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Abnormal IgG galactosylation and arthritis in MRL-*Fas*^{lpr} or MRL-*FasL*^{gld} mice are under the control of the MRL genetic background

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Abstract MRL mice bearing the *lpr* (Fas) or *gld* (Fas ligand) mutation, MRL-*Fas*^{lpr} or MRL-*FasL*^{gld}, respectively, develop arthritis similar to rheumatoid arthritis, but C3H and C57BL/6 mice bearing such mutations do not. In MRL-*Fas*^{lpr} mice, agalactosylated oligosaccharides in serum IgG increase significantly in comparison to MRL-+/+ mice without arthritis. In this study, an increased level of agalactosylation in IgG, as compared to MRL-+/+, was found in both MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice. In contrast, the incidence of IgG without galactose was comparable among C3H-*Fas*^{lpr}, C3H-*FasL*^{gld}, and C3H-+/+ mice as well as between C57BL/6-*Fas*^{lpr} and C57BL/6-+/+ mice. These results suggest that the increase in agalactosylated IgG and the development of arthritis in MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice are controlled by the MRL genetic background.

Key words: Glycosylation; IgG; Arthritis; *lpr*; *gld*; Apoptosis

1. Introduction

MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice are known to spontaneously develop an autoimmune arthritis similar to rheumatoid arthritis (RA) [1–3]. *Fas*^{lpr} (Fas gene with the *lpr* mutation) and *FasL*^{gld} (Fas ligand gene with the *gld* mutation) induce a deficit in Fas/FasL-mediated apoptosis [4,5], which is significantly related to the development of arthritis in MRL mice [6]. In contrast, C3H-*Fas*^{lpr}, C3H-*FasL*^{gld} and C57BL/6-*Fas*^{lpr} mice do not develop arthritis in spite of a deficit of Fas/FasL-mediated apoptosis [3]. Therefore, an MRL genetic background is a prerequisite for the development of arthritis in addition to abnormal apoptosis [6].

Mouse IgG oligosaccharide chains consist of a series of biantennary complex-type structures of ±Galβ1-4GlcNAcβ1-2Manα1-6(±Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc containing zero to two sialic acid residues linked to Gal residues [7]. The incidence of agalactosylated oligosaccharide chains significantly increases in serum IgG of MRL-*Fas*^{lpr} mice in comparison to that of age-matched control congenic MRL-+/+ mice with no arthritis [8]. A similar abnormality in IgG galactosylation has been observed in RA patients [9]. However, the mechanism of the aberrant existence of agalactosylated IgG and its pathogenic significance in the development of arthritis are still poorly understood.

In order to better understand the relationship between the abnormality in IgG galactosylation, the functional defects in Fas/FasL-mediated apoptosis, and the development of arthritis, we conducted a comparative study of the abnormality in IgG galactosylation in mice with different genetic backgrounds and with or without *Fas*^{lpr} and *FasL*^{gld}.

2. Materials and methods

2.1. Mice

MRL-*Fas*^{lpr}, MRL-+/+, C3H-*Fas*^{lpr}, C3H-*FasL*^{gld}, C3H-+/+, C57BL/6 (B6)-*Fas*^{lpr}, and B6-+/+ mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). MRL-*FasL*^{gld} mice were produced as described elsewhere [3]. The mice were housed under conditions free of specific pathogens. Sera (100–200 μl/mouse) were obtained from 10 mice at 6 months of age and pooled according to the mouse strain. Development of arthritis in MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice had been confirmed via histopathological study in knee and/or foot joints, characterized by synovial lining proliferation and/or granulomatous inflammation in the synovial tissue as described previously [10]. No arthritis was observed in the other mice.

2.2. Purification of IgG from sera

Each serum sample (1–2 ml) was diluted with 9 volumes of 1.5 M glycine–NaOH buffer, pH 8.9, containing 3 M NaCl, and filtered. Then, total IgG in the serum sample was bound to a protein A-Sepharose CL-4B column (1.0×6.5 cm; Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer and eluted with 0.1 M sodium citrate buffer, pH 3.0 [11], followed by further purification by gel filtration chromatography using a Sephacryl S-300 column (2.5×90 cm; Pharmacia Biotech). The purity of IgG samples was confirmed by SDS–PAGE using the Laemmli system [12] under reducing and non-reducing conditions and Ouchterlony double immunodiffusion assay using antisera against murine IgG, IgM, IgA, and whole serum. The purified IgG samples were exhaustively dialyzed against distilled water and then lyophilized.

