Primary care	Nephelometry	Dade Behring	50	ACR	Prospective	Not reported	Not reported	Yes	Yes	52	45.5	NA	Other rheumatic diseases ($n = 25$), healthy persons ($n = 50$)	30	2	2
Rheumatology clinic	Nephelometry	Dade Behring	15	ACR	Retrospective	Not reported	NA	Yes	Yes	56	86.8	13.2	Other rheumatic diseases ($n = 68$), healthy persons ($n = 60$)	77	16	5
Teaching hospital	Nephelometry	Dade Behring	Not reported	ACR	Not reported	Not reported	NA	Yes	Yes	54.8	NA	NA	Other rheumatic diseases ($n = 233$)	75	42	1
Teaching hospital	Nephelometry	Dade Behring	20	ACR	Prospective	Not reported	NA	Yes	Yes	NA	NA	NA	Other rheumatic diseases ($n = 469$)	161	89	
Teaching hospital	LA	Not reported	20	ACR	Not reported	Not reported	Not reported	Yes	No	NA	NA	NA	Progressive systemic sclerosis ($n = 32$), Wegener granulomatosis ($n = 22$)	20	32	2
Rheumatology clinic	LA	Tulip Diagnostics	8	ACR	Not reported	Not reported	Not reported	Yes	No	NA	NA	NA	Healthy persons ($n = 155$)	482	2	8
Teaching hospital	LA	Dade Behring	20	ACR	Retrospective	Not reported	Not reported	Yes	Yes			NA	OA ($n = 15$), other rheumatic diseases ($n = 10$), healthy persons ($n = 110$)	57	25	
Primary care	LA	Hitachi	9	ACR	Not reported	Not reported	Not reported	Yes	Yes	NA	NA	14.6	Other rheumatic diseases ($n = 251$)	261	54	6
Cohort study	Not reported	Not reported	Not reported	ACR	Prospective	Not reported	<12 mo	Yes	No	NA	NA	0.125	UA ($n = 98$)	56	11	4
Rheumatology clinic	LA	Mast Diagnostics	30	ACR	Prospective	Not reported	<18 mo	Yes	Yes	59.5	53.7	0.1	OA ($n = 10$), hyperlipidemia ($n = 20$), other rheumatic diseases ($n = 52$)	22	2	2
Teaching hospital	Nephelometry	Dade Behring	16.3		Prospective	Not reported	Same period	Yes	Yes	47.24	93	NA	Other rheumatic diseases ($n = 206$)	42	46	1
Rheumatology clinic	LA	Difco Laboratories	3.125	ACR	Prospective	Not reported	Same period	Yes	Yes	63.5	34.7	5	Other rheumatic diseases ($n = 146$)	93	28	2
Rheumatology clinic	Nephelometry	Dade Behring	20	ACR	Prospective	Not reported	Same period	Yes	Yes	62.9	77.9	NA	HCV infection ($n = 14$), other rheumatic diseases ($n = 28$)	32	29	
Teaching hospital	Nephelometry	Dade Behring	20	Not reported	Not reported	Not reported	Not reported	Yes	No	NA	NA	NA	Other rheumatic diseases ($n = 91$)	64	18	2
Teaching hospital	Nephelometry	Intermedico	20	ACR	Prospective	Not reported	Not reported	Yes	Yes	NA	NA	NA	UA ($n = 23$)	32	10	
Teaching hospital	Nephelometry	Dade Behring	22	ACR	Prospective	Not reported	Not reported	Yes	Yes	62.5	64.8	NA	Polymyalgia rheumatica ($n = 48$)	36	3	
Teaching hospital	Nephelometry	Dade Behring	15	ACR	Prospective	Not reported	Not reported	Yes	Yes	58.8	NA	10	HCV infection ($n = 10$)	27	6	
Teaching hospital	ELISA	Biomedical Diagnostics	20	ACR	Retrospective	Not reported	6–18 mo	Yes	No	NA	NA	NA	Other rheumatic diseases ($n = 98$), healthy persons ($n = 33$)	84	41	5
Health care centers	LA	Not reported	Not reported	Clinical diagnosis	Prospective	Yes	2 y	No	Yes	49.6	63.7	0.3	Reactive arthritis ($n = 28$), UA ($n = 10$), other arthritis ($n = 15$)	5	4	1
Teaching hospital	Nephelometry	Beckman Instruments	20	ACR	Prospective	Yes	Not reported	Yes	Yes	50.75	62	NA	Other rheumatic diseases ($n = 102$)	57	9	3
Teaching hospital	ELISA	Aesku.lab Diagnostika	15	ACR	Not reported	Not reported	Not reported	Yes	Yes	56.8	71.2	8.3	Degenerative joint disease ($n = 163$), other rheumatic diseases ($n = 103$), healthy persons ($n = 154$)	196	75	9
Cohort study	ELISA	In-house	16	ACR	Prospective	Not reported	Not reported	Yes	Yes	51.7	10.5	0.33	Other rheumatic diseases ($n = 225$)	62	11	11
Teaching hospital	ELISA	In-house	Not reported	ACR	Cross-sectional	Not reported	Not reported	Yes	Yes	62	71	NA	Other rheumatic diseases ($n = 160$), spondylo-arthropathies ($n = 79$)	143	43	5
Teaching hospital	LA	Difco Laboratories	80	ACR	Retrospective	Not reported	Not reported	Yes	Yes	47.5	79.1	NA	Other rheumatic diseases ($n = 113$), noninflammatory arthritis ($n = 23$)	73	22	2
Cohort study	ELISA	In-house	20	ACR	Nested case–control studies	Not reported	6.1 y	Yes	Yes	NA	NA	3	Healthy persons ($n = 382$)	49	23	2
Cohort study	ELISA	Not reported	Not reported	Clinical diagnosis	Prospective	Not reported	Not reported	Yes	Yes	49.4	66.6	NA	UA ($n = 157$)	35	8	5
Teaching hospital	Nephelometry	Dade Behring	15	ACR	Retrospective	Not reported	Not reported	Yes	Yes	57.18	85.2	9.4	Other rheumatic diseases ($n = 208$)	383	38	16
Rheumatology clinic	Nephelometry	Dako Diagnostics	30	Clinical diagnosis	Prospective	Not reported	Not reported	Yes	yes	57	69	NA	UA ($n = 121$)	130	8	12
Rheumatology clinic	Nephelometry	Not reported	Not reported	ACR	Prospective	Yes	Not reported	No	yes	65	89.7	NA	Other rheumatic diseases ($n = 174$), healthy persons	61	36	3

Teaching hospital	ELISA	Not reported	Not reported	ACR	Retrospective	Yes	Same period	Yes	Yes	NA	NA	NA	Other rheumatic diseases (n = 329), infectious diseases (n = 366), healthy persons (n = 120)	80	28	69
Rheumatology clinic	LA	Not reported	Not reported	ACR	Retrospective	Not reported	Not reported	Yes		NA	NA	NA	Other rheumatic diseases (n = 108), miscellaneous disorders (n = 56)	64	16	27
Teaching hospital	ELISA	In-house	Not reported	ACR	Not reported	Not reported	Not reported	Yes	Yes	NA	NA	NA	OA (n = 50), UA (n = 74), other rheumatic diseases (n = 81)	50	14	20
Rheumatology clinic	Nephelometry	Dade Behring	20	ACR	Retrospective	Not reported	Not reported	Yes		NA	NA	NA	Mixed	89	3	9
Rheumatology clinic	Rheumatoid arthritis hemaggluti-nation	Not reported	40	ACR	Prospective	Not reported	Not reported	Yes	Yes	51.1	66.6	0.66	Other arthritis (n = 21)	25	1	14
Teaching hospital	ELISA	Not reported	3	ACR	Not reported	Not reported	Not reported	Yes	Yes	42	84.5	NA	UA (n = 39)	8	8	0
Teaching hospital	LA	Pasteur Production	40	ACR	Prospective	Not reported	12–24 mo	Yes	Yes	50	75.4	0.5	UA (n = 15), other arthritis (n = 5)	20	2	29
Teaching hospital	ELISA	In-house	Not reported	Clinical diagnosis	Retrospective	Not reported	<2 mo	Yes	Yes	48	67.3	12	Mixed	157	287	78
Teaching hospital	LA	Fumouze Diagnostics	100	ACR	Prospective	Yes	Not reported	Yes		NA	NA	NA	Not reported	80	50	39
Teaching hospital	LA	Biolyon	40	ACR	Retrospective	Not reported	Not reported	Yes	Yes	51.98	59	NA	Other rheumatic diseases (n = 99)	8	8	31
Teaching hospital	LA	Not reported	Not reported	ACR	Prospective	Not reported	Not reported	Yes		NA	NA	NA	Other rheumatic diseases (n = 165), other arthritis (n = 65), healthy persons (n = 36), infectious mononucleosis (n = 10)	143	39	63
Teaching hospital	ELISA	Cogent Diagnostics	Not reported	ACR	Not reported	Not reported	Not reported	Yes		NA	NA	NA	Other rheumatic diseases (n = 100)	48	1	40
Teaching hospital	ELISA	In-house	Not reported	ACR	Not reported	Not reported	Not reported	Yes	Yes	NA	NA	NA	Healthy persons (n = 200), cancer (n = 30), infectious diseases (n = 56), other rheumatic diseases (n = 29)	36	6	41
Teaching hospital	ELISA	In-house	87	Not reported	Not reported	Not reported	Not reported	Yes		NA	NA	NA	OA (n = 56), healthy persons (n = 76)	60	8	20
Teaching hospital	ELISA	Dade MicroScan	Not reported	ACR	Prospective	Not reported	Same period	Yes		NA	70	NA	Other rheumatic diseases (n = 55)	18	3	31
Rheumatology clinic	LA	Polysciences	Not reported	Not reported	Not reported	Not reported	Not reported	Yes		NA	NA	NA	Not reported	113	19	29
Teaching hospital	ELISA	In-house	Not reported	Not reported	Not reported	Not reported	Not reported	Yes		NA	NA	NA	Not reported	163	10	28

