Lectin-based Immunoassay for Aberrant IgG Glycosylation as the Biomarker for Crohn's Disease

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Background: Easily measured and clinically useful biomarkers for inflammatory bowel disease (IBD) are required to advance patient care. We previously reported that the agalactosyl fraction among fucosylated IgG oligosaccharides is increased in IBD, especially Crohn's disease (CD). The present study aimed to establish a simple detection system for aberrant glycosylated IgG based on lectin–oligosaccharide interactions.

Methods: Lectins with higher affinity to serum IgG from IBD patients than healthy volunteers (HV) were screened by lectin microarray. Binding of selected lectins to agalactosyl IgG was definitively confirmed using step-by-step glycosidase treatment. Using the selected lectins, a lectin-enzyme-linked immunosorbent assay system was established and its clinical utility was investigated in a total of 410 (249 Japanese and 161 American) IBD patients, disease controls, and HVs.

Results: *Agaricus bisporus* Agglutinin (ABA) and *Griffonia simplicifolia* Lectin-II (GSL-II) had higher affinity for serum agalactosyl IgG from IBD patients, especially those with CD, compared to HV. Agalactosyl IgG levels measured by a lectin-enzyme immunoassay (EIA) with ABA or GSL-II were significantly increased in CD compared with HV and disease controls. Agalactosyl IgG levels significantly correlated with disease activity, showed higher predictability of therapeutic outcomes for CD than C-reactive protein levels, and exhibited higher specificity for diagnosing IBD in combination with anti-*Saccharomyces cerevisiae* antibody (ASCA). Validation analysis showed that agalactosyl IgG levels were significantly increased in Japanese and American CD patients.

Conclusions: A lectin-EIA for agalactosyl IgG is a novel biomarker for IBD, especially in patients with CD.

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Key Words: inflammatory bowel disease, Crohn's disease, biomarker, oligosaccharides, IgG

The human inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic relapsing and remitting inflammation in the digestive tract. Although genetic predisposition, environmental factors, and altered immune responses have pivotal roles in the pathogenesis of IBD, precise etiologies remain unknown. Despite recent therapeutic advances,[1,2] surgical care remains a mainstay treatment of IBD.[3] Therefore, a biomarker that reflects clinical course and therapeutic outcome is in high demand. Several serologic markers have been developed for the diagnosis of IBD such as anti-*Saccharomyces cerevisiae* antibody (ASCA),[4] peripheral antineutrophil cytoplasmic antibody,[5] or anti-outer membrane porin protein C, and anti-CBir1 flagellin.[6] However, these antibodies generally lack diagnostic and prognostic sensitivity and specificity to alter clinical decision making.

Immunoglobulin (Ig) G carries *N*-linked oligosaccharides at the Cgamma2 domain of the Fc fragment at asparagine 297, all of which are biantennary complex-type with or without bisecting *N*-acetylglucosamine (GlcNAc), core-fucose,

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TABLE 1. Patient Characteristics: Japanese Population

	CD ($N = 82$)	UC ($N = 70$)	HV ($N = 78$)	DC ($N = 19$)	SLE ($N = 29$)	RA ($N = 20$)
Male/Female	22/60	31/39	39/39	9/10	5/24	2/18
Age, yr, mean (SD)	38 (11)	39 (14)	45 (11)	35 (16)	33 (14)	52 (16)
Age at diagnosis, yr, mean (SD)	28 (10)	33 (13)				
Disease location, *N*						
Small bowel/colon/both/unknown	27/11/42/2					
Extensive/left colon/rectum/unknown		28/26/14/2				
Treatment, *N* (%)						
Salazosulfapyridine or mesalazine	72 (88)	59 (84)				
Steroids	4 (5)	9 (13)				
Immunomodulators	10 (12)	2 (3)				
Anti-TNF-alpha antibodies	7 (9)	0 (0)				
CRP, mg/dL, mean (SD)	1.0 (1.6)	0.4 (1.1)				
CDAI (CD) or CAI (UC), mean (SD)	153 (92)	3.3 (4.0)				

galactose, and sialic acid residues. Our previous analysis of IgG oligosaccharides revealed that the agalactosyl fraction among fucosylated oligosaccharides was significantly higher in patients with CD and UC than in healthy volunteers (HV) and disease controls (DC). Fucosylated agalactosyl IgG levels closely correlated with disease activity and clinical course in IBD patients, and had a significantly higher sensitivity to diagnose IBD compared with ASCA.[7] We also reported that agalactosyl IgG oligosaccharides enhanced antibody-dependent phagocytosis, suggesting that oligosaccharide alterations of IgG are not only a marker of IBD but also functionally modulate immune function.[8] The current analytic methodology, however, requires researchers to perform multistep complicated procedures to acquire an IgG oligosaccharide chart using high-performance liquid chromatography (HPLC), so the development of an easier detection system is necessary for widespread clinical application.

There have been technical obstacles in establishing simple detection systems for oligosaccharides due to difficulties in purifying an oligosaccharide-specific antibody. Although several oligosaccharide structures can be measured by lectin-antibody enzyme immunoassay (EIA),[9] lectins have a lower affinity and specificity for oligosaccharides than specific antibodies.[10] To overcome these problems, in the present study we developed a lectin-EIA system by using multiple lectins to detect agalactosyl IgG as a new serologic marker for IBD.