2.3. Analyses of structures of N-linked oligosaccharides from IgG samples

Purified IgG samples (2–3 mg) were subjected to gas-phase hydrazinolysis at 90°C for 3 h using Hydraclub S204 (Honen, Tokyo, Ja-

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Abbreviations: ABEE, *p*-aminobenzoic acid ethyl ester; B6, C57BL/6; FasL, Fas ligand; *Fas*^{lpr}, Fas gene with the *lpr* mutation; *FasL*^{gld}, Fas ligand gene with the *gld* mutation; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; HPLC, high-performance liquid chromatography; Man, mannose; RA, rheumatoid arthritis

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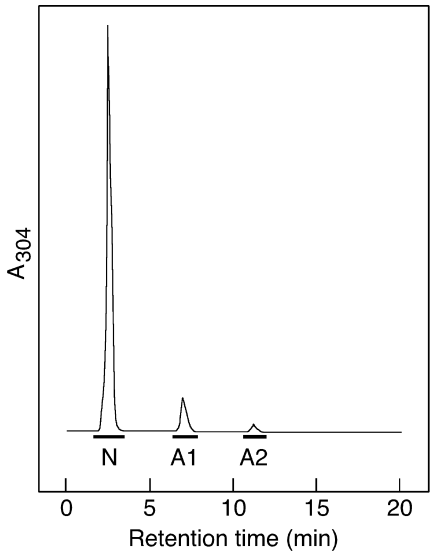

Fig. 1. Elution profile of ABEE-oligosaccharides from mouse IgG sample in anion exchange HPLC. An ABEE-oligosaccharide mixture from an IgG sample of MRL-*FasL*^{gld} mice was subjected to anion exchange HPLC with a Cosmogel DEAE column (0.75×7.5 cm).

pan) followed by *N*-acetylation to quantitatively liberate *N*-linked oligosaccharides [13]. The oligosaccharide mixture was purified with cellulose column chromatography [14] and then derivatized with *p*-aminobenzoic acid ethyl ester (ABEE; Wako Pure Chemical Industries, Osaka, Japan) by reductive amination [15,16].

Analyses of the IgG-derived ABEE-oligosaccharides were performed using high-performance liquid chromatography (HPLC). Anion exchange and reversed-phase HPLC were carried out using a Cosmogel DEAE column (0.75×7.5 cm; Nacalai Tesque, Kyoto, Japan) and a Wakosil 5C18-200 column (0.46×25 cm; Wako Pure Chemical Industries), respectively, as described previously [15,16]. Elution of ABEE-oligosaccharides was monitored at 304 nm. Mass spectrometry analysis of ABEE-oligosaccharides was performed with a MALDI-TOF mass spectrometer, model Vision 2000 (ThermoBioAnalysis, Hemel Hempsted, UK).

2.4. Oligosaccharides and enzymes

Neutral, monosialyl, and disialyl biantennary ABEE-oligosaccharides, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, were prepared from oligosaccharides of human fibrinogen [17] by ABEE derivatization as described above. ABEE-oligosaccharides 1–8, with the same structures as oligosaccharides a–h shown in Fig. 3, respectively, were prepared from oligosaccharides of human fibrinogen and human IgG [17,18] by means of ABEE-derivatization. ABEE-oligosaccharides 9 and 10 with the respective structures Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE and Manα1-6(Manα1-3)-Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE were prepared from ABEE-oligosaccharides 1 and 5, respectively, by digestion with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase. Digestion of ABEE-oligosaccharides was performed at 37°C in a toluene atmosphere for 16 h using *Arthrobacter ureafaciens* neuraminidase (Nacalai Tesque), *Diplococcus pneumoniae* β-galactosidase (Boehringer Mannheim, Mannheim, Germany), or *D. pneumoniae N*-acetyl-β-glucosaminidase (Boehringer Mannheim) as described previously [15,16].

