patients suffering from hematological malignant diseases. For the practice of autologous HSCT against those malignant diseases, graft manipulation using antibody specific for CD34, a marker of human hematopoietic stem cells, is usually essential to deplete malignant cells from the graft. On the other hand, patients treated with CD34+-selected autologous HSCT (CD34-HSCT) may have infectious complications during hematological recovery more frequently than patients treated with unselected autologous HSCT (unselected-HSCT)[11]. The graft manipulation was performed in many patients with severe autoimmune diseases treated by autologous HSCT in consideration of depleting autoreactive lymphocytes and inducing profound clinical remission. Meanwhile, it has been debated whether CD34+ cell selection in the graft is necessary or not[9,12].

The difference in conditioning regimens is not related to the clinical benefits, and about one-third of transplanted patients do not benefit from these intensive immunosuppressive treatment[9,13]. Clinical response may depend on profound qualitative immunological changes obtained by autologous HSCT in patients with systemic lupus erythematosus or multiple sclerosis[14,15]. Little is known as to why and how patients with SSc have clinical benefits of autologous HSCT. The aim of our study was to elucidate the relationship between clinical effect and alteration of immunological profiles in patients with SSc treated with autologous HSCT.

MATERIALS AND METHODS

Patients. Our study was approved by the ethical committee of Hokkaido University and written informed consent was obtained from all participants. Thirty-one patients with SSc, all of whom met the American College of Rheumatology preliminary criteria[16], were screened for our study. All patients developing SSc within the last 3 years onset fulfilled at least 1 of the following: early rapidly progressive diffuse skin sclerosis despite continuing treatment, refractory skin ulcers, interstitial lung disease confirmed by lung computed tomography (CT), reversible cardiac involvement such as arrhythmia and cardiomegaly, renal involvement with hypertension, persistent urinalysis abnormalities, and microangiopathic hemolytic anemia. Patients were excluded from the study when they were over 60 years old, or had uncontrolled arrhythmia, left ventricular ejection fraction on echocardiography below 45%, carbon dioxide diffusion lung capacity (DLCO) below 45% predicted, serum creatinine above 176.8 μmol/L (2.0 mg/dl) and glomerular filtration rate (GFR) below 40 ml/min/m^2. All enrolled patients were evaluated clinically at the time of diagnosis and on regular visits for followup.

Thirty-five healthy controls were also enrolled in the study.

Transplantation procedure and followup. The mobilization regimen comprised recombinant human granulocyte colony-stimulating factor (rhG-CSF) and intravenous cyclophosphamide (4 g/m^2). In 5 patients treated with CD34-HSCT, enriched CD34+ graft, prepared using CliniMACS® system (Miltenyi Biotec, Germany) was stored in liquid nitrate until use for transplant. Graft manipulation was not performed in the next 5 patients treated with unselected-HSCT.

We treated all SSc patients with intravenous cyclophosphamide (200 mg/kg, divided into 4 days) followed by autologous HSCT. rhG-CSF was administered from the second day of transplantation of frozen-thawed autologous enriched CD34+ grafts or frozen-thawed autologous unselected grafts. T cell depleting antibodies such as antithymocyte globulin, antilymphocyte globulin and anti-CD52 antibodies (Campath) were not administered in our patients.

We assessed the improvement of skin sclerosis by the modified Rodnan total thickness skin score (mRTSS). Electrocardiogram and echocardiography were used to evaluate the cardiac function, chest radiograph, chest high resolution CT, and spirometry to evaluate pulmonary function, renogram to evaluate renal function, and serological tests to assess other organ involvement and the presence of autoantibodies.

Lymphocyte phenotyping. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized venous blood by Ficoll-Paque Plus® (Amersham Biosciences Corp., NJ, USA).

We assessed the subpopulation of peripheral lymphocytes by immunofluorescence staining of PBMC with anti-human CD3-Cy-Chrome, CD4-fluorescein isothiocyanate (FITC), CD8-FITC, CD19-FITC, TCRγδ-FITC, CD3-phycoerythrin (PE), CD8-PE, CD45RO-PE, CD25-PE, HLA-DR-PE, and CD69-PE (BD Biosciences Pharmingen, San Diego, CA).

The expression levels of interferon (IFN)-γ and interleukin (IL)-4 were studied in the cytoplasm of peripheral CD4+ or CD8+ T cells. Briefly, we stimulated PBMC with phorbol myristate acetate (50 ng/ml) and ionomycin (250 ng/ml) for 6 h in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and monensin (2 μM) at 37°C in 5% carbon dioxide. We evaluated the IFN-γ or IL-4 expression on T cells by staining with anti-CD3-Cy-Chrome, anti-CD8-FITC and -PE, anti-IFN-γ-FITC, and anti-IL-4-PE using Cytofix/Cytoperm Plus® (BD Biosciences Pharmingen) according to the manufacturer's instructions. Immunostained cells were analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Quantification of thymic signal joint T cell receptor rearrangement excision circles (sjTREC). Thymic sjTREC on genomic DNA from PBMC was quantified by real-time quantitative polymerase chain reaction (PCR) (ABI PRISM® 7000; Applied Biosystems, Foster City, CA) according to the method of Douek, *et al*[17]. The sjTREC values were corrected by the percentage of CD3+ cells in the sample and were then expressed as numbers of sjTREC/μg of CD3+ cells DNA according to the method of Farge, *et al*[18]. Values were measured before autologous HSCT, then at 3, 6, and 12 months after autologous HSCT.

Quantification of foxp3 *gene expression levels.* Total RNA were isolated from PBMC using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed by ReverTraAce (Toyobo, Osaka, Japan), in the presence of oligo(dT)12-18 primers (Invitrogen) according to the manufacturer's instructions. We performed real-time PCR using the ABI PRISM® 7000 Sequence Detection System and specific primers for *foxp3* and *gapdh* from TaqMan® Gene Expression Assays (Applied Biosystems).

Statistical analysis. We used the Mann-Whitney U-test to analyze the difference among each value otherwise indicated. The changes in mRTSS and phenotype of lymphocytes after the autologous HSCT were compared with values at inclusion using the Wilcoxon signed rank test. Female-male ratio in each group was assessed using Fisher's exact probability test. The sjTREC values in healthy individuals were assessed using the Spearman's correlation test. Calculations were performed using the statistical software package JMP version 5.0 (SAS Institute Inc., Cary, NC). P values less than 0.05 were considered significant.

RESULTS

Between November 2000 and July 2006, 11 consecutive patients meeting the criteria in our study were enrolled and 10 patients were transplanted out of 31 screened patients with SSc for autologous HSCT treatment. One patient was not transplanted because of her mobilization failure. First 5 patients were treated with CD34-HSCT. Subsequent 5 patients were treated with unselected-HSCT. The character-

istics of patients treated with autologous HSCT are shown in Table 1. Mean age at inclusion, mean mRTSS before mobilization and mean durations from SSc onset to the treatment were similar between patients treated with CD34-HSCT and unselected-HSCT. Several treatments such as D-penicillamine, prostaglandin derivatives, and corticosteroids were not feasible for our patients. All patients were followed up until July 2007 (40.7 ± 25.6 mos).

Mean number of infused CD34+ cells was not different between CD34-HSCT and unselected-HSCT groups. Mean time needed to achieve a neutrophil count greater than 0.5 × 10^9/l and a platelet count greater than 50 × 10^9/l were not different between 2 groups. Cytomegalovirus antigenemia were shown in 3 patients out of all transplanted patients. Patient 2 had hemophagocytic syndrome on day 6. Patient 3 had adenoviral hemorrhagic cystitis on day 14 and engraftment syndrome on day 15. Patient 7 had engraftment syndrome on day 12. Hemophagocytic syndrome and engraftment syndrome responded to corticosteroid administration. Hemorrhagic cystitis was refractory to acyclovir, vidarabine, ganciclovir, or ribavirin. Patient 3 had the second autologous HSCT using unselected grafts at 3 months after first autologous HSCT using selected CD34+ cells due to recurrent infectious diseases.

Four out of 5 transplanted patients have more than a 25% fall in the skin score compared with baseline values in both groups (Figure 1). Dermal thickness assessed by skin biopsy was also improved in these patients with clinical benefits (data not shown). Additional unselected-HSCT at 3 months after CD34-HSCT did not affect Patient 3's skin manifestation. Cardiac and pulmonary functions were not altered significantly through the treatment in all patients (data not shown). Their serum level of γ-globulin almost remained normal range through autologous HSCT (data not shown). Their serum level of anti-Scl70 antibodies reduced except Patient 2 treated with CD34-HSCT (data not shown). Transplantation related complications during hospitalization are shown in Table 1. There was no significant difference in the incidence of adverse events between both groups and no transplantation related mortality.

We compared immunological reconstitution profile over time between good and poor response groups, and between CD34-HSCT and unselected-HSCT groups. First, we analyzed immunological reconstitution between good and poor response groups. Clinical response to therapy was categorized into major, partial, or no response, or disease progression or relapse according to the method of Farge, *et al*[13]. According to the observed clinical response compared to these criteria, 2 groups of patients were retrospectively constituted: good response group, consisting of 7 patients with sustained major or partial response, and poor response group, consisting of 3 patients (Patient 5, 6, and 7) with no

Table 1. Patients' profile at study inclusion and clinical findings at autologous hematopoietic stem cell transplantation (HSCT).

	Patients Treated with CD34-HSCT					Patients treated with Unselected-HSCT					Mean ± SD		
	1	2	3	4	5	6	7	8	9	10	CD34	Untreated	p
Age, yrs	57	19	54	48	52	43	19	42	30	28	46.0 ± 15.4	32.4 ± 10.1	0.094
Sex, female:male	M	F	F	F	M	M	F	F	F	F	3:2	4:1	1.000
mRTSS, 0–51	38	28	25	15	32	32	17	26	23	20	27.6 ± 8.6	23.6 ± 5.8	0.402
Disease duration, mo	21	31	21	12	36	16	24	18	8	12	24.2 ± 9.4	15.6 ± 6.1	0.141
Interstitial pneumonia	—	—	+	—	+	—	+	—	—	—	—	—	—
GFR, ml/min	76.53	121.43	101.43	114.39	99.32	139.29	120.3	101.8	82.62	103.42	102.6 ± 17.2	109.5 ± 21.3	0.465
DLCO %	83	66.8	52.2	90.9	83.8	92.5	54.7	113.4	48	94.4	75.3 ± 15.7	80.6 ± 28.0	0.465
γ-globulin, %	19.5	24.7	24.1	16.8	12.5	20.5	19.8	—	16.8	16.7	19.5 ± 5.1	18.5 ± 2.0	0.712
Anti-Scl 70, index	< 5	92.3	204.6	8.7	158.6	16.1	128.2	< 5	< 5	202	92.8 ± 90.2	69.3 ± 91.5	0.597
Prior therapies	PG	PG, D, PSL	PG, PSL	PG, PSL	D, PSL	PG	PG	D, PSL	D, PSL	PG	—	—	—
Mobilization	G	G + CYC	G + CYC	G + CYC	G + CYC	G + CYC	G + CYC	G + CYC	G + CYC	G + CYC	—	—	—
Conditioning	CYC	CYC	CYC	CYC	CYC	CYC	CYC	CYC	CYC	CYC	—	—	—
Infused CD34+ cells, × 10^6/kg	2.96	5.21	2.75	3.14	12.7	3.95	2.77	4.28	14.9	2.81	5.4 ± 4.2	5.7 ± 5.2	0.917
Purity, %	96	95	90	93.53	96.59	—	—	—	—	—	94.2 ± 2.6	—	—
Neutrophils > 0.5 × 10^9/l (day)	11	9	11	9	9	8	11	10	10	10	9.8 ± 1.1	9.8 ± 1.1	0.914
Platelets > 50 × 10^9/l (day)	15	21	16	8	11	0	8	11	11	12	14.2 ± 5.0	8.4 ± 4.9	0.138
Transplant related complications	CMV	CMV, HPS	CMV, HC, ES	—	—	—	ES	—	—	—	—	—	—

mRTSS: modified Rodnan total thickness skin score; PG: prostaglandin derivatives; D: d-penicillamine; PSL: prednisolone; G: granulocyte-colony-stimulating factor; CYC: cyclophosphamide; CMV: cytomegalovirus antigenemia; HPS: hemophagocytic syndrome; HC: hemorrhagic cystitis; ES: engraftment syndrome; GFR: glomerular filtration rate; DLCO: diffusion capacity for carbon monoxide.

