non-NPSLE patients. IFN-α production in SLE is caused, at least partially, by auto-Abs binding to RNP particles released from dead or dying cells.[15, 16] Santer et al[17] suggested that IFN-inducing activity in CSF correlates with serum anti-U1RNP Abs but not with other known anti-nuclear Abs. Therefore, anti-U1RNP Abs and/or their immune complexes (IC) in CSF may play a pathogenic role in NPSLE; however, the association between CSF anti-U1RNP Abs and IMs remains unclear. In this

study, we examined the levels of anti-U1RNP Abs and IMs in serum and CSF samples from NPSLE patients to determine whether such an association exists.

Methods

Patients

Eighty-two SLE patients (70 female, 12 male) with NP manifestations admitted to the Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, between March 2003 and October 2011 were enrolled in this study. Patient age at NP manifestation onset was 36.3 ± 15.1 years (range, 10–75 years) **(Table 1)**. SLE was diagnosed according to the ACR criteria [18, 19]. NP manifestations were determined according to case definitions for NP syndromes in SLE[20] following NP testing, electroencephalography and brain MRI. NP manifestations caused by other factors (e.g., concurrent non-SLE NP diseases such as infection, uraemia and drug-induced aseptic meningitis) were excluded from this study. After obtaining informed consent from all participants, CSF and serum samples were obtained on the same day and stored at $-80°C$ until analysis. Control CSF samples were obtained from 15 patients with other systemic rheumatic diseases including rheumatoid arthritis (4), vasculitis (7), Behçet disease (3) and TNF receptor-associated periodic syndrome (1) (all anti-U1RNP Ab negative; 5 men and 10 women with age of 42.7 ± 20.6 years). Although these patients had NP manifestations, routine laboratory analysis of CSF (e.g., protein level) was normal. The present study was carried out in compliance with the Helsinki Declaration and approved by the Kyoto University Ethics Committee Review Board (Approved #E97).

CSF IM detection

4

CSF was routinely analysed for total protein levels, cell number and IgG levels. IFN-α, IL-8 and MCP-1 levels were determined using the Procarta® Cytokine Assay Kit (Affymetrix Inc., Santa Clara, CA, USA) and quantitative multiplex cytokine analysis was performed with Bio-Plex® (Bio-Rad Laboratories, Hercules, CA, USA).

Detection of anti-RNA-binding proteins (RBPs) in serum and CSF by RNA immunoprecipitation (RNA-IPP).

RNA-IPP using HeLa-cell extracts was performed [7, 21] to detect anti-U1RNP, Sm, Ro/SSA (SSA) and La/SSB (SSB) Abs in serum and CSF samples. Briefly, 10 μl of the sample was mixed with 2 mg of protein A Sepharose[TM] CL-4B (GE Healthcare) in IPP buffer [10-mM Tris–HCl, pH 8.0; 500-mM NaCl; 0.1% Nonidet P-40 (NP-40)] and incubated for 2 h at 4°C. The IgG-coated Sepharose[TM] beads were then washed in IPP buffer and resuspended in NET-2 buffer (50-mM Tris–HCl, pH 7.5; 150-mM NaCl; 0.05% NP-40). For RNA analysis, this suspension was incubated with HeLa-cell extracts derived from 6×10^6 cells for 2 h at 4°C. The antigen-bound Sepharose[TM] was then collected, washed and resuspended in NET-2 buffer. To extract bound RNA, 30 μl of 3.0-M sodium acetate, 15 μl of 20% sodium dodecyl sulphate and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1) solution, which is saturated with 0.1M Tris-HCl pH 7.5 (about 100ml/l) and contains 0.1% 8-hydroxyquinoline, were added to the Sepharose[TM] beads. RNA was recovered in the aqueous phase after ethanol precipitation and dissolved in electrophoresis sample buffer consisting of 10-M urea, 0.025% bromophenol blue and 0.02% xylene cyanol FF (Bio-Rad) in Tris–borate–EDTA buffer (90-mM Tris–HCl, pH 8.6; 90-mM boric acid; 1-mM EDTA). The RNA samples were denatured at 65°C for 5 min and then resolved by 7-M urea–10% PAGE with silver staining (Bio-Rad). Anti-U1RNP, Sm, SSA and SSB Abs were detected as positive when U1RNA, U1-U6RNA, SSA-RNA (Y1-Y5RNA) and SSB-RNA (5S-ribosomal

RNA, 7S-RNA and Y1-Y5RNA), respectively, were precipitated.

