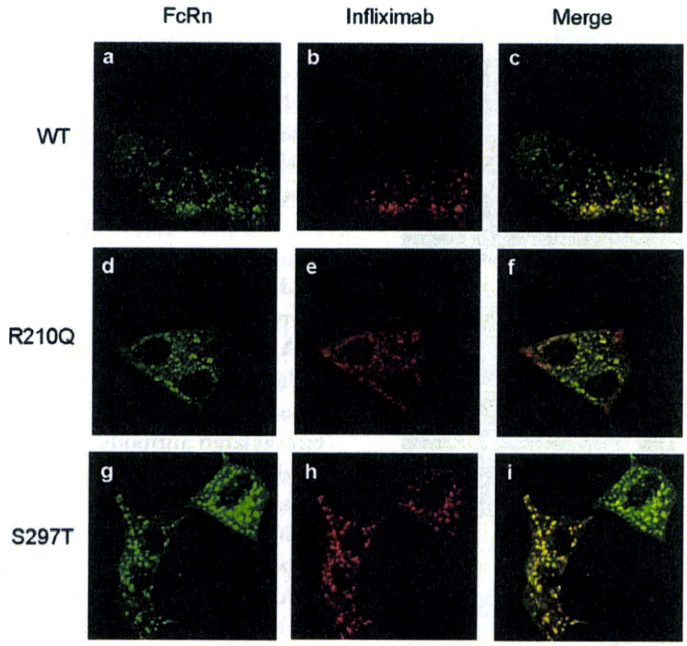


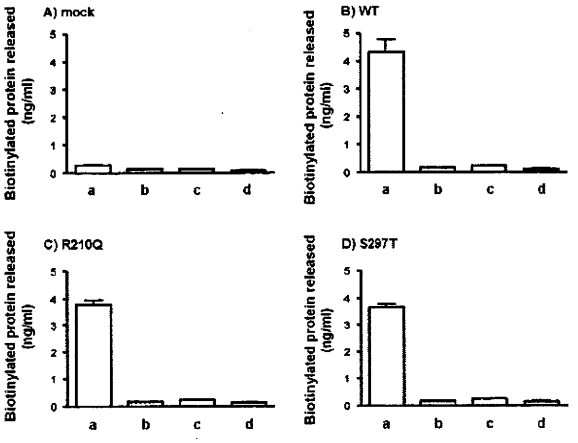
Fig. 4. Co-localization of CypHer5-labeled infliximab and FcRn in HeLa cells expressing wild-type (WT) or a variant FcRn
HeLa cells transfected with wild-type (a, b, c), or variant (d, e, f; R210Q, g, h, i; S297T) FcRn-EGFP were incubated with CypHer5-labeled infliximab in cell culture media containing sodium phosphate buffer (pH. 6.0) for 2–3 hr. After washing the cells twice with neutral pH medium, the fluorescent signal was observed. Panels (a, d, g) and (b, e, h) show the intracellular localization of FcRn-EGFP and the incorporated CypHer5-labeled infliximab, respectively. In panels (c, f, i) the fluorescent signal of FcRn-EGFP was merged with that of CypHer5-labeled infliximab.

Large interindividual variations in pharmacokinetic parameters have been reported for at least several antibody therapeutics. For example, trough concentrations in repetitive dosing of antibodies were reported to show 5.6-fold interindividual differences in 22 palivizumab-treated patients,[16] 18.2-fold differences in 16 cetuximab-treated patients,[17] and over 70-fold differences in 86 infliximab-treated patients.[18] In addition, large percent coefficients of variation were reported for $T_{1/2}$, such as 72.0% for gemtuzumab ozogamicin[19] and 76.4% for basiliximab,[20] after second dose of their treatments. We presumed that changes in FcRn expression levels and function caused by genetic variations of *FCGRT* may lead to these interindividual differences in pharmacokinetics of antibody therapeutics.

In order to identify genetic polymorphisms of *FCGRT*, we sequenced genomic DNA from 126 Japanese subjects. A total of 33 genetic variations, including 17 novel ones, were detected. A VNTR was detected in the 5′ -flanking region, as was the case in Caucasian subjects reported previously.[8] Although a recent study showed that no significant impact was observed in the rates of maternal-fetal IgG transfer,[21] VNTR3 is known to be associated with 1.66-fold higher transcriptional activity than VNTR2 *in vitro*. In addition, monocytes with VNTR3/3 showed increased binding of IgG compared to those with 2/3.[8] Thus, this variation may contribute to the interindividual differences in pharmacokinetics of antibody therapeutics. The allele frequency of VNTR2 in Japanese (0.032) was lower than that in Caucasians (0.075).[8]

In this study, two novel nonsynonymous variations were found and their functional significance was assessed *in vitro* using a mammalian expression system. However, the two FcRn variants did not show any changes in intracellular localization or recycling, suggesting that the two nonsynonymous substitutions found in a Japanese population probably do not contribute to the interindividual variations in the pharmacokinetics of antibody therapeutics. Since FcRn function is important for maintenance of IgG levels as well as maternal-fetal IgG transfer, functionally-affecting genetic variations might be few to retain its functional capability.

Amino acid residues of human FcRn that interact with IgG were reported to be E138, E139, D153 and W154, in the α2 domain.[1] (Amino acid numbers shown in this paper include the signal peptide.). The electrostatic binding of these anionic amino acid residues in FcRn with H310 and H435 in IgG, which has an isoelectric point of pH 7.6, defines the strict pH-dependent binding of IgG to FcRn.[22] The variant amino acid residues identified in this study, R210Q and S297T, are both located in the α3 domain of FcRn. According to the predicted higher order structure,[1] R210 and S297 are located very close to the



Column	a	b	c	d
Reagents	Infliximab			IgY
Loading Temp. (°C)	37	4	37	37
Recycling Temp. (°C)	37	37	4	37

Fig. 5. Recycling of biotinylated antibodies from wild-type (WT) or variant FcRn-transfected HeLa cells
HeLa cells transfected with wild-type or a variant FcRn were incubated for 1 hr with biotinylated infliximab. After washing, the cells were further incubated for 2 hr. The amount of recycled protein in the supernatant was determined by ELISA. Experimental conditions are shown in the table. For the samples shown as columns a-c, biotinylated infliximab was loaded, whereas biotinylated IgY was used for d. The temperature for antibody loading was 37°C (a, c, d) or 4°C (b). The temperature for recycling antibodies from antibody-loaded cells was 37°C (a, b, d) or 4°C (c).

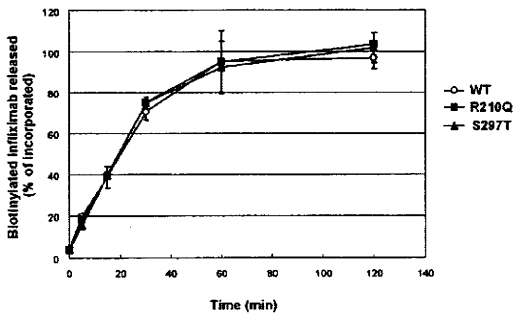


Fig. 6. Quantitative analyses of recycling of biotinylated infliximab; Time course of release of the biotinylated infliximab incorporated into the HeLa cells transfected with wild-type (WT) or variant FcRn
HeLa cells transfected with wild-type or a variant FcRn were incubated for 1 hr with biotinylated infliximab. After washing, cells were further incubated for the indicated periods of time. The amount of recycled protein was determined by ELISA. The amount of recycled antibody at each time point was expressed as a percentage of the initially incorporated antibody at time 0.

transmembrane region that is distant from the IgG binding site. Considering the results obtained here, where no difference in antibody recycling activity between wild-type and each variant FcRn was detected *in vitro*, the amino acid substitutions identified in a Japanese population may not have significant impact on structural and functional properties of FcRn. Although FcRn is known to bind with albumin as well as IgG, the albumin binding site of FcRn has been identified as H189, which also is located in the α2 domain.[23] The polymorphic sites are also far from the albumin binding site. However, the effect of amino acid substitutions R210Q and S297T on the albumin recycling activity via FcRn should be determined in a future study.

In the present study, we used HeLa cells to examine the localization and recycling activity of FcRn variants. Since endogenous expression of FcRn protein in HeLa cells has not been detected,[24] we considered HeLa cells suitable for examining the antibody recycling activity of variant FcRn since the background responses are negligible. In fact, as shown in **Figure 5**, antibody recycling was detected only in FcRn-transfected cells. Therefore, we concluded that HeLa cells can be used as a suitable model for evaluating the function of variant FcRn proteins.

Our results suggested that at least no common functional polymorphic site with amino acid change was present in *FCGRT* in our Japanese population. Since FcRn function is important for maintenance of IgG levels, there may be few functionally-affecting genetic variations. Further analysis is necessary for the functional significance of transcriptional regulatory regions.

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References

1) Andersen, J. T. and Sandlie, I.: The versatile MHC class I-related FcRn protects IgG and albumin from degradation: implications for development of new diagnostics and therapeutics. *Drug Metab. Pharmacokinet.*, **24**: 318–332 (2009).
2) Lobo, E. D., Hansen, R. J. and Balthasar, J. P.: Antibody pharmacokinetics and pharmacodynamics. *J. Pharm. Sci.*, **93**: 2645–2668 (2004).
3) Suzuki, T., Ishii-Watabe, A., Tada, M., Kobayashi, T., Kanayasu-Toyoda, T., Kawanishi, T. and Yamaguchi, T.: Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. *J. Immunol.*, **184**: 1968–1976 (2010).
4) Claypool, S. M., Dickinson, B. L., Yoshida, M., Lencer, W. I. and Blumberg, R. S.: Functional reconstitution of human FcRn in Madin-Darby canine kidney cells requires co-expressed human beta 2-microglobulin. *J. Biol. Chem.*, **277**: 28038–28050 (2002).
5) Praetor, A. and Hunziker, W.: Beta(2)-microglobulin is important for cell surface expression and pH-dependent IgG bind-

