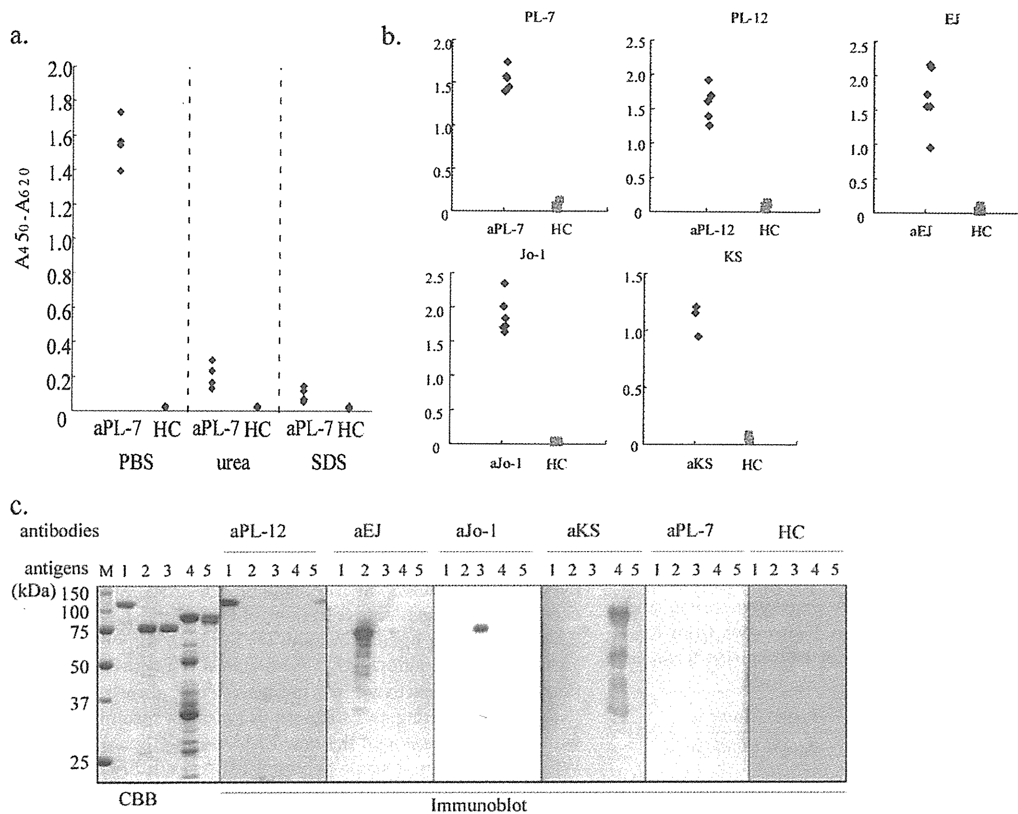

Figure 1. Antigenic activity of recombinant autoantigens a. Antigenic activity of PL-7 in various conditions. Left, purified recombinant PL-7 was eluted and diluted in PBS and coated on ELISA plates. Middle and Right, purified recombinant PL-7 was eluted in PBS and diluted in 8M urea and 2 × SDS sample buffer, respectively, and then coated onto ELISA plates. b. Five recombinant ARS antigens (His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7) were prepared as soluble polypeptides in PBS and their antigenic activity was tested in an ELISA using sera from five patients each containing corresponding autoantibodies (only GST-KS was tested using sera from three patients). Six healthy controls were used in each ELISA. c. Purified recombinant ARS antigens were electrophoresed on SDS-PAGE and transferred to a PVDF membrane followed by immunoblot analysis. CBB; Coomassie Brilliant Blue staining of gels, M; molecular weight marker, HC; healthy control, Lane 1; His-PL-12, Lane 2; His-EJ, Lane 3; GST-Jo-1, Lane 4; GST-KS and Lane 5; His-PL-7.
doi:10.1371/journal.pone.0085062.g001

not shown). There were seven anti-ARS-positive patients with other CTDs; two SSc patients were positive for anti-PL-12, two SLE patients were positive for anti-KS or anti-PL-12, and three RA patients were positive for anti-KS, anti-OJ or anti-PL-12.

Table 1 Comparison of the results between the new ELISA system and RNA-IP.

		RNA-IP		
		+	-	
anti-ARS ELISA	+	101*	1*	102
	-	0* (3)†	622* (619)†	622
	total	101* (104)†	623* (620)†	724

*The results detecting the five anti-ARS antibodies (anti-Jo-1, PL-12, EJ, KS, and PL-7) are described (sensitivity: 100%, specificity: 99.8%).
†Numbers in parenthesis are the results detecting all anti-ARS antibodies (including anti-OJ) (sensitivity: 97.1%, specificity: 99.8%).
doi:10.1371/journal.pone.0085062.t001

Clinical significance of anti-ARS ELISA in IIP

Anti-ARS antibodies were positive in 10.7% (18/168) of IIP patients. Only two patients (5.6%) with IPF were positive for anti-ARS; conversely, 16 patients (12.1%) with non-IPF were positive for anti-ARS antibodies (Tableô 2). To investigate whether the anti-ARS-positive IIP were clinically distinct from anti-ARS-negative IIP patients, we compared clinical backgrounds and treatments between anti-ARS-positive and negative non-IPF patients (Tableô 4). The anti-ARS-positive patients were significantly younger and a higher proportion was female ($p<0.01$), and they were treated more frequently with glucocorticoids (GC) or the combination of GC and immunosuppressants ($p<0.05$ and $p<0.01$, respectively).

Discussion

Among MSAs/myositis-associated autoantibodies (MAAs), anti-ARSs are the most frequently detected (28–37% [1,23,24]) in adult IIM patients, and anti-ARS-positive patients develop common characteristic symptoms known as ASS. Not only IIM but also apparent IIP patients can be positive for anti-ARS antibodies because IP often precedes myositis [1,14,20,25]. Both myopathy

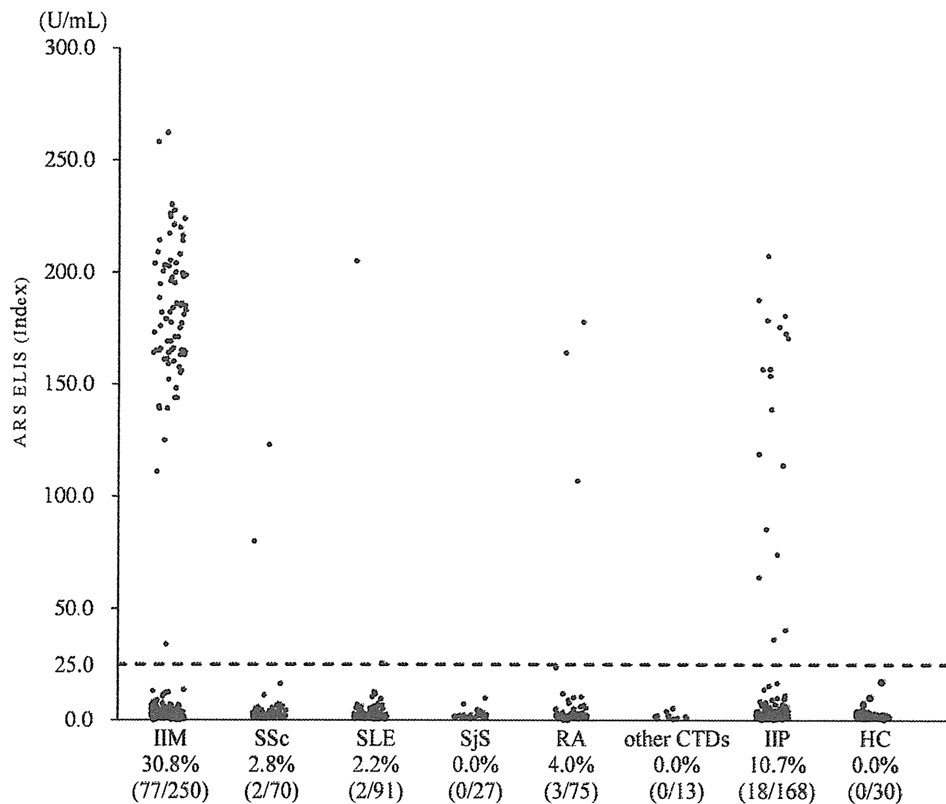

Figure 2. Confirmation of the efficiency of the ELISA system. Using the ELISA system, ARS antibodies were measured in 694 serum samples from patients with various CTDs and IIP, and 30 serum samples from healthy controls. The cutoff value (25 U/mL) is indicated by a horizontal dotted line.
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and IP anti-ARS-positive patients showed a better response to initial GC therapy but it can exacerbate the condition more often in anti-ARS-positive than in anti-ARS-negative patients [1,26]. Therefore, anti-ARS antibodies are useful not only in diagnosis, predicting the clinical course and therapy decisions in IIM, but also in classifying IP patients and predicting late-onset myopathy in IP-preceding patients.

An immunoprecipitation assay has been used to detect each anti-ARS antibody but to date, it can only be performed in a limited number of laboratories. To detect them more easily and routinely, we aimed to establish an ELISA system using the six recombinant ARS antigens to simultaneously detect anti-Jo-1, PL-7, PL-12, EJ, OJ, and KS antibodies. We did not include anti-tyrosyl or phenylalanyl synthetase because they have been reported only in one case each. However, some differences in clinical manifestations and prognoses among patients expressing different ARS antibodies, especially between anti-Jo-1 and non-anti-Jo-1 patients, have been observed [14,15]. However, different treatments for patients expressing different anti-ARSs have not been established. Currently, we treat anti-ARS-positive patients

Table 2 The frequency of each anti-ARS antibody in IIM, other CTD and IIP.

