

Low Expression Levels of Soluble CD1d Gene in Patients with Rheumatoid Arthritis

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ABSTRACT. Objective. To examine whether the expression of intact CD1d, a critical molecule for the presentation of glycolipid antigens to natural killer T (NKT) cells, and its variants differs between patients with autoimmune diseases including rheumatoid arthritis (RA) and healthy subjects. Recently, we identified 8 different CD1d variants, generated by alternative splicing. V1 lacking exon 4 (CD1d without β_2 microglobulin, β_{2m}) and V2 lacking exons 4 and 5 (soluble CD1d) may be functional molecules, because the antigen binding sites (exons 2 and 3) are intact.

Methods. Peripheral blood mononuclear cells (PBMC) from 44 patients with autoimmune disease (RA 19, systemic lupus erythematosus, SLE 10, Sjögren's syndrome, SS 15) and 15 healthy controls were separated and complementary (c)DNA was prepared. The expression of intact CD1d on PBMC was detected by flow cytometry. Alternatively spliced CD1d variants were quantified by TaqMan PCR using polymerase chain reaction with confronting 2-pair primers (PCR-CTPP) based amplification.

Results. The mean (\pm SEM) transmembrane and β_{2m} binding site deleted CD1d mRNA level in 19 patients with RA (2.0 ± 0.33) was significantly lower than in 15 controls (6.9 ± 2.08 ; $p < 0.05$), whereas there were no differences in β_{2m} deleted variants and intact CD1d mRNA.

Conclusion. Our findings suggest that low expression of soluble CD1d variants might play a role in the formation of symptoms or pathogenesis of RA. (J Rheumatol 2003;30:2524–8)

Key Indexing Terms:

AUTOIMMUNE DISEASE ALTERNATIVE SPLICING VARIANT
CD1D NATURAL KILLER T CELLS

Natural killer T (NKT) cells are a unique subset of T lymphocytes that express invariant T cell receptor (TCR) and NKR-P1A. A key feature of NKT cells is the expression of a heavily biased TCR, bearing AV24AJ18 and BV11 chains^{1,2}. NKT cells recognize glycolipid antigen presented on CD1d molecules³⁻⁵. CD1d is a nonpolymorphic major histocompatibility complex (MHC) class I-like molecule⁶. We reported the expression of 8 alternatively spliced CD1d mRNA in peripheral blood mononuclear cells (PBMC)⁷. Two of these CD1d variants (V1 and V2) were considered functional because the antigen binding site was completely conserved. V1 lacks exon 4 [β_2 -microglobulin (β_{2m}) binding site] of the gene, resulting in unstable antigen presentation. The other variant (V2) lacks both exons 4 and 5 (transmembrane domain), resulting in the soluble form of CD1d (sCD1d).

The number of NKT cells is selectively reduced in various human autoimmune diseases, such as systemic sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), and insulin-depen-

dent diabetes mellitus (IDDM)^{8,9}. Several mechanisms responsible for the low number of NKT cells in these patients have already been identified. These include inadequate presentation of the antigen, dysfunction of NKT cells, and abnormality of antigen presentation⁹. Our study was designed to examine whether there is a difference of intact CD1d, V1, and V2 CD1d variants between patients with autoimmune diseases, including RA, and healthy subjects, and thus elucidate the cause of abnormal antigen presentation and decreased NKT cells in patients. We analyzed the relative amounts of intact CD1d and isoforms, V1 and V2, using flow cytometry and real-time TaqMan polymerase chain reaction (PCR).

MATERIAL AND METHODS

Patients. The study group included 19 patients with RA diagnosed according to the American College of Rheumatology (ACR, formerly American Rheumatism Association) criteria¹⁰, 10 patients with SLE, who fulfilled ACR diagnostic criteria¹¹, and 15 patients with SS diagnosed according to the criteria¹². All patients and 15 disease-free healthy subjects were referred to the University of Tsukuba Hospital.

Preparation of cells and complementary DNA (cDNA). PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech Inc., Piscataway, NJ, USA). Total RNA was prepared from fresh PBMC with Isogen (Nippon Gene, Co., Tokyo, Japan), and reverse transcribed into cDNA using the method described by Sumida, *et al*¹³. Briefly, first-strand cDNA was synthesized in a 20 μ l reaction mixture containing oligo (dT) primer from 1 μ g of total RNA. A 0.1 μ l aliquot of the reaction mixture encoding the cDNA was used for TaqMan PCR analysis.

