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Abbreviations: CTL, cytotoxic T lymphocytes; DC, dendritic cells; HCV, hepatitis C virus; IFN, interferon; MICA, MHC class-I related chain; MDC, myeloid dendritic cells; Mo-DC, monocyte-derived dendritic cells; NK, natural killer; PDC, plasmacytoid dendritic cells; RIG-I, retinoic acid inducible gene-I, SVR, sustained virological responders; TLR, Toll-like receptors; TCR, T cell receptor; TR, transient responders

Key Words: Dendritic cells, NK cells, NKT cells, Toll-like receptor, RIG-I, pegylated interferon-alfa, ribavirin, Review

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IgG Oligosaccharide Alterations Are a Novel Diagnostic Marker for Disease Activity and the Clinical Course of Inflammatory Bowel Disease

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BACKGROUND AND AIMS: Patients with inflammatory bowel disease (IBD) share several immunologic similarities with rheumatoid arthritis (RA). Patients with RA have significantly increased levels of serum agalactosyl immunoglobulin G (IgG). Our aim was to investigate the clinical significance of analyzing the oligosaccharide structure of serum IgG in patients with IBD.

METHODS: Serum IgG oligosaccharide structures were analyzed using high-performance liquid chromatography in 60 patients with Crohn's disease (CD), 58 patients with ulcerative colitis (UC), 27 healthy volunteers (HV), and 15 disease controls (DC). The activity and mRNA level of beta-1,4-galactosyltransferase (Beta4GalT) in antibody-secreting cells were investigated in these subjects.

RESULTS: The agalactosyl fraction of the fucosylated IgG oligosaccharides (G0F/G2F) in CD and UC was significantly greater than that in HV and DC ($P < 0.001$). The percentage of subjects with a high G0F/G2F in CD, UC, HV, and DC was 72%, 33%, 0%, and 0%, respectively. G0F/G2F, which is significantly correlated with disease severity in both CD and UC, had higher sensitivity to diagnose IBD compared with anti-*Saccharomyces cerevisiae* antibody. Moreover, G0F/G2F was significantly correlated with the prognosis of UC patients: patients with a high G0F/G2F did not maintain long-term remission. The activity and mRNA level of Beta4GalT were significantly elevated in UC but not in CD.

CONCLUSIONS: G0F/G2F is a potentially effective diagnostic marker of disease activity in both CD and UC, and of the clinical course in UC. A pathophysiologic difference between CD and UC was also demonstrated.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is characterized as a chronic relapsing inflammatory process of the digestive tract. Although the precise etiology of IBD remains unknown, both genetic susceptibility (1) and dysregulation of the mucosal immune responses against enteric host flora (2) have pivotal roles in its pathogenesis. Several serologic markers related to immune responses were developed for the diagnosis of IBD (3–8). The prevalence of perinuclear antineutrophil cytoplasmic antibodies in the UC population is between 45% and 82% (3). Approximately 2–28% of patients with CD, however, also express this antibody (3). Anti-*Saccharomyces cerevisiae* antibody (ASCA), directed against oligomannan, is expressed in 48–69% of patients with CD and approximately 5–15% of patients with UC (3). Although several other antibodies have been investigated to aid in the diagnosis of IBD (3–8), the sensitivities and specificities of these antibodies are not high enough to have an essential role in the current diagnostic algorithm for IBD.

Immunoglobulin (Ig) G carries *N*-linked oligosaccharides at the Cgamma2 domain of the Fc fragment at asparagine 297 (9), all of which are biantennary complex type with or without bisecting *N*-acetylglucosamine (GlcNAc), core-fucose, galactose, and sialic acid residues (10–12). There are increased levels of agalactosyl IgG, which lacks terminal galactose in the IgG oligosaccharide, in the sera of patients with rheumatoid arthritis (RA) (13) and other chronic inflammatory diseases, including systemic lupus erythematosus, Sjögren's syndrome, and tuberculosis (14, 15). The oligosaccharide structure of IgG or its relationship to disease activity and prognosis in IBD patients, however, has not yet been investigated. In addition, the significance or incidence of anti-agalactosyl IgG autoantibodies has not been examined in IBD, although the existence of such autoantibodies has been reported in certain autoimmune diseases, including RA (16, 17).

In the present study, we analyzed the oligosaccharide structures of IgG using high-performance liquid chromatography (HPLC) and investigated the serum anti-agalactosyl IgG antibody levels. Our comprehensive oligosaccharide analysis revealed that the agalactosyl fraction in the fucosylated oligosaccharides was significantly higher in CD and UC patients than in healthy volunteers (HV) and disease controls (DC). Agalactosyl IgG was observed in UC patients with an aggressive disease phenotype. Agalactosyl IgG levels were closely correlated with disease activity and the clinical course of IBD, and had a significantly higher sensitivity to diagnose IBD compared with ASCA. Finally, investigation of the expression and activity of glycosyltransferases and glycosidases revealed a pathophysiologic difference between CD and UC.

MATERIALS AND METHODS

Subjects

Serum samples were collected from 60 patients with CD, 58 patients with UC, 27 age/gender-matched unrelated HV, and 15 patients with colonic inflammation including appendicitis, diverticulitis, and ischemic colitis (disease control, DC). All participants were Japanese, recruited at the Department of Gastroenterology and Hepatology, Osaka University Hospital (Suita, Osaka, Japan), the Department of Gastroenterology, Osaka Rosai Hospital (Sakai, Osaka, Japan), and the Department of Surgery, Rinku General Medical Center (Izumisano, Osaka, Japan). The ethics committee at each hospital approved the study protocol and written informed consent was obtained from each participant. Patients were diagnosed with CD or UC according to endoscopic, radiologic, histologic, and clinical criteria provided by the Council for International Organizations of Medical Sciences in the World Health Organization and the International Organization for the Study of Inflammatory Bowel Disease (18–20). Disease location and behavior in CD were determined based on the Vienna classification (21). Clinical activities were determined using the Crohn's Disease Activity Index (CDAI) for CD (22) or the Clinical Activity Index (CAI) for UC (23). Clinical remission was defined as CDAI of <150 in CD and CAI of <6 in UC (22, 24). Detailed patient characteristics are presented in Table 1.

IgG Purification

Serum IgG was purified using protein G sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). Briefly, half-diluted serum with phosphate-buffered saline (PBS) was loaded onto a protein G sepharose column. The column was subsequently washed with a minimum of 10 column volumes of PBS, followed by the same volume of 10 mM ammonium bicarbonate. Column-bound IgG was eluted using 0.1% trifluoroacetic acid.

Analysis of Pyridylaminated N-Linked Oligosaccharide of IgG by Reverse Phase HPLC

N-linked oligosaccharides were released from serum IgG and labeled with 2-aminopyridine, as described previously

Table 1. Patient Characteristics

	CD (N = 60)	UC (N = 58)	HV (N = 27)	DC (N = 15)
Male/female	44/16	32/26	17/10	6/9
Age, yr, mean (SD)	38 (14)	39 (15)	38 (11)	36 (17)
Age at diagnosis, yr, mean (SD)	29 (13)	34 (14)		
Bowel surgery (including appendectomy), N (%)	36 (60)*	2 (3)		
Extraintestinal manifestations, N (%)	5 (8)	2 (3)		
Treatment				
Salazosulfapyridine or mesalazine, N (%)	50 (83)	51 (88)		
Steroids, N (%)	4 (7)*	29 (50)		
Immunomodulators, N (%)	5 (8)	4 (7)		
Infliximab, N (%)	8 (13)	0 (0)		
Total parental nutrition or elemental diet, N (%)	39 (65)*	6 (10)		
Disease location (N)				
Small bowel/colon/both/unknown	11/11/37/1			
Extensive/left colon/rectum and sigmoid		31/18/9		
Disease behavior (N)				
Inflammatory/structuring/penetrating/unknown	18/22/16/4			
CRP, mg/dL, mean (SD)	1.6 (3.2)	1.7 (3.2)		
CDAI (CD) or CAI (UC), mean (SD)	197 (102)	5.9 (5.7)		

*$P < 0.001$ *versus* UC.

(12). Briefly, *N*-linked oligosaccharides were released from purified IgG samples by overnight incubation with 0.5 mU glycopeptidase F (Takara Bio Inc., Shiga, Japan) at 37°C. Oligosaccharides were further incubated with 50 mM ammonium acetate (pH 4.0) for 30 min, lyophilized, and labeled with 2-aminopyridine by GlycoTag (Takara Bio Inc.) following the manufacturer's instructions. Excess reagent was removed with a cellulose cartridge glycan preparation kit (Takara Bio Inc.) and then oligosaccharides were incubated with 2 M acetic acid at 80°C for 2 h to remove sialic acids. Pyridylamino (PA)-oligosaccharides from IgG were analyzed on reverse phase HPLC system (Waters Corp., Milford, MA) using PALPAK Type R-MB (Takara Bio Inc.) at a flow rate of 0.5 mL/min using 10 mM sodium phosphate (pH 4.4, solvent A) and the same buffer containing 0.5% 1-butanol (solvent B) at 40°C. The glycans were separated with a gradient of 0–50% solvent B for 30 min followed by 10 min of 50% solvent B. PA-oligosaccharides were detected using a fluorescence detector (Waters 2475, Waters Corp) at wavelengths of 320 nm for excitation and 400 nm for emission.

Analysis of Anti-Agalactosyl IgG Antibody

The anti-agalactosyl IgG antibody levels were measured using a lectin enzyme immunoassay kit (Eitest CARF, Eizai Co., Tokyo, Japan), according to the manufacturer's instructions (16). Briefly, diluted serum samples were added to plates precoated with human agalactosyl IgG. After rinsing with the washing buffer, biotinylated *Ricinus communis* agglutinin (RCA) 120, which recognizes the terminal galactose of anti-agalactosyl IgG antibody, was added to the plate and incubated for 1 h. After another rinse, horseradish peroxidase-conjugated streptavidin was added and the plate was incubated for 1 h. After a final rinse, the plate was incubated with a chromogen substrate solution. The reaction was stopped with 2 mM sodium azide after 30 min of incubation, and absorbance was measured at a wavelength of 405 nm with a microplate reader using a reference wavelength of 490 nm. Values greater than 6.0 AU/mL were defined as positive (16).

