dictor for the emergence of the YMDD mutant virus in LAM therapy for chronic HBV infection. This means that IVR of an on-treatment factor is very important for good therapeutic effect and the stage for the next therapeutic strategy can thus be set in a new light with this information.

Our results showed that approximately one-seventh of the patients with chronic hepatitis B treated with LAM did not achieve IVR. In the non-IVR patients, the antiviral therapeutic regimen should be amended due to the frequent emergence of LAM-resistant virus. Recently, new nucleos(t)ide analogs have become available for the treatment of chronic HBV infection. ETV has been reported to be more effective for the reduction of HBV DNA and the less frequently induced drug-resistant mutant virus than LAM in "naïve" patients with chronic hepatitis B who had not previously received nucleos(t)ide analog therapy.[10,11] ETV was also effective in patients with chronic HBV infection showing LAM resistance,[21] but the emergence rate of the ETV-resistant virus was considerably higher in LAM-resistant patients than in naïve patients.[13,22] This is because the ETV-resistant HBV strain is established by LAM-resistant YMDD mutation plus additional mutation(s) at the amino acid position(s) 184, 202 and/or 250 within the reverse transcriptase domain of HBV.[22] According to these findings, switching from LAM to ETV may be useful for treating patients who do not achieve IVR on LAM administration. This should be done before the emergence of LAM-resistant YMDD mutant virus so as not to reduce the therapeutic efficacy of ETV. In clinical practice, there are still a number of patients who have already been on continuous LAM therapy, although the current first choice drug for patients with chronic HBV infection is ETV. In our opinion, foregoing patients without IVR or YMDD mutant viruses should be switched from LAM to ETV. The therapeutic efficacy of switching from LAM to ETV in non-IVR patients should be assessed by further study with a larger number of patients.

ADV and tenofovir disoproxil fumarate (TDF) have also been shown to exert antiviral efficacy in patients with chronic HBV infection with less frequent occurrence of drug-resistant mutant virus compared to LAM.[23] In addition, unlike the case of ETV, both ADV and TDF are known to be effective in LAM-refractory patients with chronic hepatitis B, as well as naïve patients.[23] Using ADV and TDF may be helpful for the treatment of non-IVR patients, especially after the establishment of LAM-resistant mutant virus.

In conclusion, our findings indicate that IVR may be a useful factor for predicting the emergence of LAM-resistant mutant virus in patients with chronic HBV infection treated with LAM. For patients who do not achieve IVR, therapeutic options other than LAM monotherapy should be promptly implemented because of the high incidence of the subsequent emergence of the YMDD mutant virus.

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Case Report

Early emergence of entecavir-resistant hepatitis B virus in a patient with hepatitis B virus/human immunodeficiency virus coinfection

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The efficacy of entecavir for patients with hepatitis B virus/human immunodeficiency virus coinfection has not been fully elucidated. Here we examined a patient coinfected with both viruses in whom entecavir-resistant hepatitis B virus appeared. The 60-year-old Japanese male with the coinfection received antiretroviral therapy including lamivudine. The therapy initially suppressed replication of both viruses, followed by reactivation of the hepatitis B virus alone by 2 years of therapy. He subsequently received entecavir therapy in addition to the antiretroviral regimen. After entecavir administration, the hepatitis B virus DNA level was slightly reduced, but then increased after 6 months of entecavir therapy. In the sequencing analysis of hepatitis B virus, no drug resistance-associated amino acid substitutions were observed in the reverse transcriptase (rt) domain before antiretroviral therapy. The lamivudine-resistant amino acid substitutions at rt173, rt180 and rt204 were detected before entecavir administration, and further the entecavir-resistant rt202 substitution was observed after 6 months of entecavir therapy. The full-length hepatitis B sequences showed that the viral strain derived from the patient belonged to genotype H. In summary, this report describes a patient with hepatitis B virus/human immunodeficiency virus coinfection who received entecavir therapy in addition to an antiretroviral regimen and showed the early emergence of entecavir-resistance hepatitis B virus. In entecavir therapy for patients infected with both viruses, great care should be taken with respect to the emergence of entecavir-resistant hepatitis B virus, especially in patients with pre-existing lamivudine-resistant virus.