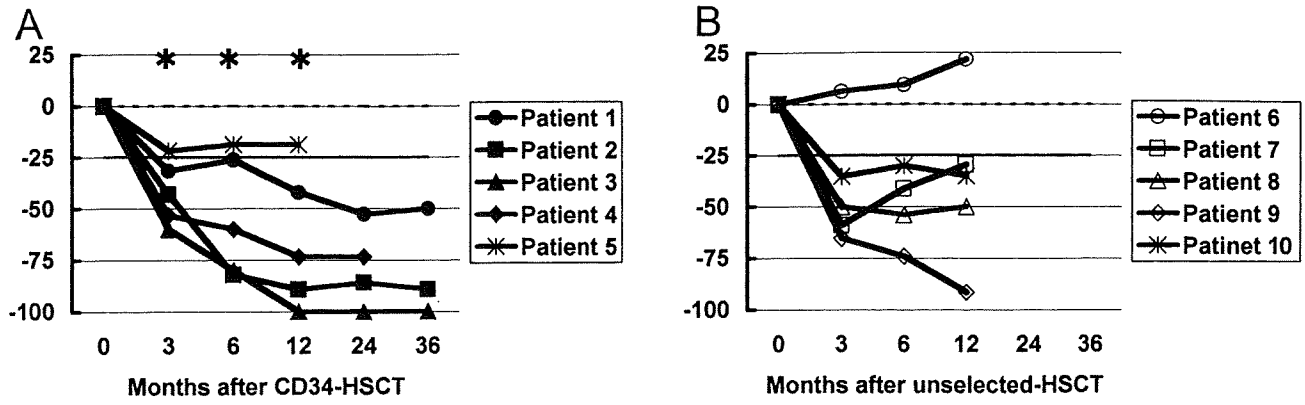

Figure 1. Evaluation of modified Rodnan total thickness skin score (mRTSS) in patients with systemic sclerosis. A. Changes of mRTSS in patients treated with CD34-HSCT. B. Changes of mRTSS in patients treated with unselected-HSCT. Proportional change from baseline measurement was calculated for each patient at each available timepoint. * $p < 0.05$.

response or with relapse of disease (Table 2). Our patients were evaluated by functional evaluation (performance status and/or health assessment questionnaire) and mRTSS with skin improvement assessed by skin biopsy. Each organ function was not altered significantly through the treatment in all patients. Mean age at inclusion, mean mRTSS before mobilization, and mean durations from onset scleroderma to treatment were similar between both groups. At inclusion, the ratio of CD4/CD8, the percentage of CD4+CD45RO+, CD4+CD45RO-, CD19+, CD4+CD25+, CD56+, CD3+TCRγδ+, IFN-γ- and IL-4-producing CD4+ and CD8+ cells were in the normal range for all patients and were not different between good and poor response groups (Table 3). After autologous HSCT, shortened CD4/CD8 ratio was sustained due to delayed CD4+ cell recovery and prompt CD8+ cell recovery in both groups. CD4+CD45RO-naive T cells remained low at 6 months after autologous HSCT in good response group, and CD4+CD45RO- cells reconstituted faster in poor response group ($p < 0.05$). CD19+ and CD56+ cells returned into the normal range at 3 months in both groups. The kinetics of other cells through autologous HSCT was not statistically different between good and poor response group in the study. To evaluate the T cell response against mitogen stimulation after autologous HSCT, mean fluorescence intensity of CD69 on CD3+ cells was investigated. CD69 expression levels on CD3+CD8+ and CD3+CD8- cells against mitogen were not different between healthy controls and patients with SSc before autologous HSCT, and its kinetics through autologous HSCT were similar in both groups (Table 3). Cytokine production in CD3+CD8- and CD3+CD8+ T cells was assessed by intracellular staining of IFN-γ and IL-4. Levels of cytokine production in CD3+CD8- and CD3+CD8+ cells were not different between both groups. IFN-γ producing CD8+ T cells increased after autologous HSCT in both groups (Table 3).

Thymic output assessed by sjTREC was analyzed to evaluate the mechanism of peripheral CD4+CD45RO- and CD4+CD25+ proliferation. In healthy controls, the sjTREC values negatively correlated with their age (Figure 2A, $p < 0.0001$, $r^2 = 0.44$). Nine out of 10 transplanted patients could be analyzed in the study. Their sjTREC values also negatively correlated with their age at inclusion of autologous HSCT (Figure 2B, $p = 0.002$, $r^2 = 0.80$). The sjTREC values were not significantly different between patients with SSc before autologous HSCT and age- and sex-matched

Table 2. Patients' profile between good and poor response groups at autologous HSCT.

	Good Response Group (n = 7)	Poor Response Group (n = 3)	p
Graft condition (CD34-HSCT: unselected)	4:3	1:2	1.000
Age, yrs	39.7 ± 14.4	38.0 + 17.1	0.819
Sex female: male	6:1	1:2	1.000
mRTSS (0–51)	25.0 ± 7.16	27.0 ± 8.66	0.568
Disease duration, mo	17.6 ± 7.72	25.3 ± 10.1	0.207
Infused CD34+ cells (× 10^6/kg)	5.15 ± 4.39	6.47 ± 5.42	0.909
Neutrophils > 0.5 × 10^9/l (day)	10.0 ± 0.82	9.33 ± 1.53	0.407
Platelets > 50 × 10^9/l (day)	13.4 ± 4.28	6.33 ± 5.69	0.064

mRTSS: modified Rodnan total thickness skin score.

Table 3. Phenotype analysis of lymphocyte population through autologous HSCT between patients with good and poor clinical response. Value are mean ± SD.

		At Inclusion		3 mo After HSCT		6 mo After HSCT		12 mo After HSCT	
	Normal Range 95% CI	Good	Poor	Good	Poor	Good	Poor	Good	Poor
CD3+, CD4+	57.57–68.89	48.16 ± 18.77	52.34 ± 6.56	20.84 ± 9.75*	34.42 ± 7.66	23.18 ± 15.00*	45.23 ± 10.74	27.65 ± 15.61***	43.07 ± 9.72
CD3+, CD8+	26.47–37.68	25.91 ± 9.32	33.92 ± 13.66	48.07 ± 21.57	49.64 ± 11.44	35.78 ± 15.44	49.69 ± 13.09	44.97 ± 13.94	47.56 ± 10.43
CD4/CD8	0.61–2.96 (ratio)	2.11 ± 1.31	1.74 ± 0.72	0.43 ± 0.13*	0.71 ± 0.15	0.66 ± 0.35*	1.01 ± 0.56	0.61 ± 0.28	0.96 ± 0.39
CD3+, TCRγδ+	0.74–9.48	3.03 ± 3.05	2.03 ± 1.19	5.11 ± 4.49	2.34 ± 0.84	2.75 ± 1.48	3.02 ± 1.86	4.18 ± 2.72	2.71 ± 1.80
CD4+, CD45RO–	5.23–42.08	28.51 ± 10.29	31.36 ± 8.40	3.43 ± 2.49*	7.89 ± 5.21	4.66 ± 2.93*	10.01 ± 8.46	7.12 ± 5.18	12.55 ± 10.78
CD4+, CD45RO+	9.00–27.97	17.08 ± 5.53	15.35 ± 3.87	15.48 ± 6.23	19.34 ± 5.87	13.68 ± 7.31	16.42 ± 4.85	14.32 ± 4.08	13.89 ± 4.10
CD4+, HLA-DR+	0.92–3.38	2.38 ± 0.86	3.95 ± 2.10	8.51 ± 4.99	12.58 ± 3.23	5.77 ± 4.58	7.12 ± 0.50	5.36 ± 4.16	5.20 ± 2.46
CD4+, CD25+	1.35–5.46	4.12 ± 3.36	5.45 ± 2.79	3.42 ± 2.21	6.12 ± 5.51	3.43 ± 2.67	7.54 ± 3.36	3.55 ± 2.28	4.62 ± 3.92
foxp3 mRNA	32.01–393.07 (copies/GAPDH 1 k copies)	563.39 ± 704.09	259.60 ± 247.27	182.74 ± 150.35	99.31 ± 29.61	201.77 ± 114.85	212.28 ± 121.62	214.00 ± 109.77	166.29 ± 133
CD3+, CD8–, IFNγ+	0.67 ± 17.49	6.05 ± 6.55	2.73 ± 0.60	12.16 ± 10.07	7.06 ± 5.20	8.21 ± 5.36	11.47 ± 8.02	9.29 ± 4.17	4.23 ± 3.66
CD3+, CD8–, IL4+	0.02–2.47	1.09 ± 0.55	1.39 ± 1.30	3.74 ± 2.63	2.50 ± 1.04	2.40 ± 2.13	4.96 ± 6.27	1.57 ± 1.24	1.20 ± 0.58
Th1/Th2	3.79–125.60 (ratio)	17.38 ± 21.04	4.98 ± 3.77	32.47 ± 52.40	107.75 ± 154.29	32.09 ± 45.55	67.05 ± 59.15	24.82 ± 23.42	23.75 ± 36.56
CD3+, CD8+, IFNγ+	0.66–41.60	5.73 ± 6.15	5.25 ± 3.11	29.83 ± 21.39*	19.80 ± 11.29	17.43 ± 13.55	25.93 ± 13.92	27.71 ± 19.13	3.29 ± 4.95
CD3+, CD8+, IL4+	0.00–1.40	0.19 ± 0.17	0.25 ± 0.38	0.86 ± 0.79	0.54 ± 0.38	1.01 ± 1.24	0.68 ± 0.68	0.72 ± 0.45	0.16 ± 0.15
Tc1/Tc2	7.83–185.08 (ratio)	89.68 ± 98.17	56.15 ± 71.29	154.35 ± 217.62	1492.65 ± 2280.82	88.36 ± 78.79	1460.06 ± 1262.52	80.27 ± 76.12	41.91 ± 71.92
CD3+, CD8–, CD69+	82.91–201.89 (MFI)	177.55 ± 90.61	121.10 ± 84.95	58.25 ± 41.65	28.34 ± 12.87	63.06 ± 37.00	67.76 ± 40.69	89.54 ± 42.85	38.52 ± 33.83
CD3+, CD8+, CD69+	54.27–119.07 (MFI)	106.82 ± 35.27	96.05 ± 64.04	30.92 ± 20.39	24.94 ± 12.97	31.00 ± 19.98	53.89 ± 30.39	61.82 ± 19.69	30.83 ± 19.29
CD19+	5.00–32.98	16.16 ± 7.54	12.69 ± 9.40	21.91 ± 22.58	14.40 ± 7.64	27.01 ± 22.93	9.50 ± 5.73	18.60 ± 10.83	11.13 ± 3.99
CD56+	8.94–22.94	13.17 ± 11.67	11.83 ± 7.68	14.11 ± 6.97	9.93 ± 4.49	8.99 ± 2.96	19.83 ± 11.87	12.05 ± 7.58	14.12 ± 7.61

* The value from the baseline measurement was calculated for each value at each timepoint. $p < 0.05$. MFI: mean fluorescence intensity.

healthy controls ($p = 0.8253$). The sjTREC values were significantly suppressed at 3 months after autologous HSCT in the good response group compared with poor responders (Figure 2C, $p = 0.0152$), although the values were not different at inclusion, 6 and 12 months after autologous HSCT between both groups.