Antibodies and flow cytometry. FITC-conjugated anti-human CD1d mono-

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clonal antibody (mAb) 42 was purchased from PharMingen (San Diego, CA, USA). The cells were resuspended in propidium iodide and then analyzed by FACSCalibur™ (Becton Dickinson, San Jose, CA, USA). A live gate was set by forward and side scatter and propidium iodide staining. Over 50,000 cells with high forward and side scatter were acquired from each sample, and data were analyzed using Cellquest™ software (Becton Dickinson).

TaqMan probe and primer design. One forward primer, 2 reverse primers, and a FAM-labeled probe were designed within the mRNA sequence of CD1d. The sequences of the primers and TaqMan probe used in this study were as follows: common CD1d forward: 5'-TGGGAGAT-
ACTCAGCAACTCTGG-3'; CD1d E4 del.reverse (E4): 5'-GGAGGTG-
TAGTCTCCACCTT-3'; CD1d E4E5 del.reverse (E45): 5'-GGACGC-
CCTGATAGGA-3'; and common CD1d PROBE FAM: 5'-FAM-
CCTGACTCAAGGAGGCCACTGACAAATT-TAMRA-3'. The antisense
primers were specific for the 5' edge of exon 5 (E4 del.) or exon 6 (E4E5 del.)
of the CD1d gene, and the 3' end of these primers (3 bp) annealed with the
end of exon 3.

Quantitative PCR. cDNA were obtained from PBMC. The real-time PCR consisted of one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in an ABI Prism 7700 Sequence Detector (Applied Biosystems Japan K.K.). All experiments were carried out in triplicate. Quantitative analysis of gene expression was performed by the comparative CT (DCt) method¹⁴. To standardize the quantification of target genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from each sample was quantified on the same plate with the target genes by using TaqMan GAPDH control reagents kit (Applied Biosystems).

Statistical analysis. All data are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) and Scheffe's post-hoc test. P values less than 0.05 were considered statistically significant.

RESULTS

Percentages of CD1d+ cells in high forward and side scatter PBMC from patients with autoimmune diseases were not significantly different from controls (Figure 1B). Cell surface expression of CD1d molecules using fluorescence intensity showed no significant difference between controls and several autoimmune diseases (Figure 1C).

To specifically detect each variant, we used confronting 2-pair primers (PCR-CTPP) based PCR amplification, a simple method designed for detection of polymorphism. In this method, allele-specific DNA products are amplified by applying appropriately designed primers. We set the primer-pair and TaqMan probe site for the specific detection of V1 and V2 variant mRNA (Figure 2A). It was confirmed that these primer pairs amplified only a specific target by the specificity of the 3' end of each antisense primer (Figure 2B). We quantified the alternatively spliced CD1d mRNA with this system. As shown in Figures 3A and B, the results indicated that the mean level of mRNA encoding the V2 variant was significantly lower in patients with RA (2.0 ± 0.33) than controls (6.9 ± 2.08 ; $p < 0.05$). In contrast, there were no significant differences in V1 mRNA between patients with autoimmune diseases and controls. The results are summarized in Table 1.

