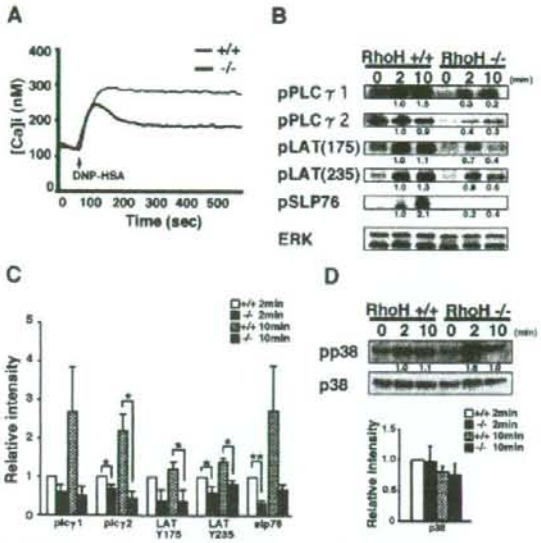

FIGURE 5. Defective FcεRI-mediated Ca^{2+} flux and adaptor phosphorylation in $RhoH^{-/-}$ mast cells. *A*, Ca^{2+} flux in $RhoH^{+/+}$ (gray line) and $RhoH^{-/-}$ (black line) BMMCs was monitored. Anti-DNP IgE sensitized BMMCs were stimulated with 100 ng/ml DNP-HSA at 70 s. Shown is representative data from two independent experiments, $n = 6$ for each group. *B*, Anti-DNP IgE sensitized BMMCs were stimulated with 10 ng/ml DNP-HSA for the indicated periods. Cell lysates were analyzed by Western blot using the indicated Abs. Numbers below the bands indicate the relative intensity of each band. Blotting for ERK was used for confirmation of equal loading to wells. Data shown is representation of three independent experiments. *C*, The bar graph represents band intensities from (*B*) relative to 2 min stimulated $RhoH^{+/+}$ in each lane. *D*, Activation of p38 in $RhoH^{-/-}$ BMMCs (*upper*). Shown is representative data from at least three independent experiments. Relative intensity of each sample is shown as in *C* (*lower*) with the mean and SE for the bar graph. Statistical significance was determined by paired *t* test; *, $p < 0.05$; **, $p < 0.01$.

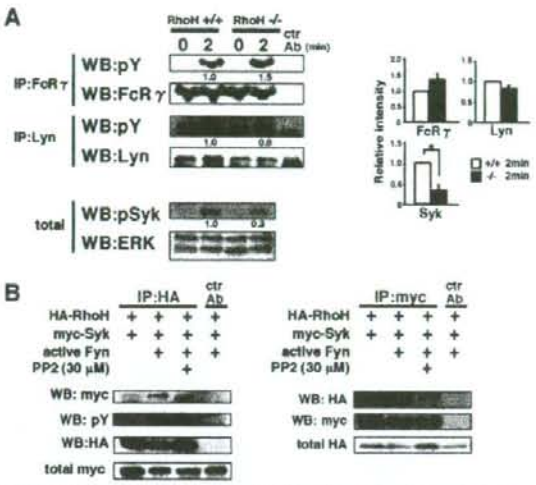

FIGURE 6. RhoH associates with Syk and regulates its activation. *A*, Anti-DNP IgE sensitized BMMCs were stimulated with 5 μg/ml anti-FcεRI for the indicated periods. Cell lysates were immunoprecipitated with anti-FcRγ Ab (*left upper*) or -Lyn Ab (*left middle*) or control Abs (ctr Ab) and analyzed by Western blotting using indicated Abs. The total lysates were analyzed by Western blotting using anti-phosphoSyk and -ERK Abs (*left lower*). The relative intensity to 2 min stimulated $RhoH^{+/+}$ BMMCs in each lane is shown in the right panel. Shown is representative data from three independent experiments and the mean and SE in the bar graph. Statistical significance was determined by paired *t* test; *, $p < 0.05$. *B*, 293T cells were transiently transfected with the indicated expression vectors. After 24 h, cells were incubated with or without PP2, lysed, and immunoprecipitated with anti-HA (*left*) or anti-myc (*right*) or control Abs (ctr Ab) followed by Western blotting with the indicated Abs. Total lysates were analyzed by Western blotting using anti-myc (*left*) or anti-HA Ab (*right*) to confirm the expression of myc-Syk or HA-RhoH, respectively.

FcεRI-mediated degranulation and cytokine production by $RhoH^{-/-}$ mast cells

The reduction in PSA without obvious developmental defects of mast cells in $RhoH^{-/-}$ mice indicated a functional deficiency in $RhoH^{-/-}$ mast cells. Therefore, we analyzed FcεRI-mediated degranulation of BMMCs from $RhoH^{-/-}$ mice in vitro. BMMCs were stimulated with varying doses of Ag, and degranulation was measured by the release of β-hexosaminidase, an enzyme found in mast cell granules. As shown in Fig. 4*A*, degranulation of RhoH-deficient BMMCs in response to Ag/IgE Ab-mediated cross-linking was significantly reduced.

Total β-hexosaminidase content from $RhoH^{+/+}$ and $RhoH^{-/-}$ BMMCs were comparable (data not shown), suggesting that RhoH is dispensable for granule biogenesis in BMMCs. We next measured gene expression of IL-6 and TNF-α (26), well-established targets of FcεRI signaling in mast cells. We stimulated $RhoH^{+/+}$ and $RhoH^{-/-}$ BMMCs for 1 h and then measured mRNA expression levels of IL-6 and TNF-α by real time RT-PCR. Expression of IL-6 and TNF-α in BMMCs was severely inhibited in the absence of RhoH (Fig. 4*B*).

Impaired Ca^{2+} influx and adapter phosphorylation in $RhoH^{-/-}$ mast cells

Engagement of FcεRI results in tyrosine phosphorylation of kinases and adaptors, and then an increase in intracellular Ca^{2+} concentration (27, 28). To explore the role of RhoH in FcεRI-dependent signal transduction, we first analyzed Ca^{2+} mobilization in RhoH-deficient BMMCs. As shown in Fig. 5*A*, Ca^{2+} concentration reached the maximum level within 150 s in cells from $RhoH^{+/+}$ and $RhoH^{-/-}$ mice after Ag challenge. The maximal Ca^{2+} concentration after the Ag challenge was, however, far less in RhoH-deficient BMMCs, indicating that the signaling defect in $RhoH^{-/-}$ cells lies upstream of Ca^{2+} mobilization. Indeed, phosphorylation of PLCγ1 and PLCγ2, which is essential for FcεRI-dependent Ca^{2+} mobilization, was significantly reduced in $RhoH^{-/-}$ BMMCs (Fig. 5, *B* and *C*). Because phosphorylation of the LAT adaptor is required for the activation of PLCγ1 and 2, we next investigated the phosphorylation status of LAT and SLP76 upon stimulation. As shown in Fig. 5, *B* and *C*, FcεRI-induced phosphorylation of both LAT (Y175 and Y235) and SLP76 were significantly inhibited. Conversely, the activation of p38 upon FcεRI engagement in RhoH BMMCs was comparable to that of $RhoH^{+/+}$ BMMCs (Fig. 5*D*).

RhoH associates with Syk and regulates its activation

These results imply the signal transduction upstream of these adaptors is defective in RhoH-deficient mast cells. The adaptors LAT and SLP-76 are phosphorylated by the kinase Syk upon antigenic stimulation (29), therefore we next examined the activation of Syk. We found that stimulation-dependent phosphorylation of Tyr 519/520 residues of Syk, a known determinant of its kinase activity, was severely reduced in RhoH-deficient BMMCs (Fig. 6*A*).

Therefore, we conclude that FcεRI-induced activation of Syk is dependent on RhoH, which is essential for the phosphorylation of LAT and SLP-76 adaptors as well as PLC phosphorylation and Ca^{2+} mobilization in mast cells.

Syk binds to phosphorylated tyrosines in the ITAM motif of the γ-subunit of FcεRI, and is phosphorylated by Lyn or by itself. We therefore examined phosphorylation of FcRγ and Lyn, which are the earliest events after FcεRI engagement and required for mast cells activation (30). As shown in Fig. 6*A*, phosphorylation of FcRγ and Lyn in $RhoH^{-/-}$ BMMCs was comparable to that in $RhoH^{+/+}$. These results indicate that cross-linking and activation of immediate downstream molecules of FcεRI occur normally in the absence of RhoH, and we concluded that the impaired phosphorylation of Syk is responsible for the defective FcεRI-dependent signal transduction in $RhoH^{-/-}$ BMMCs.

RhoH has been reported to associate with ZAP-70 in T cells (7). Because Syk and ZAP-70 belong to the same family and are functionally analogous, we hypothesized that RhoH might associate with Syk and act as an adaptor for Syk. To test this hypothesis, we coexpressed HA-tagged RhoH, myc-tagged Syk, and constitutively active Fyn in 293T cells and performed coimmunoprecipitation of RhoH and Syk. We found that RhoH associated with Syk and this association was enhanced by constitutively active Fyn (Fig. 6*B*). Concomitantly, RhoH was phosphorylated in the presence of active Fyn. Treatment of cells with PP2, an inhibitor of src family kinases, inhibited the phosphorylation of RhoH and the interaction between RhoH and Syk. Because FcεRI ligation activates Fyn in mast cells, RhoH would be able to interact with Syk more strongly, and therefore RhoH may function as an adaptor molecule for Syk in mast cells in a way analogous to that in T cells. This possibility may provide an explanation for the defective FcεRI signaling in $RhoH^{-/-}$ mast cells.

Discussion

Mast cells play important roles in initial immunological responses as well as in the clearance of parasitic infections (9, 10, 31, 32). One of the major functions of mast cells is the immediate release of histamine and other inflammatory mediators from their intracellular granules to increase vascular permeability upon antigenic cross-linking of surface FcεRI (33). In the present study, we demonstrated that the hematopoietic lineage specific atypical small GTPase RhoH, plays critical roles in mast cell function by facilitating phosphorylation of Syk in FcεRI-dependent signal transduction.