Foxp3 is a key regulatory gene for the development of regulatory T cells[19]. *Foxp3* gene expressions in PBMC were analyzed to assess the relationship between the recovery of CD4+CD25+ cells including regulatory T cells and clinical benefits in transplanted SSc patients. *Foxp3* gene expressions in PBMC were within the normal range through autologous HSCT and were not different in the 2 groups (Table 3).

Next, immunological reconstitution was analyzed between CD34-HSCT and unselected-HSCT groups to assess how graft manipulation affected immune system and clinical response. At inclusion, the ratio of CD4/CD8, the percentage of CD4+CD45RO-, CD4+CD45RO+, CD19+, CD4+CD25+, CD56+, CD3+TCRγδ+, IFN-γ, and IL-4 producing CD4+ and CD8+ cells were in the normal range for all patients and did not differ between CD34-HSCT and unselected-HSCT. After autologous HSCT, CD4/CD8 ratio remained low in both groups. In CD4+ subsets, CD4+CD45RO-, CD4+HLA-DR+, and CD4+CD25+ cells increased rapidly in unselected-HSCT compared with CD34-HSCT at 12 months ($p < 0.05$, Table 4). CD19+ and CD56+ cells returned into the normal range at 3 months in both groups. CD69 expression levels on CD3+CD8+ and CD3+CD8- cells against mitogen were not different between healthy controls and patients with SSc before autologous HSCT, and its kinetics through autologous HSCT were similar in both groups (Table 4). Levels of cytokine production in CD3+CD8- and CD3+CD8+ cells were not different between both groups. IFN-γ-producing CD3+CD8+ T cells increased after autologous HSCT in both groups (Table 4).

Cytokine production in CD8- and CD8+ T cells was assessed by intracellular IFN-γ and IL-4. Cytokine production in CD8- cells was not different between both groups. IFN-γ- and IL-4-producing CD8+ T cells increased after autologous HSCT in both groups (Table 4).

The sjTREC values recovered to the levels at inclusion between 6 to 12 months after CD34-HSCT or unselected-HSCT. There was no statistical significance through their clinical course in both groups (Figure 2D).

Foxp3 gene expressions in PBMC were within the normal range through autologous HSCT and not different in the 2 groups (Table 4).

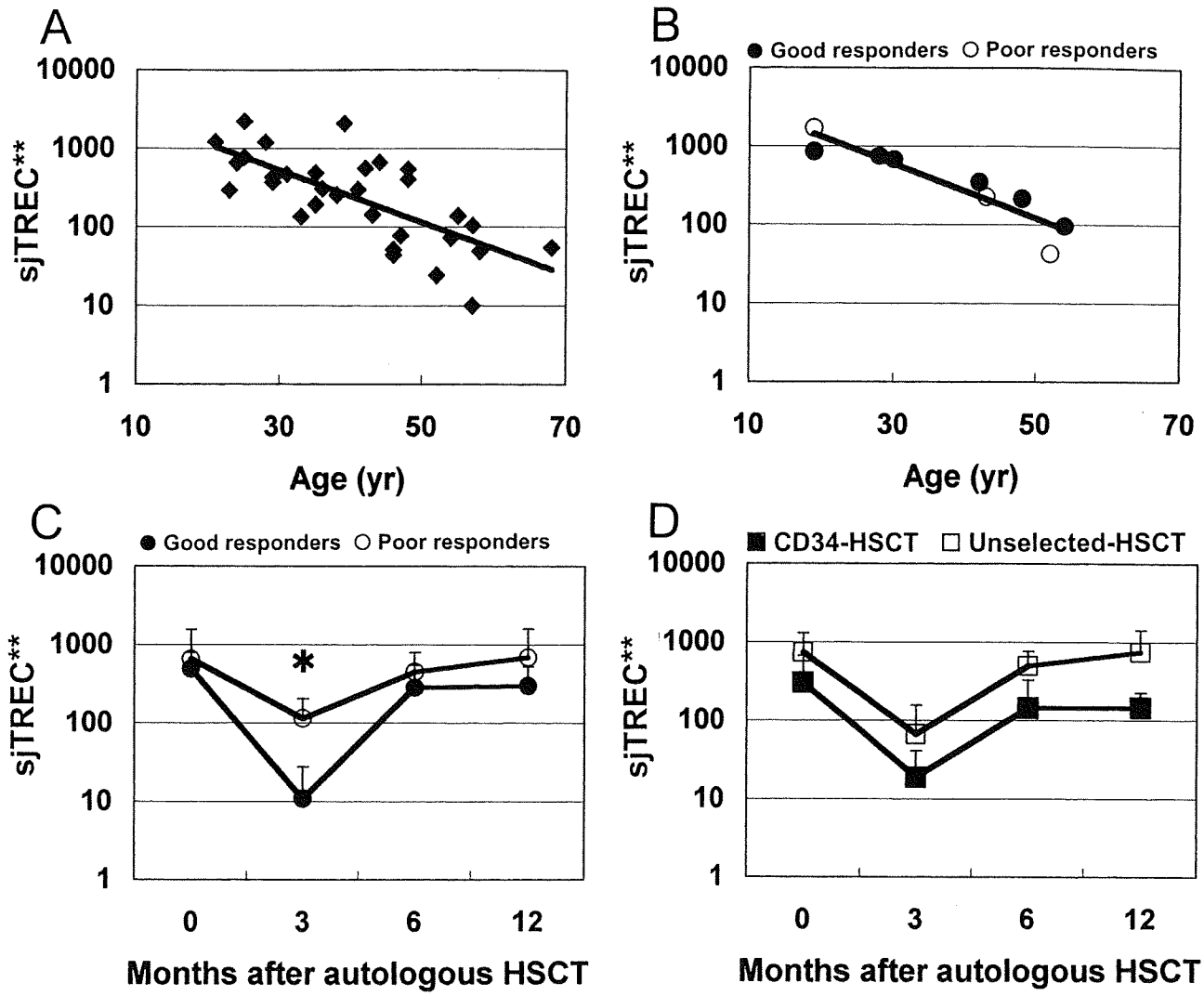

Figure 2. sjTREC values in CD3+ cells in healthy individuals and its kinetics through autologous HSCT. A. Relation between age and numbers of sjTRECs in healthy controls. B. Relation between age and numbers of sjTREC in SSc patients treated with autologous HSCT. C. sjTREC between good and poor response groups. D. sjTREC between patients treated with CD34-HSCT and unselected-HSCT. Logarithmic scales were used for y-axes to compress the figure. * p = 0.0152. **copies/μg in CD3+ cells DNA.

DISCUSSION

We described the efficacy and the safety in patients with SSc treated with autologous HSCT. More than a 25% decrease in the skin score, which correlates with patient's survival[20], was achieved in 8 out of 10 transplanted SSc patients. Skin improvement was not significantly different between CD34-HSCT and unselected-HSCT groups. In addition, additional unselected-HSCT did not lead to recurrence or adverse effect on skin manifestation in Patient 3. These results suggest that graft condition did not affect the clinical outcome on skin involvement up to 12 months after autologous HSCT in our series.

Few data on thymic function and lymphocyte phenotypes after autologous HSCT have been reported in transplanted SSc patients[13,18,21]. The TREC values might be related to clinical response in our transplanted patients. In the last decade, basic and clinical scientists have focused a role of sjTREC as a marker of human thymic function[22]. Values of sjTREC can also reflect the pathophysiology in patients with autoimmune diseases. The sjTREC values may be affected by disease activity in patients with systemic lupus erythematosus[23]. Age-inappropriate T cell senescence confirmed by decreased frequency of sjTREC may also contribute to the development of juvenile idiopathic arthritis[24]. There was no evidence to prove an age-inappropriate T cell senescence and a correlation between the sjTREC values

Table 4. Phenotype analysis of lymphocyte population through autologous HSCT between patients with CD34-HSCT and unselected-HSCT. Values are mean ± SD.

		At Inclusion		3 mo After HSCT		6 mo After HSCT		12 mo After HSCT	
	Normal Range 95% CI	CD34–HSCT	un-HSCT	CD34-HSCT	un-HSCT	CD34-HSCT	un-HSCT	CD34-HSCT	un-HSCT
CD3+, CD4+	57.57–68.89	37.84 ± 11.45	60.99 ± 9.66	17.85 ± 9.95	31.98 ± 6.69*	20.30 ± 14.73	43.31 ± 9.75	24.13 ± 14.36*	43.62 ± 8.46
CD3+, CD8+	26.47–37.68	22.20 ± 6.45	34.42 ± 11.06	36.07 ± 18.09	61.02 ± 6.96	33.69 ± 15.89	48.83 ± 11.58	42.02 ± 14.06	50.60 ± 9.08
CD4/CD8	0.61–2.96 (ratio)	1.87 ± 0.87	2.13 ± 1.45	0.49 ± 0.22*	0.54 ± 0.18*	0.62 ± 0.36*	0.97 ± 0.48	0.58 ± 0.28*	0.91 ± 0.35
CD3+, TCRγδ+	0.74–9.48	3.66 ± 3.44	1.80 ± 1.09	4.31 ± 5.13	4.24 ± 2.90	2.54 ± 1.62	3.21 ± 1.48	4.03 ± 2.78	2.85 ± 1.52
CD4+, CD45RO–	5.23–42.08	29.10 ± 7.34	29.63 ± 12.01	4.58 ± 5.44*	4.95 ± 1.91*	4.94 ± 3.36*	8.33 ± 7.59	4.29 ± 2.23*	14.73 ± 7.39
CD4+, CD45RO+	9.00–27.97	15.56 ± 4.16	17.57 ± 5.93	13.79 ± 6.90	19.48 ± 3.92	14.20 ± 7.61	15.09 ± 5.65	13.02 ± 3.83	16.32 ± 2.91
CD4+, HLA-DR+	0.92–3.38	2.90 ± 1.36	2.80 ± 1.66	7.61 ± 5.72	11.85 ± 2.72	4.36 ± 2.32	8.54 ± 4.03	2.92 ± 0.56	8.29 ± 3.34
CD4+, CD25+	1.35–5.46	2.70 ± 1.67	6.33 ± 3.28	2.30 ± 1.10	5.94 ± 4.07	3.33 ± 3.20	6.64 ± 2.98	2.02 ± 0.95	6.27 ± 2.24
foxp3 mRNA	32.01–393.07 (copies/GAPDH 1k copies)	524.44 ± 880.90	420.07 ± 199.61	110.72 ± 100.54	204.72 ± 149.57	129.22 ± 101.19	280.62 ± 51.70	123.34 ± 60.37	291.54 ± 87.8
CD3+, CD8–, IFNγ+	0.67–17.49	6.65 ± 8.28	3.58 ± 1.62	13.59 ± 10.75	7.95 ± 6.99	9.60 ± 4.19	9.27 ± 8.38	5.64 ± 3.27	17.44 ± 10.45
CD3+, CD8–, IL4+	0.02–2.47	0.85 ± 0.59	1.46 ± 0.89	4.51 ± 2.41	2.38 ± 1.75	2.98 ± 1.95	3.74 ± 5.68	1.97 ± 0.90	0.61 ± 0.31
Th1/Th2	3.79–125.60 (ratio)	20.41 ± 26.40	7.51 ± 4.67	13.88 ± 15.28	96.22 ± 120.52	31.49 ± 37.45	68.90 ± 75.09	13.81 ± 23.54	37.78 ± 25.33
CD3+, CD8+, IFNγ+	0.66–41.60	8.31 ± 6.99	3.38 ± 1.47	27.35 ± 22.64	25.80 ± 17.33	30.29 ± 10.03	10.94 ± 8.31	24.77 ± 25.94	12.34 ± 10.07
CD3+, CD8+, IL4+	0.00–1.40	0.23 ± 0.21	0.20 ± 0.28	1.11 ± 0.83	0.46 ± 0.40	1.26 ± 1.27	0.51 ± 0.65	0.81 ± 0.47	0.22 ± 0.17
Tc1/Tc2	7.83–185.08 (ratio)	49.51 ± 62.39	101.70 ± 103.52	127.14 ± 120.21	984.54 ± 1771.58	550.91 ± 974.27	654.58 ± 1136.02	60.02 ± 89.13	76.82 ± 57.32
CD3+, CD8–, CD69+	82.91–201.89 (MFI)	172.45 ± 122.20	147.75 ± 62.66	59.53 ± 53.14	39.28 ± 18.77	68.95 ± 37.16	57.96 ± 39.23	84.12 ± 47.34	47.57 ± 39.01
CD3+, CD8+, CD69+	54.27–119.07 (MFI)	82.70 ± 45.77	119.66 ± 36.51	18.72 ± 5.52	37.09 ± 20.28	33.35 ± 25.06	49.98 ± 26.42	50.73 ± 14.13	49.30 ± 40.58
CD19+	5.00–32.98	18.20 ± 8.43	12.04 ± 6.42	21.84 ± 28.33	17.47 ± 3.07	24.44 ± 24.80	14.05 ± 4.86	18.12 ± 10.94	13.93 ± 5.86
CD56+	8.94–22.94	13.42 ± 13.69	12.12 ± 6.82	12.67 ± 6.43	13.05 ± 7.12	13.79 ± 11.12	11.11 ± 4.10	14.65 ± 7.56	9.74 ± 5.19