		RNA-IP(%)					
	ARS ELISA	Jo-1	PL-7	PL-12	EJ	KS	OJ
IIM	30.8% (77/250)	13.6	13.2	2.0	6.0	0.0	0.8
other CTDs	2.5% (7/276)	0.0	0.0	1.4	0.0	0.7	0.4
IIP	10.7% (18/168)	3.6	2.4	0.6	1.2	2.4	0.0
IPF	5.3% (2/38)	0.0	0.0	2.6	0.0	2.6	0.0
non-IPF	12.3% (16/130)	4.6	3.1	0.0	1.5	2.3	0.0

doi:10.1371/journal.pone.0085062.t002

Table 3 The frequency of each anti-ARS antibody in subsets of IIM.

IIM classification	Total	Jo-1	PL-7	PL-12	EJ	KS	n (%)
I polymyositis	107	18	7	3	5	0	33 (30.8)
II dermatomyositis	93	13	10	1	9	0	33 (35.5)
III amyopathic dermatomyositis	23	0	2	0	1	0	3 (13.0)
IV malignancy-associated myositis	7	0	1	0	0	0	1 (14.3)
V juvenile myositis	1	0	0	0	0	0	0 (0)
VI overlap myositis	3	1	0	0	0	0	1 (33.3)
VII unclassified	6	2	3	1	0	0	6(37.5)

doi:10.1371/journal.pone.0085062.t003

Table 4 Comparison of clinical backgrounds between anti-ARS (+) and (−) non-IPF patients.

	non-IPF n = 130		
	anti-ARS		
	(-) n = 114	(+) n = 16	p-value
age at the onset of the disease (yr) mean	69.6±9.5	56.9±14.5	<0.01
female (n; (ratio%))	39(34.2)	12(75.0)	<0.01
chronic (n; (ratio%))	104(91.2)	13(81.3)	N.S
subacute + acute (n; (ratio%))	5(4.4)	1(6.3)	N.S
acute (n; (ratio%))	2(1.8)	1(6.3)	N.S
glucocorticoids(GC) (n;(%))	49(43)	11(68.8)	<0.05
GC + immunosuppressants(IS) (n;(%))	19(16.7)	8(50.0)	<0.01
only drugs other than IS (n;(%))	8(7.0)	2(12.5)	N.S
PaO_2 at rest (Torr) mean	75.9±14.9	86.5±37.4	N.S
SpO_2 at rest (%) mean	95.7±2.4	97.1±2.1	<0.05
SpO_2 after 6 min walk test (Torr) mean	88.6±5.5	86.9±6.0	N.S
%VC (%) mean	87.7±22.5	77.9±17.4	<0.05
%DLCO (%) mean	51.0±19.5	58.0±23.1	N.S
KL-6 (U/mL) mean	1132±949	1287±693	N.S
SP-D (ng/mL) mean	207±180	180±136	N.S

%VC: % vital capacity, %DLCO: % diffusing capacity of carbon monoxide.
doi:10.1371/journal.pone.0085062.t004

with expectation of a standard clinical course in which the disease can recur with tapering of GC and in which exacerbation of IP is associated with a poor prognosis [1,14]. Therefore, presently, we are focusing on determining whether a patient with IIM or IIP is anti-ARS positive or not for the first screening when we begin treatment. This is why we decided to use a mixture of ARS antigens and not just single antigens to detect 'multiple anti-ARS antibodies' simultaneously.

We first prepared recombinant ARSs in *E. coli*, but recombinant PL-7 and OJ did not react well with their corresponding autoantibodies either using immunoblotting or an ELISA. For PL-7, structural conformation was important for antigenic activity because the recombinant PL-7 showed good reactivity only when it was expressed in a eukaryotic Hi-5 cell and was not denatured prior to being measured in the ELISA. Conversely, when recombinant PL-7 was denatured with urea or SDS, it was weakly detected with the PL-7 antibody, although its antigenicity was not completely lost. Such antigenic characteristics have also been reported previously by others [27]. This suggests that the synthetase epitope recognized by the anti-PL-7 antibody is in its native tertiary conformation.

In contrast, recombinant OJ (isoleucyl-tRNA synthetase) was not well detected even when it was expressed in Hi-5 cells and analyzed under non-denaturing conditions. This may be due to the unique feature of this isoleucyl-tRNA synthetase, which is a component of the multi-enzyme complex containing nine ARSs with three nonenzymatic factors [28,29]. In screening tests, positivity of anti-OJ in patients' sera was determined by the pattern of immunoprecipitation using HeLa cell extracts as originally described by Targoff *et al.* [28]. But there is a possibility that some 'anti-OJ antibodies' may recognize other components of the multi-enzyme complex rather than isoleucyl-tRNA synthetase itself, or alternatively the structural conformation of the complex may be important for recognition by anti-OJ, as was previously suggested by Targoff *et al.* [10]. They examined 11 patient sera with anti-OJ for evidence of reaction with other components of the complex. Ten out of 11 sera significantly inhibited enzyme activity of isoleucyl-tRNA synthetase, but some of them also significantly inhibited other ARSs such as leucyl-, lysyl-, or arginyl-tRNA synthetases. Moreover, immunoblot analysis of anti-OJ revealed that the majority of the sera could not identify a shared band and only a few sera recognized isoleucyl-tRNA synthetase. These results suggest that most 'anti-OJ sera' may react with multiple synthetases of the multi-enzyme complex or react with conformational epitopes of the complex. For this reason, we considered that it would be difficult to prepare the immunoreactive OJ antigen as a single molecule; therefore, we developed an ELISA system using the other five recombinant ARSs. This may not significantly affect the sensitivity of the ELISA because the prevalence of anti-OJ antibodies in patients is very low among the six anti-ARS antibodies.

The efficiency of this newly established ELISA system was acceptable because the sensitivity and specificity of the system compared with RNA immunoprecipitation were 97.1% and 99.8%, respectively, even if anti-OJ-positive sera was not excluded. The prevalence of anti-ARS in our IIM cohort was comparable with previous reports [1,2]. It was noteworthy that 10.7% of IIP patients, and in particular, 12.1% of non-IPF patients were positive for anti-ARS antibodies and there were some differences between anti-ARS-positive and negative IIP patients in their clinical backgrounds and treatments. Anti-ARS-positive patients were treated significantly more frequently with GC or the combination of GC and immunosuppressants. However, we are not yet ready to recommend immunosuppressive therapy for anti-ARS-positive IIP patients because we have not yet collected enough data on their clinical response and prognosis. Although some of these anti-ARS-positive IIP patients might develop myopathy later, it suggests that the measurement of anti-ARS antibodies may be useful in stratifying patients into disease subsets, which may help in predicting their clinical course.

A line-blot assay for the detection of multiple MSAs/MAAs (EUROLINE Myositis Profile 3) has been used in which anti-Jo-1, PL-7, PL-12, EJ, and OJ are included. This system can detect and discriminate MSAs/MAAs without further anti-ARS tests, but it does not include anti-KS, which has a stronger association with IIP than myositis [30]. To address this point, our system can more efficiently detect anti-ARS and therefore, is the preferred assay to use for IIP patients than the line-blot assay, although our ELISA does not aim to discriminate specificity for each anti-ARS antibody.

In conclusion, our ELISA system using a mixture of five recombinant ARSs shows similar efficiency to RNA immunoprecipitation and makes it possible to more readily detect anti-ARS antibodies in patients with PM/DM and IIP, and can be widely applied in daily practice.

Supporting Information

Table S1 The list of approval by institutional review boards of all participating centers.
(XLSX)

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Author Contributions

Conceived and designed the experiments: TM RN. Performed the experiments: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Analyzed the data: RN YI. Contributed reagents/materials/analysis tools: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Wrote the paper: RN.

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Review Article

A New ELISA for Dermatomyositis Autoantibodies: Rapid Introduction of Autoantigen cDNA to Recombinant Assays for Autoantibody Measurement

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Advances in immunology, biochemistry, and molecular biology have enabled the development of a number of assays for measuring autoantibodies. ELISA has been widely used, because it can deal with relatively large numbers of serum samples more quickly than other immunologic methods, such as immunoblotting and immunoprecipitation. Recombinant autoantigens, which are generally produced in *E. coli* using the relevant cloned cDNA, are necessary for ELISA. Conventional clinical ELISA tests are limited in their ability to purify proteins free of bacterial contaminants, and the process is labor intensive. We recently developed new ELISA tests that utilize simple *in vitro* transcription and translation labeling of autoantigens in order to measure dermatomyositis- (DM-) specific autoantibodies, including autoantibodies to Mi-2, MDA5, NXP-2, TIF1-α, and TIF1-γ. This method may allow for the rapid conversion of cDNAs to a chemiluminescent ELISA to detect autoantibodies that are found not only in DM but also in other autoimmune diseases.

1. Introduction

Idiopathic inflammatory myopathies (IIMs) are a group of systemic autoimmune diseases that include polymyositis (PM), dermatomyositis (DM), and inclusion body myopathies. Several myositis-specific autoantibodies (MSAs) are associated with certain clinical forms of IIM, and they are useful tools for predicting the prognosis [1]. For example, anti-MDA5 antibody-positive patients demonstrate rapidly progressive interstitial lung disease (ILD), and anti-TIF1-γ antibody-positive patients are often complicated with cancer. In contrast, anti-Mi-2 antibodies are a serological marker for favorable prognosis in patients with classical DM who present with typical cutaneous manifestation and myositis. Autoantibodies to TIF1-γ are also present in juvenile DM as well as anti-MJ antibodies, and the latter recognize with NXP-2. Autoantibodies in DM tend to be mutually exclusive, thus enabling specific immune responses to differentiate between clinical subsets. It was recently clarified that anti-p155/140 antibodies, which were originally named for the molecular weight of the antigens [2], react to TIF1-γ and TIF1-α, respectively [3]. It is an exception that anti-TIF1-α antibodies appear with two mutually different prognostic markers: anti-TIF1-γ antibodies and also anti-Mi-2 antibodies [4].