*Analysis of Anti-*Saccharomyces cerevisiae *Antibody*

Serum ASCA concentrations were examined using the ASCA IgG enzyme-linked immunosorbent assay kit (Genesis Diagnostics, Cambridge, UK), according to the manufacturer's instructions. Values over 10 U/mL were defined as positive.

Isolation of B cells and Plasma Cells From Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated from the heparinized venous blood of subjects by Ficoll–Hypaque density-gradient centrifugation. B cells and plasma cells were separated from peripheral blood mononuclear cells with a B cell isolation kit II and plasma cell isolation kit, respectively (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

Real-Time Reverse Transcription-Polymerase Chain Reaction for Beta4GalT

Total cellular RNA was isolated using Isogen-LS (Wako Chemicals, Osaka, Japan), and complementary DNA was synthesized from 0.1 to 0.5 μg of total RNA using Superscript III first-strand system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. For TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR), the reaction mixture was prepared by TaqMan Universal PCR Master Mix with predesigned and prelabeled TaqMan PCR primer and probe set for human beta-1,4-galactosyltransferase (Beta4GalT) I or human beta-actin endogenous control (Applied Biosystems, Foster City, CA). Real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System instrument and software (Applied Biosystems). Each sample was run in duplicate. The relative RNA amount was calculated with the $\Delta\Delta$Ct method (25) and normalized to internal control beta-actin.

Analysis of Beta4GalT Activity in Plasma Cells

Beta4GalT activity was measured as described previously (26, 27). Isolated plasma cells were dissolved in TNE buffer (25 mM Tris-HCl [pH 7.8], 1% Nonidet P-40 [NP-40], and 1 mM ethylenediaminetetraacetic acid [EDTA]) and the supernatant was collected. The cellular supernatant was mixed with uridine diphosphate (UDP)-galactose (Sigma-Aldrich, St. Louis, MO) and PA-agalactosyl *N*-linked oligosaccharides as acceptor substrates, which were generated as previously reported (28). The mixture was incubated at 37°C for 24 h and the reaction was terminated by boiling for 1 min. The samples were then centrifuged at 12,000 *g* for 10 min and 5 μL of 25 μL supernatants were analyzed by HPLC, as described above. Beta4GalT activity was calculated as follows: the area under the peak of galactosylated oligosaccharides was measured after the reaction, and the concentration was determined using a standard galactosyl biantennary PA-oligosaccharide. Beta4GalT activity was expressed as nmol/h by dividing the concentration of galactosyl oligosaccharides by the incubation time.

Statistical Analyses

Differences between measurements and groups were tested with Mann-Whitney U-test. Either the χ^2 test, χ^2 test with Yates' correction (when sample number was less than 10), or Fischer's exact test (when sample number was less than 4), where appropriate, was used for the comparison of frequencies. Sensitivity for each test result was defined as the probability of a positive test result in a patient with the disease under investigation. Specificity was defined as the probability of a negative test result in a patient without the disease under investigation. A receiver operated characteristic (ROC) curve was generated by plotting sensitivity *versus* 1 – specificity (29, 30). Area under the curve (AUC) was calculated by StatMate software (ATMS Co., Tokyo, Japan). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Increased Agalactosyl Fraction of the Fucosylated IgG in IBD Patients

Normal oligosaccharide structures of human neutral IgG comprise 12 major structural variants (Fig. 1A) (12). We analyzed the profiles of IgG neutral oligosaccharides using HPLC in combination with fluorescent labeling of oligosaccharides. Representative profiles of HV and CD are shown in Figure 1B. We divided the oligosaccharides into three subgroups (groups I–III) according to the existence of core-fucose, bisecting GlcNAc, and both. Group II oligosaccharides, which have only core-fucose, were the major group, comprising approximately 80% of the total oligosaccharides. In fucosylated group II oligosaccharides of HV, the peak of the agalactosyl oligosaccharide (Fig. 1B) was usually lower than that of the fully galactosyl oligosaccharide (Fig. 1B). In contrast, in CD, the peak of the agalactosyl oligosaccharide (e) was higher than that of the fully galactosyl oligosaccharide (h) (Fig. 1B).

We defined the agalactosyl peak in oligosaccharides (a, e, and i) as G0 and the fully galactosyl peak (d, h, and l) as G2. The peak height ratio of G0 to G2 was calculated by dividing the peak height of G0 by that of G2 in each group. In fucosylated group II oligosaccharides, the peak height ratio of G0 to G2 in both CD and UC was significantly higher than that in HV ($P < 0.001$ for CD and UC) and the ratio in CD was significantly higher than that in UC ($P < 0.001$, Fig. 2). In order to exclude the possibility that the increase in the peak height ratio of G0 to G2 in group II is a common feature not only of IBD but also of other intestinal inflammation, we analyzed the ratio in DC. The ratio of G0 to G2 in group II in both CD and UC was also significantly higher than that in DC ($P < 0.001$ for CD and $P < 0.01$ for UC). In group III oligosaccharides, containing also core-fucose, a significant increase was also observed in the ratio of G0 to G2 in both CD and UC when compared with HV and DC. In the nonfucosylated group I oligosaccharides, however, the ratio of G0 to G2 was not increased in IBD (Fig. 2).

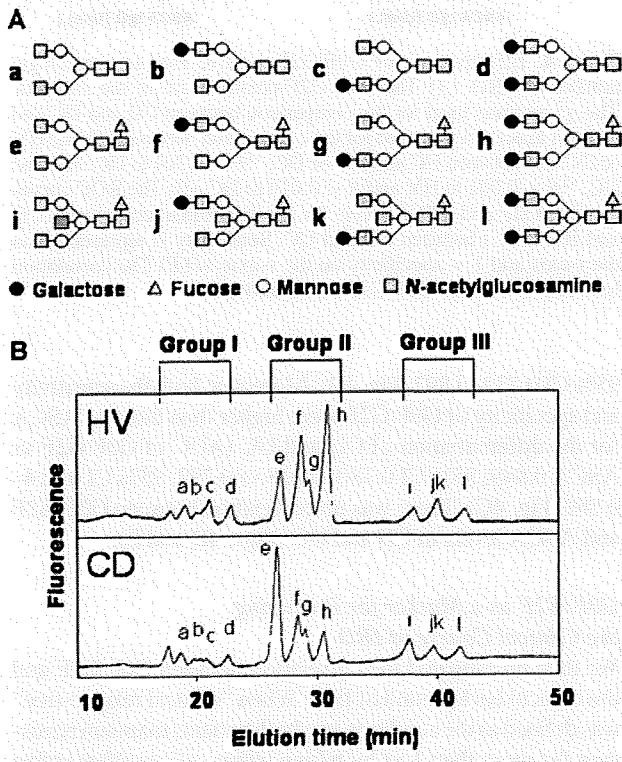

Figure 1. Structures of oligosaccharides attached to human IgG. (*A*) Structural patterns of *N*-linked neutral oligosaccharides. (*B*) Representative profiles of neutral IgG oligosaccharides purified from HV and CD patients.

Correlation Between G0F/G2F and Disease Activity

Among the three subgroups of oligosaccharides, fucosylated group II was the major group, and most clearly reflected the oligosaccharide alterations in IBD. We defined the peak height ratio of G0 to G2 of the fucosylated group II as "G0F/G2F," and G0F/G2F was used for the following clinical analysis. Because it was controversial whether the prevalence of agalactosyl IgG in IBD correlates with C-reactive protein (CRP) and disease activity (31, 32), we investigated the correlation of G0F/G2F with clinical parameters. In CD, G0F/G2F in active patients (CDAI $\geq$150) was significantly higher than that in patients in remission (CDAI <150, $P < 0.01$, Fig. 3A). G0F/G2F was also significantly higher in CD patients with extensive disease where inflammation was not limited to the terminal ileum (category L2 and L3 in Vienna Classification) (21) than in patients with inflammation in the terminal ileum alone (category L1, $P < 0.05$, Fig. 3B). Similarly, G0F/G2F was significantly higher in active UC patients (CAI $\geq$6) than in patients in remission (CAI <6, $P < 0.01$, Fig. 3C). G0F/G2F was significantly higher in UC patients with extensive disease (total colitis) than in those with only

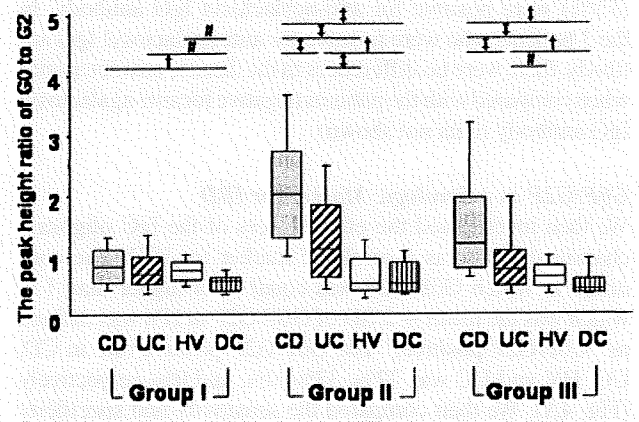

Figure 2. Increased fucosylated agalactosyl IgG in IBD patients. The peak height ratio of G0 to G2 in each subgroup was calculated for the subjects. Box plots show 50% of the relevant patient population. The line inside the box represents median value. Whiskers indicate the 90th and 10th percentiles. $^{\dagger}P < 0.001$, $^{\dagger}P < 0.01$, $^{\#}P < 0.05$ by Mann-Whitney U-test.

