immunology 2002; 23: 516-19

26. Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. Differential chemokine responses and homing patterns of murine TCR__ NKT cell subsets. *J. Immunol.* 2003; 171: 2960-9

27. Stenstrom M, Skold M, Ericsson A, Beaudoin L, Sidobre S, Kronenberg M, Lehuen A, Cardell S. Surface receptors identify mouse NK1.1 T cell subsets distinguished by function and T cell receptor type. *Eur. J. Immunol.* 2004; 34: 56-65

Figure legends

Fig. 1 Suppression of arthritis in NKT-KO mice and CD1d-KO mice. Ten NKT-KO (a, b) and ten CD1d-KO mice (c, d) were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. Tuberclosis* H37Ra. Ten C57BL/6 mice were used as the control in each examination. The incidence of CIA (a, c) and the severity of arthritis (b, d) were investigated.

"•" represents each KO mice, "■" represents C57BL/6 mice.

Fig. 2 Reduction of anti-CII Abs in NKT-KO and CD1d-KO mice. Twenty-eight days after the booster injection, the amount of anti-CII IgG antibody in the serum from NKT-KO (a) or CD1d-KO (b) mice and C57BL/6 mice was measured by ELISA.

Fig. 3 Cell proliferation and cytokine production stimulated by CII in NKT-KO mice compared with those in C57BL/6 mice. Five NKT-KO and five C57BL/6 mice were immunized with chicken CII emulsified in IFA plus *M. Tuberclosis* H37Ra. Nine days after

immunization, splenocytes were stimulated with CII. The degree of cell proliferation was evaluated by a BrdU ELISA method (a). The concentrations of IFN-γ (b) and L-4 (c) in the culture supernatants were measured by ELISA.

Fig 4. Activation level of T and B cells after the booster immunization in NKT-KO mice compared with that in C57BL/6 mice. Eight NKT-KO and eight C57BL/6 mice were immunized and boosted with chicken CII emulsified in IFA plus inactivated *M. Tuberclosis* H37Ra. Five days after the booster immunization, splenocytes were collected and stained with FITC-labeled anti-TCR_ or anti-B220, and PE-labeled anti-CD69 antibody. PI-negative cells were gated and FITC-PE double positive cells were counted (a). The proportions of CD69-positive T cells (b) or CD69-positive B cells (c) compared to the total number of T cells ($TCR_^{+}$ cells) or B cells ($B220^{+}$ cells) were calculated.

Fig 5. Cytokine mRNA expression in the spleen after the booster

immunization. Total splenic RNA was collected from three male C57BL/6 mice (•) and three male NKT-KO mice (■) 5, 10, 15, and 30 days after the booster immunization, and the relative expression levels of IFN-_ (a), IL-4 (b), or IL-1_ (c) mRNA were measured by the Taqman quantitative PCR method. * $P<0.05$; ** $P<0.01$

Clinical and Experimental Immunology (in press)

TCRVα14$^+$ NKT cells function as effector T cells in collagen-induced arthritis mice

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Keywords: NKT cells, collagen-induced arthritis, effective, cytokine

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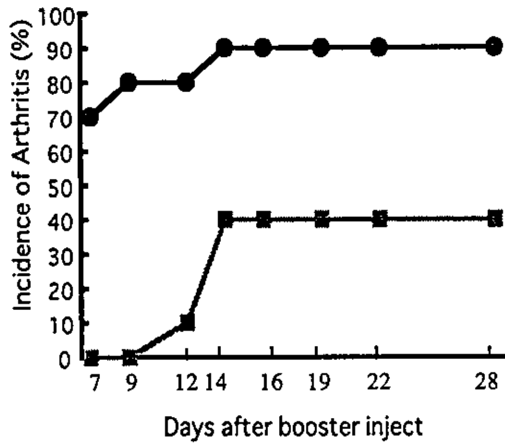
Figure 1

(a)



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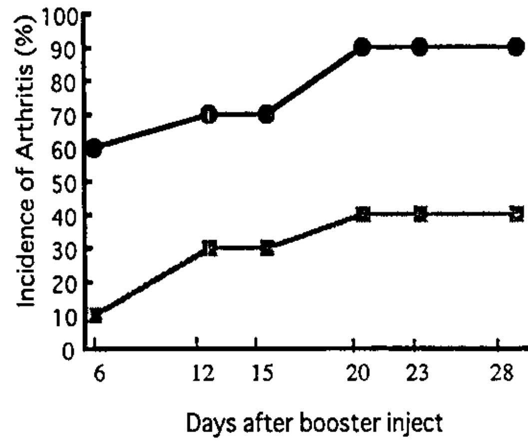

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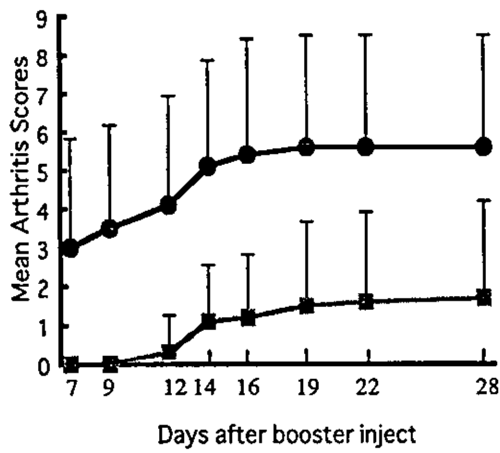

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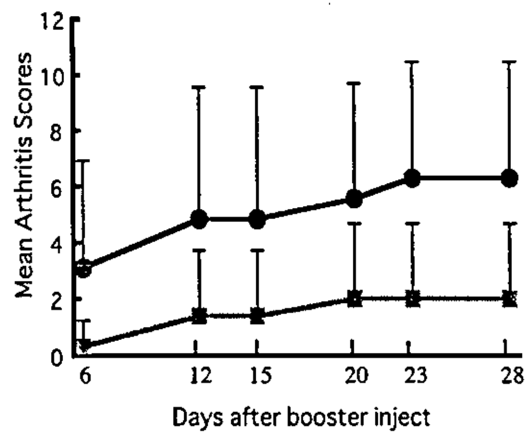
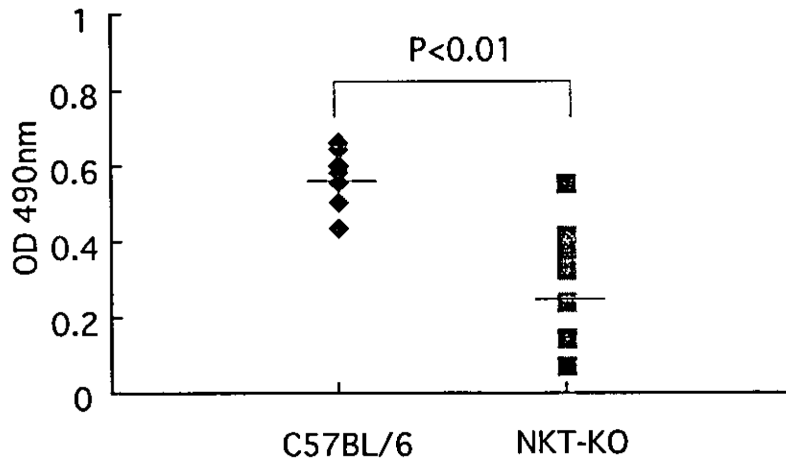


Figure 2

(a)