Laboratories have been using several methods for detecting various autoantibodies: indirect immunofluorescence, immunoprecipitation (IPP), Western blotting (WB), and enzyme-linked immunosorbent assay (ELISA). ELISA-based serologic screening is highly sensitive and efficient, but it requires highly purified recombinant protein. The efficiencies of protein expression, purification, and stability limit the development of a novel ELISA and increase the risk of false-positive antibody detection. At present, many purified recombinant proteins are commercially available; however, full-length recombinant autoantigens are not always available. Moreover, even when they are available, their prices are often very high. Recently, we have developed an ELISA for the

detection of antibodies in sera with biotinylated recombinant protein by *in vitro* translation and transcription (TnT) and have detected DM-specific autoantibodies in our DM cohort [4–6]. This review introduces our newly developed ELISA tests, which use recombinant autoantigens to measure DM-specific autoantibodies, mainly autoantibodies to Mi-2, and clarifies the clinical significance of the new assay. This method may allow for the rapid conversion of cDNAs to a chemiluminescent ELISA in order to detect autoantibodies not only in DM but also in other autoimmune diseases.

2. ELISA with Commercially Available or In-House Prepared Recombinant DM Autoantigens

Recent works have clarified new DM-specific autoantigens, MDA5, TIF1-$\alpha/\beta/\gamma$, NXP2, and SAE [1]. In some recent studies, ELISAs with some of these commercially available or in-house prepared recombinant autoantigens were used. An ELISA measuring anti-MDA5 antibodies has been used in some works [7–9]. cDNA of MDA5 was cloned by immunoscreening with a patient's sera, and its recombinant protein produced by a baculovirus expression system was used for an ELISA [7]. The analytical sensitivity and specificity of this anti-MDA5 antibody ELISA were 85% and 100%, respectively. Anti-MDA-5 antibody levels measured by this ELISA closely correlated with the severity of skin ulcerations, ILD, and the prognosis of the disease in a Chinese study [8]. In a Japanese study, the median value of the anti-MDA5 antibody titer on admission was higher in patients who later died than in those who survived [9]. The decline index of the anti-MDA5 antibody titer after treatment was lower in the subset of patients who died than in the subset of patients who lived. Sustained high levels of anti-MDA5 antibody were present in the patients who died. In light of these results, anti-MDA5 antibody ELISA is useful for evaluating the response to treatment and the status of ILD in patients with anti-MAD5 antibody-positive DM.

Fujimoto et al. used an ELISA with commercially available recombinant TIF1γ and TIF1α to investigate longitudinal changes in serum antibody titers [3]. After treatment, the titer of anti-TIF-1γ antibodies decreased in all 8 patients, while the titer of anti-TIF-1α antibodies did not always decrease. The pathological significance of the titers of TIF1-γ/α needs further investigation.

Satoh et al. used commercially available recombinant TIF1-$\alpha/\beta/\gamma$ in an ELISA [11]. They confirmed the presence of these autoantibodies by using IPP-WB, antigen-capture ELISA, and ELISA with recombinants. The results of the ELISA with recombinants were consistent with the results shown by other immunological methods.

We also tried to perform an ELISA using commercially available recombinant SAE1 [12]. Anti-SAE antibodies were screened for 110 patients with DM, and 2 patients were found to have anti-SAE antibodies. Although anti-SAE autoantibodies also react to another subunit, SAE2 [13], an ELISA with recombinant SAE2 protein has not been reported.

3. Recombinant Protein Produced by *In Vitro* Translation and Transcription

Many studies have investigated autoantibodies by using recombinant protein produced by TnT. For example, in studies on cDNA cloning of autoantigens, this eukaryotic expression system, which often uses rabbit reticulocyte lysate, has been utilized in order to confirm whether patient's sera react to candidate clone's product and whether the clone product's mobility on SDS-PAGE is the same as the mobility of the endogenous cellular antigen [14–16]. Recombinant proteins produced by TnT are generally labeled with ^{35}S-methionine. The productive efficiency is theoretically influenced by the presence of the Kozak's consensus sequence around the AUG initiation codon and the numbers of methionine residues. Recent commercial kits for TnT contain all the necessary materials, except for highly purified DNA, to produce recombinants. The recombinant protein can be used for IPP without any pretreatment, since it is generally produced in soluble form.

To eliminate the need for radioactive materials, commercial products for biotin-labeled recombinants are also available. This labeling utilizes precharged *E. coli* lysine tRNAs, which are chemically biotinylated at the ε-aminogroup. Biotinylated proteins can be detected by the binding of streptavidin-alkaline phosphatase or streptavidin-horseradish peroxidase using a colorimetric or chemiluminescent detection system. Although the presence of biotinylated lysines may affect the antigenic structure of the modified protein, in our experience, the detection of anti-DFS70 antibodies using IPP with the biotinylated recombinant protein is largely consistent with their detection by WB with bacterial recombinant protein [17]. Detections of anti-MDA-5 and anti-TIF1-γ antibodies using IPP with the biotinylated recombinant protein are also closely consistent with their detection by the standard IPP with radio-labeled cellular extract [18].

4. ELISA with Biotinylated Recombinant Protein

We applied the above recombinant protein biotinylated *in vitro* TnT system to ELISA. After cDNA inserted into a plasmid vector containing T7 promotor is purchased, it takes up to 10 days to construct an ELISA system for the measurement of autoantibodies (Figure 1). At the first attempt, biotinylated MDA5 recombinants were coated onto commercial ELISA plates to which streptavidin was covalently coupled via a spacer [5]. This procedure also enabled the recombinant protein to be purified from crude lysate. Although this measurement could have been done with a conventional optical system for ELISA, 10 μL of reaction mixture per well was necessary to get a sufficiently positive signal, at the cost of ~$5/well. If we were able to purchase a commercial purified recombinant protein for less than $100/$\mu$g, it would be more cost effective for building an ELISA. To save the cost, we used a microplate luminometer to increase the sensitivity, thereby reducing the amount of biotinylated recombinant protein

required for the assays [4, 6]. Anti-MDA5 antibody levels obtained using a luminometer had a good correlation with those obtained using spectrophotometer [19]. We were able to reduce the amount of reaction mixture of TnT from 10 μL/well to 1 μL/well. Even if the cost of streptavidin-coated plate and highly sensitive chemiluminescence substrate are added, the per-well cost will still be only around $1.

5. Anti-Mi-2 Antibodies Measured by Our ELISA

Anti-Mi-2 antibodies were the first to be identified as DM-specific marker autoantibodies [20]. Patients with DM carrying these antibodies often show classic DM skin lesions and favorable prognosis [1]. The target macromolecular protein is the Mi-2/NuRD complex, which is involved in multiple transcriptional regulatory processes [21]. Anti-Mi-2 autoantibodies mainly target Mi-2α/β that are around 240 kDa [22]. Previous epitope-mapping studies showed multiple antigenic regions on the polypeptides of Mi-2β [23]. Even the most antigenic fragment was reactive to less than 60% of anti-Mi-2-positive samples. ELISA kits for anti-Mi-2 are available from one company (Thermo Fisher Scientific Inc.), and these were validated by a single study [24]. Since full-length recombinant proteins of Mi-2α/β are not available in Japan, we tried to construct an ELISA for the measurement of anti-Mi-2 antibodies.

The full-length Mi-2β cDNA clone [25] was a kind gift from Drs. Kato and Takahashi at Nagoya University. The plasmid harboring this clone contains the T7 promotor and the HA-tag and V5/His-tag at the N-terminus and C-terminus, respectively. Biotinylated recombinant protein was produced from the cDNA, using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, USA) according to our protocol [4]. Nunc Immobilizer Streptavidin Plates (Thermo Scientific Nunc, Roskilde, Denmark) were prewashed 3 times with PBS containing 0.05% Tween 20 (T-PBS), coated with TnT product diluted with T-PBS (50 μL/well), and incubated for 1 hour at room temperature. After 3 washes with T-PBS, the wells were blocked with 200 μL of a blocking buffer of 0.5% bovine serum albumin (Wako, Osaka, Japan) in T-PBS for 1 hour. Uncoated wells were used to measure the background levels for each sample. Sample sera diluted with blocking buffer (50 μL/well) were incubated for 1 hour at room temperature, followed by incubation with antihuman IgG antibody conjugated with HRP (Dako, Glostrup, Denmark) (50 μL/well) at 1 : 30,000 dilution. After incubation for 1 hour at room temperature, the plates were washed and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, USA) (50 μL/well) as the substrate. Then, relative luminescence unit (RLU) was determined using the GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU-subtracted background was used for data analysis. The high-level anti-Mi-2 antibody-positive serum serially diluted to 1 : 5, starting from 1 : 500, was run as standard. Units correlated with the antibody titers of antibodies: 1 : 500 dilution, 625 units; 1 : 2,500 dilution, 125 units; 1 : 12,500 dilution, 25 units; 1 : 62,500 dilution, 5 units; 1 : 312,500 dilution, 1 unit.

