発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Rosen, D.B., M. Araki, J.A. Hamerman, T. Chen, T. Yamamura and L.L. Lanier	A structural basis for the association of DAP12 with mouse, but not human, NKG2D.	J. Immunol.	173	2470-2478	2004
Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura	The regulatory role of natural killer cells in multiple sclerosis.	Brain	127	1917-1927	2004
Araki, M., T. Kondo, J.E. Gumperz, M.B. Brenner, S. Miyake and T. Yamamura	Th2 bias of $CD4^+$ NKT cells derived form multiple sclerosis in remission.	Int. Immunol.	15	279-288	2003
Bedoui, S., S. Miyake, Y. Lin, K. Miyamoto, S. Oki, N. Kawamura, A. Beck-Sickinger, S. von Hoersten, and T. Yamamura	Neuropeptide Y (NPY) suppresses experimental autoimmune encephalomyelitis: NPY Y_1 receptor-specific inhibition of autoreactive Th1 responses in vivo.	J. Immunol.	171	3451-3458	2003
Stanic, A.K., R. Shashidharamurthy, J.S. Bezradica, N. Matsuki, Y. Yoshimura, S. Miyake, E.Y. Choi, T.D. Schell, L. Van Kaer, S.S. Tevethia, D.C. Roopenian, T. Yamamura and S. Joyce	Another view of T cell antigen recoginition: Co-operative engagement of glycolipid antigens by Va14Ja18 natural TCR.	J. Immunol.	171	4539-4551	2003
Miyachi K, Hirano Y, Horigome T, Mimori T, Miyakawa H, Onozuka Y, Shibata M, Hirakata M, Suwa A, Hosaka H, Matsushima S, Komatsu T, Matsushima H, Hankins RW and Fritzler MJ.	Autoantibodies from primary biliary cirrhosis patients with anti-p95c antibodies bind to recombinant p97/VCP and inhibit in vitro nuclear envelope assembly.	Clin Exp Immunol.	136 (3)	568-573	2004
Furuya T, Hakoda M, Tsuchiya N, Kotake S, Ichikawa N, Nanke Y, Nakajima A, Takeuchi M, Nishinarita M, Kondo H, Kawasaki A, Kobayashi S, Mimori T, Tokunaga K and Kamatani N.	Immunogenetic features in 120 Japanese patients with idiopathic inflammatory myopathy.	J Rheumatol.	31(9)	1768-74	2004
Kawabata D, Tanaka M, Fujii T, Umehara H, Fujita Y, Yoshifuji H, Ozaki S and Mimori T.	Ameliorative effects of follistatin-related protein/TSC-36/FSTL1 on joint inflammation in a mouse model of arthritis.	Arthritis Rheum.	50(2)	660-668	2004
Sato S, Ohosone Y, Suwa A, Yasuoka H, Nojima T, Fujii T, Kuwana M, Nakamura K, Mimori T, Hirakata M.	Effect of Intermittent Cyclical Etidronate Therapy on Corticosteroid Induced Osteoporosis in Japanese Patients with Connective Tissue Disease: 3 Year Followup.	J Rheumatol.	30	2673-2679	2003
Fujii T, Mimori T, Kimura N, Satoh S, Hirakata M.	Pseudoscleroderma associated with transforming growth factor b1-producing advanced gastric carcinoma: Comment on the article by Varga.	Arthritis Rheum.	48	1766-1767	2003

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoneda O, Imai T, Nishimura M, Miyaji M, Mimori T, Ozaki T, Domae N, Fujimoto H, Minami Y, Kono T, Bloom E.T, Umehara H.	Membrane-bound form of fractalkine induces IFN-g production by NK cells.	Eur J Immunol.	33	53-58	2003
Tanaka M, Ozaki S, Kawabata D, Kishimura M, Osakada F, Okubo M, Murakami M, Nakao K, Mimori T.	Potential preventive effects of follistatin-related protein/TSC-36 on joint destruction and antagonistic modulation of its autoantibodies in rheumatoid arthritis.	Int. Immunol.	15	71-77	2003
Fujita Y, Murakami M, Ogawa Y, Masuzaki H, Tanaka M, Ozaki S, Nakao K, Mimori T.	Leptin inhibits stress-induced apoptosis of T lymphocytes.	ClinExp Immunol.	128 (1)	21-26	2002
Kobayashi H, Hosono O, Mimori T, Kawasaki H, Dang N.H., Tanaka H, Morimoto C.	Reduction of Serum Soluble CD26/Dipeptidyl Peptidase IV Enzyme Activity and Its Correlation with Disease Activity in Systemic Lupus Erythematosus.	J. Rheumatol.	29 (9)	1858-1866	2002
Inoue H, Miyaji M, Kosugi A, Nagafuku M, Okazaki T, Mimori T, Amakawa R, Fukuhara S, Domae N, Bloom E.T, Umehara H.	Lipid rafts as the signaling scaffold for NK cell activation: tyrosine phosporylation and association of LAT with phosphatidylinositol 3-kinase and phospholipase C-g following CD2 stimulation.	Eur J Immunol.	32	2188-2198	2002
Fujii T, Okada M, Mimori T, Craft J.	The transmembrane form of TNFa drives auoantibody production In the absence of CD154: Studies using MRL/Mp-Faslpr mice.	Clin Exp Immunol.	130	224-232	2002
Naito Y, Matsumoto I, Wakamatsu E, Goto D, Sugiyama T, Matsumura R, Ito S,Tsutsumi A, Sumida T.	Muscariinic acetylcholine recepter auto antibodies in patients with Sjogren's Syndrome.	Ann. Rheum. Dis.	64	510-511	2005
Takahashi R, Tsutsumi A, Ohtani K, Muraki Y, Goto D, Matsumoto I, Wakamiya N, and Sumida T.	Association of mannose-binding lection(MBL) gene polymorphism and serum MBL concentration with characteristics and progression of systemic lupus erythematosus.	Ann. Rheum. Dis	64	311-314	2005
Tomoo T, Tsutsumi A, Yasukochi T, Ikeda T, Ochiai N, Ozawa K, Shibanaka Y, Ito S, Matsumoto I, Goto D, Sumida T.	Analysis of abnormally expressed genes in synovium from patients with rheumatoid arthritis using a column gel electrophoresis-coupled subtractive hybridization technique.	Int. J. Mol. Med.	15	453-457	2005