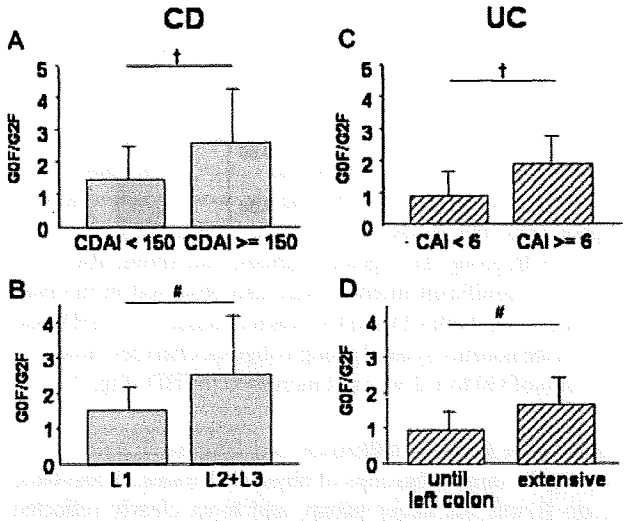

Figure 3. G0F/G2F and clinical manifestations. (*A*) G0F/G2F of CD patients in remission (CDAI <150) and in an active stage (CDAI ≥150). (*B*) G0F/G2F of CD patients with category L1 and L2 + L3. (*C*) G0F/G2F of UC patients in remission (CAI <6) and in an active stage (CAI ≥6). (*D*) G0F/G2F of UC patients with only left-side colon involvement and with extensive disease. Results are shown as mean ± SD. $^{\dagger}P < 0.01$, $^{\#}P < 0.05$ by Mann-Whitney U-test.

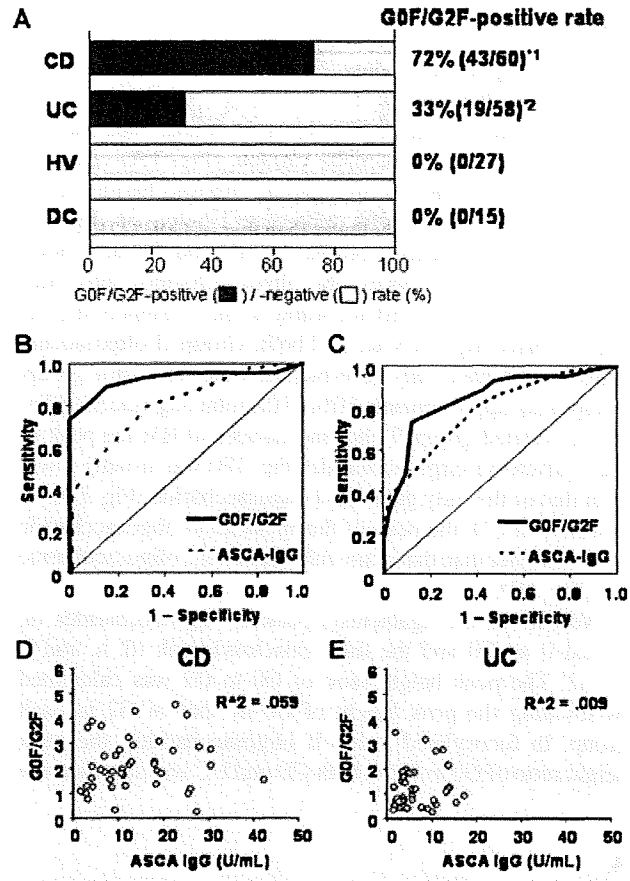

Figure 4. Effectiveness of G0F/G2F-positive rate as a serologic marker for IBD. (*A*) The G0F/G2F-positive rate in the subjects. The χ^2 test was used for the comparison between CD and UC and Fisher's exact test was used for the comparison between CD and HV or DC and between UC and HV or DC. $^{*1}P < 0.001$ to UC, HV, and DC, $^{*2}P < 0.001$ to HV, and $P < 0.010$ to DC. (B) The ROC curves for G0F/G2F and ASCA levels for the discrimination between CD and HV, or (*C*) between CD and UC. Sensitivity is represented on the *y*-axis and 1 – specificity on the *x*-axis. (*D,E*) The correlation between ASCA and G0F/G2F in CD and UC.

left-side colon involvement ($P < 0.05$, Fig. 3D). In contrast to a previous observation (32), we found no correlation between G0F/G2F and CRP level, age at onset, or disease duration (data not shown).

Infrequent Positive Rate of Anti-Agalactosyl IgG Antibody in IBD Patients

Anti-agalactosyl IgG antibody is used as an early diagnostic marker for RA, and 84% of patients with RA are positive for anti-agalactosyl IgG antibody (16, 17). Unexpectedly, only 1 of 49 patients with CD (2.0%) and 2 of 51 patients with UC (3.9%) were positive for anti-agalactosyl IgG antibody. In the 3 patients who were positive for anti-agalactosyl IgG antibody, there were no differences in the disease characteristics when compared with the patients negative for anti-agalactosyl IgG antibody (data not shown).

G0F/G2F as a Serologic Marker for IBD

We then investigated the effectiveness of the IgG oligosaccharide structure as a serologic marker for IBD. The condition in which G0F/G2F was equal to or higher than 1.4, which was the mean + 2 SD of G0F/G2F in HV, was defined as "G0F/G2F-positive." The G0F/G2F-positive rate in CD, UC, HV, and DC was 72%, 33%, 0%, and 0%, respectively (Fig. 4A). We then compared the sensitivity and specificity of G0F/G2F with those of ASCA for the discrimination of IBD by ROC curve and AUC. Both the sensitivity and specificity of G0F/G2F were higher than those of ASCA for the differentiation of CD and HV (AUC of G0F/G2F *vs* ASCA 0.926, 95% confidence interval [CI] 0.872–0.980 *vs* 0.815, 95% CI 0.732–0.897, Fig. 4B). Moreover, both the sensitivity and specificity of G0F/G2F were higher than those of ASCA for the differentiation of CD and UC (AUC of G0F/G2F *vs* ASCA 0.849, 95% CI 0.780–0.918 *vs* 0.792, 95% CI 0.714–0.869, Fig. 4C). There was no correlation between G0F/G2F and ASCA levels in CD and UC (Figs. 4D and E).

G0F/G2F as a Marker for Predicting the Clinical Course of IBD

We then investigated the correlation between G0F/G2F and the clinical background of IBD. When "clinical relapse-free" was defined as the condition in which patients maintain remission for more than 1 yr by taking either salazosulfapyridine or 5-aminosalicylic acid (without corticosteroid, antitumor necrosis factor [TNF]-α antibody, and immunomodulators), the clinical relapse-free rate of G0F/G2F-positive UC patients

Table 2. Clinical Relapse-Free Rate and G0F/G2F in Patients With CD and UC

	G0F/G2F-Negative	G0F/G2F-Positive	*P* Value
UC	77% (20/26)	11% (1/9)	<0.001
CD	50% (2/4)	6% (1/17)	0.08

(11%) was significantly lower than that of G0F/G2F-negative UC patients (77%, $P < 0.001$, Table 2). The clinical relapse-free rate of G0F/G2F-positive CD patients was lower than that of G0F/G2F-negative patients, although the difference was not significant (Table 2). Moreover, in UC patients whose CRP levels were negative at the time of blood sampling, the clinical relapse-free rate of G0F/G2F-positive UC patients (0%) was significantly lower than that of G0F/G2F-negative UC patients (90%, $P < 0.001$).

Upregulation of Beta4GalT in UC but not in CD

To investigate the mechanism underlying the increase in agalactosyl IgG in patients with IBD, we analyzed serum beta-galactosidase activity, which is responsible for the release of terminal galactose from IgG oligosaccharides. Fresh sera from patients with CD and HV were incubated with pyridylaminated biantennary oligosaccharides with an outer arm of galactose, and these oligosaccharides were subjected to HPLC analysis. The terminal galactose was not depleted in the sera of patients with CD or HV, suggesting that there is no increase in beta-galactosidase activities in the sera of patients with either CD or HV (data not shown).

We next examined the possibility that IBD patients have compromised beta-galactosyltransferase enzyme activity in both plasma cells and B cells. In plasma cells prepared from UC patients, Beta4GalT I mRNA expression was significantly higher than that in CD ($P < 0.05$) or HV ($P < 0.01$, Fig. 5A) patients. Beta4GalT I mRNA expression in B cells of UC patients was also significantly higher than that of CD or HV ($P < 0.05$, Fig. 5B) patients. Furthermore, Beta4GalT activity in the plasma cells of UC patients was higher than that of CD or HV patients (Fig. 5C).

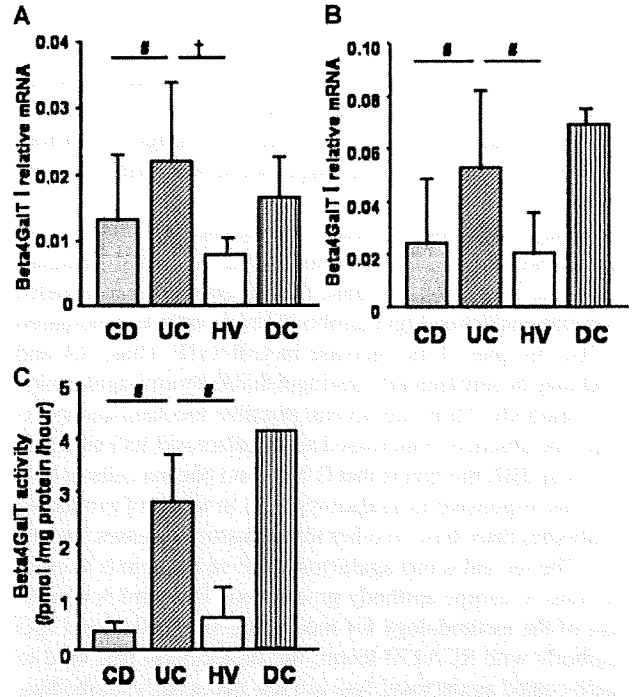

Figure 5. mRNA expression and enzyme activity of beta-1,4-galactosyltransferase (Beta4GalT). (*A*) Beta4GalT I mRNA expression in plasma cells of CD (N = 9), UC (N = 9), HV (N = 5), and DC (N = 3). (*B*) Beta4GalT I mRNA expression in B cells of CD (N = 8), UC (N = 5), HV (N = 5), and DC (N = 2). (*C*) Beta4GalT enzyme activity in plasma cells of CD (N = 3), UC (N = 3), HV (N = 3), and DC (N = 1). Results are shown as mean ± SD. $^{\dagger}P < 0.01$, $^{*}P < 0.05$.