ing of human FcRn. *J. Cell. Sci.*, **115**: 2389–2397 (2002).
6) Tesar, D. B., Tiangco, N. E. and Bjorkman, P. J.: Ligand valency affects transcytosis, recycling and intracellular trafficking mediated by the neonatal Fc receptor. *Traffic*, **7**: 1127–1142 (2006).
7) Kamei, D. T., Lao, B. J., Ricci, M. S., Deshpande, R., Xu, H., Tidor, B. and Lauffenburger, D. A.: Quantitative methods for developing Fc mutants with extended half-lives. *Biotechnol. Bioeng.*, **92**: 748–760 (2005).
8) Sachs, U. J., Socher, I., Braeunlich, C. G., Kroll, H., Bein, G. and Santoso, S.: A variable number of tandem repeats polymorphism influences the transcriptional activity of the neonatal Fc receptor alpha-chain promoter. *Immunology*, **119**: 83–89 (2006).
9) Goebl, N. A., Babbey, C. M., Datta-Mannan, A., Witcher, D. R., Wroblewski, V. J. and Dunn, K. W.: Neonatal Fc receptor mediates internalization of Fc in transfected human endothelial cells. *Mol. Biol. Cell.*, **19**: 5490–5505 (2008).
10) Ober, R. J., Martinez, C., Lai, X., Zhou, J. and Ward, E. S.: Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. *Proc. Natl. Acad. Sci. U.S.A.*, **101**: 11076–11081 (2004).
11) Ober, R. J., Martinez, C., Vaccaro, C., Zhou, J. and Ward, E. S.: Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. *J. Immunol.*, **172**: 2021–2029 (2004).
12) Ward, E. S., Martinez, C., Vaccaro, C., Zhou, J., Tang, Q. and Ober, R. J.: From sorting endosomes to exocytosis: association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. *Mol. Biol. Cell.*, **16**: 2028–2038 (2005).
13) Gan, Z., Ram, S., Vaccaro, C., Ober, R. J. and Ward, E. S.: Analyses of the recycling receptor, FcRn, in live cells reveal novel pathways for lysosomal delivery. *Traffic*, **10**: 600–614 (2009).
14) Mark, S. B., Burns, D. D., Cooper, M. E. and Gregory, S. J.: A pH sensitive fluorescent cyanine dye for biological applications. *Chem. Commun.*, **23**: 2323–2324 (2000).
15) Ternant, D. and Paintaud, G.: Pharmacokinetics and concentration-effect relationships of therapeutic monoclonal antibodies and fusion proteins. *Expert Opin. Biol. Ther.*, **5 Suppl 1**: S37–47 (2005).
16) Subramanian, K. N., Weisman, L. E., Rhodes, T., Ariagno, R., Sanchez, P. J., Steichen, J., Givner, L. B., Jennings, T. L., Top, F. H. Jr, Carlin, D. and Connor, E.: Safety, tolerance and pharmacokinetics of a humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. MEDI-493 Study Group. *Pediatr. Infect. Dis. J.*, **17**: 110–115 (1998).
17) Cèzè, N., Ternant, D., Piller, F., Degenne, D., Azzopardi, N., Dorval, E., Watier, H., Lecomte, T. and Paintaud, G.: An enzyme-linked immunosorbent assay for therapeutic drug monitoring of cetuximab. *Ther. Drug Monit.*, **31**: 597–601 (2009).
18) St Clair, E. W., Wagner, C. L., Fasanmade, A. A., Wang, B., Schaible, T., Kavanaugh, A. and Keystone, E. C.: The relationship of serum infliximab concentrations to clinical improvement in rheumatoid arthritis: results from ATTRACT, a multicenter, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.*, **46**: 1451–1459 (2002).
19) Dowell, J. A., Korth-Bradley, J., Liu, H., King, S. P. and Berger, M. S.: Pharmacokinetics of gemtuzumab ozogamicin, an antibody-targeted chemotherapy agent for the treatment of patients with acute myeloid leukemia in first relapse. *J. Clin. Pharmacol.*, **41**: 1206–1214 (2001).
20) Kovarik, J. M., Nashan, B., Neuhaus, P., Clavien, P. A., Gerbeau, C., Hall, M. L. and Korn, A.: A population pharmacokinetic screen to identify demographic-clinical covariates of basiliximab in liver transplantation. *Clin. Pharmacol. Ther.*, **69**: 201–209 (2001).
21) Freiberger, T., Ravcuková, B., Grodecká, L., Kurecová, B., Jarkovský, J., Bartonková, D., Thon, V. and Litzman, J.: No association of FCRN promoter VNTR polymorphism with the rate of maternal-fetal IgG transfer. *J. Reprod. Immunol.*, **85**: 193–197 (2010).
22) Vaughn, D. E., Milburn, C. M., Penny, D. M., Martin, W. L., Johnson, J. L. and Bjorkman, P. J.: Identification of critical IgG binding epitopes on the neonatal Fc receptor. *J. Mol. Biol.*, **274**: 597–607 (1997).
23) West, A. P., Jr. and Bjorkman, P. J.: Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complex-related Fc receptor. *Biochemistry*, **39**: 9698–9708 (2000).
24) Liu, X., Ye, L., Christianson, G. J., Yang, J. Q., Roopenian, D. C. and Zhu, X.: NF-kappaB signaling regulates functional expression of the MHC class I-related neonatal Fc receptor for IgG via intronic binding sequences. *J. Immunol.*, **179**: 2999–3011 (2007).

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Regular Article

Genetic Polymorphisms of *FCGRT* Encoding FcRn in a Japanese Population and Their Functional Analysis

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Summary: Neonatal Fc receptor (FcRn) plays an important role in regulating IgG homeostasis in the body. Changes in FcRn expression levels or activity caused by genetic polymorphisms of *FCGRT*, which encodes FcRn, may lead to interindividual differences in pharmacokinetics of therapeutic antibodies. In this study, we sequenced the 5′-flanking region, all exons and their flanking regions of *FCGRT* from 126 Japanese subjects. Thirty-three genetic variations, including 17 novel ones, were found. Of these, two novel non-synonymous variations, 629G>A (R210Q) and 889T>A (S297T), were found as heterozygous variations. We next assessed the functional significance of the two novel non-synonymous variations by expressing wild-type and variant proteins in HeLa cells. Both variant proteins showed similar intracellular localization as well as antibody recycling efficiencies. These results suggested that at least no common functional polymorphic site with amino acid change was present in the *FCGRT* of our Japanese population.

Keywords: *FCGRT*; neonatal Fc receptor (FcRn); genetic polymorphism; novel non-synonymous variation

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Introduction

Neonatal Fc receptor (FcRn) is an immunoglobulin G (IgG) receptor related to major histocompatibility (MHC) class I molecules.[1,2] Like MHC class I, FcRn consists of a heavy chain with extracellular α1, α2, and α3 domains followed by a transmembrane segment and a short cytoplasmic tail and non-covalently bound β2-microglobulin (β2m). FcRn binds the Fc region of monomeric IgG. The FcRn heavy chain is encoded by *FCGRT*, which is located in chromosome 19q13.3 and comprises 6 exons.

In humans, FcRn expression has been observed in a wide variety of tissues including placenta, liver, kidney and vascular endothelium.[1] FcRn has multiple roles in the body such as absorption or secretion of IgG across the intestinal mucosa, and IgG recycling from endothelial cells. With regard to antibody recycling, FcRn binds to the Fc domain of IgG at acidic pH in endosomes after endocytosis, and recycles it back to the extracellular space via the exocytic pathway, thereby protecting IgG from intracellular degradation in lysosomes.[2] This mechanism contributes to the long serum half-life of IgG, and thus, IgG recycling activity is an important function of FcRn and could contribute to the efficacy of antibody therapeutics. Indeed, we previously reported that affinities of antibody therapeutics to FcRn were closely correlated with the serum half-lives reported in clinical studies.[3] The relatively short serum half-life of Fc-fusion proteins such as etanercept, a fusion protein consisting of the extracellular ligand-binding portion of the human tumor necrosis factor receptor linked to the Fc portion of human IgG1, is thought to arise from low affinity to FcRn.[3]

Genetic polymorphisms of genes related to drug metabolism and transport are one of the crucial factors for low-molecular-weight drugs. Pharmacokinetics or pharmacodynamics of biologicals including antibody therapeutics may also be influenced by genetic polymorphisms of transport or target proteins. In this context, changes in FcRn expression levels or activity caused by genetic polymorphisms of *FCGRT* may lead to interindividual differences in pharmacokinetics of antibody therapeutics. However, reports on *FCGRT* genetic polymorphisms in Japanese populations are lacking.

Here we sequenced the 5′-flanking region, all exons and their flanking regions of *FCGRT* from 126 Japanese subjects. We then examined the functional properties of two detected non-synonymous variations using mammalian expression systems focusing on intracellular localization and antibody recycling activities.

Materials and Methods

Human genomic DNA samples: One hundred twenty-six Japanese cancer patients participated in this study. The ethical review boards of the National Cancer Center, Aichi Cancer Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all subjects. Genomic DNA for DNA sequencing was extracted from blood leukocytes.

PCR conditions for DNA sequencing: The following sequences obtained from GenBank were used for primer design and reference sequences: NW_927240.1 (genome) and NM_004107.3 (mRNA). For sequencing, two sets of long-range PCR were performed to amplify all 6 exons from 50 ng of genomic DNA with two sets of primers (0.5 μM) designed in the promoter or intronic regions as listed in "1st PCR" of **Table 1**. We used LA-Taq with GC buffer I (0.05 U/μl, Takara Bio Inc., Shiga, Japan) to amplify from the 5′-flanking region to exon 3 and Z-Taq (0.025 U/μl, Takara Bio. Inc.) from exons 4 to 6, as described in **Table 1**. The 1st PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min for LA-Taq, and 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec for Z-Taq. Next, each region was separately amplified in the 2nd PCR using the 1st PCR product as the template. We used LA-Taq with GC buffer I or II (0.05 U/μl) for amplifying regions from the 5′-flanking region to exon 3 and Ex-Taq (0.02 U/μl, Takara Bio. Inc.) from exons 4 to 6 as described in **Table 1**. The 2nd PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min for all regions. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and the sequencing primers listed in **Table 1** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany) and the eluates were applied to an ABI Prism 3730xl DNA Analyzer (Applied Biosystems). All relatively low frequent variations ($n \leq 5$) were confirmed by repeated sequencing analyses of PCR products generated from original (not amplified) genomic DNA. The nucleotide positions based on the cDNA sequence were numbered from the adenine of the translational initiation site or the nearest exons.

Hardy-Weinberg equilibrium and linkage disequilibrium (LD) analyses: Hardy-Weinberg equilibrium and LD analyses were performed by SNPAlyze software ver. 7 (Dynacom Co., Yokohama, Japan). Hardy-Weinberg equilibrium was assessed by the $\chi 2$ test and pairwise LDs between variations were obtained for the frequently used coefficients $|D'|$ and rho square (r^2). $|D'|$ is used to assess the probability for past recombinations, and r^2 is used as a parameter for the linkage between a pair of variations.

Table 1. Primers used for sequencing *FCGRT*

	Enzyme*	Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	LA-GI	5′-flanking to Exon 3	CTCAGGCTGGTCCTTGAACTCA	ATTAGCCAGTTATGGTGGTATG	5,244
	Z	Exons 4 to 6	CAAGTGTGGTGGTGGGCACCTA	GGGAGTTCGAGACCAGCCTGAT	3,788
2nd PCR	LA-GI	5′-flanking	CTGAACCAGCTGAACGTCCACT	CTGAGCGTGGTGGTGGGCCTGT	1,058
	LA-GII		ATAGAGGTGACAGTTGCACAGC	GGTCCAGACTGACAACAATGCC	1,477
	LA-GII	Exon 1	GAGCAGCAGCCTCCCACAGGAT	ACACAAGAGGCGACAGGTGGTT	1,017
	LA-GI	Exons 2 to 3	ATTGTTGTCAGTCTGGACCG	GCTGCAGTGGGAGGCTGATGGA	1,332
	Ex	Exons 4 to 5	CCAAGGAGGTGACATCTTGAGG	CATCTCTGGGTTTCTGTCTCCA	1,383
	Ex	Exon 6	CCGCCTTGCCGCTGCTGATCCA	GAGCTGAGATCACGCAATTGTA	1,632
Sequencing		5′-flanking	CTGAACCAGCTGAACGTCCACT	CAGGGTCTGGCTCTGTCACTCA	
			GTGCAGAATAGGCAAATCTATC	AACCACATCCTTCTGCTAGGAC	
			CGGGTTCAAGCAATTCTCCTGT	TTGAGGGTGTCTGCCGCTCAGG	
			GAGCAGCAGCCTCCCACAGGAT	CCTCCTCTCTCAGACCCAGGAA	
			CCTGGGTCTGAGGGAGGAGT	CCTCCTCGTACCTGAAGAACTT	
		Exon 1	GGACTCTCAGCCTATCAAGT	ACACAAGAGGCGACAGGTGGTT	
			CCGCGGTGTCCCGGGAGGAA		
		Exons 2 to 3	GTATCTGTCCCACTGCAGTCTA	AACTGAGGCAGGTGGGCATGAC	
		Exon 4	TGAGTCTCTGTCACCTAGGAAG	AGTTAACAGCTCTTCAGACTCA	
		Exon 5	CCGCCTTGCCGCTGCTGATCCA	GTCTCTGTCCTCCCAGGTCTGT	
		Exon 6	TCAGAGAGAGGTGGAGACAGAA	GATGTATAAAACTGGCAGGTTC	
			CCTTGGATCTCCCTTCGTGGAG	TGGCTCACACTTGTAATCCCAC	
			GACGGAGTCTTGCTCTGTTGCT		

*LA-GI: LA-Taq with GC buffer I, LA-GII: LA-Taq with GC buffer II, Z: Z-Taq, Ex: Ex-Taq.

Construction of FcRn expression plasmid: Wild-type human FcRn cDNA was originally obtained from pME18SFL3 (AK075532) (Toyobo, Osaka, Japan). The coding region of FcRn cDNA subcloned into pcDNA3 was amplified by PCR, and then inserted into the EcoRI/SalI site of pEGFP-(C) plasmid. The resulting plasmid encodes hFcRn with C-terminally fused enhanced green fluorescent protein (EGFP) containing the eight amino acid-linker peptide VDSRGSRV between the two proteins. Mutations were introduced by an inverse PCR method. Primers consisted of 5′-AAG GCC CAA CCC AGC AGC CCT GGC TTT-3′ (forward) and 5′-CAG GCG CAT GGA GGG GGG CC CTT CCA-3′ (reverse) for R210Q, 5′-TCC ACC GTC CTC GTG GTG GGA ATC GTC-3′ (forward) and 5′-CTT GGC TGG AGA TTC CAG CTC CAC CCT-3′ (reverse) for S297T. The underlines indicate the mutated nucleotides. The variant plasmids were sequenced on both strands for the entire cDNA region to confirm the introduction of the mutation only at the target sites. Human β2 microglobulin (β2m) cDNA was obtained from pME18SFL3 (FCC106E07) (Toyobo). β2m cDNA was subcloned into pcDNA3.1/
Hygro. The β2m construct was used because FcRn becomes a heterodimer with β2m, which is necessary for the proper intracellular localization of FcRn.[4,5]

Cell culture and plasmid transfection: HeLa cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Nichirei, Tokyo, Japan). The plasmids encoding the wild-type or variant FcRn fused with EGFP along with the plasmid encoding β2m were transfected into HeLa cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Plasmids encoding wild-type or variant FcRn fused with EGFP were used for all experiments, including the intracellular localization and antibody recycling activity of FcRn.

