Table 1 Continued

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Histopathological findings																								
Endomysial fibrosis	(−)	(±)	(−)	(+)	(++)	(+)	(++)	(−)	(−)	(−)	(−)	(++)	(+)	(+)	(+)	(+)	(+)	(−)	(++)	(−)	(+)	(+)	(++)	(−)
Inflammation[c]	(+)e	(±)	(±)	(+++)e	(+++)p	(+)p	(+++)p,e	(±)	(++)p	(−)	(+++)p	(++)e	(+++)p, e	(+++)p	(+++)p	(+++)p	(+++)p	(+)p	(+++)p	(+)p	(+++)p	(+++)p	(+++)p	(++)p
MHC class I[d]	(−)	(+)f	(++)di	(+)f	(+)f	(−)	(++)di	(+)f	(+++)di	(+++)di	(++)di	(+)f	(+++)di	(++)f	(−)	(+++)di	(+++)di	(+++)di	(++)di	(+++)di	(+)f	(+++)di	(+)f	(++)di
Granulomatous inflammation	(−)	(−)	(−)	(+) CD4	(+)	(−)	(+) CD4	(−)	(−)	(−)	(−)	(−)	(+) CD4	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(+) CD4	(−)

a Cardiomyopathy was defined when ejection fraction decreased by <50%.
b The normal ranges are 80–260 U/l* and 100–325 U/l**.
c Density of inflammatory cells: −, 0; ±, 1 to <5; +, 5 to <10; ++, 10 to <20; +++, 20 to <100; ++++, ⩾100.
d MHC class I antigen on the sarcolemma: −, 0%; +, <50%; ++, 50 to <75%; +++, 75 to <100%.
A = arrhythmia; Af = atrial fibrillation; AF = atrial flutter; ALP = alkaline phosphatase; Asymp. = asymptomatic PBC; AV = atrioventricular; AZP = azathioprine; C = increased serum CK level; Ca = cancer; CA = catheter ablation; CD4 = CD4-positive T cell predominance in granulomatous lesions; CK = creatine kinase; CNSDC = chronic non-suppurative destructive cholangitis; CRBBB = complete right bundle branch block; CRP = C-reactive protein; CS = corticosteroids; di = diffuse; dsDNA = anti-double-stranded DNA antibody; e = endomysium; ESR = erythrocyte sedimentation rate; f = focal; F = female; homo = homogeneous staining pattern; LE = lower extremities; M = male; MRC = Medical Research Council; Mu = muscle symptoms (weakness or atrophy); NE = not examined; NI = no information; NIPPV = non-invasive positive-pressure ventilation; NSVT = non-sustained ventricular tachycardia; nucl = nucleolar staining pattern; p = perimysium; Paf = paroxysmal atrial fibrillation; PM = pacemaker; PSVT = paroxysmal supraventricular tachycardia; PVC = premature ventricular contraction; RA = rheumatoid arthritis; RF = rheumatoid factor; RNP = anti-RNP antibody; Scl70 = anti-Scl70 antibody; SLE = systemic lupus erythematosus; sp = speckled staining pattern; SS = Sjögren syndrome; SS-A = anti-SS-A antibody; SS-B = anti-SS-B antibody; SSc = systemic sclerosis; Symp. = symptomatic PBC; UE = upper extremities; w.n.l. = within normal limit.

whether bile acid therapy had some association with any change in muscle pathology/function.

Of the 12 patients treated, all except one patient, who was treated with low-dose corticosteroids in accordance with her wishes, showed normalization of creatine kinase levels within 3 months. Improvement of muscle power was observed in six patients and it remained the same in the others. One patient, whose index of AMAs was followed up after initiation of treatment, showed a mild decrease in index, from 85.1 to 21.0 in Patient 23, but two patients did not show a decrease.

In three non-treated patients (Patients 1, 2 and 5), despite no worsening of muscle weakness during the observation period (62.0 ± 30.8; range 36–96 months), two patients (Patients 1 and 5) developed arrhythmia, one of whom (Patient 5) required an implantable pacemaker.

Histopathological features of patients with inflammatory myopathy that have anti-mitochondrial antibodies

With regard to histopathological studies, in addition to the findings of inflammatory myopathies (necrotic and/or regenerating fibres, $n = 23$; inflammatory changes, $n = 23$; positive staining of the sarcolemma with MHC class I, $n = 21$; variation of muscle fibre size, $n = 18$), endomysial fibrosis, which suggests a chronic myopathic process, was found in 15 patients. No perifascicular atrophy was observed, and the invasion of non-necrotic muscle fibres by mononuclear cells was observed in one patient (Patient 22). Intriguingly, six patients (three patients with PBC and three patients without PBC) had granulomatous inflammatory lesions and four of them showed CD4-positive T cell predominance over CD8-positive T cells in the lesions (Fig. 2).

Comparison of clinical and histopathological features between anti-mitochondrial antibody-positive and anti-mitochondrial antibody-negative patients

Table 2 shows a comparison of clinical and histopathological features between AMA-positive and AMA-negative patients, and between AMA-positive patients with myositis with and without PBC.

Regarding clinical features, the AMA-positive patients with myositis had a longer disease duration before diagnosis ($P < 0.0005$), more frequently showed muscle atrophy at the initial presentation ($P < 0.005$), showed cardiac involvement ($P < 0.005$) and less frequently showed skin rash ($P < 0.05$) than the AMA-negative patients. Regarding histopathological findings, the AMA-positive patients with myopathy more frequently showed variation in muscle fibre size ($P < 0.005$), endomysial fibrosis ($P < 0.01$) and granulomatous inflammation ($P < 0.01$) than the AMA-negative patients. In the AMA-positive patients with myositis, no significant difference was found between the patients with PBC and those without PBC, except that cardiac involvement was more frequently observed in patients with PBC.

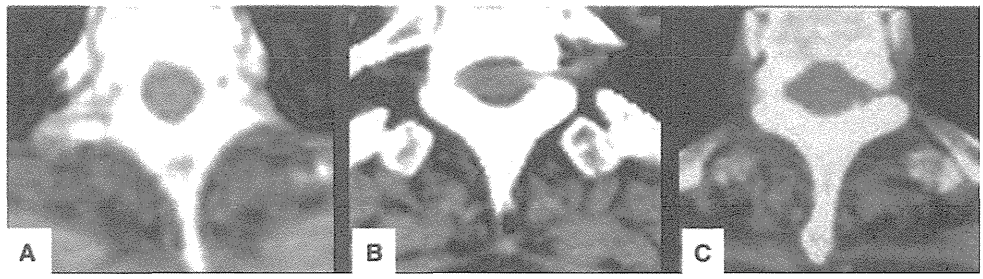

Figure 1 Muscle CT images of AMA-positive patients with the symptoms of weakness in neck flexion and lordotic posture. Paravertebral muscles at the upper thoracic level showed muscle atrophy and fatty changes in Patient 5 (**A**), Patient 7 (**B**) and Patient 23 (**C**).

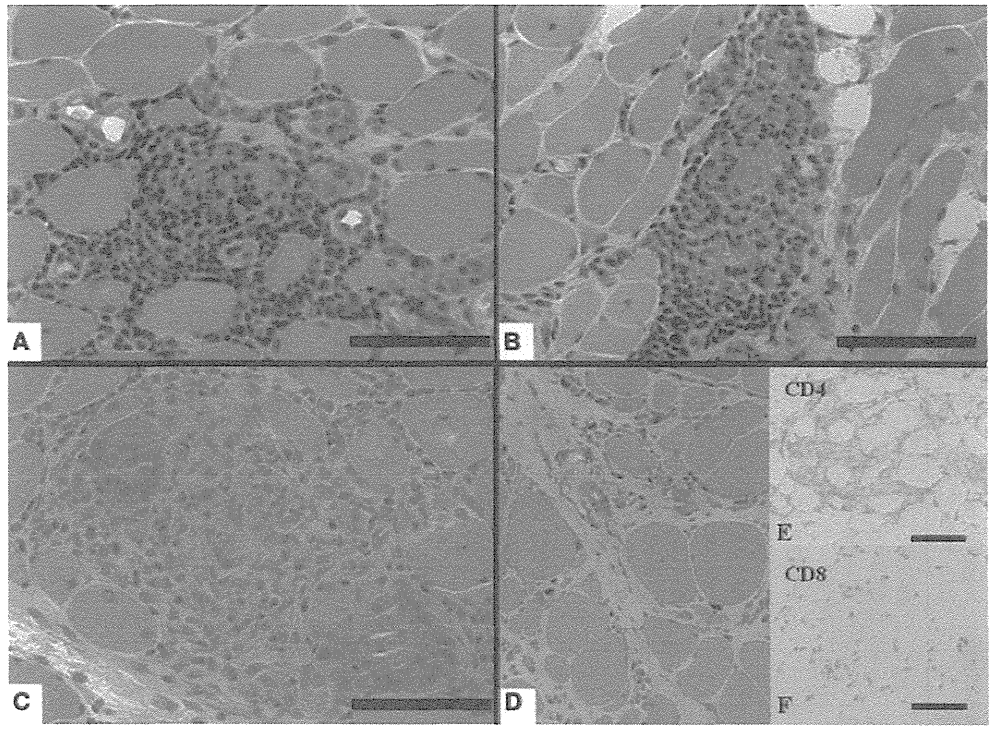

Figure 2 Muscle histopathology of AMA-positive patients with granulomatous inflammation (Patients 4, 5 and 7). Granulomatous inflammatory changes in the endomysial space are replacing muscle fibres in Patient 4 (**A**), Patient 5 (**B**) and Patient 7 (**C**). Marked fibre variation in size and increased volume of connective tissue in endomysium are also observed in Patient 7 (**D**). Surface marker analysis of infiltrating lymphocytes shows the predominance of CD4-positive lymphocytes compared with CD8-positive lymphocytes in Patient 7 (**E** and **F**). Scale bar = 100 μm.

Titre of anti-mitochondrial antibodies and clinical features

The indices of the 24 AMA-positive patients ranged from 7.4 to 124.3 (62.8 ± 43.2 index). In 13 patients with a disease duration of 12 months or longer, the index increased significantly (82.0 ± 38.6) in comparison with that of the 11 patients with a disease duration of <12 months (40.5 ± 38.8; $P < 0.05$).

A comparison of clinical and histopathological features between the patients who have high (>80) and low (≤80) indices of AMAs showed that the patients with high indices tend to have cardiomyopathies, arrhythmias and granulomatous inflammation more frequently, but differences between the two groups were not significant. However, five out of six patients with cardiomyopathies, six out of eight patients with arrhythmia and four out of six patients with the histopathological finding of granulomatous inflammation had high indices of AMAs.

In the correlation between the index of AMAs and clinical features, although the disease duration before diagnosis significantly correlated with the index of AMAs ($P < 0.05$), disease severity (modified Rankin and Medical Research Council scales) did not (Fig. 3). There was no correlation between the index of AMAs and severity or activity of PBC.