We observed impaired degranulation and cytokine production in RhoH deficient mast cells (Fig. 4) without obvious developmental defects of mast cells (Fig. 3). This result is consistent with the fact that FcεRI-dependent signaling is not required for mast cell development. As a matter of fact, the mast cell-related phenotype in $RhoH^{-/-}$ mice is similar to the one in mice deficient for Syk (16), LAT (34), SLP76 (35, 36), Fyn (18), or Btk (37, 38), which are all involved in FcεRI-related signaling. We observed both impaired Ca^{2+} influx and phosphorylation of PLCγ1 and 2, LAT (Y175, Y235), and SLP-76 (Y128) upon FcεRI stimulation in mast cells (Fig. 5). We saw a slight but reproducible decrease in ionomycin -induced degranulation in $RhoH^{-/-}$ BMMCs (Fig. 4*A*). Interestingly, a similar phenotype was seen in SLP76 mutant BMMCs, in which the calcium influx upon ionomycin treatment was lower than that of wild-type BMMCs (36). This might be because adaptor molecules like RhoH and SLP76 can affect the signaling events downstream of calcium release from the ER, or might indicate the existence for a possible positive feedback pathway from calcium signaling to the upstream of adaptor molecules.

RhoH was initially reported as an antagonist for the Rac1/RhoA/cdc42-dependent activation of NF-κB and p38 (3, 4), and has also been proposed to repress LFA-1 activation (5). More recently, RhoH has been reported to act as an adaptor for ZAP-70 by associating with ZAP-70 via its ITAM-like motif, and its association is enhanced upon TCR stimulation (7). ZAP-70 is not expressed in mast cells, but instead, Syk, which belongs to the same kinase family as ZAP-70, is expressed and plays essential roles in FcεRI signaling (16). In this study, we demonstrated that RhoH associates with Syk, and phosphorylated RhoH can interact with Syk more efficiently (Fig. 6*B*). Indeed, the reduced number of DP thymocytes in $RhoH^{-/-}$ mice cannot be explained by the functional defect of ZAP-70, because $ZAP\text{-}70^{-/-}$ mice showed normal β-selection (39). However, it is known that Syk and ZAP-70 have redundant roles in β-selection during T cell development (40). Therefore, our results showing that RhoH associates with ZAP-70 and Syk explain the impairment of β-selection in $RhoH^{-/-}$ mice. Considering the fact that Syk is critical in B cell development and BCR-dependent activation of B cells (41, 42), it is surprising that $RhoH^{-/-}$ mice showed normal B cell development and activation (data not shown). It is possible that B cells have another molecule having similar functions to RhoH, or the requirement for recruiting Syk to the membrane proximal could be different between BCR- and FcεRI-dependent signal transduction.

RhoH was reported to inhibit SDF-dependent activation of Rac1 in hematopoietic progenitor cells, and this was probably due to the inhibition of membrane targeting of Rac1 by RhoH (43). Therefore, the activity of Rac1 could be increased in the absence of RhoH. Indeed, Dorn et al. (6) showed that the PAK-binding activity of Rac1 in $RhoH^{-/-}$ T cells was increased without TCR stimulation. We did not observe enhanced phosphorylation of p38, a major downstream molecule of Rac1 in the absence or presence of FcεRI stimulation in $RhoH^{-/-}$ BMMCs (Fig. 5*D*), indicating that Rac1 is not over-activated in $RhoH^{-/-}$ mast cells. Precise function of RhoH on Rac1 regulation in mast cells should be elucidated in further studies.

Two independent groups have reported phenotypes of RhoH knockout mice (6, 7), and both of them showed defective T cell development and activation. Although both groups reported impaired phosphorylation of LAT (Y195) upon TCR stimulation, there is a discrepancy in the phosphorylation status of ZAP-70 (Y319) between the two groups. Dorn et al. (6) reported unaltered phosphorylation of ZAP-70, whereas Gu et al. (7) showed severely impaired ZAP-70 phosphorylation. Consequently, the former group hypothesized that RhoH is important for interaction between activated ZAP-70 and its substrate LAT, whereas the latter group hypothesized that RhoH is important for recruiting ZAP-70 to membrane proximal before activation. We observed impaired Syk activation upon FcεRI stimulation, evaluated by phosphorylation of Y519/520 in $RhoH^{-/-}$ mast cells (Fig. 6*A*), therefore, our results in mast cells are more consistent with the latter model in T cells. We also showed that RhoH and Syk can associate with each other without stimulation albeit very weakly, and the association was greatly enhanced by activation (Fig. 6*B*). From these results, we hypothesize that phosphorylated RhoH can associate strongly with Syk to keep Syk molecules at membrane proximal, thus facilitating effective activation of Syk. We tried to prove RhoH-dependent Syk recruitment by immunoprecipitaion and membrane subfractionation experiments. However, we were unable to detect membrane recruited Syk, possibly due to the fact that it is such a small proportion of total Syk (44). Future studies should clarify the function of RhoH in the recruitment of endogenous Syk in mast cells.

In the present study, we demonstrated that RhoH plays an important role in FcεRI-mediated activation of mast cells. RhoH$^{-/-}$ mice exhibited reduced systemic anaphylaxis in vivo, and RhoH$^{-/-}$ mast cells failed to degranulate and produce cytokines upon FcεRI stimulation in vitro. Because FcεRI-induced activation of Syk is dependent on RhoH, downstream events including phosphorylation of LAT, PLCγ1 and 2, and SLP-76 as well as Ca^{2+} mobilization in mast cells were all dependent on RhoH. RhoH associated with Syk when exogenously introduced, therefore it is possible that RhoH functions as an adaptor for Syk in mast cells, in a way analogous to its interaction with ZAP-70 in T cells (7). Collectively, these results indicate that RhoH positively regulates FcεRI signal transduction in mast cells. Because Syk is expressed in many kinds of hematopoietic lineage cells and involved in various ITAM-mediated signal transduction pathways (45), our current finding that RhoH facilitates Syk activation will shed new light on ITAM-based immune responses.

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Disclosures

The authors have no financial conflict of interest.

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Characterization of mice deficient in Melanocortin 2 receptor on a B6/Balbc mix background

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ABSTRACT

We have previously reported that Melanocortin 2 receptor ($MC2R^{-/-}$) deficient mice on B6 N5 generations exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF) and lack of detectable levels of corticosterone, and reduced serum concentrations of aldosterone and epinephrine. All $MC2R^{-/-}$ mice on B6/N8 background die within 2 days after birth, while about half of the $MC2R^{-/-}$ mice on B6/Balbc mix background survived to adulthood. Both male and female $MC2R^{-/-}$ mice were fertile, suggesting that normal development and function of reproductive organs. $MC2R^{-/-}$ mice delivered from $MC2R^{-/-}$ dams failed to survive due to lung failure, suggesting that fetal or maternal corticosterone is essential for lung maturation. $MC2R^{-/-}$ mice failed to activate the hypothalamic–pituitary–adrenal axis in response to both immune and non-immune stimuli. $MC2R^{-/-}$ mice maintained glomerular structure and achieved electrolyte homeostasis by the activation of the renin–angiotensin–aldosterone system under low aldosterone and undetectable levels of corticosterone.
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1. Introduction

The body responds to stress by activation of the hypothalamic–pituitary–adrenal (HPA) axis and release of glucocorticoids (GCs) under the control of ACTH. ACTH secreted from the anterior pituitary is in turn regulated by hypothalamic corticotropin-releasing hormone (CRH) and Arginine vasopressin (AVP). This HPA axis is regulated by negative feedback exerted by serum cortisol levels on both the hypothalamus and the pituitary gland. ACTH is also the main regulator of adrenal cortical growth. Tissue specific post-translational cleavage of the prohormone, proopiomelanocortin (POMC) gives rise to bioactive peptides including melanotropic peptides, ACTH and several endorphins. We have previously generated mice with an inactivation mutation of the Melanocortin 2 receptor (MC2R) gene, and reported that MC2R on fifth generation of backcrossing to C57/BL6 (B6/N5) leads to neonatal lethality in about three-quarters of $MC2R^{-/-}$ pups, possibly due to hypoglycemia (Chida et al., 2007). Those surviving to adulthood exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF), lack of detectable levels of GC, and reduced serum concentrations of aldosterone and epinephrine (Chida et al., 2007).

GCs are secreted into the systemic circulation from the adrenal cortex and initiate a broad range of actions throughout the organism that regulate the function of multiple organ systems including the central nervous, endocrine, and immune systems. The physiological effects of GCs are mediated by intracellular glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) that function as ligand-dependent transcription factors (Mangelsdorf et al., 1995). Even though MR has a high affinity for GCs, the majority of the physiological effects of GCs are thought to be mediated via the GR, which is expressed more ubiquitously and is a stronger transcriptional activator. Physiologically, MR is thought to act primarily as a high affinity receptor for mineralocorticoids to control the sodium/potassium balance in the kidney and large intestine.

$MC2R^{-/-}$ mice are valuable for familial GC deficiency studies, and a unique animal model for investigation of the physiological functions of GC. The physiological role of GR and MR in vivo have been extensively studied in conditional KO mice (Tronche et al., 1999; Berger et al., 2006), due to the neonatal death of

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global GR$^{-/-}$ mice and MR$^{-/-}$ mice. Comparison of MC2R$^{-/-}$ mice, which are completely deficient for GC, with GR$^{-/-}$ or MR$^{-/-}$ mice may provide valuable information on the possible roles of GC function independent of GR and/or possible roles of GR function independent of GC. In this study, by altering genetic backgrounds, we obtained MC2R$^{-/-}$ mice with improved survivability that can be used for general analyses of stress response and reproduction. We established MC2R$^{-/-}$ mice on a B6/Balbc mix background from previously established B6 congenic MC2R$^{-/-}$ mice strains. About half of them survived neonatal death and grew to adulthood. We found that MC2R$^{-/-}$ pups from MC2R$^{-/-}$ dams die due to lung failure. MC2R$^{-/-}$ mice failed to increase plasma corticosterone levels in response to restraint and LPS stimulation and MC2R$^{-/-}$ mice have preserved renal glomerular morphology.

2. Materials and methods

2.1. Animals

Generation of MC2R deficient mice was described previously (Chida et al., 2007). All the mice were kept under specific pathogen-free conditions in an environmentally controlled clean room in the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo or in the Laboratory Animal Research Center, IMCJ. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments. MC2R deficient mice were backcrossed into the C57BL/6J genetic background and intercrossed at N3, N4, N6, N7, and N8 generations to get MC2R$^{-/-}$ mice. MC2R$^{+/-}$ mice/B6 N8 mice were crossed with Balb/c mice and their pups were intercrossed and characterized in this study.

2.2. Blood analysis

12-Week-old male MC2R$^{+/-}$ or MC2R$^{-/-}$ mice were i.p. injected at 10:00 with either LPS from *Escherichia coli* serotype 0111:B4 (Sigma) (300 μg) or PBS. After 3 h, animals were sacrificed by decapitation and blood was collected. 12-Week-old male MC2R$^{+/+}$ or MC2R$^{-/-}$ mice were subjected to restraint for 30 min. Blood glucose level levels were measured by the glucose oxidase method (Terumo). Serum corticosterone levels were determined by RIA (Amersham, UK). Serum IL-6 levels were measured by ELISA (PharMingen, San Diego, CA) according to the manufacturer's instructions.