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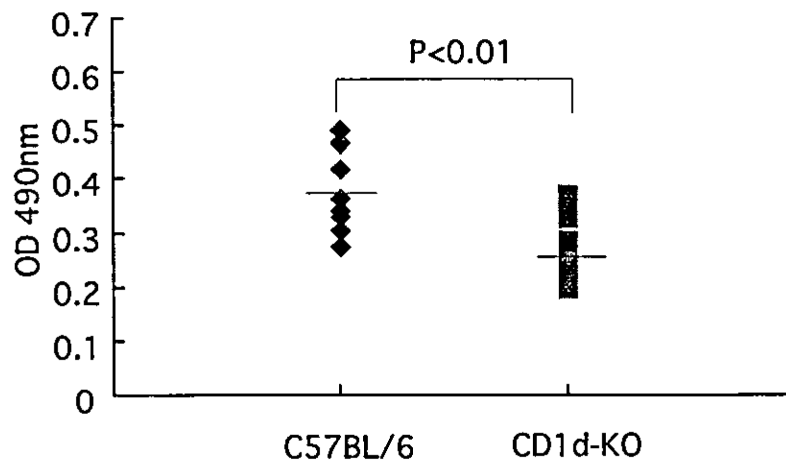
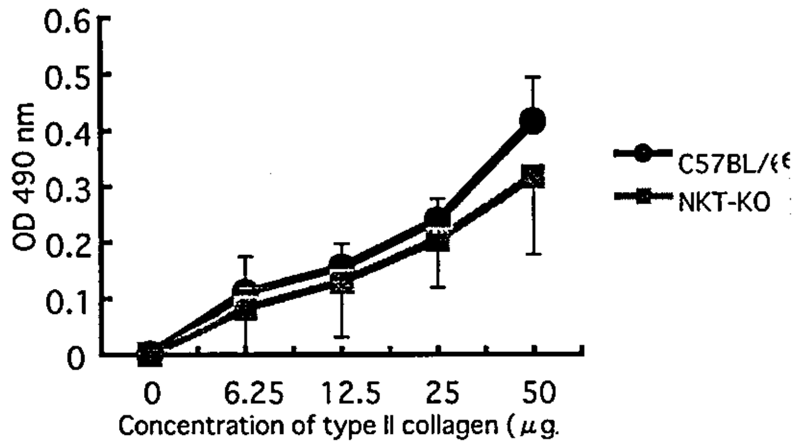


Figure 3

(a)



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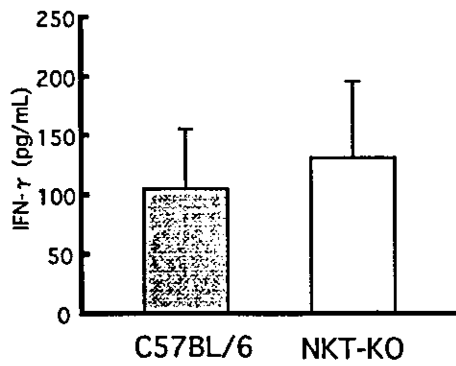

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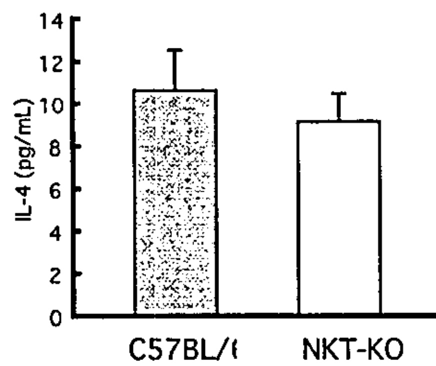


Figure 4

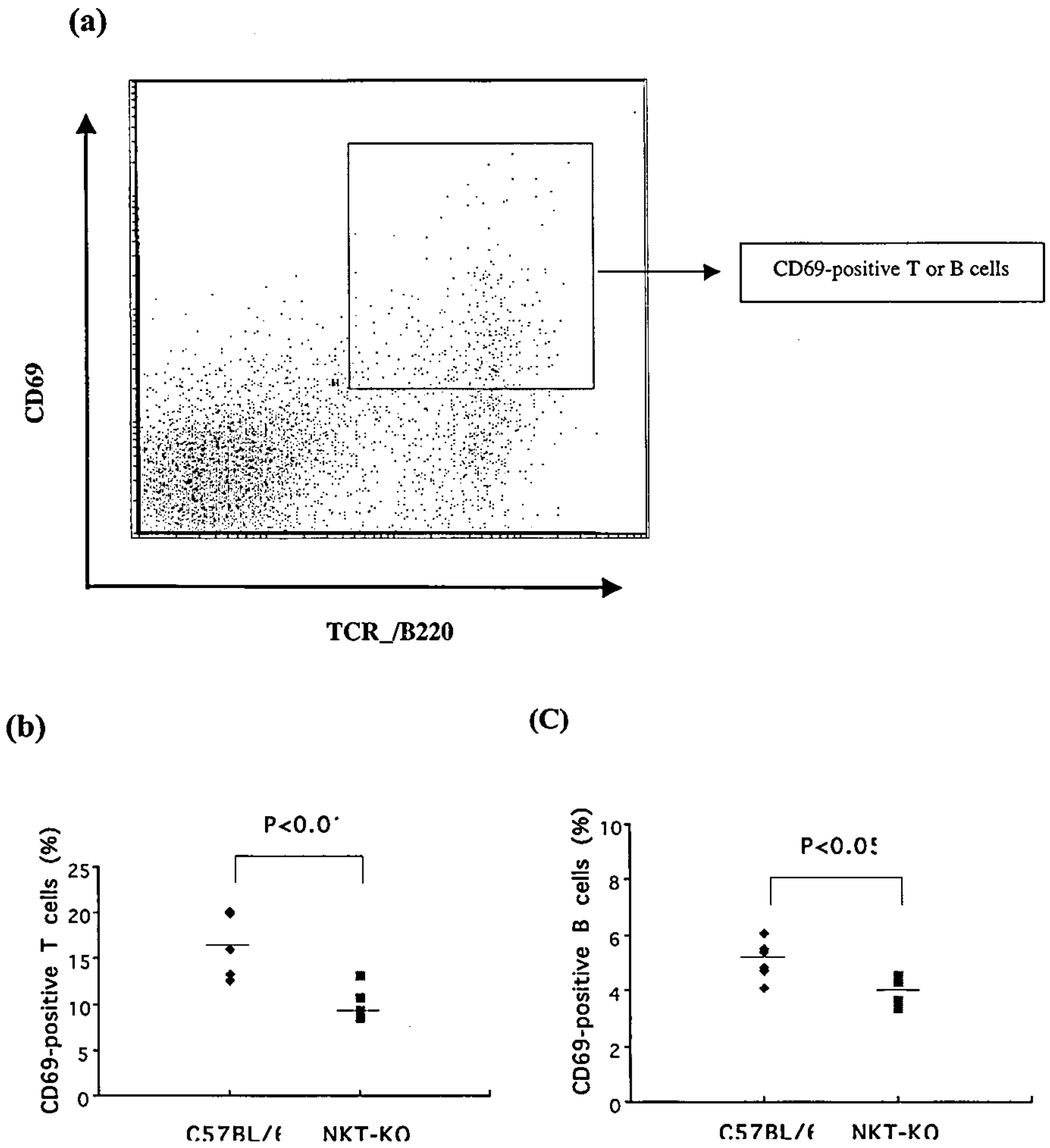
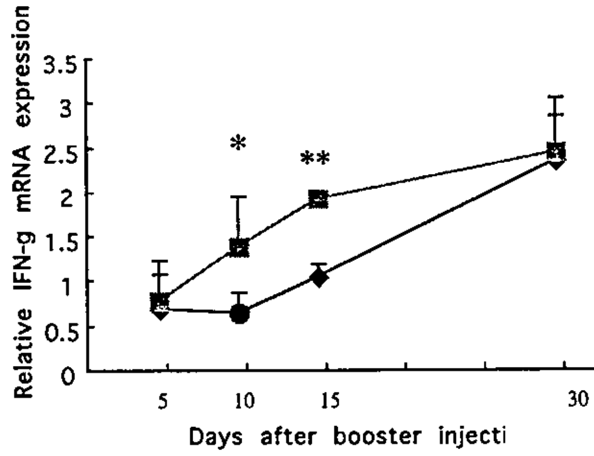


Figure 5

(a)