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we screened anti-Mi-2β antibodies in sera from 124 Japanese patients with DM (including 13 with juvenile DM, 39 with clinically amyopathic DM, and 19 with cancer-associated DM), in which 108 serum samples had been used in our previous study [4]. Five sera from patients with DM immunoprecipitated the biotinylated recombinant Mi-2β by IPP [4], and these had been confirmed to have anti-Mi-2 antibodies by IPP-WB by using anti-Mi-2α monoclonal antibody [4]. Twenty healthy individuals were assessed as normal controls. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

All 5 anti-Mi-2 positive sera identified as such by our previous study were also reactive to the recombinant in ELISA (Figure 2(a)). Using one positive serum with the high titer as the ELISA standard, all serum samples from DM patients and healthy individuals were investigated by ELISA, for which 0.5 μL/well of TnT reaction mixture was used. The cut-off level was set at 0.53 units, based on 5 standard deviations (SDs) above the mean value obtained from 20 healthy control sera. Two additional sera, which were not included in the previous study, were newly found to have anti-Mi-2 antibodies. No serum samples from the other 117 patients with DM or from the healthy individuals reached the cut-off level. One serum sample from a patient showed over 3 SDs above the mean value obtained from controls: 0.46. This serum showed no dose dependency for the amount of coated antigen (Figure 2(a)). Moreover, when only reticulocyte lysate-coated wells were used as the background for subtraction instead of uncoated wells being used, this serum unit fell below the 3SDs+mean (data not shown). Twenty ELISA-negative serum samples from DM patients were confirmed to be anti-Mi-2 negative by IPP-WB (data not shown). The clinical profiles of the 7 anti-Mi-2-positive patients are summarized and compared with data of a published Japanese multicenter study [10] in Table 1. Although ages at onset and sex ratios are different between the two studies, anti-Mi-2 positive DM is regarded as being classical DM and having a favorable prognosis without the life-threatening complication of malignancy or ILD [1].

6. Advantages and Disadvantages of the New ELISA

The results presented in this study demonstrated that a rapid, specific, sensitive, and quantitative anti-Mi-2-antibody immunoassay can be created by using commercially available *in vitro* TnT kits and precoated ELISA plates. Our newly established ELISA is a simple experimental method that does not require the use of radioisotopes. It is probably applicable for measuring various kinds of autoantibodies.

Standard IPP using cell extract has some limitations for accurate interpretation. Many DM-/PM-specific autoantigens show similar migration patterns on gel electrophoresis:

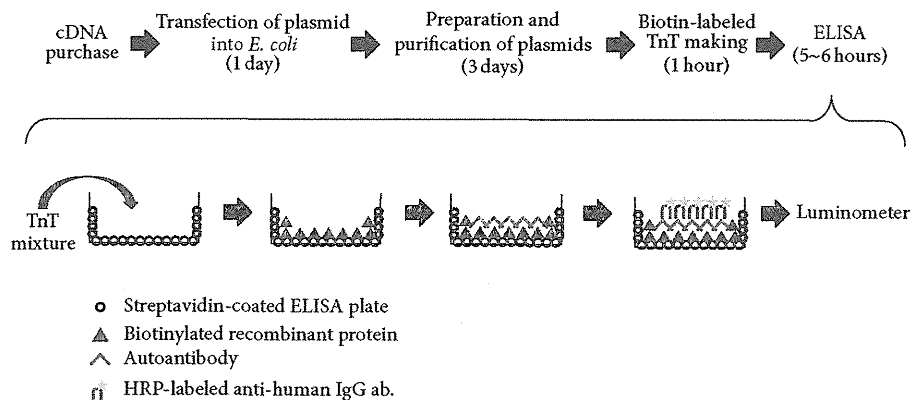

FIGURE 1: ELISA development using biotinylated recombinant protein. The process flow summarizes the method of ELISA construction, from obtaining the cDNA to obtaining the data by luminometer. We perform phenol/chloroform treatments twice to inhibit RNase for the plasmid purification.

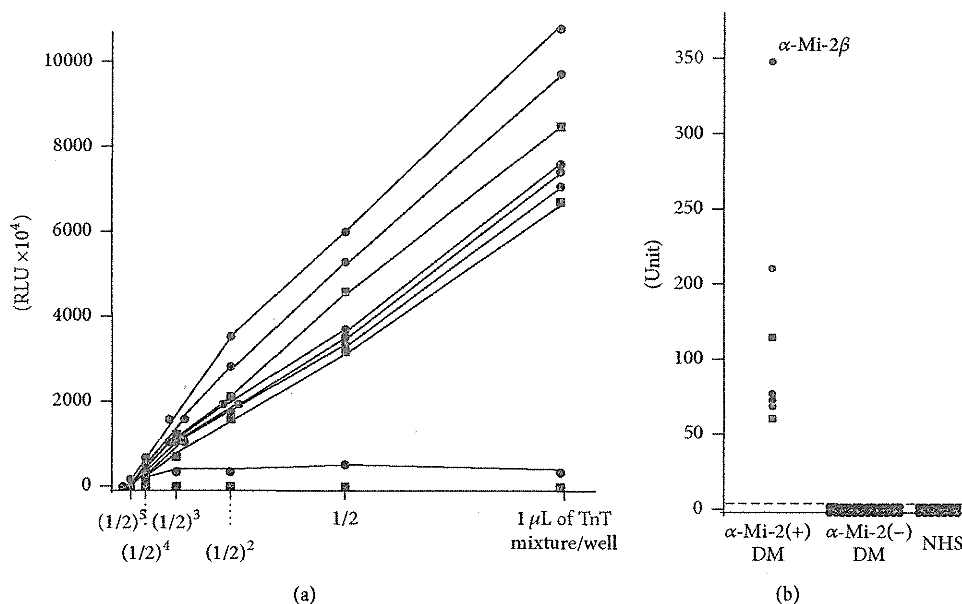

FIGURE 2: ELISA using biotinylated recombinant Mi-2β protein. (a) Serial dilution of biotinylated *in vitro* translation and transcription product for ELISA. Red circles: anti-Mi-2 positive sera defined in our previous analysis. Red squares: newly identified anti-Mi-2 positive sera. Black circles: serum from patients with DM having high background. Black squares: healthy individual serum. Recombinant protein was diluted with T-PBS to 50 μL of the final volume per well. Serum dilution was 1 : 1,000. RLU = relative luminescence unit. (b) Measurement of anti-Mi-2β antibodies in 128 serum samples from patients with DM or 20 healthy control subjects (NHS). We used the 0.5 μL/well of TnT mixture and patient serum samples diluted to 1 : 1000 for measuring all samples. Antibody units were calculated from the RLU using a standard curve obtained from serial concentrations of a serum sample containing a high titer of the anti-Mi-2β antibody. The broken line indicates the cut-off value (0.53 units).

from 100 kDa to 200 kDa. Some antigens may be insufficiently expressed in standard cultured cell lines. Although many commercial measuring kits can be purchased, commercial laboratory kits for new diagnostic autoantibodies are not readily available [26]. Traditional methods that use purified recombinant proteins are labor intensive and may take months to obtain proteins of sufficient purity and optimization prior to ELISA development. This process limits the rapid serologic analysis of novel antigens. Recently, many purified recombinant proteins have become commercially available. However, they are usually expensive, and the various kinds of epitope tags depend on each company. It is often difficult to find commercially available recombinant proteins of large molecule size as full-length proteins. For example, Mi-2 antigens are >200 kDa, and their full-length recombinants are not yet commercially available in Japan.

Table 1: Comparison of clinical data for patients with anti-Mi-2 antibody in a previous report and in the present study.

	Multicenter study*	This study
Anti-Mi-2 (+) pts., number (%)	9/376 (2.4)	7/124 (5.6)
Age at onset, median (range), y	45 (16–66)	62 (40–73)
Sex, M/F, number	6/3	1/6
Diagnosis, %		
Classical DM	100	100
Clinically amyopathic DM	0	0
Clinical features, %		
Muscle weakness	100	100
Arthritis	11	14
ILD	11	0
Malignancy	0	0
Skin eruptions, %		
Heliotrope rash	67	57
Facial erythema**	56	100
Gottron sign	89	100
Prognosis (alive), %	100	100

*Data from [10]. This cohort includes 7 patients used in this study, all of whom were negative for anti-Mi-2 antibodies. **Facial erythema other than heliotrope rash.

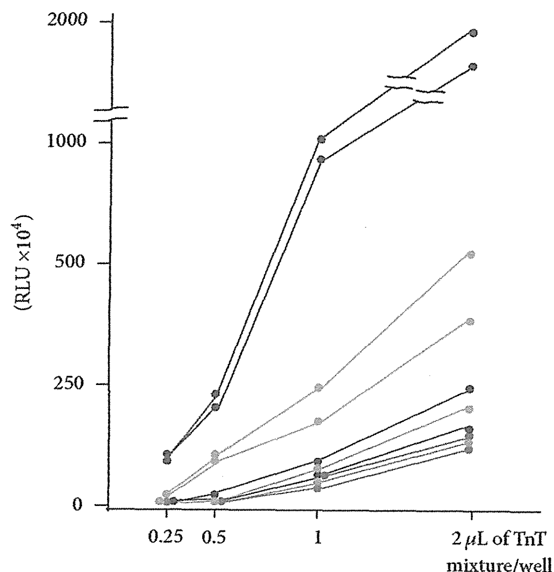

Figure 3: ELISA using biotinylated recombinant proteins of 5 different DM-specific autoantigens. Serial dilution of biotinylated *in vitro* translation and transcription product for ELISA using 2 representative positive sera from DM patients. Serum dilution was 1:1,000. Red circles: Mi-2β. Yellow circles: MJ (NXP-2). Purple circles: TIF1-α. Pink circles: MDA5. Green circles: TIF1-γ. RLU = relative luminescence unit.

In some countries, various recombinant DM autoantigens, including Mi-2β, have recently become commercially available, for example, from Diarect AG (Freiburg, Germany) and SurModics, Inc. (Eden Prairie, MN, USA).