2.3. Histology

Fetal lung tissues (E18.5), adult heart and adult kidney tissues (6 months old) were fixed overnight in 4% paraformaldehyde in PBS, embedded in paraffin, and sections at a thickness of 6 μm were collected on slide glasses and stained with hematoxylin–eosin. Bright-field images were obtained by microscopy.

2.4. Quantitative real-time polymerase-chain-reaction (QRT-PCR) analysis

For determination of relative mRNA concentrations, total fetal lung and adult kidney RNA, isolated by sepazol were subjected to reverse transcription by Superscript III (Invitrogen). cDNA was analyzed by QRT-PCR with SybrGreen (Invitrogen) using the ABI7900HT Fast Real time PCR system (ABI). RPS3 mRNA was used for normalization. Primer sequences were designed using the Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) (Roche Applied Science).

2.5. Statistical analysis

All values were calculated as means ± S.E.M. Comparisons of two groups were analyzed by the Student's *T*-test. In all analyses, a two-tailed probability of less than 5% (i.e. $p < 0.05$) was considered statistically significant.

3. Results and discussion

3.1. The effect of B6 background on the neonatal survival in MC2R$^{-/-}$ mice

We have previously reported that the majority of MC2R$^{-/-}$ mice under B6/N5 background died due to hypoglycemia before P2.5 (Chida et al., 2007). To determine whether the survival rate could be influenced by alleles present in the B6 genetic background, we analyzed the survival rate of MC2R$^{-/-}$ mice at each generation. The heterozygous mice at N3, N4, N6, N7, and N8 generations were intercrossed to generate mice homozygous for the MC2R mutation. As shown in Table 1, B6 genetic background significantly decreased the survival rate in MC2R$^{-/-}$ pups.

Table 1
The effect of B6 background on neonatal survival in MC2R$^{-/-}$ mice.

Background	+/+	+/−	−/−
N3	1	5	2 (25%)
N4	15	29	3 (6%)
N6	12	30	3 (7%)
N7	11	9	1 (5%)
N8	15	22	0 (0%)

The heterozygous mice at N3, N4, N6, N7, and N8 generations were intercrossed to generate homozygous for the MC2R mutation.

Genetic background has been shown to influence phenotype and survival of mutants in a variety of gene knockout animal models (Doetschman, 1999). Lethality for a variety of gene defects can be modified by allelic differences contributed by various inbred strain backgrounds. We have demonstrated that modifiers in the B6 genetic background reduced the survival of MC2R-null pups. Variation of disease onset and severity has been reported for familial glucocorticoid deficiency (FGD) patients with various mutations in MC2R (Clark et al., 2005). While many FGD patients show symptoms in the neonatal period and have undetectable circulating cortisol, others pass unrecognized until later childhood, and their cortisol deficiency might only be recognized after a short ACTH stimulation test. Penetrance variations in different populations suggest that genetic modifier loci or environmental factors should modulate the effect of MC2R mutation also in FGD patients.

3.2. The survival rate in MC2R$^{-/-}$ mice on B6/Balbc (BDF) background

To rescue neonatal death in MC2R$^{-/-}$ mice, we crossed MC2R$^{+/-}$ B6/N8 mice with Balb/c mice and their F1 offspring were intercrossed to generate mice homozygous for the MC2R mutation. To definitively analyze the mortality rate on B6/Balbc mix background, we collected all dead pups and analyzed their genotype and identified each pup at P14 by ear punch to analyze their genotype and follow their growth. On B6/Balbc mix background, 56% of MC2R$^{-/-}$ mice survive to adulthood. 22% died before P2.5, and 23% died between P14 and P28 (Fig. 1A). Mortality at weaning period is interesting because weaning is a crucial period where mice need to adapt to nutritional modifications. Hypoglycemia, due to an inability to adapt, may be the reason for death at weaning. To clarify this possibility, we measured blood glucose levels at P14. Levels were significantly decreased in MC2R$^{-/-}$ mice compared to MC2R$^{+/-}$ mice, while there was no significant difference in blood glucose level between dead and survived MC2R$^{-/-}$ mice (Fig. 1B). As we found aldosterone level was significantly decreased in adult MC2R$^{-/-}$ mice, it is possible that salt wasting may be the reason for death at this stage.

3.3. MC2R$^{-/-}$ mice have normal fertility

The ability of stress to interfere with reproductive functions and the association between the HPA axis and the hypothalamic–pituitary–gonadal (HPG) axis are generally recognized (Rivier and Rivest, 1991; Ferin, 1999; Kalantaridou et al., 2004). To analyze the potential effect of HPA disturbances in MC2R$^{-/-}$ mice on reproductive function, the fertility of male and female MC2R$^{-/-}$ mice was examined. Male MC2R$^{-/-}$ mice were capable of siring female mice with the average litter size. One male MC2R$^{-/-}$ mice sired 46 times

Table 2
MC2R$^{-/-}$ mice maintain normal litter size.

Mating pairs (female × male)	Litter size	n
−/− × +/−	6.8 ± 0.5	18
+/− × +/−	7.7 ± 0.5	22
+/− × −/−	7.8 ± 0.3	40

Mice carrying the MC2R mutation were mated, and their litter sizes were counted. Values are shown as the mean ± S.E.M.

in 6 months. Female MC2R$^{-/-}$ mice were also fertile and delivered their offspring with similar litter size compared to MC2R$^{+/-}$ mice (Table 2). However, we found a severely reduced survival rate among MC2R$^{+/-}$ pups delivered from MC2R$^{-/-}$ dams (D.C. unpublished observations). The reason for decreased survival observed in pups derived from MC2R$^{-/-}$ is currently under investigation. These observations indicate that the lack of MC2R does not affect fertility, however we do not necessarily deny a possible role of HPA axis in reproductive behavior (Heinrichs et al., 1997).

3.4. MC2R$^{-/-}$ mice delivered from MC2R$^{-/-}$ dams die due to lung failure

When female MC2R$^{-/-}$ mice were crossed with MC2R$^{+/-}$ males, almost all the surviving pups at P0.5 were heterozygous for the MC2R allele, suggesting that almost all the MC2R$^{-/-}$ pups were dead before P0.5. As GR$^{-/-}$ pups (Cole et al., 1995), CRHR1$^{-/-}$ pups (Smith et al., 1998), CRH$^{-/-}$ pups delivered from CRH$^{-/-}$ dams (Muglia et al., 1995) die at birth due to lung hypoplasia, we analyzed fetal lung histology at E18.5 and found that MC2R$^{-/-}$ pups from MC2R$^{-/-}$ dams had severe lung atelectasis (Fig. 2A). Consistently, the expression of surfactant apoprotein mRNAs (Sftpb, Sftpc and Sftpd) at E18.5 was reduced in MC2R$^{-/-}$ pups from MC2R$^{-/-}$ dams (Fig. 2B). As MC2R$^{-/-}$ pups from MC2R$^{+/-}$ dams and MC2R$^{+/-}$ pups from MC2R$^{-/-}$ dams are viable, either source of corticosterone, from the mother or pups, is sufficient for maturation of fetal lung, consistent with the observations for CRH$^{-/-}$ mice (Muglia et al., 1995).

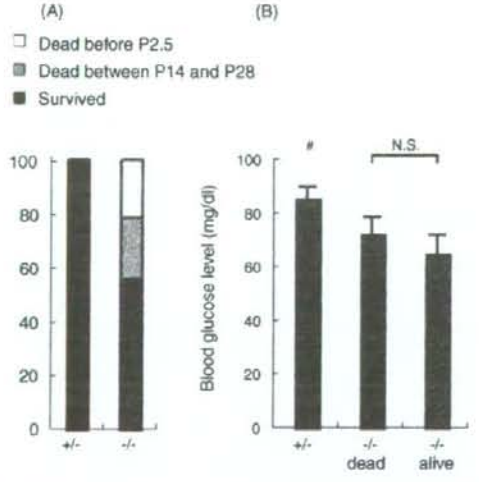

Fig. 1. Survival rate in MC2R$^{-/-}$ mice on B6/Balbc mix background. (A) On BDF background, 56% of MC2R$^{-/-}$ mice survived to adulthood. 22% of MC2R$^{-/-}$ mice died before P2.5, and 23% of MC2R$^{-/-}$ mice died between P14 and P28. The results were analyzed with (P0.5) MC2R$^{-/-}$ (n = 93) and MC2R$^{+/-}$ (n = 93) mice. (B) Blood glucose levels at postnatal day 14.5 at noon. MC2R$^{-/-}$ (n = 26) and MC2R$^{+/-}$ (n = 35) pups were analyzed. Data for MC2R$^{-/-}$ pups is shown and assessed separately based on whether they were alive (n = 19) or not (n = 7) at P28. Data are expressed as means ± S.E.M. Statistical significance was determined by ANOVA. *p < 0.05.

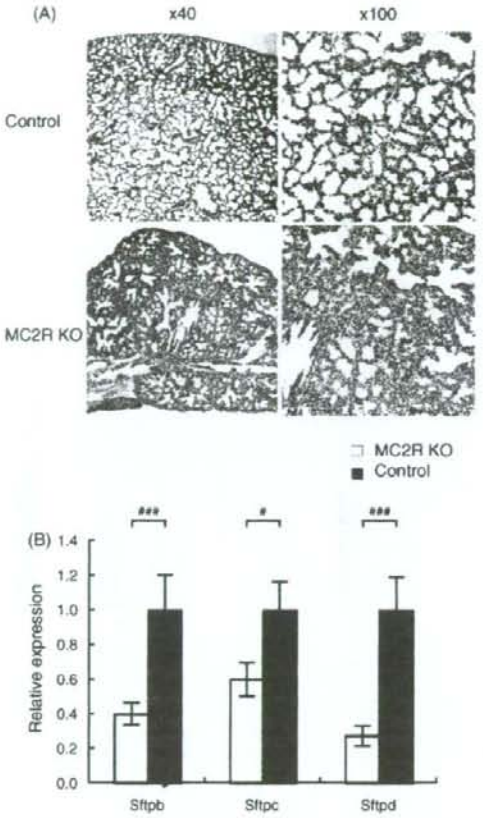

Fig. 2. Histological analysis of the fetal lung of MC2R$^{-/-}$ pups derived from MC2R$^{-/-}$ dams. (A) Hematoxylin–eosin staining of sections from the fetal lung of MC2R$^{+/-}$ or MC2R$^{-/-}$ pups from MC2R$^{-/-}$ dams. MC2R$^{-/-}$ female mice were mated with MC2R$^{+/-}$ heterozygous male and pups were obtained at E18.5 delivered by caesarean section. (B) Expression of surfactant proteins in fetal lung (E18.5) MC2R$^{-/-}$ (n = 8) and MC2R$^{+/-}$ (n = 6) mice from MC2R$^{-/-}$ dams were determined by QRT-PCR. Data are expressed as means ± S.E.M. Statistical significance was determined by T-test. ***p < 0.001; *p < 0.05.