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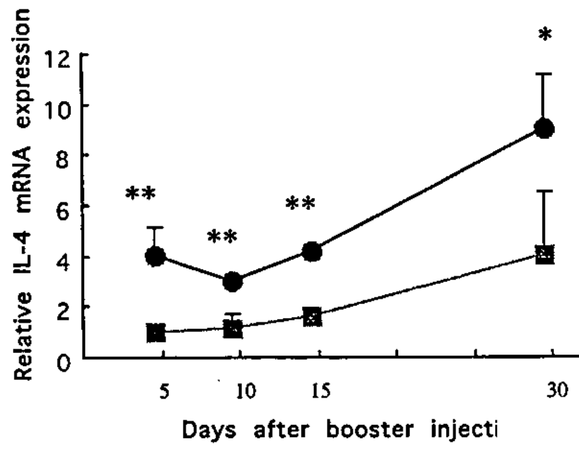
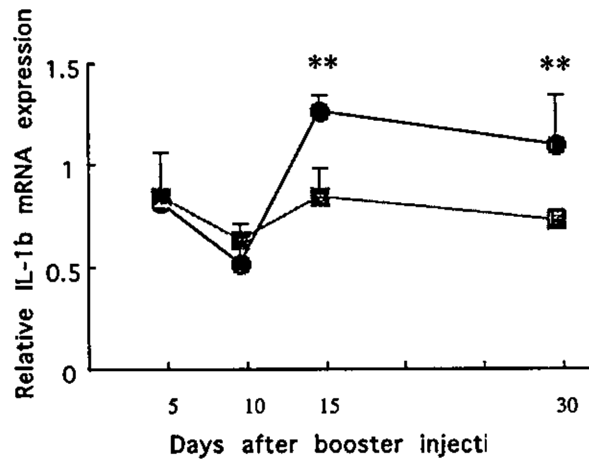


(c)



Analysis of abnormally expressed genes in synovium from patients with rheumatoid arthritis using a column gel electrophoresis-coupled subtractive hybridization technique

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Abstract. Rheumatoid arthritis (RA) is a chronic disease of unknown pathogenesis. To identify abnormally expressed genes in synovium from RA patients, we performed column gel electrophoresis-coupled subtractive hybridization (CGESH). CGESH is a newly developed subtractive hybridization technique to achieve sufficient enrichment of DNA sequences. CGESH was performed using restricted enzyme digested cDNA synthesized from mRNA of synovial tissues from one RA patient and one osteoarthritis (OA) patient. The obtained subtraction libraries (RA-OA) were screened by dot blot hybridization. The clones showing higher hybridization with the RA-OA probe were identified by sequence analysis and homology search. Their DNA sequencing revealed that the genes of HLA-DRB1, sequestosome 1, elongation factor 1α were included. Furthermore, a functionally unknown gene (FLJ00133) was also identified. It is reported that sequestosome 1 is a scaffold in the signal transduction of TNFα and interleukin 1, which are the important cytokines involved in the pathogenesis of RA. It is possible that other genes identified by the CGESH technique would be associated with the pathogenesis of RA, although there is no direct evidence yet. Our results imply that the CGESH technique is a useful tool to detect genes involved in the pathogenesis of RA. Further investigation of the functional roles of candidate genes should shed light on the pathogenesis of RA.

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Abbreviations: RA, rheumatoid arthritis; OA, osteoarthritis; CGESH, column gel electrophoresis-coupled subtractive hybridization; IGCR, in-gel competitive reassociation

Key words: rheumatoid arthritis, column gel electrophoresis-coupled subtractive hybridization

Introduction

Rheumatoid arthritis (RA) is a chronic disorder of unknown pathogenesis, that causes multiple joint destruction. Although new medications such as TNFα blocking agents are showing promising effects, deeper understanding of the disease is needed to overcome this possibly tragic condition. In order to understand the molecular mechanisms involved in the pathogenesis of RA, and to search for a possible target of RA-specific therapies, we wished to identify genes specifically expressed in the synovial tissues of patients with RA.

In the past, various strategies have been developed to examine tissue-specific differences in gene expression. We used column gel electrophoresis-coupled subtractive hybridization (CGESH) technique (1) for this purpose. The original technique which forms the basis of the CGESH, namely in-gel competitive reassociation (IGCR), was developed by Yokota *et al* (2,3). In this technique, electrophoresis size-separation of mixed restriction digested DNA samples to be compared and *in situ* denaturation-hybridization steps are carried out sequentially. These in-gel processes provide unique technical features that other batch subtractive hybridization techniques cannot. Firstly, size-dependent sequential fractionation of restriction digested DNA fragments results in an enormous reduction of sample complexity. This makes highly efficient hybridization possible. Secondly, closely related sequences will be physically separated and would not interfere with each other provided that they have different restriction fragment sizes. To overcome the drawbacks of this technique, such as its being time-consuming and too complex, Ozawa *et al* (1) made modifications to simplify the strategy of IGCR and developed the CGESH technique. While improving IGCR, this technique was originally used to detect differences between genome DNA samples (4,5), but if applied to cDNA samples, in addition to the advantages described above, it may

Table I. Oligonucleotides used for the CGESH experiment.

Name	Sequence	*HinP1* I site reconstitution
A20	5'-GACTGTCAAGGATCCCTTAG-3'	Yes
A10	3'-AGGGAATCGC-5'	
B20	5'-GATCGTGACAAGCTTCTGAC-3'	No
B10	3'-GAAGACTGGC-5'	
C20	5'-CAGACTCTGGAATTCGCATG-3'	Yes
C10	3'-AAGCGTACGC-5'	
Bio-C20	5'-biotin-CAGACTCTGGAATTCGCATG-3'	

CGESH, column gel electrophoresis-coupled subtractive hybridization. Underlining indicates *HinP1* I restriction enzyme site.