* The value from the baseline measurement was calculated for each value at each timepoint. $p < 0.05$. MFI: mean fluorescence intensity.

and disease condition in our patients with SSc. Thymic function assessed by sjTREC values is significantly suppressed at engraftment, recovers within 3 months after autologous HSCT, and is age-dependent in adults[17,25]. In our series, the lower level of sjTREC at 3 months after autologous HSCT was shown in the good response group without dependence on their age and graft condition. Longterm defects of CD3+CD4+ cells, especially CD4+CD45RO-naïve T cells, after autologous HSCT might also reflect profound suppression of thymopoiesis in the good response group. Thymus-dependent immunological reconstitution leads to the T cell precursor reeducation and renewal of the T cell repertoire, and may induce remission of autoimmunity[26,27]. Our results suggest that transient, profound suppression of thymic function might alter immune condition, leading to clinical response in patients with SSc.

Peripheral immunological reconstitution after autologous CD34-HSCT or unselected-HSCT has been well documented in patients with hematological disorders[28-30]. While CD56+ cells, followed by CD19+ cells, recover promptly after autologous HSCT, CD3+ cells, especially CD4+CD45RO- cells, remain low after autologous HSCT in CD34-HSCT and unselected-HSCT[29,30]. After the initial 2 months of autologous HSCT, IFN-γ-producing CD8+ or CD8- T cells remain normal or increased[11,30]. In our series, kinetics of lymphocytes recovery is similar to these previous results. In patients with SSc, peripheral blood T cells show a predominantly type 2 T-helper profile, and can induce fibrosis through the production of cytokines, especially IL-4[2]. Cytokine production in T cells at inclusion was not significantly different between our transplanted patients with SSc and healthy controls. The kinetics of IFN-γ- and IL-4-producing T cells after autologous HSCT was not different between CD34-HSCT and unselected-HSCT, or good and poor response groups. Therefore, the significance of cytokine production in T cells after autologous HSCT was not conclusive. In good response group with sustained major or partial response, phenotype or function of peripheral lymphocytes was not significantly different from that of poor response group through autologous HSCT. These results suggest that changes in peripheral immunity were not correlated with clinical response.

CD4+CD25+FOXP3+ regulatory T cells may play a role in the immunological reconstitution leading to the improvement of autoimmune disease or prevention of graft-versus-host disease after autologous or allogeneic HSCT[31,32]. Although CD4+CD25+ population increased at 12 months after autologous HSCT in unselected-HSCT compared with

that in CD34-HSCT, it is noted that there was no difference between good and poor response groups, and *foxp3* gene expression levels did not correlate with the clinical response or with graft condition. CD4+CD25+ populations include non-regulatory activated T cells as well as regulatory T cells[32]. Increased CD4+CD25+ population might reflect the activation of CD4+ T cells because CD4+HLA-DR+ population also increased at 12 months in unselected-HSCT group. Therefore, the role of CD4+CD25+ regulatory T cells on clinical response was not evident in our study.

Although the importance of graft manipulation in autologous HSCT for autoimmune diseases has been debated, clinical outcome may not necessarily correlate with the autoreactive clone survival after CD34-HSCT[33]. In patients with rheumatoid arthritis, a pilot study showed that clinical response and laboratory findings were also similar between CD34-HSCT and unselected-HSCT[12]. In addition, autoimmunity after autologous HSCT may result from the type of conditioning regimen rather than graft condition (i.e., CD34-HSCT or unselected-HSCT)[34]. Although peripheral immunity after autologous HSCT does not have a decisive impact on disease control in our transplanted SSc patients, further study will reveal the role of peripheral immunity after autologous HSCT. Our results suggest the relationship between clinical benefits and immunosuppression intensity sufficient to suppress thymic output by the treatment.

The results of our study suggest that immunosuppression sufficient to downregulate thymic function, rather than the graft manipulation, can lead to clinical benefits in patients with SSc. Additionally, appropriately monitoring the sjTREC values after autologous HSCT may serve to identify patients who would not achieve clinical remission by autologous HSCT and additional treatment in a more timely way.

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RESEARCH PAPER *Multiple Sclerosis* 2009; **15**: 159–173

Temporal changes and geographical differences in multiple sclerosis phenotypes in Japanese: nationwide survey results over 30 years

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Background There are two distinct phenotypes of multiple sclerosis (MS) in Asians, manifesting as optic-spinal (OSMS) and conventional (CMS) forms. In Japan, four nationwide surveys of MS have been conducted. The first three were in 1972, 1982, and 1989, and we performed the fourth in 2004.
Results The recent survey showed six main findings as follows: (1) a four-fold increase in the estimated number of clinically definite patients with MS in 2003 (9900; crude MS prevalence, 7.7/100,000) compared with 1972; (2) a shift in the peak age at onset from early 30s in 1989 to early 20s in 2003; (3) a successive proportional decrease in optic-spinal involvement in clinically definite patients with MS; (4) a significant north–south gradient for the CMS/OSMS ratio; (5) after subdivision of the mainland (30–45° North) into northern and southern parts at 37°N, northern-born northern residents (northern patients) showed a significantly higher CMS/OSMS ratio and higher frequency of brain lesions fulfilling the Barkhof criteria (Barkhof brain lesions) than southern-born southern residents (southern patients); (6) among northern patients, the absolute numbers of patients with CMS and those with Barkhof brain lesions rapidly increased with advancing birth year.
Conclusions These findings suggest that MS phenotypes are drastically altered by environmental factors, such as latitude and "Westernization." *Multiple Sclerosis* 2009; **15**: 159–173. http://msj.sagepub.com

Key words: epidemiology; Japanese; latitude; magnetic resonance imaging; multiple sclerosis; optic-spinal

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that results from a complex interplay between genetic and environmental factors [1]. MS is rare in Asians, but when it does occur, selective and severe involvement of the optic nerve and spinal cord is characteristic [2]. In 1958, Okinaka, *et al.* (1958) first reported a series of Japanese patients

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with demyelinating diseases, among whom 175 of 270 cases were described as having Devic's neuromyelitis optica (NMO), and classical MS was rare. These early researchers also found intermediate cases between NMO and classical MS [3,4]. Thereafter, only monophasic NMO has been referred to as NMO, while relapsing NMO is included within the spectrum of MS. In Japan, the latter group has been designated as having the optic-spinal MS (OSMS) and its clinical criteria, proposed in 1996 [5], have frequently been used in clinical research on Japanese patients. Recently, NMO-IgG, a newly identified marker for NMO [6], was also detected in a fraction of Japanese patients with OSMS [7,8], and OSMS is now postulated to be the same disease as relapsing NMO [9]. However, further studies are required to clarify whether MS, OSMS, and NMO are distinct diseases or whether they form a continuum [8–11].

NMO is also a major demyelinating disease in Africans [12,13]. Differences in phenotypes among races are assumed to result from genetic differences [14]. However, studies on migrants have shown changes in not only the prevalence but also the phenotype of MS, which are attributable to the early-life environment [15–21]. Asian and African descendents in the United Kingdom [17,18] and returning migrants from France to the French West Indies [21] are behind the emergence of classical MS in place of NMO in these populations, whereas Caucasian descendents in tropical Colombia show more frequent optic-spinal involvement [20]. Although these migration studies indicate the influence of exogenous factors on MS susceptibilities and phenotypes, such interpretations must be made with caution because the admixture of genes could be equally influential [14], as observed in a genetic study on Mexican mestizos, which showed that patients who presented with classical MS also harbored more Caucasian genes [22].

An alternative method for determining whether genetic or environmental factors are responsible for the manifestation of demyelinating diseases is to investigate phenotypic changes over time in genetically homogeneous and geographically isolated populations that have experienced rapid environmental changes. Japanese, for whom interracial marriage with Caucasians remains exceptional, are suitable for such a study. Because nationwide surveys of MS in Japan were conducted using essentially identical criteria in 1972, 1982, and 1989 [23,24], we decided to perform a fourth nationwide survey in 2004 to uncover any phenotypic changes in MS that have occurred during Japan's period of "Westernization."

Methods

Survey procedures

The fourth nationwide survey of MS was conducted by the Research Committees of Neuroimmunological Diseases and of Epidemiology of Intractable Diseases, sponsored by the Ministry of Health, Labour and Welfare, Japan. The study was approved by the Kyushu University Ethics Committee. The survey was undertaken in two steps. First, a preliminary survey was undertaken to ascertain the approximate number of patients with MS in Japan, and second, a survey was conducted using a questionnaire sheet for each patient. The hospitals included in the study were randomly selected from the directory of all registered hospitals throughout Japan. Selection was made according to a stratification based on the number of beds in each hospital, in which increasing numbers of beds led to increasing probabilities of being selected [25]. The sampling rates were approximately 8%, 13%, 24%, 43%, 83%, and 100% for the strata of general hospitals with 20–99 beds, 100–199 beds, 200–299 beds, 300–399 beds, 400–499 beds, and 500+ beds, respectively. All university hospitals and as well as those in which council members of the Japanese Society of Neurology and members of the Committees of Medical Facilities for Children and the Japanese Society of Child Neurology were working were also surveyed.

The questionnaire for the preliminary survey on patients with MS who visited hospitals due to disease within the period from January 1 to December 31, 2003 was mailed to 6708 departments (comprising 1933 neurology/internal medicine, 1227 orthopedics, 997 psychiatry, 945 pediatrics, 831 ophthalmology, 759 neurosurgery, and 16 rehabilitation departments) together with the diagnostic criteria in January 2004. In Japan, all patients with MS, including monophasic NMO, are requested to visit hospitals at least once every year for registration of intractable diseases with the government to be subsidized for their medical costs, which are not covered by health insurance. Following the collection and collation of the first questionnaire, a second questionnaire was forwarded to those institutions reporting patients in the first survey. The second questionnaire requested detailed clinical information on individual patients, including their ages at onset and examination, sex, birthplace, present address, symptoms based on history and signs from physical examination (Supplementary Table), laboratory findings, course, treatment, and prognosis. Patients reported by more than one hospital or department were treated as duplicates.