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Murata H, Adachi Y, Ebitsuka T, Chino Y, Takahashi R, Hayashi T, Goto D, Matsumoto I, Tsutsumi A, Akaza H, Sumida T.	Reiter's syndrome following intravesical Bacille bilie de Calmette-Guerin treatment for superficial bladder carcinoma. Report of six cases	Mod. Rheumatol.	14	82-86	2004
Muraki Y, Tsutsumi A, Takahashi R, Suzuki E, Hayashi T, Chino Y, Goto D, Matsumoto I, Murata H, Sumida T.	Polymorphisms of IL-1b gene in Japanese patients with Sjogren's syndrome and systemic lupus erythematosus.	J. Rheumatol.	31	720-725	2004
Takahashi R, Tsutsumi A, Ohtani K, Goto D, Matsumoto I, Ito S, Wakamiya N, and Sumida T.	Anti-mannose binding lection antibodies in sera of Japanese patients with systemic lupus erythematosus.	Clin.Exp.Immunol.	136	585-590	2004
Tsutsumi A, Suzuki E, Adachi Y, Murata H, Goto D, Kojo S, Matsumoto I, Zhong L, Nakamura H, and Sumida T.	Expression of tristetraprolin (GOS24) mRNA, a regulator of tumor necrosis factor-a production, in synovial tissues of patients with rheumatoid arthritis.	J. Rheumatol.	31	1044-1049	2004
Muraki Y, Matsumoto I, Chino Y, Hayashi T, Suzuki E, Goto D, Ito S, Murata H, Tsutsumi A, Sumida T.	Glucose-6-phosphate isomerase variants play a key role in the generation of anti-GPI antibodies.Possible mechanism of autoantibody production.	Biochem. Biophys. Research. Commun.	323	518-522	2004
Matsumoto I, Lee DM, Mansky RG, Sumida T, Hitchon CA, Schur PH, Anderson RJ, Coblyn JS, Weinblatt ME, Brenner M, Duclos B, Pasquali JL, Gabalawy HE, Mathis D, Benoist C.	Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and spectrum of other chronic autoimmune disorders.	Arthritis Rheum.	48(4)	944-954	2003
Tsutsumi A, Ikegami H, Takahashi R, Murata H, Goto D, Matsumoto I, Fujisawa T, Sumida T.	Mannose binding lectin gene polymorphism in patients with type I diabetes.	Human Immunol.	64	621-624	2003
Matsumoto I , Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, Mathis D, Benoist C	How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease.	Nature Immunol.	3	360-365	2002
Matsumoto I, Sumida T.	B cells and immunoglobulins dependent mechanisms in rheumatoid arthritis.	Ther. Aperesis	6	317-319	2002
Yasuda, S., Atsumi, T., Ieko, M., Matsuura, E., Kobayashi, K., Inagaki, J., Kato, H., Tanaka, H., Yamakado, M., Akino, M., Saitou, H., Amasaki, Y., Jodo, S., Amengual, O., Koike,T.	Nicked β2-glycoprotein I: a marker of cerebral infarct and a novel role in the negative feedback pathway of extrinsic fibrinolysis.	Blood.	103 (10)	3766-3772	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Bohgaki, M., Atsumi, T., Yamashita, Y., Yasuda, S., Sakai, Y., Furusaki, A., Bohgaki, T., Amengual, O., Amasaki, Y., Koike, T.	The p38 mitogen-activated protein kinase(MAPK)pathway mediates induction of the tissue factor gene in monocytes stimulated with human monoclonal anti-β2Glycoprotein I antibodies.	Int Immunol	16 (11)	1633-1641	2004
Yasuda, S., Atsumi, T., Matsuura, E., Kaihara, K., Yamamoto, D., Ichikawa, K., Koike, T.	Significance of valine/leucine247 polymorphism of β2-glycoprotein I in antiphospholipid syndrome: increased reactivity of anti-β2-glycoprotein I autoantibodies to the valine247β2-glycoprotein I variant.	Arthritis Rheum	52(1)	212-218	2004
Amengual, O., Atsumi, T., Koike, T.	Specificities properties and clinical significance of antiprothronbin antibodies.	Arthrit Rheum.	48(4)	886-895	2003
Sakai, Y., Atsumi, T., Itoh, T., Koike, T.	Uveitis,pancardotos,haemophagocytosis,and abdominal masses.	Lancet	361	834	2003
Yasuda, S., Tsustumi, A., Astumi, T., Bertolaccini, M. L., Ichikawa, K., Khamashta, M. A., HugesR. V., Koike, T.	Gene polymorphisms of tissue plasminogen activator and plasminogenactivator inhibitor-1patients with antiphospholipid antibodies.	J.Rheumatol.	29(6)	1192-1197	2002
Amborozic, A., Avicin, T., Ichikawa, K., Kveder, T., Mastuur, E., Hojnik, M., Astumi, T., Rozman, B., Koike, T.	Anti-b2-glycoprotein I antibodies in children with atopic dermatitis.	Int.Immunol.	14(7)	823-830	2002
Takeuchi, R., Atsumi, T., Ieko, M., Amasaki, Y., Ichikawa, K., Koike, T.	Supporsed intrinsic fibrinolytic activity by monoclonal anti-beta2 glycoprotein I autantibodies:possible mechanism for thrombosis in patients with antiphospholipid syndrome.	Br. J. Hematol.	119	781-788	2002

IV　研究成果刊行物・別刷

LETTERS

Muscarinic acetylcholine receptor autoantibodies in patients with Sjögren's syndrome

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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, Bacman *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 (SGSGSGSGSGSGSGSGSGSGSGS) 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.

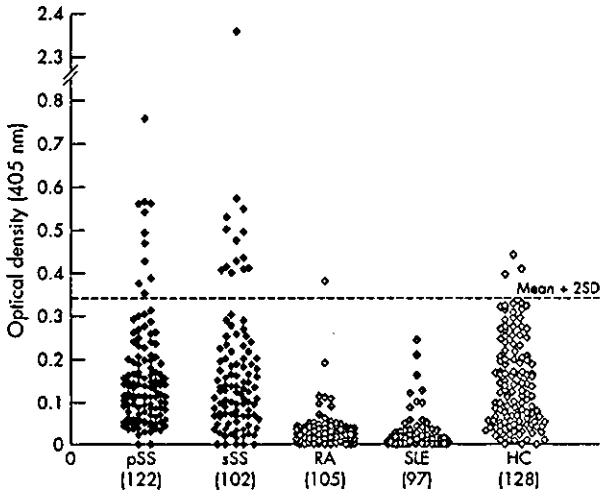

Figure 1 Optical density. pSS, primary Sjögren's syndrome; sSS, secondary Sjögren's syndrome, RA, rheumatoid arthritis, SLE, systemic lupus erythematosus, HC, healthy controls. Numbers in parentheses represent the number of patients in each group.

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Accepted 17 July 2004

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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Ann Rheum Dis 2005;64:311–314. doi: 10.1136/ard.2003.020172

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.

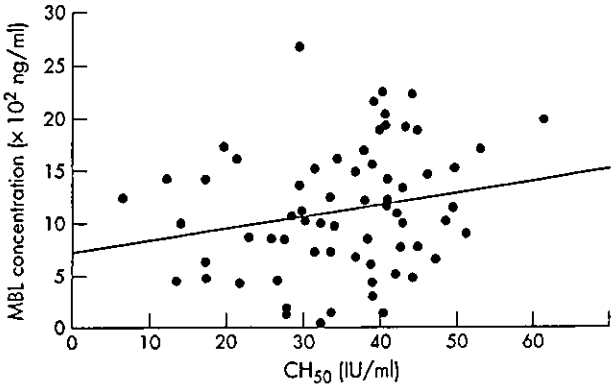

Figure 1 Relationship between serum MBL concentration and CH_{50} in genotype AA patients with SLE. $r_s = 0.253$, $p = 0.0412$ by Spearman's rank correlation test.

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

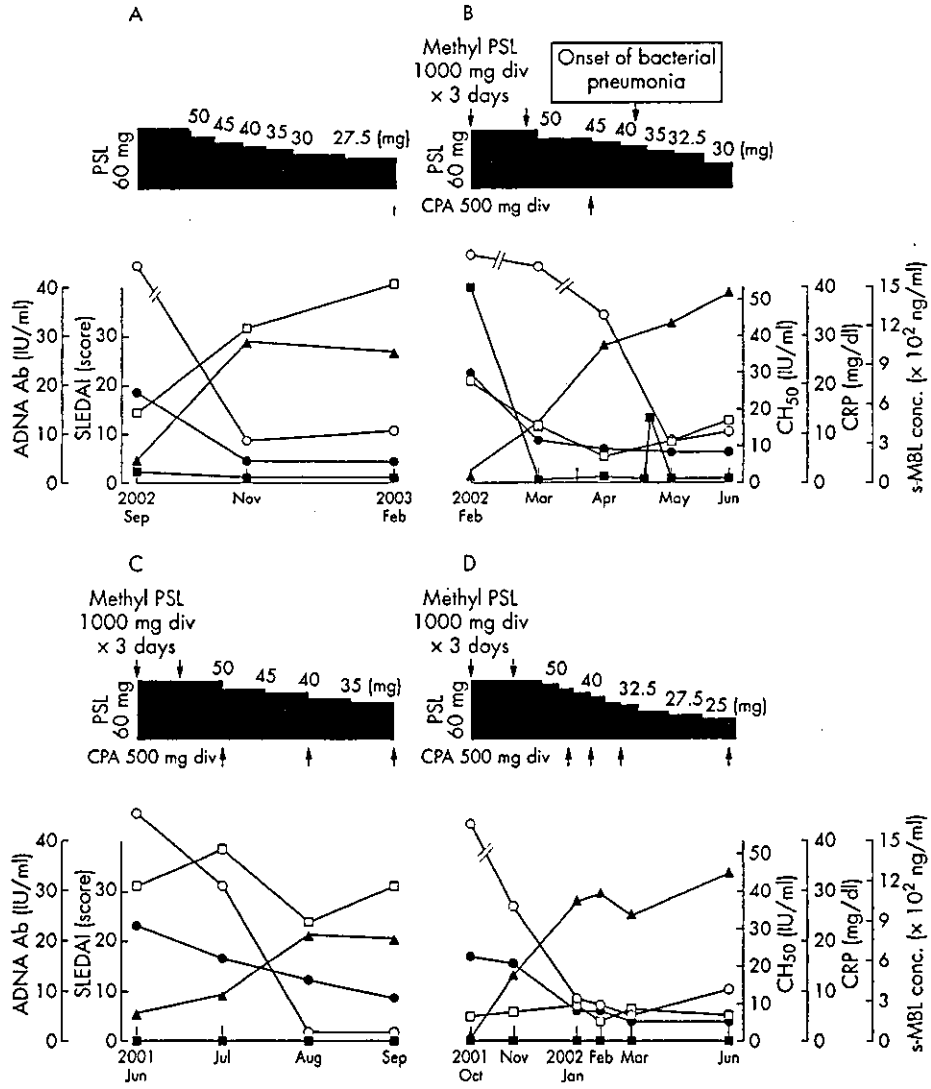

Figure 2 Fluctuation of serum MBL concentration and clinical variables during immunosuppressive treatment in patients with newly diagnosed SLE. Open squares, serum MBL concentrations (s-MBL); closed squares, C reactive protein (CRP); open circles, anti-DNA antibody (ADNA Ab); closed circles, SLE Disease 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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Accepted 9 May 2004

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.