Table 2 **Comparison of clinical and histopathological features between AMA-positive patients versus AMA-negative patients with myositis and AMA-positive patients with PBC versus AMA-positive patients without PBC**

Clinical and histopathological findings	AMA-positive myositis patients (*N* = 24)	AMA-negative myositis patients (*N* = 188)	Significance (AMA-positive versus AMA-negative)	AMA-positive myositis patients with PBC (*N* = 7)	AMA-positive myositis patients without PBC (*N* = 17)	Significance (PBC^+ versus PBC^-)
Sex (M:F)	9:15	58:130	NS	5:2	4:13	NS
Age at disease onset (years)	54 ± 13	55 ± 15	NS	54 ± 10	54 ± 15	NS
Disease duration before diagnosis (months)	20 ± 20	12 ± 36	$P < 0.0005$	26 ± 17	17 ± 21	NS
Clinical signs and symptoms, *n/N* (%)						
Severe limb muscle weakness (⩽MRC3)	7/24 (29)	55/177 (32)	NS	2/7 (29)	5/17 (29)	NS
Severe neck muscle weakness (⩽MRC3)	7/22 (32)	51/137 (37)	NS	2/6 (33)	5/16 (31)	NS
Myalgia	7/23 (30)	95/163 (58)	$P < 0.05$	2/6 (33)	5/17 (29)	NS
Muscle atrophy	13/24 (54)	40/188 (21)	$P < 0.005$	4/7 (57)	9/17 (53)	NS
Skin rash	2/24 (8)	60/188 (32)	$P < 0.05$	0/7 (0)	2/17 (12)	NS
Cardiac involvement[a]	8/24 (33)	17/188 (9)	$P < 0.005$	5/7 (71)	3/17 (18)	$P < 0.05$
Restrictive ventilatory impairment[b]	6/19 (32)	41/126 (33)	NS	2/7 (29)	4/12 (33)	NS
Dysphagia	4/21 (19)	30/121 (25)	NS	2/7 (29)	2/14 (14)	NS
Associated disorders, *n/N* (%)						
Collagen disease	7/24 (29)	43/188 (23)	NS	2/7 (29)	5/17 (29)	NS
Malignancies	3/24 (13)	28/188 (15)	NS	0/7 (0)	3/17 (18)	NS
Interstitial lung disease	6/24 (25)	75/188 (40)	NS	1/7 (14)	5/17 (29)	NS
Laboratory data						
CK level (U/l)	2322 ± 3121	3160 ± 8627	NS	1183 ± 1126	2791 ± 3567	NS
ESR (mm/h)	51 ± 26	41 ± 30	$P < 0.05$	73 ± 26	43 ± 20	$P < 0.05$
CRP level (mg/dl)	0.8 ± 0.8	1.5 ± 4.1	NS	0.7 ± 0.4	0.8 ± 0.9	NS
AMAs (index)	62.8 ± 43.2	2.4 ± 1.5	$P < 0.0001$	98.3 ± 33.6	48.2 ± 38.5	$P < 0.01$
Histopathological findings, *n/N* (%)						
Variation in muscle fibre size	18/24 (75)	75/188 (40)	$P < 0.005$	7/7 (100)	11/17 (65)	NS
Disruption of myofibrillar architecture	6/24 (25)	72/188 (39)	NS	1/7 (14)	5/17 (29)	NS
Internal nuclei[c]	9/24 (38)	59/188 (32)	NS	5/7 (71)	4/17 (24)	NS
Necrotic and/or regenerating fibres	23/24 (96)	168/188 (89)	NS	7/7 (100)	16/17 (94)	NS
Endomysial fibrosis	15/24 (63)	46/188 (25)	$P < 0.01$	5/7 (71)	10/17 (59)	NS
Perifascicular atrophy	0/24 (0)	20/188 (11)	NS	0/7 (0)	0/17 (0)	–
Inflammatory cell infiltration[d]	23/24 (96)	173/188 (92)	NS	7/7 (100)	16/17 (94)	NS
Mononuclear cells invading non-necrotic muscle fibres	1/24 (4)	8/188 (4)	NS	0/7 (0)	1/17 (6)	NS
Positive staining of the sarcolemma with MHC class I	21/24 (88)	174/188 (93)	NS	5/7 (71)	16/17 (94)	NS
Granulomatous inflammation	6/24 (25)	11/188 (6)	$P < 0.01$	3/7 (43)	3/17 (18)	NS
CD4-positive lymphocyte predominance	4/24 (17)	10/139 (7)	NS	2/7 (29)	2/17 (12)	NS

a Cardiac involvement was defined as a condition when ejection fraction was decreased by <50% or presence of arrhythmia.
b Restrictive ventilatory involvement was defined as a condition when the vital capacity was <80%.
c Positive finding of internal nuclei was defined as a finding of >5% of muscle fibres associated with internal nuclei.
d Positive inflammatory change was defined as a change when more than five inflammatory cells infiltrated the perivascular or endomysium in the muscle tissues.
CK = creatine kinase; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; F = female; M = male; MRC = Medical Research Council; NS = not significant.

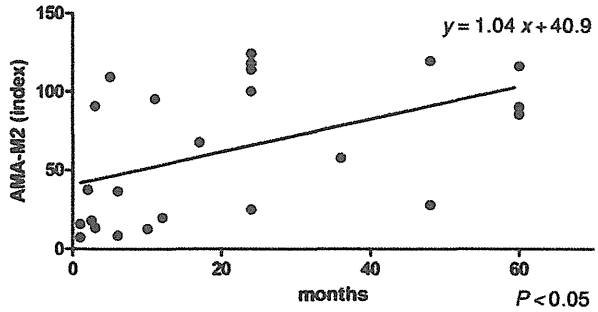

Figure 3 Correlation between titre of AMAs and disease duration before diagnosis. The index of AMA-M2 significantly correlated with the disease duration before diagnosis ($P < 0.05$).

Discussion

The association of PBC with inflammatory myopathies has been reported mainly as case reports, and comprehensive studies of the prevalence and the clinical and histopathological features of patients with inflammatory myopathies and AMAs or PBC have not been conducted thus far. In this study, we retrospectively reviewed 212 patients with inflammatory myopathies, and found 24 patients with AMAs (11.3%) and seven patients with PBC (3.3%). By analysing the clinical and histopathological features, we found that AMA-positive patients with inflammatory myopathies frequently include patients with a clinically chronic disease course, muscle atrophy, cardiac involvement and granulomatous inflammation regardless of the presence or absence of PBC. Although the comparative analysis did not show a significant difference, in our 24 patients with AMAs, two patients required respiratory support because of restrictive ventilator impairment, which is unusual for typical cases of inflammatory myopathies.

Considering the characteristic clinical features and the significant correlation between the index of AMAs and the disease duration before diagnosis, we believe that these suggest the importance of AMAs not only as markers but also as factors involved in pathogenic mechanisms. Of the 24 AMA-positive patients, despite the characteristic features of the whole group, some patients showed a subacute or acute clinical course, no muscle atrophy, and no association with cardiopulmonary involvements, indicating some variation in clinical features. Further study should be carried out to clarify the roles of AMAs in the pathogenesis of AMA-positive myositis.

Presently, the mechanisms underlying PBC pathogenesis remain unknown. In the initiation of the mechanism underlying PBC pathogenesis, the mimotopes of the vulnerable epitope of the PDC-E2 autoantigen are considered to become the autoantigens in PBC (Lleo *et al.*, 2008). In the production of the mimotopes, the modification of native proteins following the exposure to infectious microorganisms, environmental xenobiotics/chemical compounds or apoptotic biliary epithelial cells has been suggested (Selmi *et al.*, 2011). In the presence of altered regulation of self-tolerance and genetic background, it is suggested that AMAs are produced by PDC-E2 autoantigen-specific B cells and PDC-E2 autoantigen-specific T cells present in sera (Selmi *et al.*, 2011). Regarding the pathogenesis of liver damage in PBC, it has been revealed that the autoreactive CD4-positive and CD8-positive T cells infiltrating the liver in PBC recognize PDC-E2 (Hohenester *et al.*, 2009), which supports the hypothesis that T cell responses contribute to bile duct injury in PBC.

On the other hand, although AMAs are highly specific for PBC, the pathogenic role for them in this disease is uncertain since in contrast to other autoimmune diseases, PBC responds poorly to immunosuppressive agents and changes in autoantibody titre do not seem to correlate with disease severity (Selmi *et al.*, 2011).

Recently, it has been suggested that the pathogenic immune attack in PBC may be directed not only against the proteins of the 2-oxo acid dehydrogenase family (M2 antigen) but also against other antigens that become exposed during apoptosis and proliferation of biliary epithelial cells. Among these antigens, nuclear antigens, neuroendocrine compartments such as the acetylcholine receptor muscarinic M3 receptor, the α1 adrenergic receptor and proteins of the Bcl-2 family have been suggested (Berg, 2011).

Indeed, it has been known that antinuclear antibodies are detected in ~50% of serum samples from patients with PBC (Selmi *et al.*, 2011). Among them, an autoantibody against glycoprotein 201 was reported to correlate with severity of PBC.

Furthermore it was recently shown that sera from patients with PBC have functionally active anti-acetylcholine receptor muscarinic M3 autoantibodies and the author suggested the possibility of receptor desensitization due to repeated interactions of the receptor with the autoantibodies (Berg, 2011). Nicotinic but not muscarinic anti-acetylcholine receptor autoantibodies were detected in AMA-positive patients with PBC in early studies (Sundewall *et al.*, 1987; Kyriatsoulis *et al.*, 1988). Considering a previous report describing the relationships between autoimmunity against the β1-adrenergic receptor autoantibody or muscarinic acetylcholine receptor and dilated cardiomyopathy (Jahns *et al.*, 2004), in addition to autoreactive CD4-positive and CD8-positive T cells, autoantibodies against antigens other than AMAs may have some pathogenic role in patients with PBC. Further studies are necessary to reveal the mechanism of skeletal muscle damage in patients with PBC.

In our series, eight patients showed myositis associated with arrhythmia. Among them, two patients required treatment by catheter ablation and one patient required an implantable pacemaker. Furthermore, in three patients who refused any treatment, two developed arrhythmia, one of whom required an implantable pacemaker.

In previous case reports, as far as we searched, cardiac involvement in myositis in the presence of AMAs has been described in eight patients (Uhl *et al.*, 1974; Saitoh *et al.*, 1988; Harada *et al.*, 1992; Varga *et al.*, 1993; Tsai *et al.*, 1996; Kasuga *et al.*, 2004; Tanaka *et al.*, 2007). Among them, five patients had a chronic disease course (2 years, $n = 2$; 5 years, $n = 3$), and all eight patients, including one patient who required an implantable pacemaker, showed arrhythmia (supraventricular tachycardia, $n = 5$; ventricular tachycardia, $n = 2$; atrioventricular block, $n = 1$). Seven patients, including three patients who received treatment for dilated cardiomyopathies, had a decreased ejection fraction.

Interestingly, six patients with cardiac involvement and PBC have been diagnosed as having asymptomatic PBC.

From the view-point of cardiovascular system functions, abnormal autonomic nervous system regulation in patients with PBC was reported (Selmi *et al.*, 2011), and other reports show that autonomic dysfunction in PBC is associated with an increased cardiac mortality risk in non-liver chronic disease states (Neubauer *et al.*, 1997; Jackson *et al.*, 2007). Furthermore, a recent study showed impaired cardiovascular function in PBC using impedance cardiography and magnetic resonance methodologies (Jones *et al.*, 2010). Considering the above previous reports, in addition to our present results, it is possible that the frequent association of cardiac complications is a characteristic feature in inflammatory myopathies with PBC. Further study should be carried out to clarify the association between arrhythmia and AMAs, and myositis patients with AMA should be followed up carefully for cardiac complications, especially arrhythmia.

From the diagnostic view-point of myopathies, it should be noted that the 10 patients in our series showed a clinically chronic course with muscle atrophy, the findings of which are common to those of muscular dystrophy. In patients with muscle atrophy, three had paraspinal muscle involvement with lordotic posture, which is an atypical feature of inflammatory myopathies. Since myositis patients with AMA respond to treatment, as a diagnostic approach to chronic myopathies, AMAs should be evaluated particularly in patients with chronic myopathies and muscle atrophy associated with or without lordortic posture, or cardiopulmonary involvement. Moreover, the patients presenting PBC associated with AMAs with increased serum creatine kinase levels should be evaluated if they show muscle involvement or cardiomyopathy.

It has been reported that granulomatous inflammation with bile duct injury is a characteristic liver histopathological change in PBC (Ludwig *et al.*, 1978). Thus, it is interesting that six of the patients with a clinically chronic disease course showed granulomatous inflammation in muscle histopathology. In association with PBC, granulomatous extrahepatic lesions have been described in other organs such as the skin (Kishor *et al.*, 2008) and lungs (Fagan *et al.*, 1983). The presence of granulomatous inflammation in the patients with such characteristic clinical features also suggests that a pathogenic mechanism may be related to that of PBC. Further study should be carried out to clarify the exact background mechanism underlying this association.

AMAs in serum are highly sensitive and specific for PBC; they are detected in nearly 95% of patients with PBC, with specificity close to 100% when tested with recombinant antigens (Selmi *et al.*, 2011). In our study, we used an enzyme-linked immunosorbent assay for the detection of immunoglobulin G, M or A class antibodies against at least one of the 2-OADC enzymes. When performed in accordance with the manufacturer's protocol (Kadokawa *et al.*, 2003), the specificity of the detection method is 98%. In our series, seven patients were diagnosed as having inflammatory myopathies with PBC and 17 patients were diagnosed as having inflammatory myopathies with AMAs without any clinical features of PBC. In comparison of the two groups, associated autoimmune diseases and autoantibodies were observed in both groups, and no significant difference in clinical features was found between the groups except for cardiac involvement being more frequently observed in patients with PBC than in those without PBC (Table 2).