3.5. Serum corticosterone and IL-6 after acute restraint stress and LPS-induced immune challenge

The adult adrenal histology of MC2R$^{-/-}$ mice under B6/Balbc background was quite similar to B6/N5 background (data not shown). To determine whether MC2R is important for the activation of the HPA axis to stressors not associated with immune cell activation, MC2R$^{-/-}$ and MC2R$^{+/+}$ mice were restrained for 30 min followed by measurement of serum corticosterone (Fig. 3A). After restraint stress, serum corticosterone increased to 106.4 ± 16.7 ng/ml in MC2R$^{+/-}$ mice. In contrast, serum corticosterone in MC2R$^{-/-}$ mice was not detectable. To determine whether MC2R is important for activation of the HPA axis after immune system stimulation, MC2R$^{-/-}$ and MC2R$^{+/-}$ mice were injected with LPS (Fig. 3B). After LPS injection, serum corticosterone increased to 214.7 ± 7.5 ng/ml in MC2R$^{+/-}$ mice. In contrast, serum corticosterone in MC2R$^{-/-}$ mice was not detectable. As IL-6 has been

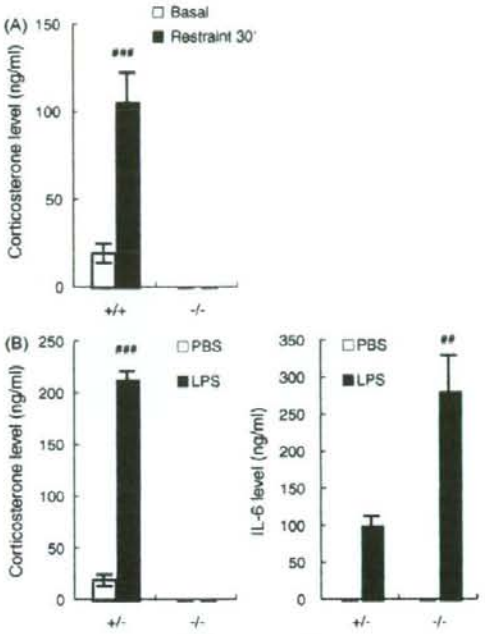

Fig. 3. Serum corticosterone and IL-6 levels after restraint or injection with LPS in MC2R$^{-/-}$ mice. (A) Basal serum corticosterone levels were measured in MC2R$^{-/-}$ ($n = 4$) and MC2R$^{+/+}$ mice ($n = 5$). Serum corticosterone levels were measured 30 min after restraint in MC2R$^{-/-}$ ($n = 4$) and MC2R$^{+/+}$ mice ($n = 5$). (B) Serum corticosterone and IL-6 levels were measured 3 h post-i.p. injection of LPS (μg/kg body weight) or PBS for MC2R$^{-/-}$ (PBS, $n = 5$; LPS, $n = 6$) and MC2R$^{+/-}$ mice (PBS, $n = 7$; LPS, $n = 8$). Statistical significance was determined by T-test. $^{**}p < 0.01$; $^{***}p < 0.001$.

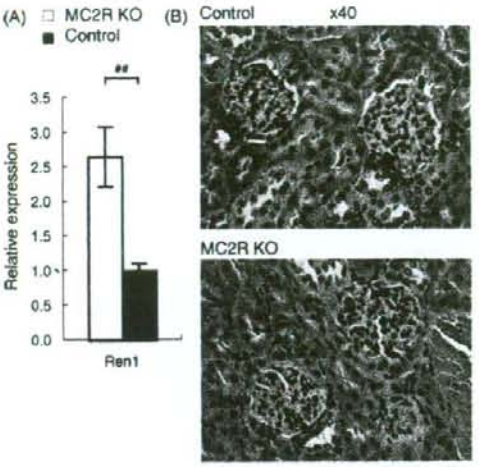

Fig. 4. Deficiency of MC2R maintained renal glomerular morphology and induced renal renin mRNA expression. (A) Expression of renal renin mRNA in adult kidney from 3-month-old MC2R$^{-/-}$ ($n = 5$) and MC2R$^{+/-}$ ($n = 4$) mice was determined by QRT-PCR. Data are expressed as means ± S.E.M. Statistical significance was determined by T-test. $^{**}p < 0.01$. (B) Hematoxylin–eosin staining of sections from kidney of MC2R$^{-/-}$ mice or MC2R$^{+/-}$ mice.

reported to increase during immune stress, we analyzed serum levels of IL-6. Serum IL-6 levels in MC2R$^{-/-}$ mice after LPS stimulation was significantly increased compared to MC2R$^{+/-}$ mice due to blunted negative feedback regulation by corticosterone, indicating that GCs are important regulators of inflammation.

3.6. *Deficiency of MC2R maintained renal glomerular morphology and induced renal renin mRNA expression*

We previously found that serum aldosterone levels were significantly decreased and the expression of angiotensin receptor 1b (AT1bR) was significantly induced in MC2R$^{-/-}$ mice (Chida et al., 2007). The observations are consistent with the recently described report that a small number of patients with homozygous nonsense mutations show biochemical evidence of mineralocorticoid deficiency (Lin et al., 2007). We found that the expression of renin mRNA was significantly increased in MC2R$^{-/-}$ mice (Fig. 4A), suggesting that the renin–angiotensin–aldosterone system (RAAS) was activated to maintain serum electrolytes and blood pressure under low aldosterone levels. As increased renin–angiotensin tone was suggested to play pathological roles in cardio-renal damage, we assessed cardiac morphology and renal glomerular structure by light microscopy in 6-month-old MC2R$^{-/-}$ mice and studied the possible effect of RAAS activation on cardiac hypertrophy and renal glomerular structure. We found that the histological parameters were not significantly different from WT mice (Fig. 4B and data not shown). These results suggest that low aldosterone level due to decreased ACTH signaling prevented cardio-renal damage under increased renin–angiotensin tones (Brown, 2003). HPA axis activation by stress may exacerbate cardiovascular disease by increasing aldosterone levels, therefore suppression of the HPA axis may be an important way to prevent the progression of the metabolic syndrome to cardiovascular disease.

In this study, we established an MC2R deficient mice line on a B6/Balbc mix background and characterized these mice for reproductive function, stress response and possible cardio-renal effects. MC2R$^{-/-}$ mice are a valuable animal model for studying familial GC deficiency, and a unique animal model to study the physiological function of GC and ACTH-MC2R signaling.

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ORIGINAL ARTICLE

Time of initial appearance of renal symptoms in the course of systemic lupus erythematosus as a prognostic factor for lupus nephritis

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Abstract The prognosis of lupus nephritis (LN) was studied retrospectively in two LN categories, LN manifested initially at systemic lupus erythematosus (SLE) onset (I-LN) and LN of delayed manifestation after SLE onset (D-LN), based on a chart review (C) of 154 SLE (85 LN) patients with a mean observation of 20.8 ± 9.3 years and a questionnaire study (Q) of 125 LN patients outside our hospital with mean observation of 17.6 ± 9.2 years. In both study groups, half of I-LN patients were relapse-free by Kaplan–Meier analysis after initial therapy, and the relapsed I-LN patients responded to retherapy at higher 5-year relapse-free rates than those of patients receiving initial therapies for D-LN. At last observation, a higher frequency of prolonged remission was shown in I-LN compared with D-LN patients (C: 22/31, 71% versus 14/49, 29%, $P < 0.01$; Q: 65/89, 73% versus 11/33, 33% $P < 0.01$) and also a higher frequency of irreversible renal damage in D-LN compared with I-LN patients (C: 25/49, 51% versus 2/31, 6%, $P < 0.001$; Q: 14/33, 42% versus 6/89, 7%, $P < 0.001$), although class IV pathology was common in patients (C) in both LN categories. Onset time of lupus nephritis in the course of SLE may affect renal prognosis.

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Introduction

Systemic lupus erythematosus (SLE) is a multiple-organ disease, and lupus nephritis (LN) is a major clinical problem because of its high morbidity and mortality rates. In accordance with the chronic nature of SLE, renal symptoms can manifest at various times in the disease course, and a physician cannot predict the future development of LN at the time of SLE onset. Furthermore, it is unclear whether there is a difference in prognosis between LN manifested at the onset of SLE and LN developed later in the course of SLE, because the clinical significance of time of LN onset in the disease course of SLE has not been clearly described in the literature, to our knowledge. Accordingly, in recent clinical trials on therapies for LN including cyclophosphamide and mycophenolate mofetil, mixtures of cases having various time intervals between SLE onset and LN onset have been studied [1, 2]. Although a prognostic impact of renal pathology and a poor prognosis of class IV disease have been established in LN based on the World Health Organization (WHO) classification [3–5] and more recent criteria [3, 6, 7], a later progression or transformation of the pathology cannot necessarily be predicted at the time of the initial biopsy [8, 9].

In our preliminary chart review, we found that numerous cases of remitted SLE had class IV LN at SLE onset. On the other hand, irreversible renal damage was precipitated in patients who initially showed no renal symptoms but later developed LN with various renal pathologies, and the LN of later development was never a case of senile-onset LN. Thus we undertook to study the possible relationship

between renal prognosis and the time of the initial renal manifestation in the course of SLE. The study consisted of two parts: a chart review in our institute and a replication study to reconfirm the results of the chart review using a questionnaire administered to LN patients outside our hospital.

Patients and methods

Chart review for patients with SLE

Hospital records of the International Medical Center of Japan were reviewed for patients with SLE having a disease duration of 5 years or more. SLE was diagnosed according to the classification criteria of the American College of Rheumatology [10]. The clinical information on 154 patients with SLE (139 females and 15 males) with mean age of 48.9 ± 12.6 (median 48) years at the last observation was available for studying the entire disease course, including 436 major therapeutic interventions for SLE and 22 deaths during 3,189 person-years or mean observation period of 20.7 ± 9.3 years. At the time of the present study, 85 patients (80 females and 5 males) had LN, and 28 of these had irreversible renal dysfunction, including 15 patients on hemodialysis therapy.

