Correspondence to: Dr H Piper, Department of Rheumatology, Cannock Chase Hospital, Brunswick Road, Cannock, Staffordshire WS11 2XY, UK; hollypiper@hotmail.com

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Altered peptide ligands regulate muscarinic acetylcholine receptor reactive T cells of patients with Sjögren's syndrome

Y Naito, I Matsumoto, E Wakamatsu, D Goto, S Ito, A Tsutsumi, T Sumida

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In the generation of Sjögren's syndrome (SS), CD4 positive αβ T cells have a crucial role. Previous studies have provided evidence about the T cell receptor (TCR) Vβ and Vα genes on these T cells, and sequence analysis of the CDR3 region indicates the presence of some conserved amino acid motifs, supporting the notion that infiltrating T cells recognise relatively few epitopes on autoantigens.[1]

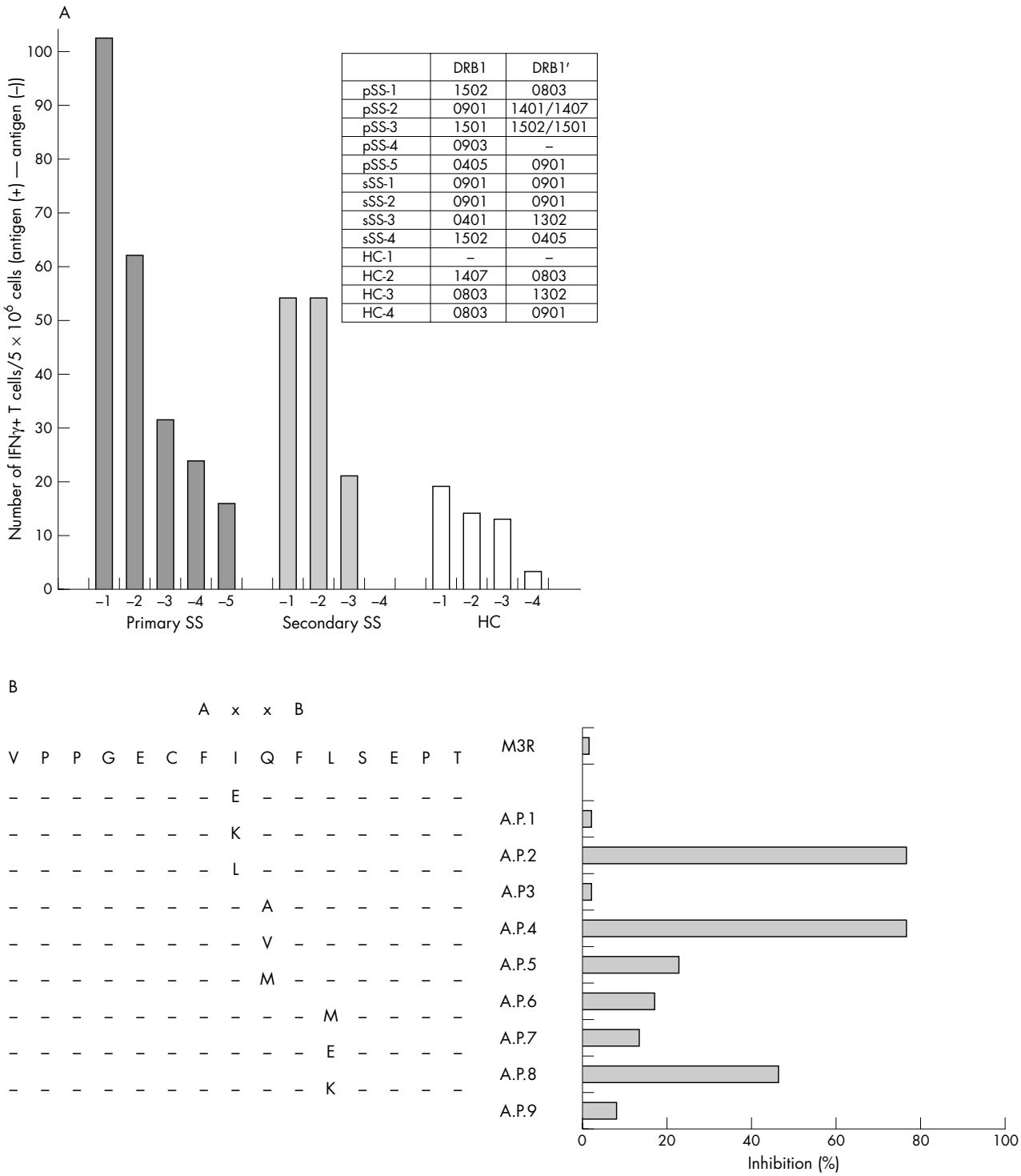
Candidate autoantigens recognised by T cells that infiltrate the labial salivary glands of patients with SS have been analysed, and Ro/SSA 52 kDa,[2] α-amylase, heat shock protein, and TCR BV6 have been identified, although Ro/SSA 52 kDa reactive T cells were not increased in peripheral blood.[3] Gordon *et al* indicated that anti-M3R autoantibodies occurred in SS and were associated with the sicca symptoms.[4] Recently, we provided evidence for the presence of autoantibodies against the second extracellular domain of muscarinic acetylcholine receptor (M3R) in a subgroup of patients with SS.[5] The M3R is an interesting molecule, because this portion has an important role in intracellular signalling,[5] although the function of anti-M3R autoantibodies remains unknown.