Measurement of anti-U1RNP Ab levels in CSF by ELISA

CSF anti-U1RNP Ab levels were determined by ELISA using recombinant U1RNP (Mesacup®-2 test RNP, Medical and Biological Laboratories (MBL) Co., Nagoya, Japan) according to the manufacturer's protocol. CSF samples were diluted 1:5 using phosphate-buffered saline (PBS). Patient CSF were incubated as primary Abs at room temperature for 2 h, and bound IgG was detected with alkaline phosphatase-conjugated anti-human IgG (Southern Biotechnology Associates Inc., Birmingham, AL, USA) at OD_{405} $_{nm}$ in a microtitre ELISA reader. All assays were performed in triplicate and $OD_{405\ nm}$ values of CSF samples were confirmed to be within the linear range of the positive control.

Statistical analysis

The non-parametric Mann–Whitney U test was used to compare levels between the means of 2 groups. Spearman's correlation coefficient by the rank test was used to evaluate the correlation between the anti-U1RNP Ab levels and IMs. $p < 0.05$ was considered to be significant.

Results

Anti-U1RNP Abs detected in serum and CSF of NPSLE patients

Auto-Abs against RBPs were examined using RNA-IPP (**Figure 1**). Serum anti-U1RNP Abs were positive in 58% patients (**Figure 2**). CSF anti-U1RNP Abs were detected in 15 patients (18% of all patients or 31% of serum anti-U1RNP Ab-positive

patients); CSF anti-SSA Abs were detected in 4 patients (5% of all patients and 8% of serum anti-SSA Ab-positive patients). Both anti-U1RNP and SSA Abs in CSF were negative in patients without serum anti-U1RNP and SSA Abs. Serum and CSF anti-Sm Abs were positive in 4 patients and 1 patient, respectively. CSF anti-SSB Abs were not detected in our patients. Because of the low frequency of CSF anti-SSA/Sm Abs and our previous data [7], we only analysed the association between anti-U1RNP Abs and CSF IMs. There was no significant association of serum or CSF anti-U1RNP Ab positivity with NPSLE forms in our patients **(Table 1)**. Also, there was no correlation between serum and CSF anti-U1RNP Ab levels (data not shown and refer to Ref. 7). Abs against ribosomal proteins such as 28S ribosome were detected in 7 patients' sera, but not in CSF.

Association of serum anti-U1RNP Abs and CSF IMs

As shown in **Figure 3**, there was no significant difference in CSF IFN-α and IL-8 levels between serum anti-U1RNP Ab-positive patients and controls. CSF MCP-1 level was significantly elevated in NPSLE than in controls.

Association of CSF anti-U1RNP Abs and CSF IMs

IFN-α levels in CSF anti-U1RNP Ab-positive patients, but not in CSF anti-U1RNP Ab-negative, were significantly higher than in controls **(Figure 4A)**. CSF MCP-1 levels in NPSLE patients were markedly elevated irrespective of CSF anti-U1RNP Ab positivity.

CSF anti-U1RNP Abs were not detected in serum anti-U1RNP-negative patients. When limited to serum anti-U1RNP Ab-positive patients, CSF levels of all 3 IMs were higher in CSF anti-U1RNP Ab-positive patients than in CSF anti-U1RNP Ab-negative patients **(Figure 4B)**.

Correlation of anti-U1RNP Ab levels with IM concentrations in CSF

Among 15 CSF anti-U1RNP Ab-positive patients, 12 of CSF samples were remained enough for determination of anti-U1RNP Ab levels by ELISA. In NPSLE patients with CSF anti-U1RNP Abs, the Ab level correlated with CSF MCP-1 concentration but not with CSF IFN-α (**Figure 5**). CSF IL-8 concentration did not correlate with CSF anti-U1RNP Ab level (data not shown).