Although AMAs serve as highly sensitive markers for the diagnosis of PBC, AMAs can frequently be detected in patients with other diseases, such as primary systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis and autoimmune hepatitis (Hu *et al.*, 2010). It has also been reported that other autoantibodies associated with PBC are rheumatoid factors (70%), anti-smooth muscle antibodies (66%), anti-thyroid (anti-microsomal, anti-thyroglobulin) antibodies (40%) and anti-nuclear antibodies (35–50%) (Talwalkar and Lindor, 2003; Selmi *et al.*, 2011). It has also been reported that AMA-positive individuals, even those without signs of cholestasis or liver inflammation, are very likely to develop PBC (Metcalf *et al.*, 1996; Hohenester *et al.*, 2009). It is not clear whether the AMA-positive patients without PBC in our series will develop PBC several years later. However, considering that inflammatory myopathies associated with AMAs are frequently observed in patients with a clinically chronic disease course, muscle atrophy, cardiopulmonary involvement and granulomatous inflammation regardless of the presence or absence of PBC, we believe that inflammatory myopathies associated with AMAs form a characteristic subgroup.

Further study, including the study about the roles of autoantibodies against antigens other than AMAs in PBC, should be carried out to clarify the exact background mechanism underlying this association.

Funding

The Health and Labour Sciences Research Grant on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan; Intramural Research Grant (23-5) for Neurological and Psychiatric Disorders of National Center of Neurology and Psychiatry (NCNP).

References

Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). N Engl J Med 1975a; 292: 344–7.

Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med 1975b; 292: 403–7.

Boki KA, Dourakis SP. Polymyositis associated with primary biliary cirrhosis. Clin Rheumatol 1995; 14: 375–8.

Benoist M, Henin D, Kahn MF, Benhamou JP. Primary biliary cirrhosis associated with rheumatoid arthritis and acute polymyositis. Nouv Presse Med 1977; 6: 2427–9.

Berg PA. The role of the innate immune recognition system in the pathogenesis of primary biliary cirrhosis: a conceptual view. Liver Int 2011; 31: 920–31.

Berg PA, Klein R, Lindenborn-Fotinos J. Antimitochondrial antibodies in primary biliary cirrhosis. J Hepatol 1986; 2: 123–31.

Charron L, Peyronnard JM, Marchand L. Sensory neuropathy associated with primary biliary cirrhosis. Histologic and morphometric studies. Arch Neurol 1980; 37: 84–7.

Dahlan Y, Smith L, Simmonds D, Jewell LD, Wanless I, Heathcote EJ, et al. Pediatric-onset primary biliary cirrhosis. Gastroenterology 2003; 125: 1476–9.

Epstein O, Burroughs AK, Sherlock S. Polymyositis and acute onset systemic sclerosis in a patient with primary biliary cirrhosis: a clinical syndrome similar to the mixed connective tissue disease. J R Soc Med 1981; 74: 456–8.

Fagan EA, Moore-Gillon JC, Turner-Warwick M. Multiorgan granulomas and mitochondrial antibodies. N Engl J Med 1983; 308: 572–5.

Greenberg SA, Amato AA. Uncertainties in the pathogenesis of adult dermatomyositis. Curr Opin Neurol 2004; 17: 359–64.

Griggs RC, Askanas V, DiMauro S, Engel A, Karpati G, Mendell JR, et al. Inclusion body myositis and myopathies. Ann Neurol 1995; 38: 705–13.

Harada N, Dohmen K, Itoh H, Ohshima T, Yamamoto H, Nagano M, et al. Sibling cases of primary biliary cirrhosis associated with polymyositis, vasculitis and Hashimoto's thyroiditis. Intern Med 1992; 31: 289–93.

Hohenester S, Oude-Elferink RP, Beuers U. Primary biliary cirrhosis. Semin Immunopathol 2009; 31: 283–307.

Honma F, Shio K, Monoe K, Kanno Y, Takahashi A, Yokokawa J, et al. Primary biliary cirrhosis complicated by polymyositis and pulmonary hypertension. Intern Med 2008; 47: 667–9.

Hoogendijk J, Amato A, Lecky B, Choy E, Lundberg I, Rose M, et al. 119th ENMC international workshop: trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis, 10-12 October 2003, Naarden, The Netherlands. Neuromuscul Disord 2004; 14: 337–45.

Hu CJ, Zhang FC, Li YZ, Zhang X. Primary biliary cirrhosis: what do autoantibodies tell us? World J Gastroenterol 2010; 16: 3616–29.

Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. N Engl J Med 2007; 357: 2153–65.

Illa I, Graus F, Ferrer I, Enriquez J. Sensory neuropathy as the initial manifestation of primary biliary cirrhosis. J Neurol Neurosurg Psychiatry 1989; 52: 1307.

Jackson H, Solaymani-Dodaran M, Card TR, Aithal GP, Logan R, West J. Influence of ursodeoxycholic acid on the mortality and malignancy associated with primary biliary cirrhosis: a population-based cohort study. Hepatology 2007; 46: 1131–7.

Jahns R, Boivin V, Hein L, Triebel S, Angermann CE, Ertl G, et al. Direct evidence for a beta 1-adrenergic receptor-directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy. J Clin Invest 2004; 113: 1419–29.

Jones DE, Hollingsworth K, Fattakhova G, MacGowan G, Taylor R, Blamire A, et al. Impaired cardiovascular function in primary biliary cirrhosis. Am J Physiol Gastrointest Liver Physiol 2010; 298: G764–73.

Kadokawa Y, Omagari K, Hazama H, Ohba K, Masuda J, Kinoshita H, et al. Evaluation of newly developed ELISA using "MESACUP-2 test mitochondrial M2" kit for the diagnosis of primary biliary cirrhosis. Clin Biochem 2003; 36: 203–10.

Kasuga K, Sato A, Kanazawa M, Kobayashi H, Tanaka K, Nishizawa M. A case with severe respiratory muscle weakness due to chronic myositis associated with PBC. Rinsho Shinkeigaku 2004; 44: 280–5.

Kishor S, Turner M, Borg B, Kleiner D, Cowen E. Cutaneous sarcoidosis and primary biliary cirrhosis: a chance association or related diseases? J Am Acad Dermatol 2008; 58: 326–35.

Kumazawa K, Kayanuma K, Kamiya T, Yomura Y, Uono M. A case of granulomatous polymyositis associated with asymptomatic primary biliary cirrhosis and immune thrombocytopenic purpura. Rinsho Shinkeigaku 1987; 27: 867–72.

Kyriatsoulis A, Manns M, Gerken G, Lohse AW, Maelicke A, Wessler I, et al. Immunochemical characterization of anti-acetylcholine receptor antibodies in primary biliary cirrhosis. J Hepatol 1988; 6: 283–90.

Lindor KD, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ. Primary biliary cirrhosis. Hepatology 2009; 50: 291–308.

Lleo A, Invernizzi P, Mackay IR, Prince H, Zhong RQ, Gershwin ME. Etiopathogenesis of primary biliary cirrhosis. World J Gastroenterol 2008; 7: 14: 3328–37.

Love LA, Leff RL, Fraser DD, Targoff IN, Dalakas M, Plotz PH, et al. A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. Medicine 1991; 70: 360–74.

Ludwig J, Dickson ER, McDonald GS. Staging of chronic nonsuppurative destructive cholangitis (syndrome of primary biliary cirrhosis). Virchows Arch A Pathol Anat Histol 1978; 379: 103–12.

Mader R, Blake R, Keystone EC. Polymyositis associated with primary biliary cirrhosis. J Rheumatol 1991; 18: 1767–8.

Manthorpe R, Permin H, Tage-Jensen U. Auto-antibodies in Sjögren's syndrome. With special reference to liver-cell membrane antibody (LMA). Scand J Rheumatol 1979; 8: 168–72.

Matsui K, Aizawa Y, Inoue K, Yaguchi H, Toda G. Polymyositis with marked paravertebral muscle atrophy in patients with primary biliary cirrhosis. Rinsho Shinkeigaku 2000; 40: 694–700.

Metcalf JV, Mitchison HC, Palmer JM, Jones DE, Bassendine MF, James OF. Natural history of early primary biliary cirrhosis. Lancet 1996; 348: 1399–402.

Milosevic M, Adams P. Primary biliary cirrhosis and polymyositis. J Clin Gastroenterol 1990; 12: 332–5.

Miyakawa H, Tanaka A, Kikuchi K, Matsushita M, Kitazawa E, Kawaguchi N, et al. Detection of antimitochondrial autoantibodies in immunofluorescent AMA-negative patients with primary biliary cirrhosis using recombinant autoantigens. Hepatology 2001; 34: 243–8.

Mutimer D, Fussey S, Yeaman S, Kelly P, James O, Bassendine M. Frequency of IgG and IgM autoantibodies to four specific M2 mitochondrial autoantigens in primary biliary cirrhosis. Hepatology 1989; 10: 403–7.

Nakasho K, Nishigami T, Sugihara A, Uematsu K, Sawada K, Shintani S, et al. A male case of polymyositis associated with asymptomatic primary biliary cirrhosis. Nippon Shokakibyo Gakkai Zasshi 1996; 93: 666–70.

Neubauer S, Horn M, Cramer M, Harre K, Newell JB, Peters W, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. Circulation 1997; 96: 2190–6.

Ono M, Niinobu T, Yamanaka Y, Yasuda O, Rakugi H, Ikegami H, et al. Primary biliary cirrhosis with polymyositis successfully treated with prednisolone and ursodeoxycholic acid. Nippon Ronen Igakkai Zasshi 1996; 33: 779–82.

Saitoh S, Yamada S, Takagi H, Abe T, Takezawa J, Nagamine T, et al. A male autopsy case of primary biliary cirrhosis associated with polymyositis and congestive cardiomyopathy. Nippon Shokakibyo Gakkai Zasshi 1988; 85: 287–91.

Selmi C, Bowlus CL, Gershwin ME, Coppel RL. Primary biliary cirrhosis. Lancet 2011; 377: 1600–9.

Simpson WG, Nickl NJ. Polymyositis associated with primary biliary cirrhosis. J Ky Med Assoc 1995; 93: 93–4.

Skopouli F, Barbatis C, Moutsopoulos H. Liver involvement in primary Sjögren's syndrome. Br J Rheumatol 1994; 33: 745–8.

Sundewall AC, Lefvert AK, Norberg R. Characterization of anti-acetylcholine receptor antibody activity in patients with anti-mitochondrial antibodies. Clin Immunol Immunopathol 1987; 45: 184–95.

Takemura M, Ohya K, Kojima K. Fundamental evaluation of "MESACUP-2 Test Mitochondrial M2". Jpn J Med Pharm Sci 2001; 46: 809–16.

Talwalkar JA, Lindor KD. Primary biliary cirrhosis. Lancet 2003; 362: 53–61.

Tanaka K, Sato A, Kasuga K, Kanazawa M, Yanagawa K, Umeda M, et al. Chronic myositis with cardiomyopathy and respiratory failure associated with mild form of organ-specific autoimmune diseases. Clin Rheumatol 2007; 26: 1917–9.

Tsai WC, Matsumura K, Sonoo M, Motoyoshi Y, Shiga J, Shimizu T. Dystrophin abnormality in a patient with polymyositis associated with primary biliary cirrhosis and myocardial injury. Rinsho Shinkeigaku 1996; 36: 876–80.

Tsuchiya K, Kakita E, Wada Y, Furukawa T, Tsukagoshi H. Two cases of polymyositis associated with liver dysfunction. Rinsho Shinkeigaku 1985; 25: 667–73.

Ueda Y, Araki K, Matsumoto M, Okanoue T, Takino T. A case of polymyositis associated with asymptomatic primary biliary cirrhosis. Nippon Naika Gakkai Zasshi 1988; 77: 115–6.

Uhl GS, Baldwin JL, Arnett FC. Primary biliary cirrhosis in systrmic sclerosis (scleroderma) and polymyositis. Johns Hopkins Med J 1974; 135: 191–8.

Varga J, Heiman-Patterson T, Munoz S, Love LA. Myopathy with mitochondrial alterations in patients with primary biliary cirrhosis and anti-mitochondrial antibodies. Arthritis Rheum 1993; 36: 1468–75.