DISCUSSION

The findings of the present study indicate that G0F/G2F, the extent of agalactosylation of fucosylated IgG, is a potential diagnostic marker for IBD. Among several serologic markers reported to have diagnostic value for IBD, ASCA is most suitable for detecting CD (3–8). Our results, however, demonstrate that G0F/G2F is a better marker than ASCA for the differentiation between CD and HV or CD and UC. In addition, G0F/G2F was not increased in patients with DC, suggesting that intestinal inflammation is not the direct cause of IgG agalactosylation. Furthermore, our results indicate that G0F/G2F reflects the clinical activity, severity, and clinical outcome of IBD. Especially in G0F/G2F-positive UC patients, the clinical relapse-free rate for a 1-yr period was significantly lower than that in the G0F/G2F-negative patients. Although this analytical method would not be useful for screening CD and UC patients in the general population, it will be useful to distinguish IBD from DC. Furthermore, it can be useful to distinguish aggressive disease from nonaggressive ones. The clinical outcome of not only UC but also CD patients for a longer period and with a larger sample size needs to be investigated and the project is currently ongoing in our laboratory. CD patients expressing an increased number of antibodies against anti-I2, antiouter membrane protein C, anti-CBir1 flagellin, and ASCA exhibit rapid disease progression (33). In addition to these reported antibodies, our study revealed that G0F/G2F has a predictive value of rapid disease phenotype in both CD and UC.

Although increased agalactosyl IgG levels are reported in approximately half of CD patients (32), the levels have not been so high, as demonstrated in the present study. Previously, anti-GlcNAc antibodies that detect galactose-uncovered terminal GlcNAc were used for measuring agalactosyl IgG. The specificity of anti-GlcNAc antibodies for the terminal agalactosylation is limited, however, because several subsets of oligosaccharides in IgG, including those with bisecting GlcNAc (Fig. 1A), can be recognized by the anti-GlcNAc antibody. In addition, the increase in

nonspecific glycosylation, such as Fab oligosaccharides or extra branches, will enhance the reactivity to this antibody. Thus, the degree of agalactosylation of IgG is likely to be underestimated. Our study clearly reevaluated the high prevalence of agalactosylation in fucosylated IgG oligosaccharides in IBD by comprehensive oligosaccharide analysis using HPLC.

Along with the increase in agalactosyl IgG, anti-agalactosyl IgG antibody is increased in several diseases, including RA (16). In contrast, the present study has revealed that anti-agalactosyl IgG antibody levels were not increased in IBD in spite of the increase in G0F/G2F. Thus, RA and IBD may be serologically distinguishable by anti-agalactosyl IgG antibody. There are several possible mechanisms to explain the absence of increased anti-agalactosyl IgG antibody levels in IBD: the first is that B cells and plasma cells in IBD are less responsive to agalactosyl IgG in terms of producing antibodies than those in other inflammatory diseases such as RA. The second is that agalactosyl IgG in IBD binds to other proteins to escape antibody production. The third is a problem of the methodology for measuring anti-agalactosyl IgG antibody with RCA120 lectin; the molecule(s) that bind to plate-coated agalactosyl IgG are not necessarily antibodies. Although the precise mechanisms are not clear, the measurement of anti-agalactosyl IgG antibody levels using the present method might not be useful for the diagnosis and treatment of IBD.

Beta4GalT has a role in conjugating galactose to the outer arm of GlcNAc in the *N*-linked oligosaccharides of IgG, and alterations in the Beta4GalT level are involved in IgG glycosylation (34). Thus, G2 oligosaccharides are generated from G0 oligosaccharides through an interim product with galactose in one of the outer branches of the oligosaccharide (G1). Therefore, the peak height ratio of G0 to G2, rather than G0 to G1 or G1 to G2, will mostly reflect the maturation status of glycosylation. However, it has not been fully proven how and where glycosylation is controlled. Furthermore, the function of agalactosyl IgG has not been clarified yet. Although Beta4GalT mRNA expression and its activity in B cells are often reported to be lower in RA, there are several conflicting reports (34, 35). In the present study, Beta4GalT I mRNA expression in B cells and plasma cells was upregulated in UC but not in CD, and Beta4GalT enzyme activity in plasma cells was increased in UC and DC but not in CD. The changes in agalactosylation observed in IBD are not clearly explained by the enzyme activities. However, elevated Beta4GalT may serve to conjugate galactose to the rapidly produced and consumed IgG during inflammation in UC and DC. In contrast, Beta4GalT was not elevated in CD in spite of the chronic inflammation. The mechanistic difference in the enzyme activity needs to be clarified. When seen from a different angle, lack of an increase in Beta4GalT in CD might be partially responsible for the increased agalactosyl IgG in CD. Besides the enzyme activity, there is another possibility that the extent of glycosylation alters the half-life of the protein. Furthermore, the half-life of glycosylated IgG might be affected by the extent of glycosylation in the inflammatory conditions, *e.g.*, UC and CD. It is important to clarify these issues and these projects are in progress.

In addition to the diagnostic value of G0F/G2F, the dramatic change in the oligosaccharide structure of IgG might be associated with the pathogenesis of IBD. In a murine collagen-induced arthritis model, agalactosyl IgG is pathogenic (36). Agalactosyl IgG either activates the complement pathway or directly activates macrophages after binding to either mannose-binding lectin or mannose receptors, respectively (37–39). Further studies are required to clarify the role of agalactosyl IgG in mucosal inflammation.

In conclusion, G0F/G2F is increased in IBD and reflects disease activity and clinical course. These findings suggest that G0F/G2F is a novel serologic marker for IBD, and a marker to predict the clinical course. Fucosylated agalactosyl IgG is a potential therapeutic target in IBD.

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STUDY HIGHLIGHTS

What Is Current Knowledge

- Patients with rheumatoid arthritis have significantly increased levels of serum agalactosyl immunoglobulin G (IgG) and anti-agalactosyl IgG antibody.
- The precise oligosaccharide structure of IgG and its relationship to disease activity and prognosis in inflammatory bowel disease (IBD) patients have not yet been investigated.
- Serologic markers for IBD, *e.g.*, anti-*Saccharomyces cerevisiae* antibody (ASCA), do not have an essential role in the current diagnostic algorithm for IBD.

What Is New Here

- The agalactosyl fraction of the fucosylated IgG oligosaccharides in IBD was significantly greater than that in healthy volunteers and a disease control.
- An infrequent positive rate of anti-agalactosyl IgG antibody was observed in IBD patients.
- Extent of agalactosylation of IgG correlated with disease activity of IBD and is a potentially effective diagnostic marker for IBD.
- mRNA expression and enzyme activity of galactosyltransferase were different between Crohn's disease and ulcerative colitis.

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CONFLICT OF INTEREST

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Upregulation of GRAIL is associated with remission of ulcerative colitis

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Egawa S, Iijima H, Shinzaki S, Nakajima S, Wang J, Kondo J, Ishii S, Yoshio T, Irie T, Nishida T, Kakiuchi Y, Yasumaru M, Yoshihara H, Kanto T, Tsujii M, Tsuji S, Hayashi N. Upregulation of GRAIL is associated with remission of ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol* 295: G163–G169, 2008. First published May 8, 2008; doi:10.1152/ajpgi.90242.2008.—Abrogating tolerance against unidentified antigens is a critical step in the pathogenesis of ulcerative colitis (UC). T cell anergy, one of the main mechanisms of tolerance, has been shown to be induced by E3 ubiquitin ligases, such as gene related to anergy in lymphocytes (GRAIL), Itch, and c-Cbl in mice. However, it is not well known whether these E3 ligases play roles in human diseases. The pathophysiological role of the E3 ligases in patients with UC was investigated. At first, the expression of GRAIL, Itch, and c-Cbl in human anergic T cells was analyzed by quantitative RT-PCR and Western immunoblotting. Next, the mRNA expression of the E3 ligases was analyzed in peripheral $CD4^+$ T cells of 20 patients with UC and 10 healthy volunteers (HV). mRNA expression was analyzed in patients with active UC before and after treatment with prednisolone and leukocytapheresis. Anergic human $CD4^+$ T cells expressed significantly higher levels of GRAIL, Itch, and c-Cbl than nonanergic cells. GRAIL expression was significantly higher in patients with UC in remission than in patients with active disease and in HV ($P < 0.01$). The level of GRAIL expression was also significantly increased in patients with active disease whose clinical activity index scores improved after treatment ($P < 0.05$). There were no significant differences in Itch and c-Cbl expression among patients with active UC, patients with UC in remission, and HV. These data suggest that GRAIL plays an important role in maintaining remission in patients with UC.