Key words: coinfection, drug-resistant hepatitis B virus, entecavir, hepatitis B virus, human immunodeficiency virus, lamivudine

INTRODUCTION

CHRONIC CARRIERS OF hepatitis B virus (HBV) number more than 350 million worldwide.[1] Chronic HBV infection is seen in approximately 10% of human immunodeficiency virus (HIV)-infected patients,[2] and coinfection with HBV and HIV is a serious health problem due to the shared mode of transmission. Since the prognosis of HIV-infected patients can be dramatically improved by highly active antiretroviral therapy (HAART), one of the major causes of mortality in HIV-infected patients is chronic liver disease due to HBV infection.[3]

Lamivudine (LAM, also abbreviated to 3TC), one of the antiretroviral drugs, has also been used for the reduction of HBV replication and improvement of HBV-related liver diseases.[4,5] However, the anti-HBV effect of LAM is hampered by the emergence of LAM-resistant mutant virus in cases of HBV monoinfection and HBV/

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HIV coinfection.[6,7] The LAM-resistant HBV strain is based on point mutation occurring within the reverse transcriptase (rt) domain of the polymerase gene. A methionine-to-valine/isoleucine amino acid substitution at rt204 (rtM204V/I) is known to confer LAM resistance.[8,9] A leucine-to-methionine substitution at rt180 (rtL180M) and a valine-to-leucine substitution at rt173 (rtV173L) have also been shown to appear in association with LAM resistance.[8,10,11] The emergence rate of LAM-resistant virus in patients coinfected with HBV and HIV has been reported to be approximately 50% after 2 years of therapy.[9]

Recently, entecavir (ETV) has been reported to be superior to LAM for the suppression of viral replication and disease activity in patients with HBV monoinfection who had not received previous treatment with other anti-HBV drugs (naïve patients).[12,13] ETV has also been shown to be effective in HBV-infected patients who had been treated with LAM and showed LAM resistance.[14] It has been demonstrated that ETV resistance occurs based with amino acid substitution(s) at rt184, rt202 and/or rt250, together with the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus after 3 years of therapy has been reported to be less than 1% in naïve patients and 15% in LAM-resistant patients with chronic HBV monoinfection.[16] However, the anti-HBV efficacy of ETV for HBV/HIV coinfection has not been fully clarified.

In this study, we examined a patient with concomitant HBV/HIV infection who underwent HAART including LAM, and showed the appearance of LAM-resistant HBV. Subsequent ETV administration did not lead to an adequate reduction of the HBV replicative level, followed by the early emergence of the ETV-resistant virus. We investigated the serial change in the drug resistance-associated mutation status within the rt domain of the HBV polymerase gene, as well as full-length nucleotide sequences of the ETV-resistant HBV strain derived from the patient.

CASE REPORT

Patient and serum sampling

A 60-YEAR-OLD JAPANESE heterosexual male first visited to the National Hospital Organization Osaka National Hospital in December 2001 due to a positive result from an HIV antibody (anti-HIV) test in voluntary HIV screening. From his anamnestic record, he had been admitted with type B acute hepatitis to another hospital 3 years earlier. Anti-HIV had been negative at that time. On his first visit, the anti-HIV positivity was confirmed by Western blot analysis. Antibodies to HIV-1 proteins, gp160, gp110/120, p68, p52, gp41, p40 and p34 were positive. As for antibodies to HIV-2 proteins, only an antibody to p68 was positive. According to these, he was judged to be infected with HIV-1. The HIV-RNA level was $10^{4.3}$ copies/mL, and the CD4+ T cell counts were 275/mm^3 (normal range, >300/mm^3). He tested positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), and negative for antibody to HBsAg (anti-HBs) and antibody to HBeAg (anti-HBe). The HBV-DNA level was $>10^{7.6}$ copies/mL, and the alanine aminotransferase (ALT) level was 106 IU/L. The patient was free of HIV-related symptoms and had no opportunistic infectious diseases. HAART with LAM (300 mg/day), zidovudine (AZT) (600 mg/day) and efavirenz (EFV) (600 mg/day) was started in April 2002. AZT and EFV were then substituted for sanilvudine (d4T) (60 mg/day) and avacavir (ABC) (600 mg/day) in July 2002 because of anemia and dizziness. By July 2002, HIV-RNA decreased to below the detection limit ($<10^{1.7}$ copies/mL), whereas the CD4+ T cell counts tended to rise up to >500/mm^3. In August 2006, fosamprenavir (FPV) (2400 mg/day) was commenced in place of d4T due to peripheral nerve palsy. Suppression of HIV-RNA below the detection limit continued at the end of follow-up, irrespective of repeated alterations in the therapeutic regimen of HAART. As for HBV status, HBV-DNA declined to $10^{3.9}$ copies/mL in April 2003 but increased again to $>10^{7.6}$ copies/mL in May 2005. To control HBV replication, ETV (0.5 mg/day) was added in October 2006. After the ETV administration, HBV-DNA slightly decreased from $>10^{7.6}$ to $10^{6.2}$ copies/mL in January 2007 but rose to $10^{7.2}$ copies/mL 3 months later. ALT remained abnormal and HBeAg continued to be positive throughout the follow-up period. The clinical course of the patient is summarized in Figure 1a.