Our assay has several limitations for detecting antibodies. When recombinant proteins are prepared by *in vitro* TnT, post-translational modification does not fully occur. Some autoantibodies are regarded as recognizing the post-translational modification of the protein [27]. Autoantibodies to insulinoma-associated protein 2 (IA-2), which are a serological marker of insulin-dependent diabetes mellitus, preferentially react to baculovirus-expressed IA-2 slightly better than *in vitro* translated IA-2 reacts [28, 29]. Autoantibodies to thyrotropin receptor (TSHR) in Graves' disease do not efficiently bind to the TSHR recombinant produced in an *in vitro* TnT system [30, 31]. The above data are probably due to the glycosylation of IA-2 and TSHR, which plays an important role in autoepitopes of these antigens and may occur in baculovirus expression system but not in TnT system.

The expected amounts of recombinant protein derived from the positive control plasmid are in the range of ~ 300 ng of protein in a standard 50 μL reaction, according to manufacturer's instructions. Protein expression with *in vitro* TnT can vary from batch to batch, and quantification of the resulting protein concentration is challenging. Estimating incorporation levels of biotinylated lysine is more difficult. Although large proteins are sometimes difficult to express as recombinant full product, we succeeded in producing Mi-2β by using a TnT system [4]. Recently, we have also succeeded in producing larger autoantigens, for example, envoplakin and periplakin, by using this system [32].

The biggest potential issue with our system is that the presence of biotinylated lysines may affect epitope recognition by the autoantibodies. We compared our ELISA results of representative positive sera for 5 different DM-marker autoantigens: Mi-2β, MJ (NXP2), MDA5, TIF1-α, and TIF1-γ (Figure 3). Recombinant Mi-2β was much more highly reactive than the other antigens. Although the reason is obscure, several possibilities are considered. The ratio of lysine content in Mi-2β (9.2%) is the highest among these antigens and, interestingly, the sequence has 3 short lysine stretches consisting of 5 or 6 residues at the N-terminus, which may incorporate biotin-labeling efficiently.

An improved method can be considered that uses TnT recombinants without biotinylation. Tag polypeptide and its ligand for coating tagged proteins, the binding of which is as strong as biotin-streptavidin binding, can be applied, such as Halo tag and its ligand [33]. We plan to investigate whether an ELISA constructed with a cDNA clone inserted into the Halo tag vector containing T7 promotor and with ligand-coated plate improves the reactivities of autoantibodies, including anti-TIF1-α and anti-TIF1-γ antibodies.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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REVIEW

Biology of the blood–nerve barrier and its alteration in immune mediated neuropathies

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ABSTRACT

The blood–nerve barrier (BNB) is a dynamic and competent interface between the endoneurial microenvironment and the surrounding extracellular space or blood. It is localised at the innermost layer of the multilayered ensheathing perineurium and endoneurial microvessels, and is the key structure that controls the internal milieu of the peripheral nerve parenchyma. Since the endoneurial BNB is the point of entry for pathogenic T cells and various soluble factors, including cytokines, chemokines and immunoglobulins, understanding this structure is important to prevent and treat human immune mediated neuropathies such as Guillain–Barré syndrome, chronic inflammatory demyelinating polyneuropathy, POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein and skin changes) syndrome and a subset of diabetic neuropathy. However, compared with the blood–brain barrier, only limited knowledge has been accumulated regarding the function, cell biology and clinical significance of the BNB. This review describes the basic structure and functions of the endoneurial BNB, provides an update of the biology of the cells comprising the BNB, and highlights the pathology and pathomechanisms of BNB breakdown in immune mediated neuropathies. The human immortalised cell lines of BNB origin established in our laboratory will facilitate the future development of BNB research. Potential therapeutic strategies for immune mediated neuropathies manipulating the BNB are also discussed.

INTRODUCTION

The presence of the blood–nerve barrier (BNB) restricts the movement of soluble mediators and leucocytes from the blood contents to the peripheral nervous system (PNS) parenchyma. As the endoneurial homeostasis protected by the BNB is a prerequisite for the proper function of the PNS,[1] pathological breakdown of the BNB may be a key event that induces various peripheral neuropathies. However, compared with its CNS counterpart, the blood–brain barrier (BBB), only limited knowledge has been accumulated with regard to the function, cell biology and clinical significance of the BNB. The aims of this article are to review the recent progress in research about the cell biology of BNB composing cells, to discuss the importance of BNB breakdown in immune mediated neuropathies to better understand their pathogenesis and to provide an overview of the development of novel therapies against these currently intractable disorders.

STRUCTURE AND FUNCTION OF THE BNB

The BNB is located at the innermost layer of the perineurium and at the endoneurial microvasculature in the PNS.[2] Although the perineurial barrier is also an important structure, this article focuses on the BNB at the endoneurial microvessels because the infiltration of mononuclear cells and leakage of soluble factors across this microvascular barrier is a key step in the development of immune mediated neuropathies, including Guillain–Barré syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), multifocal motor neuropathy (MMN), etc.

Endothelial cells composing small endoneurial vessels are normally non-fenestrated and contain few pinocytotic vesicles, and the adjacent endothelial cells are connected by complex and continuous tight junctions. These endothelial features are shared by those of the BBB and are considered to constitute the anatomical basis of the BNB that isolates the endoneurium from the intravascular component, inhibiting non-specific transcellular passage and paracellular diffusion of hydrophilic molecules. In addition, the endothelial cells forming the BNB express various receptors and transporters which remove toxic metabolites to maintain PNS homeostasis, and help to incorporate necessary compounds into the PNS parenchyma.[3] Thus the BNB is not just a 'barrier' or 'wall' but a competent interface which actively exchanges materials between the endoneurial microenvironment and the surrounding extracellular space or blood. The term 'blood–nerve interface' has also been proposed for this feature.[1]

The structure of the BNB is essentially different from that of the BBB (figure 1). The highly differentiated endothelial cells composing both the BNB and BBB are completely surrounded by a basement membrane with embedded pericytes. However, in the BBB, the entire abluminal aspect of this endothelial cell/pericyte/basement membrane complex is further ensheathed by a unique structure called the glia limitans perivascularis, consisting of a basement membrane composed of laminins, which is distinct from that of the endothelial basement membrane,[4] and by an astrocytic endfoot layer. The latter two structures, glia limitans perivascularis and astrocytic endfoot, are not present in the BNB.

As it was believed that astrocytes play a major role in BBB maintenance by regulating local water transport and by producing various growth factors and cytokines relevant for barrier maturation and maintenance, the BNB, lacking in astrocytes, has

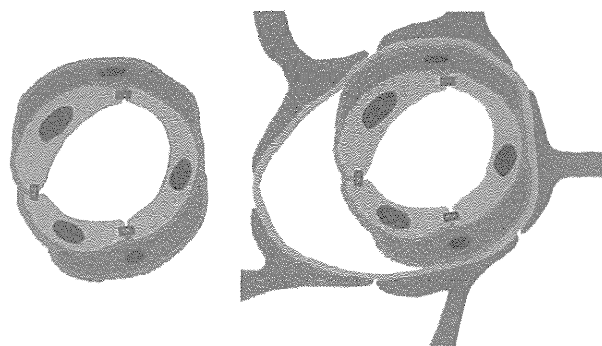

Figure 1 Basic features of the blood–nerve barrier (BNB) and blood–brain barrier (BBB). Endothelial cells (yellow) of the BNB (left) and BBB (right) are connected by tight junctions (red rectangles) and embedded in a single basement membrane (pink) with surrounding pericytes (blue). In the BBB, the second basement membrane (light green; glia limitans perivascularis) wraps the whole endothelial cell/first basement membrane/pericyte complex, and the astrocytic endfoot layer (dark green) surrounds the outer surface. The empty space between the first and second basement membranes is called the perivascular space, which is observed in postcapillary venules and drains into the CSF, and there are occasional antigen presenting cells contained in this space.

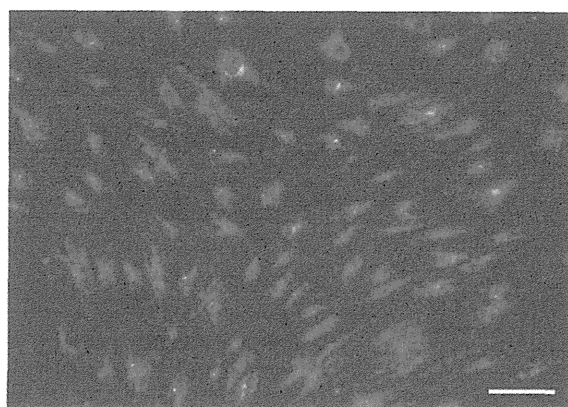

Figure 2 A human immortalised endothelial cell line of endoneurial origin. A fluorescent micrograph of the DiI-Ac-LDL incorporating cells. Bar=100 μm.

long been thought to be 'leakier' and 'weaker' than the BBB.[5] However, Poduslo *et al*[6] demonstrated that the BNB and BBB may be equally effective in excluding small and large molecules from the neural parenchyma in vivo. This means that the healthy PNS parenchyma is tightly protected by the BNB. Hence, similar to the CNS malfunction that arises due to BBB impairment, destruction of the BNB may impair endoneurial homeostasis and allow toxic substances and immunoglobulins to enter the endoneurium, leading to the onset and further worsening of immune mediated peripheral neuropathy.