Onset age, relapse ages, therapeutic doses if available, renal pathology data if any, and disease status after therapy and at the last observation in each patient were serially input in our database for SLE patients. The definition of relapse will be provided in "Results". Minor dose increases during steroid therapy for mild activities of SLE were not analyzed. Chronological profiles of the onsets and relapses of LN or extrarenal SLE flares were analyzed statistically with Stata 9.0 (Stata Corp., College Station, TX, USA).

Questionnaire study for patients with LN

To reconfirm the results of the chart review study in another LN patient group, we undertook a questionnaire study of SLE patients outside our hospital and collected individual data on LN with a SLE duration of 5 years or more. The questionnaire included ages at all of the hospitalizations due to SLE and/or LN from onset to the present time, daily doses of steroid (number of 5 mg prednisolone tablets) before and at the start of therapeutic interventions, combined use of intravenous pulse steroid or cyclophosphamide, and renal status at each hospitalization and at the present time. Renal status, which was based on the information from an attendant doctor to each patient, was expressed in terms of urine protein (negative, positive, nephrotic, or 1+ to 4+), data of serum creatinine levels if available, and clinical categories including no abnormalities, mild persistent renal disease, nephrotic syndrome, renal dysfunction or on hemodialysis.

The ethics committee of our hospital approved the present study. A questionnaire sheet including a description of the aim of our study was inserted once in the *Journal of Patients' Association of Collagen Diseases* in Japan, which was subscribed to by more than 5,000 patients, including approximately 3,000 patients with SLE. An anonymous letter in reply (datasheet) sent to the board of the above Patients' Association with a completed questionnaire was regarded as informed consent to enter the present study. We received a set of the copied datasheets from the board, which preserved the original sheets.

After removing approximately 50 cases with insufficient information in the datasheets, 7 cases having SLE without LN, and 10 cases of SLE with less than 5 years' duration, we recognized 125 datasheets that contained a sufficient description of LN. The filling of a datasheet and voluntary mailing might have resulted in a strong bias toward selecting informative repliers from among thousands of patients. The 125 patients were characterized by excellent medical compliance and were thought to have preserved written records on their own diseases.

The studied patients

Mean age of the 125 patients (121 females and 4 males) in the present study was 46.5 ± 12.5 years (median 46 years). A total of 331 hospitalizations because of therapy for SLE were mentioned during 2,200 person-years, or mean observation period of 17.6 ± 9.2 years. Chronological profiles of the relapses of LN were analyzed statistically with Stata 9.0 similarly to the analysis of the chart review study. Renal pathology in accordance with the WHO classification was mentioned in only 19 datasheets and was not analyzed.

In our SLE database, a whole chronological profile of each patient including all of the hospitalizations from onset to the last observation was specific to one subject like a fingerprint, and no overlapping cases were found by computer analysis.

Results

Definitions of initial-onset LN and delayed-onset LN in the course of SLE

In both the chart review and the questionnaire study, some of the patients had been observed with no or low-dose steroid therapy for their mild SLE conditions during the early disease course, and the initial urine proteins were documented

as negative in most of these patients or unknown in some of the referral patients, except in a small number of patients who received no therapy for their positive renal symptoms.

In the present study, we defined a SLE relapse as hospitalization in order to treat SLE after previous SLE conditions have subsided in response to treatment during hospitalization. In a small number of cases, initial steroid therapy with 20 mg/day or more of prednisolone equivalent was started at an outpatient clinic, and we classified the therapy as "treatment under hospitalization" for simplicity in the present text. We further defined "initial-onset LN" and "delayed-onset LN" as follows.

Initial-onset LN (I-LN)

I-LN was defined as LN diagnosed at the time of onset of SLE, LN diagnosed at the initial treatment under hospitalization for SLE or LN that emerged during the initial course of therapy under hospitalization. Two of the 34 I-LN patients in the chart review and 7 of the 91 I-LN patients in the questionnaire study met one of the latter two definitions of I-LN.

Delayed-onset LN (D-LN)

D-LN was defined as newly developed LN as a SLE relapse after the previous successful therapy under hospitalization. At the time of the present study, 51 patients in the chart review and 34 patients in the questionnaire study were classified into this category of LN.

Mean ages of the patients at SLE onset and D-LN onset in the present study are shown in Table 1. These data were very similar between the two study groups; the mean interval between SLE onset and D-LN onset was approximately 8.9 years in the chart review and 7.3 years in the questionnaire study.

Time course of D-LN developments in the chart review patients is shown in Fig. 1a. The Kaplan–Meier curve showed that D-LN developed in half of the SLE patients of extrarenal onset, and that half of the instances of D-LN occurred after 10 disease-years of SLE. We note that the curve might not represent a natural course of D-LN development among SLE patients because of possible sampling bias by the chart review in our single institute and based on our knowledge of the widely different frequencies of LN among SLE patients noted in the literature [11]. A comparable analysis could not be performed in the questionnaire study, because we collected only cases with a positive history of LN.

Time course of the first-time relapse of SLE after the initial treatment under hospitalization in the chart review patients (Fig. 1b)

A total of 302 SLE relapses were identified in the 154 chart review patients, and these included 110 renal involvements and 192 extrarenal SLE flares. The patients were classified into one of two groups, I-LN ($n = 34$) and others ($n = 120$), at the start point of the analysis. Patients in the "others" group, in whom LN developed later, were further classified into D-LN ($n = 51$), as defined above.

The Kaplan–Meier curve indicating those free from SLE relapse after the initial treatment under hospitalization showed a longer relapse-free period in the I-LN patients compared with the other patients (12 versus 4 years to the first relapse was expected in 50% of each patient group, $P = 0.008$) (Fig. 1b), when patients who received no or low-dose steroid were removed from the analysis.

Most of the patients without I-LN had mild SLE conditions at the time of the initial treatment under hospitalization, because neuropsychiatric involvement was not common as the initial manifestation (data not shown).

Table 1 Onset age and initial therapy for LN and/or SLE

	Onset age, years (mean ± SD)		Dose of therapy (PSL[a], mg/day)	Patient ratio that received pulse steroid[b] or IVCY[c]	
Chart review ($n = 85$)					
I-LN ($n = 34$)	SLE + LN	29.1 ± 14.8	51.3 ± 15.2 ($n = 31$)	26% (8/31)	10% (3/31)
D-LN ($n = 51$)	SLE	25.0 ± 10.9	35.0 ± 13.1 ($n = 42$)	#	
	LN	33.9 ± 12.2	41.3 ± 14.4 ($n = 45$)	64% (29/45)	2% (1/45)
Questionnaire study ($n = 125$)					
I-LN ($n = 91$)	SLE + LN	30.1 ± 12.1	44.7 ± 14.7 ($n = 84$)	29% (26/91)	12% (11/91)
D-LN ($n = 34$)	SLE	27.1 ± 11.8	39.1 ± 16.7 ($n = 32$)	#	
	LN	34.4 ± 13.5	43.0 ± 15.2 ($n = 27$)	32% (11/34)	15% (5/34)

[a] PSL, prednisolone equivalent

[b] Intravenous methylprednisolone pulse therapy

[c] Intravenous cyclophosphamide pulse therapy

Pulse steroid or IVCY was rarely used for SLE of D-LN patients at the onset of SLE

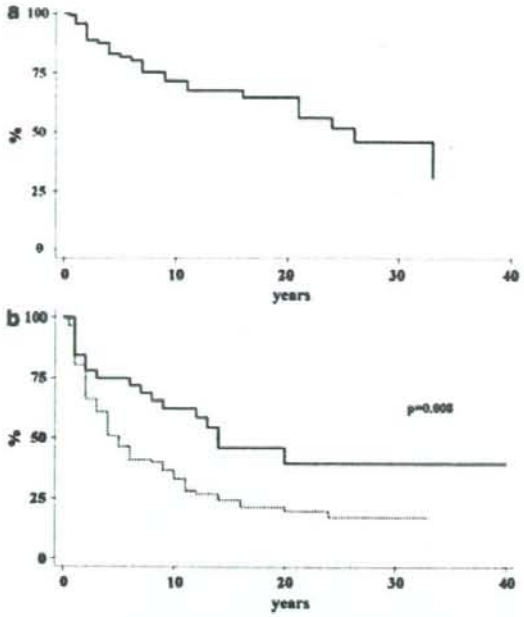

Fig. 1 **a** Kaplan–Meier curve indicating those free from LN development, for patients without initial renal involvement at onset of SLE in the chart review study. Analysis starts at the onset of SLE, and the result shows a time course of developing delayed-onset LN (D-LN). **b** Kaplan–Meier curve indicating those free from SLE relapse after the initial therapy for SLE in the chart review study. Analysis starts at the initial treatment under hospitalization for SLE. *Solid line* patients in whom the initial therapy was targeted at LN (I-LN) ($n = 31$). *Dotted line* patients in whom the initial therapy was targeted at SLE without renal involvement ($n = 95$). The log-rank test was used for statistical analysis

Accordingly, the mean initial steroid dose tended to be higher in the I-LN patients (prednisolone equivalent: 51.3 ± 15.2 mg/day, $n = 31$ versus 36.4 ± 14.1 mg/day, $n = 52$ in the non-I-LN patients of identified steroid dose). High-dose pulse steroid or cyclophosphamide was combined in a quarter of or a small number of I-LN patients (Table 1), respectively, for treating the LN, and was rarely used in non-I-LN patients. Because I-LN and non-I-LN have different organ involvement, the difference in relapse rates after therapy between the two disease categories may not be attributed to the difference in therapeutic intensities. If renal involvement at the onset of SLE indicates a severe form of SLE, a longer relapse-free period in the I-LN patients than in the non-I-LN patients (Fig. 1b) may be paradoxical, or it may be attributed to D-LN development in the non-I-LN patients. Thus, we further studied therapeutic responses in the I-LN patients and the D-LN patients.

Renal relapse-free rates in the I-LN patients (Fig. 2)

The Kaplan–Meier curve indicating freedom from LN relapse after the initial treatment under hospitalization for the I-LN is shown in Fig. 2 for the chart review patients and the questionnaire study patients. A total of five patients who received no therapeutic intervention for LN were removed from the calculation. The results in the two study groups showed consistently that half of the I-LN patients were expected to be renal-relapse-free after the initial therapy.