For the nucleotide sequencing of HBV-DNA, the serum samples were obtained in December 2001 (before HAART), August 2006 (before ETV administration), and April 2007 (after 6 months of ETV therapy). These serum sampling points were designated as P1, P2 and P3 (see Fig. 1a). Serum samples were stored at −80°C until use. Informed consent was obtained from the patient.

Virus markers and nucleotide sequencing

HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HIV were tested by chemiluminescent immunoassay. A

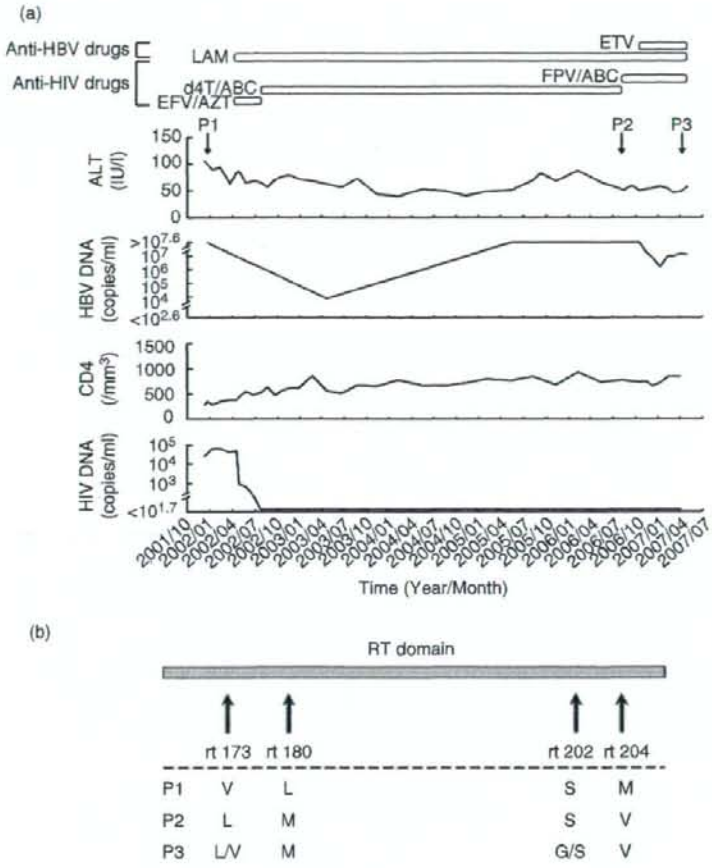

Figure 1 (a) Patient clinical course and serum sampling points. P1, P2 and P3 are the points at which serum samples were obtained. P1 was taken in December 2001 (before HAART), P2 in August 2006 (before ETV administration) and P3 in April 2007 (after 6 months of ETV therapy). ABC, avacavir; ALT, alanine aminotransferase; AZT, zidovudine; d4T, sanilvudine; EFV, efavirenz; ETV, entecavir; FPV, fosamprenavir; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine. (b) Serial change in the status of drug resistance-associated amino acid substitutions.

confirmatory anti-HIV-1/2 testing was carried out by Western blot analysis. Serum HBV-DNA was detected by means of a PCR assay (Amplicor HB monitor; Roche Diagnostics, Basel, Switzerland) with a lower detection limit of $10^{2.6}$ (=400) copies/mL. Plasma HIV-RNA was quantified by a PCR assay (Amplicor HIV-1 monitor; Roche) whose lower detection limit was $10^{1.7}$ (=50) copies/mL.