Discussion

We previously showed that CSF anti-U1RNP Abs detected by RNA-IPP are more specific markers for primary central NPSLE than CSF IL-6 concentration and IgG index in serum anti-U1RNP Ab-positive patients.[7] However, how CSF anti-U1RNP Abs are correlated with the NPSLE pathogenesis remains unclear. To solve this question, we collected CSF samples of SLE patients with NP manifestations widely, including patients whose NP symptoms were not so severe. Therefore, positivity of CSF anti-U1RNP Abs in the present study was less frequent than that in the previous study. [7]

This study is the first one to show a significant association between CSF anti-U1RNP Abs and particular subsets of IMs in NPSLE patients. CSF IFN-α levels were increased only in patients with CSF anti-U1RNP Abs, suggesting anti-U1RNP Ab-mediated IFN-α production in CNS. Santer et al[17] reported that IC-containing CSF-IgG of NPSLE patients had abnormally high IFN-α-inducing activity. This phenomenon was most prominent in serum anti-U1RNP Ab-positive patients, which supports our results. IFN-α was detected in CSF of NPSLE patients; therefore, it might be induced by intrathecal complexes of anti-U1RNP Ab and IC.   IFN-α production has a pathogenic significance in SLE [22]; therefore, CSF anti-U1RNP Ab-associated NP manifestations should be analysed in future studies. Although the percentages of diffuse psychiatric and neuropsychological syndromes

were similar in our patients, symptoms of NPSLE patients with CSF anti-U1RNP Abs appeared to be relatively limited to headache and psychosis. In most of the patients with these diagnoses, however, CSF anti-U1RNP Abs are negative. Recently, Fragoso–Loyo et al[23] reported that CSF IFN-α was not a useful biomarker of central NPSLE. Our data suggested that no elevation of CSF IFN-α levels occurred in patients without CSF anti-U1RNP Abs and that an increased CSF IM levels may depend on the specificity of CSF auto-Abs. IL-8 and MCP-1 synthesis might be regulated by other auto-Abs in CSF. The reason why CSF IFN-α concentrations did not correlate with CSF anti-U1RNP Ab levels could not be elucidated in the present study. In our NPSLE patients, IgG index is significantly higher in CSF anti-U1RNP Ab-positive than in CSF anti-U1RNP Ab-negative patients (unpublished data); therefore, intrathecal polyclonal B-cell activation might also induce nonspecific IgG production in CNS, which inhibits FcγR IIa-mediated interferogenic activity.[17, 24] Alternatively, CSF IFN-α might be regulated by other immunologic factors than anti-U1RNP Abs in NPSLE.

Several reports have shown the clinical importance of other IMs in NPSLE patients. CSF MCP-1 has been reported as a useful biomarker that differentiates SLE-attributable NP manifestations from non-SLE-related manifestations.[9] Our results indicated that the elevated CSF MCP-1 level was not specific to CSF anti-U1RNP Ab-positive patients. When limited to NPSLE patients with serum anti-U1RNP Abs, however, CSF anti-U1RNP Ab positivity appeared to be positively correlated with the elevation of CSF MCP-1 levels. Therefore, CSF MCP-1 activation resulting from CNS injuries may stimulate the anti-U1RNP Ab production in CNS. We previously suggested the possibility of intrathecal anti-U1RNP Ab production by showing U1-70K different autoepitope patterns between serum and CSF anti-U1RNP Abs.[7]

To date, only our group[7, 25] and a German group[26] have published reports of

CSF anti-U1RNP Ab-positive cases. A critical point of our study is the use of RNA-IPP to detect anti-U1RNP Abs in CSF. RNA-IPP is the most sensitive and specific immunological method for detecting Abs, especially against 'native' RBPs.[27] Because less than 20 μL of sample is enough for assay, RNA-IPP is more suitable than ELISA for detecting anti-RBP Abs in CSF, which usually cannot be obtained in a large amount from patients.

However, the present study has some limitations. First, because we did not have CSF of SLE patients without NP manifestation, it is unclear whether CSF anti-U1RNP Abs exist in those patients. Second, other NPSLE-associated auto-Abs such as anti-ribosomal P were not investigated. Although we could find Abs against ribosomal antigens such as 28S-ribosomal protein in the present study, anti-ribosomal P Abs could not be specifically detected by RNA-immunoprecipitation assay. Previous studies demonstrated that IL-6 levels in CSF may be associated with anti-NR2 Abs.[28] IL-8 and MCP-1 levels appear to be associated with other auto-Ab levels more than anti-U1RNP Ab levels. Auto-Abs against DNA-binding or associated antigens cannot be detected by RNA-IPP; therefore, an association study of anti-non RBP Abs and CSF IMs using other assays is needed.