2.5. Statistical analysis

Statistical analysis, Student's *t*-test, was performed with a Macintosh computer (PowerBook G4 from Apple Japan, Tokyo, Japan) using software titled StatMate III for Macintosh (ATMS, Tokyo, Japan).

3. Results

3.1. Analysis of murine IgG oligosaccharides by charge

The total IgG fractions were purified from sera of MRL-*Fas*^{lpr}, MRL-*FasL*^{gld}, MRL-+/+, C3H-*Fas*^{lpr}, C3H-*FasL*^{gld}, C3H-+/+, B6-*Fas*^{lpr}, and B6-+/+ mice. Oligosaccharides of these IgG samples were then prepared and derivatized with ABEE as described in Section 2. To determine the charges of IgG oligosaccharides, ABEE-oligosaccharide fractions obtained from all IgG samples were subjected to ion exchange HPLC with a Cosmogel DEAE column. Each ABEE-oligosaccharide fraction yielded one major neutral (N) and two minor acidic (A1 and A2) fractions as shown in Fig. 1. Since the elution positions of A1 and A2 corresponded to those of authentic monosialyl and disialyl biantennary ABEE-oligosaccharides, respectively, and since they were converted to neutral oligosaccharides by sialidase treatment (data not shown), A1 and A2 were respectively noted to be monosialylated and disialylated ABEE-oligosaccharides. Molar ratios of N, A1, and A2 calculated on the basis of their absorbance at 304 nm have been summarized in Table 1. MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice with arthritis displayed a slightly higher incidence of neutral oligosaccharides in comparison to MRL-+/+ mice without arthritis. All other mice with or without *Fas*^{lpr} or those having *FasL*^{gld} with no arthritis displayed a comparable ratio.

3.2. Structural analyses of desialylated oligosaccharides from murine IgG

To analyze the oligosaccharide structure of IgG samples, desialylated neutral ABEE-oligosaccharide mixtures obtained by exhaustive sialidase treatment followed by purification with anion exchange HPLC were subjected to reversed-phase

Table 1
Percent molar ratios of various oligosaccharide structures of serum IgG from various strains of mice

Mice	Arthritis	Sialylated glycoforms (%)			Galactosylated glycoforms (%)		
		N	A1	A2	G0	G1	G2
MRL-+/+	−	84.7±0.5	13.0±0.3	2.3±0.1	29.2±0.3	48.6±0.6	22.2±0.5
MRL-*Fas*^{lpr}	+	92.2±0.6	6.5±0.5	1.3±0.2	51.0±3.0	41.7±1.4	7.3±3.0
MRL-*FasL*^{gld}	+	91.4±1.8	7.0±1.2	1.6±0.5	46.2±3.0	41.8±0.6	12.0±3.6
C3H-+/+	−	90.3±1.0	6.8±0.8	2.9±0.2	34.5±3.0	47.3±0.5	18.2±2.6
C3H-*Fas*^{lpr}	−	91.4±0.8	7.4±0.8	1.2±0.1	38.8±3.3	47.9±2.6	13.3±0.8
C3H-*FasL*^{gld}	−	91.5±0.9	7.3±0.6	1.2±0.3	37.5±0.8	49.2±0.6	13.3±0.3
B6-+/+	−	84.4±1.0	12.9±1.2	2.7±0.3	31.6±1.7	39.9±0.9	28.5±0.9
B6-*Fas*^{lpr}	−	86.9±1.6	11.5±1.4	1.6±0.4	34.1±1.2	43.6±0.8	22.3±1.2

N, A1, and A2 denote non-, mono-, and disialyloligosaccharides, respectively. G0, G1, and G2 denote oligosaccharides with zero, one, and two galactose residue(s), respectively. The percentages of sialylated or galactosylated glycoforms were calculated on the basis of their absorbance at 304 nm in ion exchange or reversed-phase HPLC, respectively. This study was repeated three times and values are indicated as the mean± S.E.M. of the three pooled sera.