Van de Water J, Cooper A, Surh C, Coppel R, Danner D, Ansari A, et al. Detection of autoantibodies to recombinant mitochondrial proteins in patients with primary biliary cirrhosis. N Engl J Med 1989; 320: 1377–80.

Van de Water J, Gershwin M, Leung P, Ansari A, Coppel R. The autoepitope of the 74-kD mitochondrial autoantigen of primary biliary cirrhosis corresponds to the functional site of dihydrolipoamide acetyltransferase. J Exp Med 1988; 167: 1791–9.

Willson RA. Therapeutic quandary: asymptomatic primary biliary cirrhosis associated with polymyositis. Dig Dis Sci 1981; 26: 372–5.

Yasuda Y, Nakano S, Akiguchi I, Tanaka M, Kameyama M. A case of polymyositis associated with primary biliary cirrhosis. Rinsho Shinkeigaku 1990; 30: 88–93.

ARTHRITIS & RHEUMATISM
Vol. 64, No. 11, November 2012, pp 3741–3749
DOI 10.1002/art.34629

T Lymphocytes and Muscle Condition Act Like Seeds and Soil in a Murine Polymyositis Model

Naoko Okiyama,[1] Takahiko Sugihara,[1] Takatoku Oida,[2] Junko Ohata,[2] Hiroo Yokozeki,[3] Nobuyuki Miyasaka,[3] and Hitoshi Kohsaka[1]

Objective. **It has been reported that polymyositis (PM) is driven by CD8+ cytotoxic T lymphocytes. The C protein–induced myositis (CIM) model we have established is similar to PM in pathology except that it undergoes spontaneous remission. We undertook the present study to delineate the roles of innate and acquired immunity in myositis.**

Methods. **C57BL/6 mice were immunized with recombinant C protein fragments together with Freund's complete adjuvant (CFA) and Toll-like receptor (TLR) ligands at hind leg footpads and tail bases. CIM mediated by adoptive transfer of T cells to naive mice was treated with cytokine antagonists.**

Results. **Second immunization with C protein fragments revealed no induction of tolerance. Injection of CFA and TLR ligands at the hind leg footpads reinduced myositis in the same legs. Interestingly, initial myositis was observed only in the CFA-treated forelegs. Transfer of C protein fragment–specific T cells from mice with CIM induced myositis in CFA- and TLR ligand–treated legs of recipient mice. CFA treatment resulted in the recruitment of macrophages producing inflammatory cytokines. Induction of myositis was inhibited by blocking interleukin-1 receptor or tumor necrosis factor α.**

Conclusion. **Myositis development requires activation of autoaggressive T cells and conditioning of muscle tissue. CIM regression is due to attenuation of local CFA-induced immune activation. These results are in accordance with a "seed and soil" model of disease development and might offer clues to decipher clinical aspects of PM.**

Polymyositis (PM) is a chronic inflammatory myopathy of unknown etiology. It affects striated muscles and induces varying degrees of muscle weakness, especially in the proximal muscles (1). Dysphagia and respiratory muscle weakness with choking episodes and/or recurrent aspiration pneumonia can lead to premature death of patients. Current standard treatment is administration of high-dose glucocorticoids and/or immunosuppressants, which do not address the specific pathology of PM. Some patients have unwanted side effects, while other patients' disease is refractory to these drugs.

Immunohistochemical analysis of biopsy specimens of muscle tissue in PM suggested that muscle injury is driven primarily by CD8+ cytotoxic T lymphocytes (CTLs) (2). Thus, the disease process derives from systemic autoimmune reactions to muscle autoantigen(s), which could incite systemic muscle inflammation. However, the entire muscle system is not necessarily

Supported by a grant-in-aid from the Ministry of Health, Labor, and Welfare, Japan, a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan, a Basic Dermatological Research grant from the Japanese Dermatological Association, funded by the Shiseido Company, a Young Investigator Rheumatology Research grant to Dr. Okiyama from the Japan Rheumatism Foundation, and a Global Center of Excellence Program grant from the Japan Society for the Promotion of Science to the International Research Center for Molecular Science in Tooth and Bone Diseases at Tokyo Medical and Dental University. Dr. Okiyama is recipient of a Research Fellow grant-in-aid from the Japan Society for the Promotion of Science, funded by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

[1]Naoko Okiyama, MD, PhD (current address: National Cancer Institute, NIH, Bethesda, Maryland), Takahiko Sugihara, MD, PhD, Hitoshi Kohsaka, MD, PhD: Tokyo Medical and Dental University, Tokyo, Japan, and RIKEN Yokohama Institute, Yokohama City, Kanagawa, Japan; [2]Takatoku Oida, PhD, Junko Ohata, MD, PhD (current address: Chugai Pharmaceutical, Tokyo, Japan): RIKEN Yokohama Institute, Yokohama City, Kanagawa, Japan; [3]Hiroo Yokozeki, MD, PhD, Nobuyuki Miyasaka, MD, PhD: Tokyo Medical and Dental University, Tokyo, Japan.

Dr. Miyasaka has received honoraria from Chugai Pharmaceutical (more than $10,000).

Address correspondence to Hitoshi Kohsaka, MD, PhD, Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: kohsaka.rheu@tmd.ac.jp.

Submitted for publication August 2, 2011; accepted in revised form July 5, 2012.

subject to inflammation in PM. This is evident especially when the muscles of the whole body are scanned with magnetic resonance imaging (MRI). The affected muscles are often separated clearly from unaffected muscles (3). Moreover, peripheral blood of PM patients in clinical remission still contained expanding CD8+ cytotoxic T cell clones (4,5). These clones appeared autoreactive since they were found in the inflamed muscles prior to the remission induction (5).

In order to gain mechanistic insights into the pathology of PM, we have established a murine PM model, skeletal muscle C protein–induced myositis (CIM) (6). CIM can be induced by single immunization with recombinant mouse or human skeletal muscle C protein fragments in Freund's complete adjuvant (CFA). Our previous studies demonstrated that CIM is primarily mediated by CD8+ CTLs (6,7). This is in sharp contrast with the classic myosin-induced experimental autoimmune myositis model, which is driven by CD4+ T cells and humoral immunity (8,9). While experimental autoimmune myositis is induced in SJL/J mice carrying a mutated dysferlin gene that causes spontaneous muscle necrosis and secondary inflammation (10), CIM can be induced in C57BL/6 (B6) and other strains of mice.

As with most other models of autoimmune disease that are inducible by immunization with autoantigens, CIM resolves spontaneously. The myositis peaks 2 to 3 weeks after the immunization with C protein fragments and regresses in 10 weeks (6,11). Since muscle fibers regenerate rapidly, no histologic abnormality is observed after the resolution of disease.

Spontaneous regression of inducible autoimmune disease models has been intensively investigated because the results might grant insight into the development of new treatment modalities. For example, disease regression in experimental autoimmune encephalomyelitis (EAE; a model of multiple sclerosis) in rats induced by immunization with myelin basic protein is associated with the appearance of immunosuppressive cytokines including transforming growth factor β and interleukin-4 (IL-4) (12). Recently, accumulating evidence suggests that naturally arising CD25+CD4+ Treg cells actively act to maintain peripheral self tolerance (13). CD25+ cell–depleted mice had significantly more severe diseases in murine models of multiple sclerosis, rheumatoid arthritis (RA), myasthenia gravis, and Hashimoto thyroiditis (14–18).

In the present studies, extensive investigation was directed at addressing the mechanistic processes involved in spontaneous regression of CIM. The results show that attenuated activity of autoaggressive T cells was not exclusively responsible for the spontaneous regression of CIM. Instead, attenuation of innate immunity in the muscles also contributed to disease regression. These facts led us to propose a "seed and soil" theory of autoimmune tissue damage, in which autoaggressive T cells and activation of innate immunity act as seed and soil, respectively. Combination therapies that address pathomechanisms of both aspects involved in autoimmune disease damage may be an effective approach to explore in the treatment of disease.

MATERIALS AND METHODS

Mice. B6 mice were purchased from Charles River Japan. All experiments were carried out under specific pathogen–free conditions in accordance with the ethics and safety guidelines for animal experiments of Tokyo Medical and Dental University and RIKEN.

CIM induction and recurrence. To induce CIM, female B6 mice ages 8–10 weeks were immunized intradermally with 200 μl of an emulsion consisting of 200 μg of murine C protein fragments emulsified in CFA containing 100 μg of heat-killed *Mycobacterium butyricum* (Difco) (11). The immunogens were injected at the hind leg footpads and tail bases. At the same time, 0.2–2 μg of pertussis toxin (Seikagaku Kogyo) in phosphate buffered saline (PBS) was injected intraperitoneally. Some mice were subjected to additional intradermal injections at the front paws of 100 μl of an emulsion consisting of PBS/CFA or PBS/Freund's incomplete adjuvant (IFA). In a modified protocol, mice were immunized intradermally with 200 μl of the C protein fragment/CFA emulsion only at the left hind leg footpads and tail bases together with intraperitoneal injection of 0.2–2 μg of pertussis toxin. These mice were treated after 42 days with an intradermal injection of 100 μl of a C protein fragment/CFA emulsion, a CFA emulsion containing PBS vehicle, a C protein fragment/CFA emulsion containing 1 mg of polymyxin B (Sigma-Aldrich), an IFA emulsion containing PBS vehicle, an IFA emulsion containing 100 μg of poly(I-C) sodium salt (Sigma-Aldrich), or an IFA emulsion containing 100 μg of lipopolysaccharide (LPS; Sigma-Aldrich) at the contralateral hind leg foot pads and tail bases.

Hematoxylin and eosin–stained 10-μm sections of the hamstrings and quadriceps and the brachial triceps were examined histologically for the presence of mononuclear cell infiltration and degeneration of muscle fibers. Histologic severity of myositis in each muscle block was graded as follows (6): grade 1 = involvement of a single muscle fiber or <5 muscle fibers; grade 2 = a lesion involving 5–30 muscle fibers; grade 3 = a lesion involving a muscle fasciculus; grade 4 = diffuse, extensive lesions. When multiple lesions with the same grade were found in a single block, 0.5 points was added to the grade. The stained sections were evaluated by 2 independent observers (NO and TS), who reported comparable results.

Cell proliferation assay and interferon-γ (IFNγ) detection in culture supernatants. Bone marrow cells from B6 mice were treated with granulocyte–macrophage colony-

stimulating factor to prepare bone marrow–derived dendritic cells (BMDCs) (19). More than 70% of the treated cells were positive for CD11c staining. Lymph node (LN) cells were prepared from the inguinal and popliteal LNs from the mice with CIM 21 days after the immunization. One hundred thousand LN cells and 1×10^4 C protein fragment–pulsed or untreated mature BMDCs were cultured for 3 days. Proliferation was evaluated with incorporation of ^{3}H-thymidine during the last 8 hours of the incubation. The culture supernatants were examined for the concentration of IFNγ with an enzyme-linked immunosorbent assay (ELISA) kit (Mouse IFN-gamma DuoSet; R&D Systems).

Adoptive transfer of CIM. LN cells were prepared from the inguinal and popliteal LNs of the mice with CIM 21 days after the immunization. Three million LN cells and 1.5×10^6 C protein fragment–pulsed mature BMDCs were cultured with 100 IU/ml of recombinant human IL-2 (Shionogi Pharmaceuticals) for 3 days. Eight million LN cells were adoptively transferred to naive mice that were treated simultaneously with subcutaneous hind leg footpad injection of 50 μl of CFA emulsion, IFA emulsion, or IFA emulsion containing 100 μg of poly(I-C), LPS, or CpG-containing oligonucleotide (CpG ODN) 1826 (InvivoGen). Their muscles were examined histologically after 14 days.

Immunohistochemical analysis. Cryostat frozen sections (6 μm) fixed in cold acetone were stained with anti-CD68 monoclonal antibodies (mAb) (FA-11; AbD Serotec). Nonspecific staining was blocked with 4% Blockace (DS Pharma Biomedical). Bound antibodies were visualized with peroxidase-labeled anti-rat IgG antibodies and associated substrates (Histofine Simple Stain Max PO; Nichirei Biosciences). Isotype controls were used as a negative control.