10 **Okada M**, Ogasawara H, Kaneko H, Hishikawa T, Sekigawa I, Hashimoto H, *et al*. Role of DNA methylation in transcription of human endogenous retrovirus in the pathogenesis of systemic lupus erythematosus. *J Rheumatol* 2002;29:1678–82.
11 **Sasaki K**, Tsutsumi A, Wakamiya N, Ohtani K, Suzuki Y, Watanabe Y, *et al*. Mannose-binding lectin polymorphisms in patients with hepatitis C virus infection. *Scand J Gastroenterol* 2000;35:960–5.
12 **Ohtani K**, Suzuki Y, Eda S, Kawai T, Kase T, Keshi H, *et al*. High-level and effective production of human mannan-binding lectin (MBL) in Chinese hamster ovary (CHO) cells. *J Immunol Methods* 1999;222:135–44.
13 **Atkinson JP**. Complement deficiency: predisposing factor to autoimmune syndromes. *Clin Exp Rheumatol* 1989;7:S95–101.
14 **Mevorach D**, Zhou JL, Song X, Elkon KB. Systemic exposure to irradiated apoptotic cells induces autoantibody production. *J Exp Med* 1998;188:387–92.
15 **Hisano S**, Matsushita M, Fujita T, Endo Y, Takebayashi S. Mesangial IgA2 deposits and lectin pathway-mediated complement activation in IgA glomerulonephritis. *Am J Kidney Dis* 2001;38:1082–8.
16 **Steinfeld S**, Penaloza A, Ribai P, Decoestecker C, Danguy A, Gabius HJ, *et al*. D-mannose and N-acetylglucosamine moieties and their respective binding sites in salivary glands of Sjögren's syndrome. *J Rheumatol* 1999;26:833–41.
17 **Thiel S**, Holmskov U, Hviid L, Laursen SB, Jensenius JC. The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. *Clin Exp Immunol* 1992;90:31–5.

Clin Exp Immunol 2004; 136:585–590 doi:10.1111/j.1365-2249.2004.02477.x

Anti-mannose binding lectin antibodies in sera of Japanese patients with systemic lupus erythematosus

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(Accepted for publication 16 March 2004)

SUMMARY

Mannose-binding lectin (MBL) is a key element in innate immunity with functions and structure similar to that of complement C1q. It has been reported that MBL deficiency is associated with occurrence of systemic lupus erythematosus (SLE). We hypothesized that anti-MBL antibodies, if present, would affect the occurrence or disease course of SLE, by reduction of serum MBL levels, interference of MBL functions, or binding to MBL deposited on various tissues. To address this hypothesis, we measured the concentration of anti-MBL antibodies in sera of 111 Japanese SLE patients and 113 healthy volunteers by enzyme immunoassay. The titres of anti-MBL antibodies in SLE patients were significantly higher than those in healthy controls. When the mean + 2 standard deviations of controls was set as the cut off point, individuals with titres of anti-MBL antibodies above this level were significantly more frequent in SLE patients (9 patients) than in controls (2 persons). One SLE patient had an extremely high titre of this antibody. No associations of titres of anti-MBL antibodies and (i) genotypes of MBL gene, (ii) concentrations of serum MBL, or (iii) disease characteristics of SLE, were apparent. Thus, we have confirmed that anti-MBL antibodies are indeed present in sera of some patients with SLE, but the significance of these autoantibodies in the pathogenesis of SLE remains unclear.

Keywords Lupus/ systemic lupus erythematosus autoantibodies MBL C1q polymorphisms

INTRODUCTION

Both genetic and environmental factors are important in the development of systemic lupus erythematosus (SLE), a systemic autoimmune disease of unknown origin [1,2]. With respect to genetic background, deficiencies in components of the classical pathway of complements (C1q, C1r, C1s, C4 or C2) are known to be major predisposing risk factors for SLE [3–6]. In complement deficiencies, an abnormal clearance of not only immune complexes [3], but also apoptotic cells, has been suggested as contributive towards the occurrence of SLE [7]. Inappropriate levels of apoptotic nuclei are suggested to be a source of autoantigens in SLE [8].

Mannose-binding lectin (MBL) comprises a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q [9]. The MBL gene is located on the long arm of chromosome 10 at 10q11.2-q21 and contains 4 exons [10]. Several polymorphisms have been reported for the MBL gene, and a large interindividual difference in serum MBL concentration among test subjects is caused by the possession of variant alleles. Codon 52, 54 and 57 polymorphisms are all on exon 1, and the presence of any of the minority alleles results in a significant reduction of the serum MBL concentration. Furthermore, homozygosity for minority alleles results in almost complete deficiency of serum MBL [11,12]. This has been attributed to increased degradation of the mutated protein [12]. In the promoter region of the MBL gene, polymorphisms are reported at positions −550, −221 and + 4, and they also greatly influence the levels of serum MBL [13,14]. MBL mediates lectin-dependent activation of the complement pathway [9], and plays an important role in host defense against microorganisms by phagocytosis. Individuals lacking this protein could develop severe episodes of bacterial infections from early life [15–17].

Recently, several studies have suggested that MBL deficiency, or low serum MBL levels caused by polymorphisms in the structural portion or promoter region of the MBL gene, may be associated with occurrence of SLE [18–22]. Two possible explanations for the associations between MBL deficiency and the occurrence of SLE are suggested. Firstly, MBL can bind to and initiate uptake of apoptotic cells by macrophages [23], and an abnormal

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clearance of apoptotic cells caused by MBL deficiency may result in the overexpression of autoantigens. Alternatively, viral infection is believed to be one of causes of SLE [24–26], and MBL deficiency may lead to more frequent infections. On the other hand, deposits of MBL were found in glomerular tissues of SLE patients [27,28], and D-mannose and N-acetylglycosamine, both possible ligands for MBL, are present in the salivary glands of patients with Sjögren's syndrome [29]. In this situation, MBL may have a pathogenic role during the course of SLE.

It has been reported that autoantibodies to C1q are associated with hypocomplementemia and glomerulonephritis [30]. If autoantibodies to MBL, a molecule similar to C1q in structure and functions, are present in patients with SLE, they may: reduce MBL levels; interfere with MBL functions; or bind to MBL deposited to various diseases. We investigated whether anti-MBL antibodies are indeed present in sera of Japanese patients with SLE.

PATIENTS AND METHODS

Patients and controls

Samples used for the study were taken from 111 Japanese patients with SLE, at Division of Rheumatology, Department of Internal Medicine, University Hospital of Tsukuba, Japan. All patients fulfilled the 1997 American College of Rheumatology (ACR) Classification Criteria for SLE. Patients with drug-induced lupus were excluded. The study was approved by the local ethics committee, and written informed consent was obtained from all participants of this study. Medical information including clinical manifestations, and laboratory data were collected simultaneously with sampling. Samples from 113 Japanese healthy volunteers served as controls.

Detection of immunoglobulin G (IgG) binding to MBL

Sumilon S plates (Sumitomo Bakelite, Tokyo Japan) were coated overnight at 4°C with 100 μl/well of recombinant MBL [31] in a carbonate/bicarbonate-buffer (pH 9·6) at a concentration of 1 μg/ml. The plates were washed three times with tris-buffered saline (TBS, pH 7·4) containing 0·05% Tween-20 (TBS/Tw). Unoccupied binding sites were blocked by incubation with 1% bovine serum albumin (BSA) in TBS for 1 h at 37°C. One hundred μl/well of serum samples diluted to 1 : 50 in TBS/Tw containing 0·3% BSA and 1 mM EDTA were added to the wells, and the plates were incubated overnight at 4°C. EDTA was included to inhibit the Ca^{2+} dependent binding of MBL to carbohydrates present on the Fc portion of IgG. All samples were analysed in triplicates. After incubation, 100 μl/well alkaline phosphate (AP)-conjugated goat antihuman IgG, specific for Fab fragment (Sigma, St Louis, MO, USA) diluted 1 : 5000 in TBS/Tw, was added to each well. The microtiter plates were incubated for 1 h at room temperature. Subsequently, alkaline phosphate substrate (Sigma) was added to each well. The plates were incubated for 2 h at room temperature. Optical densities (OD) were measured at 405 nm. The concentration of IgG reactive with MBL is expressed in units/ml of serum (U/ml), where the concentration in a standard sample was defined as 1000 U/ml. Standard curves were generated in all assays performed.