LISA = enzyme-linked immunosorbent assay; FN = false negative; FP = false positive; HCV = hepatitis C virus; LA = latex agglutination; LR = likelihood ratio; NA = not available; OA = osteoarthritis; RF = rheumatoid factor; TN = true negative; TP = true positive; UA
h Diagnostika (Wendelsheim, Germany), Beckman Instruments (Fullerton, California), Biolyon (Lyon, France), Biomedical Diagnostics (Marne-la-Vallée, France), Cogent Diagnostics (Penicuik, United Kingdom), Dade Behring (Marburg, Germany), Dade MicroScan (West Sacr
ostics (Asnières, France), Hitachi (Tokyo, Japan), Intermedico (Markham, Ontario, Canada), Mast Diagnostics (Bootle, United Kingdom), Pasteur Production (Paris, France), Polysciences (Northampton, United Kingdom), Tulip Diagnostics (Goa, India).

	cohort	study		other ACR criteria							
;lish	Population-based cohort	Retrospective cohort study	79 blood donors with RA	Cumulative percentage of positive test results before onset of symptoms	7.5 y	15 (maximum)	79/79	Not reported	51.4	62	ACR
;lish	Population-based cohort	Prospective cohort study	69 patients with RA	PPV for rheumatoid factor	3 mo	2	69/69	Not reported	49.6	63.7	Clinical judgment
;lish	Population-based cohort	Nested case–control studies	83 blood donors with RA adjusted in multivariate logistic regression	OR for RA	3	10.9	Not reported	Not reported	Not available	Not available	ACR
;lish	Primary care	184 patients with UA	Prediction of RA	<1	2.5 (median)	Not reported	Not reported	49.5	68.9	ACR	Prospective cohort study

KA = antikeratin antibody; CCP = cyclic citrullinated peptide; ELISA = enzyme-linked immunosorbent assay; OR = odds ratio; PPV = positive predictive value; RA = rheumatoid arthritis; RF = rheumatoid factor; UA = undifferentiated arthritis.

	cohort study	study								
English	Population-based cohort study	Prospective cohort study	Sharp–van der Heijde score	4.3	5	99/99	MTX (n = 38), sulfasalazine (n = 31), both MTX and sulfasalazine (n = 27), corticosteroids (n = 33)	50	73	ACR
English	Teaching hospital	Prospective cohort study	Sharp–van der Heijde score	<1	10	114/130	DMARDs (95%), MTX (35%), sulfasalazine (47%), bucillamine (13%), gold (7%), auranofin (2%)	54	69	ACR
English	Rheumatology clinic		Larsen erosive scores at 3 y	<2	3	866/866	Followed UK guidelines for RA	NA	64	ACR
English	Teaching hospital	Prospective cohort study	Sharp–van der Heijde score	<1	2	94/111	Followed the algorithm created by the authors	51.5	70.3	ACR
English	Population-based cohort study	Prospective cohort study	Larsen score	<1	2	333/379	DMARDs (66%), MTX (36%), sulfasalazine (51%)	55	65	ACR
English	Teaching hospital	Prospective cohort study	Progression of total Sharp score Progression of erosion Sharp score Progression of total Sharp score Progression of erosion Sharp score	<1	5	156/191	DMARDs or NSAIDs (100%)	50.5	73	ACR
English		Prospective cohort study	Larsen score progression >10 vs. <10 Larsen score progression >10 vs. <10 Larsen score progression >10 vs. <10 Larsen score progression >10 vs. <10	<2	2	104/104	Not reported	NA	NA	ACR
English	Population-based cohort study	Prospective cohort study	Difference of Sharp–van der Heijde score from multiple logistic regression	0.25	1	114/130	Not reported	64	68	ACR
English	Rheumatology clinic	Prospective cohort study	Larsen score progression >20	<1	3	63/63	Gold (83%), sulfasalazine (12%), hydroxychloroquine (5%)	43.5	0.83	Clinical judgment
English	Rheumatology clinic	Prospective cohort study	Larsen score progression/y	Not reported	12 (by exploration of linear regression model)	Not reported	Not reported	59	71.6	ACR
English	Teaching hospital	Prospective cohort study	Sharp–van der Heijde score	<1	6	Not reported	Not reported	51.5	65.9	ACR
English	Teaching hospital	Prospective cohort study	Modified Sharp score	<1	3	Not reported	Not reported	NA	NA	ACR
English	Teaching hospital	Retrospective cohort study	Probability of predicting erosion (Larsen score ≥ grade 2)	3	1	175/175	Not reported	59	71	ACR
English	Teaching hospital	Prospective cohort study	Physician opinion	1.6	6 (median)	Not reported	Not reported	NA	NA	ACR

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Specificity of enzyme-linked immunosorbent assay for IgG anti-NR2 glutamate receptor antibodies: comment on the concise communication by Yoshio et al

To the Editor:

We read with interest the concise communication by Yoshio et al (1) describing an association of IgG anti-NR2 glutamate receptor antibodies in cerebrospinal fluid (CSF) with neuropsychiatric systemic lupus erythematosus (NPSLE). DeGiorgio et al first demonstrated that IgG anti-NR2 glutamate receptor antibodies caused neuronal death in mice, when injected into the brain (2). They also reported the presence of these antibodies in CSF from an SLE patient with progressive cognitive decline (2). It was further demonstrated by investigators in that group that the presence of IgG anti-NR2 glutamate receptor antibodies within the brain resulted in cognitive decline in mice (3). These findings suggested that these antibodies might cause diffuse psychiatric/neurologic syndromes in human SLE. However, Yoshio and colleagues found that, compared with lupus patients without NPSLE, CSF IgG anti-NR2 glutamate receptor antibody levels were increased in lupus patients with neurologic syndromes of the central nervous system, but not in those with diffuse psychiatric/neuropsychological syndromes alone (1).

To measure IgG anti-NR2 glutamate receptor antibodies in sera and CSF, Yoshio et al used an enzyme-linked immunosorbent assay (ELISA) with the synthetic DWEYSVWLSN peptide conjugated to bovine serum albumin (BSA) as antigen (1). Our group previously demonstrated that sera from SLE patients frequently express antibodies to human serum albumin (HSA), BSA, and ovalbumin (4). Therefore, subtracting binding activity to these carrier proteins would be mandatory in order to determine specific activities of antibodies to peptides conjugated to carrier proteins, including BSA and HSA (4). However, Yoshio and colleagues did not subtract

Table 1. Measurement of IgG anti-NR2 glutamate receptor antibodies (optical density at 492 nm) using enzyme-linked immunosorbent assay*

Sample	CSF or serum	Maxisorp		Pro-Bind	
		HSA–NR2	HSA	HSA–NR2	HSA
1	CSF	0.146	0.065	0.090	0.067
2	CSF	0.223	0.068	0.147	0.124
3	CSF	0.206	0.278	0.053	0.090
4	CSF	0.428	0.521	0.050	0.060
5	CSF	0.974	0.480	0.088	0.082
6	CSF	0.205	0.323	0.224	0.280
7	CSF	0.248	0.139	0.259	0.249
8	CSF	0.044	0.135	0.101	0.139
9	CSF	0.332	0.137	0.030	0.020
10	Serum	0.628	0.686	0.094	0.116
11	Serum	0.434	0.795	0.100	0.180
12	Serum	0.272	0.093	0.050	0.044
13	Serum	0.181	0.209	0.082	0.099
14	Serum	0.333	0.339	0.057	0.221
15	Serum	0.280	0.139	0.051	0.050
16	Serum	0.878	0.464	0.616	0.522

* Cerebrospinal fluid (CSF) or serum samples from systemic lupus erythematosus patients were assayed for IgG anti-NR2 glutamate receptor antibodies by enzyme-linked immunosorbent assay as described in the text. Peroxidase-conjugated $F(ab')_2$ goat anti-human IgG was used at 1:10,000 (Maxisorp plates) and at 1:5,000 (Pro-Bind plates). HSA–NR2 = human serum albumin conjugated with synthetic DWEYSVWLSN peptide.

BSA binding activities. It appears that all sera with anti-HSA also contain antibodies to BSA (4). We therefore reexamined whether CSF and sera from SLE patients contain antibodies to HSA, using the method described by Yoshio et al.

Wells of a 96-well microtiter plate (Falcon Pro-Bind; Becton Dickinson, Lincoln Park, NJ or Nunc-immuno module F8 Maxisorp; Nunc, Roskilde, Denmark) were coated with HSA (Miles, Elkhart, IN) or HSA conjugated (at a 1:1 weight ratio) with highly purified synthetic DWEYSVWLSN peptide (purity >95%) (HSA–NR2 peptide), at 20 μg/ml in phosphate buffered saline (PBS), overnight at 4°C. The wells were blocked with Block Ace (Dainippon, Osaka, Japan) for Falcon Pro-Bind plates or with PBS containing 1% BSA (Miles) for Nunc Maxisorp plates, for 2 hours at room temperature. Before being added to the wells, serum and CSF samples were diluted 1:200 and 1:2, respectively, in PBS containing 1% BSA. After incubation at 37°C for 1 hour, bound IgG anti-NR2 glutamate receptor antibody was detected with peroxidase-conjugated $F(ab')_2$ fragments of goat anti-human IgG (Cappel, Cochranville, PA). Binding activity was expressed as optical density at 492 nm (OD_{492}) as measured in a 2-wavelength microplate photometer (MTP-450; Corona Electric, Ibaraki, Japan).

As seen in Table 1, all 16 samples exhibited positive binding to HSA–NR2 peptide in Falcon Pro-Bind plates (Yoshio and colleagues' method) as well as in Nunc Maxisorp plates. However, 8 of the 16 samples showed higher binding activity (OD_{492}) to HSA alone than to HSA–NR2, indicating that those samples would yield false-positive results for IgG anti-NR2 glutamate receptor antibodies unless nonspecific binding to HSA alone were subtracted. In addition, the levels of binding activity obtained with Falcon Pro-Bind plates were ~20–30% of those with Nunc Maxisorp plates, even though peroxidase-conjugated $F(ab')_2$ goat anti-human IgG was used at 1:5,000 in the former plates and at 1:10,000 in the latter. Therefore, it would be preferable to use Nunc Maxicorp plates. Nonetheless, these results confirm that ~50% of serum and CSF samples contain antibodies to HSA (and presumably to BSA as well, since it was previously shown that all sera positive for anti-HSA contained antibodies to BSA [4]).