The nucleotide sequences of HBV-DNA were determined by a method based on nested PCR and direct sequencing, as described elsewhere.[17] In this study, primers BF5-2 (5′-TCC TCA GGC CAT GCA GTG GA-3′, nt 3201-20) and BR8 (5′-TTG CGT CAG CAA ACA CTT GG-3′, nt 1195-76) were also used. Nucleotide sequences of the entire rt domain in the polymerase gene were examined in HBV strains derived from the P1 and P2 serum samples (GenBank accession nos. AB353765 and AB353766), whereas the full-length HBV-DNA was determined in the strain derived from the P3 serum sample (GenBank accession no. AB353764). The full-length HBV strain obtained in this study (designated as HBDI03), the seven representative HBV strains of genotypes A–G and the eight previously isolated HBV strains of genotype H were aligned, and the phylogenetic tree was constructed. These analyses were done at the homepage of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Results of sequencing analysis of HBV

The serial change in the nucleotide sequences in the rt domain of the HBV polymerase gene was first examined

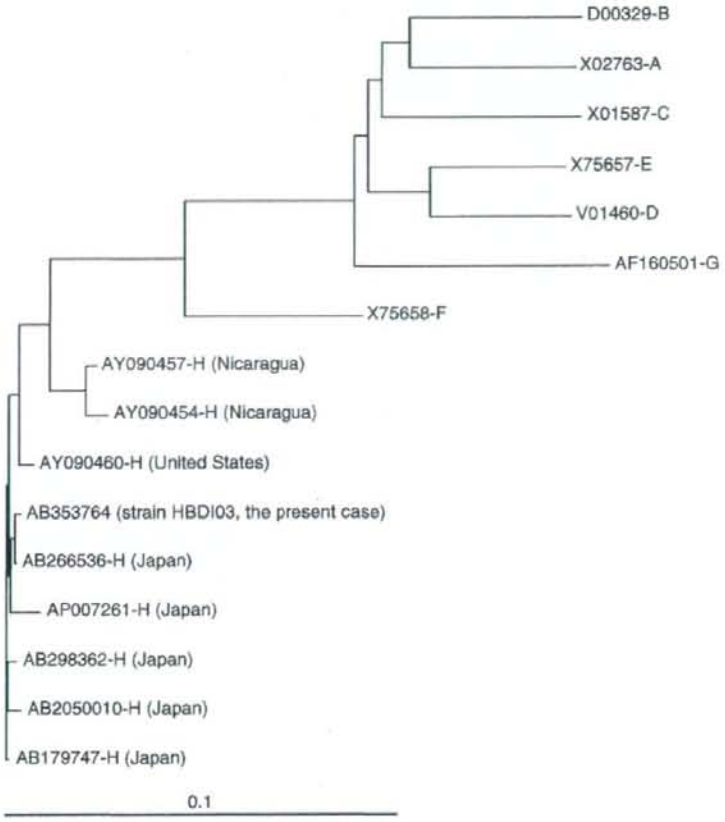

Figure 2 Phylogenetic tree analysis including the HBV strain HBDI03 obtained in this study, the seven representative HBV strains of genotypes A–G, and the eight previously isolated HBV strains of genotype H.

using serum samples obtained at P1–P3 (Fig. 1b). At point P1, no drug resistance-associated mutations were found in the rt domain, but three LAM resistance-associated substitutions, rtM204V, rtL180M and rtV173L, emerged at point P2. A serine-to-glycine substitution at rt202 (rtS202G), which has been shown to be one of the ETV resistance-associated substitutions,[15] was further observed at point P3, although rtS202G and rtV173L substitutions occurred incompletely. No other amino acid substitutions were seen in the rt domain of the HBV polymerase gene from point P1 to P3. Thus, in the patient with HBV/HIV coinfection, the emergence of the drug resistance-associated amino acid substitutions revealed a close relationship with the poor anti-HBV efficacy of LAM and ETV.

Next, the full-length nucleotide sequences of HBV were determined from the P3 serum sample of the patient with HBV/HIV coinfection showing ETV resistance. The full-length HBV strain HBDI03 comprised a total of 3215 nucleotide lengths. The phylogenetic tree was depicted using the HBV strain HBDI03, the seven representative HBV strains of genotypes A–G and the eight previously identified genotype H HBV strains. As shown in Figure 2, the HBV strain HBDI03 obtained in this study was classified as genotype H. When the nucleotide sequences of the strain HBDI03 were compared with the eight reported genotype H HBV strains, the strain HBDI03 showed a 97.2–99.8% identity with these strains. The unique amino acid substitutions in the strain HBDI03 were further investigated in comparison with these eight genotype H HBV strains. As shown in Table 1, four drug resistance-associated substitutions within the rt domain were observed, as described above. The two amino acid substitutions in the S gene were also caused by the same mutations of the drug resistance-associated rtV173L and rtM204V