Inhibition assays

Anti-MBL positive sera diluted to 1 : 50 were preincubated with TBS or recombinant MBL at concentrations from 0·1563 μg/ml to 10 μg/ml at room temperature for 1 h. The samples were then put onto MBL-coated plates, and IgG binding to MBL was measured as described above.

Typing of the MBL gene

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 using the polymerase chain reaction- restriction fragment length polymorphism method according to the methods of Madsen *et al.* [11]. The wild-type allele was designated as allele A, and codon 54 substitution (glycine to aspartic acid) was designated as allele B. Previous studies have shown that codon 52 and 57 polymorphisms are not present or extremely rare in the Japanese population [32,33].

Measurement of the serum MBL concentration by enzyme immunoassay

Serum concentration of MBL was measured by a specific enzyme immunoassay utilizing two rabbit polyclonal anti-MBL antibodies as described previously [31]. All samples were stored at −80°C and no previous freeze/thaw was done.

Statistics

Mann–Whitney *U*-test, Fisher's exact test, chi-square analysis and Spearman's rank correlation test were used. *P*-values of <0.05 were considered to be statistically significant.

RESULTS

Detection of autoantibodies to MBL in patients with SLE

Titers of IgG reactive with human MBL in patients with SLE were significantly higher than those in healthy controls: $P < 0.0001$, median MBL concentration ± standard deviation (s.d.); 47·4 ± 49·3 and 30·6 ± 29·2, in SLE patients and healthy controls, respectively (Fig. 1). The assay was performed in the presence of EDTA in order to inhibit the binding between the carbohydrate recognition domain of MBL and carbohydrates on the Fc portion of IgG. Furthermore, selected samples were digested with pepsin and $F(ab')_2$ fragments were purified. $F(ab')_2$ fragments did bind to MBL coated plates, indicating that IgG–MBL interaction detected in this assay is indeed antigen-antibody binding (results not shown). We found a patient with an extremely high level of serum anti-MBL, and the titre of anti-MBL antibodies in the serum of this patient was designated 1000 U/ml. The number of subjects having a titre of more than 2 sd. above the average of healthy controls (89·5, indicated by dotted line in Fig. 1) was 9 of the patients with SLE, and 2 of the healthy controls. This difference was statistically significant ($P = 0.0341$ by Fisher's exact test).

A titration curve could be adequately drawn using serial dilutions of the standard serum (Fig. 2a). In addition, adding excess amounts of recombinant MBL to diluted standard serum inhibited the binding of IgG to solid phase MBL in a dose dependent manner (Fig. 2b).

Associations between levels of anti-MBL antibodies, and MBL gene genotypes or serum concentrations of MBL in patients with SLE

Serum MBL concentrations reflected the MBL genotype of the individual in accordance with previous reports (Fig. 3) [11,12].

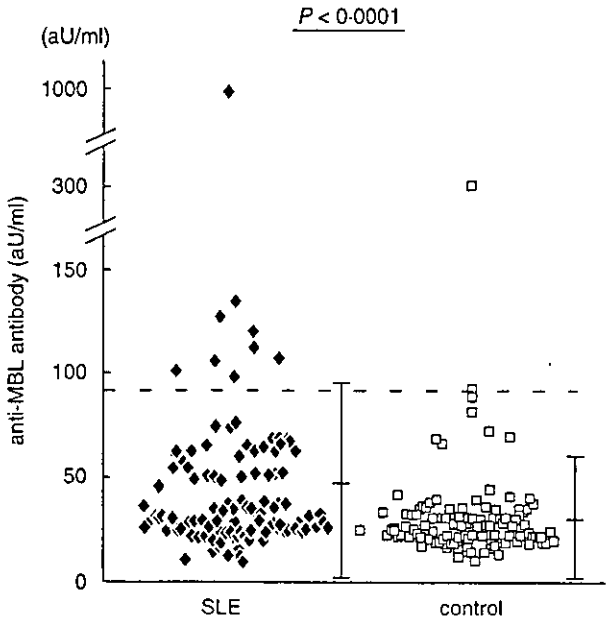

Fig. 1. Autoantibodies to mannose-binding lectin (MBL) in serum samples. Anti-MBL antibodies were measured in 111 samples from patients with systemic lupus erythematosus (SLE) and in 113 samples from healthy controls. in the presence of EDTA (1 mM). Dotted line indicates 2 standard deviation (s.d.) above average in healthy controls. *P*-value by Mann–Whitney *U*-test. aU, arbitrary units.

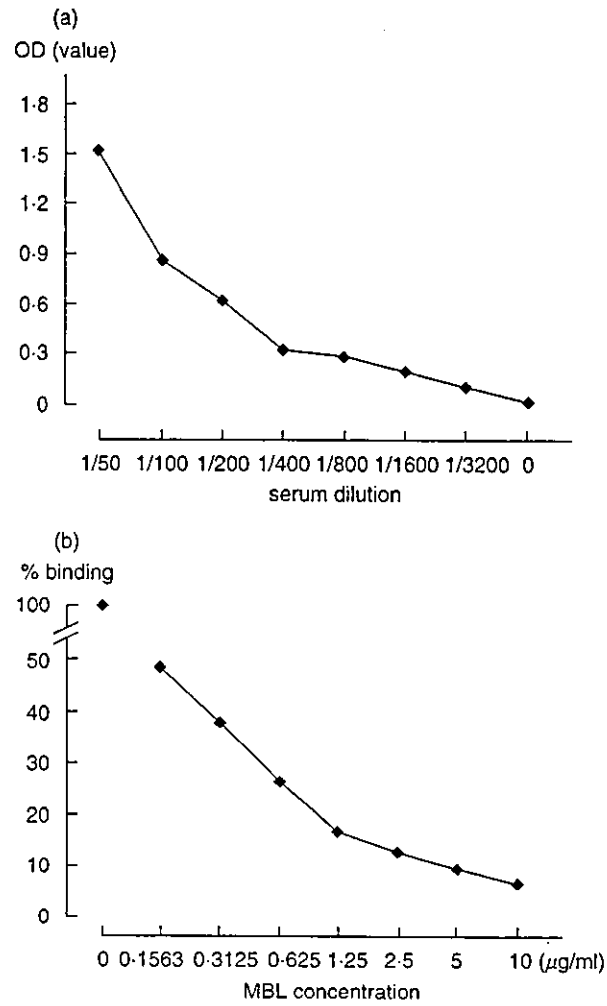

Fig. 2. Titration curve and inhibition assay for autoantibodies to mannose-binding lectin (MBL). (a) Titration curve for anti-MBL antibodies using serial dilutions of the standard serum in the presence of EDTA (1 mM). (b) Inhibition assay for anti MBL antibodies adding excess amount of recombinant MBL to diluted standard serum in the presence of EDTA (1 mM).

Serum MBL concentrations in SLE patients were not significantly different from those in healthy individuals ($P = 0{\cdot}5296$). Among individuals with the same genotype, SLE patients tended to have higher MBL concentrations than controls, but without statistical significance (AA; $P = 0{\cdot}3385$, AB; $P = 0{\cdot}5556$, BB; $P = 0{\cdot}1573$ by Mann–Whitney's *U*-test).

We next examined whether genotypes of the MBL gene in patients with SLE are associated with levels of anti-MBL antibodies (Fig. 4). Titres of anti-MBL antibodies tended to be lower in patients with allele B (AA; $60{\cdot}15 \pm 133{\cdot}3$, AB; $50{\cdot}10 \pm 26{\cdot}95$, BB; $38{\cdot}23 \pm 18{\cdot}88$), but no significant differences were observed.

Finally, we compared the serum concentrations of MBL and titres of anti-MBL antibodies in patients with SLE. We found no significant relationship between them (Fig. 5).

Relationships between the presence of anti-MBL antibodies in sera, and clinical characteristics or disease parameters of SLE

We investigated whether patients having anti-MBL antibodies at titres above 2 sd. of the average in healthy controls had some significant clinical characteristics (Table 1). No significant associations were observed. However, patients with higher serum concentration of anti-MBL antibodies tended to have a lower occurrence of anti-DNA antibodies, although statistical significance was not achieved. The incidence of infections requiring hospitalization during their course of SLE was not significantly higher in patients with higher serum concentration of anti-MBL antibodies.