These findings raise serious concern about the specificity of the ELISA used by Yoshio et al. It is highly likely that the presence of anti-BSA antibodies would have significantly influenced their results and conclusions. Therefore, their conclusion that IgG anti-NR2 glutamate receptor antibodies in CSF may cause focal neurologic damage such as seizure disorders, aseptic meningitis, and transverse myelopathy is not supported.

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Research article

Association of cerebrospinal fluid anti-ribosomal P protein antibodies with diffuse psychiatric/neuropsychological syndromes in systemic lupus erythematosus

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Abstract

We explored the relationship of antibodies to the whole ribosomal P proteins (P0, P1, and P2) in cerebrospinal fluid (CSF) with diffuse psychiatric/neuropsychological syndromes in systemic lupus erythematosus (SLE). CSF samples were obtained from 71 SLE patients (52 patients with diffuse psychiatric/neuropsychological syndromes [diffuse NP-SLE] and 19 patients with neurological syndromes or peripheral neuropathy [focal NP-SLE]) as well as from 24 patients with non-inflammatory neurological disease. Immunoglobulin G (IgG) antibodies to the C-terminal 22-amino acid ribosomal P synthetic peptide (anti-P_{C22}) and those to purified bovine ribosomal P proteins (P0, P1, and P2) (anti-whole P) were determined by enzyme-linked immunosorbent assay; affinity-purified IgG anti-P_{C22} were used as the standard. The concentrations of antibodies to epitopes other than the C-terminal 22 amino acids of ribosomal P proteins were calculated by subtracting anti-P_{C22} from anti-whole P (anti-$P_{EX.C22}$). CSF anti-whole P levels were significantly elevated in diffuse NP-SLE compared with focal NP-SLE or control patients. By contrast, there were no significant differences in CSF anti-P_{C22} levels among the three groups. Of note, CSF anti-$P_{EX.C22}$ levels were significantly elevated in diffuse NP-SLE compared with the other two groups. CSF anti-$P_{EX.C22}$ levels were not significantly correlated with CSF anti-P_{C22} levels, but with CSF antibodies against the recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids (C22-depleted rP0). Moreover, levels of CSF anti-$P_{EX.C22}$ or CSF anti-C22-depleted rP0, but not CSF anti-P_{C22}, were significantly correlated with CSF anti-neuronal cell antibodies (anti-N). These results indicate that CSF IgG antibodies to the epitopes other than the C-terminal 22 amino acids of ribosomal P proteins, which might contain one of the major targets of CSF anti-N, are associated with the development of diffuse NP-SLE.

Introduction

Central nervous system (CNS) involvement is a relatively common and serious complication of systemic lupus erythematosus (SLE) [1,2]. Previous studies have demonstrated the association of serum antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein (anti-P_{C22}) with CNS involvement in patients with SLE (neuropsychiatric SLE [NP-SLE]), especially diffuse psychiatric/neuropsychological syndromes (diffuse NP-SLE) [3-5]. However, the mechanism by which serum anti-P_{C22} leads to the development of diffuse NP-SLE has not yet been elucidated. In fact, the role of anti-P_{C22} in the cerebrospinal fluid (CSF) in the

ACR = American College of Rheumatology; anti-C22-depleted rP0 = antibodies directed against recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids; anti-N = anti-neuronal cell antibodies; anti-P_{C22} = antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein; anti-$P_{EX.C22}$ = autoantibodies directed against the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence; anti-whole P = antibodies to the whole ribosomal P proteins; C22-depleted rP0 = recombinant ribosomal P0 fusion protein lacking the C-terminal 22 amino acids; CNS = central nervous system; CSF = cerebrospinal fluid; ELISA = enzyme-linked immunosorbent assay; HSA = human serum albumin; IgG = immunoglobulin G; IL-6 = interleukin-6; NMDA = *N*-methyl-d-aspartate; non-CNS SLE = systemic lupus erythematosus without neuropsychiatric manifestations; NP-SLE = neuropsychiatric systemic lupus erythematosus; OD_{492} = optical density at 492 nm; PBS = phosphate-buffered saline; SLE = systemic lupus erythematosus.

pathogenesis with diffuse NP-SLE or even their presence in the CSF remains uncertain. Thus, Golombek and colleagues [6] detected the presence of CSF anti-P_{C22} in all four of the patients with lupus psychosis in their studies, whereas others did not [3,4,7].

On the other hand, autoantibodies, which react with the neuronal cell lines or brain tissue, have been reported in the sera of patients with NP-SLE [8-10]. However, they have been shown to be present in SLE patients with no clinical evidence of CNS involvement [10]. In fact, in a cross-sectional study of SLE patients, no significant association was found between serum lymphocyte/brain cross-reacting antibodies and NP-SLE (present in 32% of cases with NP-SLE and 23% of those without NP-SLE) [10]. Of note, using a radioimmunoassay with the SK-N-SH neuroblastoma cell as a target, Bluestein and colleagues [11] demonstrated that immunoglobulin G (IgG) anti-neuronal cell antibodies (anti-N) were present in much higher concentrations in the CSF from patients with active NP-SLE than in the CSF from SLE patients without active CNS involvement. Using a cell enzyme-linked immunosorbent assay (ELISA) with SK-N-MC neuroblastoma cell lines fixed with paraformaldehyde, we also confirmed that CSF IgG anti-N levels were significantly elevated in patients with diffuse NP-SLE compared with those in SLE patients without diffuse NP-SLE [7]. However, the fine epitopes to which CSF anti-N were directed have not yet been delineated.

The presence of the immunodominant C-terminal epitope of ribosomal P proteins was demonstrated to be present on the surface of human neuroblastoma cells [12]. However, CSF anti-P_{C22} could be detected in only a fraction of patients with diffuse NP-SLE, whereas almost all the patients with diffuse NP-SLE expressed CSF anti-N [7]. Of note, previous studies also demonstrated the presence of a 38-kDa protein that is closely related to, or identical with, ribosomal P0 protein in purified human plasma membranes [12]. In addition, it has been shown that autoantibodies directed against the ribosomal P proteins are not only directed against the common C-terminal 22 amino acids, but against the N-terminal sequence of the ribosomal P2 or P1 proteins [13]. In fact, recent studies have revealed that measurement of CSF IgG anti-ribosomal P protein antibodies with Western blotting using purified ribosomes, containing whole ribosomal P0, P1, and P2 proteins, was more sensitive [14]. Because ribosomal P0 protein contains epitopes other than the C-terminal 22 amino acids, it is possible that CSF from patients with diffuse NP-SLE contains antibodies to such epitopes. The current studies, therefore, were carried out to compare the CSF levels of antibodies to the whole ribosomal P proteins (anti-whole P) in patients with diffuse NP-SLE and in patients with focal NP-SLE or non-SLE non-inflammatory neurological disorders.

Materials and methods

Patients and samples

One hundred and three patients with SLE were included in the present study. All patients fulfilled the American College of Rheumatology (ACR) 1982 revised criteria for the classification of SLE [15]. Of the 103 patients with SLE, 52 showed diffuse psychiatric/neurological syndromes (diffuse NP-SLE) according to the 1999 ACR definition of NP-SLE [16], 19 patients showed CNS manifestations other than diffuse NP-SLE (focal NP-SLE), and 32 patients showed no CNS manifestations (non-CNS SLE). Ten of the 52 patients with diffuse NP-SLE also presented seizures. Because of the difficulties in confirming the neurological diagnosis and in assigning the cause to SLE, we defined NP-SLE as (a) the presence of neuropsychiatric manifestations and (b) the elevation of CSF Ig indices [17,18] and/or the elevation of CSF interleukin-6 (IL-6) levels [19]. Thus, the 52 patients all showed increased CSF Ig indices and/or CSF IL-6 in the present study. In addition, 24 patients with non-SLE non-inflammatory neurological diseases (9 cerebrovascular diseases, 8 cervical spondylosis, 4 degenerative diseases, 2 diabetic neuropathy, and 1 epilepsy) were studied as a control. The 127 patients all gave informed consent, and the study was approved by the institutional ethical committee of Teikyo University School of Medicine (Tokyo). The detail and demographic features of the 127 patients are shown in Table 1. CSF specimens were obtained by a lumbar puncture when the patients showed active disease. These samples were kept frozen at -20°C until assayed. All assays were performed without knowledge of the diagnosis or clinical presentations.

Human anti-P_{C22} sera and affinity purification of anti-P_{C22}

IgG fractions were purified from the anti-P_{C22}-positive sera of SLE patients by means of a protein G-Sepharose 4FF column (Amersham Pharmacia Biotech, now part of GE Healthcare, Little Chalfont, Buckinghamshire, UK). Anti-P_{C22} were purified from the IgG fractions of SLE sera by means of an *N*-hydroxysuccinimide-activated Sepharose HP column (GE Healthcare) coupled with synthetic ribosomal P peptide-human serum albumin (HSA) conjugates as previously described [20]. Anti-P_{C22} thus purified reacted strongly with ribosomal P peptide-HSA conjugates, but not with HSA alone in an ELISA. It was also confirmed on Western blot analysis that purified anti-P_{C22} reacted with native ribosomal P proteins (P0, P1, and P2) (data not shown).

Measurement of autoantibodies to ribosomal P proteins

Antibodies for the C-terminal 22-amino acid ribosomal P synthetic peptide (anti-P_{C22}) in sera and CSF and those for purified whole ribosomal P proteins (anti-whole P) in CSF were determined by specific ELISA using the highly purified synthetic C-terminal 22-amino acid ribosomal P peptide conjugated to HSA as an antigen as previously described [5] and highly purified bovine ribosomal P proteins (P0, P1, and P2) (purity of more than 90%) (Arotec Diagnostics Limited, Wel-

Table 1

Profiles of the patients studied

Diagnosis	Number of patients	Gender (male/female)	Age in years (mean ± SD)
SLE	103		
Diffuse NP-SLE	52	4/48	37.8 ± 14.2
Acute confusional state	20		
Anxiety disorder	3		
Cognitive dysfunction	10[a]		
Mood disorder	12[b]		
Psychosis	7		
Focal NP-SLE	19	2/17	39.0 ± 14.8
Cerebrovascular disease	6		
Headache	2		
Movement disorder	1		
Seizure disorder	6		
Polyneuropathy	4		
Non-CNS SLE	32	3/29	42.7 ± 13.9
Non-SLE control	24	22/2	48.0 ± 13.7

[a]One patient also presented mood disorder. [b]One patient also presented cognitive dysfunction. Non-CNS SLE, systemic lupus erythematosus without neuropsychiatric manifestations; NP-SLE, neuropsychiatric systemic lupus erythematosus; SD, standard deviation; SLE, systemic lupus erythematosus.

lington, New Zealand). Antibodies for the epitope representing regions of the ribosomal P proteins other than P_{C22} were similarly determined by ELISA using recombinant ribosomal P0 fusion protein lacking the C-terminal 22 amino acids (C22-depleted rP0) as previously described [21].