DISCUSSION

We concluded from our results that the numerical change in

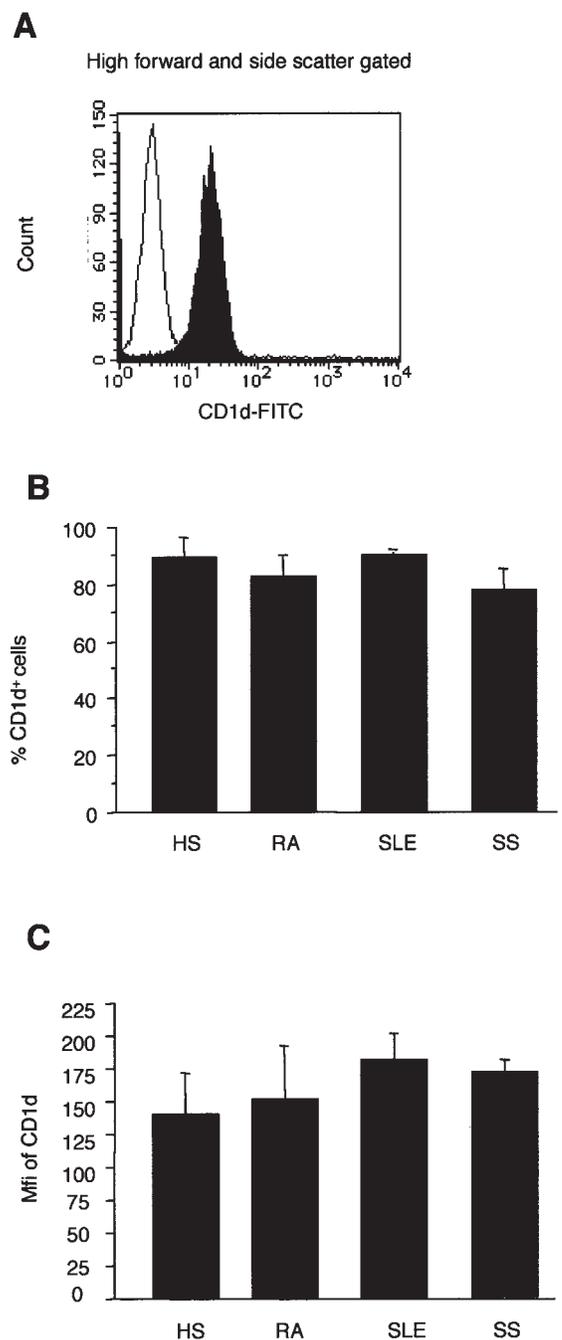


Figure 1. Relative expression of cell surface CD1d. PBMC from 15 patients with autoimmune diseases (7 RA, 6 SLE, 2 SS) and 4 healthy controls (HS) were stained with anti-CD1d mAb. (A) Typical staining pattern of CD1d in high forward and side scatter cells (black histogram: anti-CD1d mAb; white histogram: control mAb). (B) Percentage of CD1d positive cells. (C) Mean fluorescence intensity of CD1d. Data are mean ± SEM.

CD1d positive cells does not affect the NKT cell population commonly observed in RA, SLE, and SS. In comparison, Takahashi, *et al*¹⁵ reported that the percentage of CD1a-positive cells was lower in monocyte-derived dendritic cells (Mo-DC) from patients with IDDM. CD1a molecule is a

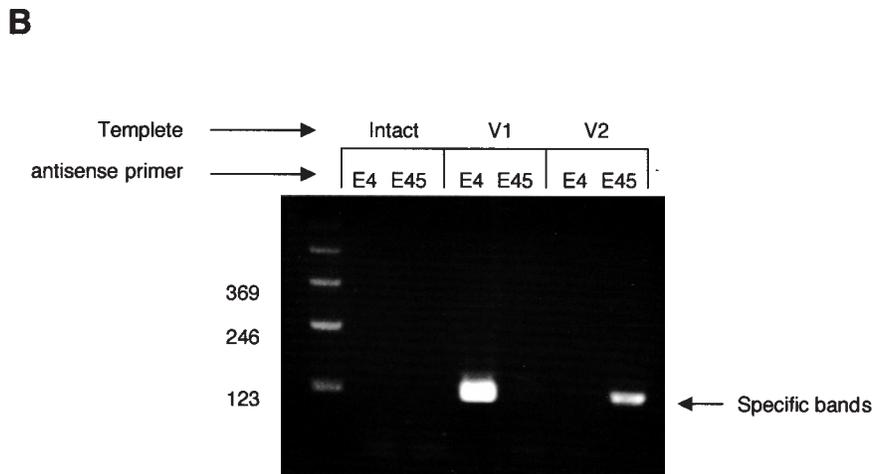
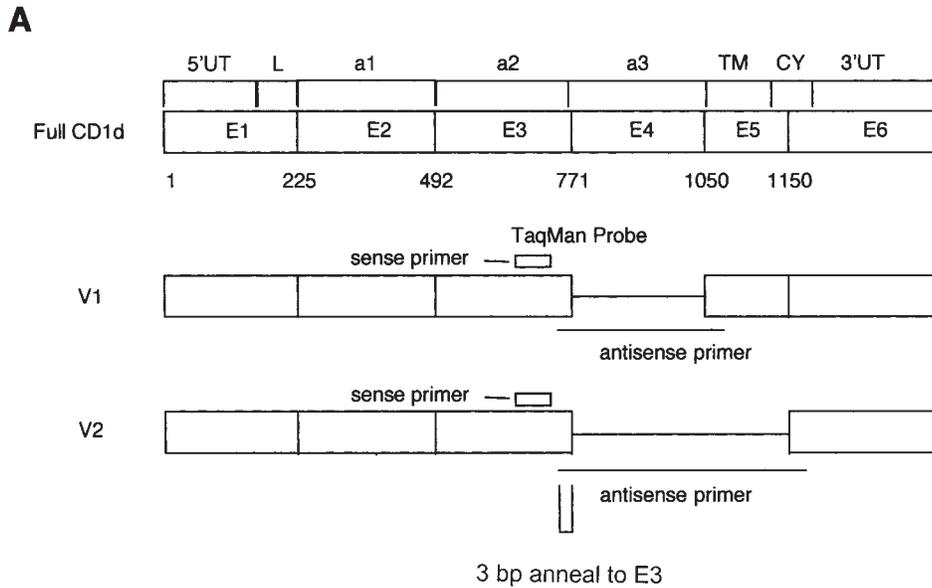