In conclusion, CSF anti-U1RNP Abs are associated with the elevated CSF IFN-α levels in NPSLE. CSF MCP-1 concentrations correlate with CSF anti-U1RNP Ab levels, whereas increased CSF MCP-1 is not specific for anti-U1RNP Ab-positive NPSLE.

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Figure legends

Figure 1. Serum and CSF anti-U1RNP and SSA Abs detection by RNA-IPP

Auto-Abs against RBPs in serum and CSF of NPSLE patients were examined. (1) non-SLE

control without anti-RBP Abs, (2, 3) NPSLE patients with both serum and CSF anti-U1RNP

Abs, (4) NPSLE patient with both serum and CSF anti-U1RNP/Sm Abs, (5) secondary

NPSLE patient with serum anti-U1RNP Abs, (6) NPSLE patients with serum anti-SSA Abs

and (7) NPSLE patients without anti-RBP Abs.

T = total RNA, S = serum, C = CSF

Figure 2. Frequency of anti-U1RNP and SSA Abs in serum and CSF of NPSLE patients

CSF anti-U1RNP Abs are not detected in serum anti-U1RNP Ab-negative patients. CSF

anti-SSA Abs are infrequently found even in serum anti-SSA Ab-positive NPSLE patients.

Figure 3. Association between serum anti-U1RNP Ab-positivity and CSF IM levels

CSF levels of IFN-α, IL-8 and MCP-1 were compared among non-NPSLE patients without

serum anti-U1RNP Abs (control) (n = 15), NPSLE patients without serum anti-U1RNP Abs

(anti-U1RNP Ab (−), n = 34), and NPSLE patients with serum anti-U1RNP Abs

(anti-U1RNP Abs (+), n = 48).

n.s., not significant, ** $p < 0.01$ (compared with control)

Figure 4. Association between CSF anti-U1RNP Ab-positivity and CSF IM levels

(A) CSF levels of IFN-α, IL-8 and MCP-1 were compared among non-NPSLE patients

without CSF anti-U1RNP Abs (control) (n = 15), CSF anti-U1RNP Abs-negative NPSLE

patients (anti-U1RNP Abs (−), including serum anti-U1RNP Ab-negative patients, n = 67),

and NPSLE with CSF anti-U1RNP Abs (anti-U1RNP Abs (+), n = 15).

n.s., not significant, *$p < 0.05$, **$p < 0.01$ (compared with control)

(B) CSF levels of IFN-α, IL-8 and MCP-1 were compared between NPSLE patients without

(CSF −, n = 33) and with CSF anti-U1RNP Abs (CSF +, n = 15). Serum anti-U1RNP

Ab-negative patients were not included.

n.s., not significant, *$p < 0.05$ (compared with CSF anti-U1RNP Ab-negative patients)

Figure 5. Correlation of CSF anti-U1RNP Ab levels and CSF IM concentrations

Correlation of CSF anti-U1RNP Ab levels with CSF IFN-α or MCP-1 concentrations is

shown. CSF anti-U1RNP Ab levels were examined by ELISA after a 1:5 dilution.

n.s., not significant

Table 1. Anti-U1RNP Abs and NPSLE forms

		Total	Serum anti-U1RNP		CSF anti-U1RNP	
			(+)	(-)	(+)	(-)
	Patient number	82	48	34	15	67
	age at onset (years old)	35.4±14.3	35.1±15.7	38.0±14.3	30.3±13.2	37.6±15.3
	female (%)	85	92	76	87	94
	Serum anti-U1RNP (+) (%)	59	100	0	100	49
	CSF anti-U1RNP (+) (%)	18	31	0	100	0
Neurologic syndrome of the CNS	Aseptic meningitis	4	3	1	1	3
	Cerebrovascular disease	5	2	3	0	5
	Seizure disorders	6	2	4	0	6
	Myelopathy	4	3	1	2	2
	Transverse myelopathy	1	0	1	0	1
	Headache	18	12	6	5	13
	(Subtotal)	38 (46%)	22 (46%)	16 (47%)	8 (53%)	30 (45%)
Diffuse psychiatric/ neuropsychological syndrome	Mood disorder	3	2	1	0	3
	Cognitive dysfunction	7	3	4	0	7
	Psychosis	12	7	5	4	8
	Acute confusional state	2	2	0	0	2
	(Subtotal)	24 (29%)	14 (29%)	10 (29%)	4 (27%)	20 (30%)
Others	Neuropathy, cranial	9	5	4	1	8
	Movement disorder	1	0	1	0	1
	Polyneuropathy	1	1	0	1	0
	Mononeuropathy, single/multiplex	8	5	3	1	7
	Acute inflammatory polyradiculoneuropathy	1	1	0	0	1
	(Subtotal)	20 (24%)	12 (25%)	8 (24%)	3 (20%)	17 (25%)