Supplementary Table Neurological symptoms or signs during the course of illness in clinically definite cases of multiple sclerosis

	Clinically definite MS[a]	
	1989 (n = 861)	2004 (n = 1493)
Mental impairment	20.4	17.4
Aphasia, apraxia, agnosia	5.1	4.1
Generalized convulsion	8.3	3.8
Visual loss	70.4	56.1
Optic atrophy	52.2	32.3
Visual field defect	33.4	27.8
Diplopia	28.4	21.3
Internuclear ophthalmoplegia	6.1	7.9
Nystagmus	36.5	27.1
Dysarthria	30.5	21.9
Dysphagia	17.7	10.4
Facial palsy	18.3	13.3
Quadriparesis	38.3	18.4
Paraparesis	48.3	43.4
Hemiparesis	37.5	35.5
Spasticity	55.9	47.6
Babinski reflex	64.1	58.7
Sensory disturbance		
Face	25.6	21.2
Segmental	36.8	34.5
Below a certain level	31.3	37.9
Hemi	33.8	33.7
Transverse myelitis	36.7	27.4
Recurrent	22.2	15.4
Limb ataxia	37.4	26.3
Truncal ataxia	33.5	30.5
Disturbance in urination	61.1	49.6
Painful tonic spasm	28.7	18.1
Lhermitte sign	32.5	29.7

[a]% of all cases for which information could be obtained regarding each of these items.

Diagnostic criteria

The diagnostic criteria used for the present survey were based on those used for the first nationwide survey in 1972 [23], except that the limitation of age at onset was removed, as it was in the third survey [24]. The criteria required multiplicity in time and space and were essentially the same as Schumacher's criteria [26]. Briefly, the criteria used for relapsing–remitting MS in the present survey consisted of three items for clinically definite MS: (1) symptoms and signs due to multifocal lesions in the CNS (more than two lesions in the CNS); (2) remissions and exacerbations (multiplicity in time); and (3) exclusion of other diseases, such as tumors, syphilis, cerebrovascular accident, cervical spondylosis, angiomas, subacute myelo-optico-neuropathy, neuro-Behçet, cerebellar degeneration, human T-lymphotropic virus-I-associated myelopathy/tropical spastic paraparesis, and collagen diseases. Clinically definite MS fulfilled all of the criteria, while a diagnosis of possible MS was made when all three criteria for clinically definite MS could not be fulfilled, but the signs were suggestive. The criteria for primary progressive MS (PPMS) were taken from McDonald's criteria [27]. Data from cases with monophasic NMO were also collected. The criteria for monophasic NMO were as follows: acute bilateral visual impairment (optic neuritis) and transverse myelitis occurring successively within several weeks.

Classification of clinical phenotypes

The classification of MS subtypes was solely based on the clinically estimated sites of the lesions. The second questionnaire requested the responders to check the clinically estimated sites of the lesions according to the symptomatology during the entire clinical course among the following sites: optic nerve, cerebrum, cerebellum, brainstem, and spinal cord. Moreover, the questionnaire also requested the responders to check for the presence of any of the signs and symptoms listed in Supplementary Table during the entire clinical course. The survey center classified each case into the following clinical subtypes based on the clinically estimated lesion sites reported by each institution: OSMS involving the optic nerve and the spinal cord; optic-brainstem-spinal MS (OBSMS) involving the optic nerve, brainstem, and spinal cord; brainstem-spinal MS (BSMS) involving the brainstem and the spinal cord; spinal MS (SMS) involving only the spinal cord, which was identical to recurrent myelitis without any known cause; and conventional MS (CMS), which involved multiple sites of the CNS, including the cerebrum or cerebellum. If there was no information on the lesion sites or the symptoms and signs during the entire course were incompatible with the lesion sites, the cases were placed into the unclassified category. As SMS is generally regarded as a limited form of OSMS [28], OSMS and SMS were grouped together and compared with other forms (CMS, OBSMS, and BSMS) that involved multiple sites of the CNS (such as the cerebrum, cerebellum, and brainstem) in some analyses. Because many intermediate cases between relapsing NMO and classical MS [3] have been reported in Japanese, the term "relapsing NMO" was not used in the four nationwide surveys. Thus, relapsing NMO was considered to be included in the OSMS subgroup in the present survey, according to the confined involvement of the optic nerve and the spinal cord. This was distinct from CMS presenting with multiple sites of CNS involvement including the cerebrum or cerebellum.

Statistical analysis

The estimated total number of patients with MS in Japan was calculated by summing the figures for the

total reported number of patients in each stratum divided by the ratio of responding institutions to the number of surveyed institutions in each stratum. The formulas used to estimate the total number of patients and the 95% confidence intervals (CIs) have been described in detail elsewhere [29,30]. The crude prevalence rate per 100,000 people was determined from the population of Japan in 2003. Statistical analyses of numerical variables were initially performed using the Kruskal–Wallis H test. When statistical significance was found, the Mann–Whitney *U* test was used to determine the statistical significance of differences among subgroups. Uncorrelated *P* values were multiplied by the number of comparisons (Bonferroni–Dunn's correction) to calculate corrected *P* values. Differences in the ratios between two groups were tested for significance by the χ^2 test or Fisher exact test when the criteria for the χ^2 test were not fulfilled. All statistical analyses were performed by three committee members (MO, TM, and KS).

Results

Comparisons with previous survey results

In the preliminary survey, 3749 institutions (55.9%) responded and reported 4827 patients with MS, including 849 patients with possible MS. In the second questionnaire, detailed data were collected for 1919 patients (39.3% of the preliminary survey), including 30 duplicate cases. There were no significant regional differences in the response rates. Specifically, there was no significant correlation between the response rate and the northern latitude of present residence in either the preliminary ($r = 0.067$, $P = 0.6522$) or second ($r = 0.048$, $P = 0.7431$) survey by Spearman's rank correlation test. Thus, the estimated number of clinically definite patients with MS in 2003 was 9900 (95% CI: 9100–10,700), representing a four-fold increase compared with the first nationwide survey in 1972 (Table 1) [23]. The estimated crude prevalence was 7.7/100,000 (95% CI: 7.1–8.4). The proportions of patients with clinically definite MS and female patients had increased since the first survey. The percentage of patients with monophasic NMO among all patients with MS as well as the absolute number of patients with this subtype had progressively decreased over time (Table 1; absolute numbers as follows: 82 in 1972, 77 in 1982, 46 in 1989, and 22 in 2004). Compared with the third nationwide survey, the peak age at onset had decreased and the second peak in the early 50s had disappeared (Figure 1A). Visual loss at onset and during the entire clinical course and optic atrophy during the entire course had decreased over the period of the four surveys. When the small numbers of patients with PPMS ($n = 40$) in the present survey were omitted, all statistical analyses gave essentially the same results as those described in the following sections (data not shown).

Clinical features and classification of patients with MS

Based on the clinically estimated sites of lesions, 1493 patients with clinically definite MS and completed questionnaires were classified as having CMS

Table 1 Comparison of demographic features among the four nationwide survey

Year of survey	1972	1982	1989	2004[a]
Estimated number of clinically definite patients with MS	2280	ND	3700	9900
Number of cases collated for the final survey	1084	1518	1270	1889
Sex ratio (male:female)	1:1.7	1:2.3	1:2.6	1:2.9
Clinically definite MS (%)[b]	46.9	55.9	67.8	84.5
Monophasic NMO (%)[b]	7.6	5.1	3.6	1.2
Age at onset (mean ± SD, years)[b]	33 ± 13	32 ± 13	34 ± 13	32 ± 13
Age at examination (mean ± SD, years)[b]	39 ± 13	40 ± 13	41 ± 14	42 ± 14
Mean disease duration (years)[b]	6	8	8	10
Familial occurrence (%)	1	1.3	ND	1.1
Visual loss at onset (%)	41.8	34.6	36.6	29.5
Visual loss during entire course (%)	79	ND	70.4	56.1
Optic atrophy during entire course (%)	62	ND	52.2	32.3
Quadriparesis during entire course (%)	ND	ND	38.3	18.4
Transverse myelitis during entire course (%)	ND	ND	36.7	27.4

MS, multiple sclerosis; ND, not determined; NMO, neuromyelitis optica.
[a]Interferon beta-1b, the only disease-modifying drug available in Japan since 2001, was administered in 37.2% of the cases in 2003.
[b]Data from all patients with MS including possible MS and monophasic NMO are shown. When the small numbers of primary progressive patients with MS ($n = 40$) in the present survey were omitted, all statistical analyses in the following sections gave essentially the same results.

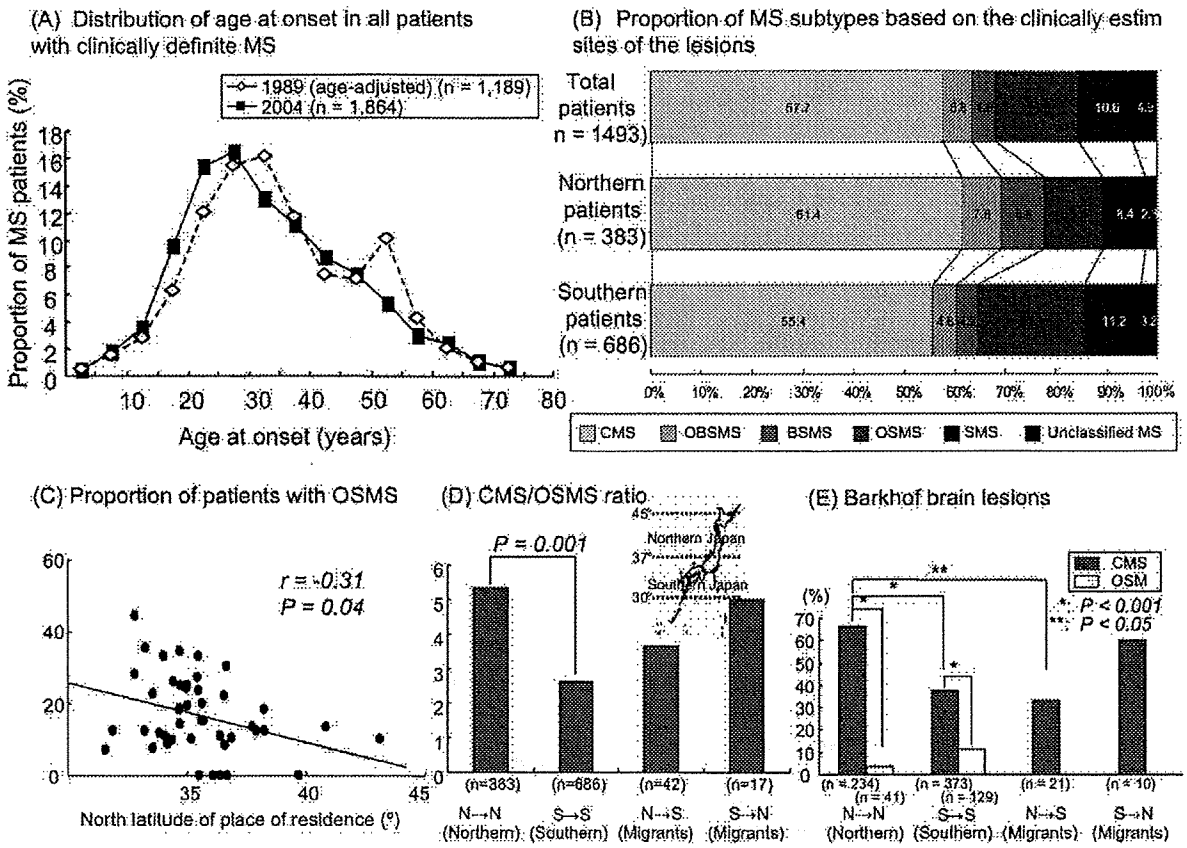


Figure 1 (A) Distribution of ages at onset in all patients with clinically definite MS. (B) Proportions of MS subtypes based on the clinically estimated sites of lesions. (C) Relationships between the OSMS percentages in the 46 prefectures of Japan and the northern latitudes of prefecture office locations. (D) CMS/OSMS ratios in relation to place of birth and residence. (E) Frequencies of brain magnetic resonance imaging lesions fulfilling the Barkhof criteria in relation to place of birth and residence. In (A), the proportions of patients with MS at each age at onset in 1989 have been adjusted to the age distribution of the Japanese population in 2003. Note that the age at onset curve shifts toward the younger side in 2004, whereas the second peak around the early 50s seen in 1989 is no longer evident. In (B), only those patients with known birthplace and present residence were analyzed. Here, the proportions of patients with MS showing the CMS, OBSMS, and BSMS phenotypes are higher among northern patients than among southern patients, whereas the OSMS and SMS phenotypes show the reverse trend. In (C), there is a significant positive correlation between the OSMS percentage and the latitude of the place of residence ($P < 0.05$). The same is true for the birthplace ($P < 0.05$) (data not shown). In (D), the Japanese mainland (inset in D), located from 30° North to 45° North, is arbitrarily divided into northern and southern parts at a latitude of 37° North. The respective CMS/OSMS ratios are shown for northern-born northern residents (N→N), southern-born southern residents (S→S), northern-born southern residents (N→S) and southern-born northern residents (S→N). The CMS/OSMS ratio is significantly higher in northern-born northern residents (northern patients) than in southern-born southern residents (southern patients) ($P < 0.001$). In (E), among patients with CMS, brain lesions fulfilling the Barkhof criteria are significantly more common in northern patients than in southern patients. Among patients with CMS, the frequency of Barkhof brain lesions is significantly lower in northern-born southern residents than in northern-born northern residents. The frequencies of Barkhof brain lesions in the OSMS groups are not shown in migrants because the sample numbers were too small. BSMS, brainstem-spinal form of multiple sclerosis; CMS, conventional form of multiple sclerosis; n, number of patients whose information was obtained; OBSMS, optic-brainstem-spinal form of multiple sclerosis; OSMS, optic-spinal form of multiple sclerosis; SMS, spinal form of multiple sclerosis.