Antiinflammatory cytokine treatment. Hamster/mouse chimeric anti-murine IL-1 receptor (IL-1R) IgG1 mAb (M147) (20), rat/mouse chimeric anti–tumor necrosis factor α (anti-TNFα) IgG2a mAb (cV1q) (21), and rat anti–IL-6R IgG1 mAb (MR16-1) (22) were provided by Amgen, Centocor, and Chugai Pharmaceutical, respectively. Bovine serum albumin (Sigma-Aldrich) or rat antidinitrophenol IgG1 mAb (KH-5; Chugai Pharmaceutical) was used as a control.

Statistical analysis. Histologic scores were analyzed statistically using the Mann-Whitney U test.

RESULTS

No C protein–specific tolerance induction after spontaneous regression of CIM. As is the case in most inducible models of autoimmune disease, our model of CIM regresses spontaneously (6,11). To study whether immunologic tolerance to C protein fragments had been established after the regression, we rechallenged mice with CIM with C protein fragments emulsified in CFA. First, B6 mice were immunized with a C protein fragment/CFA emulsion at only the left hind leg footpads and tail bases with intraperitoneal injections of pertussis toxin. This treatment resulted in myositis development in the muscles of the ipsilateral hind legs 3 weeks after initial immunization. Then, after disease regression, these mice were reimmunized with the same antigen/CFA emulsion in the footpads and tail bases. Contralateral legs were used for repeat immunization due to skin damage in previously immunized ipsilateral legs. IFA alone was injected as a vehicle control. Histologic evaluation of the muscles of the contralateral hind legs 14 days after the repeat C protein fragment/CFA immunization revealed that repeat immunization with C protein fragment/CFA had reinduced the myositis, while control IFA treatment had not (Figure 1A). These results showed that tolerance to the C protein fragment was not established following disease regression.

Notably, CFA injection without C protein fragments, but not IFA, at hind leg footpads and tail bases induced myositis after disease regression. Myositis reinduced with CFA alone was less severe than that reinduced with C protein fragment/CFA reimmunization, suggesting that attenuated activity of the autoaggressive T cells may also partly account for disease regression. As a control, CFA alone was used in the first and second treatments, and we found no inflammation that damaged muscles (Figure 1A).

To study the mechanism of CFA-induced recurrence more carefully, we tried to inhibit the recurrence by adding polymyxin B, an inhibitor of LPS (a Toll-like receptor 4 [TLR-4] ligand), in CFA emulsion. Polymyxin B partially inhibited myositis recurrence (Figure 1B). The partial inhibition was likely attributable to activators of innate immunity other than LPS contained within CFA (23). The effect of TLR ligands was examined directly with injection of LPS- and poly(I-C) (a TLR-3 ligand)–containing emulsions without C protein fragments as the second treatment. Injection of these TLR ligands induced recurrence of myositis (Figure 1B). Thus, redevelopment of myositis requires activation of local innate immunity, but not necessarily T cell reactivation.

Requirement of local CFA treatment for the development of myositis. Previously, we immunized mice at their hind leg footpads and tail bases and examined their femoral muscles for histologic changes. The results of the reimmunization experiments prompted us to examine the brachial muscles of the immunized animals. We found that they did not develop myositis at the brachial muscles (Table 1). We then injected CFA at the right front paws and IFA at the left front paws in mice at the same time that they were immunized in the conventional way to induce CIM. These mice developed myositis only in the CFA-treated forelegs and hind legs. The brachial muscles of the IFA-treated forelegs had no myositis (Table 1). No myositis was observed in the

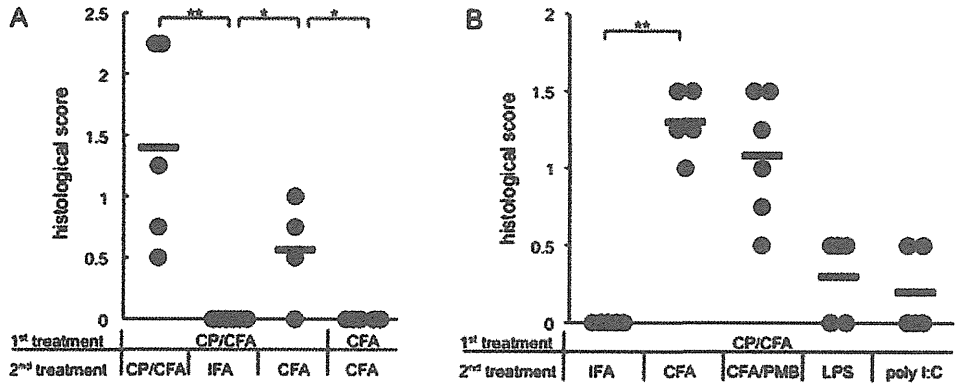

Figure 1. No establishment of tolerance to C protein fragments (CP) after disease regression. **A**, C57BL/6 mice were immunized with C protein fragments/Freund's complete adjuvant (CFA) emulsion only at the footpads of left hind legs and tail bases (first treatment: CP/CFA). Treatment with C protein fragments/CFA resulted in myositis development in the muscles of the ipsilateral legs followed by spontaneous regression that was confirmed 42 days after the initial immunization. After the myositis regression, these mice were reimmunized with C protein fragments/CFA or treated with Freund's incomplete adjuvant (IFA) or CFA emulsion (second treatment). Another group of mice was treated with CFA alone twice (first and second treatment: CFA). **B**, In a separate experiment, the same C protein fragment/CFA–immunized mice were treated afterward with CFA, CFA together with polymyxin B (PMB), lipopolysaccharide (LPS), or poly(I-C) emulsions at the footpads of the contralateral hind legs. IFA was injected as a vehicle control. Histologic evaluation of the muscles of the right legs was performed 14 days after the second treatment. Horizontal bars indicate the mean. * = $P < 0.05$; ** = $P < 0.01$.

forelegs of naive mice treated in the same way. These findings reinforced the fact that effector T cell development and local activation of innate immunity are both critical for myositis development.

Requirement of local activation of innate immunity for adoptive transfer of CIM. To explore the mechanism of effector T cell activation (a component of acquired immunity) in muscle cells engaged in local innate immunity, we used an adoptive transfer CIM model (7). Prior to the transfer to naive mice, the LN cells from mice with CIM were cocultured for 5 days with C protein fragment–pulsed BMDCs together with recombinant IL-2. Upon coculture with C protein fragment–pulsed BMDCs, LN cells showed enhanced proliferation and IFNγ induction compared with LN cells cocultured with untreated BMDCs (Figure 2A). Thus, transferred LN cells contained T cells reactive specifically to C protein fragments. Because we had confirmed that intradermal injection of CFA at leg footpads did not cause myositis (Figure 2B), we injected CFA intradermally at the right hind leg footpads and IFA intradermally at the left hind leg footpads simultaneously upon transfer of LN cells. Two weeks later, we examined the muscles of the hind legs histologically and found myositis only in the hind legs that were injected with CFA, but not in the hind legs that were injected with IFA (Figure 2B).

Table 1. Requirement of local CFA treatment for myositis induction*

Foreleg status, muscle, footpad treatment	Histologic score, mean ± SD
Untreated	
Femoral	
CP/CFA	1.88 ± 0.75
Brachial	
None	0
Treated	
Femoral	
CP/CFA	1.00 ± 0.42
Brachial	
CFA	0.83 ± 1.03
IFA	0

* Four mice that were treated to induce C protein–induced myositis (CIM) at the hind leg footpads and tail bases (foreleg untreated) did not develop myositis of the brachial muscles. In 6 mice, at the same time as treatment to induce CIM, Freund's complete adjuvant (CFA) and Freund's incomplete adjuvant (IFA) emulsions were injected additionally into the right and left footpads, respectively, of forelegs (foreleg treated), and myositis developed in the brachial muscles of the CFA-treated legs. Myositis of the femoral and the right/left brachial muscles was histologically assessed 21 days after the immunization. CP = C protein fragments.

As in the aforementioned studies on CFA-induced recurrence, TLR ligands, including poly(I-C), LPS, and CpG ODN, successfully primed muscle tissues as well as CFA and facilitated adoptive transfer of myositis (Figure 2C). Thus, it was confirmed that not only effector T cells, but also conditioning of the muscle tissues via activation of innate immunity, is essential for the development of myositis.

Local activation of innate immunity as a therapeutic target in CIM. Activation of local innate immunity with CFA or other TLR ligands was crucial to

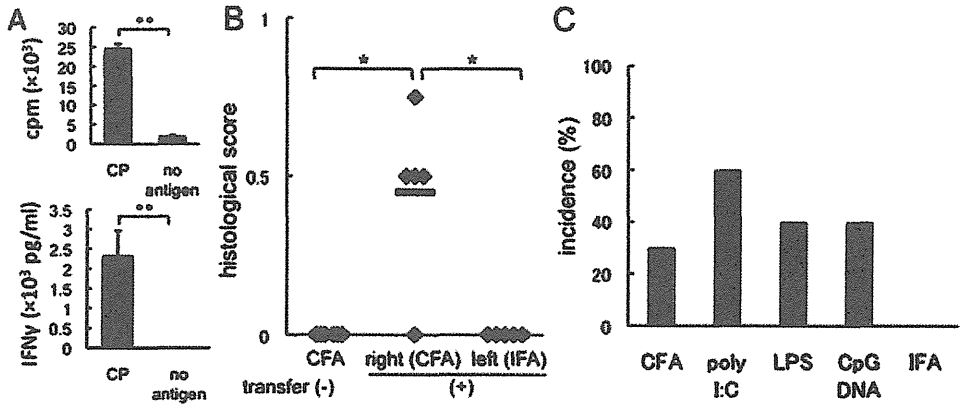

Figure 2. Selective transfer of myositis to CFA/Toll-like receptor (TLR) ligand–treated legs. A, Lymph node (LN) cells from mice with C protein–induced myositis (CIM) were stimulated with C protein fragment–pulsed mature bone marrow–derived dendritic cells (BMDCs) or untreated mature BMDCs (no antigen) for 3 days. Their proliferation was determined by ^{3}H-thymidine incorporation (top). Interferon-γ (IFNγ) in the culture supernatants was quantified by enzyme-linked immunosorbent assay (bottom). Values are the mean ± SD of 3 independent experiments. ** = $P < 0.01$. B, LN cells from mice with CIM stimulated with interleukin-2 (IL-2) and C protein fragment–pulsed mature BMDCs were transferred to 5 naive mice with their right hind leg footpads treated with CFA and their left hind leg footpads treated with IFA. Six naive mice were treated with CFA on their hind leg footpads without adoptive transfer. Myositis of the bilateral femoral muscles was histologically assessed 14 days after the transfer. Horizontal bars indicate the mean. * = $P < 0.05$. C, Upon adoptive transfer of LN cells from mice with CIM stimulated with IL-2 and C protein fragment–pulsed mature BMDCs, the legs of the recipient mice were treated with the TLR ligands poly(I-C), LPS, and CpG-containing oligonucleotide (CpG ODN; CpG DNA). CFA and IFA were included as positive and negative controls, respectively. The incidence of myositis resulting from the transfer is shown. Each group included 5 mice. See Figure 1 for other definitions.

facilitate autoaggressive T cell attack of muscle fibers. To elucidate the distal effects of CFA treatment, muscles were histologically examined 14 days after CFA treatment. In comparison with normal muscles, muscle tissue 14 days after CFA treatment contained more mononuclear cells but showed no signs of damage. Pathologic scores remained grade 0 since no muscle damage was observed. Mononuclear cells were positive for CD68 (Figure 3A), but not for CD4, CD8, or B220 (data not shown). Thus, macrophages were recruited into the muscles of the CFA-treated limbs without tissue damage.

Triggering of macrophage TLRs induces production of type I IFNs and inflammatory cytokines including IL-1, TNFα, and IL-6 (24). Since a separate experiment showed that IFNα/β/ω receptor 1–null B6 mice, lacking type I IFN receptors, were as susceptible to CIM induction as wild-type mice (data not shown), it is unlikely that macrophage type I IFN contributed to autoaggressive T cell infiltration into muscle tissue. We thus assumed that alternative cytokines, including IL-1, TNFα, and IL-6, were crucial to recruit autoaggressive T cells.