MATERIALS AND METHODS

Subjects

Serum samples were collected from 82 patients with CD, 70 patients with UC, 72 age/gender-matched unrelated HVs, and 19 patients with colonic inflammation including appendicitis, diverticulitis, and ischemic colitis (DCs). These participants were Japanese recruited at the Department of Gastroenterology and Hepatology, Osaka University Hospital (Suita, Osaka, Japan), and the Department of Gastroenterology, Osaka Rosai Hospital (Sakai, Osaka, Japan). Serum samples of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) were provided by the Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Hospital. Patient characteristics are presented in Table 1. On the validation analysis, American serum samples were collected from 103 patients with CD, 39 patients with UC, and 19 HVs, who were recruited at the Department of Medicine, University of North Carolina Hospital (Chapel Hill, NC). Patient characteristics are presented in Table 2. 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.[11–13] Patients with CD were classified by age at diagnosis (A1, below 16 years old; A2, between 17 and 40 years old; A3, above 40 years old), location (L1, ileal; L2, colonic; L3, ileocolonic; L4, isolated upper disease), and behavior (B1, nonstricturing and nonpenetrating; B2, stricturing; B3, penetrating) according to the Montreal Classification.[14] Clinical activities were determined using the Crohn's Disease Activity Index (CDAI) for CD[15] or the Clinical Activity Index (CAI) for UC.[16] Infliximab maintenance therapy[17] was performed on biologic therapy-naïve CD patients. Clinical responders by infliximab therapy were defined as the patients whose CDAI scores at week 30 were less than 150 or decreased more than 70 from the pretreatment scores.

IgG Oligosaccharide Analysis by HPLC

Serum IgG oligosaccharide analysis was performed as described previously.[7] Briefly, IgG was purified using Protein

TABLE 2. Patient Characteristics: U.S. Population

	CD ($N = 103$)	UC ($N = 39$)	HV ($N = 19$)
Male/Female	49/54	23/16	11/8
Age, yr, mean (SD)	39 (14)	43 (18)	31 (4)
Age at diagnosis, yr, mean (SD)	27 (9)	33 (7)	
Duration of disease, yr, mean (SD)	12 (9)	10 (7)	
Disease location (*N*)			
Small bowel/colon/both/others	28/24/49/3		
Extensive/left colon/rectum/unknown		24/11/3/1	
Treatment, N (%)			
Salazosulfapyridine or mesalazine	25 (24)	20 (51)	
Steroids	15 (14)	9 (23)	
Immunomodulators	35 (34)	4 (10)	
Anti-TNF-alpha biologics	23 (22)	11 (28)	

G sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). *N*-linked oligosaccharides were released from serum IgG by Glycopeptidase F (Takara Bio, Shiga, Japan) and labeled with 2-aminopyridine by GlycoTag (Takara Bio). Pyridylamino- (PA-) oligosaccharides from IgG were analyzed on a reverse-phase HPLC system (Waters, Milford, MA).

Lectin Microarray

Total pattern of oligosaccharide structures in serum IgG was investigated with evanescent-field fluorescence-assisted lectin microarray.[18] Forty-five kinds of lectins were immobilized on the glass slide in triplicate and ≈250 ng/mL of IgG in phosphate-buffered saline (PBS) with 1% Triton X-100 was applied to the array. To label target glycoproteins, Cy3 monoreactive dye (GE Healthcare Biosciences, Chalfont St Giles, UK) was used in this analysis. Detailed procedures were described previously.[18] Fluorescence intensity of all lectins and that of lower signal-lectins were analyzed independently.

Step-by-step Glycosidase Treatment Followed by Lectin Blotting

Purified serum IgG was obtained and incubated with sialidase, beta-galactosidase, and glucosaminidase by enzymatic carborelease kit (QA Bio, Palm Desert, CA), according to the manufacturer's instructions. Lectin blotting was performed in each step of glycosidase treatment.

Lectin Blotting

Eight micrograms of purified serum IgG was separated 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and then transferred to a nitrocellulose membrane (GE Healthcare Biosciences). The membrane was incubated overnight at 4°C with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), and then for 1 hour at room temperature with biotinylated *Agaricus bisporus* Agglutinin (ABA) (1 μg/mL, Seikagaku biobusiness, Tokyo, Japan), *Griffonia simplicifolia* Lectin-II (GSL-II) (2 μg/mL, Vector Laboratories, Burlingame, CA), *Concanavalin A* (ConA) (0.5 μg/mL, Seikagaku biobusiness), *Sambucus sieboldiana* Agglutinin (SSA) (1 μg/mL, Seikagaku biobusiness), or *Ricinus communis* Agglutinin I (RCA120) (1 μg/mL, Seikagaku biobusiness) in 3% bovine serum albumin in TBS-T. After washing with TBS-T three times, the membrane was incubated with Vectastatin ABC kit (Vector Laboratories) for 20 minutes, then washed with TBS-T three times. Staining was performed with ECL reagent (GE Healthcare, Milwaukee, WI).

Lectin-EIA

Purified IgGs from sera of patients and HVs (adjusted to 5 μg/well) were prepared with sample diluents (PBS containing 0.1% bovine serum albumin [BSA] and 0.05% Tween 20) containing SDS (final concentration is 0.05%) for 1 hour. Flat-bottomed 96-well streptavidin-precoated microtiter plate (Nalge Nunc International, Tokyo, Japan) was coated with 50 μL/well of biotinylated ABA (0.33 μg/mL) and/or biotinylated GSL-II (0.67 μg/mL) with 0.05% BSA in PBS for 1 hour at room temperature. After washing the plate four times with PBS containing 0.05% Tween 20 (PBS-T), 50 μL/well of the prepared IgG was added. After incubation for 1 hour, the plate was washed four times with PBS-T, then 100 μL/well of 0.1% BSA in PBS was added to block nonspecific protein binding sites. After incubation for 1 hour, the plate was washed four times with PBS-T, then 50 μL/well of a 1/2000-diluted solution of alkaline phosphatase-conjugated antibody against human IgG (SouthernBiotech, Birmingham, AL) was added. After incubation for 1 hour, the plate was washed four times with PBS-T and added 50 μL/well of 0.1M glycine buffer (pH 10.4) with phosphate substrate (Sigma-Aldrich, St. Louis, MO), and then incubated for 20 minutes. The optical density (OD) was measured at 405 nm. All experiments were run in duplicate and the median was used as the final value for each sample. Agalactosyl IgG standard was kindly provided by Sanko Junyaku (Tokyo, Japan).[19]

Statistical Analyses

Differences between measurements and groups were tested with Mann–Whitney *U*-test. A *P* value less than 0.05 was considered statistically significant. A receiver operated characteristic (ROC) curve was generated by plotting sensitivity versus 1 – specificity for every possible cutoff score[20,21] and area under the ROC curve (AUC) was calculated. The optimal cutoff scores were determined by ROC curve, and then sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) were measured.