The mechanism through which a peptide is recognised by a TCR is flexible. If the amino acid residue of the peptide ligands for TCR is substituted by a different amino acid and can still bind to major histocompatibility complex molecules (altered peptide ligand), such an altered peptide ligand could regulate the activation of T cells. Several studies have shown that an altered peptide ligand could induce differential cytokine secretion, anergy, and antagonism of the response to the wild-type antigens.[6 7] The altered peptide ligand has the potential of being used therapeutically against T cell mediated diseases such as autoimmune diseases and allergic disorders.

As an extension to our previous study,[5] we focused in the present study on M3R reactive T cells and analysed T cell epitopes and their altered peptide ligands with the aim of regulating T cell proliferation and autoantibody production. The 25mer synthetic amino acids encoding the second extracellular domain of M3R (KRTVPPGECFIQFLSEPTITF GTAI, AA213-237) were used as the antigen for T cells, and the number of interferon (IFN) γ producing T cells was counted by flow cytometry using a magnetic activated cell sorting (MACS) secretion assay. The proportion of IFNγ-producing T cells among peripheral blood mononuclear cells (PBMCs) was high in two of five patients with primary SS (pSS) and two of four patients with secondary SS (sSS), compared with the level in four healthy control subjects (HC) (fig 1A). Three patients with SS and M3R reactive T cells (pSS-2, and sSS-1, 2) had the HLA-DR B1*0901 allele and the other patient (pSS-1) had HLA-DR B1*1502 and *0803 alleles. The 25mer amino acids contain the anchored motifs that bind to HLA-DR B1*0901. Thus, IFNγ production by T cells should be due to the recognition of antigen on the HLA molecule by the TCR on T cells.

The results shown in fig 1 were obtained as follows. Blood samples were collected from five Japanese patients with pSS and four Japanese patients with sSS followed up at the University of Tsukuba Hospital. All patients with SS satisfied both the Japanese Ministry of Health criteria for the classification of SS[8] and the revised EU-US criteria[9]. We also recruited four HC from our university. Approval for this study was granted from the local ethics committee, and written informed consent was obtained from all patients and HC who participated in this study.

Their HLA-DR allele was examined by the SSOP-PCR method, as described elsewhere. A 15mer peptide (VPPGECFIQFLSEPT) (M3R AA216–230) corresponding to the sequence of the second extracellular loop domain was also synthesised (Kurabo Industries, Osaka, Japan). PBMCs were purified with Ficoll-Paque and 5×10^6 cells were cocultured with 10 μg of M3R peptide (25mer) in 1 ml of RPMI-1640 with 10% of human AB serum (Sigma, St Louis, MO) for 12 hours at 37℃. As a positive control, 1 μg of staphylococcal enterotoxin B (Toxin Technology Inc, USA) was used. IFNγ-producing cells were identified by the MACS cytokine secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the cells were incubated with 20 μg of IFNγ detection antibody (Ab; Miltenyi Biotec), 20 μg of



	DRB1	DRB1′
pSS-1	1502	0803
pSS-2	0901	1401/1407
pSS-3	1501	1502/1501
pSS-4	0903	–
pSS-5	0405	0901
sSS-1	0901	0901
sSS-2	0901	0901
sSS-3	0401	1302
sSS-4	1502	0405
HC-1	–	–
HC-2	1407	0803
HC-3	0803	1302
HC-4	0803	0901

Figure 1 (A) M3R reactive T cells. (B) Selection of altered peptide ligands.

anti-CD4-FITC Ab (Becton Dickinson, Franklin Lakes, NJ, USA), and 5 μg of anti-CD3-APC Ab (Becton Dickinson) for 10 minutes at 4℃. After double washing with a cold buffer (phosphate buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were incubated with 20 μg of anti-phycoerythrin microbeads (Miltenyi Biotec) for 15 minutes at 4℃. After double washing, the cells were resuspended with 500 μl buffer and then passed through an MS column (Miltenyi Biotec), which was set to mini-magnet (Miltenyi Biotec). The column was set on the Falcon tube (Becton Dickinson), bead-binding cells were eluted by 1 ml of cold buffer, and IFNγ-producing cells were analysed by FACSCalibur (Becton Dickinson).