To study the role of IL-1, TNFα, and IL-6, anti–IL-1R, anti-TNFα, and anti–IL-6R mAb were employed to block individual cytokines in the CIM adoptive transfer model. Intraperitoneal administration of these reagents was initiated when activated LN cells from mice with CIM were transferred to naive mice that were treated simultaneously with CFA at the hind leg footpads. Anti–IL-1R, anti-TNFα, and anti–IL-6R mAb were administered until 14 days following transfer of LN cells from mice with CIM, when muscles of the recipient mice were histologically evaluated. Blockade of both IL-1R and TNFα suppressed the transfer, while anti–IL-6R mAb, a dose of which suppressed development of conventional CIM (11), failed to suppress induction of myositis (Figure 3B). Thus, IL-1 and TNFα from macrophages may be responsible for the conditioning of muscle tissues that mediates attack by autoaggressive T cells.

DISCUSSION

By investigating spontaneous regression of mononuclear cell infiltration and muscle injury in CIM, we found that activation of autoaggressive effector T cells and that of innate immunity at the target tissues are required for autoimmune tissue injury. TNFα and IL-1 are essential in the activation of innate immunity. Presumably, local macrophages are the source of TNFα and IL-1 in muscles that are activated by CFA. CFA may have dual roles since it should provoke systemic T cell responses specific to C protein fragments by activating

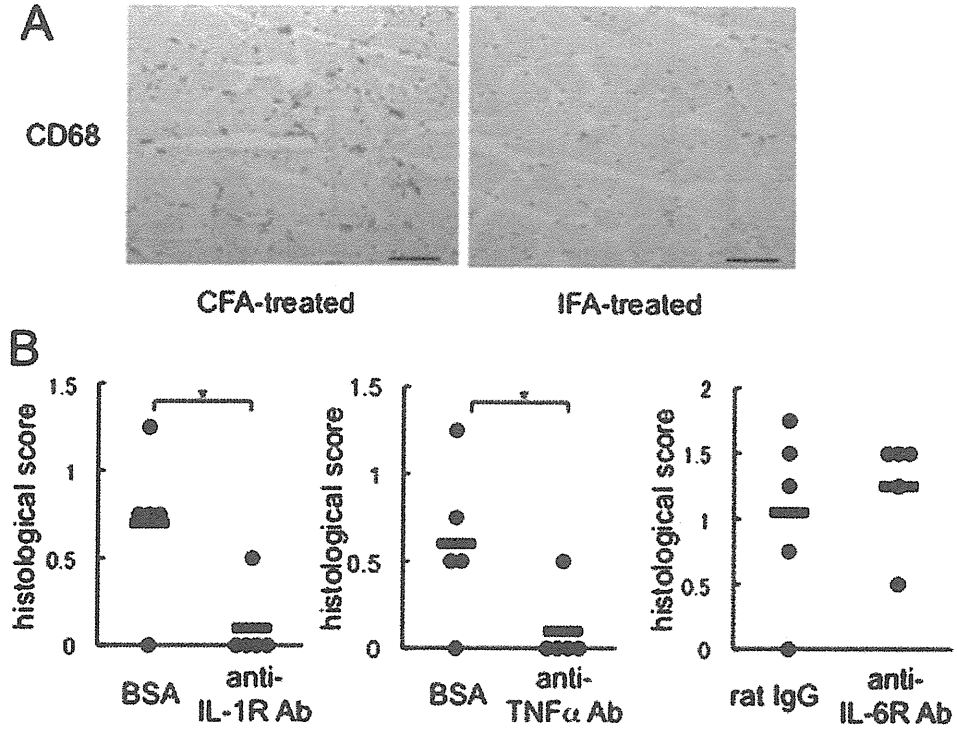

Figure 3. Effect of blockade of inflammatory cytokines on adoptive transfer of C protein–induced myositis (CIM). A, Femoral muscles from mice treated with intradermal CFA injection at the footpads and from mice treated in the same way with IFA were stained immunohistochemically with anti-CD68 antibodies. Although CD68+ macrophages were increased in CFA-treated mice, no muscle damage was observed. Bars = 50 μm. B, Activated lymph node cells from mice with CIM were transferred to naive recipient mice that were treated with CFA at the hind leg footpads to induce myositis. At the same time, the recipient mice were subjected to intraperitoneal injection of 100 μg of anti–interleukin-1 receptor (anti–IL-1R) antibodies (Ab), which was followed by repeat administration every 3 days; 100 μg of anti–tumor necrosis factor α (anti-TNFα) antibodies, which was followed by repeat administration 3 times a week; or 4 mg of anti–IL-6R antibodies, which was followed by 0.3-mg injections twice a week. Bovine serum albumin (BSA) or rat IgG was used as a control. The proximal muscles were examined for histologic scoring 14 days after transfer. Each group included 5 mice. Horizontal bars indicate the mean. * = $P < 0.05$. See Figure 1 for other definitions.

DCs. Once T cell responses against muscles are established, local injection of CFA and TLR ligands at the footpads can cause myositis to recur in legs otherwise free from myositis. The effect of CFA in activating local innate immunity appeared more potent than individual TLR ligands and could not be inhibited fully by a TLR-4 inhibitor. This is most likely because CFA contains activators of innate immunity other than TLR-4 ligands (23,25–27). Thus, CIM depends on systemic T cell autoimmunity as well as activation of innate immunity, especially that of TLR signaling in the muscles.

In CIM, TNFα and IL-1 production from macrophages stimulated with CFA or TLR ligands is a key event to activate innate immunity. Activation of innate immunity per se is insufficient for development of myositis, because single and repeat CFA injections increased local macrophages in number while not inducing muscle damage. The other essential event is activation of C protein fragment–reactive T cells. In contrast to LN cells from mice with CIM cocultured with C protein fragment–pulsed BMDCs, LN cells cocultured with untreated BMDCs did not proliferate or produce IFNγ. Moreover, they failed to transfer myositis to a naive recipient mouse with footpad CFA injection (data not shown). Histologic scores were set at zero to indicate no muscle damage. Involvement of activated T cells is crucial for muscle damage; therefore, histologic scores greater than zero suggested that CIM and its recurrence were mediated by C protein fragment–reactive T cells inducing muscle damage.

These observations echo the "seed and soil" model, which had been proposed in metastatic processes of tumor cells. Metastasis depends on tumor cells liberated from tumor mass as seeds and also depends on target tissues as soil that accepts the tumor cells readily for their local growth. Analogously, autoreactive CD8+

T cells act as seeds while muscle tissues act as soil. Requirement of the 2 factors was demonstrated for the first time in animal models of autoimmunity.

Molecular events of the "soil" activation in muscle tissues in CIM may include up-regulation of adhesion molecules on endothelial cells, chemokine release from macrophages, and costimulatory molecule expression by myofibers, which can be promoted by TNFα and/or IL-1 (28). To address this point, we used immunohistochemistry to study the expression of intercellular adhesion molecule 1 and class I major histocompatibility complex in the muscles from the CFA- and IFA-treated legs, and we found no difference in their expression levels (data not shown). Although more intensive studies will be required to draw definitive conclusions, other molecules should be of importance for recruitment of autoaggressive T cells.

It was reported previously that collagen-induced arthritis, a murine model of RA, could be reactivated by oral administration of LPS (29). Since the reactivation accompanied increased levels of antibodies against the immunizing antigen (type II collagen), the authors concluded that B cell activation by LPS might be responsible for the reactivation. EAE was also reactivated by intravenous injection of LPS (30). It appeared to be mediated by proliferation and cytokine production in a fraction of effector/memory CD4+ T cells. While these studies implied that systemic delivery of LPS stimulated pathogenic lymphocytes in the systemic pool, our studies revealed the contribution of local innate immunity activated by LPS.

It is still an open question whether the same "seed and soil" model applies to human PM. Apparently, CFA is not involved in PM. Since the recurrent myositis and the T cell adoptive transfer in the present studies showed that TLR ligands were sufficient to prime the muscles, stimulation by endogenous TLR ligands might account for production of IL-1 and TNFα, both of which were reportedly expressed by infiltrating cells in muscles in PM/dermatomyositis (DM) and CIM (6,11,31). Alternatively, aberrant production of inflammatory cytokines and chemokines might be responsible for attraction of autoaggressive T cells. It is known that muscle fibers can produce these cytokines under physiologic conditions, especially during exercise (32–35). Etiologic studies revealed heavy muscular exertion as a risk factor for development of PM/DM (36). As was reviewed earlier, MRI scans typically show that some muscle fascicles are affected and that others remain unaffected (3). Cytotoxic CD8+ T cells, which appeared specific to the muscles, were present for a long time after successful treatment of the disease (4). These facts could be explained by the 2 requirements: activation of effector T cells and that of the muscle tissues.

Experiments of repeat antigen/CFA immunization and CFA treatment have demonstrated that tolerance was not induced during spontaneous regression of disease and that activation of local innate immunity alone can cause recurrence of myositis. We also noted that reimmunization of repeat antigen/CFA provoked more severe myositis than CFA treatment alone. Thus, the T cell activation level following regression must have waned, which could also contribute to the disease regression. In this regard, contribution of Treg cells was studied with antibody-mediated CD25+ T cell depletion, which did not alter the disease course of CIM (data not shown). Genetic absence of IL-10, which is one of the key effector molecules of Treg cells, did not interfere with the regression (data not shown). It was thus suggested that no active suppression of autoreactive T cells operates in the regression phase.

Waning T cell activation might partially account for the fact that myositis was modest in the transfer model, which was assessed histologically 2 weeks after the T cell transfer. Incidence of myositis transfer varied among experiments (25–80%), which might depend on the frequency and activation levels of C protein fragment–specific T cells. We had to compare the severity and incidence of transferred myositis in the same set of experiments. Nonetheless, the adoptive transfer model has enhanced the value of CIM since it allows us to evaluate the effector phase of myositis. Together with conventional CIM, we can dissect the pathologic processes involved in the induction and effector phases of myositis. In this regard, IL-6 inhibition was effective for treating conventional CIM (11), while IL-6 inhibition in the recipient animals did not prevent adoptive transfer of CIM. It is likely that IL-6 plays a more prominent role in the induction phase of myositis. Of special interest are interventions targeting the myositis effector phase, since we always have to treat patients after disease has been established.

At the moment, histologic evaluation is the only reliable way to assess severity of rodent models of PM, a problem shared by all murine myositis models. Serum levels of creatinine kinase or other muscle-derived proteins are unreliable. They are often high in normal mice, presumably because of their physical activity. The rotarod test is difficult because some mice become accustomed to it and avoid falling off. Other mice are willing to drop off from the device. We believe that we need

devices that directly quantify muscle strength of rodents to follow clinical disease course.

Glucocorticoids, which are a first-line medication in the current therapeutic approaches to PM, should suppress activation of T cells as well as innate immune cells. They are effective, but have quite a few side effects. On the other hand, most small-molecule immunosuppressants target primarily lymphocyte activation. The results of the present study suggest that combinatorial approaches that address activation of T cells and innate immunity could be optimal for treating autoimmune myositis and suggest that glucocorticoids could become dispensable if activation of innate immunity in the muscles is suppressed by alternative treatment. This implication appears interesting for designing future clinical trials to treat PM.

ACKNOWLEDGMENTS

We thank Eri Yoshimoto for her technical assistance, and Centocor R&D (Radnor, PA), Amgen (Seattle, WA), and Chugai Pharmaceutical (Tokyo, Japan) for providing monoclonal antibodies.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kohsaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Okiyama, Yokozeki, Miyasaka, Kohsaka.

Acquisition of data. Okiyama, Sugihara, Oida, Ohata.

Analysis and interpretation of data. Okiyama, Sugihara, Oida, Ohata, Kohsaka.

ADDITIONAL DISCLOSURE

Author Ohata is an employee of Chugai Pharmaceutical.