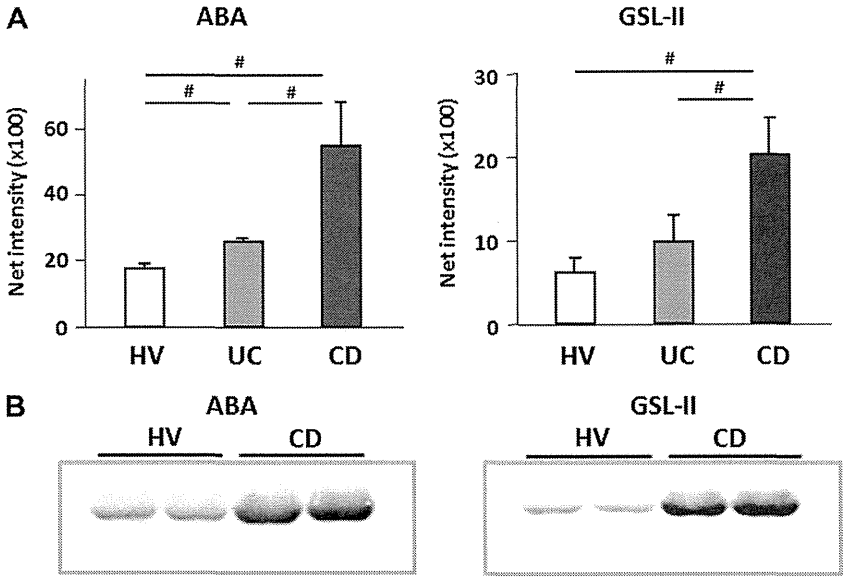

FIGURE 1. Binding levels of ABA and GSL-II to IgG *N*-linked oligosaccharides are higher in patients with IBD than in HVs. (A) Lectin microarray for serum IgGs from HV, patients with UC, and those with CD (five cases each). Among 45 lectins on the lectin microarray, results for ABA and GSL-II are shown. #$P < 0.05$. (B) Lectin blotting for serum IgGs from HV and CD (two cases each). Each blot is representative of three independent experiments with similar results.

All analyses were performed using JMP v.8 software for Windows (SAS, Cary, NC).

RESULTS

Lectin Microarray Analysis for Serum IgG Oligosaccharides

To identify the most relevant lectin(s) characteristic for detecting agalactosyl IgG in IBD, we first screened serum by lectin microarray. Serum IgGs from patients with CD, UC, and HV were purified by protein G column liquid chromatography and a lectin-microarray was performed for each sample (Fig., Supplemental Digital Content 1, http://links.lww.com/IBD/A0). Among 45 lectins, only two lectins had higher affinity for IBD and potentially recognize *N*-linked agalactosyl oligosaccharides. Lectin microarray showed that the signal intensities of the lectins, ABA and GSL-II, were significantly higher in IBD patients, especially those with CD, than in HV (Fig. 1A). Lectin blot analysis, performed to confirm the results obtained from lectin-microarray, clearly showed that serum IgGs from CD patients had higher affinity for both ABA and GSL-II compared to those from HV (Fig. 1B).

Both ABA and GSL-II Recognize "Agalactosyl IgG"

Previous reports showed that both ABA and GSL-II recognize agalactosyl *N*-linked oligosaccharides.[22,23] These studies were performed with frontal affinity chromatography using fluorescence-labeled oligosaccharides that are not attached to proteins. To confirm that both lectins recognize agalactosyl oligosaccharides attached to IgG, namely, "agalactosyl IgG," we first investigated whether IgG itself was recognized by these lectins using IgG treated with a set of glycopeptidases to detach oligosaccharides from IgG. Depletion of oligosaccharides was confirmed by Coomassie brilliant blue (CBB) staining, which showed a slight decrease in the molecular weight of IgG (Fig. 2A). Glycopeptidase-treated IgG showed decreased affinity for ConA, which recognizes most *N*-linked oligosaccharides except those with a bisecting GlcNAc structure,[24] as well as to both ABA and GSL-II (Fig. 2A). These results indicate that ABA and GSL-II accurately recognize oligosaccharides attached to IgG. Next, a step-by-step glycosidase treatment followed by lectin blotting was performed to determine the characteristic oligosaccharide structure. First, IgG was treated by sialydase, which detaches sialic acid from sugar chains. The removal of sialic acid was confirmed by sialic acid binding lectin, SSA. Binding of ABA and GSL-II to the IgG, however, was unchanged (Fig. 2B). Next, sialydase-treated IgG was incubated with the galactose remover beta-galactosidase. Affinities of ABA and GSL-II to IgG were increased after the removal of galactose, whereas the affinity of RCA120, an *N*-linked galactose binder, was decreased (Fig. 2C). Finally, galactosidase-treated IgG was incubated with glucosaminidase, which removes GlcNAc from mannose. Both ABA and GSL-II lectin blotting showed decreased binding affinities for IgG oligosaccharides (Fig. 2D). These findings indicate that both ABA and GSL-II recognize *N*-linked GlcNAc attached to IgG, namely "agalactosyl IgG."