Figure 2. The human alternative splicing forms of CD1d transcript and the primer and probe sites for the TaqMan PCR. (A) The primer and probe sites. The CD1D transcript comprises 6 exons (E1–E6) encoding the leader (L), $\alpha 1$, $\alpha 2$, $\alpha 3$ domains, transmembrane (TM), and cytoplasmic (CY) domains. (B) Specificity of these primer sets was confirmed by PCR using previously cloned intact and variant CD1d cDNA as PCR templates.

member of the human CD1 gene family, and a marker for human immature Mo-DC¹⁶. Dendritic cells are antigen-presenting cells that efficiently activate NKT cells^{17,18}, therefore a reduction in dendritic cell population may result in reduction of NKT cells. However, we found no significant differences in percentage of CD1d-positive cells among patients with various autoimmune diseases including RA, SLE, and SS and controls.

We have reported the presence of alternatively spliced isoform mRNA on CD1d molecule⁷. Although the function of these alternatively spliced forms remains unclear, we

assumed that these variants alter the antigen presentation of antigen-presenting cells to NKT cells. Therefore, we quantified the alternatively spliced CD1d mRNA by the TaqMan PCR method^{19,20}. Our results indicated that mRNA encoding the V2 variant was significantly lower in patients with RA than controls; in contrast, there were no significant differences in V1 mRNA between patients with autoimmune diseases and controls.

Previous studies reported the presence of high serum levels of soluble MHC class I molecules in patients with active SLE, RA, and multiple sclerosis^{21,22}. Our results