CELLULAR BIOLOGY OF THE BNB

To understand the biological basis of the BNB, immortalised BNB cell lines that possess in vivo characteristics are the most potent tool. However, compared with the recent developments in BBB research based on primary cultured and immortalised endothelial cells of BBB origin,[7 8] in vitro studies of the BNB have fallen far behind. In the 1990s, two reports concerning the primary culture of rat[9] and bovine[10] endoneurial endothelial cells were published, and we conducted in vitro studies using bovine primary culture cells.[11 12] However, bovine primary culture cells apparently have some limitations with regard to evaluating the molecular evolution of BNB composing cells, and we have moved forward to using cells from rodents and humans. Using rat or human sciatic nerve tissue as a starting material, we sequentially established an immortalised rat endothelial cell line (TR-BNBs[3]), followed by a rat pericyte cell line,[13] a human endothelial cell line[14 15] (figure 2) and a human pericyte cell line.[16]

The endothelial cells forming the BBB express many tight junction associated molecules, including occludin,[17] claudin-5,[18] claudin-12,[18] ZO-1, ZO-2 and JAM-A.[19] These molecules compose tight junctions and limit the paracellular permeability in order to maintain the brain microenvironment, thus leading to barrier properties. Our human and rat endothelial cell lines of BNB origin share these tight junction molecules. Compared with the rat and human immortalised BBB cell lines also established in our laboratory,[8 20] most of the molecular (tight junction proteins, transporters, etc) and physiological (permeability measured by ^{14}C-inulin and transendothelial electrical resistance) characteristics were similar, except that the BNB endothelial cells did not express efflux transporters OAT3 and Oatp2, which, respectively, carry homovanillic acid and dehydroepiandrosterone from the nervous system to the blood. The absence of OAT3 and Oatp2 in endothelial cells of BNB origin seems reasonable, and may reflect an important difference between the microenvironments of the CNS and PNS—namely, that the former has synapses while the latter does not. This finding also supports the concept that the BNB is a distinct structure that differs from the BBB at the cellular and molecular levels.

Pericytes are polygonal cells located at the periphery of the microvessel wall and wrap it with their processes (figure 1). Although having largely been ignored in the clinical literature for a long time, brain pericytes are now recognised as important cellular constituents of the BBB and actively communicate with other cells of the neurovascular unit, such as endothelial cells, astrocytes and neurones.[21] Pericytes also exhibit phagocytic activity and may function as pluripotent stem cells, capable of forming neurones and glia.[22] Hence peripheral nerve pericytes may take part in BNB maintenance, tissue repair and revascularisation after PNS injury. Another important piece of information confirmed by these immortalised BNB cell lines is that peripheral nerve pericytes maintain the physiological BNB function and enhance the expression of endothelial tight junction molecules through their secretion of various soluble factors, especially basic fibroblast growth factor.[16] It can thus be concluded that in the BNB, peripheral nerve pericytes play the same role as astrocytes in the BBB. Peripheral nerve pericytes also produce several neurotrophic factors, such as nerve growth factor, brain derived neurotrophic factor and glial cell derived neurotrophic factor,[16] which may facilitate axonal regeneration in peripheral neuropathy. These neurotrophic factors easily gain access to axons and Schwann cells because pericytes are located just behind the continuous endothelial cells and nothing interferes with the diffusion of neurotrophic factors released by pericytes. This means that early and complete recovery of BNB function in immune mediated neuropathy by immunomodulatory therapy could be beneficial in two aspects: in stopping the inflammatory cell/humoral factor intrusion into the PNS parenchyma and in facilitating axonal regeneration after the recovery in the *milleu intérieur* of the PNS, with the aid of neurotrophic factors secreted from healthy pericytes.

BNB ALTERATION IN IMMUNE MEDIATED NEUROPATHIES

Because the healthy PNS is tightly sealed by the BNB, intrusion of pathogenic T cells, as well as humoral factors (including immunoglobulin), into the PNS parenchyma follows BNB impairment. In particular, microvessels in several important structures in the PNS, including the dorsal root ganglia and nearby spinal roots[1 23] as well as neuromuscular junctions (NMJ),[24] are devoid of barrier properties; hence these sites are believed to be especially vulnerable in inflammatory neuropathies[25 26] because macromolecules such as immunoglobulins can easily penetrate into the PNS parenchyma through their leaky microvasculatures. However, in NMJ, antibodies against presynaptic axons were promptly incorporated into axons and underwent retrograde transport.[27] This rapid clearance of autoantibodies, which has rarely occurred in nodes of Ranvier, may result in relative sparing of NMJ as the site of complement mediated injury in immune mediated neuropathies, including Guillain–Barré syndrome,[28] despite the absence of BNB at NMJ.

Biomarkers that enable detection of BNB breakdown are not well established. Although elevated levels of soluble adhesion molecules,[29] chemokines[30] and matrix metalloproteinases[31] in serum and CSF observed in patients with neuropathy may be indicative of T cell migration across the BNB, only pathological examination and MRI are reliable methods that can presently be used to assess BNB derangements in human immune mediated neuropathies. Enhancement of the spinal root, cauda equina and peripheral nerve trunk in T1 weighted MRI with gadolinium enhancement[32] is occasionally observed in immune mediated neuropathies, and is interpreted as a hallmark of BNB breakdown. This finding should be carefully interpreted in the spinal root because the BNB is physiologically lacking in the dorsal root ganglia and nearby spinal roots, as mentioned above.

PATHOLOGY OF BNB DERANGEMENTS

Morphological abnormalities of endothelial cells constituting the BNB have occasionally been reported in various neuropathies. As autopsy specimens are not adequate to evaluate the fine ultrastructural changes of the BNB, including endothelial fenestration and tight junction impairment, most of the reliable documentation has been based on nerve biopsy specimens. In immune mediated neuropathies, fenestration of endoneurial microvessels,[33 34] gaps between adjacent endothelial cells[35] and the disappearance of tight junctions have been reported in CIDP[34] and macroglobulinaemic neuropathy.[33 35 36] Monoclonal IgM in two[35 36] out of these three cases showed antimyelin associated glycoprotein (MAG)/sulfoglucuronosyl paragloboside (SGPG) activity. As we previously reported that anti-MAG/SGPG IgM was toxic and induced permeability change in primary cultured bovine endothelial cells of BBB origin,[37] the detrimental effect of anti-MAG/SGPG antibody against BNB is highly plausible. We also found that autoimmune demyelinating neuropathy patients with anti-glycosphingolipid (GSL) antibodies showed more severe BNB disruption than those without anti-GSL antibodies or non-autoimmune neuropathy patients.[34] The most commonly observed change in endoneurial microvessels in anti-GSL antibody positive autoimmune demyelinative neuropathy patients was the finding of continuous spaces lacking tight junctions between endothelial cells. These morphological abnormalities may be the result of direct attacks by anti-GSL antibodies against GSL epitopes on the luminal surface of the endothelial cells forming the BNB, because primary cultured bovine endothelial cells of BNB origin are known to contain various GSLs, including GM1, GD1a and GD1b.[10] The effect of anti-GSL antibodies[11] and the sera from Guillain–Barré syndrome patients[12] on opening the BNB was also confirmed by in vitro studies using bovine BNB derived endothelial cells.

These pathological changes of endoneurial microvessels are likely the result of alterations of the tight junction proteins in endothelial cells. We found downregulation of claudin-5 and altered localisation of ZO-1 in biopsied sural nerves obtained from CIDP patients.[38] So far, that is the only report that has confirmed changes in tight junction proteins in immune mediated neuropathies using human materials. The changes may have been due to upregulation of proinflammatory cytokines and vascular endothelial growth factor (VEGF) in the sera of CIDP patients[39] because the endothelial cells forming the BNB are the only cells in the PNS directly exposed to the serum contents. We recently confirmed downregulation of claudin-5 in a human BNB derived endothelial cell line by VEGF and proinflammatory cytokines,[14 16] and demonstrated its upregulation after corticosteroid treatment.[15]

Multifocal motor neuropathy

T1 weighted MRI with gadolinium enhancement is the most reliable method to evaluate BNB breakdown in the peripheral nerve trunk where the tightness of the BNB is normally guaranteed. Kaji *et al*[32] described the enhancement of the enlarged median nerve at the site of conduction block in one MMN patient and showed clustering of denuded or thinly myelinated axons around the endoneurial microvessel in another MMN patient. Although the ultrastructural alterations of endoneurial microvessels have not been described in detail, it may reflect focal BNB derangements in MMN. The presence of anti-GM1 autoantibodies in the sera of MMN patients may be related because GM1 is present in endothelial cells forming the BNB,[10] and anti-GM1 monoclonal antibodies can open the BNB without the need for complement components.[11] One of the most intriguing findings in this disorder is the presence of multifocal persistent conduction block which is not related to the common compression sites; however, no clear explanation of this multifocality has been provided so far. We speculate that putative humoral factor(s) present in patients' sera firstly reacts with the endothelial cell composing BNB, followed by a vicious cycle of local cytokine release, upregulation of adhesion molecules and T cell recruitment, which may result in persistent conduction block at the site of broken BNB. Screening of MMN sera for compounds that lead to BNB breakdown is now underway in our laboratory.

POEMS (Crow–Fukase) syndrome

In the context of microvascular changes in immune mediated neuropathies, POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein and skin changes) syndrome cannot be ignored. In POEMS syndrome, hyperplasia of endoneurial microvessels[40] and endoneurial oedema[41] have frequently been reported. These pathological alterations may be closely related to the high concentration of serum VEGF, a potent multifunctional cytokine that induces angiogenesis and microvascular hyperpermeability.[11] Second, the microangiopathy caused by the above VEGF effect reduces the oxygen supply, which leads to the robust expression of HIF-1α by all of the constituents of the nerve, with a secondary increase in local VEGF expression causing a self-perpetuating VEGF mediated toxic gain of function.[42] Serum VEGF induced BNB

dysfunction may occur throughout the whole PNS, not necessarily confined to the sites of weak BNB: this may explain the neurophysiological findings that the nerve trunk is predominantly affected in POEMS syndrome.[43 44] Scarlato *et al* also showed a 'gap' between adjacent endoneurial endothelial cells, suggesting BNB impairment in one case, but this change is different from the change that we previously reported in inflammatory neuropathies,[34] and pathological alterations directly indicating BNB destruction (loss of tight junctions, gaps between adjacent endothelial cells, etc) are relatively rare in biopsied sural nerve specimens obtained from patients with POEMS syndrome (unpublished observations).