In the chart review, 18 (58%) of 31 treated patients had no LN relapse throughout the observation period, and all of these patients achieved complete renal remission, although extrarenal SLE flares were observed in 5 patients. Renal relapse was defined as rehospitalization in order to treat SLE accompanied by emerging or increasing proteinuria. Renal remission was defined by normal serum creatinine levels and no urine abnormalities

In the questionnaire study, 52 (58%) out of 89 treated patients had no LN relapse throughout the observation. Of these, 90% (47/52) of the patients achieved complete renal

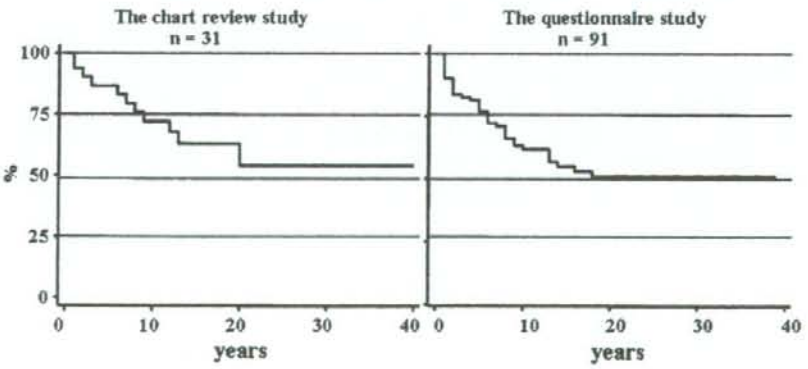

Fig. 2 Kaplan–Meier curve indicating patients free from LN relapse after the initial therapy for I-LN in the two study groups. Analysis starts at the initial treatment under hospitalization for I-LN

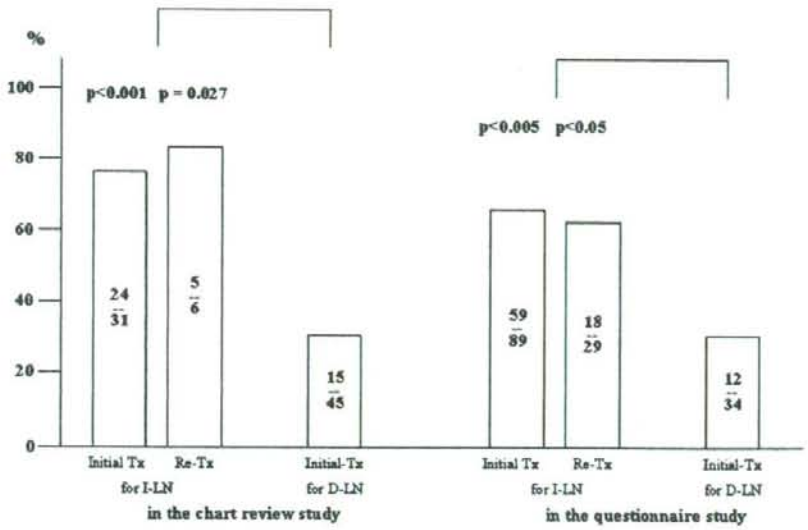

Fig. 3 Response rate to therapy for LN. Initial therapy for I-LN, retherapy for relapsed I-LN, and initial therapy for D-LN were studied by historical follow-up for 5 years; ratios of patients who responded to therapy for LN are shown. Definition of therapeutic response is given in the text. Chi-squared or Fisher's exact probability test was used for statistical analysis

remission. Of the remaining five patients with no relapse but no remission, three patients had massive urine protein and elevated serum creatinine levels at SLE onset that did not respond to initial therapies, and renal deterioration progressed without further therapeutic interventions, and the other two patients had mild persistent urine protein at the last observations.

These results regarding renal relapse suggested that renal remission was common in I-LN only in response to the initial therapy. We further studied prognoses of relapsed I-LN patients in comparison with those of D-LN patients, because first-time relapse of I-LN and onset of D-LN are similarly defined under "relapse of SLE."

Comparison of renal response to therapy between I-LN and D-LN (Fig. 3)

Initial therapies for I-LN or D-LN, respectively, were begun based on renal biopsy in most of the patients of the chart review study, as described later, and positive urine protein was found to be a major reason for the therapeutic intervention in all of the chart review and the questionnaire study patients. Retherapy for relapsed I-LN was begun at the time of rehospitalization in order to treat SLE accompanied by emerging or increasing proteinuria.

Response criteria

Responses to therapy for LN were estimated by 5-year historical follow-up after the initial therapy for I-LN or D-LN, and after the retherapy for I-LN of the first-time relapse. Positive renal response was defined as no relapse, no progressive renal dysfunction, and no nephrotic levels of urine protein during 5-year observation after therapy. The patients in the chart review study who responded to therapy also met response criteria similar to those in the literature [12], i.e., serum creatinine not exceeding the lowest level during treatment under hospitalization, proteinuria less than 3+ or a urine-protein/creatinine ratio less than 1, and no nephritic findings in the urine sediment for at least 6 months after therapy. In the questionnaire study patients, the responses to therapy were identified based on the mention of no renal dysfunction and no or less than 3+ proteinuria after therapy.

Patients who received no therapeutic intervention for renal deterioration were removed from the calculation. In the descriptions below, we classified a patient who showed both renal abnormalities and renal relapse after therapy into a category of renal relapse.

Chart review study

A better renal response to initial therapy was observed in the I-LN patients compared with the D-LN patients (Fig. 3). Initial steroid therapies for LN in the I-LN and the D-LN are shown in Table 1. The difference in the therapeutic doses did not seem large enough to explain the different response rates between the I-LN and the D-LN patients.

WHO class IV histology was documented at the renal biopsy before therapy for LN with similar frequencies (I-LN: 14/21, 64% versus D-LN: 22/39, 56%) in the two LN categories, and the chronic lesions were each identified in only a small number of patients. The total results of

histology identified in 21 I-LN versus 39 D-LN were: II, 1 versus 5; III, 0 versus 1; IVa or b, 6 versus 10; IVc, 2 versus 4; IVd, 0 versus 0; IV of unknown subclass, 6 versus 8; V, 6 versus 11; and there was no significant difference in the distribution of the histology between I-LN and D-LN.

Serological data before therapy (the number of patients) were identified in most of the patients treated for I-LN (31) or D-LN (45). Serum anti-DNA antibody levels were elevated in 89% (24/27) of I-LN patients and 93% (37/40) of D-LN patients; the mean titer in I-LN patients was 100.5 ± 20.1 IU/ml ($n = 11$) by enzyme-linked immunosorbent assay (ELISA) for anti-double-stranded DNA IgG antibodies (normal range <20), 10 ± 2 IU/ml ($n = 10$) by radioimmunoassay (RIA) for anti-DNA antibodies (normal range <6), or unknown except positive results ($n = 3$), whereas that in D-LN patients was 56.7 ± 11.2 IU/ml ($n = 12$) by ELISA, 12 ± 2 IU/ml ($n = 15$) by RIA, or unknown except positive results ($n = 10$). Hypocomplementemia was observed in 83% (20/24) of I-LN patients and 93% (38/41) of D-LN patients based on the data of serum C3 and/or CH50. Of these, the comparable assays for C3 (normal range 60–95 mg/dl) showed mean levels 40.2 ± 5.6 mg/dl ($n = 13$) in I-LN patients and 43.5 ± 9.9 mg/dl ($n = 27$) in D-LN patients, respectively. The above data showed that the two patient groups I-LN and D-LN had similar serological abnormalities before initial therapy for LN.

Renal status (number of patients) during 5 years after initial therapy for LN

The 31 I-LN patients showed one of the following: 5-year remission (22), mild persistent proteinuria (2) including accompanied renal dysfunction (1) with serum creatinine >1 mg/dl after rapidly progressing glomerulonephritis (RPGN), or renal relapse (6). A negative response was found in seven patients.

The 45 D-LN patients showed one of the following: 5-year remission (10), mild persistent proteinuria (5), or persistent proteinuria of nephrotic levels (5), including accompanying renal dysfunction (3) with serum creatinine >1 mg/dl after RPGN, or renal relapse (25). A negative response was found in 30 patients.

Renal status during 5 years after retherapy for the first relapse of I-LN

LN relapse occurred in 11 of the 31 I-LN patients after the initial therapy. Of these, nine patients received therapeutic interventions at the mean prednisolone-equivalent dose of 44.4 ± 15.1 mg/day, and a methylprednisolone pulse was added in two patients. By the time of the present study, six patients had been followed for more than 5 years after the retherapy for LN; 5-year remission (three), mild persistent proteinuria (two) or a second renal relapse (one) during 5 years were found in the six patients. A negative response was found in one patient. The renal response to the retherapy was again better than that following the initial therapy for D-LN (Fig. 3).

Serum anti-DNA antibody levels before therapy were elevated in 89% (8/9) of the patients treated for relapsed I-LN, and the mean titer was 59.5 ± 22.1 IU/ml ($n = 5$) by ELISA or 8 ± 2 IU/ml ($n = 3$) by RIA. Hypocomplementemia was observed in 78% (7/9) of the patients, and the mean serum C3 level was 48.1 ± 5.1 mg/dl ($n = 7$).

The questionnaire study

A better renal response to initial therapy was observed in the I-LN patients compared with the D-LN patients (Fig. 3). Initial steroid therapies for LN in the I-LN and the D-LN are shown in Table 1. The difference in the therapeutic doses did not seem large enough to explain the different response rates between the I-LN and the D-LN patients.

Renal status (the number of patients) during 5 years after initial therapy for LN

The 89 I-LN patients showed one of the following: 5-year remission (32), probable remission or mild proteinuria (16), mild persistent proteinuria (11), chronic proteinuria of nephrotic levels accompanying renal dysfunction (3) after RPGN, or renal relapse (27). "Probable remission or mild proteinuria" was defined based on no subsequent relapse and the mention of no renal abnormalities at the last observation but no mention of the early renal status after therapy. A negative response was found in 30 patients.

The 34 D-LN patients showed one of the following: 5-year remission (9), mild persistent proteinuria (3), persistent proteinuria of nephrotic levels and/or renal dysfunction (12), or renal relapse (10). At least five patients had RPGN before therapy. A negative response was found in 22 patients.