We next analysed whether or not titres of anti-MBL antibodies are associated with various disease parameters of SLE in 111 SLE patients. Anti-DNA antibodies and total IgG tended to be positively related with anti-MBL antibodies, but statistical significance was not achieved. No other correlation was observed (Table 2).

DISCUSSION

In this study, we found the presence of autoantibodies against MBL in some patients with SLE. This is in accordance with the study by Seelen *et al.* [34], which was published very recently.

We confirmed that we were indeed detecting anti-MBL antibodies by; addition of EDTA in the enzyme immunoassay, thereby inhibiting the Ca^{2+} dependent binding of carbohydrate recognition domain on MBL to carbohydrates on IgG; digesting IgG with pepsin, and confirming that the binding region of IgG was on $F(ab')_2$; and detecting an inhibition of aqueous MBL to the binding of IgG to solid phase MBL. These methods and results are similar to those reported by Seelen *et al.* [34], except that we did detect dose dependent inhibition by our inhibition assay. The reason for this discrepancy is unclear, but may possibly be due to

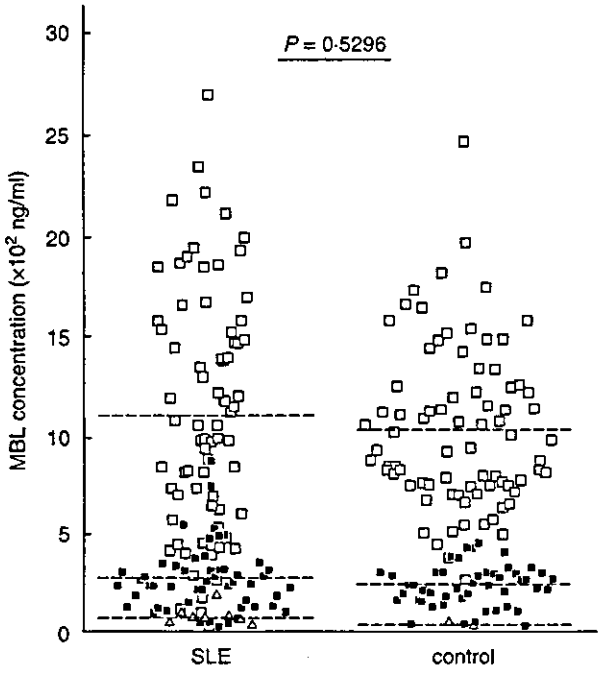

Fig. 3. Serum mannose-binding lectin (MBL) concentrations in 111 patients with systemic lupus erythematosus (SLE) and 113 healthy controls. Subjects with homozygosity for the codon 54 wild-type allele (□), subjects with heterozygosity for the codon 54 variant (■), and subjects with homozygosity for the codon 54 variant allele (Δ) are indicated in both patients with SLE and healthy controls. Dotted lines indicate average of titres of serum MBL concentrations in each genotype on both groups. *P*-value by Mann–Whitney *U*-test.

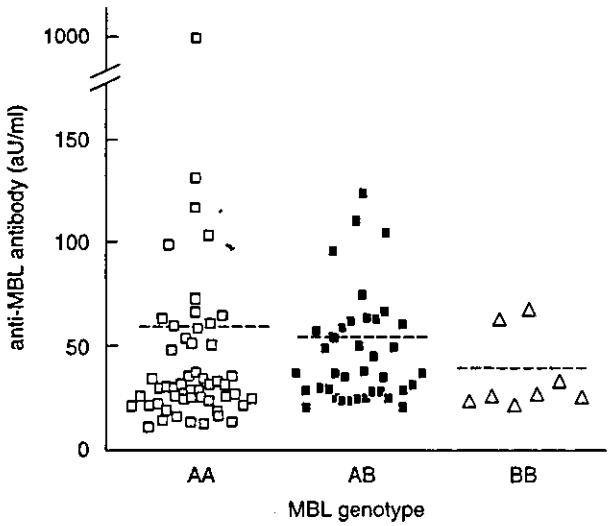

Fig. 4. Association between genotypes of the mannose-binding lectin (MBL) gene and levels of anti-MBL antibodies in patients with systemic lupus erythematosus (SLE). AA; homozygosity for the codon 54 wild-type allele, AB; heterozygosity for the codon 54 variant, BB; homozygosity for the codon 54 variant allele. Dotted lines indicate average of titres of anti-MBL antibodies in each genotype. aU, arbitrary units.

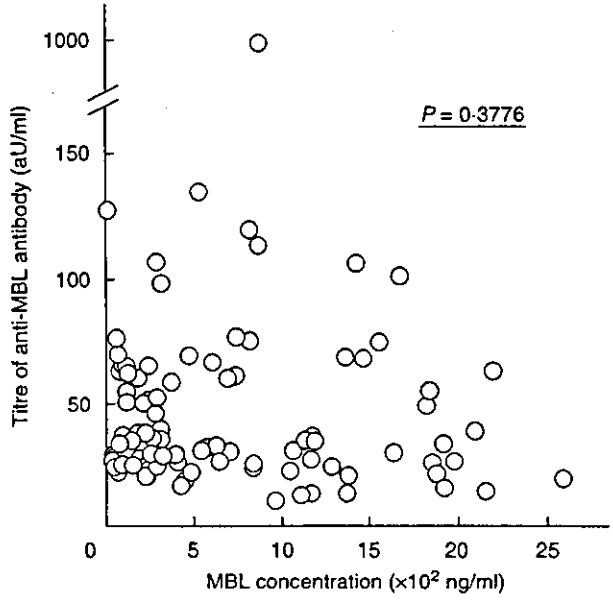

Fig. 5. Association between titres of anti mannose-binding lectin (MBL) antibodies and concentrations of MBL in systemic lupus erythematosus (SLE) patients. *P*-value by Spearman's rank correlation test. aU, arbitrary units.

Table 1. Disease characteristics of 111 patients with systemic lupus erythematosus (SLE) categorized by positivity of anti-mannose-binding lectin (MBL) antibody

	Positive (n = 9)	Negative (n = 102)	P-value
Malar rash	3	44	0·7309
Discoid lupus	0	13	0·5951
Photosensitivity	1	22	0·6821
Oral ulcers	2	20	0·9999
Arthritis	5	59	0·9999
Serositis	4	22	0·2099
Renal disorder	1	29	0·4399
Neurological disorder	0	9	0·9999
Haematologic disorder			
Haemolytic anemia	0	8	0·9999
leukopenia	4	52	0·7422
lymphopenia	4	48	0·9999
thrombocytopenia	1	27	0·4447
Anti-ds DNA Ab	4	74	0·1225
Anti-Sm Ab	0	8	0·9999
Antiphospholipid Ab	3	18	0·3673
ANA	8	95	0·5033
Infections requiring hospitalization	3	29	0·7155

Anti-MBL antibody positive was defined as having a titre higher than mean +2 s.d. of 113 healthy individuals. Serositis, pleuritis or pericarditis; renal disorder, proteinuria or cellular casts; neurological disorder, seizures or psychosis; Anti-ds DNA Ab, anti-double strand DNA antibody; Anti-Sm Ab, anti-Sm antibody; Antiphospholipid Ab, antiphospholipid antibody. P = AA + AB versus BB by chi-square analysis.

Table 2. Associations of titres of anti-mannose-binding lectin (MBL) antibody and various disease parameters of systemic lupus erythematosus (SLE) in 106 SLE patients

Disease parameters of SLE	*P*-value[a]
Anti-DNA antibody	0·2173
C3	0·8844
C4	0·2131
CH50	0·7919
IgG	0·0665
IgA	0·9026
IgM	0·1637

[a]Spearman's rank correlation test.

the nature of anti-MBL antibodies in individual patients, or concentrations or conformations of MBL used in the assays.

Similarities in structure and function exist between MBL and C1q, and it is known that C1q-deficient or anti-C1q antibody positive individuals have a high probability of developing SLE [5,30,35,36]. It has been reported that MBL deficiency may be associated with the occurrence of SLE [18–22], although deficiency of MBL is not an extremely high risk factor, in contrast to deficiencies of other complement molecules such as C1q. The presence of autoantibodies against MBL may cause similar pathological conditions to those found in MBL deficiency, as with the case of anti-C1q antibodies. In this context, it is noteworthy that a previous study has shown that anti-C1q antibodies do not recognize MBL [37], which suggests that anti-MBL and anti-C1q antibodies are not identical.

In accord to previous studies, serum MBL concentrations were closely associated with the MBL genotypes of the individuals studied (Fig. 3). However, in this study, no significant differences in serum MBL concentrations were observed between SLE patients and healthy controls, when individuals with the same genotype were compared. This is different from the study by Seelen *et al.* [34], where they found that serum MBL concentrations were higher in SLE patients than in controls. This difference may be due to differences in MBL genotype distributions or disease activities of SLE in the individuals studied, or other unknown factors.