The 15mer peptide (M3R 216–230) and its nine altered peptide ligand candidates were synthesised (Sigma) (fig 1B). The purity of each peptide was >90%. The anchor positions binding to HLA-DR B1*0901 are AA222 and AA225, which are indicated as A and B in fig 1B. PBMCs from patient pSS-2 were used in this experiment; 1×10^6 cells were cultured with 10 μg of each peptide in 1 ml of RPMI-1640 with 10% human

AB serum. IFNγ-producing T cells were identified using MACS secretion assay as described in fig 1A.

To determine the altered peptide ligands of M3R in patients with SS, we synthesised nine 15mer peptides (VPPGECFI→E/K/LQFLSEPT, VPPGECFIQ→A/V/MFLSEPT, VPPGECFIQFL→M/E/KSEPT, M3R216–230), in which the anchored motif binding to the HLA-DR B1*0901 molecule is conserved, although one amino acid to TCR was different. Altered peptide ligands were selected based on inhibition of IFNγ production by M3R reactive T cells. Figure 1B shows that M3R 223I→K and M3R 224Q→A significantly suppressed the number of IFNγ-producing T cells, suggesting that they are candidates for selection as altered peptide ligands. The inhibition of IFNγ by other cytokines may not be likely, because interleukin 4 producing T cells were not increased (data not shown).

In conclusion, we have provided evidence for the presence of M3R reactive T cells in the serum of patients with SS and shown that VPPGECFKQFLSEPT (M3R 223I→K) and VPPGECFIAFLSEPT (M3R 224Q→A) are candidate altered peptide ligands of the second extracellular domain of M3R. Our findings may provide the basis of a potentially useful antigen-specific treatment for SS using altered peptide ligands of autoantigens recognised by autoreactive T cells.

Authors' affiliations
Y Naito, I Matsumoto, E Wakamatsu, D Goto, S Ito, A Tsutsumi, T Sumida, Department of Internal Medicine, University of Tsukuba, Tsukuba, Japan

Correspondence to: Professor T Sumida, Department of Internal Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8575, Japan; tsumida@md.tsukuba.ac.jp

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IL1RN genotype as a risk factor for joint pain in hereditary haemochromatosis?

E J Walker, J Riddell, H J Rodgers, M L Bassett, S R Wilson, J A Cavanaugh

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Hereditary haemochromatosis is a genetically determined disease of disrupted iron metabolism caused predominantly by the C282Y mutation in the HFE gene on chromosome 6.[1] If detected early, the symptoms of haemochromatosis can usually be prevented by venesection to remove excess iron and maintain normal iron stores.

One of the most common symptoms of haemochromatosis is joint pain (arthralgia) with or without associated joint disease (arthropathy),[2 3] and it has been generally assumed that arthritis in haemochromatosis is caused by iron deposition in the joints. Nevertheless, studies have failed to demonstrate any correlation between the level of iron overload and the presence or absence of arthritis or arthralgia,[4–6] nor does venesection ameliorate joint symptoms.[6] The interleukin cluster on chromosome 2 includes the interleukin (IL) 1α, IL1β, and IL1RN genes, and an association has been demonstrated between an IL1RN polymorphism and several inflammatory disorders, including osteoarthritis[7] but not rheumatoid arthritis.[8] This 86 bp polymorphism with five known alleles (a, b, c, d, and e) in intron 2 of the IL1RN gene has also been shown to be associated with expression levels.[9]

As far as we know, this study is the first to investigate genetic associations underlying joint pain in haemochromatosis. All participants gave informed written consent and the study was conducted with institutional ethics approval. In preliminary studies, we found that the frequency of the IL1IN polymorphism was significantly different ($p \leqslant 0.01$) between patients with haemochromatosis ($n = 313$) and controls ($n = 349$), and the IL1RN*a allele was more common in patients than in controls (data not shown).

We assessed 66 HFE C282Y homozygote patients (29 female, 37 male) and 52 healthy volunteers (25 women, 27 men) who were all wild type for the HFE mutation. All were available for clinical assessment of joint pain. Blood was collected for extraction of DNA and determination of serum ferritin (Immulite, 2000) and serum IL1RN levels (enzyme linked immunosorbent assay (ELISA), R&D, Minneapolis, USA). Clinical data, including the presence of joint pain, were collected through a questionnaire and by subsequent consultation with a physician (JR), who was unaware of their genetic status. Patient and control data were then divided according to whether they experienced joint pain or not. People who experienced joint pain as a result of injury