REFERENCES

1. Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet 2003;362:971–82.
2. Engel AG, Arahata K, Emslie-Smith A. Immune effector mechanisms in inflammatory myopathies. Res Publ Assoc Res Nerv Ment Dis 1990;68:141–57.
3. Tomasova Studynkova J, Charvat F, Jarosova K, Vencovsky J. The role of MRI in the assessment of polymyositis and dermatomyositis. Rheumatology (Oxford) 2007;46:1174–9.
4. Nishio J, Suzuki M, Miyasaka N, Kohsaka H. Clonal biases of peripheral CD8 T cell repertoire directly reflect local inflammation in polymyositis. J Immunol 2001;167:4051–8.
5. Benveniste O, Cherin P, Maisonobe T, Merat R, Chosidow O, Mouthon L, et al. Severe perturbations of the blood T cell repertoire in polymyositis, but not dermatomyositis patients. J Immunol 2001;167:3521–9.
6. Sugihara T, Sekine C, Nakae T, Kohyama K, Harigai M, Iwakura Y, et al. A new murine model to define the critical pathologic and therapeutic mediators of polymyositis. Arthritis Rheum 2007;56:1304–14.
7. Sugihara T, Okiyama N, Suzuki M, Kohyama K, Matsumoto Y, Miyasaka N, et al. Definitive engagement of cytotoxic CD8 T cells in C protein–induced myositis, a murine model of polymyositis. Arthritis Rheum 2010;62:3088–92.
8. Rosenberg NL, Kotzin BL. Aberrant expression of class II MHC antigens by skeletal muscle endothelial cells in experimental autoimmune myositis. J Immunol 1989;142:4289–94.
9. Matsubara S, Okumura S. Experimental autoimmune myositis in SJL/J mice produced by immunization with syngeneic myosin B fraction: transfer by both immunoglobulin G and T cells. J Neurol Sci 1996;144:171–5.
10. Bittner RE, Anderson LV, Burkhardt E, Bashir R, Vafiadaki E, Ivanova S, et al. Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B. Nat Genet 1999;23:141–2.
11. Okiyama N, Sugihara T, Iwakura Y, Yokozeki H, Miyasaka N, Kohsaka H. Therapeutic effects of interleukin-6 blockade in a murine model of polymyositis that does not require interleukin-17A. Arthritis Rheum 2009;60:2505–12.
12. Khoury SJ, Hancock WW, Weiner HL. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor β, interleukin 4, and prostaglandin E expression in the brain. J Exp Med 1992;176:1355–64.
13. Sakaguchi S. Naturally arising Foxp3–expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005;6:345–52.
14. Montero E, Nussbaum G, Kaye JF, Perez R, Lage A, Ben-Nun A, et al. Regulation of experimental autoimmune encephalomyelitis by $CD4^+$, $CD25^+$ and $CD8^+$ T cells: analysis using depleting antibodies. J Autoimmun 2004;23:1–7.
15. Morgan ME, Sutmuller RP, Witteveen HJ, van Duivenvoorde LM, Zanelli E, Melief CJ, et al. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum 2003;48:1452–60.
16. Liu R, La Cava A, Bai XF, Jee Y, Price M, Campagnolo DI, et al. Cooperation of invariant NKT cells and $CD4^+CD25^+$ T regulatory cells in the prevention of autoimmune myasthenia. J Immunol 2005;175:7898–904.
17. Allenbach Y, Solly S, Gregoire S, Dubourg O, Salomon B, Butler-Browne G, et al. Role of regulatory T cells in a new mouse model of experimental autoimmune myositis. Am J Pathol 2009;174:989–98.
18. Morris GP, Brown NK, Kong YC. Naturally-existing $CD4^+CD25^+Foxp3^+$ regulatory T cells are required for tolerance to experimental autoimmune thyroiditis induced by either exogenous or endogenous autoantigen. J Autoimmun 2009;33:68–76.
19. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 1999;223:77–92.
20. Rogers HW, Sheehan KC, Brunt LM, Dower SK, Unanue ER, Schreiber RD. Interleukin 1 participates in the development of anti-Listeria responses in normal and SCID mice. Proc Natl Acad Sci U S A 1992;89:1011–5.
21. Kadokami T, Frye C, Lemster B, Wagner CL, Feldman AM, McTiernan CF. Anti-tumor necrosis factor-α antibody limits heart failure in a transgenic model. Circulation 2001;104:1094–7.
22. Okazaki M, Yamada Y, Nishimoto N, Yoshizaki K, Mihara M. Characterization of anti-mouse interleukin-6 receptor antibody. Immunol Lett 2002;84:231–40.
23. Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, et al.

Adjuvant-enhanced antibody responses in the absence of Toll-like receptor signaling. Science 2006;314:1936–8.
24. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 2006;117:979–87.
25. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801.
26. Matsunaga I, Moody DB. Mincle is a long sought receptor for mycobacterial cord factor. J Exp Med 2009;206:2865–8.
27. Ishikawa E, Ishikawa T, Morita YS, Toyonaga K, Yamada H, Takeuchi O, et al. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J Exp Med 2009;206:2879–88.
28. Chevrel G, Granet C, Miossec P. Contribution of tumour necrosis factor α and interleukin (IL) 1β to IL6 production, NF-κB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors. Ann Rheum Dis 2005;64:1257–62.
29. Yoshino S, Sasatomi E, Mori Y, Sagai M. Oral administration of lipopolysaccharide exacerbates collagen-induced arthritis in mice. J Immunol 1999;163:3417–22.
30. Nogai A, Siffrin V, Bonhagen K, Pfueller CF, Hohnstein T, Volkmer-Engert R, et al. Lipopolysaccharide injection induces relapses of experimental autoimmune encephalomyelitis in non-transgenic mice via bystander activation of autoreactive $CD4^+$ cells. J Immunol 2005;175:959–66.
31. Lundberg I, Ulfgren AK, Nyberg P, Andersson U, Klareskog L. Cytokine production in muscle tissue of patients with idiopathic inflammatory myopathies. Arthritis Rheum 1997;40:865–74.
32. Gomez-Merino D, Drogou C, Guezennec CY, Chennaoui M. Effects of chronic exercise on cytokine production in white adipose tissue and skeletal muscle of rats. Cytokine 2007;40:23–9.
33. Lira FS, Koyama CH, Yamashita AS, Rosa JC, Zanchi NE, Batista ML Jr, et al. Chronic exercise decreases cytokine production in healthy rat skeletal muscle. Cell Biochem Funct 2009;27:458–61.
34. Girgenrath M, Weng S, Kostek CA, Browning B, Wang M, Brown SA, et al. TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. EMBO J 2006;25:5826–39.
35. Griffin CA, Apponi LH, Long KK, Pavlath GK. Chemokine expression and control of muscle cell migration during myogenesis. J Cell Sci 2010;123:3052–60.
36. Lyon MG, Bloch DA, Hollak B, Fries JF. Predisposing factors in polymyositis-dermatomyositis: results of a nationwide survey. J Rheumatol 1989;16:1218–24.

ARTHRITIS & RHEUMATISM
Vol. 64, No. 11, November 2012, pp 3736–3740
DOI 10.1002/art.34657

BRIEF REPORT

Association of HLA–DRB1*0101/*0405 With Susceptibility to Anti–Melanoma Differentiation–Associated Gene 5 Antibody–Positive Dermatomyositis in the Japanese Population

Takahisa Gono,[1] Yasushi Kawaguchi,[1] Masataka Kuwana,[2] Tomoko Sugiura,[1] Takefumi Furuya,[1] Kae Takagi,[1] Hisae Ichida,[1] Yasuhiro Katsumata,[1] Masanori Hanaoka,[1] Yuko Ota,[1] and Hisashi Yamanaka[1]

Objective. **The complication of interstitial lung disease (ILD) in polymyositis/dermatomyositis (PM/DM) is associated with anti–aminoacyl–transfer RNA synthetase (anti-aaRS) antibody or anti–melanoma differentiation–associated gene 5 (anti–MDA-5) antibody positivity. Anti–MDA-5 antibody is associated with clinically amyopathic DM and fatal outcome due to rapidly progressive ILD in Asian populations. The association between genetic factors and anti–MDA-5 antibody–positive DM is unclear. This study was undertaken to investigate the HLA–DRB1 genotype in patients with anti–MDA-5 antibody–positive DM.**

Methods. **We examined genetic differences among 17 patients with anti–MDA-5 antibody–positive DM, 33 patients with anti-aaRS antibody–positive PM/DM, 33 patients with PM/DM without anti-aaRS antibody or ILD, and 265 healthy controls.**

Results. **The frequencies of HLA–DRB1*0101 and DRB1*0405 were 29% and 71%, respectively, in patients with anti–MDA-5 antibody–positive DM, which were higher than the frequencies in healthy controls (10% and 25%, respectively). Among the 17 patients with anti–MDA-5 antibody–positive DM, 16 (94%) harbored either the DRB1*0101 or DRB1*0405 allele. The combined frequency of the DRB1*0101 allele and the DRB1*0405 allele was significantly higher in patients with anti–MDA-5 antibody–positive DM than in patients with PM/DM without anti-aaRS antibody or ILD, with an odds ratio (OR) of 42.7 (95% confidence interval [95% CI] 4.9–370.2) ($P = 1.1 \times 10^{-5}$), or in patients with anti-aaRS antibody–positive PM/DM (OR 13.3 [95% CI 1.6–112.6], $P = 4.5 \times 10^{-3}$).**

Conclusion. **Our findings indicate that HLA–DRB1*0101/*0405 is associated with susceptibility to anti–MDA-5 antibody–positive DM in the Japanese population.**

Dermatomyositis (DM) is characterized by inflammation of the skin and muscle (1) and is occasionally complicated by interstitial lung disease (ILD). In particular, rapidly progressive ILD is an intractable and life-threatening complication. Clinically amyopathic DM (CADM) includes typical skin lesions with amyopathy or hypomyopathy (2). It has recently been reported that patients with CADM who are positive for the anti–melanoma differentiation–associated gene 5 (MDA-5) antibody frequently have complications with rapidly progressive ILD, especially in the Japanese population (3–5). In general, anti–MDA-5 antibody is specific for rapidly progressive ILD associated with CADM and is not detected in patients with CADM or DM without ILD or in patients with polymyositis (PM). The MDA-5 protein plays a role in the innate immune system. MDA-5 initially recognizes picornaviruses, such as coxsackievirus, and induces antiviral responses by producing type I interferons and tumor necrosis factor α (6). Hyperferritinemia is complicated by rapidly progressive ILD in anti–MDA-5 antibody–positive DM (4,5). Although the pathogenesis of rapidly progressive ILD associated with anti–MDA-5 antibody–positive DM has been tentatively attributed to a cytokine storm triggered

Supported by a research grant from the Ministry of Health, Labor, and Welfare of Japan.

[1]Takahisa Gono, MD, PhD, Yasushi Kawaguchi, MD, PhD, Tomoko Sugiura, MD, PhD, Takefumi Furuya, MD, PhD, Kae Takagi, MD, PhD, Hisae Ichida, MD, Yasuhiro Katsumata, MD, Masanori Hanaoka, MD, Yuko Ota, MD, Hisashi Yamanaka, MD, PhD: Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan; Masataka Kuwana, MD, PhD: Keio University School of Medicine, Tokyo, Japan.

Dr. Kuwana holds a patent on an anti–MDA-5 antibody measuring kit.

Address correspondence to Yasushi Kawaguchi, MD, PhD, Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-Ku, Tokyo 162-0054, Japan. E-mail: y-kawa@ior.twmu.ac.jp.

Submitted for publication March 24, 2012; accepted in revised form July 26, 2012.

by viral infection, especially in the skin and lungs, its exact mechanism is unknown.

In PM/DM, complication with ILD is associated with the anti–aminoacyl–transfer RNA synthetase (anti-aaRS) antibody or anti–MDA-5 antibody. It has been reported that 90% of Caucasian patients with the anti-aaRS antibody are carriers of HLA–DRB1*03 (7). In the Japanese population, HLA–DRB1*0405 is associated with susceptibility to anti-aaRS antibody–positive PM/DM (8). However, associations between genetic factors and anti–MDA-5 antibody–positive DM have remained unclear.

Therefore, we investigated the HLA–DRB1 gene in patients with anti–MDA-5 antibody–positive DM. In addition, we compared genetic differences in HLA among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD.

Table 1. Clinical characteristics and HLA–DRB1 genotype of the patients with anti–MDA-5 antibody–positive DM*

Patient/age/sex	Genotype	Phenotype	ILD type
1/48/M	DRB1*0101/1602	CADM	Rapidly progressive
2/25/F	DRB1*0101/1501	CADM	Chronic
3/53/F	DRB1*0101/0803	CADM	Rapidly progressive
4/18/M	DRB1*0101/1502	CADM	Rapidly progressive
5/47/F	DRB1*0101/0405	DM	Rapidly progressive
6/58/M	DRB1*0405/1406	CADM	Rapidly progressive
7/16/F	DRB1*0405/0401	CADM	Rapidly progressive
8/53/F	DRB1*0405/1501	CADM	Rapidly progressive
9/53/F	DRB1*0405/0410	CADM	Rapidly progressive
10/44/F	DRB1*0405/1406	CADM	Chronic
11/45/F	DRB1*0405/1202	CADM	Chronic
12/39/M	DRB1*0405/0401	CADM	Chronic
13/47/F	DRB1*0405/1201	CADM	Chronic
14/76/F	DRB1*0405/0802	CADM	Rapidly progressive
15/56/F	DRB1*0405/1502	CADM	Rapidly progressive
16/43/M	DRB1*0405/0901	CADM	Chronic
17/66/F	DRB1*0901/1502	CADM	Rapidly progressive

* Anti–MDA-5 = anti–melanoma differentiation–associated gene 5; DM = dermatomyositis; ILD = interstitial lung disease; CADM = clinically amyopathic DM.