T cell anergy; E3 ubiquitin ligase; tolerance; inflammatory bowel disease

IMMUNOLOGICAL STATUS AGAINST luminal bacteria and food antigens plays a critical role in the development and healing of ulcerative colitis (UC), a chronic colorectal inflammatory disease (22). In healthy subjects, mucosal tolerance in the gut lumen prevents bacteria and food antigens from evoking T cell activation by eliminating activated antigen-specific T cells through clonal deletion, clonal anergy, and active suppression (8). In contrast, patients with active UC react to their own bacterial flora and various dietary antigens due to abrogation of tolerance (6, 11, 20). Recently, disruptions in oral tolerance in patients with active inflammatory bowel disease (IBD) were further confirmed with the use of keyhole limpet hemocyanin as a model antigen (13). According to reports of studies using animal models, genetic predisposition also contributes to abrogation of immune tolerance against normal enteric flora (4, 7, 27).

Clonal anergy is one of the important mechanisms governing immune tolerance. During anergy, lymphocytes are intrinsically and functionally inactivated following an antigen encounter and remain in a hyporesponsive state for an extended period (23). Anergic cells do not proliferate or express interleukin (IL)-2 following T cell receptor-specific stimulation by their cognate antigens, even in the presence of adequate costimulation. Unresponsiveness is reported to be correlated with an increase in intracellular Ca^{2+}; however, the mechanism underlying induction of T cell anergy is poorly understood (10).

Recently, several E3 ubiquitin ligases have been demonstrated to be essential for induction of T cell anergy(19). Ubiquitin is a highly conserved 76-amino acid globular protein that attaches to substrate proteins, thereby affecting multiple cellular processes, including cellular trafficking, transcriptional activation, and proteasomal- and lysosomal-mediated degradation. Ubiquitination is accomplished through a series of enzymatic steps catalyzed by a ubiquitin-activating enzyme (called E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), which mediates the transfer of ubiquitin from the E2 protein to a lysine residue on the target protein (29). Only one E1 has been identified in mammals compared with over 30 E2 enzymes and many more E3 ligases. E3 ubiquitin ligases provide substrate specificity in ubiquitination reactions and thus toward the many cellular processes controlled by ubiquitin modification. The gene related to anergy in lymphocytes (GRAIL), one of the well-characterized E3 ubiquitin ligases, is a type I transmembrane protein that localizes to the endocytic pathway and contains a really interesting new gene (RING) finger motif (1, 26). Constitutive retroviral expression of GRAIL has been shown to render naïve $CD4^+$ T cells anergic to antigenic challenge (24), and the expression of GRAIL in retrovirally transduced T cell hybridoma cells significantly inhibits activation-induced IL-2 and IL-4 cytokine production (1). Remarkably, overexpression of an enzymatically inactive form of GRAIL that inhibits endogenous GRAIL function successfully blocks the development of anergy (24). Itch is also reported to be an E3 ubiquitin ligase related to T cell anergy in mice (9). Itch-deficient mice develop a progressive autoimmune-like disease characterized by lymphoproliferation in the lymphoid organs, such as spleen, lymph nodes, and a medulla of the thymus. c-Cbl was identified from the genome of a transforming retrovirus in mouse pre-B lymphoma cells (15)

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and was the first E3 ubiquitin ligase linked to the development of the clonal anergy state (5).

The correlation between anergy and these E3 ubiquitin ligases has been well described in mouse models. Also, the correlation of GRAIL expression and T cell function has been recently reported in human peripheral T cells (12); however, the expression of the E3 ligases in human diseases has not been reported. In this study, we examined the expression of GRAIL, Itch, and c-Cbl in human $CD4^+$ T cells isolated from patients with active and remitting UC and identified the role of these E3 ubiquitin ligases in patients with UC.

MATERIALS AND METHODS

Subjects. Twenty patients with UC who visited or were hospitalized in Osaka University Hospital and Osaka Rosai Hospital were enrolled in this study (Table 1). Ten age-matched healthy volunteers (HV) were recruited and served as the control group. The diagnosis of UC was based on conventional clinical, endoscopic, and histopathological criteria. The clinical activity index (CAI) of UC was determined according to the criteria of Rachmilewitz et al. (21), and patients with UC whose CAI scores were equal or greater than 6 were considered active (14). Eight patients who had been asymptomatic and whose CAI score had been zero for longer than 3 mo without using corticosteroids or immunomodulators (azathioprimes or 6-Mercapto prime) were enrolled as patients in remission. All of the subjects provided written informed consent, and the study protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine and Osaka Rosai Hospital.

Reagents and antibodies. Unlabeled anti-human CD3 and anti-CD28 monoclonal antibodies (mAbs), phycoerythrin (PE)-conjugated anti-CD25 (M-A251), CD45RA (HI100), FITC-conjugated anti-CD4 (RPA-T4), CD45RO (UCHL1), PECy5-conjugated anti-CD4 (RPA-T4), and isotype control Abs were purchased from BD Biosciences (San Jose, CA).

Induction of anergy in human $CD4^+$ T cells in vitro. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized peripheral blood of HV by density-gradient centrifugation using Ficoll-paque PLUS (GE Healthcare Bio-Sciences, Piscataway, NJ). $CD4^+$ T cells were purified by positive selection using CD4 microbeads and a magnetic cell sorting system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The purified $CD4^+$ T cells (2×10^6/ml) were treated with 1.5 μM ionomycin (Sigma-Aldrich, St. Louis, MO) at 37°C for 6 h (mRNA) or 18 h (protein). Cells were washed, and cell pellets were prepared for quantitative RT-PCR or Western blot analysis. To confirm the induction of anergy, 5×10^5 cells were examined for proliferation. After 18 h of anergic stimuli, the cells were cultured in vitro in 96-well plates precoated with anti-CD3 mAb (2 μg/ml) together with soluble anti-CD28 mAb (1 μg/ml) for 72 h. The cells were pulsed for the final 16 h with [^{3}H]thymidine, and the radioactivity was counted in a β-counter.

Table 1. *Characteristics of the subjects enrolled in this study*

	UC Active	UC Remission	HV
Age, years	38±17	41±16	34±2.6
Gender, male/female	8/4	3/5	7/3
Type, pancolitis/left-sided	7/5	1/7	
CAI	8.5±4.0	0	
CRP, mg/l	31±42	<2.0	
Previous medication, yes			
5-Aminosalicylic acid	12	7	
Corticosteroid	9	4	
Immunomodulator	5	1	
Cytapheresis	0	0	
Current medication, yes			
5-Aminosalicylic acid	12	7	
Corticosteroid	9	0	
Immunomodulator	4	0	
Cytapheresis	6	0	

Applicable values are means ± SE. UC, ulcerative colitis; HV, healthy volunteers; CAI, clinical activity index; CRP, C-reactive protein.

Cell purification. Memory T cells and $CD4^+CD25^+$ T cells were isolated using the Memory $CD4^+$ T cell isolation kit and $CD4^+CD25^+$ regulatory T cell isolation kit, respectively (Miltenyi Biotec), according to the manufacturer's protocol. Naïve T cells were isolated by positive selection of PE-conjugated anti-human CD45RA with anti-PE microbeads from unlabeled $CD4^+$ T cells. Before positive selection of naïve T cells, the unlabeled $CD4^+$ T cells were purified by negative selection with the $CD4^+$ T cell isolation kit II (Miltenyi Biotec). The purity of the cells was >97%, as analyzed by flow cytometry.

Real-time quantitative RT-PCR. Total RNA was extracted from 25 ml of peripheral blood or collected materials during centrifugal leukocytapheresis (CFLA) using guanidine thiocyanate-phenol solution (ISOGEN; Wako, Osaka, Japan) and subsequently reverse transcribed with the use of random hexamer primers and the SuperScript 3 First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time quantitative RT-PCR for GRAIL, Itch, c-Cbl, and β-actin was performed using ready-to-use assays (Applied Biosystems, Foster City, CA) in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Probes and primer sets specific for the GRAIL, Itch, and c-Cbl cDNA were purchased from Applied Biosystems. All reactions were performed in duplicate. The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantities of each E3 ligase were expressed as the E3 ligase/β-actin arbitrary units (AU).

Immunoblot analysis. Cells were washed with cold PBS and lysed in RIPA buffer (1× Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.004% sodium azide). Lysates were centrifuged at 12,000 revolution/min for 20 min, and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Cleared lysates were resolved by SDS-PAGE and absorbed to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Proteins were detected with GRAIL (IMGENEX, San Diego, CA), Itch, and c-Cbl (BD Biosciences)-specific Abs. Blots were developed with horseradish peroxidase (HRP)-conjugated secondary Abs and enhanced chemiluminescence (GE Health Care).

Treatment and blood sampling by CFLA. CFLA was performed in eight patients with active UC with a Haemonetics CCS (Haemonetics, Braintree, MA) (Table 2). The access and return line was connected to the patients' cubital vein, and apheresis was performed at a blood flow rate of 50 ml/min. In each session, the leukocyte-rich fractions were removed from 2,000 to 2,400 ml of patients' peripheral blood. Mononuclear cells were isolated from the buffy coat using Ficoll-paque. Each patient was treated with CFLA once per week, and the treatment continued for 4–5 wk.

Flow cytometry. The mononuclear cells of patients with active UC obtained at CFLA were subjected to flow cytometry using specific surface antibodies. For flow cytometry, single-cell suspensions were stained for 30 min at 4°C with PE-, FITC-, and PECy5-conjugated mAbs. The stained cells were washed twice in fluorescence-activated cell sorting (FACS) buffer, and the cells were resuspended in PBS containing 1% paraformaldehyde. The fixed cells were then analyzed on a FACScan flowcytometer (BD Biosciences). Data from 10^4 cells were analyzed with the Cell Quest software (BD Biosciences).

Statistical analysis. The Student's *t*-test, Mann-Whitney *U*-test, the Kruskal-Wallis test followed by the Mann-Whitney *U*-test with Bonferroni correction, and the Wilcoxon signed-rank test were used for statistical analysis, using Stat View software Version 5.0 (SAS Institute, Cary, NC). A P value <0.05 was considered statistically significant.