RNP, ribonucleoprotein; NPSLE, neuropsychiatric systemic lupus erythematosus; CNS, central nervous system; CSF, cerebrospinal fluid
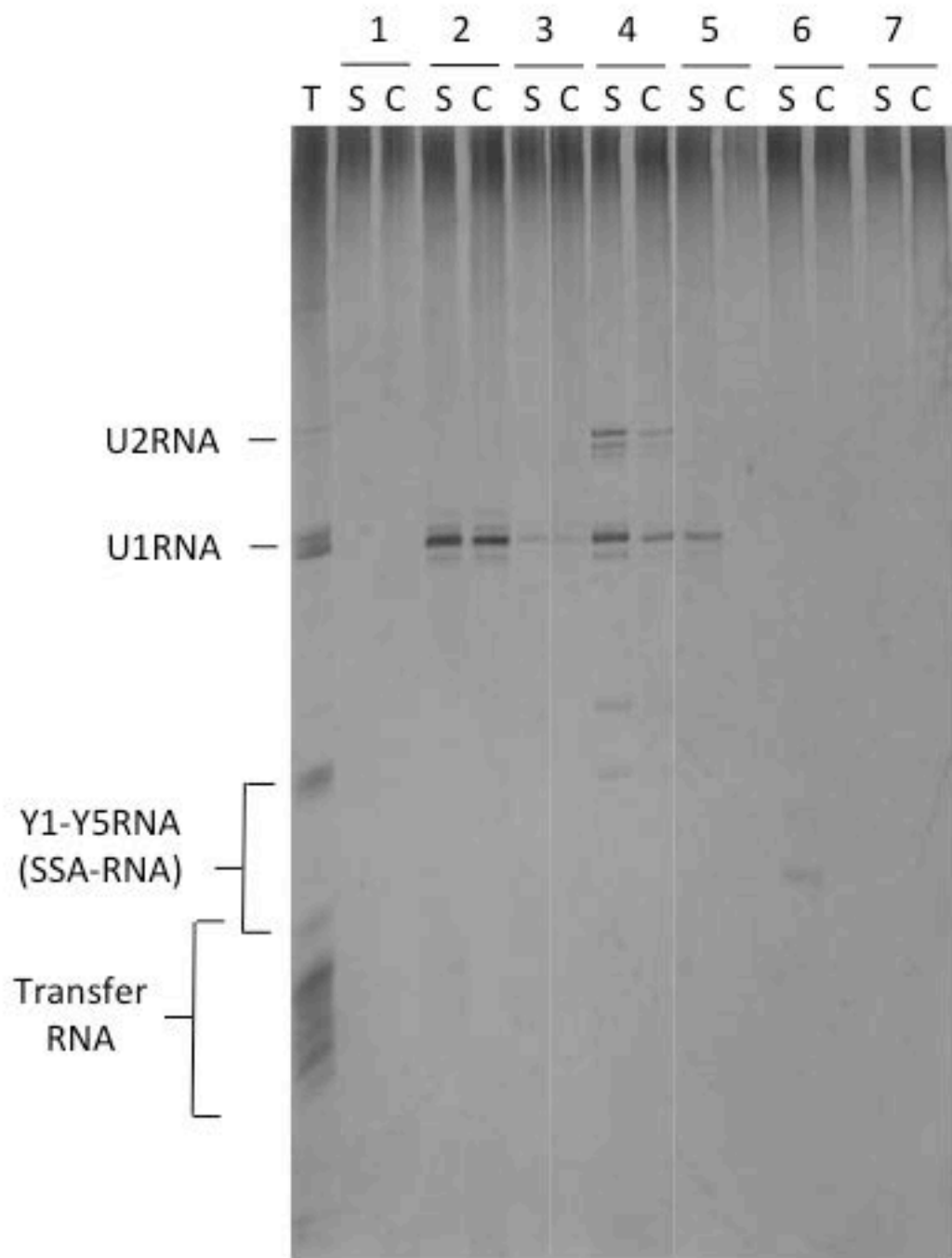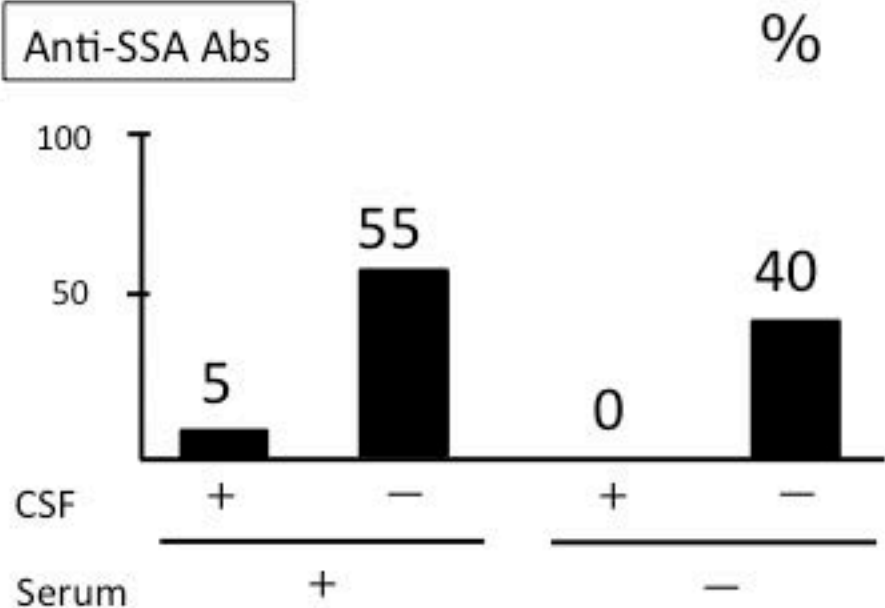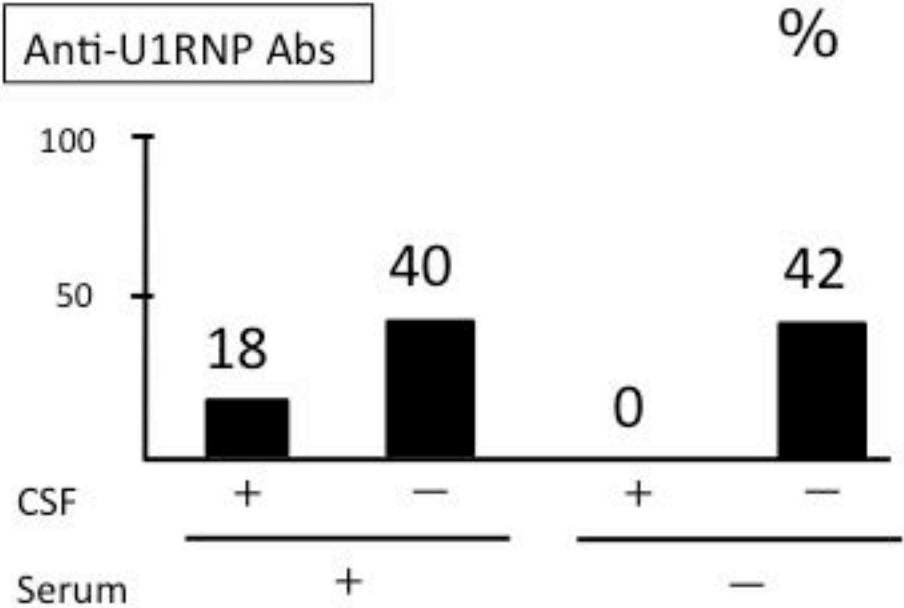
Figure 1