We next asked whether there is any association between levels of anti-MBL antibodies and MBL genotypes. No such correlation was observed (Fig. 4). However, levels of anti-MBL antibodies in patients having genotype AB were higher than those in patients with genotype AA, if we excluded one patient with genotype AA with an extremely high level of anti-MBL antibodies (Fig. 4). In addition, some genotype BB patients had anti-MBL antibodies (Fig. 4). We went on to study the relationship between serum MBL concentration and levels of anti-MBL antibodies. There was no statistically significant relationship (Fig. 5). These findings support the notion that elevated serum MBL is not a causative factor for anti-MBL antibody production, and other factors should contribute to the production of these autoantibodies. One possible factor is the production of mutated MBL protein in genotype AB or BB individuals. Individuals with genotype AB or BB produce a mutated MBL protein which is degraded in sera, since they are unable to form a stable oligomerized structure [12,38]. These degraded MBL protein products may have a role in the occurrence of anti-MBL antibodies. However, at this point, this remains only a speculation. Other factors must be important as well, since some patients with genotype AA also have anti-MBL antibodies. Many questions need to be solved, before the mechanisms of autoantigen recognition and autoantibody production including anti-MBL antibodies could be clarified.

We examined the disease characteristics of SLE in anti-MBL antibodies positive patients (Table 1). There were no significant relationships between the possession of a significantly high titre of anti-MBL antibodies, and the characteristics or parameters of SLE. This is in accord with the report by Seelen *et al.* [34], which showed no difference between anti-MBL levels in sera of patients with active disease and inactive disease, especially concerning renal involvement. However, among patients having high titre of anti-MBL antibodies, smaller number of patients tended to have anti-DNA antibodies, and more patients (3 of 9 patients, 33%) developed intestinal pneumonitis, which usually occur in less than 10% of SLE patients [39]. Thus, we felt that some cases had somewhat atypical features of SLE. Whether this is only a coincidence or not is unclear. A study of larger number of patients should be done to clarify the clinical significance of anti-MBL antibodies in SLE.

It has been reported that individuals lacking MBL are prone to severe episodes of bacterial infections from early life [15–17]. A recent study has shown that presence of MBL minority alleles is a risk factor for infection in patients undergoing bone marrow transplantation [40]. It is also reported that the MBL deficiency, resulting from the possession of the variant alleles of the MBL gene, is a risk factor in patient receiving immunosuppressive therapy [19,20]. Although we anticipated that decreased MBL function caused by anti-MBL antibodies might lead to more frequent infections during the course of SLE, we could not find, in the present study, any significant associations between the presence of anti-MBL antibodies and the occurrence of infections requiring hospitalization after initiation of therapy of SLE. The effect of anti-MBL antibodies to increased susceptibility to infections in individuals under immunosuppressive therapy may not be as large as that caused by MBL gene polymorphisms. Since only 9 patients had significantly high titre of serum anti-MBL antibodies, a larger study is necessary to confirm this observation.

In conclusion, we detected anti-MBL antibodies in sera of patients with SLE. However, we could not find any significant relationships with MBL genotype, clinical characteristics and parameters of SLE in this study. Further studies are necessary to elucidate the actual functions of autoantibodies to MBL in the pathogenesis of SLE, and to determine the value of measuring these autoantibodies in clinical practice.

REFERENCES

1 Winchester RJ, Nunez-Roldan A. Some genetic aspects of systemic lupus erythematosus. Arthritis Rheum 1982; 25:833–7.
2 Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, Walker A, Mack TM. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum 1992; 35:311–8.
3 Atkinson JP. Complement deficiency: predisposing factor to autoimmune syndromes. Clin Exp Rheumatol 1989; 7:S95–101.
4 Arnett FC, Reveille JD. Genetics of systemic lupus erythematosus. Rheum Dis Clin North Am 1992; 18:865–92.
5 Bowness P, Davies KA, Norsworthy PJ, Athanassiou P, Taylor-Wiedeman J, Borysiewicz LK, Meyer PA, Walport MJ. Hereditary C1q deficiency and systemic lupus erythematosus. QJM 1994; 87:455–64.

6 Walport MJ. Complement deficiency and disease. Br J Rheumatol 1993; 32:269–73.
7 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.
8 Mevorach D, Zhou JL, Song X. Elkon KB. Systemic exposure to irradiated apoptotic cells induces autoantibody production. J Exp Med 1998; 188:387–92.
9 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.
10 Sastry K, Herman GA, Day L, Deignan E, Bruns G, Morton CC, Ezekowitz RA. The human mannose-binding protein gene. Exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and localization to chromosome 10. J Exp Med 1989; 170:1175–89.
11 Madsen HO, Garred P, Kurtzhals JA, Lamm LU, Ryder LP, Thiel S, Svejgaard A. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics 1994; 40:37–44.
12 Sumiya M, Super M, Tabona P, Levinsky RJ, Arai T, Turner MW, Summerfield JA. Molecular basis of opsonic defect in immunodeficient children. Lancet 1991; 29:1569–70.
13 Madsen HO, Garred P, Thiel S, Kurtzhals JA, Lamm LU, Ryder LP, Svejgaard A. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J Immunol 1995; 15:3013–20.
14 Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P. Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J Immunol 1998; 161:3169–75.
15 Koch A, Melbye M, Sorensen P *et al.* Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA 2001; 285:1316–21.
16 Summerfield JA, Ryder S, Sumiya M, Thursz M, Gorchein A, Monteil MA, Turner MW. Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet 1995; 345:886–9.
17 Summerfield JA, Sumiya M, Levin M, Turner MW. Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. Br Med J 1997; 314:1229–32.
18 Davies EJ, Snowden N, Hillarby MC, Carthy D, Grennan DM, Thomson W, Ollier WE. Mannose-binding protein gene polymorphism in systemic lupus erythematosus. Arthritis Rheum 1995; 38:110–4.
19 Garred P, Madsen HO, Halberg P, Petersen J, Kronborg G, Svejgaard A, Andersen V, Jacobsen S. Mannose-binding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. Arthritis Rheum 1999; 42:2145–52.
20 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.
21 Ip WK, Chan SY, Lau CS, Lau YL. Association of systemic lupus erythematosus with promoter polymorphisms of the mannose-binding lectin gene. Arthritis Rheum 1998; 41:1663–8.
22 Tsutsumi A, Sasaki K, Wakamiya N *et al.* Mannose-binding lectin gene: polymorphisms in Japanese patients with systemic lupus erythematosus, rheumatoid arthritis and Sjögren's syndrome. Genes Immun 2001; 2:99–104.
23 Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM. 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.
24 James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest 1997; 15 (100):3019–26.
25 James JA, Neas BR, Moser KL, Hall T, Bruner GR, Sestak AL, Harley JB. Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure. Arthritis Rheum 2001; 44:1122–6.
26 Okada M, Ogasawara H, Kaneko H *et al.* Role of DNA methylation in transcription of human endogenous retrovirus in the pathogenesis of systemic lupus erythematosus. J Rheumatol 2002; 29:1678–82.
27 Hisano S, Matsushita M, Fujita T, Endo Y, Takebayashi S. Mesangial IgA2 deposits and lectin pathway-mediated complement activation in IgA glomerulonephritis. Am J Kidney Dis 2001; 38:1082–8.
28 Lhotta K, Wurzner R, Konig P. Glomerular deposition of mannose-binding lectin in human glomerulonephritis. Nephrol Dial Transplant 1999; 14:881–6.
29 Steinfeld S, Penaloza A, Ribai P *et al.* D-mannose and N-acetylglucosamine moieties and their respective binding sites in salivary glands of Sjögren's syndrome. J Rheumatol 1999; 26:833–41.
30 Siegert C, Daha M, Westedt ML, van der Voort E, Breedveld F. IgG autoantibodies against C1q are correlated with nephritis, hypocomplementemia, and dsDNA antibodies in systemic lupus erythematosus. J Rheumatol 1991; 18:230–4.
31 Ohtani K, Suzuki Y, Eda S *et al.* High-level and effective production of human mannan-binding lectin (MBL) in Chinese hamster ovary (CHO) cells. J Immunol Meth 1999; 222:135–44.
32 Hakozaki Y, Yoshiba M, Sekiyama K *et al.* Mannose-binding lectin and the prognosis of fulminant hepatic failure caused by HBV infection. Liver 2002; 22:29–34.
33 Sasaki K, Tsutsumi A, Wakamiya N, Ohtani K, Suzuki Y, Watanabe Y, Nakayama N, Koike T. Mannose-binding lectin polymorphisms in patients with hepatitis C virus infection. Scand J Gastroenterol 2000; 35:960–5.
34 Seelen MA, Trouw LA, van der Hoorn JW, Fallaux-van den Houten FC, Huizinga TW, Daha MR, Roos A. Autoantibodies against mannose-binding lectin in systemic lupus erythematosus. Clin Exp Immunol 2003; 134:335–43.
35 Slingsby JH, Norsworthy P, Pearce G, Vaishnaw AK, Issler H, Morley BJ, Walport MJ. Homozygous hereditary C1q deficiency and systemic lupus erythematosus. A new family and the molecular basis of C1q deficiency in three families. Arthritis Rheum 1996; 39:663–70.
36 Walport MJ, Davies KA, Botto M. C1q and systemic lupus erythematosus. Immunobiology 1998; 199:265–85.
37 Martensson U, Thiel S, Jensenius JC, Sjoholm AG. Human autoantibodies against Clq: lack of cross reactivity with the collectins mannan-binding protein, lung surfactant protein A and bovine conglutinin. Scand J Immunol 1996; 43:314–20.
38 Garred P, Larsen F, Madsen HO, Koch C. Mannose-binding lectin deficiency – revisited. Mol Immunol 2003; 40:73–84.
39 Eisenberg H, Dubois EL, Sherwin RP, Balchum OJ. Diffuse interstitial lung disease in systemic lupus erythematosus. Ann Intern Med 1973; 79:37–45.
40 Rocha V, Franco RF, Porcher R *et al.* Host defense and inflammatory gene polymorphisms are associated with outcomes after HLA-identical sibling bone marrow transplantation. Blood 2002; 100:3908–18.