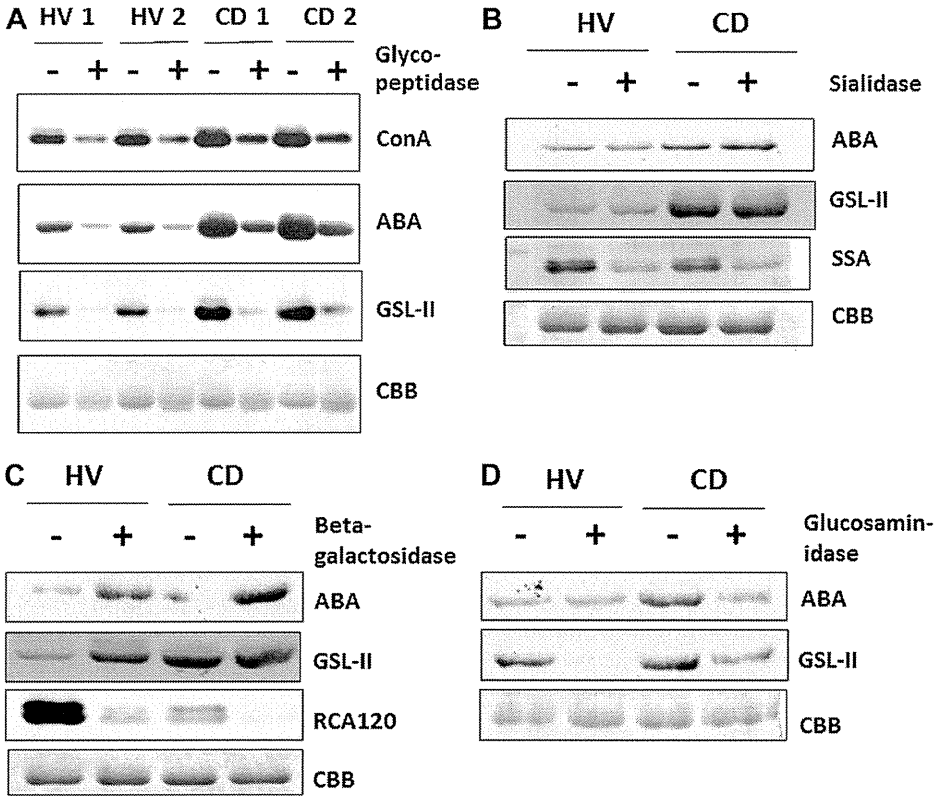

FIGURE 2. ABA and GSL-II recognize the *N*-linked oligosaccharide structure. (A) Purified serum IgGs from HV and CD (two cases each) were treated with glycopeptidase F to remove oligosaccharides from IgG. Binding of ABA, GSL-II, and ConA (binds *N*-linked oligosaccharides) to enzymatically treated or untreated IgGs was analyzed by lectin blotting. (B–D) Serum IgGs from HV and CD (two cases each) were treated with or without (B) sialidase, (C) beta-galactosidase, and (D) glucosaminidase in a step-by-step manner followed by lectin blotting. Each blot is representative of three independent experiments with similar results.

Agalactosyl IgG Is Increased in IBD Patients by Lectin-EIA

To quantify agalactosyl IgG by EIA, a standard curve using agalactosyl IgG was generated. First, oligosaccharide analysis of agalactosyl control IgG was performed by conventional HPLC methods.[7] The outer arm galactose was confirmed to be almost completely absent in control agalactosyl IgG (Fig. 3A). Using this control IgG as an EIA standard, a lectin-EIA system for agalactosyl IgG (Agal-IgG) was developed by immobilizing both ABA and GSL-II on the same plate, by which an accurate standard curve with high R^2 ratio (0.9991) was generated (Fig. 3B). Using the standard curve, Agal-IgG levels were investigated in sera from human subjects. The levels of Agal-IgG in patients with CD were significantly increased than those with UC, HV, and DC, and the levels in patients with UC were also significantly higher than those with HV (Fig. 3C). Agal-IgG levels in patients with SLE or RA were also significantly higher than those with HV. Agal-IgG levels in patients with CD were significantly higher than those with SLE and relatively higher than those with RA. In addition, certain cases of CD showed dramatic increases in Agal-IgG compared to other IBD and autoimmune diseases (Fig. 3C). These results indicate that Agal-IgG is increased in patients with IBD, especially with CD.

Agal-IgG Reflects Disease Activity and Clinical Course in CD

We next investigated whether values obtained by lectin-EIA (Agal-IgG) reflect disease classification, activity, and clinical course in IBD. Agal-IgG levels were significantly higher in CD patients whose onset age was below 16 (category A1) or between 17 and 40 (category A2) than in those above 40 (category A3) years of age at time of diagnosis (Fig. 4A). There were no significant age-related increases in Agal-IgG in either HV and CD patients (Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A1), suggesting that the influence of age-related change in agalactosyl IgG is very small because the participants are relatively young, and that the increase in Agal-IgG in CD patients is not related to aging but to younger