Diabetic neuropathy

While diabetic neuropathy is not usually categorised as an immune mediated neuropathy, microvascular changes are one of the pathological hallmarks of this disorder, and autoimmune abnormalities are evident in at least some patients with diabetic neuropathy. Microvascular abnormalities in diabetic neuropathy patients have long been reported, including loss of tight junctions,[45] hypertrophy of the microvascular basement membrane[46] and loss of microvascular pericytes.[46] Among these changes, basement membrane hyperplasia around endoneurial microvessels possessing BNB function is the most conspicuous pathological abnormality in diabetic patients. This may cause BNB derangement as well as hypoxia in the endoneurial space, which result in further worsening of peripheral neuropathy; however, the pathogenesis remains unclear. Using our human in vitro BNB model, we recently found that advanced glycation end products induce basement membrane hypertrophy and BNB disruption by increasing the autocrine secretion of VEGF, as well as by augmenting transforming growth factor β signalling from microvascular pericytes under diabetic conditions.[14]

FUTURE STRATEGIES TO MANIPULATE THE BNB

The basic structure of the BNB is less complicated than that of the BBB because it lacks the glia limitans perivascularis and astrocytic endfoot. This has two major implications from a therapeutic point of view: pathogenic T cells can enter the PNS parenchyma more easily than the CNS because there is no acellular barrier (glia limitans) present to prevent non-activated T cell intrusion. As the glia limitans contains laminin isoforms which are different from those of the endothelial basement membrane,[4 47] immature encephalitogenic T cells cannot bind to the glia limitans and thus cannot enter the CNS parenchyma. To breach this acellular barrier provided by the glia limitans, T cells need to re-encounter a cognate antigen in the context of major histocompatibility complex class II bearing antigen presenting cells in the perivascular space (the space between the endothelial basement membrane and the glia limitans) and acquire matrix metalloproteinase -2 and -9 activity.[48] To enter the PNS parenchyma, T cells do not need to undergo this complex process, so blockade of T cell invasion just at the endothelial monolayer may be more important in the treatment of immune mediated neuropathies such as CIDP.

Recruitment of circulating T cells into the tissue parenchyma has been shown to begin by the sequential interaction of different adhesion and/or signalling molecules on T cells and endothelial cells in a multistep cascade.[49] These sequential steps include rolling, adhesion, crawling and diapedesis of lymphocytes, and each process has its own molecular pathway. The same cascades may be relevant in the T cell/endothelial cell interaction in the BNB although there is no direct evidence that these processes are identical in the BNB and BBB or other organs.

Natalizumab, a humanised monoclonal antibody against the α4 integrin which strongly interferes with the 'adhesion' process, is now widely used for the treatment of relapsing forms of multiple sclerosis. This drug is theoretically also effective against immune mediated neuropathies, including CIDP,[50] but a therapeutic trial in 61-year-old intractable CIDP failed.[51] This result does not necessarily indicate that natalizumab will not be effective against immune mediated neuropathies, and further controlled studies are necessary. We recently found that corticosteroids, the firstline treatment for CIDP, enhance BNB function via upregulation of claudin-5.[15] Further screening of candidate drugs that can upregulate claudin-5 and augment BNB integrity using our in vitro human BNB model are now underway.

The second implication of this simpler structure is that only pericytes should be considered as the influential cell population affecting endothelial function. Endothelial cells and pericytes are embedded together in one basement membrane (endothelial basement membrane) and thus these two cells are in close proximity so the paracrine secretion of growth factors and cytokines from nearby biologically active pericytes may strongly influence endothelial function. These factors may be key to maintaining BNB function.[13 16] The paracrine secretion of transforming growth factor β from endoneurial pericytes disrupts the BNB and causes basement membrane hypertrophy around the endoneurial microvessels in diabetic neuropathy.[14] A possible method to manipulate the BNB for therapeutic purposes is to modify endothelial function using oligonucleotides, siRNAs and virus vectors. Because endothelial cells forming the BNB are the only cells that come into direct contact with the blood constituents in the PNS, endothelial cells can be easily manipulated via the system circulation. Another possible method is to modify BNB pericytes: small hydrophobic substances that can reach the pericyte membrane through the endothelial monolayer and strengthen pericytic activity, including release of various cytokines/chemokines that influence endothelial function, may also be useful as drug candidates to control BNB function. The latter strategy may be more promising because indirect manipulation of endothelial cells, the principal regulators of BNB integrity, via humoral factors from pericytes would be more physiologically relevant than their direct modification.

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ORIGINAL ARTICLE

Age at onset and gender distribution of systemic lupus erythematosus, polymyositis/dermatomyositis, and systemic sclerosis in Japan

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Abstract
Objectives The aim of this study was to describe age, gender distribution, and age at onset of systemic lupus erythematosus (SLE), polymyositis/dermatomyositis (PM/DM), and systemic sclerosis (SSc) in Japan.
Methods We used epidemiological information on 21,405, 6,327, and 10,058 patients with SLE, PM/DM, and SSc, respectively, in a Japanese nationwide registration database of patients with intractable diseases.
Results All three diseases occur predominantly in women, with the female-to-male ratio being 8.2:1, 2.6:1, and 7.7:1 for SLE, PM/DM, and SSc, respectively. The most susceptible age for SLE is 15–44 and 20–39 years for males and females, respectively. For PM/DM it is 45–64 and 40–64 years and for SSc, 50–69 and 40–59 for men and women, respectively.
Conclusions The basic descriptive epidemiological characteristics of SLE, PM/DM, and SSc in Japan, such as gender distribution, present age, and age at onset, were surveyed nationwide for fiscal 2007. It was found that these characteristics were similar to those in Western populations. Our finding provides new information on the natural history of disease development.

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Keywords Age at onset · Epidemiology · Polymyositis/dermatomyositis (PM/DM) · Systemic lupus erythematosus (SLE) · Systemic sclerosis (SSc)

Introduction

Systemic lupus erythematosus (SLE), polymyositis/dermatomyositis (PM/DM), and systemic sclerosis (SSc) are systemic autoimmune diseases. Their chronic intractable nature has a significant impact on medical care utilization, activity of daily living, and quality of life. As these diseases are relatively rare, their epidemiological characteristics have not yet been described in detail in Japan. In such rare diseases, accumulation of large numbers of patients from the entire country is necessary for informative epidemiological studies.

The National Programme on Rare and Intractable Diseases was launched in Japan in 1972. Since then, the government has promoted research and expanded support for patients with a number of such diseases [1]. This programme established a nationwide registration system for patients with intractable diseases, including SLE, PM/DM, and SSc. Here, we describe age, gender distribution, and age at onset of these diseases using data from the registration system.

Materials and methods

Data sources

The Japanese government has established a nationwide registration system for patients with intractable diseases

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under which registered patients are eligible for financial aid from the government for their treatment. Details of the registration system have been described elsewhere [1]. Most patients with SLE, PM/DM, or SSc are expected to be registered as having a designated intractable disease, although not all registered patients' data have been converted to electronic form. The electronic database has been effectively utilizable for epidemiological research since 2003. After obtaining permission from the Ministry of Health, Labour and Welfare (MHLW) of Japan, we used data of fiscal year 2007 consisting of patient sex, age, birth year, and disease-onset year.

Statistical analysis

We calculated the electronic data entry rate as the number of patients whose data was converted into electronic form divided by the total number of patients enrolled in the registration system. The latter information is contained in MHLW's Report on Public Health Administration and Services [2]. We ascertained age at disease onset as onset year minus birth year. Using onset age, we determined the most susceptible age as the minimum range that includes peak onset age and 50 % of onsets. All statistical analyses were performed with SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

Ethical considerations

All data provided by the MHLW are anonymous, and researchers cannot access personal information about any patient.

Results

Table 1 shows the number of patients with SLE, PM/DM, and SSc whose data was converted into electronic form and the electronic data entry rate in fiscal 2007. We used electronic data for 21,405, 6,327, and 10,058 SLE, PM/DM, and SSc cases, respectively. The numbers of all patients registered with the database for the MHLW's Report on Public Health Administration and Services were 55,021 SLE and 37,975 PM/DM and SSc (PM/DM and SSc were not reported separately) [2]. The proportion of all patients with electronic data entered was therefore 39 % for SLE and 43 % for PM/DM and SSc. Estimating the number of all PM/DM- and SSc-registered patients separately, the number would be 14,714 (6,327/0.43) PM/DM and 23,391 (10,058/0.43) SSc. These can be considered to be the total number of registered patients in the entire Japanese (population 126 million).

Table 1 Number of patients with SLE, PM/DM, and SSc whose data was converted into electronic form, and the electronic data entry rate in fiscal 2007

Diseases	No. of electronic entries [a]	Total No. of patients [b]	Electronic data entry rate (a/b)
SLE	21,405	55,021	0.39
PM/DM	6,327	37,975	0.43
SSc	10,058		

SLE systemic lupus erythematosus, *PM/DM* polymyositis/dermatomyositis, *SSc* systemic sclerosis

[a] The number of patients whose data was converted into electronic form

[b] Total numbers of patients enrolled in the registration system was obtained from the 2007 Report on Public Health Administration and Services [2]

Table 2 Number of male and female patients with SLE, PM/DM, and SSc in fiscal 2007

Diseases	Total	Male	Female	Sex ratio
SLE	21,405	2,336	19,069	8.2
PM/DM	6,327	1,735	4,592	2.6
SSc	10,058	1,157	8,901	7.7

Sex ratio (female/male)

SLE systemic lupus erythematosus, *PM/DM* polymyositis/dermatomyositis, *SSc* systemic sclerosis

Table 2 shows the number of patients with SLE, PM/DM, and SSc stratified by sex. All three diseases predominantly affect women, with a female-to-male ratio of 8.2:1, 2.6:1, and 7.7:1 for SLE, PM/DM, and SSc, respectively.