(57.7%), OBSMS (5.8%), BSMS (4.6%), OSMS (16.5%), SMS (10.6%), or unclassified MS (4.9%) (Figure 1B). There were no significant differences in disease durations among the subtypes other than a significantly shorter disease duration in patients with SMS compared with patients with CMS and OSMS (Table 2). Comparisons of the clinical features between patients with CMS and OSMS showed significant differences in many aspects, similar to previous findings in Japanese patients [2]. Compared with patients with CMS, patients with OSMS showed a significantly higher

Table 2 Clinical characteristics among each multiple sclerosis subgroup classified according to the clinically estimated sites of the lesions

	OSMS ($n = 246$)	CMS ($n = 862$)	P value	SMS ($n = 158$)	BSMS ($n = 68$)	OBSMS ($n = 86$)
Sex ratio (male:female)	1:4.5	1:2.4	<0.001	1:2.3‡	1:3.5	1:4.4
Age at onset (years)	35.4 ± 12.9	29.3 ± 12.5	<0.001	38.3 ± 13.5†	31.6 ± 11.8	31.1 ± 11.7‡
Age at examination (years)	47.1 ± 14.1	39.9 ± 13.6	<0.001	45.3 ± 13.5†	41.1 ± 11.8‡	41.8 ± 12.7‡
Disease duration (years)	11.7 ± 9.1	10.6 ± 8.4	NS	7.0 ± 6.4*,†	9.5 ± 6.9	10.9 ± 7.3
EDSS scores	4.3 ± 2.7	3.5 ± 2.9	<0.001	3.4 ± 2.3‡	2.6 ± 2.3*	4.0 ± 2.8
Symptoms during entire course						
Bilateral visual loss	131/246 (53.3%)	260/851 (30.6%)	<0.001	0/158 (0.0%)*,†	0/68 (0.0%)*,†	46/84 (54.8%)†
Transverse myelitis	113/231 (48.9%)	170/823 (20.7%)	<0.001	52/152 (34.2%)†,‡	24/68 (35.3%)§	24/82 (29.3%)‡
Paraparesis	139/239 (58.2%)	319/839 (38.0%)	<0.001	85/151 (56.3%)†	32/68 (47.1%)	41/83 (49.4%)
Quadriparesis	46/238 (19.3%)	160/848 (18.9%)	NS	20/151 (13.2%)	14/66 (21.2%)	19/83 (22.9%)
Sensory impairment below a certain level	141/224 (62.9%)	223/777 (28.7%)	<0.001	75/148 (50.7%)†	30/65 (46.2%)§	31/75 (41.3%)*
Sphincter disturbance	155/238 (65.1%)	393/848 (46.3%)	<0.001	75/154 (48.7%)*	35/68 (51.5%)	48/81 (59.3%)
Severe motor disability at the time of last examination[a]	57/231 (24.7%)	142/805 (17.6%)	0.017	15/148 (10.1%)*	8/63 (12.7%)	18/80 (22.5%)
Secondary progression	19/246 (7.7%)	131/861 (15.2%)	0.003	11/158 (7.0%)§	5/68 (7.4%)	9/86 (10.5%)
Cerebrospinal fluid findings						
Marked pleocytosis (≥50 WBC/mm^3) or neutrophilia (≥5 neutrophils/mm^3)	21/191 (11.0%)	51/730 (7.0%)	NS	11/134 (8.2%)	6/60 (10.0%)	3/65 (4.6%)
Increased IgG index	31/106 (29.2%)	240/397 (60.5%)	<0.001	35/68 (51.5%)‡	21/38 (55.3%)‡	22/47 (46.8%)
Brain MRI findings						
≥ 1 Gd-enhanced lesion or ≥9 T2 brain lesions	41/226 (18.1%)	507/840 (60.4%)	<0.001	31/146 (21.2%)†	30/66 (45.5%)*	33/79 (41.8%)*,†
≥ 9 T2 brain lesions	37/226 (16.4%)	390/840 (46.4%)	<0.001	24/146 (16.4%)†	22/66 (33.3%)‡	31/79 (39.2%)*
≥ 1 Gd-enhanced lesion	10/199 (5.0%)	292/688 (42.4%)	<0.001	12/133 (9.0%)†	19/62 (30.6%)*	15/72 (20.8%)*,†
≥ 1 juxtacortical lesion	28/218 (12.8%)	303/786 (38.5%)	<0.001	11/138 (8.0%)†	9/66 (13.6%)†	17/78 (21.8%)§
≥ 3 periventricular lesions	59/221 (26.7%)	526/806 (65.3%)	<0.001	34/143 (23.8%)†	27/66 (40.9%)†	37/80 (46.3%)*,†
≥ 1 infratentorial lesion	42/219 (19.2%)	539/827 (65.2%)	<0.001	27/145 (18.6%)†	45/67 (67.2%)*	57/81 (70.4%)*
Lesions fulfilling the Barkhof criteria	19/223 (8.5%)	382/844 (45.3%)	<0.001	16/145 (11.0%)†	18/67 (26.9%)*,§	25/81 (30.9%)*
No cranial lesion	94/223 (42.2%)	16/844 (1.9%)	<0.001	68/145 (46.9%)†	7/67 (10.4%)*,†	8/81 (9.9%)*,†
Spinal cord MRI findings						
≥ 1 T2 lesion	203/223 (91.0%)	508/724 (70.2%)	<0.001	145/153 (94.8%)†	60/66 (90.9%)†	69/74 (93.2%)†
LESCL	93/223 (41.7%)	121/724 (16.7%)	<0.001	46/153 (30.1%)†	15/66 (22.7%)‡	24/74 (32.4%)†
Gd-enhanced lesion	99/181 (54.7%)	187/653 (28.6%)	<0.001	74/127 (58.3%)†	37/63 (58.7%)†	29/67 (43.3%)

BSMS, brainstem form of multiple sclerosis; CMS, conventional form of multiple sclerosis; EDSS, expanded disability status scale of Kurtzke; Gd, gadolinium; LESCLs, longitudinally extensive spinal cord lesions extending 3 or more vertebral segments; MRI, magnetic resonance imaging; NS, not significant; OSMS, optic-spinal multiple sclerosis; OBSMS, optic-brainstem-spinal multiple sclerosis; SMS, spinal form multiple sclerosis (recurrent myelitis of unknown cause).

[a]Chair-bound or worse.

*$P < 0.01$ (vs OSMS), correlated P values multiplied by the number of comparisons (6 times).

†$P < 0.01$ (vs CMS).

‡$0.01 \leq P < 0.05$ (vs OSMS).

§$0.01 \leq P < 0.05$ (vs CMS).

age at onset, greater proportion of women, higher expanded disability status scale (EDSS) of Kurtzke score [31], and higher frequencies of bilateral visual loss, transverse myelitis, paraparesis, sensory impairment below a certain level, and sphincter disturbance. By contrast, patients with OSMS had significantly lower frequencies of secondary progression and increased IgG index in the cerebrospinal fluid (CSF) than patients with CMS. The occurrences of brain lesions fulfilling the Barkhof criteria [32] (Barkhof brain lesions) and each item of the criteria were significantly higher in patients with CMS than in patients with OSMS ($P < 0.001$), whereas longitudinally extensive spinal cord lesions (LESCLs) extending across three or more vertebral segments and gadolinium-enhanced spinal cord lesions showed the reverse trend ($P < 0.001$) (Table 2).

Regarding the other subtypes classified on the basis of the clinically estimated lesion sites, although the disease durations did not differ significantly among patients with OSMS, OBSMS, and BSMS, patients with OBSMS and BSMS showed lower frequencies of transverse myelitis and sensory levels, higher frequencies of brain magnetic resonance imaging (MRI) lesions and less frequent occurrence of LESCLs than patients with OSMS (Table 2). Patients with OBSMS and BSMS showed some similarities to patients with CMS due to their higher frequencies of brain lesions, although patients with BSMS had lower EDSS scores and patients with OBSMS had more frequent bilateral visual impairment and LESCLs than patients with CMS. By contrast, patients with SMS were similar to patients with OSMS in many aspects, including higher age at onset, fewer brain MRI lesions, and higher frequency of LESCLs, although the severity of the spinal cord lesions represented by the EDSS scores was milder and an increased IgG index was more frequently observed in patients with SMS than in patients with OSMS, suggesting the possibility that SMS is, for the most part, an incomplete form of OSMS during the early course of disease. Therefore, in the following sections, the comparisons between patients with CMS and OSMS by geography and year of birth are supplemented by additional analyses in which OSMS and SMS patients were combined into one group (27.1% of all patients with MS) and patients with CMS, BSMS, and OBSMS were combined into another group (68.1% of all patients with MS).

Differences in clinical phenotypes by latitude and year of birth

A significant negative correlation was found between the proportion of patients with OSMS and the northern latitude of present residence ($r = -0.31$, $P = 0.04$) (Figure 1C). When the mainland (30–45° North) was subdivided into northern and southern parts at approximately the midpoint (37° North), northern-born northern residents (defined as northern patients) showed a significantly higher CMS/OSMS ratio than southern-born southern residents (southern patients) (Figure 1D). Both the patients with OBSMS and BSMS showed some similarities to patients with CMS in terms of their clinical features, and these forms of the disease were twice as common in northern patients than in southern patients, whereas SMS with similar demographic features to OSMS was more common in southern patients than in northern patients (Figure 1B). Thus, the CMS+OBSMS+BSMS/OSMS+SMS ratio was also significantly higher in northern patients than in southern patients (data not shown). Migrants, especially northern-born southern residents, showed intermediate CMS/OSMS ratios between those of the northern and southern patients (Figure 1D).

The CMS/OSMS ratio increased dramatically with advancing year of birth among northern patients, but such a trend was more modest among southern patients (Figure 2A, B). There was a steady increase in the absolute numbers of patients with CMS among both northern and southern patients with advancing year of birth, whereas the numbers of patients with OSMS showed a modest tendency to decrease with advancing year of birth among northern patients, but had no definite trend among southern patients.