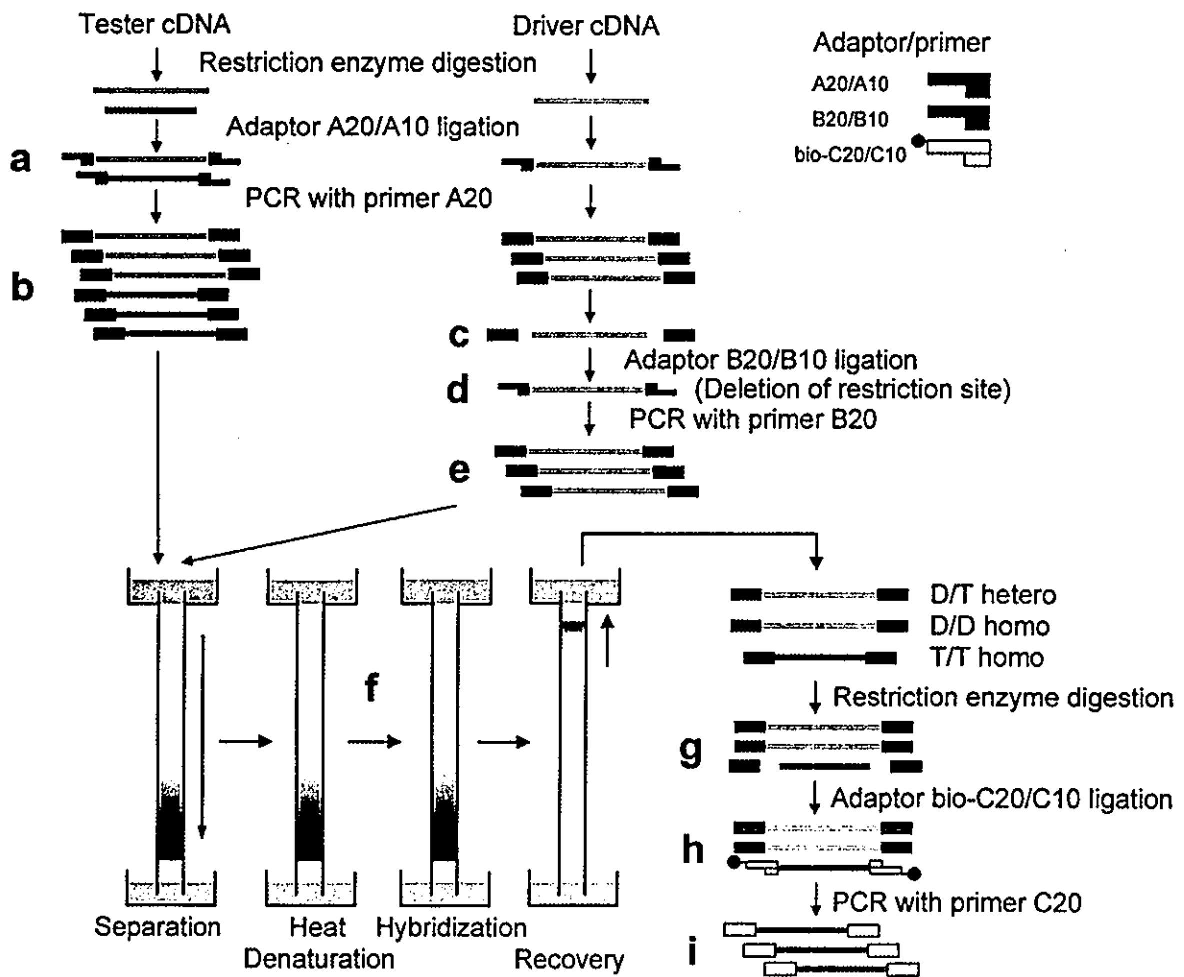

Figure 1. Summarized illustration of the steps of the column gel electrophoresis-coupled subtractive hybridization (CGESH) technique. *HinP1* I-digested driver or tester cDNA fragments were ligated with adaptor A20/A10, which preserves the *HinP1* I restriction enzyme site (a) and preamplified by PCR using primer A20 (b). Adaptor was removed from driver cDNA fragments by *HinP1* I digestion (c) and replaced with a new adaptor B20/B10, which does not reconstitute the *HinP1* I recognition site (d), and reamplified by PCR using primer B20 (e). Gel electrophoresis, denaturation, hybridization and recovery were carried out sequentially (f). Only perfectly reannealed tester-derived fragments can reconstitute the *HinP1* I site and the A20/A10 adaptor sequence are removed again by restriction digestion (g). A new adaptor, consisting of an oligonucleotide pair bio-C20/C10, is attached to both ends of the tester-derived fragments (h) and trapped by Streptavidin Sepharose beads (h). After removing unbound cDNA fragments by washing the beads, specifically captured cDNA fragments were amplified by PCR using primer C20 (i). See Table I for adaptor and primer sequences.

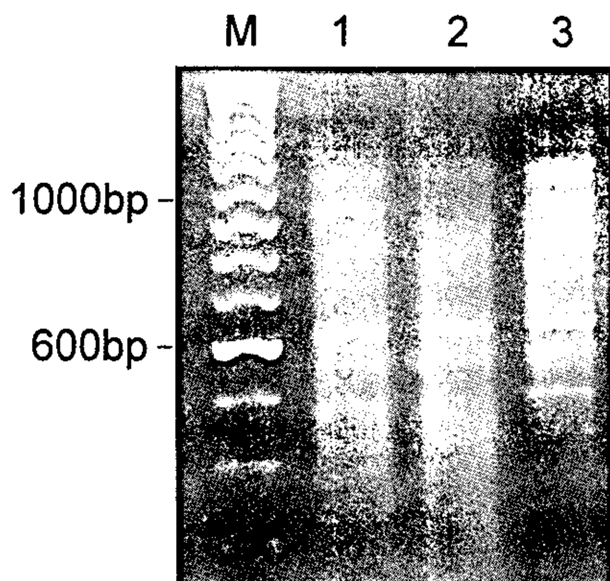

Figure 2. Electrophoresis pattern of the subtracted cDNA. Lane 1, driver cDNA amplified with primer A20, adaptor replaced and amplified with primer B20; Lane 2, tester cDNA amplified with primer A20; Lane 3, product of 1st round subtraction amplified with primer C20; Lane M, 100 bp DNA ladder.

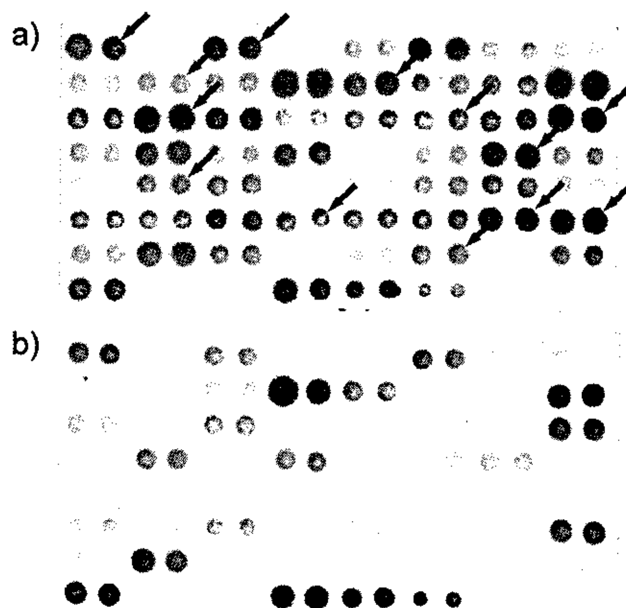

Figure 3. Screening of subtraction library by dot blot hybridization. Two identical cDNA blots of PCR-amplified inserts derived from the subtracted cDNA library were separately hybridized with DIG-labeled subtracted probe (a) and reverse-subtracted probe (b). Arrows indicate clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) measured by a densitometer.

be possible to detect size variants such as those produced by alternative splicing, which are not detectable by conventional subtraction techniques. Thus, CGESH can be a powerful tool to identify known or unknown genes specifically expressed in a given tissue. Our objective was to detect genes specifically expressed in synovial tissues from RA patients using this newly developed technique.

Materials and methods

Synovial tissue was obtained under informed consent from patients with rheumatoid arthritis (RA) who met the criteria of the American Collage of Rheumatology (6), and patients with osteoarthritis (OA). Total RNA from synovial tissue was isolated using Isogen (Nippon Gene, Tokyo, Japan), and poly (A)$^+$ RNA was isolated from total RNA using a Oligotex-dt30 <Super> mRNA Purification Kit (Takara Bio, Shiga, Japan), following the protocols supplied by the manufacturers. The list of the oligonucleotide primers used in this study is provided in Table I.

CGESH technique. CGESH was performed essentially following the methods described by Ozawa *et al* (1), with some alterations (Fig. 1). Poly (A)$^+$ RNA (1 μg) from synovial tissues from RA or OA patients were subjected to cDNA synthesis. cDNA was synthesized using cDNA synthesis kit (Takara Bio) according to the manufacturer's instructions. cDNA from RA was designated as tester and that from OA as driver. Tester and driver cDNA were digested by a restriction enzyme *HinP1* I (New England Biolabs, Beverly, MA, USA), and ethanol-precipitated. Digested DNA fragments were ligated to oligonucleotide adaptor A20/A10 with T4 DNA ligase (New England Biolabs). The DNA was amplified using primer A20 by PCR using mini cycler PTC-150 (MJ Research, Waltham, MA, USA) under the following cycling conditions: 72°C for 1 min, 30 cycles of 94°C for 1 min and 68°C for 5 min, followed by 68°C for 20 min as a final extension step. Amplified driver DNA was digested with *HinP1* I, ligated with adaptor B20/B10, then amplified by PCR with primer B20 as described above. Tester DNA (10 ng) and driver DNA (1 μg) were mixed with formamide (10% final) to a final amount of 20 μl, and loaded on the top of the formamide-containing poly-acrylamide gel in a glass column (inner diameter: 5 mm; length: 130 mm), and electrophoresis was performed in the presence of a phosphate buffer. After gel separation, the column was incubated at 80°C for 10 min to denature dsDNA fragments in the gel. Then, the temperature was gradually lowered to hybridization temperature (37°C), and kept at 37°C overnight. After hybridization, DNA fragments were recovered by electrophoresis in the reverse direction, and collected by absorbing with Q-sepharose (Amersham Biosciences, Piscataway, CA, USA). Gel-recovered DNA was again digested with *HinP1* I, ligated with biotinylated adaptor bio-C20/C10. Biotinylated adaptor-ligated DNA fragments were absorbed with Streptavidin Sepharose (Amersham Bio-sciences). Amplification reaction was performed by adding the DNA-bound Streptavidin Sepharose slurry into a PCR reaction containing primer C20. The thermal cycling conditions were as described above.

Screening of differential expression. PCR products from the subtracted libraries were subcloned into pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA). cDNA inserts of the cloned cDNA library were amplified by subjecting an aliquot of the bacterial culture directly to PCR.

Table II. Genes found to be up-regulated in RA compared to OA using the CGESH technique.

Clone No.	Accession No.	Identified gene
H237	NM_002124.1	Major histocompatibility complex, class II, DR ß 1 (HLA-DRB1)
H19, 180	NM_003900.2	Sequestosome 1 (SQSTM1) (p62)
H13, 167	NM_001402.4	Elongation factor 1 α 1 (EEF1A1)
H91, 129, 165, 190, 224, 252	NM_203339.1	Clusterin (CLU)
H95	NM_000852.2	Glutathione S-transferase pi (GSTP1)
H231	XM_059482.6	FLJ00133 protein (FLJ00133)

CGESH, column gel-electrophoresis subtractive hybridization.