Figure 1 shows the age distribution of male and female SLE, PM/DM, and SSc patients. The prevalence of SLE in women showed two peaks, at age 35–39 and 55–59 years, with a wide age distribution. The distribution of PM/DM was similar to SSc, with only a small number of patients <50 years, and peak prevalence at 55–59 years for PM/DM and 65–69 years for SSc. SLE distribution in men showed no significant age peak, and PM/DM and SSc were similar to that seen in women.

Figure 2 shows the distribution of age of onset. SLE onset peaked at 25–29 years in women, decreasing thereafter. Onset of both PM/DM and SSc in women also had one peak, but later, at 50–54 years of age, with PM/DM tending to have a younger onset than SSc. Age of SLE onset showed no peak in men, and again, PM/DM and SSc was similar in men and women.

These data on age at onset are summarized in Table 3. For SLE patients, age at the 50th percentile was 35 and

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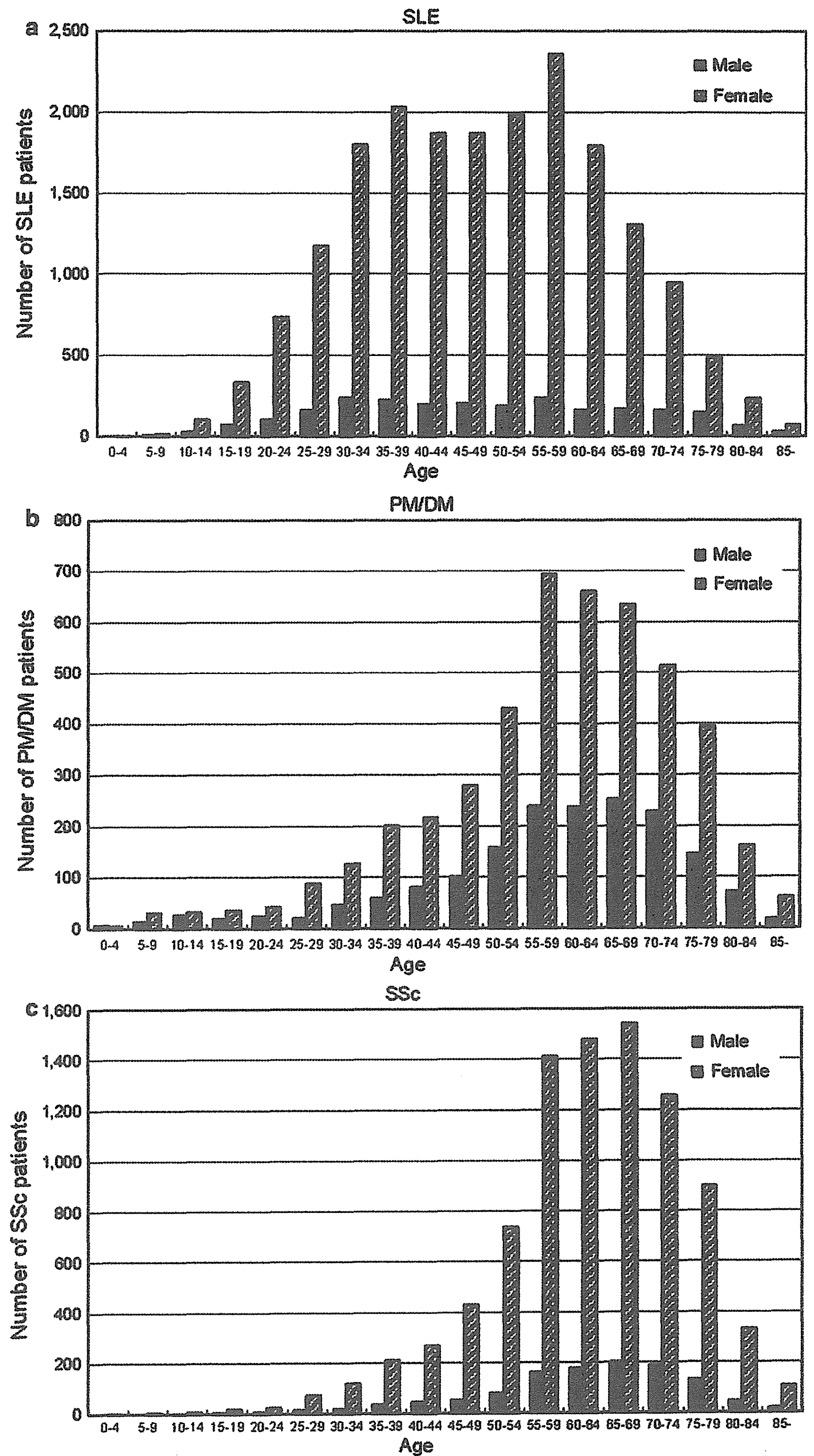

Fig. 1 Age distribution of SLE, PM/DM, and SSc in fiscal 2007. **a** *SLE* systemic lupus erythematosus, **b** *PM/DM* polymyositis/dermatomyositis, **c** *SSc* systemic sclerosis. *Solid bars* show the number of male patients, and *bars* with *right-slanting lines* show the number of female patients, by age

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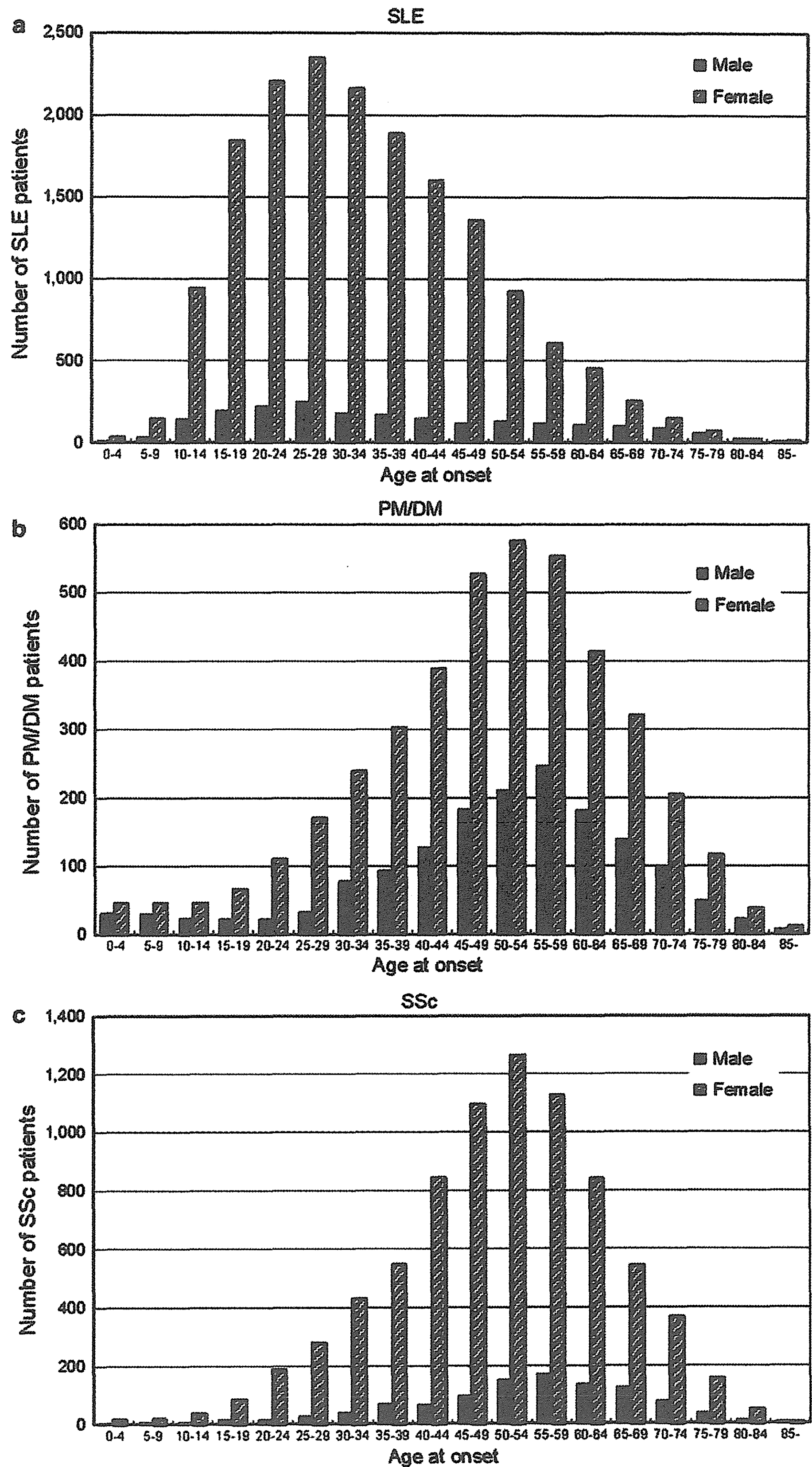

Fig. 2 Distribution of age at onset of SLE, PM/DM, and SSc in fiscal 2007. **a** *SLE* systemic lupus erythematosus, **b** *PM/DM* polymyositis/dermatomyositis, **c** *SSc* systemic sclerosis. *Solid bars* show the number of male patients, and *bars* with *right-slanting lines* show the number of female patients, by age at onset

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