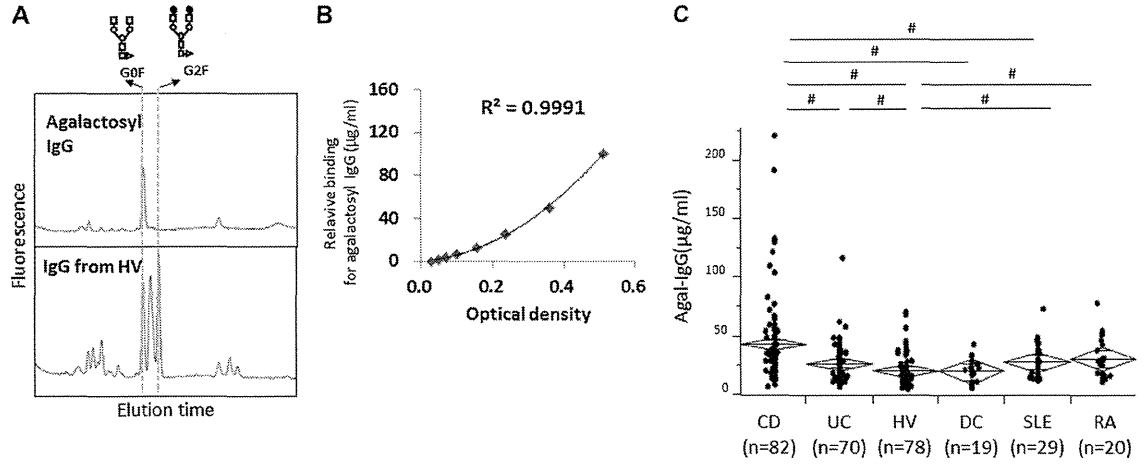

FIGURE 3. Agalactosyl IgG is increased in IBD patients by lectin-EIA. (A) Oligosaccharide structures from control agalactosyl IgG or serum IgG in HV were analyzed by HPLC. The outer arm galactose was almost completely absent in control agalactosyl IgG. This result is representative of three independent experiments. (B) To generate a standard curve, control agalactosyl IgG was diluted step-by-step and assayed for reactivity with ABA and GSL-II (Agal-IgG) by EIA. This result is representative of three independent experiments. (C) Agal-IgG levels of purified serum IgG from CD, UC, HV, DC, SLE, and RA by EIA. #$P < 0.05$.

onset of disease. There were no significant differences in the location (category L) or behavior (category B) of disease based on Agal-IgG levels (Fig. 4B,C). Agal-IgG levels were also not correlated with C-reactive protein (CRP) levels (data not shown). Moreover, Agal-IgG levels were significantly higher in patients with active CD (CDAI ≥150) than in patients in remission (CDAI <150, Fig. 4D). When CD patients treated with infliximab scheduled maintenance therapy were divided into responders and nonresponders at 30 weeks after the initial infusion, the difference between Agal-IgG levels at week 6 and week 0 (ΔAgal-IgG) was significantly higher in responders than in nonresponders (Fig. 4E), although the difference between CRP levels at week 6 and week 0 (ΔCRP) was unchanged in both groups (Fig. 4F). These results indicate that Agal-IgG could be a biomarker not only for detecting disease activity but also for predicting the clinical course of CD.

Combination of Agal-IgG and ASCA Increases Diagnostic Accuracy for CD

We then investigated the diagnostic accuracy of Agal-IgG for IBD by comparing with ASCA. We compared the sensitivity and specificity of Agal-IgG with those of ASCA for the discrimination of IBD by ROC curve and AUC analyses. The AUC was unchanged between Agal-IgG and ASCA for the differentiation of CD and HV (AUC of Agal-IgG vs. ASCA = 0.79 vs. 0.81; Fig. 5A), as well as CD and UC (AUC of Agal-IgG vs. ASCA = 0.69 vs. 0.72; Fig. 5B). The ROC curve, however, showed different patterns between Agal-IgG and ASCA; Agal-IgG had higher specificity than ASCA under high sensitivity conditions. There was no correlation between Agal-IgG and ASCA levels among all subjects (Fig. 5C). We then calculated the sensitivity and specificity of Agal-IgG/ASCA double-positive CD patients. By the ROC curve, the combination of Agal-IgG and ASCA positivity showed higher specificity than Agal-IgG or ASCA alone for the diagnosis of CD (Table 3).

Agal-IgG Levels Are Also Increased in American IBD Patients

To determine whether Agal-IgG levels are increased in a different geographic population, we investigated Agal-IgG levels in U.S. IBD patients and healthy volunteers. Agal-IgG levels were also significantly higher in IBD patients, especially in CD patients, than in healthy volunteers (Fig. 6A). In CD patients, Agal-IgG levels were significantly higher in patients with earlier onset category A1 or A2 than in those with category A3 (Fig. 6B). No significant differences were observed in category L or category B (Fig. 6C,D). These results were in excellent concordance with analyses for Japanese IBD patients (Figs. 3C, 4A–C), indicating Agal-IgG could be a biomarker for IBD patients irrespective of geography and consequently genetic background.

DISCUSSION

We previously reported that fucosylated agalactosyl IgG is increased in the sera of patients with IBD, and the extent of agalactosylation of fucosylated IgG is a potential diagnostic marker for IBD.[7] In the present study, we showed that both ABA and GSL-II preferentially recognize agalactosyl IgG, and affinities of both lectins to IgG were significantly increased in CD patients compared to HV. We also generated a lectin-EIA system in which an increase in agalactosyl IgG could be detected