Electrophoresis of the amplified product in agarose gels was always done to confirm that all clones were single. The amplified materials were then dot blotted onto nitrocellulose membranes in duplicates. Two identical membranes were prepared, and they were hybridized with either the subtracted probe (RA-OA) or the reverse-subtracted probe (OA-RA). Probes were labeled with a DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's recommended protocol. Hybridization and detection via a chemiluminescence reaction were carried out employing a DIG luminescent detection kit (Roche diagnostics), according to the supplier's standard protocol. The amount of hybridization was semiquantified with a densitometer (LAS-3000; Fuji Photo Film, Tokyo, Japan). Clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) were selected and plasmids were purified using a Qiagen plasmid mini kit (Qiagen, Hilden, Germany). Inserts were sequenced by chain termination reaction using an automated sequencer (ABI prism 310 genetic analyzer; Perkin Elmer, Wellesley, MA, USA). Nucleic acid homology searches were performed using the BLAST program at the National Center of Biotechnology Information (National Institutes of Health).

Results and Discussion

Using CGESH, we constructed one subtracted library using RA synovial tissue sample as tester and OA synovial sample as driver (RA-OA) (Fig. 2). In parallel, a reverse-subtracted library was prepared (OA-RA) to make a control probe for dot blot hybridization screening. This subtracted library was cloned, and a total of 273 clones were obtained. Thirteen clones with a hybridization ratio of 2:1 or larger (RA-OA vs OA-RA) measured by a densitometer in the dot blot hybridization were selected (Fig. 3). These clones were sequenced, and 6 genes (HLA-DRB1, sequestosome 1, elongation factor 1 α 1, clusterin, glutathione S-transferase pi and FLJ00133) were identified. Six clones (H91, 129, 165, 190, 224, 252) were derived from the clusterin gene, two clones (H19, 180) were sequestosome 1, and the other two clones (H13, 167) were elongation factor 1 α 1. This suggests that the dot blot hybridization worked well for selecting genes from the CGESH library, although the true frequency of this gene expression was not clarified. The sequences of the clones were identical to those submitted in the BLAST program (Table II).

HLA-DRB1, a class II MHC molecule, is important for antigen presentation, and would be important in the pathogenesis of RA (7,8). Increased expression of MHC class II molecules in RA synovial tissue is also reported (9). Sequestome 1, also known as p62, is reported to be a scaffold in the signal transduction of TNFα and interleukin-1 leading to the activation of NF-κB (10). These two important cytokines are well known to be involved in the pathogenesis of RA (11). Recently, it has been shown that p62 is an important mediator during osteoclastogenesis and induced bone remodeling (12). p62 may have a function in the joint destruction process in RA patients. Elongation factor 1 α 1 is reported to increase at the mRNA level in patients with RA synovial samples compared to OA synovial samples (13). Autoantibody against this protein has been reported to be present in patients with Felty's syndrome (14). The function of this protein is not fully understood, but may have a role in the pathogenesis of RA. Both clusterin and glutathione S-transferase pi genes are reported to be involved in the signal transduction of NF-κB (15,16). FLJ00133, which was identified in the NEDO human cDNA sequencing project at Kazusa DNA Research Institute (17), was also one of the genes identified in our study. The function of this gene product is yet to be clarified.

We used the dot hybridization technique to efficiently screen the clones obtained by CGESH. This method allows us to easily identify genes with higher expression in the tester sample than in the driver. However, it is true that dot blot hybridization cannot identify size variants such as alternatively spliced gene products. CGESH is theoretically potent for screening such variants because a size fractionation step is included in the procedure. A large number of clones that were considered non-specific in dot blot assays may contain such variants. By combining an efficient method to identify such clones, we would be able to make the most of the CGESH technique and be able to show that it is a powerful tool for identifying genes that are differently expressed between two tissues.

In summary, we have identified a number of genes possibly involved in the pathogenesis of RA by means of the CGESH technique, with RA samples as tester and OA samples as driver.

References

1. Ozawa K, McElgunn CJ, Yasukochi T and Shibanaka Y: A column gel electrophoresis-coupled genomic DNA subtractive hybridization technique. Electrophoresis 25: 2193-2200, 2004.
2. Yokota H and Oishi M: Differential cloning of genomic DNA: cloning of DNA with an altered primary structure by in-gel competitive reassociation. Proc Natl Acad Sci USA 87: 6398-6402, 1990.
3. Yokota H, Amano S, Yamane T, Ataka K, Kikuya E and Oishi M: A differential cloning procedure of complex genomic DNA fragments. Anal Biochem 219: 131-138, 1994.
4. Kakinuma N, Sato M, Yamada T, Kohu K, Nakajima M, Akiyama T, Ohwada S and Shibanaka Y: Improved in-gel competitive reassociation technique can detect epstein-barr virus genome in gastric cancer. J Electrophoresis 48: 25-30, 2004.
5. Kakinuma N, Kohu K, Sato M, Yamada T, Nakajima M, Akiyama T, Ohwada S and Shibanaka Y: Candidate regions of tumor suppressor locus on chromosome 9q31.1 in gastric cancer. Int J Cancer 109: 71-75, 2004.
6. Arnett FC, Edworthy SM, Bloch DA, *et al*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315-324, 1988.
7. Wakitani S, Murata N, Toda Y, Ogawa R, Kaneshige T, Nishimura Y and Ochi T: The relationship between HLA-DRB1 alleles and disease subsets of rheumatoid arthritis in Japanese. Br J Rheumatol 36: 630-636, 1997.
8. Ohnishi Y, Tsutsumi A, Sakamaki T and Sumida T: T cell epitopes of type II collagen in HLA-DRB1*0101 or DRB1*0405-positive Japanese patients with rheumatoid arthritis. Int J Mol Med 11: 331-335, 2003.
9. Zanders ED, Goulden MG, Kennedy TC and Kempsell KE: Analysis of immune system gene expression in small rheumatoid arthritis biopsies using a combination of subtractive hybridization and high-density cDNA arrays. J Immunol Methods 233: 131-140, 2000.
10. Geetha T and Wooten MW: Structure and functional properties of the ubiquitin binding protein p62. FEBS Lett 512: 19-24, 2002.
11. Choy EH and Panayi GS: Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 344: 907-916, 2001.
12. Duran A, Serrano M, Leitges M, Flores JM, Picard S, Brown JP, Moscat J and Diaz-Meco MT: The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev Cell 6: 303-309, 2004.
13. Justen HP, Grunewald E, Totzke G, Gouni-Berthold I, Sachinidis A, Wessinghage D, Vetter H, Schulze-Osthoff K and Ko Y: Differential gene expression in synovium of rheumatoid arthritis and osteoarthritis. Mol Cell Biol Res Commun 3: 165-172, 2000.
14. Ditzel HJ, Masaki Y, Nielsen H, Farnaes L and Burton DR: Cloning and expression of a novel human antibody-antigen pair associated with Felty's syndrome. Proc Natl Acad Sci USA 97: 9234-9239, 2000.
15. Santilli G, Aronow BJ and Sala A: Essential requirement of apolipoprotein J (clusterin) signaling for IkappaB expression and regulation of NF-kappaB activity. J Biol Chem 278: 38214-38219, 2003.
16. Morceau F, Duvoix A, Delhalle S, Schnekenburger M, Dicato M and Diederich M: Regulation of glutathione S-transferase P1-1 gene expression by NF-kappaB in tumor necrosis factor alpha-treated K562 leukemia cells. Biochem Pharmacol 67: 1227-1238, 2004.
17. URL:http://www.kazusa.or.jp/NEDO

LETTERS

Muscarinic acetylcholine receptor autoantibodies in patients with Sjögren's syndrome