Western blot analysis: Wild-type and variant FcRn-EGFP transfected into HeLa cells in 35-mm-diameter dishes were lysed with 500 μL of RIPA buffer [50 mM Tris HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 and 0.25% sodium deoxycholate] supplemented with protease inhibitors (Nacalai Tesque, Kyoto, Japan). After incubation on ice for 30 min, the lysates were centrifuged at 15,000 rpm at 4°C for 20 min. An aliquot (3 μL) of the supernatant was diluted in SDS-sample buffer and applied to 10% SDS-polyacrylamide gel. After electrophoresis, separated proteins were transferred onto polyvinylidene fluoride membrane. Immunochemical detection of FcRn-EGFP proteins was performed using rabbit anti-human FcRn antibody raised against a peptide antigen (residues 135–148, LNGEEFMNFDLKQG). Visualization of the proteins was achieved with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, USA) and the ECL Plus Western blotting detection reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protein band densities measured by LAS-3000 (Fuji Film, Kanagawa, Japan) were quantified with Multi Gauge software (Fuji Film).

The relative expression levels are shown as means ± SD of three separate transfection experiments. To verify that the samples were evenly loaded, the blot was reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antibody (R&D Systems, Minneapolis, MN, USA).

Fluorescent labeling of antibodies: As a model antibody, we used infliximab, a clinically used chimeric anti-human TNFα antibody which has the Fc domain of human IgG1. The binding of infliximab to human FcRn was shown by surface plasmon resonance analysis in our previous study.[3] Infliximab, kindly provided by Tanabe Pharmaceutical Co. Ltd. (Osaka Japan), was labeled with CypHer5 (GE Healthcare Bio-Sciences, Uppsala, Sweden) by incubating with CypHer5E mono NHS ester in PBS containing 0.5 M Na_2CO_3 (pH 8.3) for 1 hr at room temperature. After the reaction, unbound dye was removed by dialysis in PBS. The protein concentration and degree of labeling were determined by spectrophotometry. IgY (Jackson Immuno Research Laboratories, West Grove, PA, USA) was also labeled with CypHer5 and used in control experiments.

Imaging with fluorescence microscopy: HeLa cells transfected with wild-type or variant FcRn-EGFP cDNA and the β2m cDNA were cultured on 35-mm poly-L-lysine-coated glass-bottom dishes (0.08–0.12 mm thickness) (Matsunami, Osaka, Japan) for 2–4 days. The intracellular localization analyses of wild-type and variant FcRn-EGFP were carried out by confocal laser scanning fluorescence microscopy using a Carl Zeiss LSM510 system (Carl Zeiss, Jena, Germany). For co-localization experiments, wild-type or variant FcRn-EGFP-transfected HeLa cells were incubated with CypHer5-labeled infliximab diluted in cell culture medium containing 200 mM sodium phosphate buffer (pH 6.0) for 2–3 hr at 37°C. Note that throughout this study, the cell culture media used for incubation with the labeled antibody was acidified (pH 6.0) to obtain enhanced incorporation of antibodies into the cells, as reported previously.[6,7] The fluorescent signal was observed in neutral pH medium after washing the cells twice. The 488- and 633-nm laser lines were used to image FcRn-EGFP and CypHer5 labeled-infliximab, respectively.

Biotin labeling of antibodies: Infliximab and IgY were labeled with biotin using EZ-link sulfo-NHS-biotin (Pierce, Rockford, IL, USA). Antibodies and sulfo-NHS-biotin were mixed at the molar ratio of 1:20 and incubated for 60 min at room temperature. Biotinylated antibodies were purified using Zeba desalt spin column (Pierce). Protein concentration was determined by BCA protein assay (Pierce) using bovine serum albumin as a standard.

Recycling assay: HeLa cells were transfected with the wild-type or variant FcRn-EGFP construct along with the β2m construct. The day after transfection, cells were seeded on 96-well plates at 4×10^4 cells/well. After further culturing for one day, recycling assays were performed. Hanks' balanced salt solutions (HBSS) (pH 6.0 and 7.4) were prepared supplemented with 10 mM MES (pH 6.0) and 10 mM Hepes (pH 7.4). The cells were washed with HBSS (pH 7.4) and pre-incubated with HBSS (pH 7.4) for 30 min at 37°C. After washing with HBSS, 10 μg/ml of biotinylated infliximab diluted in HBSS (pH 6.0) containing 0.5% fish gelatin was added to each well. The cells were incubated at 37°C for 1 hr to allow the antibody to be incorporated into the cells. Cells were then washed five times with HBSS (pH 7.4). Then, HBSS (pH 7.4) supplemented with 2% ultra-low IgG FCS (Invitrogen) was added to each well and incubated at 37°C for the indicated periods of time. The supernatant was collected and subjected to ELISA for quantitating the recycled antibody. In order to determine the amount of biotinylated infliximab incorporated into the cells during the 1-hr incubation at 37°C, cells were lysed using RIPA buffer supplemented with protease inhibitors (Nacalai Tesque, Kyoto, Japan) after washing five times with HBSS, and the lysate was subjected to ELISA. Biotinylated IgY was also used as a negative control in some experiments.

Enzyme linked immunosorbent assay (ELISA) for biotinylated antibody: NeutrAvidin (Pierce, Rockford, IL) was bound on Maxisorp 96-well black plates (Thermo Fisher Scientific, Roskilde, Denmark) using IMMUNO-TEK ELISA construction system (ZeptoMetrix, Buffalo, NY, USA). Supernatants or lysates obtained from the recycling assay were applied on the wells and incubated for 16 hr at 4°C. The plates were washed three times with Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBST). Peroxidase-conjugated goat anti-human IgG (Pierce) diluted with TBST was added to the plate and incubated for 1 hr at room temperature. After washing three times with TBST, chemiluminescent reagent (SuperSignal ELISA Femto, Pierce) was added and incubated for 1 min at room temperature. The chemiluminescent signal was detected using an ARVO 1420 multilabel counter (Perkin Elmer, Waltham MA, USA). When the amount of biotinylated IgY was measured, peroxidase-conjugated rabbit anti-chicken IgY (Promega, Madison, WI, USA) was used. For generation of a standard curve, 0.1 to 10 ng/ml of biotinylated corresponding protein was used.

Results

***FCGRT* variations found in a Japanese population:** Thirty-three genetic variations were found, including 17 novel ones, in 126 Japanese subjects (**Table 2**). Of these variations, 14 were located in the 5′-flanking region, 4 (2 synonymous and 2 non-synonymous) in the coding exons, 13 in the introns, 1 in the 3′-untranslated region (UTR), and 1 in the 3′-flanking region. All detected variations were in Hardy-Weinberg equilibrium

Table 2. Summary of *FCGRT* variations detected in this study

SNP ID		Location	Position		Nucleotide change	Amino acid change or known VNTR	Frequency	
This Study	dbSNP (NCBI) or reference		NW_927240.1	From the translational initiation site or from the end of the nearest exon				95% Confidence interval
MPJ6_FRT001[a]		5′-flanking	1557122	−2230	agaacctgaactA > Ccctgaccagcag		0.004	0.000–0.012
MPJ6_FRT002[a]			1557195	−2157	gggtgtcttgcaC > Actgtcatcccag		0.008	0.000–0.019
MPJ6_FRT003	rs78889190		1557207	−2145	cctgtcatcccaG > Ctgctttgggagg		0.020	0.003–0.037
MPJ6_FRT004[a]			1557221	−2131	gctttgggaggcC > Taaggtgggaggc		0.004	0.000–0.012
MPJ6_FRT005[a]			1557498_1557505	−1854_−1847	ggaaggaaggaaGGAAGGAA/-ggaggcaaggaa		0.024	0.005–0.043
MPJ6_FRT006	rs60964075		1557502_1557505	−1850_−1847	ggaaggaaggaaGGAA/-ggaggcaaggaa		0.103	0.066–0.141
MPJ6_FRT007	rs60964075		1557505_1557506	−1847_−1846	ggaaggaaggaaggaa-/GGAAggaggcaaggaa		0.099	0.062–0.136
MPJ6_FRT008[a]			1557505_1557506	−1847_−1846	ggaaggaaggaaggaa-/GGAAGGAAggaggcaaggaa		0.020	0.003–0.037
MPJ6_FRT009[a]			1557506	−1846	ggaaggaaggaaG > Agaggcaaggaag		0.004	0.000–0.012
MPJ6_FRT010[a]			1557540_1557547	−1812_−1805	aaggaaggaaggAAGGAAGG/-aggcaaggaagg		0.004	0.000–0.012
MPJ6_FRT011	rs2335534		1557671	−1681	tctgggagcagcG > Agctgtttaacgg		0.028	0.007–0.048
MPJ6_FRT012[a]			1558366	−986	gatacagaggggT > Gaggaggaggatc		0.004	0.000–0.012
MPJ6_FRT013	ref. 8		1558963_1558999	−389_−353	cgagggtagagcGGTTGGGGGCCCGGACTCCTGG GTCCGAGGGTAGAGC/-ggttgggggccc	VNTR3 > VNTR2	0.032	0.010–0.053
MPJ6_FRT014[a]			1559173	−179	actgagatccagT > Gtcaggggtgaaa		0.028	0.007–0.048
MPJ6_FRT015	rs59774409	Intron 1	1559442	IVS1 + 18	ggccgctccgggC > Tcagggccctgct		0.028	0.007–0.048
MPJ6_FRT016[a]			1559453	IVS1 + 29	gccagggccctgC > Ttgcaggcgggcg		0.147	0.103–0.191
MPJ6_FRT017	rs11551281	Exon 2	1559885	126[b]	ctcgcctgccccG > Tgggactccctgcc	Pro42Pro	0.044	0.018–0.069
MPJ6_FRT018	rs2878342	Exon 3	1560418	582[b]	ggagaggggccgC > Tggaaacctggag	Arg194Arg	0.028	0.007–0.048
MPJ6_FRT019[a]		Exon 4	1570485	629[b]	gcctgaaggcccG > Aacccagcagccc	Arg210Gln	0.004	0.000–0.012
MPJ6_FRT020	rs3810194	Intron 4	1570734	IVS4 + 7	agctgggtgaggT > Ccccgccaggtgg		0.048	0.021–0.074
MPJ6_FRT021	rs1132990		1570857	IVS4 + 130	gccttgaacctcA > Gcgcctgtcagtg		0.048	0.021–0.074
MPJ6_FRT022[a]			1570915	IVS4 + 188	ccaactgccttcC > Tgtctcctgctgc		0.020	0.003–0.037
MPJ6_FRT023	rs10525267		1571020_1571025	IVS4 + 293_ + 298	tgctgctgctgcTGCTGC/-gggtcttcctgg		0.083	0.049–0.117
MPJ6_FRT024[a]			1571170	IVS4–238	ctggcacagcccC > Tgccttgccgctg		0.020	0.003–0.037
MPJ6_FRT025	rs73582442		1571235	IVS4–173	gctggttcttacG > Atccaacctgggg		0.048	0.021–0.074
MPJ6_FRT026	rs73582446		1571314	IVS4–94	gctggaatctccG > Aaggctgggaggg		0.048	0.021–0.074
MPJ6_FRT027[a]		Exon 5	1571425	889[b]	ccagccaagtccT > Accgtgctcgtgg	Ser297Thr	0.020	0.003–0.037
MPJ6_FRT028	rs55662447	Intron 5	1571614_1571615	IVS5 + 90_ + 91	agagacccagagAG/-ggggacagaga		0.028	0.007–0.048
MPJ6_FRT029[a]			1571615	IVS5 + 91	gagacccagagaG > Tggggacagaga		0.004	0.000–0.012
MPJ6_FRT030	rs77741672		1571691	IVS5 + 167	gagaggggacgG > Cagacagagaccc		0.151	0.107–0.195
MPJ6_FRT031[a]			1571915	IVS5–46	gtcagacccagaG > Acgcctcagagat		0.020	0.003–0.037
MPJ6_FRT032	rs14769	3′-UTR	1572276	1304 (*206)[c]	taacacgagtttG > Aggcccgaatcag		0.044	0.018–0.069
MPJ6_FRT033[a]		3′-flanking	1572364	1312 + 80 (*214 + 80)[d]	tgggcctcggatC > Ttctcctacaggt		0.004	0.000–0.012

[a]Novel variations detected in this study.
[b]Positions in cDNA (NM_004107.3).
[c]Numbered from the termination codon TGA.
[d]Positions were shown as 1312 (*214) (final base of exon 6) + bases from the end of exon 6.