Table 2. *Characteristics of the subjects received CFLA*

No.	Gender	Age	Type	Corticosteroid	Immunomodulator	CRP, mg/l	CAI
1	Male	37	pancolitis	yes	yes	29.0	15
2	Female	40	left-sided	yes	no	5.0	9
3	Male	18	left-sided	yes	no	<2.0	7
4	Male	16	pancolitis	yes	no	25.0	7
5	Male	46	left-sided	yes	no	12.0	9
6	Female	67	pancolitis	yes	yes	<2.0	8
7	Male	42	pancolitis	yes	yes	<2.0	13
8*	Female	43	left-sided	yes	no	116.5	15

*CAI score was increased after centrifugal leukocytapheresis (CFLA).

RESULTS

GRAIL, Itch, and c-Cbl mRNA and protein expression are upregulated in human anergic CD4$^+$ T cells. Human CD4$^+$ T cells freshly isolated from HV were cultured in the anergic or activated condition, and mRNA expression of these E3 ubiquitin ligases was analyzed. The calcium ionophore ionomycin has previously been used to mimic early events in the induction of T cell anergy (10, 16). The mRNA levels of the E3 ubiquitin ligases (GRAIL, Itch, and c-Cbl) were significantly higher in the ionomycin-treated CD4$^+$ T cells than in the nontreated cells (Fig. 1*A*). In addition, protein expression of these E3 ubiquitin ligases in ionomycin-treated cells was upregulated compared with untreated cells, as determined by Western immunoblotting (Fig. 1*B*). The anergic state was confirmed by proliferation assay; the CD4$^+$ T cells cultured in vitro with ionomycin showed significantly lower proliferation rates than the nontreated cells (Fig. 1*C*). Alternatively, T cell anergy can be induced by stimulation with anti-CD3 mAb in the absence of anti-CD28 mAb stimulation, whereas T cells are activated by the costimulation with anti-CD3 mAb and anti-CD28 mAb (25). Under anergic conditions (induced by anti-CD3 mAb treatment alone), GRAIL mRNA expression in human T cells was significantly higher than that of cells costimulated with anti-CD3 mAb and anti-CD28 mAb ($P < 0.05$, data not shown).

Expression of GRAIL, Itch, and c-Cbl in CD4$^+$ T cell subsets. To determine the cell types that express GRAIL, Itch, and c-Cbl in human PBMCs of HV, we isolated CD4$^+$ T cells from PBMCs and compared the expression of the E3 ligases in CD4$^+$ T cells and CD4$^-$ cells. GRAIL was more highly expressed on CD4$^+$ T cells than on CD4$^-$ cells ($P < 0.05$, Fig. 2*A*). However, the levels of Itch and c-Cbl mRNA were not higher in CD4$^+$ T cells than in CD4$^-$ cells (Fig. 2*A*). Because GRAIL was highly expressed in CD4$^+$ T cells, we analyzed the expression of GRAIL in memory/naïve and regulatory/nonregulatory T cell subsets of CD4$^+$ T cells. However, there were no significant differences in the levels of GRAIL mRNA expression between the naïve CD4$^+$CD45RA$^+$ T cell subset and the memory CD4$^+$CD45RO$^+$ T cell subset (Fig. 2*B*). In contrast, the expression of Itch and c-Cbl in naïve CD4$^+$CD45RA$^+$ T cells was significantly higher than that in memory CD4$^+$CD45RO$^+$ T cells. Because murine regulatory CD4$^+$CD25$^+$ T cells are reported to be anergic (28), the level of GRAIL mRNA in human peripheral CD4$^+$CD25$^+$ T cells was examined and compared with that in CD4$^+$CD25$^-$ T cells. However, there was no significant difference in the level of GRAIL expression between these two subsets (Fig. 2*C*). The expression of Itch and c-Cbl also revealed no significant difference between the two subsets.

Patients with UC in remission express high levels of GRAIL mRNA. We next analyzed the expression of GRAIL, Itch, and c-Cbl mRNA in human CD4$^+$ T cells isolated from patients with UC. The patients' profile and disease characteristics are presented in Table 1. When we compared the levels of GRAIL, Itch, and c-Cbl expression between all UC patients (both active and in remission) and HV, we found no significant differences in the levels of E3 ligases between the two groups (data not shown). However, the level of GRAIL expression was significantly higher in UC patients in remission than in HV (median 1.25 AU vs. 0.23 AU, $P = 0.0021$, Fig. 3*A*). In addition,

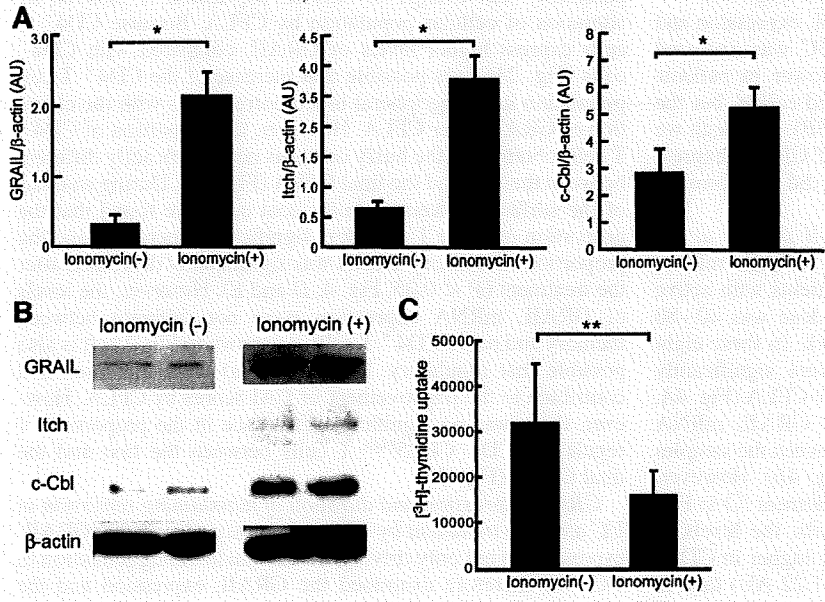

Fig. 1. E3 ligase is expressed on anergic human T cells. CD4$^+$ T cells obtained from peripheral blood of healthy volunteers (HV) were cultured with the calcium ionophore ionomycin. *A*: mRNA levels of gene related to anergy in lymphocyte (GRAIL), Itch, and c-Cbl were analyzed by real-time RT-PCR. Data are presented as the arbitrary units (AU) of GRAIL, Itch, and c-Cbl normalized to β-actin. GRAIL expression in ionomycin-treated T cells (+) was significantly higher (*$P < 0.05$) than in nontreated T cells (−) (n = 5–10). *B*: expression of GRAIL, Itch, and c-Cbl protein was analyzed by Western immunoblot analysis. Representative images are shown. *C*: cellular proliferation of ionomycin-treated cells was analyzed by [^{3}H]thymidine uptake. [^{3}H]thymidine uptake of the cells without ionomycin treatment was significantly higher than in ionomycin-treated cells (**$P < 0.01$).

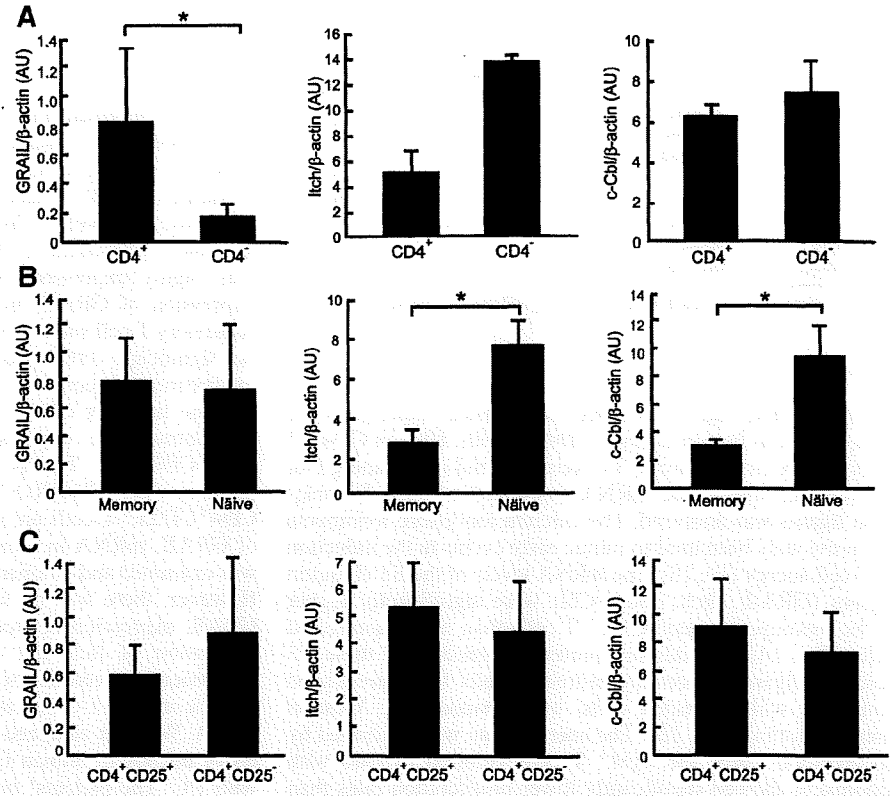

Fig. 2. E3 ligase expression in subsets of peripheral blood T cells. *A*: quantitative real-time PCR analysis of GRAIL, Itch, and c-Cbl expression in purified $CD4^+$ T cells and $CD4^-$ cells freshly isolated from peripheral blood of HV ($n = 6$). *B*: quantitative real-time PCR analysis of GRAIL, Itch, and c-Cbl expression in $CD4^+CD45RO^+$ memory and $CD4^+CD45RA^+$ naive T cells ($n = 5$). *C*: quantitative real-time PCR analysis of GRAIL, Itch, and c-Cbl expression in $CD4^+CD25^+$ regulatory T cells and $CD4^+CD25^-$ nonregulatory T cells of HV ($n = 4$). Data are presented as the AU of GRAIL, Itch, and c-Cbl normalized to β-actin. The statistical significance was determined by Mann-Whitney *U*-test. $^*P < 0.05$.