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

Ann Rheum Dis 2005;000:1–2. doi: 10.1136/ard.2004.025478

Sjögren's syndrome (SS) is an autoimmune disease characterised by lymphocytic infiltration into the lachrymal and salivary glands, leading to dry eyes and mouth. Infiltration is also found in the kidneys, lungs, thyroid, and liver. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lachrymal glands, and kidneys are CD4 positive αβ T cells. Previous studies with polymerase chain reaction provide evidence about the T cell receptor Vβ and Vα genes on these T cells, and sequence analysis of the CDR3 region indicates 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 infiltrating the labial salivary glands of patients with SS have been analysed, and Ro/SSA 52 kDa, α-amylase, heat shock protein, and T cell receptor BV6[2] have been identified. However, there is no direct evidence that these reactive T cells really attack and destroy the salivary glands. In contrast, the presence of autoantibodies (Abs) against M3 muscarinic acetylcholine receptor (M3R) has been reported, and it is suggested that an immune reaction to M3R plays a crucial part in the generation of SS.[3–5] Robinson, *et al* demonstrated that human anti-M3R Abs reduce the secretory function in NOD.Igμnull mice.[3] Moreover, Baeman *et al* clearly showed that human Abs against the second extracellular loop of M3R could activate nitric oxide synthase coupled to the lachrymal gland M3R, suggesting that anti-M3R Abs are a new marker of dry eye SS.[4] The M3Rs are expressed on salivary and lachrymal glands, and thus they should be key receptors involved in the production of saliva and tears after stimulation of acetylcholine. Thence, autoantibodies against M3R could interfere with the production of saliva and tears. To test this hypothesis we analysed the prevalence of anti-M3R Abs in patients with SS.

Approval for this study was obtained from the local ethics committee and written informed consent was obtained from all patients and volunteers who participated in this study.

METHODS

Serum samples were collected from 122 Japanese patients with primary SS and 102 Japanese patients with secondary SS followed up at the Department of Internal Medicine, University of Tsukuba Hospital, Japanese Red Cross Mito Hospital, and Shimosizu National Hospital. All patients with SS satisfied the Japanese Ministry of Health criteria for the classification of SS. We also recruited 105 patients with rheumatoid arthritis, 97 with systemic lupus erythematosus, and 128 healthy subjects from our University.

A 25mer peptide (KRTVPPGECFIQFLSEPTITFGTAI) corresponding to the sequence of the second extracellular loop domain of the human M3R was synthesised (Kurabo Industries, Osaka, Japan). As a negative peptide, a 25mer peptide (SGSGSGSGSGSGSGSGSGSGSGSGS) was also synthesised (Kurabo Industries). Peptide solution (100 μl/well at 10 μg/ml) in 0.1 M Na_2CO_3 buffer, pH 9.6, was adsorbed to a Nunc-Immuno plate (Nalge Nunc International, Rochester, NY) at 4°C overnight, and blocked with 5% bovine serum albumin (Wako Pure Chemical Industries, Osaka) in phosphate buffered saline (PBS) for 1 hour at 37°C. Serum at 1:50 dilution in blocking buffer was incubated for 2 hours at 37°C. The plates were then washed three times with 0.05% Tween 20 in PBS, and 1 μl of alkaline phosphatase conjugated goat antihuman IgG (Fc; American Qualex, San Clemente, CA) diluted 1:1000 in PBS was added for 1 hour at room temperature. After extensive washing, 100 μl of *p*-nitrophenyl phosphate (Sigma, St Louis, MO) solution (final concentration 1 mg/ml) was added as alkaline phosphatase substrate. Plates were incubated for 1 hour at room temperature and the optical density at 405 nm was measured by plate spectrophotometry (Bio-Rad Laboratories, Hercules, CA; fig 1). Determinations were performed in triplicate and standardised between experiments.

RESULTS AND DISCUSSION

The 25mer synthetic amino acid encoding the second extracellular domain of M3R was used as the antigen, because this portion has an important role in intracellular signalling.[6] The binding activity of Abs to the second extracellular domain of M3R is dependent on the concentration of Abs using serial-diluted quantitative assay (data not shown). Figure 1 shows that Abs against M3R were more commonly detected in the serum of patients with primary (11/122 (9%), $p<0.05$) and secondary SS (14/102 (14%), $p<0.05$) than in those with other autoimmune diseases such as rheumatoid arthritis (1/105 (1%)) and systemic lupus erythematosus (0/97 (0%)), or healthy subjects (3/128 (2%)). These results clearly showed that autoantibodies against M3R are specifically present in SS, suggesting that anti-M3R Abs could be used as a diagnostic marker in a subgroup of patients with SS (9–14%). The proportions of patients positive for anti-M3R Ab and anti-SSA Ab, anti-SSB Ab, rheumatoid factor, and antinuclear factor were 68%, 29%, 57%, and 83%. In contrast, the proportions of patients negative for anti-M3R Ab with these autoantibodies were 65%, 6%, 59%, and 76%, respectively. Thus, anti-SSB Ab is strongly associated with anti-M3R Ab ($p<0.05$), although the homology between SSB and the M3R molecule is very low and the detailed mechanism remain unclear. The clinical feature is not significantly different between in patients with SS positive for anti-M3R Ab and negative patients.

In conclusion, we detected autoantibodies against M3R in a subgroup of patients with SS, suggesting that anti-M3R Ab could be used as a new diagnostic marker for SS. Further experiments on the functional analysis of anti-M3R Abs in SS using M3R transfectant cell lines should shed light on the relationship between the presence of anti-M3R autoantibodies and the pathogenesis of SS.

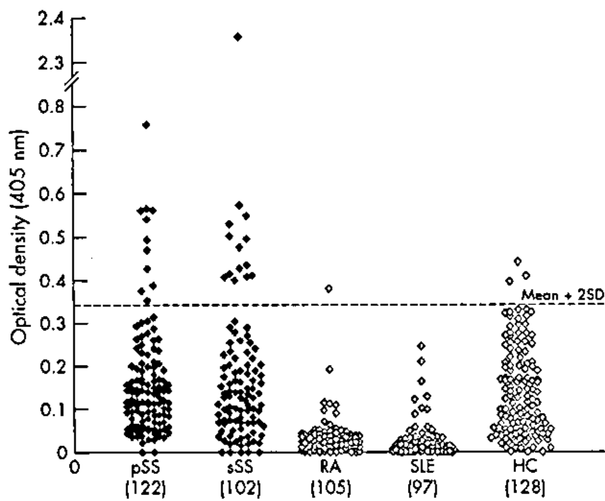

Figure 1 Optical density. pSS, primary Sjögren's syndrome; sSS, secondary Sjögren's syndrome, RA, rheumatoid arthritis, SLE, systemic lupus erythematosus, HC, healthy controls. Numbers in parentheses represent the number of patients in each group.

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REFERENCES

1 **Sumida T**, Matsumoto I, Maeda T, Nishioka K. T-cell receptor in Sjögren's syndrome. *Br J Rheumatol* 1997;36:622–9.
2 **Sumida T.** T cells and autoantigens in Sjögren's syndrome. *Mod Rheumatol* 2000;10:193–8.
3 **Robinson CP**, Brayer J, Yamachika S, Esch TR, Peck AB, Stewart CA, *et al.* Transfer of human serum IgG to nonobese diabetic Igμnull mice reveals a role for autoantibodies in the loss of secretory function of exocrine tissues in Sjögren's syndrome. *Proc Natl Acad Sci USA* 1998;95:7538–43.
4 **Bacman S**, Berra A, Sterin-Borda L, Borda E. Muscarinic acetylcholine receptor antibodies as a new marker of dry eye Sjögren's syndrome. *Invest Ophthalmol Vis Sc* 2001;42:321–7.
5 **Nguyen K**, Brayer J, Cha S, Diggs S, Yasunori U, Hilal G, Peck AB, Humphreys-Beher MG. Evidence for anti-muscarinic acetylcholine receptor antibody-mediated secretory dysfunction in NOD mice. *Arthritis Rheum* 2000;43:2297–306.
6 **Kostenis E**, Zeng FY, Wess J. Structure-function analysis of muscarinic receptors and their associated G proteins. *Life Sci* 1999;64:355–62.

CONCISE REPORT

Association of mannose binding lectin (MBL) gene polymorphism and serum MBL concentration with characteristics and progression of systemic lupus erythematosus

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Objective: To determine whether occurrence, characteristics, and progression of systemic lupus erythematosus (SLE) are associated with polymorphism of the mannose binding lectin (MBL) gene and with serum MBL concentration.