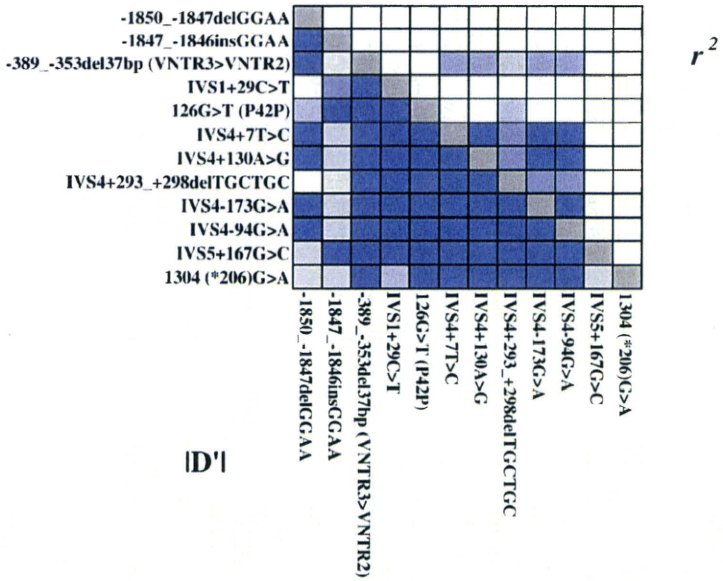


Fig. 1. Linkage disequilibrium (LD) analysis of *FCGRT*
Pairwise LD is expressed as r^2 (upper right) and |D′| (lower left) values (from 0 to 1) by 10-graded blue colors. A denser color represents closer linkage.

($p \geq 0.05$). Two novel non-synonymous variations, 629G > A (R210Q) and 889T > A (S297T), were found as heterozygotes. The allele frequencies were 0.004 for R210Q and 0.020 for S297T. The functional significance of these non-synonymous variations was explored *in vitro* in the following sections. The other coding variations were previously reported synonymous variations. A variable number of tandem repeats (VNTR) was detected in the 5′-flanking region as was found in Caucasian subjects,[8] and the frequencies of VNTR3 (with 3 repeats) and VNTR2 were 0.968 and 0.032, respectively. A short tandem repeat of GGAA was also detected in the 5′-flanking region with a repeat number of 8 (frequency: 0.024), 9 (0.103), 10 (0.754), 11 (0.099) and 12 (0.020). With the 12 detected variations with ≥ 0.03 frequencies, linkage disequilibrium (LD) was analyzed using |D′| and r^2 values (**Fig. 1**). Because of relatively weak linkage between the variations in r^2 values, haplotype analysis was not performed.

Intracellular localization of FcRn variants: Two novel non-synonymous variations, R210Q and S297T, were functionally tested using a mammalian expression system. First, relative expression levels of wild-type and variant FcRn proteins were evaluated by Western blotting. As shown in **Figure 2**, similar levels of the proteins were detected in the three FcRn constructs, and we did not find any statistically significant differences ($p > 0.05$) between the wild-type and the two variants assessed by Dunnett's multiple comparison test when normalized by the expression levels of glyceraldehyde-3-phosphate dehydrogenase as a control. When the wild-type levels were set as 100%, R210Q and S297T levels were 95.08 ± 12.38% and 93.94 ± 13.24%, respectively.

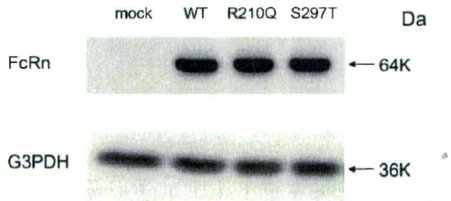


Fig. 2. Western blotting of wild-type and variant FcRns
Cell lysates obtained from the HeLa cells transfected with wild-type or either of the two variant FcRn-EGFP plasmids were subjected to electrophoresis, followed by transfer to the membrane. Detection of FcRn-EGFP was performed as described in Materials and Methods. One representative data of three independent transfections is shown. The FcRn band (64 KDa) consists of 37 KDa of FcRn and 27 KDa of EGFP. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) levels were used for normalization of the lysate proteins applied to electrophoretic gels.

In order to examine the differences of intracellular localization between wild-type FcRn and its variants, each EGFP fusion construct together with a human β2m construct was transfected into HeLa cells, and fluorescent images were observed by confocal microscopy. There have been several studies reporting the intracellular localization or trafficking of FcRn using fluorescent protein-tagged FcRn.[9-12] N- and C-terminally tagged FcRn showed similar localization.[13] Since FcRn is a type I membrane protein, N-terminal amino acid residues including R210 and S297 were located in the extracellular

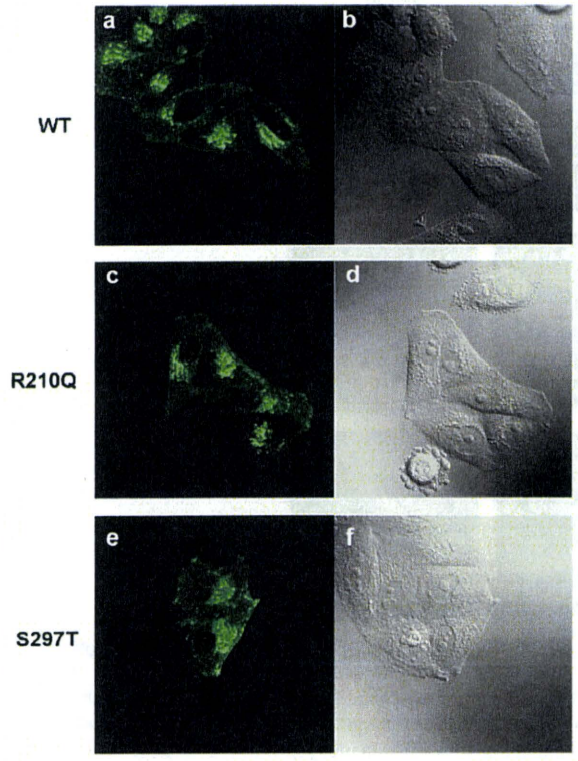


Fig. 3. Intracellular localization of wild-type (WT) and variant FcRns in HeLa cells
HeLa cells were transfected with wild-type (a) or variant (c; R210Q, e; S297T) FcRn-EGFP. The intracellular localization of FcRn-EGFP was observed by confocal laser scanning fluorescence microscopy. Differential interference contrast images of the field are also shown (b, d, f).

or intraluminal region. Therefore, we chose a C-terminal EGFP tag located in the cytoplasmic region of FcRn in order to minimize the effect of the fluorescent tag on the structural environment around the mutation sites.

As shown in **Figure 3a**, the fluorescent signal of wild-type FcRn-EGFP was located primarily in intracellular vesicular components, especially in the perinuclear region. Similar localization was observed for R210Q and S297T variants (**Figs. 3c** and **3e**), suggesting that these amino acid mutations do not affect the intracellular localization of FcRn.

Intracellular co-localization of FcRn variants and incorporated antibody: We then examined the co-localization of the incorporated CypHer5-labeled infliximab and FcRn-EGFP. The binding of CypHer5-labeled infliximab to FcRn was confirmed beforehand (data not shown).

As shown in **Figure 4**, co-localization of FcRn-EGFP and CypHer5-labeled infliximab in intracellular vesicular compartments was observed in HeLa cells expressing wild-type or variant FcRn. Since the fluorescence intensity of CypHer5 increases in acidic pH,[14] the observed fluorescent signal can indicate that CypHer5-labeled infliximab is localized in intracellular acidic compartments such as endosomes. Since the fluorescent images were obtained by confocal microscopy from cells which were washed with neutral pH media, the fluorescence is thought to be derived from incorporated antibodies and not from cell surface-bound antibodies. Therefore, these results showed that both types of FcRn variant, as well as wild-type FcRn, were in acidic endosomes in which incorporated antibodies localized.

Antibody recycling activity of FcRn variants: In order to elucidate the antibody recycling activity of wild-type and variant FcRn, we established the ELISA for biotinylated antibody (infliximab in this study), and measured the amount of recycled antibody from wild-type or variant FcRn-transfected cells. The binding of biotinylated infliximab to FcRn was confirmed by surface plasmon resonance (SPR) analysis (data not shown).

As shown in **Figure 5b**, recycled biotinylated infliximab was detected when the biotinylated infliximab had been loaded to the HeLa cells transfected with wild-type FcRn. The recycling was not detected in mock-transfected cells (**Fig. 5a**), showing that recycling was dependent on expression of FcRn. When the cells were incubated at 4°C for incorporation or recycling, the antibody was not detected in the supernatant. Therefore, recycling was mediated by intracellular trafficking of antibody and not by nonspecific mechanisms. As shown in **Figures 5c** and **5d**, similar levels of antibody recycling were also observed in HeLa cells transfected with either variant FcRn, suggesting similar IgG binding and intracellular trafficking properties of variant FcRns to those of wild-type FcRn. **Figure 6** shows the time course of antibody recycling from cells transfected with wild-type or variant FcRn. The amount of incorporated antibody was measured using the cell lysate at 0 min, and it is noteworthy that no statistical differences assessed by Dunnett's multiple comparison test were observed in the amount of incorporated antibodies between wild-type and either variant FcRn at time 0 (data not shown). The amount of recycled antibody at each time point was expressed as a percentage of the initially incorporated antibody. There was no significant difference between wild-type and the variant FcRns in the amount of recycled antibody, suggesting that these amino acid substitutions do not affect the antibody recycling activity of FcRn.

Discussion

In general, antibody therapeutics have longer half-lives than those of chemical drugs, and the $T_{1/2}$ of IgGs, except for IgG3, in humans is around 21 days. IgG1, IgG2 and IgG4, which are currently used isoforms for antibody therapeutics, have high affinities for FcRn.[15] Escaping from intracellular degradation by binding to FcRn has shown to contribute to this long half-life of the IgGs.

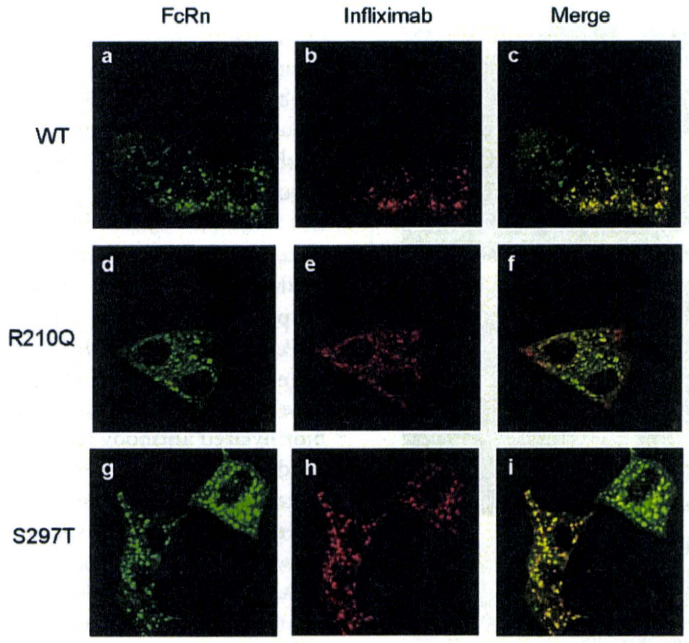


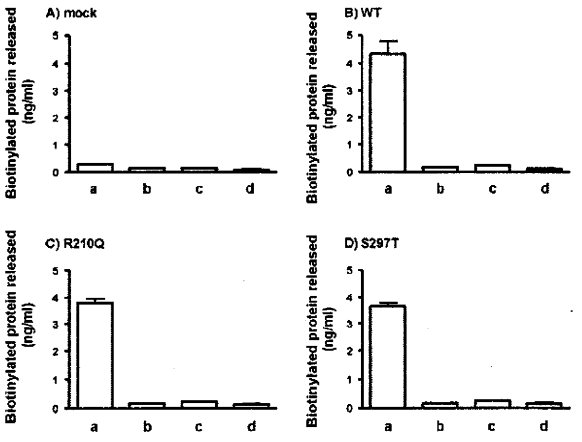
Fig. 4. Co-localization of CypHer5-labeled infliximab and FcRn in HeLa cells expressing wild-type (WT) or a variant FcRn
HeLa cells transfected with wild-type (a, b, c), or variant (d, e, f; R210Q, g, h, i; S297T) FcRn-EGFP were incubated with CypHer5-labeled infliximab in cell culture media containing sodium phosphate buffer (pH. 6.0) for 2–3 hr. After washing the cells twice with neutral pH medium, the fluorescent signal was observed. Panels (a, d, g) and (b, e, h) show the intracellular localization of FcRn-EGFP and the incorporated CypHer5-labeled infliximab, respectively. In panels (c, f, i) the fluorescent signal of FcRn-EGFP was merged with that of CypHer5-labeled infliximab.