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Glucose-6-phosphate isomerase variants play a key role in the generation of anti-GPI antibodies: possible mechanism of autoantibody production

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Received 16 July 2004
Available online 2 September 2004

Abstract

Glucose-6-phosphate isomerase (GPI), recognized as an autoantigen in the K/BxN arthritis model, is a ubiquitous cytoplasmic enzyme. Anti-GPI antibodies (Abs) are also detected in the serum of patients with arthritic diseases including rheumatoid arthritis (RA). So far, 24 GPI variants have been reported and most of these variants relate to non-spherocytic hemolytic disease. To understand the mechanisms of anti-GPI Ab production, cDNAs from peripheral blood mononuclear cells of subjects with or without anti-GPI Abs were cloned and sequenced. We identified 39 new GPI variants (57–1596 bp). The frequency of GPI variants in healthy control subjects (HS) with anti-GPI Abs (27/73, 31.5%) was significantly higher than that in anti-GPI Ab-negative HS (5/78, 6.4%, $p < 0.001$). The frequency of GPI variants in anti-GPI Ab-positive RA patients (22/77, 28.6%) was more significantly higher than in anti-GPI Ab-negative patients (1/63, 1.6%, $p < 0.0001$). Our results suggest that GPI variants may play a crucial role in the production of autoantibodies against ubiquitous GPI autoantigens.

Keywords: Glucose-6-phosphate isomerase; Autoantibodies; Autoantigens; Rheumatoid arthritis

Autoantigens in chronic inflammatory arthritides such as rheumatoid arthritis (RA) are still unknown [1]. Several candidate autoantigens have been identified in RA patients, although they have not been confirmed because of the heterogeneity of the disease. The K/BxN T cell receptor transgenic mouse spontaneously develops a joint disorder with many of the clinical, histolopathological, and immunological features of RA in human [2]. The disease in the model mouse is initially provoked by T cell recognition of ubiquitously expressed self-antigen, and the consequent arthritic events in the joint are mainly driven by autoantibodies [3].

Glucose-6-phosphate isomerase (GPI), a second glycolytic enzyme, is the target of both initiating T cells and pathogenic immunoglobulins [4,5]. Several groups reported the presence of anti-GPI antibodies (Abs) in the serum of patients with severe forms of arthritic diseases including RA [6–8], suggesting their pathogenic role in human arthritides. With regard to autoantigen GPI, the gene locus is located on chromosome 19 [9], and the gene spans more than 40 kbp, including 18 exons and 17 introns [10–12]. GPI is also known as an extracelluar cytokine [autocrine motility factor (AMF), neuroleukine, or maturation factor], and probably increases the susceptibility to certain autoimmune diseases or cancer metastasis [13–16]. So far, 24 GPI variants have been identified and most of these variants are associated with a decrease in isomerase activity on

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.08.123

erythrocytes, resulting in non-spherocytic hemolytic disease [17–22]. Moreover, 10 single nucleotide polymorphisms (SNPs) of GPI gene have been reported [23]. Based on the above background, we hypothesized that the amino acid mutations or deletion of GPI autoantigens results in the generation of autoantibodies against GPI, with subsequent development of arthritis.

In the present study, to clarify the relevance between GPI and anti-GPI Abs, we cloned full-length GPI complementary DNAs (cDNAs) derived from subjects with and without anti-GPI Abs, sequenced them, and compared them with intact GPI cDNA [24]. We observed numerous GPI variants in anti-GPI Ab-positive subjects compared with anti-GPI Ab-negative individuals. These findings support the notion that variant molecules of autoantigens are essential for production of autoantibodies.

Materials and methods

Subjects and cDNA synthesis. Peripheral blood mononuclear cells (PBMC) derived from 10 patients with RA (including 5 anti-GPI Ab-positive) and healthy volunteers (including 5 anti-GPI Ab-positive) were used. These subjects were defined as anti-GPI Ab-positive or -negative, after GPI enzyme-linked immunosorbent assay (ELISA) by using recombinant human GPI and rabbit muscle GPI as antigens [7]. Total RNA was prepared from PBMC by using ISOGEN method (Nippon gene, Tokyo, Japan) according to the instructions provided by the manufacturer. For first strand cDNA synthesis, 0.5 μg of total RNA with 1 μl of oligo(dT)$_{18}$ primer was incubated with 2 μg of 10 nM dNTP mix, 20 U RNase inhibitor, 4 μg of 5× reaction buffer, and 200 U M-MuLV reverse transcriptase (RT) (Revert Aid First Strand cDNA Synthesis Kit, Fermentas, Hanover, MD) in a total volume of 20 ml for 1 h at 42 °C.

RT-PCR and GPI cDNA cloning. Primer sets for full-length GPI cDNA including first codon ATG and stop codon TAA were 5′-TCAGTGTACCTTCTAGTCCCG-3′ and 5′-GTCCAAGCCCACA ACCAGA-3′ (Table 1) [17]. Full-length GPI cDNAs were prepared with 1 μl first strand cDNA, 15 pmol primer sets, 2 μg of 25 mM $MgSO_4$, 5 μg of 2 mM dNTP mixture, 5 μg of 10× reaction buffer, and 2.5 U KOD *Taq* polymerase (Toyobo, Osaka, Japan) in total 50 μl reaction volume by using PCR Gene Amp system 9700 (Applied Biosystems, Foster City, CA). The reaction mixture was incubated at 94 °C for 2 min and amplified for 30 cycles at 94 °C for 15 s, 59 °C for 30 s, and 72 °C for 2 min. Next, 5 ml of mixture was loaded onto 1% agarose gel to check for amplification. The amplified cDNAs were subcloned into pCR-Blunt II TOPO Vector by Zero blunt TOPO PCR Cloning kit (Invitrogen, San Diego, CA). To confirm the insert, over 20 clones per subject were checked by colony PCR using M13 reverse primer and T7 primer on the vector following 1% agarose gel electrophoresis. The clone with a shorter fragment than the intact length (2-kbp) was sequenced by using approximately primers (Table 1) with BigDye terminator system (AB) after purification of the plasmid (GFX Micro Plasmid Prep Kit, Amersham Life Science, Buckinghamshire, UK). The sequence of insert cDNA was compared and confirmed with intact GPI mRNA [24].