Methods: Codon 54 MBL gene polymorphism of 147 patients with SLE and 160 healthy controls was determined by polymerase chain reaction-restriction fragment length polymorphism. Serum concentration of MBL was measured by enzyme immunoassay. Fluctuations of serum MBL were analysed with respect to disease characteristics and activity.

Results: Frequency of homozygosity for codon 54 minority allele was 6% (9/147) in patients with SLE, and significantly higher than in controls ($p = 0.0294$, Fisher's exact test). MBL polymorphism in patients with SLE was not significantly associated with disease characteristics or immunological phenotypes. Patients homozygous for the B allele tended to have a higher risk of infection during treatment. Levels of C3 and CH_{50} were slightly, but significantly, associated with serum MBL concentration in patients with SLE homozygous for the majority allele. During the course of SLE, serum MBL concentration increased in 6/14 patients, and decreased in 7 after initiation of immunosuppressive treatment.

Conclusions: MBL gene polymorphism influences susceptibility to SLE, but has no direct effect on disease characteristics. Serum MBL levels fluctuate during the course of SLE in individual patients. MBL genotyping may be useful in assessing the risk of infection during treatment of SLE.

Mannose binding lectin (MBL) is a molecule that shares many features with C1q. MBL comprises a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure.[1] MBL mediates lectin dependent activation of the complement pathway,[1] and has an important role in host defence against micro-organisms. People lacking this protein could develop severe episodes of bacterial infections from early life.[2] Several polymorphisms have been reported for the MBL gene, and a large interindividual difference in serum MBL concentration is caused by the possession of variant alleles. Codon 52, 54, and 57 polymorphisms are all on exon 1 of the MBL gene, and the presence of any of the minority alleles significantly reduces serum MBL concentration. Furthermore, homozygosity for minority alleles results in almost complete deficiency of serum MBL.[3] This has been attributed to increased degradation of the mutated protein.[4]

Recently, several studies have suggested that possession of MBL minority alleles may be associated with occurrence of systemic lupus erythematosus (SLE).[5,6] It is known that C1q deficiency is associated with severe symptoms of SLE.[7] Two possible explanations for associations between MBL or C1q deficiency and occurrence of SLE can be proposed: (*a*) MBL and C1q can bind to and initiate uptake of apoptotic cells into macrophages,[8,9] and abnormal clearance of apoptotic cells caused by MBL or C1q deficiency may result in overexpression of autoantigens; (*b*) viral infection is believed to be one of the causes of SLE,[10] and MBL or C1q deficiency may lead to more frequent infections.

This study was conducted on the premise that occurrence, characteristics, and progression of SLE are associated with polymorphism of the MBL gene and with serum MBL concentration. To our knowledge, this is the first study that has measured serum MBL concentration before and after immunosuppressive treatment in patients with newly diagnosed SLE.

PATIENTS AND METHODS

Samples from 147 Japanese patients with SLE followed up at our hospital, were used for the study. All patients fulfilled the 1997 American College of Rheumatology Classification Criteria for SLE. Samples from 160 Japanese healthy volunteers served as controls.

Genomic DNA was purified from peripheral blood leucocytes using the DNAQuick DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan), and stored at −30°C. Typing of the MBL gene allele was performed by polymerase chain reaction-restriction fragment length polymorphism, according to the method of Madsen *et al*.[3] The wild-type allele was designated allele A, and codon 54 substitution (glycine to aspartic acid) was designated allele B. Previous studies have shown that codon 52 and 57 polymorphisms are not present or extremely rare in the Japanese population.[11] Serum concentration of MBL was measured by a specific enzyme immunoassay using two rabbit polyclonal anti-MBL antibodies as described previously.[12]

Table 1 Codon 54 genotypes of the MBL gene in patients with SLE and healthy controls

	SLE	Healthy controls	p Value
AA + AB	138 (AA; 84, AB; 54)	158 (AA; 101, AB; 57)	0.0294
BB	9	2	
Total	147	160	

Allele A, codon 54 wild type majority allele; allele B, codon 54 variant minority allele.
p Value by Fisher's exact test.

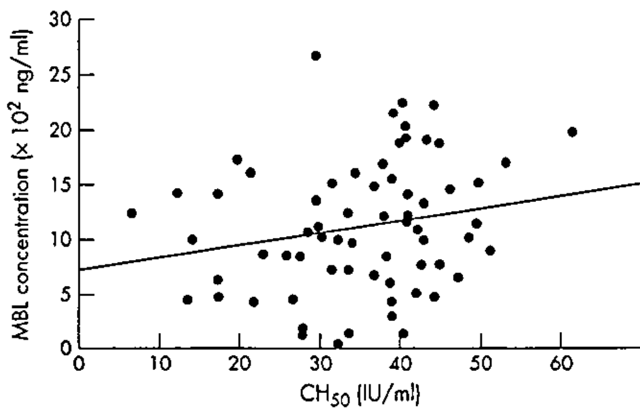

Figure 1 Relationship between serum MBL concentration and CH_{50} in genotype AA patients with SLE. $r_s = 0.253$, $p = 0.0412$ by Spearman's rank correlation test.

Fisher's exact test was used to compare the frequencies of genotypes AA/AB and BB, between disease and control groups, and to compare clinical characteristics between patients with genotypes AA/AB and those with BB. Mann-Whitney's U test was used to compare ages at diagnosis of SLE between patients with genotypes AA/AB and those with BB, and to compare serum MBL concentration between patients and controls of the same genotype. Spearman's rank correlation test was used to compare serum MBL concentration and the levels of anti-DNA antibody, C3, C4, and CH_{50}. Values of $p \leqslant 0.05$ were considered significant.

RESULTS

MBL gene genotypes were studied in patients with SLE and healthy controls (table 1). Among 147 patients with SLE, 9 were homozygous for allele B, which was significantly increased compared with controls ($p = 0.0294$).

We analysed the difference in disease characteristics among patients with SLE categorised by MBL genotypes. Ages (mean (SD)) at diagnosis of SLE tended to be younger in patients with allele B (AA: 32.5 (14.8); AB: 30.7 (15.2); BB: 23.4 (13.3)), but no significant differences were seen ($p = 0.0681$). Clinical characteristics, serological, and immunological measures did not significantly differ between genotype BB patients and other patients with SLE. This is