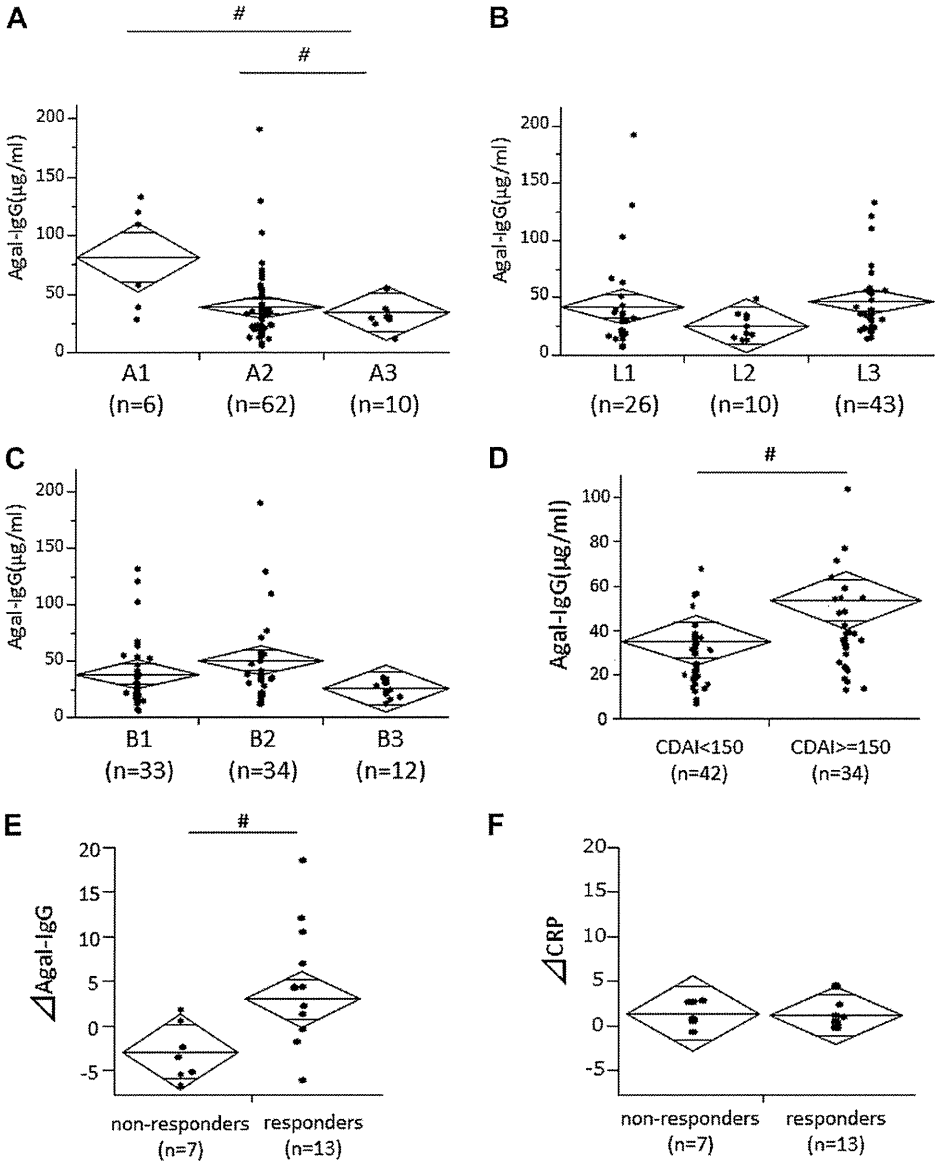

FIGURE 4. Agalactosyl IgG reflects disease activity and clinical course in CD. (A) Agal-IgG levels and age of disease onset (category A) in CD patients. Agal-IgG levels were significantly higher in patients with category A1 than A2 or A3. (B,C) Agal-IgG levels and disease location (category L, (B)) and disease behavior (category B, (C)) in CD patients. (D) Agal-IgG levels and disease activity in CD patients. Agal-IgG was higher in active patients (CDAI ≥150, $n = 42$) than in patients in remission (CDAI <150, $n = 34$). (E,F) Agal-IgG or CRP levels and clinical response to infliximab. Infliximab-naïve CD patients were treated with infliximab scheduled maintenance therapy. (E) The difference between Agal-IgG levels at week 6 and week 0 (ΔAgal-IgG) was higher in responders than in nonresponders. (F) The difference between CRP levels at week 6 and week 0 (ΔCRP) was unchanged in both groups. #$P < 0.05$

and determined that agalactosyl IgG is a useful diagnostic marker that reflects disease activity and clinical course of CD.

GSL-II binds to agalactosyl *N*-linked oligosaccharides with primary recognition of a GlcNAc residue and major specificity for tri- or tetra-antennary structures.[22] ABA has been widely used to detect and capture *O*-linked oligosaccharides containing Galβ1-3GalNAc.[25,26] However, ABA also has substantial affinity for mono-, bi-, and tri-antennary agalactosyl *N*-linked oligosaccharides.[23] Although these previous reports demonstrated affinity between lectin and agalactosyl *N*-linked oligosaccharides, the investigators used fluorescence-binding oligosaccharides as a substrate and did not confirm the affinity

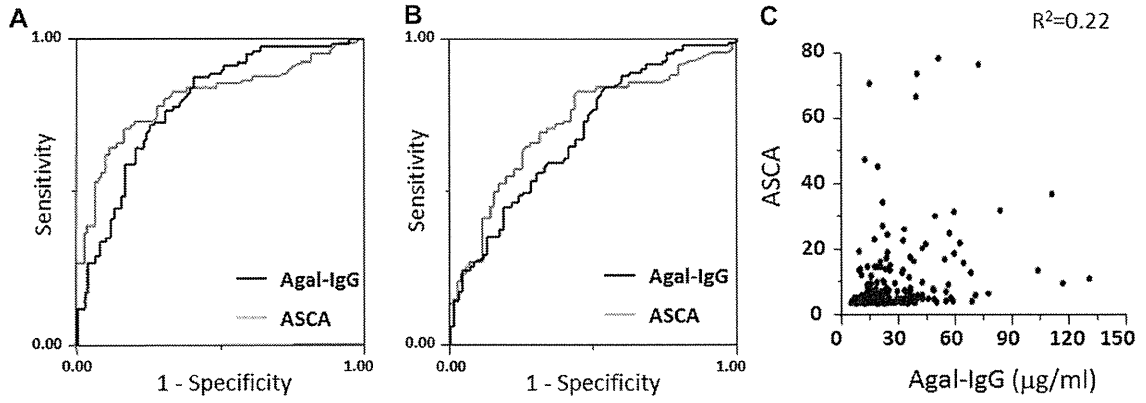

FIGURE 5. The combination of Agal-IgG and ASCA more efficiently differentiates CD. (A) The ROC curves for Agal-IgG and ASCA levels for discrimination between CD and HV, or (B) between CD and UC. (C) Correlation between Agal-IgG and ASCA levels in all subjects (n = 230).

for glycoproteins, i.e., IgG. In the present study we therefore demonstrate for the first time that both ABA and GSL-II recognize native biantennary GlcNAc on *N*-linked IgG oligosaccharides without destroying the crystal structure of the IgG glycoprotein. Lectin microarray is a powerful tool for screening such types of lectins.