Table 1 The unique amino acid substitutions in strain HBDI03 in comparison with eight previously isolated genotype H hepatitis B virus strains

Amino acid position	Consensus residue of genotype H	Residue unique to strain HBDI03
Polymerase		
519 (rt173)	V	L/V
526 (rt180)	L	M
548 (rt202)	S	G/S
550 (rt204)	M	V
Surface		
164	E	D/E
195	I	M
X		
32	W	G

Consensus residues of genotype H were from the eight reported hepatitis B virus (HBV) strains (GenBank accession nos. AY090454, AY090457, AY090460, AP007261, AB179747, AB205010, AB266536 and AB298362).

changes. As for the remaining one amino acid substitution in the X gene, the substituted glycine residue observed in the HBDI03 strain was a common one in the representative HBV strains of genotypes A–G at the corresponding codon position. Taken together, the HBDI03 strain did not appear to have any distinctive features other than the presence of the drug-associated amino acid substitutions.

DISCUSSION

RECENTLY, ETV HAS been widely accepted as an effective drug for the treatment of HBV monoinfection because of its stronger inhibitory effect on HBV replication and lower emergence rate of drug-resistant mutant virus compared to LAM.[12–14] ETV-resistant HBV has been demonstrated to be established by amino acid substitution(s) at rt184, rt202 and/or rt250, in addition to the LAM-resistant rtM204V/I and rtL180M substitutions.[15] The emergence rate of ETV-resistant virus has been reported to be higher in LAM-resistant patients than in naïve patients.[16] There has so far been little evidence concerning the anti-HBV efficacy of ETV for patients with HBV/HIV coinfection. In particular, LAM-resistant HBV has been shown to emerge frequently in patients with HBV/HIV coinfection who received LAM therapy as a component of HAART.[7] The therapeutic efficacy of ETV on LAM-resistant HBV should be assessed in patients with HBV/HIV coinfection. In this study, we examined a patient with HBV/HIV coinfection who had LAM-resistant HBV induced by HAART including LAM, and underwent subsequent ETV therapy. The patient showed a rather weak suppressive effect of ETV on HBV replication, followed by the emergence of ETV-resistant HBV in the early phase of therapy.

In the sequence analysis of the HBV genome, no drug-resistant HBV mutations were detected before HAART, but continuous LAM administration induced the LAM-resistant mutant HBV with rtM204V, rtL180M and rtV173L amino acid substitutions. Subsequent ETV therapy resulted in the emergence of an ETV-resistant virus possessing the rtS202G substitution in addition to the three LAM resistance-associated substitutions after no more than 6 months of ETV therapy, although the rtS202G and rtV173L substitutions were incomplete. In LAM-resistant patients with HBV monoinfection, the emergence rate of the ETV-resistant mutation has been reported to be merely 15% after 3 years of therapy.[16] In comparison with this, ETV-resistant HBV appeared in an extremely early phase of therapy in our patient with HBV/HIV coinfection. According to this, ETV resistance is speculated to be established earlier in patients with HBV/HIV coinfection than in those with HBV monoinfection, although concomitant HIV infection has not thus far been suggested to result in a higher incidence of the drug-resistant HBV strain in the treatment with other anti-HBV drugs in chronic HBV infection. The latent immune deficiency caused by HIV infection might prevent HBV eradication through a host immune response, resulting in poor anti-HBV efficacy of ETV. Alternatively, simultaneous usage of multiple antiretroviral drugs might in some way contribute to the emergence of ETV-resistant HBV.

Very recently, it has been shown that ETV possesses modest anti-HIV activity both *in vitro* and *in vivo* and can induce the drug-resistant mutant HIV strain in patients with HBV/HIV coinfection.[16] This suggests that ETV may not be appropriate for the treatment of patients with HBV/HIV coinfection in whom HAART is not needed. On the other hand, ETV is considered to be beneficial for patients with HBV/HIV coinfection undergoing a stable continuation of HAART. In particular, the therapeutic efficacy of ETV may be more promising in patients without LAM-resistant HBV than in those with it. Although the present case of the patient under discussion, who already displayed LAM-resistant HBV due to the preceding HAART, did not support the usefulness of ETV therapy because of the early emergence of ETV-resistant HBV, further studies with a large number of

patients should be completed to assess the antiviral efficacy and deliberate clinical application of ETV therapy for HBV/HIV coinfection.