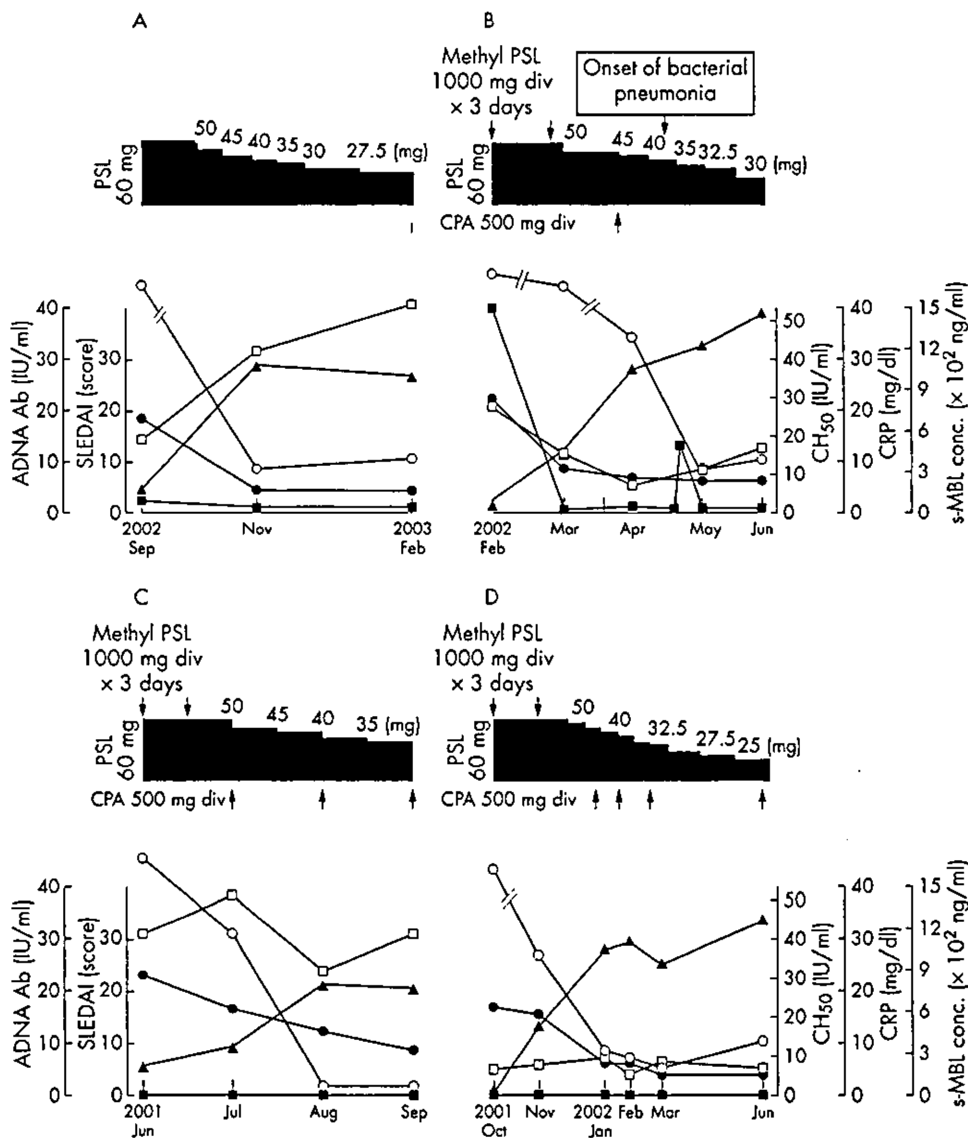

Figure 2 Fluctuation of serum MBL concentration and clinical variables during immunosuppressive treatment in patients with newly diagnosed SLE. Open squares, serum MBL concentrations (s-MBL); closed squares, C reactive protein (CRP); open circles, anti-DNA antibody (ADNA Ab); closed circles, SLE Disease Activity Index (SLEDAI); closed triangles: CH_{50}. PSL, prednisolone; CPA, cyclophosphamide.

most probably because of the small size of the BB cohort. However, incidence of infections requiring admission to hospital was significantly higher in patients with genotype BB than in other patients (genotype AA +AB; 35/132 patients, BB; 5/8 patients, p = 0.0287).

Serum MBL concentration reflected the MBL genotype of the individual subject, in accordance with previous reports[3] (data not shown). Among subjects with the same genotype, patients with SLE tended to have a higher MBL concentration than controls, but without statistical significance. The level of CH_{50} was weakly but significantly associated with serum MBL concentration in patients with SLE with genotype AA (p = 0.0412) (fig 1). In genotype AA patients, C3 was also associated with serum MBL concentration, although C4 was not (C3; p = 0.0494, C4; p = 0.4265). No significant relationship between anti-DNA antibody titre and serum MBL was found. In patients with other genotypes, no significant association was seen between serum MBL concentration and levels of anti-DNA antibody or complement components (data not shown).

We studied fluctuation of serum MBL concentration during immunosuppressive treatment in patients with newly diagnosed SLE (fig 2). In patient 1 with genotype AA (fig 2A), serum MBL increased in parallel with CH_{50} after initiation of methylprednisolone treatment, while the SLE Disease Activity Index (SLEDAI) and anti-DNA antibody decreased. In patient 2 with genotype AA (fig 2B), serum MBL concentration decreased after initiation of methylprednisolone pulse therapy, while CH_{50} increased. After CRP decreased to normal levels, MBL gradually increased in parallel with CH_{50}. In patient 3 with genotype AA (fig 2C), serum MBL did not show a clear trend, although disease activity steadily decreased. In patient 4 with genotype AB (fig 2D), serum MBL was low throughout, reflecting the MBL genotype. When the serum MBL concentration before and after immunosuppressive treatment was compared in 14 newly diagnosed patients, it increased in 6/14 patients (genotype AA: 2, AB: 4), and decreased in 7 patients (genotype AA: 5, AB: 2). There was no significant association between increase or decrease of serum MBL concentration and genotypes and clinical phenotypes in patients with SLE (data not shown).

DISCUSSION

Several studies have indicated that MBL gene polymorphism influences susceptibility to SLE.[5 6] When the components of the classical pathway of complement (C1q, C1r, C1s, C4, or C2) are deficient, it has been suggested that abnormal clearance of not only immune complexes[13] but also apoptotic cells[8] contributes to the occurrence of SLE. It has been indicated that inappropriate levels of apoptotic nuclei may be a major source of autoantigens in SLE.[14] Recently, it was reported that MBL can bind to apoptotic cells and initiate their uptake by macrophages,[9] and thus, abnormal clearance of apoptotic cells due to MBL deficiency may provide a source of autoantigens in SLE. However, deficiency of MBL is not an extremely high risk factor, in contrast with deficiencies of other complement molecules such as C1q.[7] The precise consequences of MBL deficiency for the onset and progression of SLE remain unclear. The lag time between occurrence of the first symptom attributable to SLE and diagnosis of definite SLE was reported to be significantly shorter for variant allele carriers than in those with genotype AA.[6] Therefore, the MBL gene may be a disease modifier locus rather than a true SLE susceptibility locus. Although no significant correlation between disease characteristics and MBL genotypes was seen, genotype BB was significantly associated with occurrence of infection in our patients, in accord with a previous report.[6] MBL genotyping may help in assessment of the risk of opportunistic infections in patients with SLE.

The balance of MBL production and consumption determines serum MBL levels. As the presence of MBL deposits in tissues of autoimmune patients has been demonstrated,[15 16] we expected that MBL would be consumed during active disease, and that serum MBL concentration might reflect disease activity and pathological features of SLE in individual patients. To test this hypothesis, we measured serum MBL concentration during immunosuppressive treatment in patients with newly diagnosed disease. As shown in fig 2, serum MBL concentration did fluctuate during the course of immunosuppressive treatment in patients with SLE, especially in genotype AA patients. In patients 1 and 2, the increasing phase of serum MBL concentration may reflect the decreased consumption of MBL while SLE activity gradually decreased, and the decreasing phase may reflect reduced production of MBL because MBL is an acute phase inflammatory protein.[17] Thus, MBL levels appear to reflect disease activity in some patients. The weak but significant association between serum MBL concentration and serum C3 or CH_{50} levels supports this view.

In conclusion, frequency of homozygosity for a minority allele of the MBL gene was increased in patients with SLE compared with controls, confirming previous studies. MBL gene polymorphism may have no direct effect on disease characteristics, but patients homozygous for the minority allele had significantly more frequent episodes of infections. Serum MBL levels did fluctuate during the course of SLE in individual patients, although the mechanism of their fluctuation and their consequences in SLE are unclear. The value of serum MBL monitoring in clinical practice should be determined in future studies.

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REFERENCES

1 **Holmskov U**, Malhotra R, Sim RB, Jensenius JC. Collectins: collagenous C-type lectins of the innate immune defense system. *Immunol Today* 1994;**15**:67–74.

2 **Summerfield JA**, Ryder S, Sumiya M, Thursz M, Gorchein A, Monteil MA, *et al.* Mannose binding protein gene mutations associated with unusual and severe infections in adults. *Lancet* 1995;**345**:886–9.

3 **Madsen HO**, Garred P, Kurtzhals JA, Lamm LU, Ryder LP, Thiel S, *et al.* A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. *Immunogenetics* 1994;**40**:37–44.

4 **Sumiya M**, Super M, Tabona P, Levinsky RJ, Arai T, Turner MW, *et al.* Molecular basis of opsonic defect in immunodeficient children. *Lancet* 1991;**29**:1569–70.

5 **Davies EJ**, Snowden N, Hillarby MC, Carthy D, Grennan DM, Thomson W, *et al.* Mannose-binding protein gene polymorphism in systemic lupus erythematosus. *Arthritis Rheum* 1995;**38**:110–14.

6 **Garred P**, Voss A, Madsen HO, Junker P. Association of mannose-binding lectin gene variation with disease severity and infections in a population-based cohort of systemic lupus erythematosus patients. *Genes Immun* 2001;**2**:442–50.

7 **Walport MJ**, Davies KA, Botto M. C1q and systemic lupus erythematosus. *Immunobiology* 1998;**199**:265–85.

8 **Korb LC**, Ahearn JM. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J Immunol* 1997;**158**:4525–8.

9 **Ogden CA**, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, *et al.* C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 2001;**194**:781–95.