Large interindividual variations in pharmacokinetic parameters have been reported for at least several antibody therapeutics. For example, trough concentrations in repetitive dosing of antibodies were reported to show 5.6-fold interindividual differences in 22 palivizumab-treated patients,[16] 18.2-fold differences in 16 cetuximab-treated patients,[17] and over 70-fold differences in 86 infliximab-treated patients.[18] In addition, large percent coefficients of variation were reported for $T_{1/2}$, such as 72.0% for gemtuzumab ozogamicin[19] and 76.4% for basiliximab,[20] after second dose of their treatments. We presumed that changes in FcRn expression levels and function caused by genetic variations of *FCGRT* may lead to these interindividual differences in pharmacokinetics of antibody therapeutics.

In order to identify genetic polymorphisms of *FCGRT*, we sequenced genomic DNA from 126 Japanese subjects. A total of 33 genetic variations, including 17 novel ones, were detected. A VNTR was detected in the 5′ -flanking region, as was the case in Caucasian subjects reported previously.[8] Although a recent study showed that no significant impact was observed in the rates of maternal-fetal IgG transfer,[21] VNTR3 is known to be associated with 1.66-fold higher transcriptional activity than VNTR2 *in vitro*. In addition, monocytes with VNTR3/3 showed increased binding of IgG compared to those with 2/3.[8] Thus, this variation may contribute to the interindividual differences in pharmacokinetics of antibody therapeutics. The allele frequency of VNTR2 in Japanese (0.032) was lower than that in Caucasians (0.075).[8]

In this study, two novel nonsynonymous variations were found and their functional significance was assessed *in vitro* using a mammalian expression system. However, the two FcRn variants did not show any changes in intracellular localization or recycling, suggesting that the two nonsynonymous substitutions found in a Japanese population probably do not contribute to the interindividual variations in the pharmacokinetics of antibody therapeutics. Since FcRn function is important for maintenance of IgG levels as well as maternal-fetal IgG transfer, functionally-affecting genetic variations might be few to retain its functional capability.

Amino acid residues of human FcRn that interact with IgG were reported to be E138, E139, D153 and W154, in the α2 domain.[1] (Amino acid numbers shown in this paper include the signal peptide.). The electrostatic binding of these anionic amino acid residues in FcRn with H310 and H435 in IgG, which has an isoelectric point of pH 7.6, defines the strict pH-dependent binding of IgG to FcRn.[22] The variant amino acid residues identified in this study, R210Q and S297T, are both located in the α3 domain of FcRn. According to the predicted higher order structure,[1] R210 and S297 are located very close to the



Column	a	b	c	d
Reagents	Infliximab			IgY
Loading Temp. (°C)	37	4	37	37
Recycling Temp. (°C)	37	37	4	37

Fig. 5. Recycling of biotinylated antibodies from wild-type (WT) or variant FcRn-transfected HeLa cells
HeLa cells transfected with wild-type or a variant FcRn were incubated for 1 hr with biotinylated infliximab. After washing, the cells were further incubated for 2 hr. The amount of recycled protein in the supernatant was determined by ELISA. Experimental conditions are shown in the table. For the samples shown as columns a-c, biotinylated infliximab was loaded, whereas biotinylated IgY was used for d. The temperature for antibody loading was 37°C (a, c, d) or 4°C (b). The temperature for recycling antibodies from antibody-loaded cells was 37°C (a, b, d) or 4°C (c).

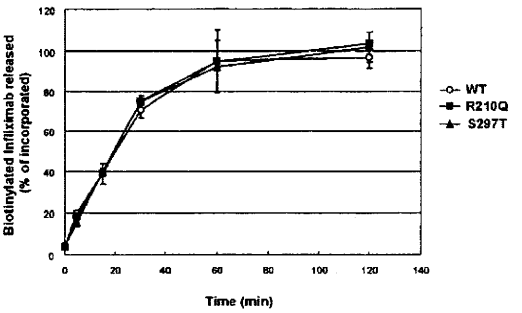


Fig. 6. Quantitative analyses of recycling of biotinylated infliximab; Time course of release of the biotinylated infliximab incorporated into the HeLa cells transfected with wild-type (WT) or variant FcRn
HeLa cells transfected with wild-type or a variant FcRn were incubated for 1 hr with biotinylated infliximab. After washing, cells were further incubated for the indicated periods of time. The amount of recycled protein was determined by ELISA. The amount of recycled antibody at each time point was expressed as a percentage of the initially incorporated antibody at time 0.

transmembrane region that is distant from the IgG binding site. Considering the results obtained here, where no difference in antibody recycling activity between wild-type and each variant FcRn was detected *in vitro*, the amino acid substitutions identified in a Japanese population may not have significant impact on structural and functional properties of FcRn. Although FcRn is known to bind with albumin as well as IgG, the albumin binding site of FcRn has been identified as H189, which also is located in the α2 domain.[23] The polymorphic sites are also far from the albumin binding site. However, the effect of amino acid substitutions R210Q and S297T on the albumin recycling activity via FcRn should be determined in a future study.

In the present study, we used HeLa cells to examine the localization and recycling activity of FcRn variants. Since endogenous expression of FcRn protein in HeLa cells has not been detected,[24] we considered HeLa cells suitable for examining the antibody recycling activity of variant FcRn since the background responses are negligible. In fact, as shown in **Figure 5**, antibody recycling was detected only in FcRn-transfected cells. Therefore, we concluded that HeLa cells can be used as a suitable model for evaluating the function of variant FcRn proteins.

Our results suggested that at least no common functional polymorphic site with amino acid change was present in *FCGRT* in our Japanese population. Since FcRn function is important for maintenance of IgG levels, there may be few functionally-affecting genetic variations. Further analysis is necessary for the functional significance of transcriptional regulatory regions.

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References

1) Andersen, J. T. and Sandlie, I.: The versatile MHC class I-related FcRn protects IgG and albumin from degradation: implications for development of new diagnostics and therapeutics. *Drug Metab. Pharmacokinet.*, **24**: 318–332 (2009).
2) Lobo, E. D., Hansen, R. J. and Balthasar, J. P.: Antibody pharmacokinetics and pharmacodynamics. *J. Pharm. Sci.*, **93**: 2645–2668 (2004).
3) Suzuki, T., Ishii-Watabe, A., Tada, M., Kobayashi, T., Kanayasu-Toyoda, T., Kawanishi, T. and Yamaguchi, T.: Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. *J. Immunol.*, **184**: 1968–1976 (2010).
4) Claypool, S. M., Dickinson, B. L., Yoshida, M., Lencer, W. I. and Blumberg, R. S.: Functional reconstitution of human FcRn in Madin-Darby canine kidney cells requires co-expressed human beta 2-microglobulin. *J. Biol. Chem.*, **277**: 28038–28050 (2002).
5) Praetor, A. and Hunziker, W.: Beta(2)-microglobulin is important for cell surface expression and pH-dependent IgG bind-

ing of human FcRn. *J. Cell. Sci.*, **115**: 2389–2397 (2002).
6) Tesar, D. B., Tiangco, N. E. and Bjorkman, P. J.: Ligand valency affects transcytosis, recycling and intracellular trafficking mediated by the neonatal Fc receptor. *Traffic*, **7**: 1127–1142 (2006).
7) Kamei, D. T., Lao, B. J., Ricci, M. S., Deshpande, R., Xu, H., Tidor, B. and Lauffenburger, D. A.: Quantitative methods for developing Fc mutants with extended half-lives. *Biotechnol. Bioeng.*, **92**: 748–760 (2005).
8) Sachs, U. J., Socher, I., Braeunlich, C. G., Kroll, H., Bein, G. and Santoso, S.: A variable number of tandem repeats polymorphism influences the transcriptional activity of the neonatal Fc receptor alpha-chain promoter. *Immunology*, **119**: 83–89 (2006).
9) Goebl, N. A., Babbey, C. M., Datta-Mannan, A., Witcher, D. R., Wroblewski, V. J. and Dunn, K. W.: Neonatal Fc receptor mediates internalization of Fc in transfected human endothelial cells. *Mol. Biol. Cell.*, **19**: 5490–5505 (2008).
10) Ober, R. J., Martinez, C., Lai, X., Zhou, J. and Ward, E. S.: Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. *Proc. Natl. Acad. Sci. U.S.A.*, **101**: 11076–11081 (2004).
11) Ober, R. J., Martinez, C., Vaccaro, C., Zhou, J. and Ward, E. S.: Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. *J. Immunol.*, **172**: 2021–2029 (2004).
12) Ward, E. S., Martinez, C., Vaccaro, C., Zhou, J., Tang, Q. and Ober, R. J.: From sorting endosomes to exocytosis: association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. *Mol. Biol. Cell.*, **16**: 2028–2038 (2005).
13) Gan, Z., Ram, S., Vaccaro, C., Ober, R. J. and Ward, E. S.: Analyses of the recycling receptor, FcRn, in live cells reveal novel pathways for lysosomal delivery. *Traffic*, **10**: 600–614 (2009).
14) Mark, S. B., Burns, D. D., Cooper, M. E. and Gregory, S. J.: A pH sensitive fluorescent cyanine dye for biological applications. *Chem. Commun.*, **23**: 2323–2324 (2000).
15) Ternant, D. and Paintaud, G.: Pharmacokinetics and concentration-effect relationships of therapeutic monoclonal antibodies and fusion proteins. *Expert Opin. Biol. Ther.*, **5 Suppl 1**: S37–47 (2005).
16) Subramanian, K. N., Weisman, L. E., Rhodes, T., Ariagno, R., Sanchez, P. J., Steichen, J., Givner, L. B., Jennings, T. L., Top, F. H. Jr, Carlin, D. and Connor, E.: Safety, tolerance and pharmacokinetics of a humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. MEDI-493 Study Group. *Pediatr. Infect. Dis. J.*, **17**: 110–115 (1998).
17) Cèzè, N., Ternant, D., Piller, F., Degenne, D., Azzopardi, N., Dorval, E., Watier, H., Lecomte, T. and Paintaud, G.: An enzyme-linked immunosorbent assay for therapeutic drug monitoring of cetuximab. *Ther. Drug Monit.*, **31**: 597–601 (2009).
18) St Clair, E. W., Wagner, C. L., Fasanmade, A. A., Wang, B., Schaible, T., Kavanaugh, A. and Keystone, E. C.: The relationship of serum infliximab concentrations to clinical improvement in rheumatoid arthritis: results from ATTRACT, a multicenter, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.*, **46**: 1451–1459 (2002).
19) Dowell, J. A., Korth-Bradley, J., Liu, H., King, S. P. and Berger, M. S.: Pharmacokinetics of gemtuzumab ozogamicin, an antibody-targeted chemotherapy agent for the treatment of patients with acute myeloid leukemia in first relapse. *J. Clin. Pharmacol.*, **41**: 1206–1214 (2001).
20) Kovarik, J. M., Nashan, B., Neuhaus, P., Clavien, P. A., Gerbeau, C., Hall, M. L. and Korn, A.: A population pharmacokinetic screen to identify demographic-clinical covariates of basiliximab in liver transplantation. *Clin. Pharmacol. Ther.*, **69**: 201–209 (2001).
21) Freiberger, T., Ravcuková, B., Grodecká, L., Kurecová, B., Jarkovský, J., Bartonková, D., Thon, V. and Litzman, J.: No association of FCRN promoter VNTR polymorphism with the rate of maternal-fetal IgG transfer. *J. Reprod. Immunol.*, **85**: 193–197 (2010).
22) Vaughn, D. E., Milburn, C. M., Penny, D. M., Martin, W. L., Johnson, J. L. and Bjorkman, P. J.: Identification of critical IgG binding epitopes on the neonatal Fc receptor. *J. Mol. Biol.*, **274**: 597–607 (1997).
23) West, A. P., Jr. and Bjorkman, P. J.: Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complex-related Fc receptor. *Biochemistry*, **39**: 9698–9708 (2000).
24) Liu, X., Ye, L., Christianson, G. J., Yang, J. Q., Roopenian, D. C. and Zhu, X.: NF-kappaB signaling regulates functional expression of the MHC class I-related neonatal Fc receptor for IgG via intronic binding sequences. *J. Immunol.*, **179**: 2999–3011 (2007).