Both adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF) have recently been shown to effectively inhibit HBV replication in patients with HBV/HIV coinfection, irrespective of LAM resistance.[19,20] ADV exerts only anti-HBV activity and is available for patients with HBV/HIV coinfection who have no need for HAART or who are receiving a stable HAART regimen. In contrast, TDF can be used as a component of HAART because of its valuable antiviral activity against both HBV and HIV. Accordingly, ADV and TDF are currently useful drugs for patients with HBV/HIV coinfection and may be subsequent therapeutic options for the patient reported in this study.

Our patient was found to be infected with HBV of genotype H, a globally rare genotype. To date, the full-length sequences of eight genotype H HBV strains have been reported from the USA, Nicaragua and Japan (see Fig. 2). Of them, one strain has been obtained from a Japanese patient with chronic HBV monoinfection who underwent ETV therapy as a naïve patient and showed ETV resistance later.[21] The relevance of the genotype frequency to the therapeutic efficacy of ETV should be studied extensively in HBV-infected patients treated with ETV.

In Japan, genotypes B and C are prevalent in chronic HBV carriers who acquire the infection mainly through the mother-to-child transmission route. In contrast, the foreign HBV strains other than genotypes B and C have been shown to be involved in a considerable proportion of patients with acute HBV infection.[22] Infection of such foreign types of HBV possibly occurs through sexual contacts in Japan. In our patient with HBV/HIV coinfection who had genotype H HBV of foreign origin, it is speculated that acute HBV infection occurring 3 years before his first visit led to the transition to chronicity. The time of HIV infection cannot be defined due to the lack of HIV-RNA testing during the period of acute HBV infection. The possibility of simultaneous infection with HBV and HIV cannot be excluded, despite the negative result of anti-HIV at that time, because the test may have taken place during the immunological window period of HIV infection.

In summary, we have introduced a patient with HBV/HIV coinfection who underwent ETV therapy in addition to the HAART regimen and showed ETV resistance in the early phase of therapy. Our finding suggests that, in ETV therapy for patients with HBV/HIV infection, great care should be taken against the emergence of ETV-resistant HBV, especially in patients with pre-existing LAM-resistant HBV.

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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).

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

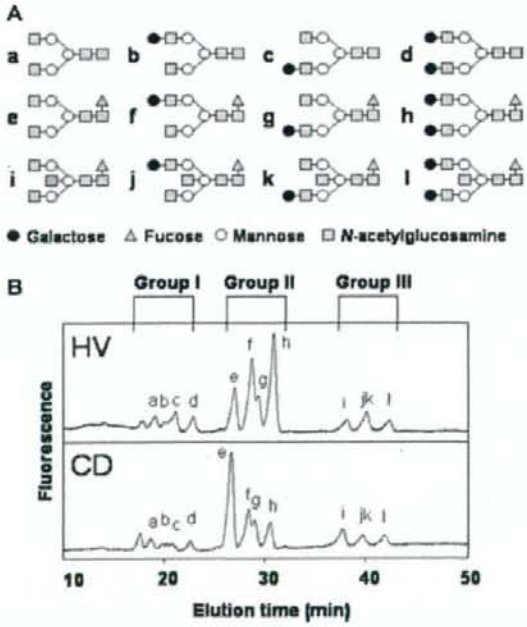

Figure 1. Structures of oligosaccharides attached to human IgG. (*A*) Structural patterns of *N*-linked neutral oligosaccharides. (*B*) Representative profiles of neutral IgG oligosaccharides purified from HV and CD patients.

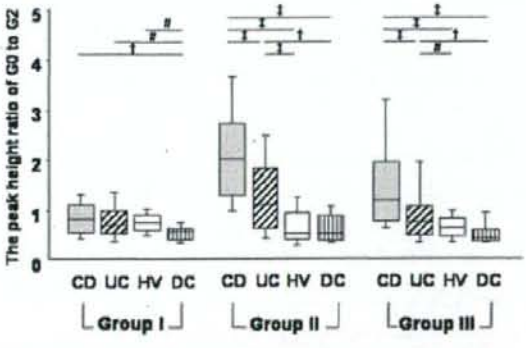

Figure 2. Increased fucosylated agalactosyl IgG in IBD patients. The peak height ratio of G0 to G2 in each subgroup was calculated for the subjects. Box plots show 50% of the relevant patient population. The line inside the box represents median value. Whiskers indicate the 90th and 10th percentiles.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