GRAIL expression was significantly higher in patients with UC in remission than in patients with active UC (median 1.25 AU vs. 0.16 AU, $P = 0.0015$). The levels of GRAIL expression in patients with active UC were similar to those of HV. Although there was a trend toward increased levels of Itch and c-Cbl expressions in patients in remission compared with other groups, there were no significant differences in the levels of Itch and c-Cbl mRNA among the three groups (Fig. 3*A*).

The correlation between the level of GRAIL expression and extension of the disease in patients with UC was analyzed further. GRAIL expression was relatively lower in patients with pancolitis than in patients with left-sided colitis, but the results were not statistically significant (Fig. 3*B*). Although we analyzed the correlation between the level of GRAIL expression and severity of UC, GRAIL expression did not correlate with CAI (data not shown).

CAI scores, GRAIL expression, and T cell subsets at the initial and final CFLA. We next analyzed GRAIL mRNA expression during treatment of UC. Eight patients with active UC were treated with prednisolone (initial dose was 40–80 mg/day) and CFLA (once per wk) for 4–5 wk. In these eight patients, CAI scores at the final CFLA were significantly decreased compared with the scores at the first CFLA (Fig. 4*A*, $P < 0.05$). In these patients, the levels of GRAIL mRNA expression were not significantly different between the samples obtained at the first and the final CFLA (Fig. 4*B*). However, when a patient who did not respond to the treatment (○ in Fig. 4, *A* and *B*) was eliminated from the analysis, the levels of GRAIL mRNA expression were significantly higher in $CD4^+$ T cells obtained at the final CFLA (average 1.22 AU) than in those obtained at the first CFLA (average 0.98 AU) in patients who responded to the treatment (● in Fig. 4, *A* and *B*, $P < 0.05$). In a patient whose CAI increased despite treatment, GRAIL expression was decreased after the treatment (○ in Fig. 4, *A* and *B*).

It has been reported that $\sim 10^9$ cells can be eliminated from the blood circulation in each CFLA session (3), and there is a possibility that change of GRAIL expression is induced by alteration of cellular population by CFLA. Because $CD4^+$ T cells express higher levels of GRAIL compared with $CD4^-$ cells (Fig. 2*A*), it is possible that increase of the $CD4^+$ T cell population in the peripheral blood corresponds with the elevation of GRAIL after CFLA. However, the proportion of $CD4^+$ T cells obtained in the buffy coat was not significantly different between the first and the final CFLA (Fig. 4*C*). During analysis of the surface markers of buffy coat cells, we found that the proportion of naïve T cells was significantly increased and the proportion of memory T cells was significantly decreased after the treatment ($P < 0.05$, Fig. 4, *D* and *E*). However, the levels of GRAIL mRNA expression were not different between memory and naïve $CD4^+$ T cells, as shown in Fig. 2*A*. It is also possible that regulatory T cells (Tregs) were increased and contribute to the improvement of CAI scores by CFLA. However, there was no significant difference in the proportion of regulatory $CD4^+CD25^{high}$ T cells between the first and the final CFLA (Fig. 4*F*).

GRAIL expression and duration of maintaining remission in UC after the treatment by CFLA. One may suspect that GRAIL expression is not only driving but also maintaining remission in UC. We therefore examined the GRAIL expression and the

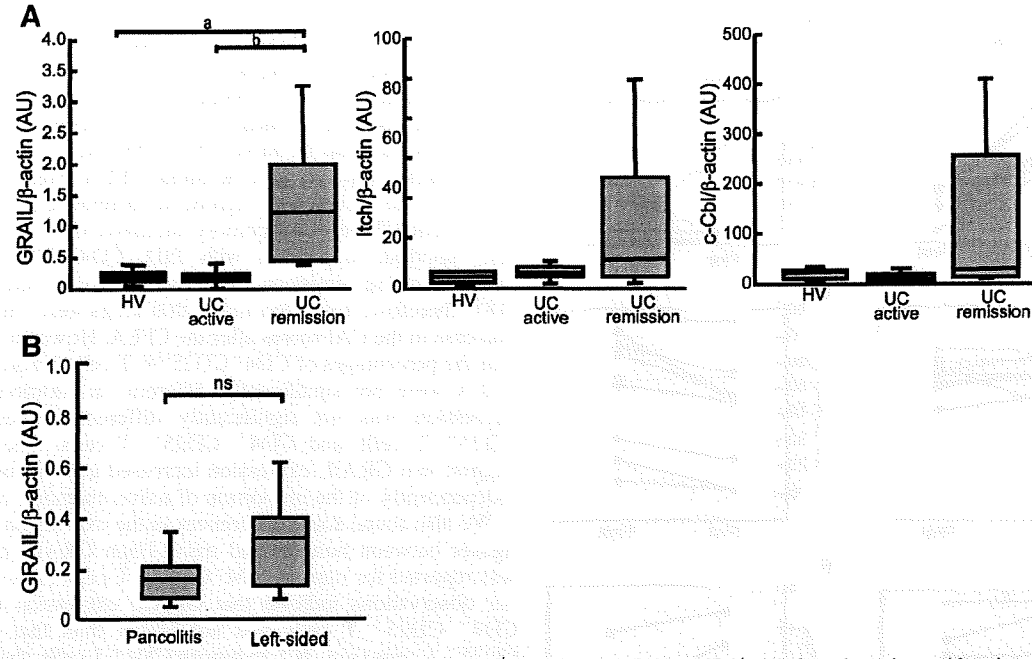

Fig. 3. E3 ligase expression in peripheral CD4$^+$ cells. *A*: levels of GRAIL, Itch, and c-Cbl mRNA in CD4$^+$ cells from the patients with active ulcerative colitis (UC), UC in remission, and HV were compared by quantitative real-time PCR. The expression of GRAIL in the patients with UC who maintained remission was significantly higher ($^aP = 0.0021$; $^bP = 0.0015$) than in patients with active UC or in HV. *B*: level of GRAIL expression in patients with active UC was analyzed according to disease extension. No significant difference was detected in the level of GRAIL expression between patients with pancolitis and those with left-sided disease (ns, not significant). The statistical significance of the difference between the medians was determined by the Mann-Whitney *U*-test.

duration of remission in the patients with UC after the treatment by CFLA. GRAIL expression was relatively higher in those who maintained long-term remission than those who encountered early relapse, although not statistically significant (Fig. 5). The average of GRAIL expression levels was 0.18 AU in patients who maintained remission less than 6 mo and was 1.95 AU in patients who maintained remission more than 6 mo.

DISCUSSION

Induction of clonal anergy in T cells is associated with global defects in T cell receptor (TCR) signaling, including reduced phosphorylation of TCR-ξ and -ϵ chains, poor activation of p56^{Lck}, 70-kDa zeta-associated protein (Zap70), Ras, c-JNK, ERK, and defective transactivation at the *IL2* gene by NF-κB, activating protein 1 (AP-1), and NF-AT(19). E3 ubiquitin ligases, such as GRAIL, Itch, and c-Cbl, have been shown to be involved in the process of T cell anergy in animals (1, 19, 24). In this study, we found that GRAIL, Itch, and c-Cbl mRNA and protein were highly expressed on anergic human CD4$^+$ T cells. These E3 ubiquitin ligases appear to reflect the status of T cell anergy not only in mice but also in human CD4$^+$ T cells. We found that GRAIL is dominantly expressed in CD4$^+$ T cell subsets, whereas Itch and c-Cbl are also expressed in CD4$^-$ cells. Recently, Jagged-1-induced Notch signaling in human CD4$^+$ T cells has been shown to be associated with upregulation of GRAIL, but not with expression of Itch or c-Cbl (12). In addition, the expression pattern on naïve and memory T cells was different among GRAIL, Itch, and c-Cbl. These results suggest that the expression of these different E3 ligases is regulated differently among the T cell subsets.

We demonstrated that GRAIL expression in CD4$^+$ T cells of patients with UC in remission was significantly higher than that in patients with active UC or in HV (Fig. 3*A*). Because the anergic CD4$^+$ T cells express high levels of GRAIL (Fig. 1), it is possible that patients in remission have more profoundly anergic CD4$^+$ T cells than do patients with active UC or HV. In addition, because the level of GRAIL expression was not significantly different regardless of the inflamed location (Fig. 3*B*) or activity of UC, low GRAIL expression is suggested to be involved even in the mild inflammation of UC. The expression of Itch and c-Cbl in UC patients in remission was relatively higher than in patients with active UC and in HV; this result further supports the involvement of anergy in the remission of UC. It was difficult to evaluate the status of T cell anergy in patients with UC because the antigen(s) responsible for the induction of inflammation in UC is (are) unknown. However, our data, in addition to other previous information about E3 ligases (1, 19), suggest that analyzing the E3 ubiquitin ligases will provide information about T cell anergic status. In addition to the driving property of GRAIL to remission of UC, GRAIL might be important for maintaining remission of UC. Considering clinical relevance of maintaining remission in UC, we further examined GRAIL level and duration of the remission in the patients treated with CFLA. Although the number of the subjects was limited to confirm this hypothesis, the expression of GRAIL was relatively higher in UC patients who maintained long-term remission than those who encountered early relapse. These preliminary results suggest the association of GRAIL with remission of UC, which remained to be investigated in further study involving the larger number of patients with UC.