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総説

抗体医薬品の体内動態制御に関わる受容体：FcRn

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要約：腫瘍や自己免疫疾患等の治療を目的とした分子標的薬として，抗体医薬品の研究開発が国内外で活発に行われている．抗体医薬品の特徴は標的分子に高い親和性をもって極めて特異的に結合することであるが，他のバイオ医薬品と比較して血中半減期が長いことも特筆すべき点である．ペプチドあるいはタンパク質を医薬品として応用する場合には血中半減期が実用化のためのハードルとなることが少なくない．しかし，多くの抗体医薬品は，生体内IgGの分解抑制に関わるneonatal Fc receptor（FcRn）を介したリサイクリング機構を利用することができるため，数日～数週間という長い血中半減期を有している．FcRnは齧歯類の新生児小腸に高発現し，乳汁に含まれる母親由来IgGの吸収に関与する受容体として同定された．その後の研究により，FcRnが成体においても種々の組織に発現し，IgGのリサイクリングやトランスサイトーシス等に関与していることが報告され，母子免疫以外にも様々な側面でIgGの体内動態制御に関わっていることが明らかにされている．我々は，既承認抗体医薬品のFcRn結合親和性を解析し，ヒトでの血中半減期とFcRn結合親和性の相関，および抗体医薬品のFcRn結合親和性を規定する構造特性の一端を明らかにした．近年の創薬研究では，FcRn結合親和性を改変した抗体医薬品等の開発が進んでいる他，FcRnのもう1つのリガンドであるアルブミンを利用することにより体内動態特性を改変したタンパク質医薬品の開発も進んでいる．FcRnは，抗体医薬品をはじめとするバイオ医薬品の体内動態制御に関わる鍵分子の1つと言えるであろう．

はじめに

2010年9月までに日米欧で29品目のモノクローナル抗体医薬品が承認されている（図1）．既承認抗体医薬品の中には顕著な有効性が認められているものも少なくなく，例えば，抗TNF α 抗体が奏功している関節リウマチの治療では“The era of biological therapy has arrived.”と言われるほどである(1)．現在臨床開発段階にある抗体医薬品は140品目に上り，今後さらに承認品目数が増加する可能性が高い．本稿では，抗体医薬品および抗体医薬品に類似した性質を持つFc融合タンパク質医薬品の概略を述べた後，抗体医薬品の体内動態制御に関わる受容体FcRnについて，発見の経緯，構造と機能，および抗体医薬品の体内動態との関連に関して，既承認抗体医薬品のFcRn結合親和性を解析した我々の知見を含めて紹介する．

1. 抗体医薬品

抗体医薬品の多くは抗腫瘍作用あるいは免疫調節作用を持つ医薬品である（図1）．これらは作製法に起因するアミノ酸配列の相違により，マウス抗体，キメラ型抗体，ヒト化抗体，ヒト抗体に分類される(2)．1975年にKöhlerとMilsteinによりマウスモノクローナル抗体作製技術(3)が開発された当初，ミサイル療法が現実のものになると抗体医薬品の開発に大きな期待が寄せられた．しかし，マウス抗体をヒトに投与すると高頻度で抗体産生が起こりアナフィラキシー反応が懸念されるために繰り返し投与が困難であること，および半減期が短いことが障壁となり(4)，多くの開発が失敗に終わった．ヒト生体内IgGの半減期が約20日(5)であるのに対して，ヒトに投与されたマウス抗体の半減期は数時間～3日程度である(6)．1980年代に承認された抗体医薬品は，腎移植後の急性拒絶反応の治療に用いられるマウス抗CD3抗体のみであったが，その後，キメラ型抗体(7)やヒト化抗体(8)の作製技術が確立されてヒトIgG骨格を持った抗体を遺伝子組換えにより作製・製造することが可能になり，1990年代半ば以降，抗体医薬品の開発が急伸した．マウス抗体の可変領域あるいは相補性決定領域以外をヒトIgG由来の配列に置き換えることで，免疫原性を低下させ，

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分類	承認年	名称	標的	主な適応疾患	血中半減期
マウス抗体	1986	ムロモナブ-CD3	CD3	腎移植後の急性拒絶反応	0.75日
	2002	イブリツモマブ チウキセタン	CD20	非ホジキンリンパ腫	1.1日
	2003	トシツモマブ	CD20	非ホジキンリンパ腫	2.7日
キメラ型抗体	1997	リツキシマブ	CD20	非ホジキンリンパ腫	9.4日
	1998	バシリキシマブ	CD25	腎移植後の急性拒絶反応	4.1日
	1998	インフリキシマブ	TNFα	関節リウマチ	9.5日
	2004	セツキシマブ	EGFR	頭頸部癌，結腸・直腸癌	4.8日
ヒト化抗体	1997	ダクリズマブ	CD25	腎移植後の急性拒絶反応	20日
	1998	パリビズマブ	RSV F protein	RSウイルス感染	19～27日
	1998	トラスツズマブ	HER2	転移性乳癌	2.7～10日
	2001	アレムツズマブ	CD52	B細胞性慢性リンパ性白血病	12日
	2003	オマリズマブ	IgE	喘息	20日
	2003	エファリズマブ	CD11	尋常性乾癬	5.5～10.5日(*)
	2004	ベバシズマブ	VEGF	結腸・直腸癌	11.7～13.4日(*)
	2005	トシリズマブ	IL-6R	キャッスルマン病，関節リウマチ	5.5日(*)
ヒト抗体	2002	アダリムマブ	TNFα	関節リウマチ	14.7～19.3日
	2009	ゴリムマブ	TNFα	関節リウマチ	14日(*)
	2009	ウステキヌマブ	IL12, IL23 p40	乾癬	14.9～45.6日(*)
	2009	カナキヌマブ	IL-1β	クリオピリン関連周期性症候群	26日(*)
	2009	オファツムマブ	CD20	慢性リンパ性白血病	14日(*)
Fc融合タンパク質	1998	エタネルセプト	TNFα, LTα	関節リウマチ	4日
	2003	アレファセプト	CD2	尋常性乾癬	11.3日(*)
	2005	アバタセプト	CD80/CD86	関節リウマチ	13.1日(*)
	2008	リロナセプト	IL-1	クリオピリン関連周期熱症候群	6.3～7.5日(*)
	2008	ロミプロスチム	TPOR	血小板減少性紫斑病	1～34日(*)

図1　既承認抗体医薬品の例（マウス抗体およびヒト IgG1 の Fc 領域を持つ抗体と Fc 融合タンパク質）
血中半減期は文献 6 より引用（*は製品添付文書より引用）

血中半減期を延長できたことが抗体医薬品の実用化におけるブレークスルーであったと言える．

抗体分子は，抗原との結合を担う Fab 領域と Fc 受容体や補体との結合に関与する Fc 領域から構成される．近年，抗体に類似した特徴を持つ医薬品として，標的分子特異的結合能を持つ受容体タンパク質等を抗体の Fc 領域と融合させた Fc 融合タンパク質医薬品の開発も進んでおり，これまでに日米欧で 5 品目が承認されている（図 1）．例えば，TNF 受容体の細胞外領域と Fc 領域の融合タンパク質であるエタネルセプトは，TNF を中和する作用を持ち，関節リウマチ等の治療に用いられる．これらは，標的分子結合部位のみでは医薬品とすることが難しいタンパク質あるいはペプチドを Fc 領域との融合タンパク質として血中安定化を図ることにより臨床応用することに成功した例と言える．本稿では，抗体医薬品として，モノクローナル抗体医薬品および Fc 融合タンパク質医薬品を指すこととする．

2.　FcRn の発見の経緯および構造と機能

1）FcRn 発見の経緯

IgG の体内動態制御に関わる受容体は，IgG の代謝や輸送に特異性と飽和が存在することを根拠に 1964 年に Brambell によりその存在が提唱され，Brambell receptor とも言われていたものである(9)．その後，新生児小腸での IgG 吸収に関わる受容体（neonatal gut transport receptor）として“FcRn”，IgG 分解抑制に関わる受容体（IgG protection receptor）として“FcRp”の存在が考えられるようになっていた(10)．1989 年になり，ラット新生児小腸から FcRn がクローニングされて一次構造が解明され，β2-microglobulin（β2m）とヘテロダイマーを形成する受容体であることも明らかになった(11)．キメラ型抗体医薬品やヒト化抗体医薬品が承認され始めていた 1996 年，β2m ノックアウトマウスを用いた実験により，FcRn が IgG の半減期制御に関わる受容体 FcRp としての機能も合わせ持つことが明らかにされた(12-14)．IgG の半減期制御における FcRn の寄与については FcRn（α鎖）のノックアウトマウスを用いた検証も行われている(15)．これらの研究により，FcRn および FcRp と考えられていた受容体の実体は同じ分子であり，“FcRn”として同定された受容体が IgG の輸送と血中濃度維持の両方の機能を担っていることが明らかになった．

2）FcRn の構造と機能

ヒト FcRn は MHC クラス I 分子に類似した構造を持ち，342 アミノ酸残基からなるα鎖（図 2）と 99 アミノ酸残基からなるβ鎖（β2m）により構成される．β2m は FcRn α鎖の細胞内局在に関与する(16, 17) ほか，IgG 結合にも関与している(17)．

FcRn と IgG は酸性条件下（pH6～6.5）で結合し，中性条件下（pH7.4）では解離する．FcRn との結合には，

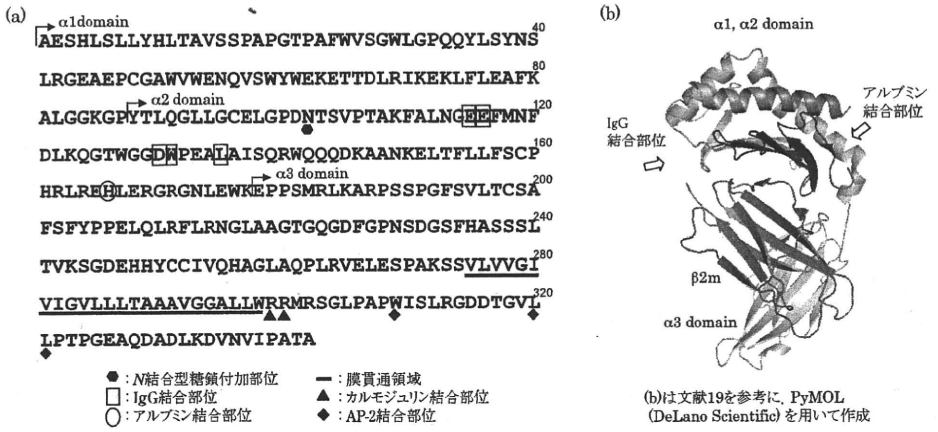

図2 ヒトFcRn（α鎖）のアミノ酸配列（a）および細胞外領域の立体構造（b）

IgGの高次構造上CH2領域とCH3領域の間に位置するLeu253，His310，His435が関与し，IgGのHis残基とFcRnの酸性アミノ酸残基の静電的相互作用がpH依存性の結合に寄与しているとされている(18)．IgG結合に関与するFcRn上のアミノ酸残基は，FcRnα2ドメインのGlu115，Glu116，Asp130，Trp131，Leu135とされている(19)が，この部位のアミノ酸配列には種による相違がみられ，FcRnとIgGの結合に種差があることの一因と考えられている(18)．現在では，マウスIgG1およびIgG2aはヒトFcRnに結合しないことが明らかにされており(20)，ヒトに投与されたマウス抗体が短半減期であることの科学的根拠となっている．