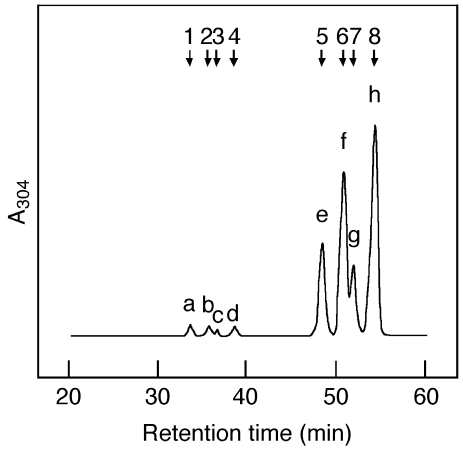

Fig. 2. Elution profile in reversed-phase HPLC of desialylated neutral ABEE-oligosaccharides from mouse IgG sample. A desialylated neutral ABEE-oligosaccharide mixture from the IgG sample of MRL-*FasL*^{gld} mice was subjected to reversed-phase HPLC with a Wakosil 5C18-200 column (0.46×25 cm). The numbered arrows at the top of the figure indicate the elution positions of authentic ABEE-oligosaccharides. Structures of these authentic oligosaccharides are described in Section 2.

HPLC with a Wakosil 5C18-200 column. As shown in Fig. 2, the ABEE-oligosaccharide mixtures of an IgG sample obtained from MRL-*FasL*^{gld} yielded eight oligosaccharide fractions (a–h in Fig. 2). The ABEE-oligosaccharide mixtures of those obtained from all other strains also yielded eight oligosaccharide fractions, although the ratios of the fractions differed among the IgG samples. The elution positions of oligosaccharide fractions a–h corresponded to those of a series of authentic biantennary complex-type oligosaccharides 1–8 with the structures ±Galβ1-4GlcNAcβ1-2Manα1-6(±Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(±Fucα1-6)GlcNAc-ABEE, respectively.

The structures of the respective IgG-derived ABEE-oligosaccharides were analyzed using sequential exoglycosidase digestion followed by reversed-phase HPLC. Conversion of the eight ABEE-oligosaccharide fractions a–h into two oligosaccharide fractions d and h was observed upon incubation with β-galactosidase (data not shown). After sequential digestion with β-galactosidase and *N*-acetyl-β-glucosaminidase, eight ABEE-oligosaccharide fractions were converted into two oligosaccharide fractions eluting at the same positions as authentic ABEE-oligosaccharides 9 and 10 with the structures Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-ABEE and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-ABEE, respectively (data not shown). The behavior of the oligosaccharides in fractions a–h on the reversed-phase HPLC column and the results of the sequential exoglycosidase treatment suggested that structures of the ABEE-oligosaccharides a–h derived from each IgG sample were as shown in Fig. 3. These results were also confirmed by analysis of these eight ABEE-oligosaccharide fractions with MALDI-TOF mass spectrometry (data not shown).

The molar ratios of oligosaccharides a–h in each IgG sample were calculated on the basis of their absorbance at 304 nm. Levels of incidence of fucosylated oligosaccharides were similar (90–95%) among IgG samples. In contrast, those of galactosylated oligosaccharides differed (see below). Incidences (in percent) of oligosaccharides G0 (d and h in Fig. 3), G1

Fractions	Structures of ABEE-oligosaccharides
a:	Galβ1→4GlcNAcβ1→2Manα1↘6 Galβ1→4GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
b:	Galβ1→4GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
c:	GlcNAcβ1→2Manα1↘6 Galβ1→4GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
d:	GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
e:	Fucα1↓6 Galβ1→4GlcNAcβ1→2Manα1↘6 Galβ1→4GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
f:	Fucα1↓6 Galβ1→4GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
g:	Fucα1↓6 GlcNAcβ1→2Manα1↘6 Galβ1→4GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE
h:	Fucα1↓6 GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3Manβ1→4GlcNAcβ1→4GlcNAc−ABEE

Fig. 3. Structures of ABEE-oligosaccharides a–h derived from IgG samples.

(b, c, f, and g in Fig. 3), and G2 (a and e in Fig. 3) with zero, one, and two galactose residues, respectively, are summarized in Table 1.