Renal status during 5 years after retherapy for the first relapse of I-LN

LN relapse occurred in 39 of the 91 I-LN patients, and 36 patients received therapeutic interventions. The identified mean prednisolone-equivalent dose was 45.5 ± 13.3 mg/day ($n = 30$), and the combination use of steroid pulse was mentioned by ten patients, and that of cyclophosphamide by six patients. The 29 patients who received retherapy and 5-year follow-up showed one of the following: 5-year remission (8), mild persistent proteinuria

Table 2 Renal outcomes of I-LN or D-LN in the two study groups: renal status over 3 years at time of the last observation

	n	Remission	MPD	N + CRF + HD	Flare	Observation period of LN (years)
Chart review						
I-LN	31	22 (71%)*	7	0 + 0 + 2 (6%)	0	19.6 ± 9.2
D-LN	49	14 (29%)	8	4 + 11 + 10 (51%)**	2	13.0 ± 8.1
Questionnaire study						
I-LN	89	65 (73%)*	8	0 + 4 + 2 (7%)	10	17.5 ± 9.1
D-LN	33	11 (33%)	5	6 + 4 + 4 (42%)**	3	12.1 ± 7.8

Remission: no renal abnormalities but including SLE conditions of serologically active clinically quiescent (SACQ) disease for at least 3 years. MPD (mild persistent disease; positive urine proteins less than nephrotic levels). N (nephrotic levels of urine proteins): prolonged massive urine proteins without apparent renal dysfunction. CRF (chronic renal failure but no uremia): prolonged elevation of serum creatinine levels (>1 mg/dl) or mention of renal dysfunction in the questionnaire based on information from attendant doctors. HD (hemodialysis): on maintenance HD or having a history of receiving renal implantation irrelevant to the present renal status. Flare: flare of SLE and/or LN within 3 years by the last observation

* $P < 0.01$, ** $P < 0.001$ (chi-squared test)

(3), probable remission or mild proteinuria (7), persistent proteinuria of nephrotic levels and/or renal dysfunction (2), or a second renal relapse (9). A negative response was found in 11 patients. The renal response to the retherapy was again better than that following the initial therapy for D-LN (Fig. 3).

The results in the two study groups were consistent, and suggested that renal response to both the initial therapy for I-LN and the retherapy for relapsed I-LN were better than that to the initial therapy for D-LN.

Renal outcomes: renal status over 3 years at the time of the last observation (Table 2)

Renal status was classified according to definitions described in the footnotes of Table 2, and we classified a patient that showed both of chronic renal damage and recent renal flare into a category of chronic renal damage but not flare. A higher frequency of renal remission was observed in I-LN compared with D-LN, and a higher frequency of irreversible renal damage including nephrotic levels of prolonged urine proteins or chronic renal failure was observed in D-LN compared with I-LN.

The above results were consistent in the two study groups and suggested a good renal prognosis of I-LN patients and a poor renal prognosis of D-LN patients. In most of the patients with irreversible renal damage in the present study, frequent relapse resulted in renal deterioration.

Patients whose irreversible renal damage was established before the initial therapy were removed from the estimation of renal outcomes shown in Table 2. This included three I-LN patients and two D-LN patients in the chart review and two I-LN patients and one D-LN patient in the questionnaire study. Some of the remaining patients in the chart review did not receive an appropriate therapeutic intervention for renal deterioration that developed in the course of SLE for various reasons, including a referral delay, poor medical compliance, and accompanying chronic infection.

Except in the case of receiving insufficient therapy as described above, most of the patients in the chart review and the questionnaire study were treated for LN appropriately with 30–60 mg/day prednisolone-equivalent doses of steroid at the onset of LN and at the first LN relapse. For repeated SLE relapses, however, intensities of therapeutic interventions were widely different from case to case.

Renal outcomes in reference to the renal pathology before therapy in the chart review study

Renal pathology before initial therapy for LN (the first-time biopsy; Fig. 4)

Renal outcomes in the chart review patients were collated according to the renal pathology data at biopsy before initial therapy (Fig. 4), which were available in 61 out of 85 LN patients based on the reports by pathologists in our hospital or the referral description of other hospitals. In addition, one I-LN and three D-LN patients manifested RPGN at the onset of LN and had probable class IV-LN.

The descriptions of renal pathology found by chart review were mostly in accordance with the WHO classification (3, 4), although the information on subclass was not comprehensive. In the present study (Fig. 4), we classified "a description of membranous LN" or "class V without description of combined III or IV" into "V", and "diffuse proliferative LN without subclass-description" into "IV-" (Fig. 4). In addition, "IV + V" documented in some of the case records was also denoted as "IV-" but not Vd. Thus, "IV-" in Fig. 4 might contain various types of class IV.

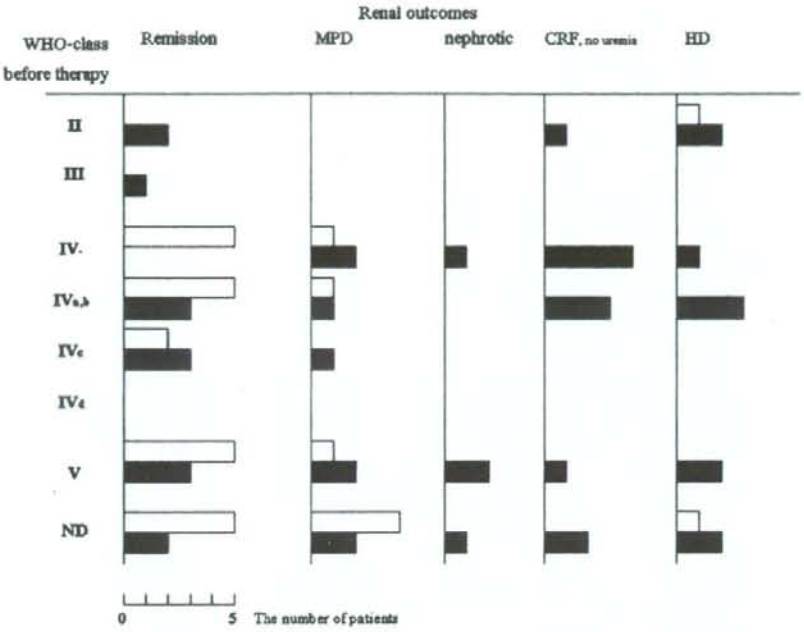

Fig. 4 Renal outcomes in 31 I-LN patients and 47 D-LN patients in the chart review study in reference to renal pathology at biopsy before initial therapy for LN. Each *bar* denotes the number of patients (*white bar* I-LN, *black bar* D-LN). "IV-" includes diffuse proliferative LN when lacking information regarding activity, necrosis or sclerosis findings. The definition of "V" is given in the text. *ND* not done, *MPD* mild persistent disease, *CRF* chronic renal failure without uremia, *HD* hemodyalysis

Patients with I-LN Class IV was common in these patients but rarely associated with the irreversible renal damage at the time of the last observation. Two patients had end-stage renal disease: a patient with RPGN at SLE onset followed by repeated renal relapses, and another patient with class II-LN at SLE onset and suffering from later developed thrombotic thrombocytopenic purpura (TTP) that caused uremia.

Patients with D-LN Irreversible renal damage was common in these patients having various renal pathology classes including class IV before initial therapy for the LN. TTP was involved in two patients and directly caused chronic renal failure in at least one of the patients.

Renal pathology before therapy for relapsed LN (change of the histology)

The renal pathology at time of the LN relapse was documented in five I-LN patients and nine D-LN patients. These findings are described below in reference to those at first biopsy (in parentheses) and clinical renal outcomes in terms defined in Table 2.

Patients with I-LN In four patients, retherapy for relapsed LN of class II (V), IVa (V), IVa (ND) or IVc (V) led to a clinical remission at the last observation. In another patient, LN of class IVc + V (IVa) manifested after self-discontinuation of maintenance therapy, and responded to retherapy and led to mild persistent disease (MPD) that was relapse-free for 16 years by the present time.

Patients with D-LN In two patients, each of the retherapies for relapsed LN of class IVc (V) using combined cyclophosphamide improved nephrotic syndrome to MPD. In another three patients, relapsed LN of class IVa (II), IVd (IVb) or IVd (ND) resulted in CRF without uremia despite retherapy. In the remaining four patients, relapsed LN of class IVd (V), IVd (II), IVc (IVa) or Va (V) accompanied by intractable nephrotic syndrome resulted in CRF on hemodialysis despite retherapy.

As described above, class IV with sclerosing lesions was commonly uncovered at the second biopsy in the D-LN patients who resulted in CRF. On the other hand, a transformation of renal histology observed in most of the examined cases of relapsed I-LN showed a small impact on clinical prognosis.

Discussion

The present chart review study and questionnaire study consistently showed a relatively better prognosis of I-LN patients compared with D-LN patients. Half of the I-LN patients were expected to be relapse-free after the initial therapy (Fig. 2) and most of the relapse-free patients

achieved renal remission throughout the observation period. In the I-LN cases of relapsed LN, most patients responded to retherapy (Fig. 3). Consistent with these findings, more than 70% of the I-LN patients had obtained prolonged renal remission at the last observation (Table 2). In contrast, a poor prognosis of D-LN patients was shown, and irreversible real damage was precipitated in this category of LN. The resulting data (Figs. 2, 3 and Table 2) in the two study groups were surprisingly similar, and strongly suggested the prognostic impact of the difference between the two chronological categories I-LN and D-LN. A pathological transformation towards class IV with sclerosing lesions was found at the second biopsy in most of the examined D-LN cases in the chart review.

Despite a similarity in the patients' demographics between the present two study groups (Table 1), there was a large difference in the ratio of D-LN to I-LN patients: 51/34 (1.5) in the chart review and 34/91 (0.37) in the questionnaire study ($P < 0.00001$). The chart review study in our hospital included all of the deceased patients and numerous numbers of referral patients because of intractable disease, and thus patients with severe forms of SLE may be overrepresented in the chart review study. The result of the higher D-LN/I-LN ratio in the chart review than that in the questionnaire study may be consistent with the putative poorer prognosis of D-LN compared with I-LN.

The present study suggested that D-LN tended to progress in renal damage despite steroid therapy, in contrast to the good therapeutic response of I-LN even having renal pathology class IV. Cyclophosphamide, which has been included recently in a standard regimen for treating LN in our hospital, had not been widely used for the cases in the present study that included only therapies occurring more than 5 years ago. Therapy using steroid and combined cyclophosphamide may improve the prognosis of D-LN patients, and the efficacy of therapy for I-LN and that for D-LN should be estimated separately because of a probable difference in therapeutic response between the two LN categories.

Conclusions

The time of the initial appearance of renal symptoms in the course of SLE may have a prognostic impact on lupus nephritis.