EIA is widely used as a simple system to quantify specific proteins; however, several problems make it difficult to apply the "protein-specific" EIA to an "oligosaccharide-specific" system. First, the lectin-oligosaccharide interaction is approximately one-tenth the affinity of antigen–antibody binding,[10] so lectin-oligosaccharide complexes easily dissociate during the EIA procedure. Second, the recognition of an oligosaccharide by a lectin is not always specific for a single structure. Third, oligosaccharides are sometimes sterically encumbered by the surrounding protein so that lectins do not bind to glycoproteins compared to oligosaccharide structures without proteins. To overcome these problems, we adopted a simultaneous detection system by two lectins. We showed that both lectins recognize agalactosyl IgG oligosaccharides, but the binding affinity of each lectin to oligosaccharide might be subtly influenced by the surrounding protein structure.[27] The Agal-IgG EIA may have achieved a high sensitivity because each lectin contributes to bind to agalactosyl IgG. Another reason the dual lectin Agal-IgG EIA may be a sensitive and specific method is that nonspecific binding for each lectin may decrease due to the requirement for reduced concentrations: A higher concentration of a single lectin is necessary to generate a standard curve for agalactosyl IgG.

We previously reported on the significance of agalactosyl fraction of the fucosylated IgG oligosaccharides (G0F/G2F) in patients with IBD. It is interesting that fucosylated G0F/G2F is more specific than nonfucosylated agalatosyl IgG in IBD.[7] Agal-IgG should be consistent with G0F/G2F by HPLC, because Agal-IgG theoretically indicates whole agalactosyl IgG, and because G0F/G2F is a major fraction of IgG oligosaccharides. In contrast, fucosylation of IgG alters biological activity of IgG including antibody-dependent

TABLE 3. Accuracy of Agal-IgG and ASCA to Differentiate (A) CD from HV, or (B) CD from UC

	Sensitivity	Specificity	PPV	NPV
(A)				
ASCA (+)	71% (58/82)	83% (65/78)	82% (58/71)	73% (65/89)
Agal-IgG (+)	88% (72/82)	59% (46/78)	69% (72/104)	82% (46/56)
ASCA (+) /Agal-IgG (+)	67% (55/82)	94% (72/78)	90% (55/61)	73% (72/99)
(B)				
ASCA (+)	70% (57/82)	69% (48/70)	72% (57/79)	66% (48/73)
Agal-IgG (+)	83% (68/82)	47% (33/70)	65% (68/105)	58% (33/57)
ASCA (+) / Agal-IgG (+)	62% (51/82)	84% (59/70)	82% (51/62)	66% (59/90)

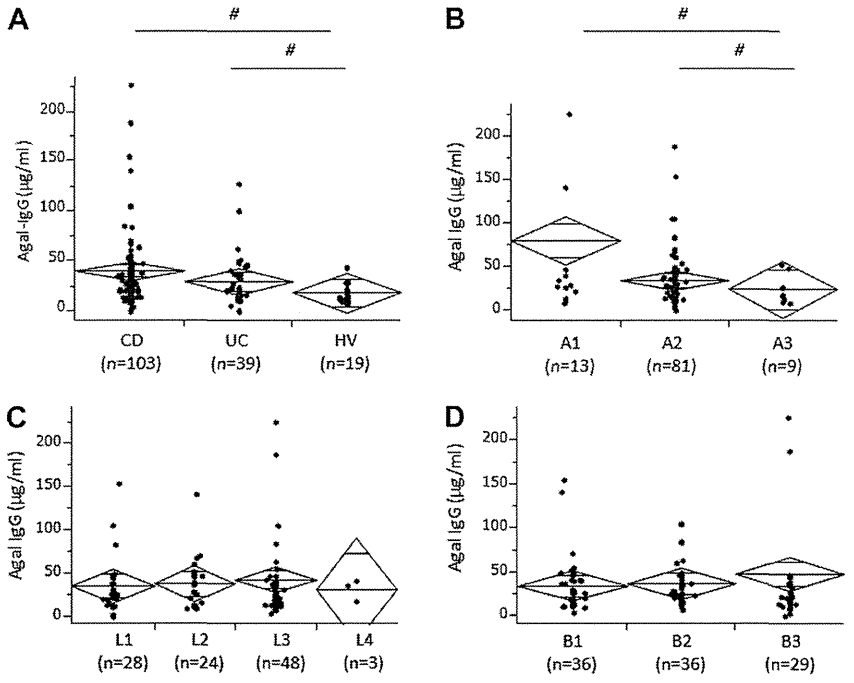

FIGURE 6. Agal-IgG levels are also increased in American IBD patients. (A) Agal-IgG levels of each purified serum IgG from CD, UC, and HV by EIA. (B) Agal-IgG levels and onset age (category A) in CD patients. Agal-IgG levels were significantly higher in patients with category A1 than with A2 or A3. (C,D) Agal-IgG levels and disease location (category L, (B)) and behaviors (category B, (C)) in CD patients. #$P<0.05$.

cellular cytotoxicity, which might be due to conformation changes in the IgG Fc portion.[28] In our system ABA and GSL-II can recognize both the oligosaccharide structure and the 3D structure of the IgG Fc portion, whereas the HPLC system purely analyzes the oligosaccharide structure of IgG. The sensitivity and specificity of Agal-IgG for diagnosis of CD might, therefore, be slightly different from our previous study. However, Agal-IgG could be a marker for disease activity of CD and the combination of Agal-IgG and ASCA is a better marker for diagnosing IBD than ASCA alone, as discussed below. Furthermore, certain cases of CD showed dramatic increases in Agal-IgG measured by lectin-EIA, compared to other IBD and autoimmune diseases. Although we started the present study to establish more convenient methods to measure agalactosyl IgG levels than the HPLC system, Agal-IgG by lectin-EIA can possibly help elucidate the novel pathogenesis of CD. Further studies from both clinical and basic approaches will be required for CD patients showing extremely high Agal-IgG levels.