Figure 2

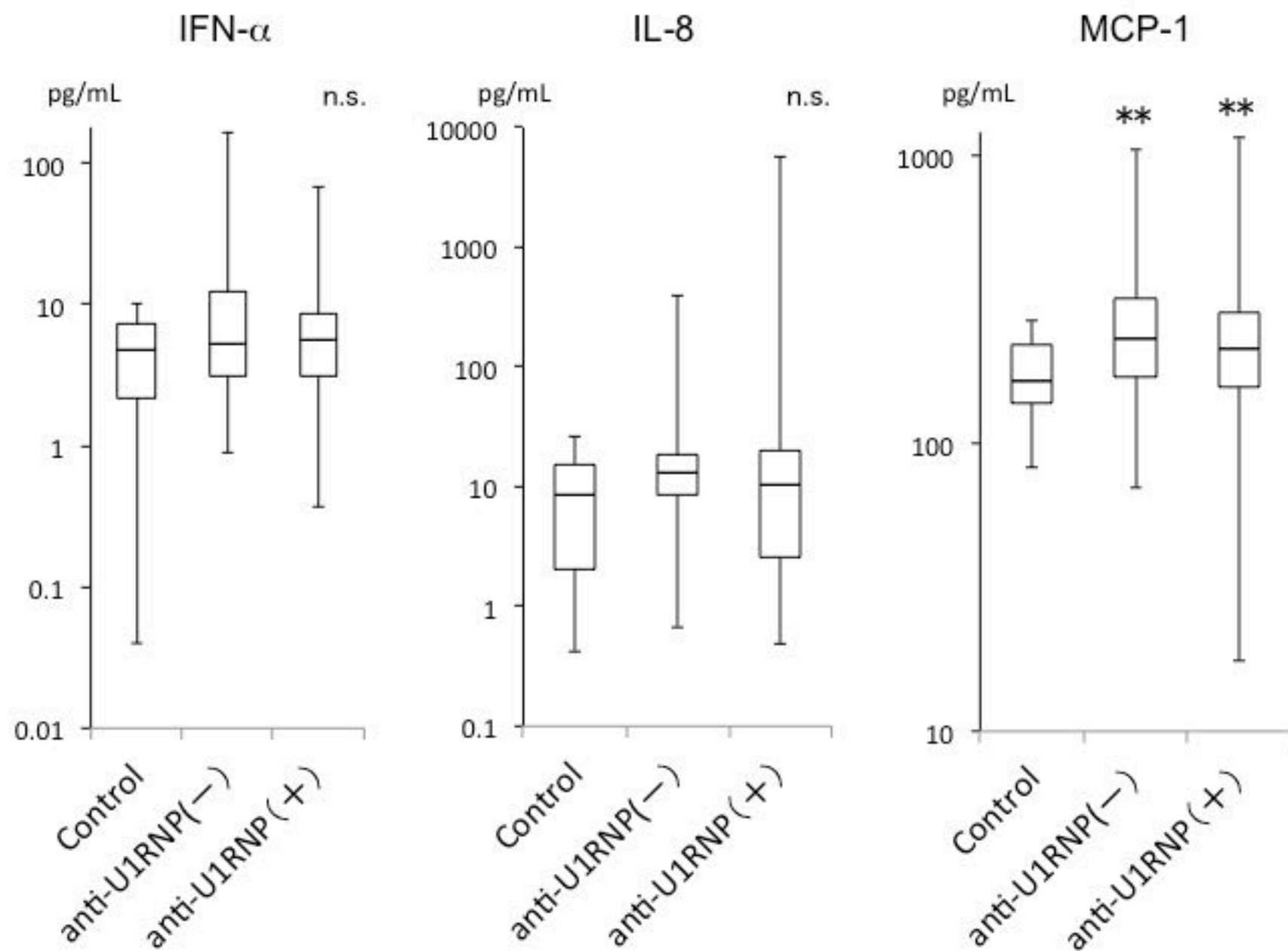**Anti-U1RNP Abs**   **%**

100
50

	18	40	0	42

CSF   +   —   +   —

Serum   +   —

Anti-SSA Abs   **%**

100
50

	5	55	0	40

CSF   +   —   +   —

Serum   +   —

Figure 3

IFN-α

IL-8

MCP-1

Figure 4

Figure 5