3.3. IgG galactosylation in various strains of mice

Relative contents of the agalactosylated oligosaccharides were compared among IgG samples from strains with the same genetic background to elucidate the relationship in terms of the presence of *Fas*^{lpr} or *FasL*^{gld} and the development of arthritis. The incidences of oligosaccharides without a galactose residue, G0, in IgG samples derived from MRL mice bearing *Fas*^{lpr}, which have arthritis, increased significantly ($P < 0.002$) in comparison to those derived from MRL-+/+ mice without arthritis (Table 1). The same significant increase ($P < 0.005$) in the incidence of G0 oligosaccharides was found in MRL mice bearing *FasL*^{gld}, which also have arthritis (Table 1). However, no significant difference in the incidence of G0 oligosaccharides was observed among C3H-*Fas*^{lpr}, C3H-*FasL*^{gld}, and C3H-+/+ ($P > 0.38$), and between B6-*Fas*^{lpr} and B6-+/+ ($P > 0.29$), all of which do not develop arthritis (Table 1). The incidence of oligosaccharides with two galactose residues, G2, in IgG samples from C3H-*Fas*^{lpr} and C3H-*FasL*^{gld} mice decreased to about 70% of that in C3H-+/+ mice as in the case of MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice, where the incidence decreased to one-third and half of that in MRL-+/+ mice. However, statistical analyses indicated that the decrease in G2 incidence in C3H mice was not significant ($P > 0.13$) while the decrease in MRL-*Fas*^{lpr} and MRL-

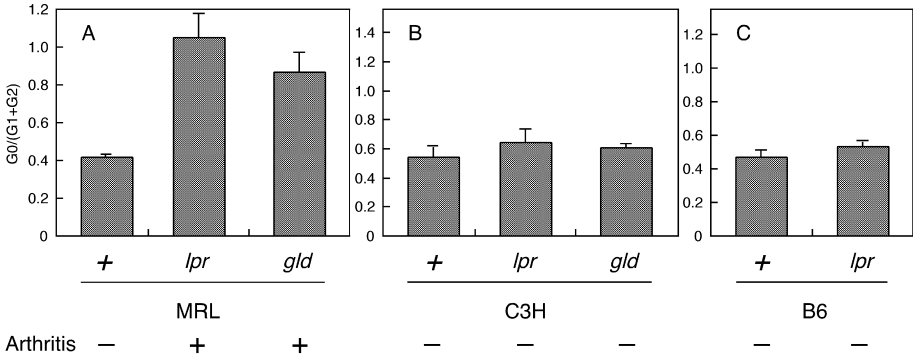

Fig. 4. Comparison of IgG agalactosylation levels among various mice with different genetic backgrounds. Agalactosylation levels, G0/(G1+G2), of serum IgG in MRL (A), C3H (B), and B6 (C) mice without (+) or with the *Fas*^{lpr} gene (*lpr*) or the *FasL*^{gld} gene (*gld*) were calculated as described in the text. Values are indicated as the mean ± S.E.M. of the three pooled sera. The presence or absence of the development of arthritis in each strain of mice is shown at the bottom of the figure.

FasL^{gld} was significant ($P < 0.01$ and $P < 0.05$, respectively). The decrease in the incidence of sialylated oligosaccharides as found in MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice (Table 1) may be explained by the decrease in galactosylation of IgG oligosaccharides.

When the level of agalactosylation in each IgG sample was calculated as the ratio of non-galactosylated oligosaccharide G0 to galactosylated oligosaccharides G1 plus G2, MRL-*Fas*^{lpr} mice displayed a marked increase ($P < 0.01$) in the level of agalactosylation when compared to MRL-+/+ mice (Fig. 4A); this result was consistent with previous reports [8]. The level of IgG agalactosylation in MRL-*FasL*^{gld} mice also increased ($P < 0.02$) comparably to that in MRL-*Fas*^{lpr} mice (Fig. 4A), indicating that MRL-*FasL*^{gld} mice, as well as MRL-*Fas*^{lpr} mice, exhibit an abnormality in IgG galactosylation. On the other hand, no significant difference ($P > 0.38$) was observed in the level of agalactosylation for C3H-+/+, C3H-*Fas*^{lpr}, and C3H-*FasL*^{gld} mice; similarly, no significant difference ($P > 0.29$) was observed for B6-*Fas*^{lpr} and B6-+/+ mice (Fig. 4B,C).