Differences in MRI findings by latitude and year of birth

Among the patients with CMS, northern patients showed a significantly higher frequency of Barkhof brain lesions than southern patients ($P < 0.001$) and northern-born southern residents ($P = 0.02$) (Figure 1E), whereas southern patients had significantly more LESCLs than northern patients ($P < 0.001$) (Supplementary Figure 1A). When the CMS+OBSMS+BSMS and OSMS+SMS groups were analyzed, essentially the same results were obtained (data not shown). The proportions and absolute numbers of patients with Barkhof brain lesions steadily increased with advancing year of birth among both northern and southern patients, but the trend was far more marked in northern patients (Figure 2C, D). The proportions of patients with LESCLs successively decreased with advancing year of birth among both northern and southern patients, whereas the absolute numbers remained largely unchanged over the wide range of birth

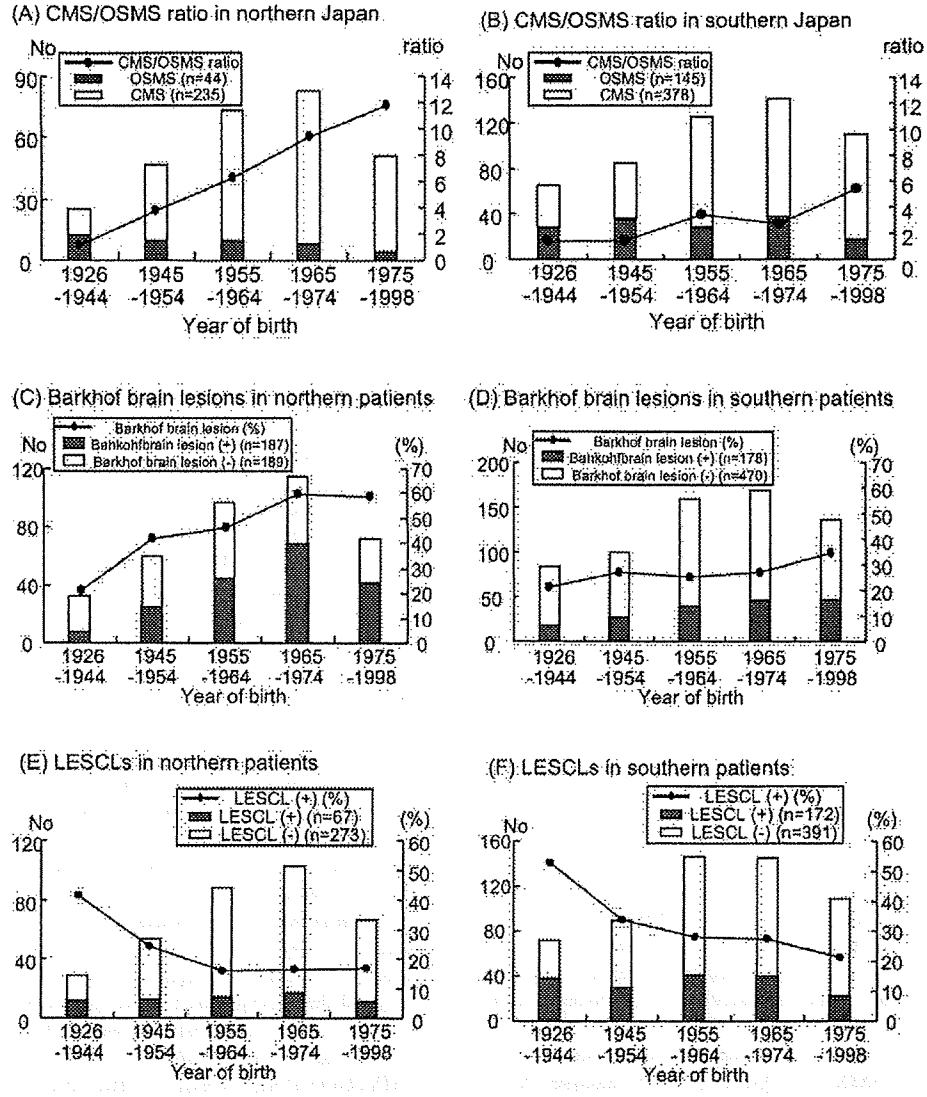


Figure 2 Changes in the clinical phenotypes of patients with clinically definite MS in relation to year of birth. CMS/OSMS ratios and absolute numbers of patients with each phenotype in relation to year of birth among northern (A) and southern (B) patients. Changes in the proportions and absolute numbers of patients with Barkhof brain lesions in relation to year of birth among northern (C) and southern (D) patients. Proportions and absolute numbers of patients with LESCLs in relation to year of birth among northern (E) and southern (F) patients. In (A) and (B), the CMS/OSMS ratios are compared between northern and southern patients and configured according to the year of birth. The ratio steadily increases with advancing year of birth. Each bar indicates the absolute number in each group in the indicated birth years while each dotted line shows the changes in the ratios or percentages. In (C-F), the proportions and absolute numbers of patients with brain lesions fulfilling the Barkhof criteria are increased among northern patients with descending year of birth, while only the proportions of patients with LESCLs decrease with descending year of birth among both northern and southern patients. Bars indicate the absolute numbers in each group with the indicated birth years, while dotted lines show the changes in the positive percentages of the indicated groups. BSMS, brainstem-spinal multiple sclerosis; CMS, conventional multiple sclerosis; LESCLs, longitudinally extensive spinal cord lesions extending for three or more vertebral segments on MRI; *n*, number of patients whose information was obtained; OBSMS, optic-brainstem-spinal multiple sclerosis; OSMS, optic-spinal multiple sclerosis; SMS, spinal multiple sclerosis.

years (Figure 2E, F). The frequency of Barkhof brain lesions was significantly higher in patients born after 1955 than in those born before 1954 among northern patients ($P < 0.001$), but not among southern patients. The frequencies of patients with LESCLs were significantly higher among patients born before 1954 than among those born after 1955 ($P < 0.001$).

Differences in the distributions of MS subtypes by latitude and year of birth

The proportions and absolute numbers of patients with CMS with Barkhof brain lesions, a classical form of Western-type MS, increased steadily with advancing year of birth in both northern and southern patients, but these increases were more pronounced in northern patients than in southern patients (Supplementary Figure 1B, C). The proportions of OSMS+SMS patients with LESCLs, representing a prototypic form of Asian-type MS, decreased among recently born patients in both the northern and southern groups, but decreases in absolute numbers were only modestly seen in those born after 1955 and only in the northern group (Supplementary Figure 1D, E). On the other hand, the absolute numbers of OSMS+SMS patients without LESCLs increased among northern patients born after 1955 (Supplementary Figure 1D). The same was also true for patients with OSMS (data not shown). By contrast, there was a trend toward increases in the proportion and absolute number of patients with LESCLs with advancing year of birth among northern patients with CMS, but the increase in absolute number was minimal among southern patients with CMS (Supplementary Figure 1F, G).

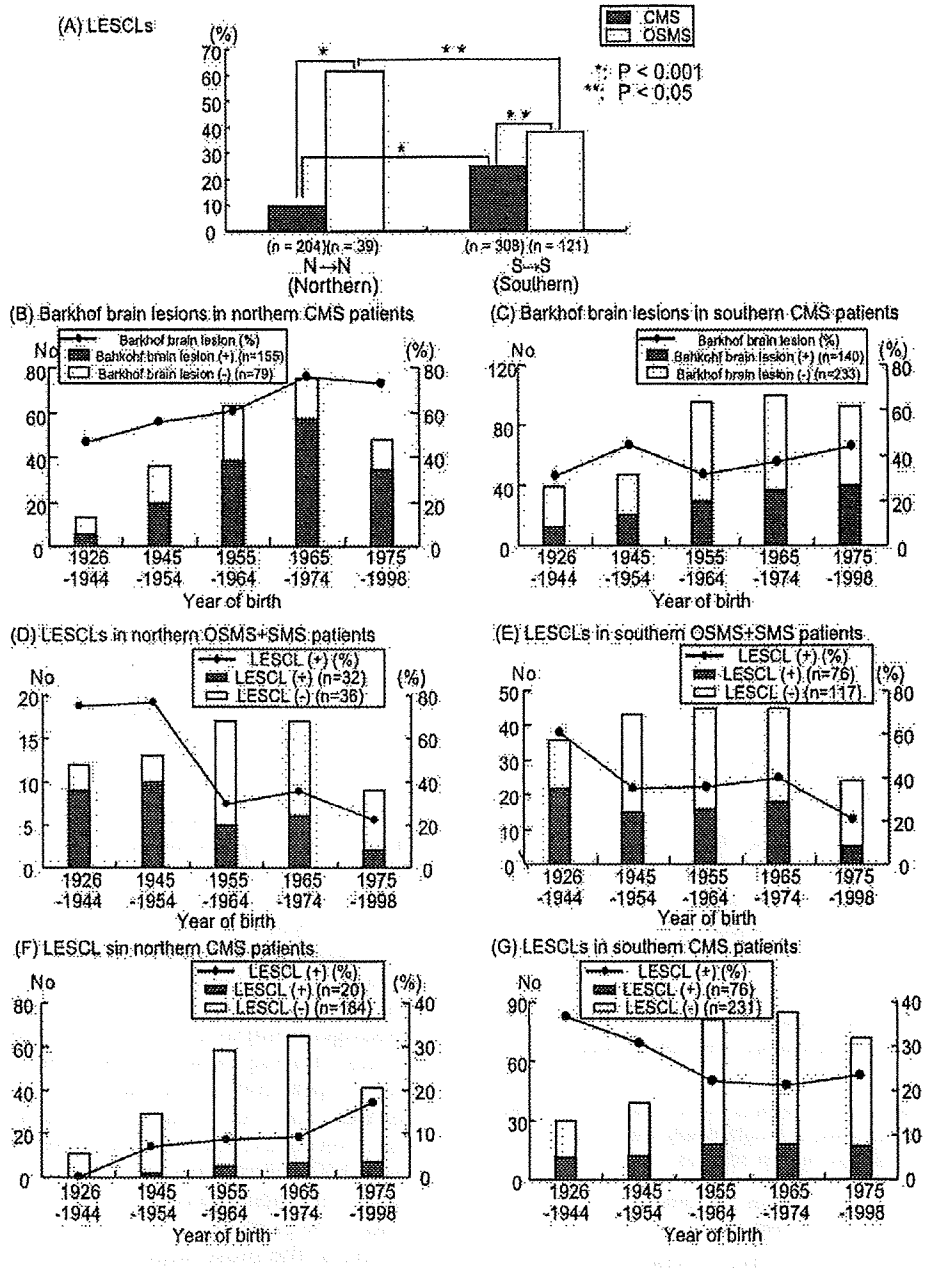
Multiple logistic analyses

Multiple logistic analyses showed that CMS phenotype ($P < 0.0001$), northern residence ($P < 0.0001$), increased CSF IgG index ($P = 0.0064$), and EDSS score ($P = 0.0380$) had significant positive associations with Barkhof brain lesions, whereas marked CSF pleocytosis was negatively associated with these measures ($P = 0.0026$) (Table 3). By contrast, EDSS score ($P < 0.0001$), marked CSF pleocytosis ($P = 0.0007$), OSMS ($P = 0.0007$), and disease duration ($P = 0.0284$) were positively associated with LESCLs, whereas increased IgG index was negatively associated ($P = 0.0398$) (Table 4). In analyses for either Barkhof brain lesions or LESCLs, substitution of CMS with CMS+OBSMS+BSMS, OSMS with OSMS+SMS, northern birth with northern residence, and CSF oligoclonal bands with increased IgG index gave essentially the same results (data not shown).