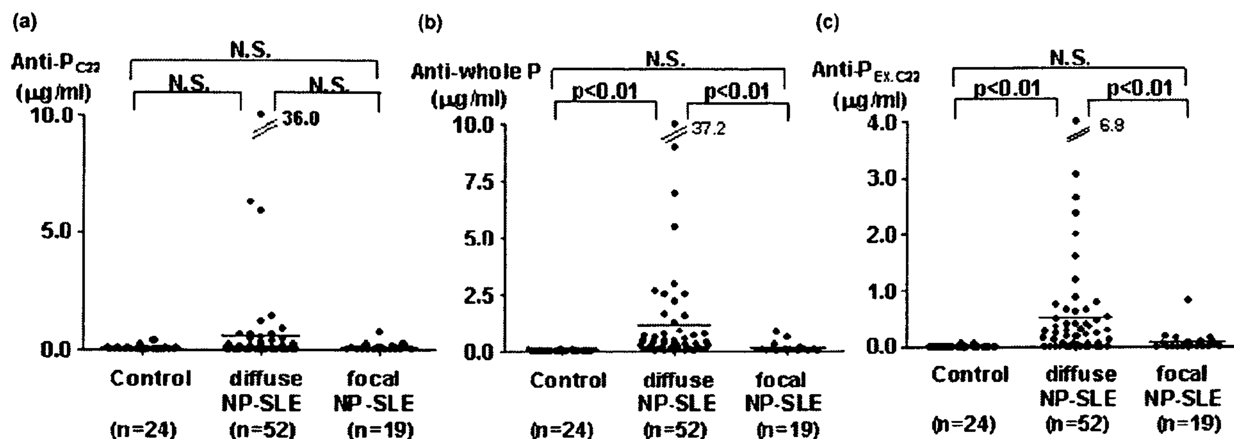
Briefly, wells of a 96-well microtiter plate were coated with ribosomal P peptide-HSA conjugates at 15 μg/ml or highly purified bovine ribosomal P proteins at 1.0 μg/ml in phosphate-buffered saline (PBS) (pH 7.2) or C22-depleted rP0 at 5 μg/ml in 6 M urea/10 mM Tris-HCl (pH 7.5) with 2 mM 2-mercaptoethanol (coating buffer) at 4°C overnight. Each well was then overcoated with Block Ace (Dainippon Pharmaceutical, Osaka, Japan), diluted 1:4 with PBS. Prior to being added to the antigen-coated wells, serum and CSF samples were usually diluted 1:200 and 1:2, respectively, in PBS containing 1% bovine serum albumin (Miles, now part of Bayer Corp., Emeryville, CA, USA). Bound antibody was detected with peroxidase-conjugated F $(ab')_2$ fragments of goat anti-human IgG (MP Biochemicals, Solon, OH, USA). After incubation with substrate solution containing 60 mg of *o*-phenylenediamine and 10 μl of 30% H_2O_2 in 100 ml of 0.05 M citrate phosphate buffer (pH 4.8) at 37°C for 30 minutes, the reaction was stopped by addition of 5 N H_2SO_4, and the absorbance (optical density) at 492 nm (OD_{492}) was read with a two-wavelength microplate photometer (MTP-120; Corona Electric Co., Ltd., Ibaraki, Japan). Determinations of OD_{492} were normalized to affinity-purified anti-P_{C22} such that anti-P_{C22} and anti-whole P activity might be converted to micrograms per milliliter of IgG. Antibodies directed against C22-depleted rP0 (anti-C22-depleted rP0) were expressed by arbitrary unit designation using a standard serum.

Non-specific binding activities to HSA for anti-P_{C22} or those to wells with PBS alone or coating buffer alone for anti-whole P or anti-C22-depleted rP0 were also determined in reference to the standard curves for binding activities to ribosomal P peptide (P_{C22})-HSA conjugates, highly purified ribosomal P proteins, or C22-depleted rP0. The specific anti-P_{C22}, anti-whole P, or anti-C22-depleted rP0 activities were thus determined by subtracting the values for the non-specific binding activity from those for binding activity to P_{C22}-HSA conjugates or to highly purified ribosomal P proteins or C22-depleted rP0. The intra-assay and interassay variances (coefficient of variation values) for anti-whole P were 13.8% and 15.7%, respectively, and those for anti-P_{C22} were previously described [7].

Measurement of anti-N

Anti-N in the CSF samples were determined by a cell ELISA using human neuroblastoma cell line SK-N-MC as previously

Figure 1



Cerebrospinal fluid antibodies to various components of ribosomal P proteins. CSF antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}), highly purified ribosomal P proteins (anti-whole P), and epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$). Anti-P_{C22} **(a)**, anti-whole P **(b)**, and anti-$P_{EX.C22}$ **(c)** in CSF from patients with non-inflammatory neurological diseases (Control), with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE), or with focal NP-SLE were compared. Horizontal lines indicate the mean values. Statistical analysis was performed by Kruskal-Wallis test with multiple comparisons (Scheffé's method). CSF, cerebrospinal fluid; N.S., not significant.

described [7]. Briefly, SK-N-MC cells were seeded at a density of 5 × 10^4 per well in wells of a flat-bottomed 96-well tissue culture plate (no. 3596; Costar, now part of Corning Life Sciences, Acton, MA, USA) for 48 hours, after which the cells were fixed with 1% paraformaldehyde in PBS for 5 minutes at 37°C. After three washes with PBS containing 0.05% Tween 20, 50 µl of the appropriately diluted samples or various concentrations of standard sera were added and the plates were incubated for 1 hour at 37°C. Bound IgG anti-N were detected with peroxidase-conjugated $F(ab')_2$ fragments of goat anti-human IgG as previously described [7]. Determination of OD_{492} was normalized to standard sera for anti-N obtained from patients with diffuse NP-SLE such that anti-N activity might be converted to an arbitrary unit scale. The concentration of anti-N that produced half of the maximal absorbance at 492 nm, given by the saturating concentration of anti-N in the cell ELISA plate, was arbitrarily defined as 1 U/ml [7].

Statistical analysis

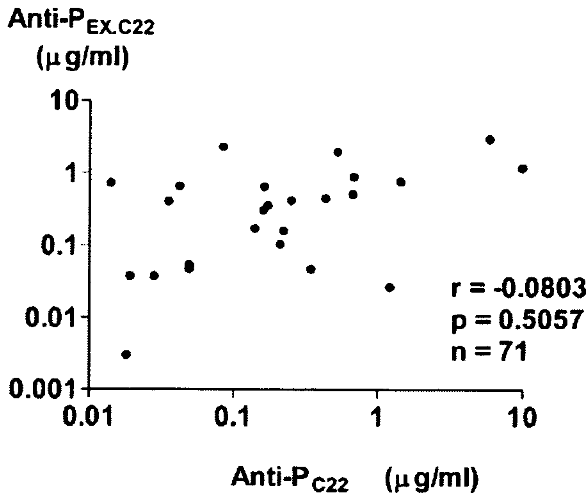
Differences in CSF anti-P_{C22}, anti-whole P, anti-$P_{EX.C22}$, and anti-C22-depleted rP0 among various groups were analyzed by Kruskal-Wallis test with multiple comparison (Scheffe's method). The correlation of anti-P_{C22} levels with anti-$P_{EX.C22}$ or anti-C22-depleted rP0 levels and the correlation of anti-N levels with anti-P_{C22}, anti-$P_{EX.C22}$, or anti-C22-depleted rP0 levels were evaluated by Spearman rank correlation test. Differences in serum anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ levels between non-CNS SLE and NP-SLE were analyzed by Welch's *t* test.

Results

Initial experiments examined CSF anti-P_{C22} levels in the three groups of patients. Although anti-P_{C22} levels in CSF appeared to be higher in diffuse NP-SLE, there were no significant differences in their levels among the three groups, including diffuse NP-SLE, focal NP-SLE, and non-inflammatory neurological control (Figure 1a). The results therefore confirm the previous observation that CSF anti-P_{C22} might not be prevalent in diffuse NP-SLE. By contrast, anti-whole P levels in CSF from patients with diffuse NP-SLE were significantly elevated compared with those from patients with focal NP-SLE or with non-inflammatory neurological diseases (Figure 1b). In addition, it should be noted that CSF anti-whole P levels were significantly higher than CSF anti-P_{C22} levels in 67 patients with diffuse NP-SLE and focal NP-SLE ($P < 0.0001$ as evaluated by Wilcoxon signed rank test). These results suggest that in addition to anti-P_{C22}, CSF from patients with NP-SLE might contain autoantibodies that recognize ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence.

To explore in detail the prevalence of the autoantibodies directed against the ribosomal P protein, epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) were calculated by subtracting anti-P_{C22} from anti-whole P. As can be seen in Figure 1c, anti-$P_{EX.C22}$ levels in CSF from patients with diffuse NP-SLE were significantly elevated compared with those from patients with focal NP-SLE or with non-inflammatory neurological diseases. As shown in Figure 2, there was no significant correlation between CSF anti-P_{C22} and CSF anti-$P_{EX.C22}$ levels, obviating the possibility that CSF anti-$P_{EX.C22}$ activities might result from contamination of CSF anti-P_{C22} in patients with SLE. These results indicate that autoantibodies directed against ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence are strongly associated with the development of diffuse NP-SLE. Moreover, the data indicate that the expression of such

Figure 2



Correlation between autoantibodies to various components of ribosomal P proteins. The correlation between antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}) and those to the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) in cerebrospinal fluid from patients with systemic lupus erythematosus (SLE), including 52 patients with diffuse neuropsychiatric SLE (NP-SLE) and 19 patients with focal NP-SLE, was analyzed. Statistical analysis was performed by Spearman rank correlation test.

autoantibodies in CSF is not related to the presence of anti-P_{C22} in CSF.