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特集 リウマチ性疾患診療に関連した神経病変の診断と治療

進行性多巣性白質脳症(PML)*

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Key Words : progressive multifocal leukoencephalopathy, PML, JC virus, rheumatic diseases, SLE

はじめに

進行性多巣性白質脳症(progressive multifocal leukoencephalopathy；PML)は，JCウイルス(JCV)感染によって起きる中枢神経系での脱髄性疾患で，主に血液系の悪性疾患患者，免疫抑制剤投与を受けている移植患者など，いわゆるimmunocompromised hostに発症し，近年はHIV感染患者の増加とともに発症例が増加している．その予後は不良で，患者は初期の神経症状の出現から通常数か月で死亡に至る．

一方，その多くが免疫抑制治療を受けている自己免疫性疾患での発症例は報告が乏しかったが，最近のrituximab投与中の全身性エリテマトーデス(SLE)患者や，natalizumab投与中の多発性硬化症とクローン病患者でのPML発症例の報告以来，報告が増加している．自己免疫疾患での報告例には，軽微な免疫抑制療法中の症例が多く含まれており，自己免疫疾患自体がPMLを発症しやすい要因をもっている可能性が示唆されている．

JCVとPML

PMLはミエリンを形成しているオリゴデンドロサイトがJCVに侵され，脱髄を生じる疾患である．JCVは1971年にPML患者の脳組織から初めて分離された，ポリオーマウイルス科に属する環状2本鎖DNA構造をもつウイルスで，塩基構造はウイルス蛋白をコードする領域と，その発現調節にかかわる調節領域に分けられる[1]．

大部分のヒトは小児期にJCVに感染し，成人の80%以上が抗体陽性を示す．初感染時には明らかな症状は起こさず，体内に入ったJCVは完全に排除されずに主に腎組織に持続感染する．腎臓のほかにはBリンパ球にも感染することが確認されている．

PML患者の中枢神経組織より分離されるJCVは尿路に持続感染しているものと異なり，調節領域に多様な変化がみられる(以下，尿路に持続感染している原型JCVに対してPML型JCVと呼ぶ)[2]．世界各地で得られた原型JCVとPML型JCVの塩基配列を比較した研究では，PML型JCVが自分自身の系統をもたないことが示された[3]．このことより，PML型JCVが直接ヒトの間で感染するのではなく，原型JCVが感染初期に患者体内で調節領域に変化を起こしてPML型JCVとなりリンパ球内に持続感染し，宿主の免疫低下などをきっかけに中枢神経組織で増殖するのではないかと推測されている[3][4]．

* Progressive multifocal leukoencephalopathy in rheumatic diseases.

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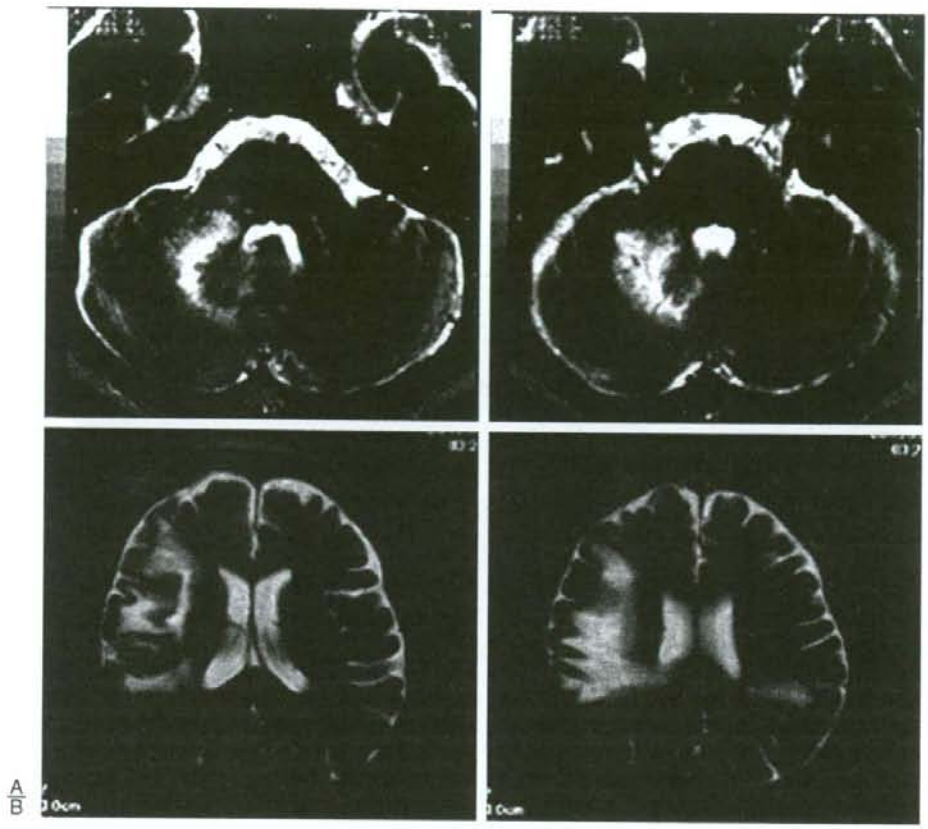

図1　PML患者のMRI像
A：非典型的にSLE患者の右小脳と橋に生じたPML病変，
B：SLE患者の右前側頭葉と左側頭葉白質のPML病変．ともにT2強調画像（文献[7]より引用）

PMLの臨床像

1．神経症状

発症時の病変部位によって初発症状は異なる．症状は運動麻痺がもっとも多く，通常片麻痺から四肢麻痺に至る．その他，仮性球麻痺，失調，痙攣などが続き，痴呆，意識障害も高頻度に生じる．また，PMLの病変は後頭葉から発症しやすく，半盲や羞明などの視野障害も高率にみられる．感覚麻痺の報告は少ないが，意識障害によりマスクされていると考えられる．自律神経障害の報告も少ない．髄膜刺激症状は欠如し，頭痛の訴えも少ない[5]．

2．髄液所見

約半数の症例で軽度の蛋白上昇を示すほかは，髄液圧，細胞数，糖などすべて正常である[5][6]．SLEなどの自己免疫性疾患をはじめ，中枢神経系の障害を生じる他の疾患との大きな鑑別点である．

3．MRIによる画像診断

もっとも診断価値が高いのはMRIである．典型的な画像はT1強調画像で低信号，T2強調画像で高信号を示す，大脳皮質，基底核を避け，大脳半球白質に広がる特徴的な病変を呈する．しかし，膠原病例での報告では，脳幹部や小脳から発症する例も多くみられる[7]（図1）．

ウイルス学的検査

前記のように，成人の大部分がJCVに自然感染していることより，抗JCV抗体の血清検査は臨床的価値に乏しい．

杉本らはnested PCR法を用いて髄液中のPML型JCVの調節領域を増幅する方法がPMLの診断に有用であることを報告している[8]．この方法の優れている点は，自然感染している原型JCVの混入

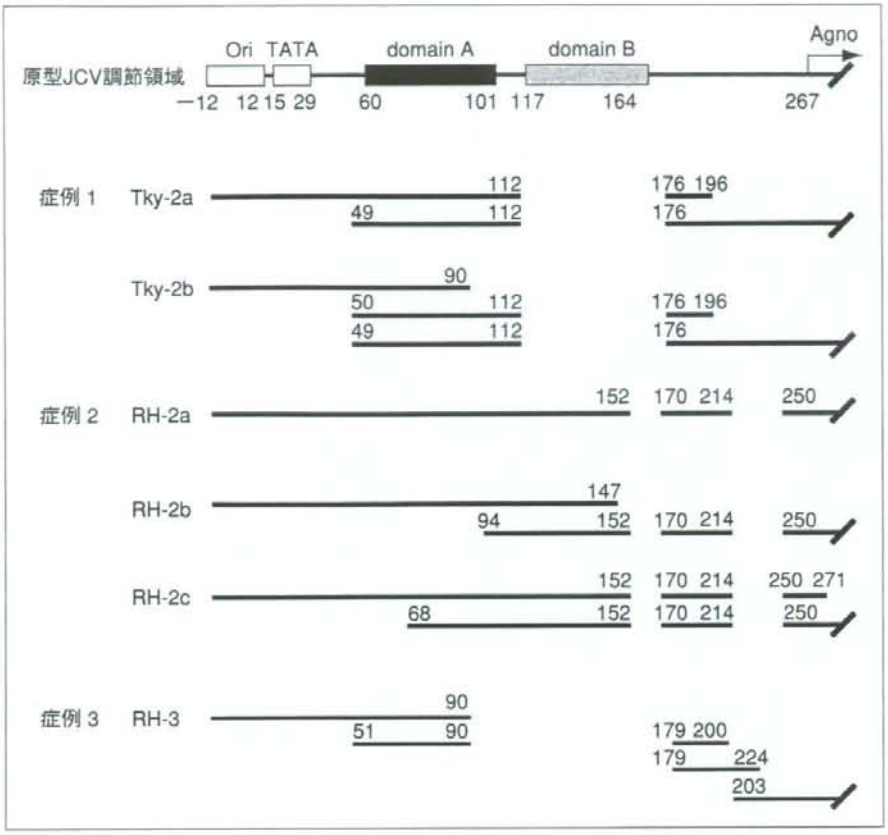

図2　原型JCVと筆者らの施設でSLE患者3例から得られたPML型JCVの調節領域の比較
原型調節領域と同じ配列を直線で表し，欠失はブランクで，重複配列は開始位置へ段を変えて表している．Ori：複製開始点，TATA：TATA配列，Agno：翻訳開始点（文献[7]より引用，一部改変）

を除外でき，またPML型の調節領域は患者個々に特異的であるため，得られた塩基配列を解析することによってコンタミネーションなどによる擬陽性の可能性を排除できることである．著者らの施設でも，東京大学医学部附属病院泌尿器科・男性科の余郷嘉明先生のご協力で2例のSLE患者髄液よりPML型JCVを検出し，PMLの確定診断を行った[7]（図2—症例2,3．症例1は検出法が異なる）．

髄液でPML型JCVが検出されなくても，他の所見からPMLが疑われる場合は脳生検を行う．脳の脱髄病変部では髄液に比べJCVの量がはるかに多いため，免疫組織染色を用いたJCV蛋白検出のほか，前記のPCR法を用いれば診断の可能性は高くなる．

PMLの治療

PMLの予後はきわめて不良である．HIV以前の時代ではPML患者の平均余命は2.6か月で，80％の患者が9か月以内に死亡している[9]．その後，HIVの領域ではHAART療法により生存率の改善が認められているが，免疫抑制治療の継続が必要な疾患群には導入が困難である[10]．自己免疫性疾患との合併例でも，これまで免疫抑制療法の解除に加え，cidofovir, interferon, cytarabineなどの投与が試みられ，効果があったとする報告はあるが，明らかな効果が証明されている治療法はない．これまでの抗ウイルス療法とは異なる方法論では，JCVが細胞に感染するときにセロトニン受容体を用いることより，5-hydroxytryptamine 2A receptor（5-HT2AR）阻害の有用性