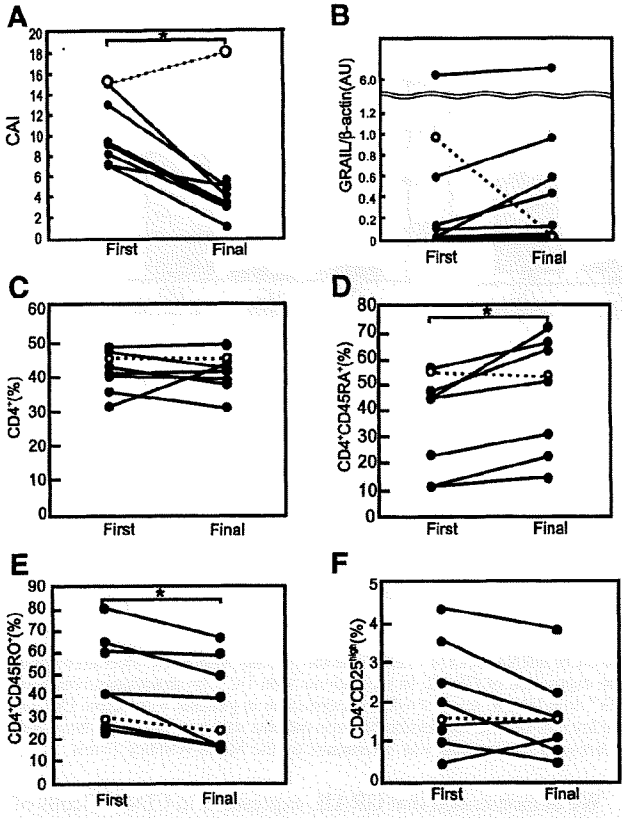

Fig. 4. Changes in clinical activity index (CAI), GRAIL, and other surface markers at the initial and the final treatment. *A*: CAI was evaluated at the initial and final centrifugal leukocytapheresis (CFLA). *B*: mRNA levels of GRAIL in $CD4^+$ T cells isolated during the initial and the final CFLA were determined by quantitative RT-PCR. Flow cytometry was performed for $CD4^+$ T cells (*C*), $CD45RA^+$ naïve T cells (*D*), $CD45RO^+$ memory T cells (*E*), and regulatory $CD4^+CD25^{high}$ T cells (*F*). The ○ corresponds with Case No.8 in Table 2 who did not respond with CFLA. The statistical significance of the differences between the means was determined by the Wilcoxon signed-rank test. $*P < 0.05$; bars indicate means ± SE of the mean.

It is also necessary to analyze the expression of the E3 ligases in the colonic mucosa to further clarify the roles of the E3 ligases in the mucosal T cell anergy. However, we could not detect the E3 ligase mRNA expressions either from intestinal biopsy samples or from peripheral blood samples from patients with Crohn's disease by the conventional method. Evaluation of the E3 ligases in the mucosa and blood samples of Crohn's disease by modified methods is ongoing.

It was expected that $CD4^+$ T cells of HV show higher levels of anergic status than that of patients with active UC. However, in this study, GRAIL expression in $CD4^+$ T cells of HV was not significantly higher than that of patients with active UC. One potential explanation is that HV do not encounter anergic or pathogenic stimulation from the environment. The exact mechanisms regulating the expression levels of the E3 ligases are unknown and need further investigation.

In addition to the observation of high GRAIL expression in patients with UC in remission, GRAIL expression was significantly increased after treatment in patients with active UC whose CAI scores improved after treatment with CFLA and prednisolone. Previous reports demonstrated that the number of memory $CD4^+CD45RO^+$ T cells of patients with UC decreases after leukocytapheresis with the use of a leukocyte removal filter (2). We obtained similar results showing a significant decrease in memory $CD4^+CD45RO^+$ T cells and significant increase in naïve $CD4^+CD45RA^+$ T cells at the final CFLA compared with the initial CFLA (Fig. 4, *D* and *E*). However, we observed no significant difference in the expression of GRAIL between memory and naïve $CD4^+$ T cells. It is also reported, in patients with IBD, $CD4^+CD25^{high}$ Tregs increase during remission but decrease during active disease (18). Therefore, it was speculated that Tregs were attributed for decrease in the CAI scores after the CFLA. However, we found that the percentages of $CD4^+CD25^{high}$ T cells before and after CFLA were not significantly different. In addition, GRAIL expression was not significantly different between $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^-$ T cells. These results suggest that GRAIL expression increased in patients with UC independently of the proportion of naive, memory, and Tregs.

We also found some differences in the expression of the E3 ligases between humans and mice. High GRAIL expression was reported for murine $CD4^+CD25^+$ T cells (17). However, our observations indicate that GRAIL expression in human $CD4^+$ $CD25^+$ T cells was not higher than that in $CD4^+$ $CD25^-$ T cells (Fig. 2*C*). In human $CD4^+$ T cells, there was no significant difference in the levels of GRAIL expression between naïve and memory $CD4^+$ T cell subsets (Fig. 2*B*). In contrast, GRAIL expression in murine memory $CD4^+$ T cells was significantly higher than that in naïve $CD4^+$ T cells (S. Egawa, unpublished observations). The E3 ligase expression variations could be caused by species-specific differences or by differences in antigen exposure between humans and mice, since mice used for experimentation are exposed to a limited number of antigens in pathogen-free animal facilities.

Our present study demonstrated that GRAIL was upregulated in human $CD4^+$ T cells following the induction of anergy in vitro, and GRAIL expression was enhanced in patients with remission stage of UC compared with patients with active disease. Previous basic research has shown that the anergic phenotype can be obtained by introducing GRAIL into murine T cells (27), indicating that GRAIL is not only a marker for T

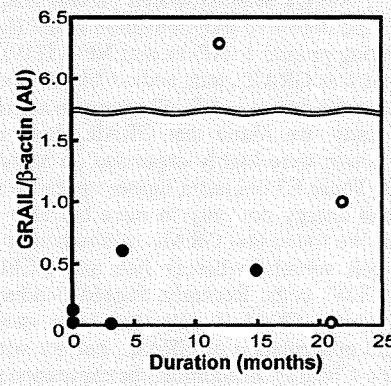

Fig. 5. Correlation of GRAIL expression and duration of maintaining remission. The duration of maintaining remission in patients with UC after the treatment by CFLA was determined. ○, patients who continued to maintain remission at the time of the analysis; ●, patients who relapsed before the analysis.

cell anergy but also plays a key role in inducing anergy in $CD4^+$ T cells. Taken together, GRAIL is suggested to be useful to reflect the status of human T cell anergy in UC and also to be a potential therapeutic target of UC.

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B ウイルスによるがん

1 ウイルス感染とがん

a ウイルス感染によるがんの発見

ウイルス感染とがんとの関連は1908年にデンマークの病理学者によりニワトリの白血病が“filterable agents（濾過性因子）”によることが示されたことに端を発します．その3年後にはラウス（Peyton Rous）がニワトリのサルコーマがウイルスにより伝染性に形成されることを報告しています．現在ではこの現象はレトロウイルス発がんであることは周知のことですが，がん原遺伝子の発見，細胞がん化の機構解明にきわめて重要な発見でした[1]．しかし，がんが伝染性であるという概念は基本的には肯定されず，特にヒトのがん発症へのウイルスの関与は1964年にバーキットリンパ腫（Burkitt lymphoma：BL）細胞内のエプスタイン-バーウイルス（Epstein-Barr virus：EBV）の発見まで待たなければなりませんでした．

現在ヒトのがんの15％前後（およそ100万～150万症例くらい）が何らかのウイルスもしくは感染性因子に起因すると考えられています．ヒト腫瘍ウイルスとしては**表1**に掲げたものが考えられます[2-4]．また，昨今，ヒトポリオーマウイルスであるBKウイルスやJCウイルス，本来サルのウイルスであるSV40（simian virus 40）がある種の脳腫瘍や中皮腫の発症にかかわっているとの報告もみられますが，これらに関してはさらに厳密な解析が必要です．

b ウイルス感染と発がんとの関連性

ウイルス感染と発がんとの関連性の証明は広くヒト界に蔓延しているウイルスではきわめて難しい問題です．古典的にはコッホ（Koch）の原則が満たされていなければなりません（**表2**）[5]が，ヒトに感染するウイルスすべてに培養細胞系や小動物における感染系が存在するわけではなく，個体レベルでの実証は実際上不可能です．また，ウイルスによってはhit-and-run的に相互作用するものもあると思われます．

現状では感染–疾患（がん）の因果関係は科学的に妥当である機構が実験的に証明されていることに加え，疫学的に関連性，一貫性，特異性，時間的因果関係，量依存性などが証明される必要があります．

c ウイルスによる発がんメカニズム

ヒト腫瘍ウイルスによる発がんは持続感染や潜伏感染といった慢性感染症の結果として生じる経過の長い多段階プロセスです．この機構の解明は本来ヒトでは腫瘍を形成しないSV40，アデノウイルスによる*in vitro*での初代培養細胞を不死化する機構の解明にありました．それはSV40のT抗原（T-Ag）やアデノウイルスの*E1A*あるいは*E1B*遺伝子産物が細胞増殖を負に制御する遺伝子産物，*pRb*や*p53*といった抑制性がん遺伝子と相互作用してその機能を不活化するという画期的な報告でした．ヒトパピローマウイルス（human papillomavirus：HPV）の*E6*や*E7*遺伝子にも同様の活性があることもまもなく示され，ヒト腫瘍ウイルスによるがん化機構の一般性を引き出しました．

最近ではシグナル伝達を含めた細胞周期の促進・抑制にかかわる因子の活性化・不活化，アポトーシスの抑制やテロメア伸長因子の活性化による細胞寿命の延長など，細胞の増殖性の獲得・維持に関する多くの報告があります[2,3]（**図1**）．