To confirm the presence of autoantibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence, IgG antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids (C22-depleted rP0) were examined in CSF from 65 SLE patients with neuropsychiatric manifestations. Affinity-purified anti-P_{C22} reacted with ribosomal P peptide-HSA conjugates, but not with C22-depleted rP0, confirming the lack of the C-terminal 22-amino acid sequence in the C22-depleted rP0 (Figure 3). As shown in Figure 4, CSF anti-C22-depleted rP0 levels were significantly correlated with CSF anti-$P_{EX.C22}$ levels in these 65 patients. In addition, anti-C22-depleted rP0 levels in CSF from patients with diffuse NP-SLE were significantly elevated compared with those from patients with focal NP-SLE or with non-inflammatory neurological diseases (Figure 5). Accordingly, the frequency of positive expression of anti-C22-depleted rP0 in CSF from patients with diffuse NP-SLE was higher than that in CSF from patients with focal NP-SLE or with non-inflammatory neurological diseases (Table 2). These results confirm the presence of autoantibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence.

We next examined whether CSF anti-whole P might account for anti-N activities in CSF from patients with NP-SLE. As shown in Table 3, levels of CSF anti-whole P and anti-PC22 as well as CSF anti-N were decreased when CSF was incubated with paraformaldehyde-fixed SK-N-MC cells for 120 minutes at room temperature, confirming that CSF anti-whole P or anti-PC22 are constituents of CSF anti-N. However, as shown in Figure 6a, CSF anti-N levels were not significantly correlated with CSF anti-PC22 levels in SLE patients, including those with diffuse NP-SLE and focal NP-SLE. By contrast, CSF anti-N levels were significantly correlated with CSF anti-PEX.C22 or CSF anti-C22-depleted rP0 levels (Figure 6b,c

Finally, we examined serum levels of anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ in patients with non-CNS SLE or with NP-SLE. The values of anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ in 24 patients with non-SLE non-inflammatory neurological diseases were 2.44 ± 2.92 μg/ml, 4.92 ± 6.51 μg/ml, and 3.41 ± 6.06 μg/ml (mean ± standard deviation), respectively. As shown in Figure 7, serum anti-P_{C22} as well as anti-whole P levels in NP-SLE were significantly elevated compared with those in non-CNS SLE, which is consistent with previous studies [3-5]. Serum anti-P_{C22} and anti-whole P levels appeared to be higher in diffuse NP-SLE than those in focal NP-SLE, although

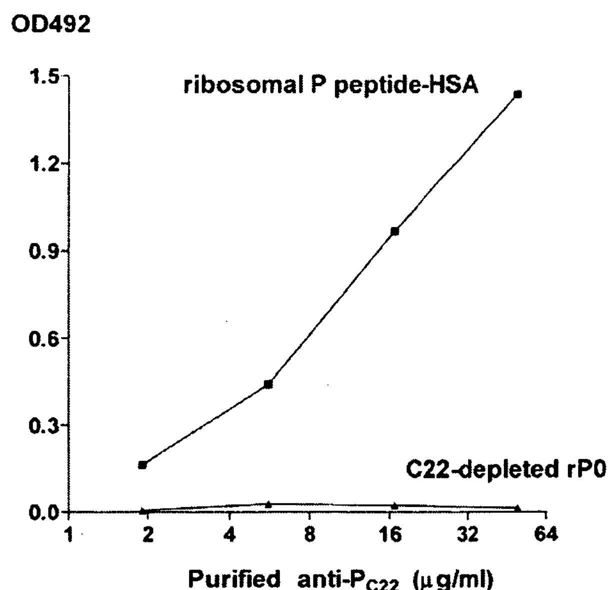
Table 2

Summary of the frequency of positive expression of antibodies to various ribosomal P protein components in cerebrospinal fluid[a]

	Percentage positive[b]		
	Control	Diffuse NP-SLE	Focal NP-SLE
Anti-P_{C22}	4.2% (1/24)	23.1% (12/52)	5.3% (1/19)
Anti-whole P	0% (0/24)	78.8% (41/52)	31.6% (6/19)
Anti-$P_{EX.C22}$	4.2% (1/24)	65.4% (34/52)	26.3% (5/19)
Anti-C22-depleted rP0	5.3% (1/19)	44.7% (21/47)	5.6% (1/18)

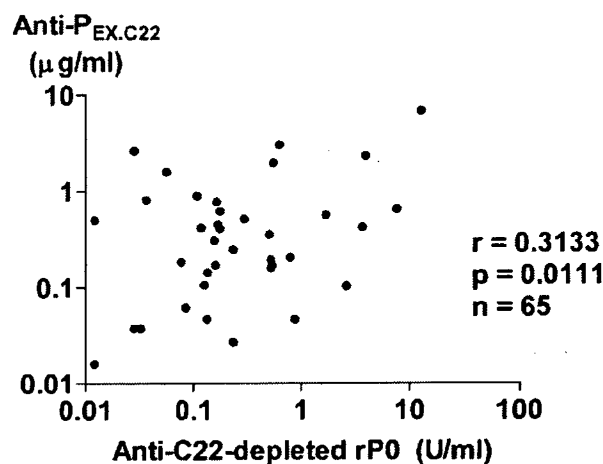
[a]Antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}), to highly purified ribosomal P proteins (anti-whole P), to the epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$), and to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) in cerebrospinal fluid from patients with non-inflammatory neurological diseases (Control), with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE), or with focal NP-SLE were compared. [b]Cutoff values were set as the mean + 3 standard deviations of the values in control group. Values in parenthesis mean (numbers of patients with positive results/total patient numbers) in each group.

Figure 3



Differential reactivity of purified antibodies to the C-terminal 22 amino acids of ribosomal P protein. Differential reactivity of purified antibodies to the C-terminal 22-amino acid sequence of ribosomal P protein (anti-P_{C22}) with ribosomal P peptide-human serum albumin (HSA) conjugates and with recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (C22-depleted rP0). Purified anti-P_{C22} react with ribosomal P peptide-HSA conjugates, but not with C22-depleted rP0 on enzyme-linked immunosorbent assay plates. OD492 (optical density at 492 nm) values that are subtracted by non-specific binding activities are plotted.

Figure 4



Correlation between autoantibodies to various components of ribosomal P proteins. The correlation between antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) and those to the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) in cerebrospinal fluid patients, including 47 patients with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE) and 18 patients with focal NP-SLE, was analyzed. Statistical analysis was performed by Spearman rank correlation test.

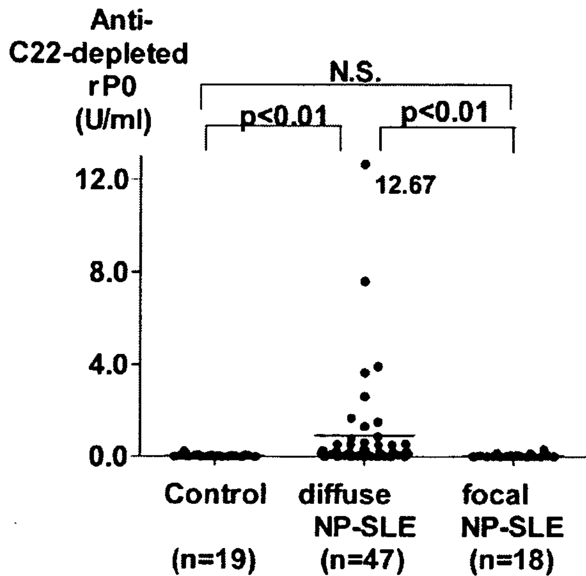
there were no statistical significances by Kruskal-Wallis test with multiple comparisons. Of note, there were no significant differences in serum anti-$P_{EX.C22}$ levels between non-CNS SLE and NP-SLE. These results suggest that in contrast with the CSF results, serum anti-P_{C22}, but not serum anti-P). The data therefore suggest that C22-depleted rP0 might contain one of the major targets, against which CSF anti-N are directed.$_{EX.C22}$, are associated with NP-SLE, especially diffuse NP-SLE.

Discussion

A number of studies have suggested that CSF anti-N play an important role in the pathogenesis of diffuse NP-SLE [7,11]. However, the epitopes to which CSF anti-N are directed have not been delineated. Of note, previous studies have demonstrated that epitopes antigenically related to ribosomal P proteins are present on the surface of SK-N-MC neuroblastoma cells [12]. Although anti-P_{C22} have been shown to be major autoantibodies to ribosomal P proteins [3,4,22], the frequency of their detection in CSF from patients with diffuse NP-SLE was not high enough to ensure their involvement in the pathogenesis of this disease [3,4,7]. Therefore, it was suggested that anti-P_{C22} might not be a major constituent of anti-N in CSF from patients with diffuse NP-SLE. Consistently, the data in the current studies indicated that CSF anti-P_{C22} levels were not significantly elevated in patients with diffuse NP-SLE compared with those in patients with focal NP-SLE or with non-inflammatory neurological diseases. However, it was still possible that CSF autoantibodies directed to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence were more prevalent. Thus, the results in the current studies have also demonstrated that levels of CSF anti-whole P as well as CSF anti-$P_{EX.C22}$ were significantly higher in patients with diffuse NP-SLE than in patients with focal NP-SLE or non-inflammatory neurological diseases. The data therefore indicate that CSF antibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence are associated with diffuse NP-SLE.

To confirm the presence of antibodies for the epitopes representing regions of the ribosomal P proteins other than the C-terminal 22-amino acid sequence, antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22 amino acids (C22-depleted rP0) [21] were evaluated. The results clearly demonstrate that CSF anti-C22-depleted rP0 levels were significantly correlated with CSF anti-$P_{EX.C22}$ levels. In addition, levels of CSF anti-C22-depleted rP0 as well as CSF anti-$P_{EX.C22}$ were significantly elevated in diffuse NP-SLE. The data therefore confirm that CSF antibodies to ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence play a role in the pathogenesis of diffuse NP-SLE, but further studies are required to identify the fine epitopes.