FcRnにはIgGの他にアルブミンもpH依存的に結合する(21)．FcRnにおけるアルブミン結合部位はHis166であり，IgG結合部位とは異なっているため，IgGとアルブミンは拮抗することなくFcRnに結合する(22)．ヒトにおけるアルブミンの半減期は約20日であり，IgGと同様である(22)．

FcRnの膜貫通領域直下の2つのArg残基はカルモジュリンの結合に関わっており，カルモジュリンとの結合がFcRnタンパク質の半減期やIgGの輸送効率に関与していることが報告されている(23)．また，ヒトを含む多くの種で保存されているTrp309，Leu320，Leu321がadaptor protein AP-2との結合およびFcRnのエンドサイトーシスに関与することが報告されている(18)．

3. 生体各組織におけるFcRnの役割

FcRnは新生児期のみならず成体においても種々の組織に発現していることが明らかにされており(21)，IgGの分解抑制による血中濃度維持，および細胞内経路を介したIgGの輸送により，IgGによる生体防御機構に関与していると考えられる．

1) IgG血中濃度維持

FcRnは，主として細胞内に局在している(24)．FcRnは細胞内に取り込まれたIgGとエンドソーム内で結合して，IgGがリソソームに輸送されて分解されるのを抑制し，IgGを細胞外にリサイクルすることによってIgGの血中濃度を維持していると考えられている(21)（図3a）．IgG血中濃度維持には血管内皮細胞および血球系細胞に発現しているFcRnが関与していることが，組織特異的ノックアウトマウスを用いた実験等により示されている(25, 26)．IgGの細胞への取り込みはピノサイトーシスによるとされているが(21)，in vitro実験ではFcRn発現量とIgG取り込み量が相関するという報告(27)がある他，Na^+/H^+交換輸送体の活性が高い上皮細胞やがん細胞あるいは炎症部位などでは細胞表面の微小環境の酸性化により細胞表面でのIgGとFcRnの結合が可能になり，FcRnがIgG取り込みに関与している可能性も考えられている(18)．

2) IgG輸送

FcRnは上皮細胞等においてトランスサイトーシスによりIgGを輸送する（図3b）．多量体免疫グロブリン受容体pIgRによる二量体IgAの輸送が基底膜側から管腔側への単方向であるのと対照的に，FcRnは双方向性にIgGを輸送し得ることが報告されている(16, 28)．各組織でのFcRnの役割については未解明の点が多いが，齧歯類の新生児小腸における乳汁中IgGの吸収の他，ヒト，サル，およびウサギの胎盤における母親由来IgGの胎児への輸送，成体の小腸におけるIgGの管腔側への分泌と基底膜側への抗原の取り込み，および抗原提示細胞への輸送(29)，腎糸球体基底膜からのIgG除去(30)等にFcRnが関与しているとされている．また，好中球における抗体結合細菌の貪食(31)や，抗原提示細胞での抗原抗体複合体の取り込みと抗原提示にもFcRnが関わっている(32)．

4. 抗体医薬品の体内動態とFcRn

1) 抗体医薬品の血中半減期とFcRn

図1に抗体医薬品のヒトにおける血中半減期を示し

た(6)．マウス抗体の血中半減期が短いこと，また，表に記載したキメラ型，ヒト化，ヒト抗体および Fc 融合タンパク質は全てヒト IgG1 由来の Fc 領域を持つが，その血中半減期は製品により異なっていることが分かる．一般に，バイオ医薬品の血中半減期制御には，分子量や等電点，受容体結合性など多くの要素が関わっている．我々は，抗体医薬品の血中半減期と FcRn 結合親和性の関連を明らかにする目的で，11 種類の代表的な既承認抗体医薬品について，表面プラズモン共鳴法を用いてヒト FcRn との結合親和性を解析した．その結果，ヒト IgG1 由来 Fc 領域を持つ抗体医薬品の FcRn 結合親和性は製品により異なっており，特に Fc 融合タンパク質では FcRn 結合親和性が低いことが明らかになった．また，一部の抗体を除いて，FcRn 結合親和性とヒトでの血中半減期（文献値）に相関が認められ，FcRn 結合親和性が血中半減期制御に関わる重要な要素の 1 つであることが示された(33)．

さらに，評価に用いた抗体医薬品の中には，アロタイプの違いにより Fc 領域のアミノ酸 2 残基の配列が異なる製品があることや，Fc 領域に結合している糖鎖の構造も製品により異なっていると考えられることから，これらの限定的な差が FcRn 結合親和性に影響している可能性を考え，パパイン消化により Fc 領域を Fab 領域あるいは受容体領域から分離して FcRn との結合親和性を測定した．興味深いことに，FcRn 結合親和性が相対的に低い抗体医薬品の FcRn 結合親和性は，パパイン消化により上昇し，FcRn に高親和性を示す抗体医薬品と同程度になることが明らかになった（図 4）．すなわち，アロタイプや糖鎖構造の相違が FcRn 結合親和性に影響しているのではなく，Fab 領域あるいは受容体領域の構造が Fc 領域内の FcRn 結合部位の高次構造に影響する結果，各抗体医薬品が固有の FcRn 結合親和性を有していることが示唆された(33)．

2）抗体医薬品の生体内分布と FcRn

3. の 2）で述べたように，FcRn を介した生体局所での IgG 動態制御に関する知見が集積され，IgG を介し

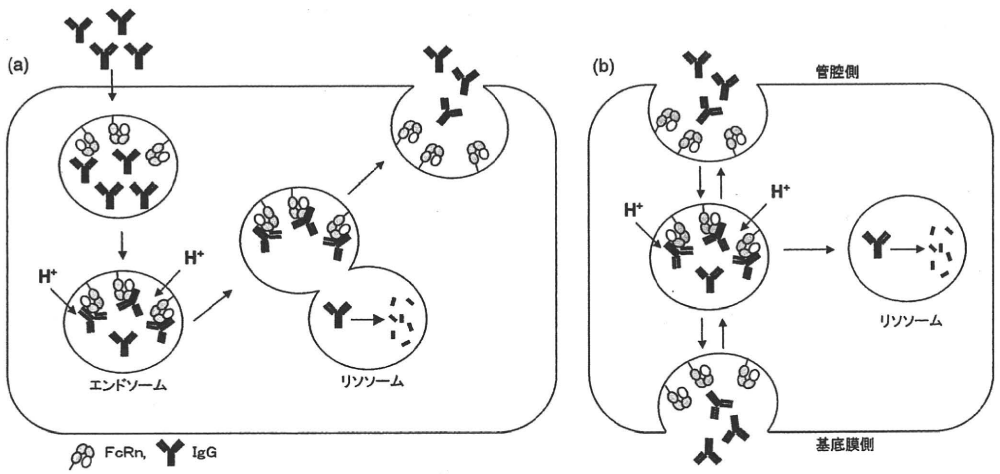

図 3　FcRn を介した IgG のリサイクリング（a）およびトランスサイトーシス（b）
(a) リサイクリング：血管内皮細胞等に発現している FcRn は IgG 分解抑制を担っている．ピノサイトーシスにより細胞内に取り込まれた IgG は，H^+の流入により酸性化したエンドソーム内で FcRn に結合する．FcRn に結合しなかった IgG はリソソームで分解されるが，FcRn と結合した IgG は分解を免れ，細胞外に輸送されて FcRn から解離することにより，リサイクルされる．（文献 21 より改変）
(b) トランスサイトーシス：小腸上皮細胞等に発現している FcRn は細胞内経路を介した IgG 輸送を担っている．

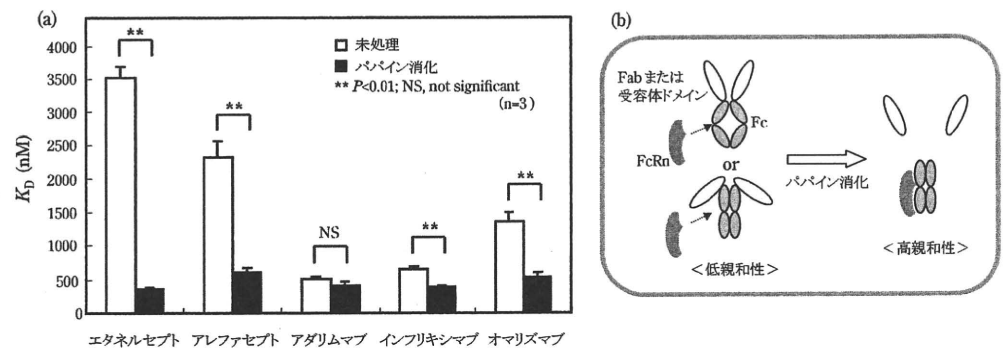

図 4　抗体医薬品の FcRn 結合親和性のパパイン消化による変化（a）およびデータから想定されるモデル（b）
(a) Suzuki T. et al.（文献 33 より一部改変）

た免疫応答におけるFcRnの役割が解明されつつある．投与された抗体医薬品も生体内IgGと同様の機構により局所で輸送されると考えられるが，血中から組織への抗体医薬品の移行は主として対流や拡散等の受動的な機構によるものと考えられており(34)，抗体医薬品の生体内分布とFcRnの関連に関する知見は限られている．しかし，FcRnを介した抗体の胎盤通過が抗体医薬品の安全性を考える上で重要であるという点は明確であり(35)，ヒトでは胎盤にFcRnが発現する妊娠第3期以降には母親に投与された抗体医薬品が胎児に移行する可能性が高い．また，非臨床生殖発生毒性試験の実施に際しては，試験に用いる動物のFcRnと被験薬の結合親和性や，動物におけるIgG母子免疫の機構を十分に考慮する必要がある．この他，アルツハイマー病治療薬として開発が進められている抗βアミロイド抗体の作用機構についてはまだ一定の見解が得られていないが，FcRnの関与を示唆する報告もあり(36)，抗体医薬品の生体内分布とFcRnの関連の解明は今後の課題である．

5. FcRnとの結合性を利用したバイオ医薬品の開発動向

現在までに，FcRnとの結合親和性を上昇させ，血中半減期の延長と有効性の向上を目的とした改変型抗体(37)の開発が進められている他，ホルモンやサイトカイン，酵素などをFc領域と融合させることにより血中半減期を延長することが試みられている(38)．また，エリスロポエチンあるいは卵胞刺激ホルモンをFc領域と融合させたタンパク質では，FcRnを介した肺上皮細胞におけるトランスサイトーシスを利用し，経肺的に投与する試みが報告されている(39, 40)．FcRnはアルブミンの半減期制御にも関わっているが，Fc融合タンパク質の場合と同様，有効成分となるタンパク質をアルブミン融合タンパク質(41)あるいはアルブミン結合性タンパク質(42)に改変することにより，血中半減期の延長を図っている例もある．

一方，FcRnを介したリサイクリングには，結合のpH依存性が保たれていることが重要であるため，pH非依存的にFcRnと結合する改変型抗体はFcRnの阻害薬として作用する．このような抗体は内因性抗体のクリアランスを亢進させるため，自己免疫疾患治療に有用である可能性が考えられており(43)，今後，FcRn阻害薬が自己免疫疾患治療薬として開発される可能性もある(44)．ヒト血漿由来IgGを大量に静注する免疫グロブリン製剤の有効性メカニズムにもFcRnの飽和による内因性自己抗体のクリアランス亢進が関わっている可能性が考えられている(45)．

おわりに

バイオ医薬品の開発には，生命科学の進歩による新たな知見の蓄積と，医薬品製造技術の進展が関わっており，抗体医薬品は両者が相乗的に作用して発展を続ける好例と言える．ペプチドおよびタンパク質医薬品では，体内動態の改善が開発の鍵となっている例が少なくないが，IgGのFc領域やアルブミンは，種々の生理活性タンパク質に血中安定性を付与し得るドメインとして今後も活用されると予想される．FcRnを含め，タンパク質医薬品の体内動態制御に関わる分子的基盤の解明が進み，新薬の分子設計や関連する医薬品の有効性・安全性確保に寄与することが期待される．