4. Discussion

The present study led to two significant conclusions with regard to the expression of the abnormality in IgG galactosylation. First, the expression of the abnormality in IgG galactosylation in MRL-*Fas*^{lpr} mice is not due to the *lpr* genotype itself but to the functional defect of Fas/FasL-mediated apoptosis since this abnormality was also observed in MRL-*FasL*^{gld} mice. Second, the MRL genetic background is involved in the expression of the abnormal IgG galactosylation in addition to the presence of *Fas*^{lpr} or *FasL*^{gld} since this abnormality was not observed in IgG samples from C3H-*Fas*^{lpr}, C3H-*FasL*^{gld}, or B6-*Fas*^{lpr} mice. It has been demonstrated that the development of arthritis in MRL-*Fas*^{lpr} and MRL-*FasL*^{gld} mice is controlled by the MRL genetic background [6]. Taken together with the present results, abnormal IgG galactosylation, like arthritis, occurs with a combination of a deficit in Fas/FasL-mediated apoptosis and an MRL genetic background and is thus related to the development of arthritis in MRL mice with abnormal apoptosis.

It has been reported that IgG was galactosylated differently among the IgG subclasses in mice with collagen-induced arthritis [19]. It was also indicated that degalactosylation of IgG delayed the clearance of IgG, particularly IgG2a, from sera of several strains of mice including MRL mice [20]. It should be noted that the IgG samples used in this study were the total IgG fractions of murine sera, which were eluted from the protein A-Sepharose column with sodium citrate buffer, pH 3.0, according to the methods described [11]. Therefore, agalactosylation levels in these IgG samples may have been affected by the delay in clearance of the particular subclass of IgG which may be involved in the pathogenesis. Analyses of the oligosaccharide structures with regard to IgG subclasses would be helpful in elucidating the possible effects of this delay, although the current study did not determine the population of IgG subclasses and their oligosaccharide structures.

Agalactosylated IgG can activate complements via interaction with mannose-binding lectin [21], which can bind to nonreducing terminal GlcNAc residues in agalactosylated IgG oligosaccharides [22] and lectin is a ligand of complement receptor 1 [23], which is involved in phagocytosis and clearance of immune complexes [24]. These processes might be effective for opsonization and elimination of immune complexes in host immune responses. However, the excessive and dysregulated existence of agalactosylated IgG in serum occurring in MRL mice with abnormal apoptosis and in patients with RA could have significant immunopathological consequences. Autoantibodies produced by activated autoreactive B cells that escaped from Fas/FasL-mediated apoptosis, aberrantly agalactosylated under the control of MRL background genes, may cause significant development of tissue lesions. A recent report has demonstrated that oligosaccharides of IgG rheumatoid factor derived from patients with RA were aberrantly agalactosylated [16]. In light of this fact, the aberrant amount of agalactosylated IgG autoantibodies in serum that is characteristic of MRL mice might be responsible for the disease development occurring selectively in an MRL strain.

Recent genetic studies of MRL-*Fas*^{lpr} mice have indicated that several gene loci with the MRL alleles are related to the development of autoimmune diseases in a polygenic manner [10,25–28], although the natures of their gene products remain unclear. Among the genes involved in the MRL genetic background, a certain gene may be present to affect the expression and activity of galactosyltransferase involved in IgG galactosylation in cooperation with the *Fas*^{lpr} and *FasL*^{gld} genes.

Recently, we found the occurrence of abnormal IgG galac-

tosylation, similar to that in control MRL-*Fas*^{lpr} mice with arthritis, in MRL-*Fas*^{lpr} mice depleted of CD4^{+} T cells, which failed to develop arthritis, suggesting that the abnormality in IgG galactosylation does not arise as a consequence of disease development [29]. Rather, this abnormality may act as an accelerator in the development of arthritis, when considering that arthritis in MRL-*Fas*^{lpr} mice is a multigenic disease [10]. Further study from the viewpoint of genomics is necessary to elucidate the mechanism of the aberrant existence of agalactosylated IgG and the role of agalactosylated IgG with regard to disease progression in MRL mice with a deficit in Fas/FasL-mediated apoptosis.

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