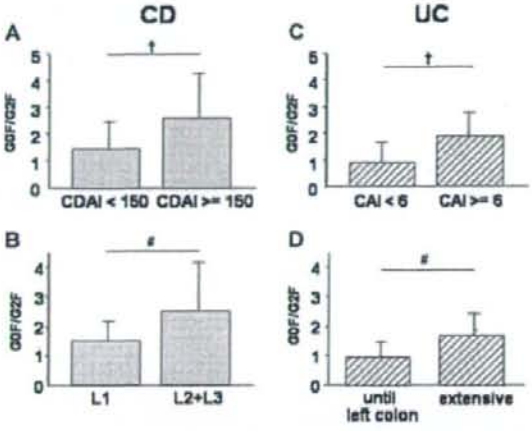

Figure 3. G0F/G2F and clinical manifestations. (*A*) G0F/G2F of CD patients in remission (CDAI <150) and in an active stage (CDAI ≥150). (*B*) G0F/G2F of CD patients with category L1 and L2 + L3. (*C*) G0F/G2F of UC patients in remission (CAI <6) and in an active stage (CAI ≥6). (*D*) G0F/G2F of UC patients 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.

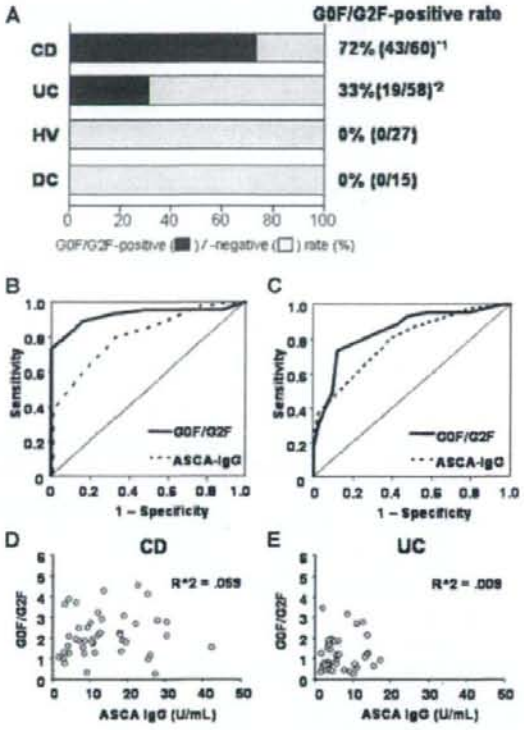

Figure 4. Effectiveness of G0F/G2F-positive rate as a serologic marker for IBD. (*A*) The G0F/G2F-positive rate in the subjects. The χ^2 test was used for the comparison between CD and UC and Fisher's exact test was used for the comparison between CD and HV or DC and between UC and HV or DC. $^{*1}P < 0.001$ 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).

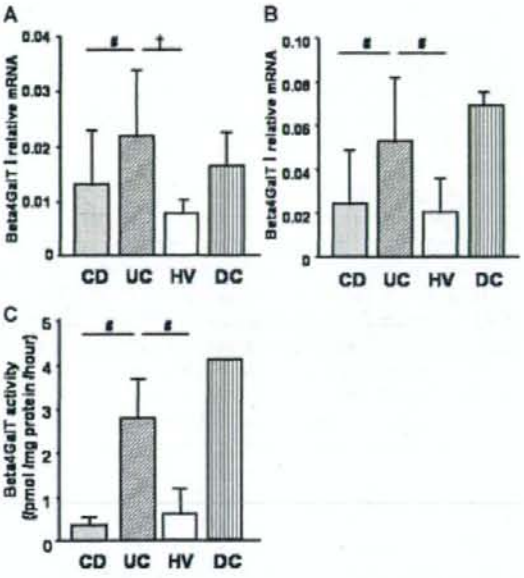

Figure 5. mRNA expression and enzyme activity of beta-1,4-galactosyltransferase (Beta4GalT). (*A*) Beta4GalT I mRNA expression in plasma cells of CD (N = 9), UC (N = 9), HV (N = 5), and DC (N = 3). (*B*) Beta4GalT I mRNA expression in B cells of CD (N = 8), UC (N = 5), HV (N = 5), and DC (N = 2). (*C*) 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.