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文　献

1) Isenberg DA. Nat Clin Pract Rheumatol. 2006;2:229.
2) 川西 徹. 日薬理誌. 2008;131:102-108.
3) Köhler G, et al. Nature. 1975;256:495-497.
4) 野口浩. 医学のあゆみ. 1993;167:457-462.
5) Morell A, et al. J Clin Invest. 1970;49:673-680.
6) Lobo ED, et al. J Pharm Sci. 2004;93:2645-2668.
7) Morrison SL, et al. Proc Natl Acad Sci U S A. 1984;81:6851-6855.
8) Jones PT, et al. Nature. 1986;321:522-525.
9) Brambell FW, et al. Nature. 1964;203:1352-1354.
10) Junghans RP. Immunol Res. 1997;16:29-57.
11) Simister NE, et al. Nature. 1989;337:184-187.
12) Ghetie V, et al. Eur J Immunol. 1996;26:690-696.
13) Israel EJ, et al. Immunology. 1996;89:573-578.
14) Junghans RP, et al. Proc Natl Acad Sci U S A. 1996;93:5512-5516.
15) Roopenian DC, et al. J Immunol. 2003;170:3528-3533.
16) Claypool SM, et al. J Biol Chem. 2002;277:28038-28050.
17) Praetor A, et al. J Cell Sci. 2002;115:2389-2397.
18) Ward ES, et al. Adv Immunol. 2009;103:77-115.
19) Andersen JT, et al. Drug Metab Pharmacokinet. 2009;24:318-332.
20) Andersen JT, et al. J Biol Chem. 2010;285:4826-4836.
21) Roopenian DC, et al. Nat Rev Immunol. 2007;7:715-725.
22) Anderson CL, et al. Trends Immunol. 2006;27:343-348.
23) Dickinson BL, et al. Mol Biol Cell. 2008;19:414-423.
24) Lencer WI, et al. Trends Cell Biol. 2005;15:5-9.
25) Akilesh S, et al. J Immunol. 2007;179:4580-588.
26) Montoyo HP, et al. Proc Natl Acad Sci U S A. 2009;106:2788-2793.
27) Goebl NA, et al. Mol Biol Cell. 2008;19:5490-5505.
28) McCarthy KM, et al. J Cell Sci. 2000;113(Pt 7):1277-1285.
29) Yoshida M, et al. J Clin Invest. 2006;116:2142-2151.
30) Akilesh S, et al. Proc Natl Acad Sci U S A. 2008;105:967-972.
31) Vidarsson G, et al. Blood. 2006;108:3573-3579.
32) Qiao SW, et al. Proc Natl Acad Sci U S A. 2008;105:9337-9342.
33) Suzuki T, et al. J Immunol. 2010;184:1968-1976.
34) Tabrizi M, et al. Aaps J. 2009;12:33-43.
35) Pentsuk N, et al. Birth Defects Res B Dev Reprod Toxicol. 2009;86:328-344.
36) Deane R, et al. J Neurosci. 2005;25:11495-11503.
37) Zalevsky J, et al. Nat Biotechnol. 2010;28:157-159.
38) Jazayeri JA, et al. BioDrugs. 2008;22:11-26.
39) Bitonti AJ, et al. Proc Natl Acad Sci U S A. 2004;101:9763-9768.
40) Low SC, et al. Hum Reprod. 2005;20:1805-1813.
41) Subramanian GM, et al. Nat Biotechnol. 2007;25:1411-1419.
42) Dennis MS, et al. J Biol Chem. 2002;277:35035-35043.
43) Vaccaro C, et al. Nat Biotechnol. 2005;23:1283-1288.
44) Sesarman A, et al. Cell Mol Life Sci. 2010;67:2533-2550.
45) Akilesh S, et al. J Clin Invest. 2004;113:1328-1333.

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総説

抗体医薬品の体内動態制御に関わる受容体：FcRn

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要約：腫瘍や自己免疫疾患等の治療を目的とした分子標的薬として，抗体医薬品の研究開発が国内外で活発に行われている．抗体医薬品の特徴は標的分子に高い親和性をもって極めて特異的に結合することであるが，他のバイオ医薬品と比較して血中半減期が長いことも特筆すべき点である．ペプチドあるいはタンパク質を医薬品として応用する場合には血中半減期が実用化のためのハードルとなることが少なくない．しかし，多くの抗体医薬品は，生体内IgGの分解抑制に関わるneonatal Fc receptor（FcRn）を介したリサイクリング機構を利用することができるため，数日～数週間という長い血中半減期を有している．FcRnは齧歯類の新生児小腸に高発現し，乳汁に含まれる母親由来IgGの吸収に関与する受容体として同定された．その後の研究により，FcRnが成体においても種々の組織に発現し，IgGのリサイクリングやトランスサイトーシス等に関与していることが報告され，母子免疫以外にも様々な側面でIgGの体内動態制御に関わっていることが明らかにされている．我々は，既承認抗体医薬品のFcRn結合親和性を解析し，ヒトでの血中半減期とFcRn結合親和性の相関，および抗体医薬品のFcRn結合親和性を規定する構造特性の一端を明らかにした．近年の創薬研究では，FcRn結合親和性を改変した抗体医薬品等の開発が進んでいる他，FcRnのもう1つのリガンドであるアルブミンを利用することにより体内動態特性を改変したタンパク質医薬品の開発も進んでいる．FcRnは，抗体医薬品をはじめとするバイオ医薬品の体内動態制御に関わる鍵分子の1つと言えるであろう．

はじめに

2010年9月までに日米欧で29品目のモノクローナル抗体医薬品が承認されている（図1）．既承認抗体医薬品の中には顕著な有効性が認められているものも少なくなく，例えば，抗TNF α 抗体が奏功している関節リウマチの治療では“The era of biological therapy has arrived.”と言われるほどである(1)．現在臨床開発段階にある抗体医薬品は140品目に上り，今後さらに承認品目数が増加する可能性が高い．本稿では，抗体医薬品および抗体医薬品に類似した性質を持つFc融合タンパク質医薬品の概略を述べた後，抗体医薬品の体内動態制御に関わる受容体FcRnについて，発見の経緯，構造と機能，および抗体医薬品の体内動態との関連に関して，既承認抗体医薬品のFcRn結合親和性を解析した我々の知見を含めて紹介する．

1．抗体医薬品

抗体医薬品の多くは抗腫瘍作用あるいは免疫調節作用を持つ医薬品である（図1）．これらは作製法に起因するアミノ酸配列の相違により，マウス抗体，キメラ型抗体，ヒト化抗体，ヒト抗体に分類される(2)．1975年にKöhlerとMilsteinによりマウスモノクローナル抗体作製技術(3)が開発された当初，ミサイル療法が現実のものになると抗体医薬品の開発に大きな期待が寄せられた．しかし，マウス抗体をヒトに投与すると高頻度で抗体産生が起こりアナフィラキシー反応が懸念されるために繰り返し投与が困難であること，および半減期が短いことが障壁となり(4)，多くの開発が失敗に終わった．ヒト生体内IgGの半減期が約20日(5)であるのに対して，ヒトに投与されたマウス抗体の半減期は数時間～3日程度である(6)．1980年代に承認された抗体医薬品は，腎移植後の急性拒絶反応の治療に用いられるマウス抗CD3抗体のみであったが，その後，キメラ型抗体(7)やヒト化抗体(8)の作製技術が確立されてヒトIgG骨格を持った抗体を遺伝子組換えにより作製・製造することが可能になり，1990年代半ば以降，抗体医薬品の開発が急伸した．マウス抗体の可変領域あるいは相補性決定領域以外をヒトIgG由来の配列に置き換えることで，免疫原性を低下させ，

キーワード：FcRn，バイオ医薬品，抗体医薬品，Fc融合タンパク質医薬品，体内動態
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分類	承認年	名称	標的	主な適応疾患	血中半減期
マウス抗体	1986	ムロモナブ-CD3	CD3	腎移植後の急性拒絶反応	0.75日
	2002	イブリツモマブ チウキセタン	CD20	非ホジキンリンパ腫	1.1日
	2003	トシツモマブ	CD20	非ホジキンリンパ腫	2.7日
キメラ型抗体	1997	リツキシマブ	CD20	非ホジキンリンパ腫	9.4日
	1998	バシリキシマブ	CD25	腎移植後の急性拒絶反応	4.1日
	1998	インフリキシマブ	TNFα	関節リウマチ	9.5日
	2004	セツキシマブ	EGFR	頭頸部癌，結腸・直腸癌	4.8日
ヒト化抗体	1997	ダクリズマブ	CD25	腎移植後の急性拒絶反応	20日
	1998	パリビズマブ	RSV F protein	RSウイルス感染	19～27日
	1998	トラスツズマブ	HER2	転移性乳癌	2.7～10日
	2001	アレムツズマブ	CD52	B細胞性慢性リンパ性白血病	12日
	2003	オマリズマブ	IgE	喘息	20日
	2003	エファリズマブ	CD11	尋常性乾癬	5.5～10.5日(*)
	2004	ベバシズマブ	VEGF	結腸・直腸癌	11.7～13.4日(*)
	2005	トシリズマブ	IL-6R	キャッスルマン病，関節リウマチ	5.5日(*)
ヒト抗体	2002	アダリムマブ	TNFα	関節リウマチ	14.7～19.3日
	2009	ゴリムマブ	TNFα	関節リウマチ	14日(*)
	2009	ウステキヌマブ	IL12, IL23 p40	乾癬	14.9～45.6日(*)
	2009	カナキヌマブ	IL-1β	クリオピリン関連周期性症候群	26日(*)
	2009	オファツムマブ	CD20	慢性リンパ性白血病	14日(*)
Fc融合タンパク質	1998	エタネルセプト	TNFα, LTα	関節リウマチ	4日
	2003	アレファセプト	CD2	尋常性乾癬	11.3日(*)
	2005	アバタセプト	CD80/CD86	関節リウマチ	13.1日(*)
	2008	リロナセプト	IL-1	クリオピリン関連周期熱症候群	6.3～7.5日(*)
	2008	ロミプロスチム	TPOR	血小板減少性紫斑病	1～34日(*)

図1　既承認抗体医薬品の例（マウス抗体およびヒトIgG1のFc領域を持つ抗体とFc融合タンパク質）
血中半減期は文献6より引用（*は製品添付文書より引用）

血中半減期を延長できたことが抗体医薬品の実用化におけるブレークスルーであったと言える．

抗体分子は，抗原との結合を担うFab領域とFc受容体や補体との結合に関与するFc領域から構成される．近年，抗体に類似した特徴を持つ医薬品として，標的分子特異的結合能を持つ受容体タンパク質等を抗体のFc領域と融合させたFc融合タンパク質医薬品の開発も進んでおり，これまでに日米欧で5品目が承認されている（図1）．例えば，TNF受容体の細胞外領域とFc領域の融合タンパク質であるエタネルセプトは，TNFを中和する作用を持ち，関節リウマチ等の治療に用いられる．これらは，標的分子結合部位のみでは医薬品とすることが難しいタンパク質あるいはペプチドをFc領域との融合タンパク質として血中安定化を図ることにより臨床応用することに成功した例と言える．本稿では，抗体医薬品として，モノクローナル抗体医薬品およびFc融合タンパク質医薬品を指すこととする．

2. FcRnの発見の経緯および構造と機能

1) FcRn発見の経緯

IgGの体内動態制御に関わる受容体は，IgGの代謝や輸送に特異性と飽和が存在することを根拠に1964年にBrambellによりその存在が提唱され，Brambell receptorとも言われていたものである(9)．その後，新生児小腸でのIgG吸収に関わる受容体（neonatal gut transport receptor）として“FcRn”，IgG分解抑制に関わる受容体（IgG protection receptor）として“FcRp”の存在が考えられるようになっていた(10)．1989年になり，ラット新生児小腸からFcRnがクローニングされて一次構造が解明され，β2-microglobulin（β2m）とヘテロダイマーを形成する受容体であることも明らかになった(11)．キメラ型抗体医薬品やヒト化抗体医薬品が承認され始めていた1996年，β2mノックアウトマウスを用いた実験により，FcRnがIgGの半減期制御に関わる受容体FcRpとしての機能も合わせ持つことが明らかにされた(12-14)．IgGの半減期制御におけるFcRnの寄与についてはFcRn（α鎖）のノックアウトマウスを用いた検証も行われている(15)．これらの研究により，FcRnおよびFcRpと考えられていた受容体の実体は同じ分子であり，“FcRn”として同定された受容体がIgGの輸送と血中濃度維持の両方の機能を担っていることが明らかになった．

2) FcRnの構造と機能

ヒトFcRnはMHCクラスI分子に類似した構造を持ち，342アミノ酸残基からなるα鎖（図2）と99アミノ酸残基からなるβ鎖（β2m）により構成される．β2mはFcRnα鎖の細胞内局在に関与する(16, 17)ほか，IgG結合にも関与している(17)．

FcRnとIgGは酸性条件下（pH6～6.5）で結合し，中性条件下（pH7.4）では解離する．FcRnとの結合には，