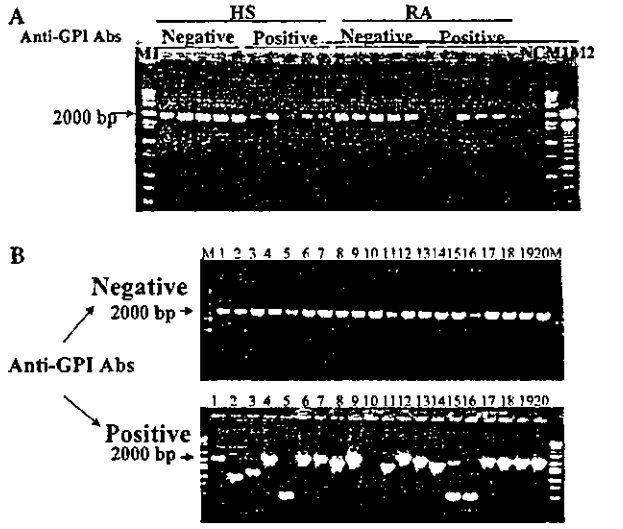

Fig. 1. (A) RT-PCR for full-length GPI cDNA. One microliter of synthesized cDNA template was used in each 50 μl PCR system. Three microliters of PCR products was loaded in 1.0% agarose gel. NC, negative control. M1, wide Range DNA Ladder (50–10,000 bp) (TAKARA); M2, 100 bp DNA Ladder (Invitrogen). Five samples were subcloned into each group, respectively. (The left one was not done in RA positive). (B) Colony PCR for insert check. M13 RV and T7 primer on the pCRII vector were used in each 10 μl PCR system. The band at 2000 bp showed an intact size of full-length GPI cDNA. Three microliters of PCR products was loaded in 1.0% garose gel. M1. Wide Range DNA Ladder (50–10,000 bp) (TAKARA). In case of anti-GPI Ab-positive, clone Nos. 2, 3, 5, 8, 9, 11, 13, 14, 15, and 16 were sequenced. Clone Nos. 11, 14, and 15 were found deletion variants after sequencing analysis. About 20 colony PCR for insert check was done in each group, respectively, to total 291.

Table 1
Oligonucleotides used in this study

No.	Sequence	Position in NM_000175
GPI-5′	5′-TCAGTGACCTTCTTAGTCCCG-3′	81–101
GPI-5′-1	5′-CGGACGTCATCAACATTGGCAT-3′	552–573
GPI-5′-2	5′-GAGTGGTTTCTCCAGGCGGCCA-3′	782–803
GPI-5′-3	5′-GTATCTGGTACATCAACTGC-3′	1074–1093
GPI-5′-4	5′-ATCGACGGAGGAGGCCCGAA-3′	1423–1442
GPI-3′	5′-GTCCAAGCCCACAACCAGA-3′	1901–1882
GPI-3′-1	5′-CTGGACTCTTGCCCGCAGCC-3′	1472–1452
GPI-3′-2	5′-GGTGCAGGTACTGGTCATA-3′	1142–1123
GPI-3′-3	5′-CAGGGCAACAAAGTGCTTCG-3′	840–821
GPI-3′-4	5′-CCATGAGGGGTCCCAGGTC-3′	602–584

The set of GPI-5′and GPI-3′ was used for RT-PCR. Others were used for sequencing.

Results and discussion

To examine whether variant forms of GPI exist in PBMC of anti-GPI Ab-positive individuals, DNAs encoding the GPI gene were amplified by RT-PCR with primers specific for GPI (Table 1). As shown in Fig. 1A, the amount of DNA encoding intact GPI gene at 1820 bp in anti-GPI Ab-positive (+) subjects was less than in anti-GPI Ab-negative (−) subjects. This tendency was almost equally found in both RA patients and healthy subjects (HS). Amplified GPI DNAs were cloned and several different GPI genes were more frequently detected in anti-GPI Abs (+) than anti-GPI Abs (−) HS and RA patients (Fig. 1B). Sequence analysis of different length clones showed many GPI variants (Fig. 2); 39 GPI variants were identified in 291 clones. The frequency of GPI variants in anti-GPI (+) HS (23/73, 31.5%) was significantly higher compared to those in anti-GPI (−) HS (5/78, 6.4%, $p < 0.001$, Table 2). Furthermore, GPI variants in anti-GPI (+) RA (22/77, 28.6%) were also significantly more frequent than in anti-GPI (−) RA (1/63, 1.6%, $p < 0.0001$, Table 2). There was significant relationship between the presence of GPI variants and anti-GPI Abs ($p < 0.0001$). However, the proportion of GPI variants in RA did not correlate with that in HS (Table 2). These findings suggest that the newly presented B cell epitopes on altered GPI molecules are recognized as antigens and that

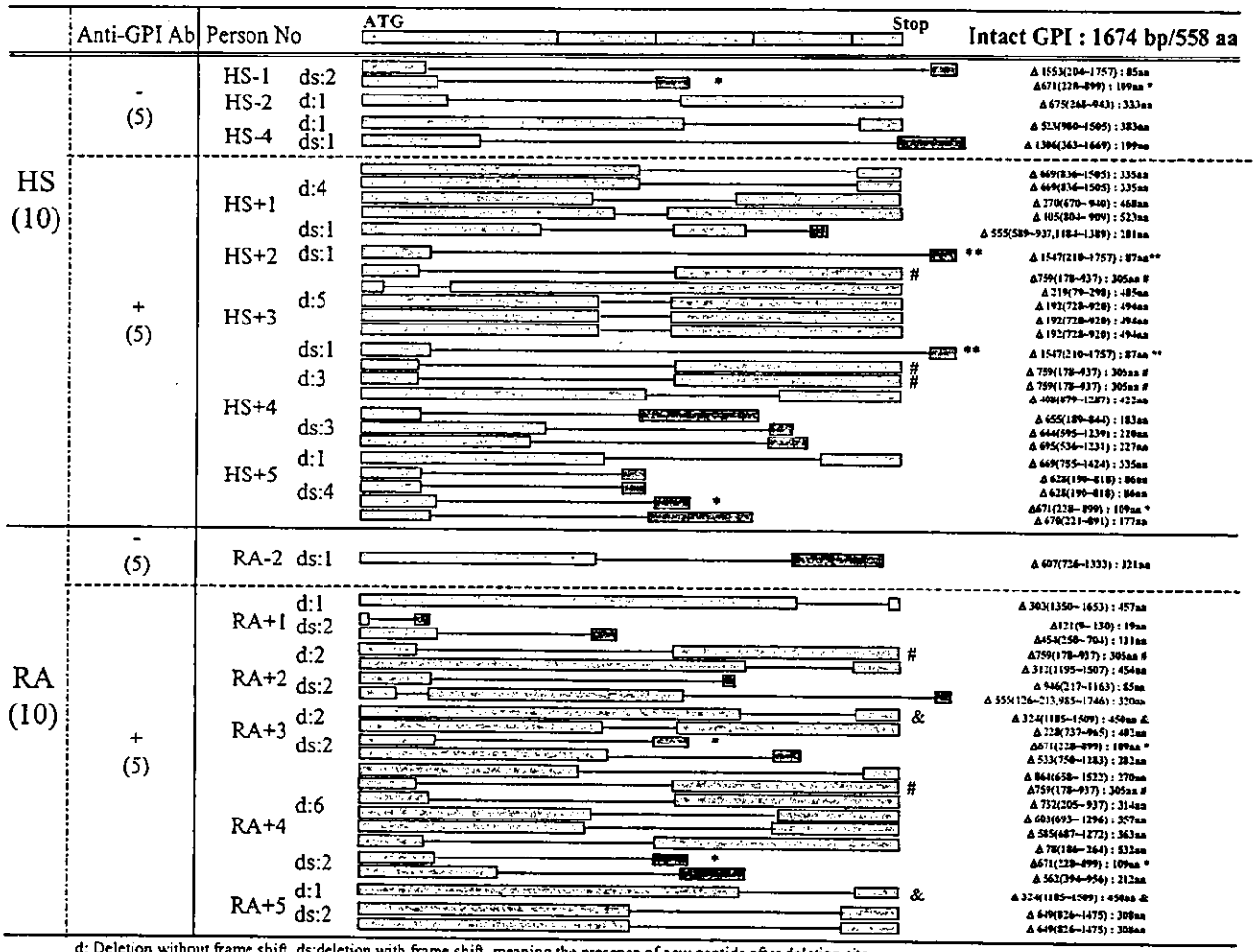

Fig. 2. Mapping of GPI deletions. Sequencing of GPI deletions was confirmed by comparison with intact GPI mRNA [14] after sequencing analysis of the plasmid having different length cDNAs. Thirty-nine kinds of GPI variants (totally 51 clones) were identified. In the most upper column, green bar showed 1674 bp/558 aa length of intact GPI/aa sequence from start codon (ATG) to stop codon. In the following column, green bar showed the part of intact GPI/aa sequence. Binding line between green bar and dark bar showed the part of deletion. Dark bar showed the part of new peptide which occurred by flame shift resulting in deletion. d, Deletion with intact peptide (without flame shift); ds, deletion with new peptide (with flame shift) including new stop codon; Δ1553(204–1757): 85aa, 1553 bp deletion between 204 and 1757 bp from start codon on intact GPI mRNA and it results in 85 aa length protein; *, **, &, and #, indicating the same sequencing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)