Discussion

The present study had some limitations because the questionnaires were answered by many different clinicians across the country, and the response rate in the second survey was not high. Concerning the relatively low response rate to this type of nationwide epidemiological survey in Japan, the assumption that the mean number of patients among responding hospitals is equal to that among non-responding hospitals has already been validated [29]. Moreover, there were no significant regional differences in the response rates in the present study and no significant differences between the northern and southern response rates (59% vs 55% in the preliminary survey and 46% vs 39% in the second survey, $P > 0.1$). Differences in the ascertainment rates between the northern and southern parts of Japan in previous surveys, undertaken 15 to 30 years ago, was unclear; however, as the previous surveys were carried out using practically the same methodology as the present one, it is unlikely that there were large ascertainment differences between northern and southern parts of Japan in those surveys. Therefore, we consider that our results would not be seriously distorted by the relatively low response rates. In addition, 88% of the questionnaires were collected from neurologists, 70% of whom had previously participated in a randomized controlled trial of interferon beta-1b [33], which increases the quality of the data on one hand, but produces a selection bias on the other hand. However, the validity of the data is indirectly supported by two aspects. First, although the present figure derived from clinical symptomatology-based criteria could be an underestimate compared with estimates based on recent MRI-based diagnostic criteria [27], the estimated number of patients with MS is close to the number of patients with MS (10,391) registered in the government's health care system for intractable diseases in 2003. Second, the increased MS prevalence revealed by the present study is concordant with the results of two recent epidemiological surveys carried out in the northernmost island of Japan, namely 8.57/100,000 [34] and 10.2/100,000 [35]. Both studies showed a four-fold increase in MS prevalence over a 30-year period and one of them also disclosed a similar frequency of OSMS (16% at 42° North) to that found in the present survey [34].

The steady rise in the prevalence of clinically definite MS can be explained by the increased availability of MRI, which helps with the exclusion of other diseases, and the increased number of practicing neurologists. Increased survival due to improved case ascertainment could be inferred from the prolongation of mean disease duration shown in more recent surveys. Thus, the increase in MS prevalence appears to be partly attributable to improved case ascertainment. However, the younger age at onset and two-fold increase in the proportion of females, which corresponds to a worldwide increase in the number of female patients with MS [36–38], in the latest survey, cannot be fully explained by improved case ascertainment. Orton, *et al.* [39] reported that year of birth is a significant predictor for sex ratio; the



Supplementary Figure 1 Frequencies of longitudinally extensive spinal cord lesions (LESCLs) on MRI (A) in northern-born northern residents (N→N) (northern patients), southern-born southern residents (S→S) (southern patients), northern-born southern residents (N→S), and southern-born northern residents (S→N). The difference in the frequencies of LESCLs between patients with OSMS and patients with CMS is marked in northern patients, but rather small in southern patients. Changes in the proportions and absolute numbers of CMS patients with Barkhof brain lesions in relation to birth year among northern (B) and southern (C) patients. Changes in the proportions and absolute numbers of OSMS+SMS patients with LESCLs in relation to birth year among northern (D) and southern (E) patients. Proportions and absolute numbers of CMS patients with LESCLs among northern (F) and southern (G) patients. In (B)–(G), bars indicate the absolute number in each group with the indicated birth years, while dotted lines show the changes in the positive percentages of the indicated groups. CMS, conventional multiple sclerosis; LESCLs, longitudinally extensive spinal cord lesions extending for three or more vertebral segments on MRI; *n*, number of patients whose information was obtained; OSMS, optic-spinal multiple sclerosis; SMS, spinal multiple sclerosis.

Table 3 Multiple logistic analysis for possible factors contributing to the development of Barkhof brain lesions in patients with multiple sclerosis

	Odds ratio	95% CI	*P* value
CMS	5.389	3.616–8.031	<0.0001
Female	0.784	0.518–1.187	0.2501
Age at onset (years)	0.990	0.974–1.007	0.2689
Disease duration (years)	1.019	0.991–1.048	0.1863
Northern residence	2.205	1.513–3.214	<0.0001
EDSS score	1.090	1.005–1.182	0.0380
Increased CSF IgG index	1.678	1.156–2.435	0.0064
Marked CSF pleocytosis	0.097	0.021–0.443	0.0026

CI, confidence interval; CSF, cerebrospinal fluid; EDSS, expanded disability status scale of Kurtzke; CMS, conventional multiple sclerosis.
Clinically definite multiple sclerosis patients were divided into those with or without Barkhof brain lesions.

female to male ratio increases rapidly with advancing year of birth in Canada, implicating the importance of environmental factors in early life for causing a disproportionate increase in the number of female patients with MS. An "anticipation" of age at onset was also observed in Sardinia [40], where rapid increases in the incidence and prevalence of MS over recent decades were noted [41]. Intriguingly, when individuals with ancestors originating from regions where MS is rare are raised in a region of high MS prevalence, the age at onset of MS is reported to decrease [42]. Therefore, it is possible that susceptibility to MS has increased among younger Japanese females raised in a modern Westernized environment, resulting in earlier ages at onset.

Subtype classification of MS based on symptomatology inevitably results in some ambiguity in the classification of individual cases by different institutions, and such arbitrariness in the classification may produce equivocal results. To minimize this limitation, clinical classifications were performed in all cases by the central office reviewing the collated information. Concerning the grouping of clinical symptomatology-based subtypes, patients with SMS had similarities to patients with OSMS in terms of demographic features, including MRI characteristics, whereas patients with BSMS and patients with OBSMS showed similarities to patients with CMS. In addition, comparison of patients with OSMS+SMS and patients with CMS+BSMS+OBSMS gave practically the same results as the comparison of patients with OSMS and CMS in terms of their distributions by latitude and year of birth. Therefore, we consider that although ambiguity and limitation inherent to such clinical classification and grouping should be cautiously judged, the methodology used in the present study is generally adequate for dealing with the large number of patients with MS collated from all over the country. The four nationwide surveys in Japanese included NMO according to the identical diagnostic criteria within the MS spectrum. However, the nosological positions of NMO and OSMS are less certain [9,10]. The present survey could not incorporate testing for either NMO-IgG or antibodies against the relevant autoantigen, aquaporin-4 (AQP4), because it had not been discovered when this survey was initiated. However, recent studies from Japan have shown that approximately 50% of patients with OSMS with LESCLs are negative for anti-AQP4 antibodies [8,43] and NMO-IgG or anti-AQP4 antibody-positive patients with MS frequently have periventricular ovoid lesions in the brain and short spinal cord lesions in addition to LESCLs, suggesting that there is still some overlap between NMO-IgG-positive and -negative patients with MS, at least among Japanese [8]. Therefore, although future epidemiological surveys of demyelinating diseases need to include an anti-AQP-4 antibody assay, successive nationwide surveys encompassing the NMO phenotype within the MS spectrum, based on their identical inclusion criteria, still seem to be relevant.

Table 4 Multiple logistic analysis for possible factors contributing to the development of longitudinally extensive spinal cord lesions in patients with multiple sclerosis

	Odds ratio	95% CI	*P* value
OSMS	2.931	1.578–5.444	0.0007
Female	1.726	0.949–3.137	0.0735
Age at onset (years)	0.999	0.977–1.022	0.9399
Disease duration (years)	0.958	0.922–0.995	0.0284
Northern residence	0.723	0.436–1.199	0.2087
EDSS score	1.436	1.282–1.608	<0.0001
Increased CSF IgG index	0.578	0.343–0.975	0.0398
Marked CSF pleocytosis	19.533	3.475–109.798	0.0007

CI, confidence interval; CSF, cerebrospinal fluid; EDSS, expanded disability status scale of Kurtzke; LESCLs, longitudinally extensive spinal cord lesions extending three or more vertebral segments; OSMS, optic-spinal multiple sclerosis.
Clinically definite multiple sclerosis patients were divided into those with or without LESCLs.

One of the most important findings in the present study is the large increase in the number of patients with classical type MS harboring Barkhof brain lesions because this increase showed distinctive patterns according to geography and year of birth. The differential increase in the prevalence of the CMS phenotype based on year of birth is consistent with the results of two small regional-scale studies performed in Japan that revealed increased numbers of cases of CMS relative to the numbers of OSMS cases [44,45] and cannot be explained by ascertainment bias alone. The present nationwide survey showed, for the first time, that the amount

of this increase in the prevalence of CMS differed with latitude, and that the emergence of MS-like brain lesions is also affected by latitude and year of birth. Geographically, the higher CMS/OSMS ratio and brain lesion loads in northern patients indicate the presence of environmental factors predisposing people in the north to Western-type MS. The results of the multiple logistic analyses strongly support the presence of factors predisposing people in the north to the development of MS-like brain lesions. Although the timing of migration was not specified in the present survey, the reduction in the prevalence of the CMS phenotype and brain lesion burden owing to migration from north to south may indicate the existence of environmental factors predisposing people in the north to brain lesion development that operate continuously until early adulthood or the existence of exogenous factors providing resistance to the CMS phenotype and brain lesion development in the south. Both the excess of CMS over OSMS and the increased number of patients with Barkhof brain MRI lesions with descending year of birth indicate phenotypic changes in MS associated with the year of birth, a fact that first became apparent in those born after World War II in the north, and only recently became apparent in the south. These findings point to a corresponding change in the distribution of environmental factors in Japan, especially in the north.

Another important issue is the trend toward decreased proportions of patients with monophasic NMO as well as patients with MS showing optic-spinal involvement across the four nationwide surveys over the 30-year period. Regarding patients with monophasic NMO, not only patients with onset during the survey year but also those who were previously diagnosed and visited hospitals for a regular follow-up during the survey year were enrolled. Therefore, the period of observation of these patients in the present survey was 9.5 ± 9.2 years, which is assumed to be long enough to distinguish between monophasic NMO and relapsing OSMS in most patients. Thus, the decreases in the proportions of patients with monophasic NMO and the absolute number of monophasic NMO patients over the four survey periods are considered to indicate a real decrease in the prevalence of this condition, possibly resulting from environmental changes during the rapid "Westernization" of Japan. Since the classification criteria for relapsing OSMS were first proposed in 1996 [5], this phenotype was not classified separately in the previous three surveys, meaning that the frequencies of the relapsing OSMS phenotype could not be compared among the four surveys. However, the fourth nationwide survey showed that the absolute numbers of patients with OSMS were actually unchanged over the wide range of birth years among southern patients, whereas only a modest decrease was observed with advancing year of birth among northern patients. These findings suggest the possibility that the changes in environmental factors responsible for the drastic decrease in the prevalence of monophasic NMO may not have equally lessened the occurrence of relapsing OSMS among Japanese. Although the relationships among monophasic and relapsing NMO and OSMS are still obscure, it has been reported that patients with monophasic NMO and those with relapsing NMO have significantly different autoimmune backgrounds [46]. Thus, it seems feasible that patients with monophasic NMO and those with relapsing OSMS have distinct susceptibilities to changes in environmental factors.

The present survey confirmed the frequent occurrence of LESCLs in Japanese patients with MS. Specifically, these lesions were much more common in patients with OSMS, but were also clearly evident even among patients with CMS. LESCLs are exceptional in MS in Western patients [47] but are more common in Asian patients [8,48,49]. Indeed, in the present study, multiple logistic analyses showed that factors characteristic of Asian type MS, such as marked CSF pleocytosis, OSMS phenotype, and higher EDSS score [2], were strongly related to the development of LESCLs, whereas heightened CSF IgG response, a consistent finding in patients with Western type MS [1], was negatively associated. These findings indicate that LESCLs are one of the decisive denominators of Asian type MS. In the present study, the presence of LESCLs was assessed during the entire course, which possibly resulted in an underestimation of their frequency because not all spinal cord MRI scans were taken during the acute phase. However, we consider that the approximately three-fold difference in LESCL frequency between patients with OSMS and patients with CMS would not be largely distorted by this underestimation.

Regarding the differences in the MS phenotypes associated with year of birth, although the absolute numbers of patients with OSMS and those with LESCLs as a whole were largely unchanged over the wide range of birth years, the absolute numbers of patients with OSMS+SMS with LESCLs showed a tendency to decrease with advancing year of birth, whereas the numbers of patients with CMS with LESCLs increased with advancing year of birth among northern patients; these trends were minimal among southern patients. Anti-AQP4 antibody-positive NMO patients can exhibit extra-optic-spinal manifestations, which may account for the presence of patients with CMS with LESCLs. However, because anti-AQP4 antibody-positive patients usually show a higher age at onset [8], the increased prevalence of CMS with LESCLs among