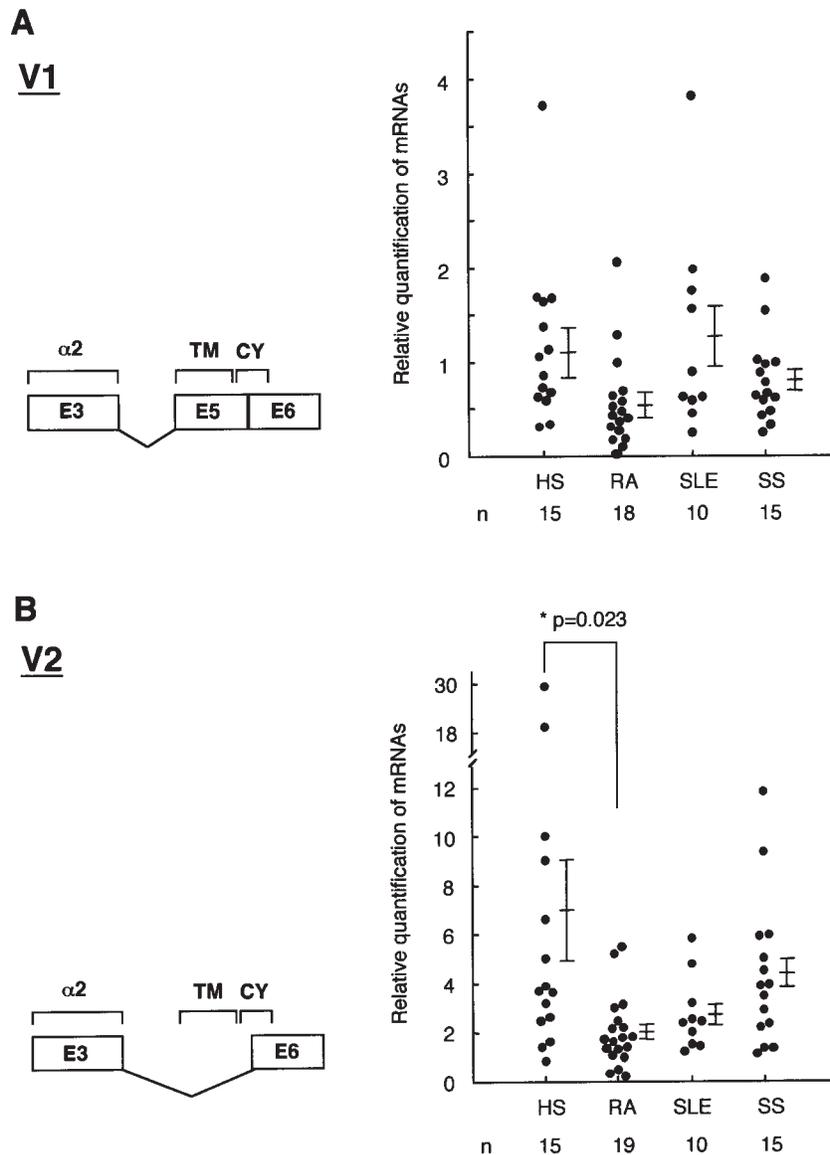


Figure 3. Reduction of sCD1d (V2) mRNA in patients with RA. The relative amounts of V1 (A) and V2 (B) were measured using the real-time TaqMan PCR method. V2 mRNA were significantly reduced in patients with RA compared with healthy controls (HS). Data represent mRNA levels of individuals, together with the mean \pm SEM values of the indicated number of subjects (n). E3: exon 3; E5: exon 5; E6: exon 6; α 2: α 2 domain; TM: transmembrane domain; CY: cytoplasmic tail.

suggest that soluble MHC molecules may be useful as a marker of activity of rheumatic disease, and may function as immunoregulators of the autoimmune response. On the other hand, we found a reduction of sCD1d at the mRNA level in patients with RA. Here, we speculate the following 2 functions for the sCD1d molecule. First, sCD1d could directly stimulate and activate NKT cells. Several studies have reported that soluble MHC molecule binds to TCR and stimulates alloreactive T cells²³. Hence, reduction of sCD1d might consequently lead to a reduction of NKT cells in RA patients. Second, sCD1d could inhibit hyperactivation of

NKT cells by occupying T cell receptors. NKT cells are highly sensitive to activation-induced cell death (AICD) (e.g., induced by stimulation with anti-CD3e antibody), compared with conventional T cells²⁴. Thus, low levels of sCD1d molecules cannot produce a sufficient inhibition of AICD of NKT cells, resulting in a reduction of NKT cells.

Our findings indicated significantly low expression of V2 variants lacking both exon 4 and 5 genes in patients with RA and the reduction of V2 molecules might be associated with decreased numbers of NKT cells in patients with RA. However, little is known about the mechanism of secretion

Table 1. Summary of V1 and V2 (sCD1d) mRNA expression in patients and healthy subjects (HS). Results are expressed as mean \pm SEM.

	n	Relative Expression of mRNAs	p
V1			
HS	15	1.2 \pm 0.23	
RA	18	0.5 \pm 0.12	0.110
SLE	10	1.3 \pm 0.34	0.994
SS	15	0.8 \pm 0.11	0.595
V2			
HS	15	6.9 \pm 2.08	
RA	19	2.0 \pm 0.33	0.023
SLE	10	2.7 \pm 0.48	0.162
SS	15	4.3 \pm 0.78	0.473

or the immunoregulatory role of sCD1d. Further examination of the sCD1d molecule (V2) at a protein level is necessary.

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