Figure 5



Cerebrospinal fluid antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence. Antibodies to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) (U/ml) in cerebrospinal fluid from patients with non-inflammatory neurological diseases (Control), with diffuse neuropsychiatric systemic lupus erythematosus (NP-SLE), or with focal NP-SLE were compared. Horizontal lines indicate the mean values. Statistical analysis was performed by Kruskal-Wallis test with multiple comparisons (Scheffé's method). N.S., not significant.

In has been demonstrated that purified human plasma membranes contain a 38-kDa protein that is closely related or identical to ribosomal P0 proteins [12]. Therefore, it was suggested that autoantibodies to ribosomal P proteins, especially those directed to epitopes other than the C-terminal 22-amino acid sequence, might be involved (at least in part) in CSF anti-N activities. In fact, levels of CSF anti-$P_{EX.C22}$ as well as CSF anti-P_{C22} or CSF anti-whole P were decreased after incubation of CSF with paraformaldehyde-fixed SK-N-MC cells, confirming that CSF anti-$P_{EX.C22}$ as well as anti-P_{C22} are constituents of CSF anti-N. However, CSF anti-P_{C22} levels were not significantly correlated with CSF anti-N levels in the present study. By contrast, CSF anti-$P_{EX.C22}$ or CSF anti-C22-depleted rP0 levels were significantly correlated with CSF anti-N levels. These results indicate that ribosomal P0 proteins contain one of the major targets of CSF anti-N in their portions other than the C-terminal 22-amino acid sequence. Of note, recent studies have demonstrated that autoantibodies directed against the *N*-methyl-d-aspartate (NMDA) receptor mediated apoptotic death of neurons *in vivo* and *in vitro* in murine systems [23]. Of note, anti-NMDA receptor antibodies were also detected in CSF from a single patient with SLE [22]. It is therefore likely that anti-NMDA receptor antibodies might also be involved in CSF anti-N activities and thus play a pivotal role in the pathogenesis of diffuse NP-SLE. Further studies with a large number of patients are required to confirm the involvement of anti-NMDA receptor antibodies in diffuse NP-SLE and to explore its relationship with anti-N.

A number of studies have indicated that serum anti-ribosomal P protein antibodies, including anti-P_{C22} or anti-whole P, are frequently observed in patients with NP-SLE [3-5,24]. Consistently, the results in the current studies have also disclosed that levels of serum anti-P_{C22} as well as serum anti-whole P are significantly higher in NP-SLE than those in non-CNS SLE. Of

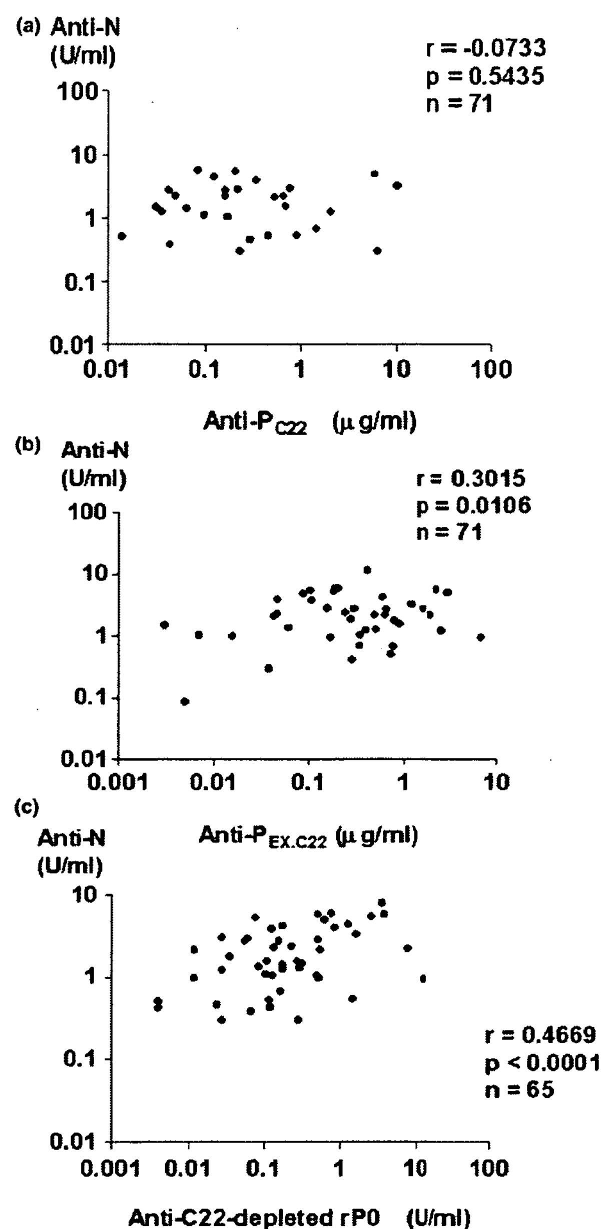
Table 3

Absorption of CSF autoantibodies to various components of ribosomal P proteins by neuronal cells

Patient	Autoantibodies	Without absorption	With absorption
1	Anti-whole P (μg/ml)	7.243	1.435
	Anti-P_{C22} (μg/ml)	3.456	1.019
	Anti-$P_{EX.C22}$ (μg/ml)	3.787	0.416
	Anti-N (U/ml)	4.083	1.950
2	Anti-whole P (μg/ml)	0.140	0.050
	Anti-P_{C22} (μg/ml)	0.070	0.042
	Anti-$P_{EX.C22}$ (μg/ml)	0.070	0.008
	Anti-N (U/ml)	0.789	0.588

Cerebrospinal fluid (CSF) samples (50 μl/well) were incubated in wells of a 96-well flat-bottomed microtiter plate with or without confluent SK-N-MC cells fixed with 1% paraformaldehyde at room temperature for 2 hours. After the incubation, CSF samples were recovered and were examined for anti-whole P, anti-P_{C22}, anti-$P_{EX.C22}$, and anti-N as described in Materials and methods. Anti-N, anti-neuronal cell antibodies; anti-P_{C22}, antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein; anti-$P_{EX.C22}$, autoantibodies directed against the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence; anti-whole P, antibodies to the whole ribosomal P proteins.

Figure 6



Correlation between autoantibodies to ribosomal P proteins and anti-neuronal cell antibodies. The correlation of antibodies to the C-terminal 22-amino acid sequence of ribosomal P proteins (anti-P_{C22}) **(a)**, those to the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence (anti-$P_{EX.C22}$) **(b)**, or those to recombinant ribosomal P0 protein lacking the C-terminal 22-amino acid sequence (anti-C22-depleted rP0) **(c)** with anti-neuronal cell antibodies (anti-N) in cerebrospinal fluid from systemic lupus erythematosus (SLE) patients, including 52 patients **(a,b)** or 47 patients **(c)** with diffuse neuropsychiatric SLE (NP-SLE) and 19 patients **(a,b)** or 18 patients **(c)** with focal NP-SLE, was analyzed. Statistical analysis was performed by Spearman rank correlation test.

note, serum anti-$P_{EX.C22}$ levels were not significantly elevated in NP-SLE compared with those in non-CNS SLE. These findings contrast sharply with the results of CSF studies. Thus, in CSF, anti-$P_{EX.C22}$, but not anti-P_{C22}, were significantly associated with diffuse NP-SLE, whereas in serum, anti-P_{C22}, but not anti-$P_{EX.C22}$, were associated with NP-SLE.

The mechanism by which anti-whole P cause neuronal damage remains unclear. We previously reported that the expression of IL-6 mRNA in neurons was upregulated in the brain of an SLE patient who died of active diffuse NP-SLE [25]. Of note, we recently disclosed that anti-P_{C22} upregulate the expression of mRNAs for IL-6 and tumor necrosis factor-alpha in human peripheral blood monocytes [20]. It should be pointed out that anti-$P_{EX.C22}$ as well as anti-P_{C22} might be able to bind the ribosomal P protein on neuronal cells [12]. Taken together, these results suggest that anti-whole P or anti-$P_{EX.C22}$ might also upregulate the expression of IL-6 mRNA in neurons and thus result in the alteration of their functions. Further studies to explore the targets and the effects on their functions of anti-P_{C22} and anti-$P_{EX.C22}$ (or anti-P_{AA9}) would improve our understanding of the pathogenesis of NP-SLE.

In summary, the current studies have demonstrated that the expression of autoantibodies directed against the epitopes of ribosomal P proteins other than the C-terminal 22-amino acid sequence is increased in CSF from patients with diffuse NP-SLE. The presence of such autoantibodies might account for CSF anti-N activities, although there might be other antibodies that bind to neuronal cells, such as anti-NMDA receptor antibodies. Further studies to explore the whole spectrum of epitopes of neurons to which autoantibodies are directed as well as the mechanism by which such autoantibodies cause damage to neurons are needed for a complete understanding of the pathogenesis of diffuse NP-SLE.

Conclusion

The present study has disclosed that CSF IgG antibodies to the epitopes of ribosomal P0 proteins other than the C-terminal 22 amino acids are associated with the development of diffuse NP-SLE as one of the major CSF anti-N components.