PATIENTS AND METHODS

Patients. This retrospective study included patients admitted to Tokyo Women's Medical University Aoyama Hospital or Keio University Hospital from August 1992 to February 2010. Medical records were obtained for 142 and 57 patients diagnosed as having DM and CADM, respectively. The anti–MDA-5 antibody was detected in 31 patients. DNA samples were available for 17 patients with the anti–MDA-5 antibody, and all of these patients were enrolled in the study. All of the enrolled patients had skin rashes, myopathy, or respiratory symptoms (or a combination thereof) at admission. The patients were diagnosed as having DM or CADM based on the criteria of Bohan and Peter (9) or Sontheimer (10), respectively. Specific rashes, including heliotrope rash, Gottron's sign, or Gottron's papules, were used to define DM or CADM. In general, CADM patients present with typical skin lesions and amyopathy or hypomyopathy with a duration of >6 months. A subset of the CADM group included patients who developed fatal ILD within the first 6 months of this study. Clinical data were obtained from hospital admission records.

To investigate the characteristics of the HLA–DRB1 genotype in anti–MDA-5 antibody–positive DM, HLA data were obtained in patients with anti-aaRS antibody–positive PM/DM, patients without anti-aaRS antibody or ILD, and healthy controls. These HLA genotype databases have been described previously (8). All of the subjects in the present study were Japanese. None of the subjects had rheumatoid arthritis (RA) or other connective tissue diseases. This study was approved by the ethics committee of Tokyo Women's Medical University and was performed in accordance with the Declaration of Helsinki.

Evaluation of autoantibodies. Anti–MDA-5 antibody was detected by immunoprecipitation (IP) assay and enzyme-linked immunosorbent assay using recombinant MDA-5 as an antigen, as previously described (3). Anti-aaRS antibodies, including Jo-1, EJ, PL-7, PL-12, and OJ; anti–signal recognition particle (anti-SRP) antibody; anti-Ku antibody; and anti–U1 small nuclear RNP (anti-U1 snRNP) antibody were assessed by RNA IP assays.

Classification of ILD. Patients were evaluated for ILD by chest radiography and computed tomography (CT) or high-resolution CT of the chest. Rapidly progressive ILD was defined as a progressive ILD within 3 months of the onset of respiratory symptoms. Chronic ILD was defined as ILD that was asymptomatic and non–rapidly progressive or slowly progressive over 3 months (11).

HLA–DRB1 genotyping. HLA–DRB1 genotyping was performed using polymerase chain reaction–reverse sequence-specific oligonucleotide techniques and standard methods. The DNA for the HLA–DRB1 genotyping of the patients was extracted from peripheral blood mononuclear cells using standard methods.

Statistical analysis. The chi-square test was used for the comparison of frequencies, and Fisher's exact test was used when appropriate. Data were analyzed using JMP software (SAS Institute). *P* values were adjusted by Bonferroni correction when appropriate.

RESULTS

Clinical characteristics and HLA–DRB1 genotype of patients with anti–MDA-5 antibody–positive DM. As shown in Table 1, 17 patients with anti–MDA-5 antibody–positive DM were enrolled in the study. Their mean ± SD age was 46 ± 16 years. Seventy-one percent were women. The HLA–DRB1*0101 and DRB1*0405 alleles were identified in 5 patients (29%) and 12 patients (71%), respectively. The HLA–DRB1*0101 or *0405 allele was identified in 16 (94%) of the 17

Table 2. Comparison of HLA–DRB1 genotypes among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD*

Genotype	Patients with anti–MDA-5 antibody–positive DM (n = 17)	Patients with anti-aaRS antibody–positive PM/DM		Patients with PM/DM without anti-aaRS antibody or ILD		Healthy controls (n = 265)
		PM/DM (n = 33)	DM (n = 19)	PM/DM (n = 33)	DM (n = 21)	
DRB1*0101	29	12	11	12	14	10
DRB1*0401	12	0	0	3	5	2
DRB1*0403	0	9	5	6	5	5
DRB1*0405	71†	42	53	18	24	25
DRB1*0406	0	6	5	3	5	7
DRB1*0407	0	0	0	0	0	2
DRB1*0410	6	3	5	9	5	2
DRB1*0802	6	18	21	9	10	7
DRB1*0803	6	24	21	27	19	14
DRB1*0901	12	24	21	18	19	30
DRB1*1101	0	3	0	0	0	2
DRB1*1201	6	9	16	3	5	7
DRB1*1202	6	6	0	0	0	4
DRB1*1301	0	0	0	3	0	0
DRB1*1302	0	6	5	21	29	19
DRB1*1401	0	3	5	9	10	5
DRB1*1403	0	3	0	6	10	4
DRB1*1405	0	3	5	3	5	6
DRB1*1406	12	0	0	3	5	3
DRB1*1501	12	12	16	9	5	11
DRB1*1502	18	6	11	21	24	20
DRB1*1602	6	0	0	6	0	3
Other	0	0	0	0	0	5

* Values are the percent of subjects. Anti-aaRS = anti–aminoacyl-transfer RNA synthetase; PM = polymyositis (see Table 1 for other definitions).
† $P = 0.0003$ versus patients with PM/DM without anti-aaRS antibody or ILD; $P = 0.00018$ versus healthy controls.

patients. No patients had homoalleles of HLA–DRB1*0101 or DRB1*0405. One patient had both DRB1*0101 and DRB1*0405. With respect to the clinical phenotype, 16 patients had CADM. ILD complication was observed in all of the patients. Moreover, the frequency of rapidly progressive ILD was high (65%). No patients had RA or other connective tissue diseases as complications.

Comparison of the HLA–DRB1 genotype in patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD. To investigate the characteristics of the HLA–DRB1 genotype in anti–MDA-5 antibody–positive DM, the frequency of the HLA–DRB1 genotype was compared among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, patients with PM/DM without anti-aaRS antibody or ILD, and healthy controls (Table 2).

Data previously obtained at our institution indicated that 33 PM/DM patients (14 patients with PM and 19 with DM) exhibited anti-aaRS antibody, as follows: 8 PM patients and 8 DM patients had anti–Jo-1; 4 PM patients and 6 DM patients had anti-EJ; 2 PM patients and 2 DM patients had anti–PL-7; 0 PM patients and 3 DM patients had anti–PL-12; and none of the patients had anti-OJ. Of the 33 patients with anti-aaRS antibody–positive PM/DM, 24 (73%) had ILD. Moreover, 33 PM/DM patients (12 PM patients and 21 DM patients) had neither anti-aaRS antibody nor ILD, and in all 21 of these DM patients, the clinical phenotype was classic DM, not CADM. In patients with PM/DM without anti-aaRS antibody or ILD, anti-SRP antibody, anti-U1 snRNP antibody, and anti-Ku antibody were detected in 3 PM patients, 1 DM patient, and 0 patients, respectively.

As shown in Table 2, the frequency of HLA–DRB1*0101 was ~30% in anti–MDA-5 antibody–positive DM and ~10% in the other subsets, although the difference was not significant ($P = 0.012$ versus

Table 3. Frequency of the HLA–DRB1*0101/0405 alleles in patients with anti–MDA-5 antibody–positive DM*

	Patients with anti–MDA-5 antibody–positive DM (n = 17)	Patients with anti-aaRS antibody–positive PM/DM		Patients with PM/DM without anti-aaRS antibody or ILD	
		PM/DM (n = 33)	DM (n = 19)	PM/DM (n = 33)	DM (n = 21)
DRB1*0101 or DRB1*0405, %	94	55	63	27	33
P†	–	4.5×10^{-3}	4.4×10^{-2}	1.1×10^{-5}	2.0×10^{-4}
OR (95% CI)	–	13.3 (1.6–112.6)	9.3 (1.0–86.4)	42.7 (4.9–370.2)	32 (3.5–293.1)

* Anti-aaRS = anti–aminoacyl–transfer RNA synthetase; PM = polymyositis; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Versus patients with anti–MDA-5 antibody–positive DM.

healthy controls, adjusted P value not significant). P values with Bonferroni correction for multiple comparisons less than 0.0023 were considered significant; this was determined by dividing the P value of 0.05 by 22 (the number of HLA genotypes). The inadequate statistical power may be attributed to small sample sizes. Moreover, the frequency of HLA–DRB1*0405 was significantly higher in the patients with anti–MDA-5 antibody–positive DM than in the patients with PM/DM without anti-aaRS antibody or ILD ($P = 0.0003$) or in the healthy controls ($P = 0.00018$). The frequency of HLA–DRB1*0405 was also high in patients with anti-aaRS antibody–positive PM/DM, although it was not significantly different from that in the other subsets. No significant differences were found regarding the frequencies of the other alleles.

Frequency of HLA–DRB1*0101/*0405 in patients with anti–MDA-5 antibody–positive DM compared with other PM/DM patient subsets. In this study, the HLA–DRB1*0101 or *0405 allele was identified in all but 1 of the 17 anti–MDA-5 antibody–positive patients. In the HLA–DRB1 alleles, residues 70–74 of the DRβ chain form the third hypervariable region, an important region for antigen presentation. This amino acid sequence is QRRAA, which is a shared epitope motif in both DRB1*0101 and DRB1*0405. We speculated that QRRAA may be a critical sequence in the pathophysiology of anti–MDA-5 antibody–positive DM. We considered the role of both DRB1*0101 and DRB1*0405 in anti–MDA-5 antibody–positive DM. Therefore, the combined frequency of the DRB1*0101 allele and the DRB1*0405 allele was compared among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD.

As shown in Table 3, the combined frequency of DRB1*0101 and *0405 was significantly higher in patients with anti–MDA-5 antibody–positive DM than in patients with PM/DM without anti-aaRS antibody or ILD, with an odds ratio (OR) of 42.7 (95% confidence interval [95% CI] 4.9–370.2, $P = 1.1 \times 10^{-5}$), or in patients with DM without anti-aaRS antibody or ILD (OR 32 [95% CI 3.5–293.1], $P = 2 \times 10^{-4}$). The combined frequency of DRB1*0101 and *0405 was also higher in patients with anti–MDA-5 antibody–positive DM than in patients with anti-aaRS antibody–positive PM/DM (OR 13.3 [95% CI 1.6–112.6], $P = 4.5 \times 10^{-3}$) and patients with anti-aaRS antibody–positive DM (OR 9.3 [95% CI 1.0–86.4], $P = 4.4 \times 10^{-2}$). Moreover, the frequency of these alleles was higher in patients with anti-aaRS antibody–positive PM/DM than in patients with PM/DM without anti-aaRS antibody or ILD (OR 3.2 [95% CI 1.1–8.9], $P = 2.4 \times 10^{-2}$).

DISCUSSION

We have demonstrated an association between a genetic factor and anti–MDA-5 antibody–positive DM. Specifically, this study shows that HLA–DRB1*0101/*0405 is associated with susceptibility to anti–MDA-5 antibody–positive DM. HLA–DRB1*0301 is associated with susceptibility to anti-aaRS antibody–positive PM/DM in Caucasians. In contrast, the frequency of HLA–DRB1*0301 is low, but the frequency of HLA–DRB1*0405 is relatively high, at ~20%, in the Japanese population. HLA–DRB1*0405 is associated with susceptibility to anti-aaRS antibody–positive PM/DM in the Japanese population, whereas HLA–DRB1*0101 is not (8). In the present study, the frequency of HLA–DRB1*0405 was high in both anti–MDA-5 antibody–positive DM and anti-aaRS antibody–positive PM/DM. In contrast, the frequency of HLA–DRB1*0405 among patients with PM/DM without anti-aaRS antibody or ILD was similar to that in healthy controls. Type 1