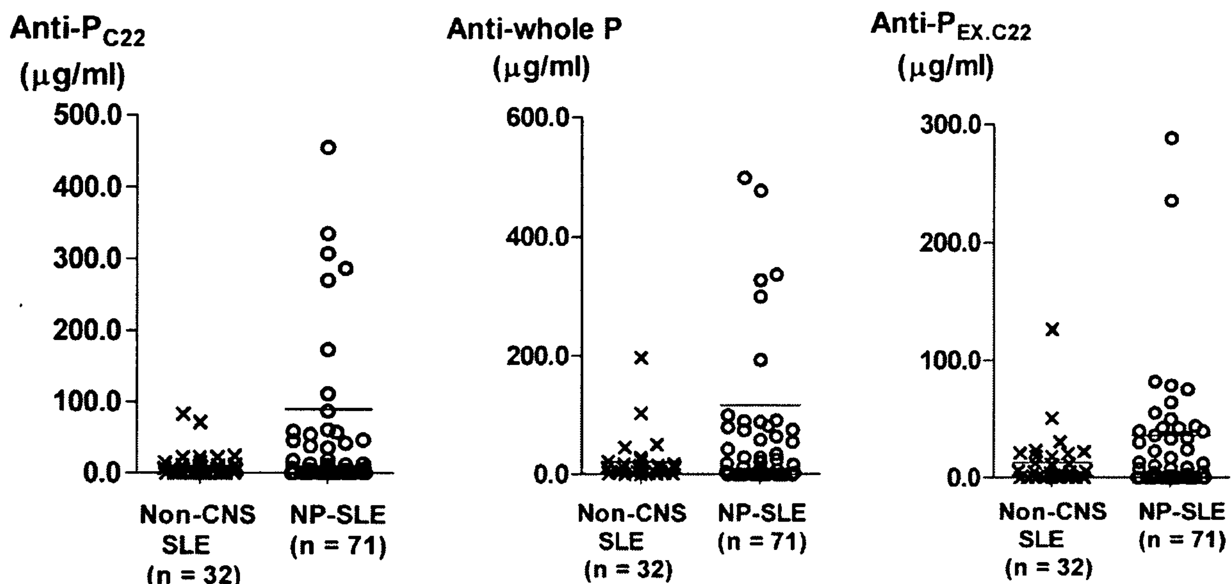
Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SH designed the study and participated in experimental procedures, collection, analysis, and interpretation of data and manuscript preparation. YA and MT contributed to the collection and analysis of data. TY helped to prepare C22-depleted rP0 and to develop ELISA for anti-C22-depleted rP0. All authors read and approved the final text before submission of the manuscript.

Figure 7



Serum autoantibodies to various components of ribosomal P proteins. Anti-P_{C22}, anti-whole P, and anti-$P_{EX.C22}$ in sera from SLE patients without neuropsychiatric manifestations (non-CNS SLE) (cross), with diffuse NP-SLE (open circle), or with focal NP-SLE (closed circle) were compared. Horizontal lines indicate the mean values. Statistical analysis between non-CNS SLE versus NP-SLE (focal + diffuse) was performed by Welch's *t* test. Anti-P_{C22}, antibodies directed against the C-terminal 22-amino acid sequences of ribosomal P protein; anti-$P_{EX.C22}$, autoantibodies directed against the ribosomal P protein epitopes other than the C-terminal 22-amino acid sequence; anti-whole P, antibodies to the whole ribosomal P proteins; non-CNS SLE, systemic lupus erythematosus without neuropsychiatric manifestations; NP-SLE, neuropsychiatric systemic lupus erythematosus; SLE, systemic lupus erythematosus.

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CASE REPORT

Lymphomatoid granulomatosis and diffuse alveolar damage associated with methotrexate therapy in a patient with rheumatoid arthritis

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Abstract We report on a patient of rheumatoid arthritis (RA) who sequentially developed an axillary mass and a fatal interstitial pneumonia during a 2-year course of methotrexate (MTX) therapy. Autopsy revealed a systemic lymph node involvement and the diagnosis of Epstein–Barr virus (EBV)-related lymphoproliferative disease (LPD) with the features of lymphomatoid granulomatosis was made. The lung tissue specimens revealed a typical diffuse alveolar damage (DAD), and small nodules consisting of atypical B lymphocytes showing positive staining for EBV were sparsely recognized only in basal lungs. This is the first report of a RA patient receiving MTX therapy sequentially developing MTX-associated lymphomatoid granulomatosis and DAD.

Keywords Diffuse alveolar damage · Epstein–Barr virus · Lymphomatoid granulomatosis · Methotrexate · Rheumatoid arthritis

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Introduction

Methotrexate (MTX) is a key drug in the treatment of rheumatoid arthritis (RA), and it has been shown to delay the progression of radiographic changes of the joints, halt the worsening of the quality of life, and reverse the shortening of the life span in patients with RA [1, 2].

However, a proportion of patients receiving MTX therapy may develop potentially life-threatening adverse events, e.g., interstitial pneumonia [3–9], severe bone marrow suppression [10, 11], and lymphoproliferative disease (LPD), including malignant lymphoma [12–15].

We report on a patient of RA who sequentially developed Epstein–Barr virus (EBV)-related LPD with the features of lymphomatoid granulomatosis and interstitial pneumonia presenting diffuse alveolar damage (DAD) on histological examination during a 2-year course of MTX therapy.

Case report

A 71-year-old Japanese man was admitted to our hospital on July 29, 2002, with worsening dyspnea. He had first developed polyarthralgia in December 1996, and visited an orthopedic clinic. A diagnosis of RA was made, based on the presence of symmetrical polyarthritis involving hands, elbows, and knees, and a positive test for serum rheumatoid factor. Treatment with prednisolone (10 mg/day), as well as sulfasalazine (1000 mg/day) and actarit (200 mg/day), another disease-modifying antirheumatic drug (DMARD), was initiated immediately. Intramuscular gold injection was also added up to a total dose of 675 mg. In February 2000, MTX was substituted for those three DMARDs at the dose of 6 mg/week, which was soon increased to 8 mg/week.

The patient showed gradual resolution of his articular symptoms in response to MTX.

In January 2002, he noticed a mass about 3 cm in diameter in his right axilla, which increased rapidly in size over the next few months; thus, he was referred to our hospital on July 15. While he has been scheduled to undergo biopsy of the mass, he was emergently admitted to our hospital on July 29 with worsening dyspnea.

A hard mass, approximately 12 cm in diameter, was palpable in his right axilla, and there was right supraclavicular lymphadenopathy. There was no evidence of active synovitis. Chest X-ray showed diffuse infiltration which showed a rapid aggravation since July 15 (Fig. 1c,d). The patient needed emergent endotracheal intubation and mechanical ventilation because of severe hypoxia. The peripheral blood leukocyte count was 11,700/mm^3 (eosinophol, 3%; and atypical lymphocyte, 6%), the hemoglobin was 11.8 g/dl, and the platelet count was 503,000/mm^3. Blood biochemical examination revealed the following: serum total protein, 4.9 g/dl; serum alanine aminotransferase, 19 IU/l; serum lactate dehydrogenase, 577 IU/l; and serum creatinine, 1.2 mg/dl. The serum C-reactive protein (CRP) level was markedly elevated to 20.0 mg/dl. Urine examination was normal.

Intravenous pulse therapy with methylprednisolone (1,000 mg daily for 3 days), followed by treatment with oral prednisolone at 60 mg/day was immediately initiated, along with empiric antibiotic therapy with panipenem/betamipron, fluconazole, and sulfamethoxazole/trimethoprim. However, the patient showed no signs of improvement.

Serological examination revealed a positive test for rheumatoid factor (up to 1:80 dilution in the passive hemagglutinin test) and negative test for antinuclear antibodies. The serum immunoglobulin (Ig) levels were as follows: IgG: 912 mg/dl; IgE: 979 IU/ml. The serum level of KL-6 (Krebs von den Lungen-6), a MUC1 mucin which is a useful marker for various interstitial lung diseases [16], was 849 U/ml (normal range <500 U/ml). The serum carcinoembryonic antigen (CEA) level was 22.5 ng/ml (normal range, 1.0–6.7 ng/ml), squamous cell carcinoma (SCC) antigen level was 5.4 ng/ml (normal range, <1.5 ng/ml), neuron-specific enolase (NSE) level was 18 ng/ml (normal range <10 ng/ml), and carbonic anhydrase15-3 (CA15-3) level was 40 U/ml (normal range, <30 U/ml). The soluble interleukin 2 receptor level was markedly elevated to 7,700 U/ml (normal range, 220–530 U/ml). Notably, serum IgG reaction with EBV-viral capsid antigen was positive (1/160), whereas that with the nuclear antigen (EBNA) was negative. Real-time polymerase chain reaction (PCR) for EBV using whole blood demonstrated 250,000 copies of EBV per 100,000 leukocytes. Serum beta-D-

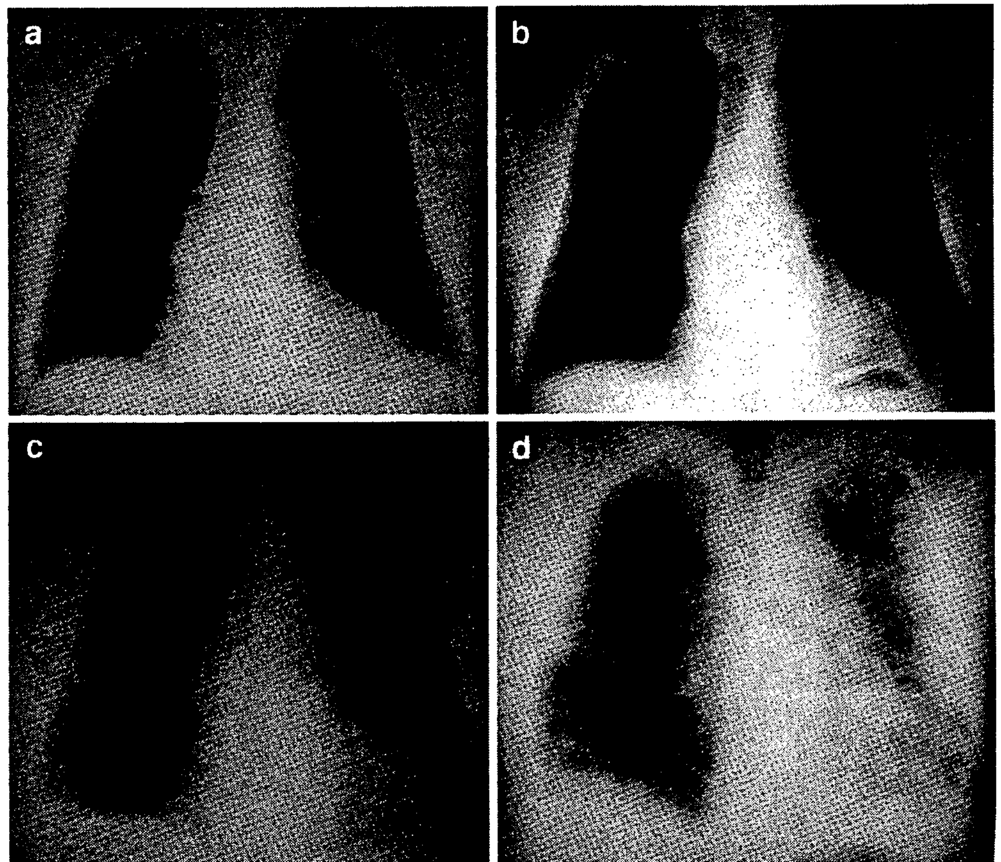

Fig. 1 Chest radiographs obtained on January 26, 2000 (**a**; before MTX therapy), January 18, 2002 (**b**), July 15, 2002 (**c**; at the first visit to our hospital), and July 29, 2002 (**d**; on admission)

glucan, antineutrophil cytoplasmic antibody (for either against myeloperoxydase or proteinase 3), sputum PCR test for Pneumocystis jerovicii, as well as the tests for influenza virus, mycoplasma, legionella, and other bacteria, were all negative.

Examination of hematoxylin–eosin-stained sections of the biopsy specimen obtained from the mass in his right axilla on the day of admission revealed findings highly suggestive of malignant lymphoma. Therefore, combined chemotherapy with cyclophosphamide 860 mg, doxorubicin 55 mg, and vincristine 1.5 mg was added to prednisolone (CHOP therapy) on August 3. However, despite our best efforts, the patient expired after 2 days.

Autopsy was performed, and histopathologically, the axillary tumor, which was 12 cm in diameter with irregular shaped necrotic foci, showed an angiocentric and angiodestructive polymorphous lymphoid infiltrate (Fig. 2a). Large-sized atypical lymphoid cells were CD20-positive (Fig. 2b) and EBER-positive by in situ hybridization (Fig. 2c). CD3-positive T cells were also observed. For rearrangement analysis of the Ig heavy chain gene, 2-step seminested PCR was carried out, which showed several bands (Fig. 3). Thus, monoclonal expansion of B lymphocytes was not demonstrated. Systemic lymph node involvement was noted and nodular lesions were observed in the lungs, spleen, and adrenal glands. The diagnosis of EBV-related LPD with the features of lymphomatoid granulomatosis was made.

On the other hand, the lung tissue specimens revealed diffuse deposition of hyaline membrane along the alveolar

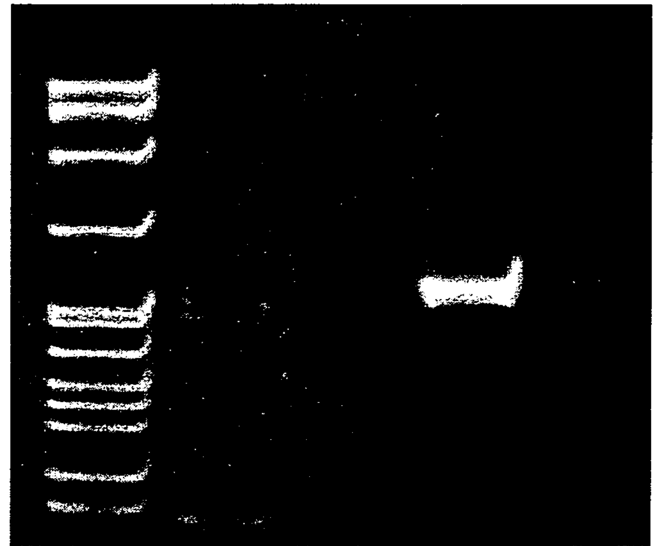

Fig. 3 Examination of the immunoglobulin heavy chain gene rearrangement by two-step seminested PCR using DNA extracted from paraffin-embedded sections. The primers used for the seminested PCR were FR1 family-specific primer cocktail and LJH for the first round, and FR2 family-specific primer cocktail and VLJH for the second round. Several polyclonal bands were observed in the sample, while a single monoclonal band appeared in the positive control demonstrating monoclonality

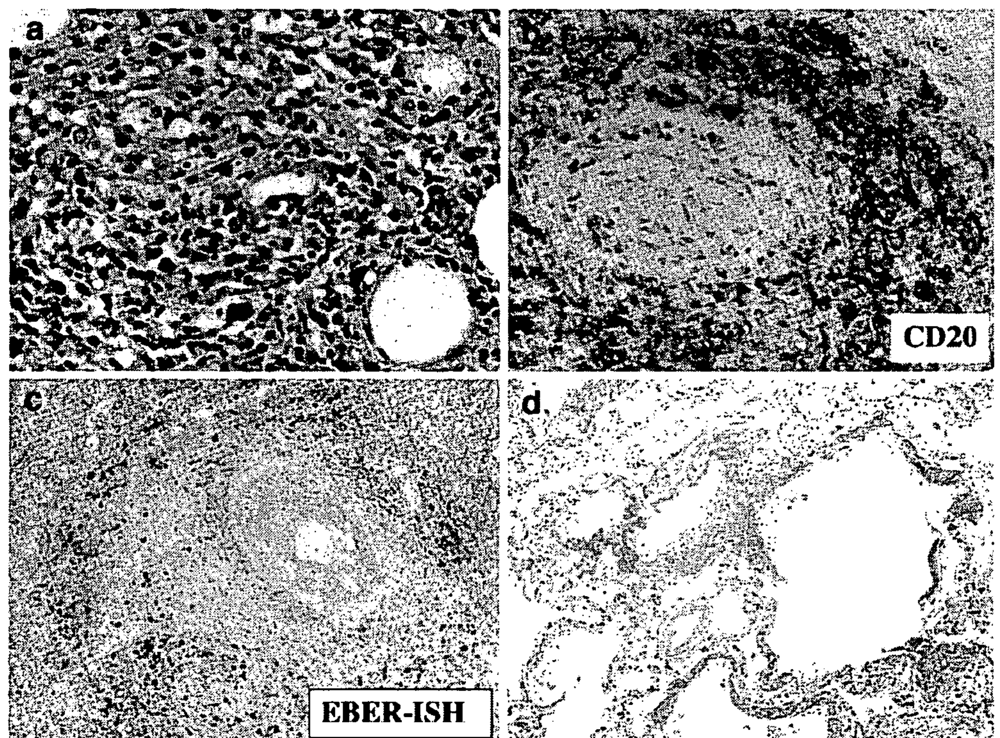

Fig. 2 Histopathological examinations. **a** Tumor section stained with hematoxylin–eosin under high power magnification demonstrating polymorphous proliferation of medium- to large-sized tumor cells with irregular nuclei. **b** Tumor cells showing an angiophilic tendency and positive staining with anti-CD20 antibody. **c** In situ hybridization to check for the presence of EBV demonstrated positive staining with the EBER-1 probe in the tumor cells. **d** Postmortem examination of the lung revealed organizing DAD

walls, indicative of diffuse alveolar damage (DAD; Fig. 2d). Small nodules consisting of atypical B lymphocytes showing positive staining for EBV were very sparsely recognized only in basal lungs. Other infections were denied. The lymphocyte stimulation test for MTX was positive, with a maximal stimulation index of 4.7 (normal range <1.8). Therefore, the patients fulfilled the diagnostic criteria for MTX pneumonia [3, 4, 7].

Discussion

This is the first report of a RA patient receiving MTX therapy developing both lymphomatoid granulomatosis and interstitial pneumonia with the pathological features of DAD. Numerous case studies have revealed a nearly 100% increase in the risk of development lymphoma in patients with RA [12]. The increased risk of lymphoma has been attributed to the severity of RA, secondary Sjögren's syndrome, MTX, and/or antitumor necrosis factor (TNF) therapy [12]. Patients receiving MTX therapy for RA could to be at a particularly increased risk, as compared with the general population, of developing EBV-associated LPD probably via p38 mitogen-activated protein (MAP) kinase, phosphatidylinositol 3-kinase, and MAP kinase pathways and specific *cis*-acting motifs in the two viral immediate-early promoters [13]. More importantly, discontinuation of MTX therapy is followed by the disappearance, or decrease in size, of lymphomatous tumors in some RA patients, prompting the condition to be referred to as "reversible lymphoma" [14]. However, three large epidemiological studies failed to find an overall association between MTX exposure and the development of non-Hodgkin's lymphoma in RA patients. [17–19].

EBV is a ubiquitous human herpesvirus that establishes a lifelong persistent infection of B cells in more than 90% of the human adult population. It is thus a B-lymphocytotropic virus causing a variety of B-cell disorders, including infectious mononucleosis and LPD. In the present patient, immunohistochemical analysis showed a B-cell phenotype of CD20+, CD79a+, CD3−, CD5−, CD10−, with a latency 2 pattern of EBV infection, which is characterized by results positive for EBV (by in situ hybridization) and latent membrane protein (LMP)-1, and negative for Epstein–Barr nuclear antigen (EBNA)-2 (data not shown) [13].

Interstitial pneumonia in our patient developed possibly as a result of RA disease activity, an adverse event of MTX, and lymphomatoid granulomatosis of the lung. Among those possibilities, MTX-associated interstitial pneumonia is most likely because RA disease activity had been well controlled with MTX, and the involvement of lymphomatoid granulomatosis was very limited by histological examination.

MTX pneumonia is a serious adverse event affecting about 1% of RA patients receiving MTX treatment. Male sex, smoking, and preexisting interstitial lung disease (ILD), as well as hypoproteinemia, renal dysfunction, prior use of DMARDs and diabetes, have been listed as the risk factors for the development of MTX pneumonia [3–8]. Our patient was a smoker with hypoproteinemia, renal dysfunction, and prior use of DMARDs. Bibasilar ILD had been modestly observed in January 2000 (Fig. 1a), which did not show a significant progression, at least, until January 2002 (Fig. 1b). MTX pneumonia has been histopathologically characterized by the presence of interstitial pneumonia, bronchiolitis, and giant cell formation [8], although a recent review indicated that DAD, as in our case, could be sometimes demonstrated as well [9]. Pulmonary involvement associated with LPD, reported previously [15], was concomitantly observed in our patient, although the fatal respiratory failure was attributed to DAD because the lesion showing the features of lymphomatoid granulomatosis were very localized. In addition, our case fulfilled all of the diagnostic criteria for MTX pneumonia proposed by Searles and McKendry [3], Carson et al. [4], and Kremer et al. [7].

In conclusion, serious adverse events such as LPD and DAD may even develop concomitantly in patients with RA receiving MTX, and a prompt identification of the complications followed by an immediate institution of an adequate treatment is necessary to avoid a fatal outcome.

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