Here we demonstrated that Agal-IgG is a useful diagnostic marker in combination with ASCA. ASCA is one of the most well-established serologic markers for diagnosing CD,[4] but ASCA alone does not possess enough power to diagnose CD with sufficient sensitivity or specificity. Therefore, ASCA has been combined with other markers, such as peripheral antineutrophil cytoplasmic antibody[5] or anti-outer membrane porin protein C, and anti-CBir1 flagellin.[6] In the present study, Agal-IgG itself showed almost the same sensitivity and specificity as ASCA for the discrimination of CD and HV or CD and UC; however, Agal-IgG was not strongly correlated with ASCA. The combination of Agal-IgG and ASCA could augment the specificity as a diagnostic marker partly because Agal-IgG might identify different subgroups of patients within CD than ASCA. Further investigation is necessary to assess whether the combination of Agal-IgG and ASCA is a better marker for diagnosing IBD than Agal-IgG or ASCA alone. Moreover, we showed that Agal-IgG may have higher predictability for response to infliximab compared with CRP (Fig. 4E,F). Agal-IgG can reflect different inflammatory conditions from CRP, because Agal-IgG is not increased in patients with acute intestinal inflammation (Fig. 3C), and because IgG has a long serum half-life of 3 weeks, whereas that of CRP is 4–6 hours. The decrease of Agal-IgG in 6 weeks of infliximab treatment may be, therefore, a novel marker for sustained response. If confirmed in larger prospective analyses, this finding could have important clinical implications.

In a validation study, we showed that Agal-IgG levels were also significantly increased in a non-Asian, U.S. cohort of IBD patients, especially in early onset (category A1/A2) CD patients. A previous report showed that disease location and

clinical course are severer in patients whose onset is younger, and that the A3 group had a lower incidence of fistulas and fewer requirements for immunomodulators and corticosteroids.[29] Higher levels of Agal-IgG in category A1/A2 patients may therefore reflect disease severity in younger-onset patients. Although the mechanism has not been clarified, Agal-IgG can be useful especially for screening pediatric patients for whom invasive studies are hard to perform. Although clinical manifestations appear to be similar in all geographies, ethnic differences in genetic associations have been reported. For instance, mutations in the leucine-rich repeats (LRRs) of nucleotide-binding oligomerization domain containing 2 (NOD2) are associated with an increased risk for CD in many Caucasian populations,[30–32] but not in Japanese patients.[33] Therefore, it is of interest that increases in Agal-IgG levels are observed in Japanese and American IBD patients because of the clinical implications as a diagnostic marker, but also as a reason to explore IgG glycosylation as a global defect in the pathogenesis of IBD.

An increase in serum agalactosyl IgG is also reported in other diseases such as RA,[34] SLE,[35] and tuberculosis.[36] In the present study we showed that Agal-IgG levels were significantly increased in patients with RA and SLE. Agal-IgG might be an effective serological marker for other immunological disorders such as SLE and RA. In addition, Agal-IgG levels in patients with CD are significantly higher than those with SLE and relatively higher than those with RA. Agalactosyl IgG seems to have different functions in each disease, because our recent studies show that the levels of anti-agalactosyl IgG antibodies are increased in the sera of RA[37] but not IBD patients.[7] Moreover, the lectin-complement pathway is activated through agalactosyl IgG in RA[38] but not IBD.[8] Therefore, the availability of an Agal-IgG lectin-EIA will help in translational studies to elucidate mechanisms through which agalactosyl IgG contributes to pathogenesis in different diseases.

In conclusion, the Agal-IgG lectin-EIA system for agalactosyl IgG may represent a novel biomarker assay for IBD. The presence of Agal-IgG in numerous autoinflammatory diseases has potential significance as a diagnostic marker that may fill important clinical needs, and may provide further information about pathogenesis.

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ORIGINAL ARTICLE

Clinical characteristics and risk factors for *Pneumocystis jirovecii* pneumonia in patients with rheumatoid arthritis receiving adalimumab: a retrospective review and case–control study of 17 patients

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Abstract
Objectives To investigate the clinical characteristics and risk factors of *Pneumocystis jirovecii* pneumonia (PCP) in rheumatoid arthritis (RA) patients treated with adalimumab.

Methods We conducted a multicenter, retrospective, case–control study to compare RA patients treated with adalimumab with and without PCP. Data from 17 RA patients who were diagnosed with PCP and from 89 RA

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patients who did not develop PCP during adalimumab treatment were collected.

Results For the PCP patients, the median age was 68 years old, with a median RA disease duration of eight years. The median length of time from the first adalimumab injection to the development of PCP was 12 weeks. At the onset of PCP, the median dosages of prednisolone and methotrexate were 5.0 mg/day and 8.0 mg/week, respectively. The patients with PCP were significantly older ($p < 0.05$) and had more structural changes ($p < 0.05$) than the patients without PCP. Computed tomography of the chest revealed ground-glass opacity without interlobular septal boundaries in the majority of the patients with PCP. Three PCP patients died.

Conclusions PCP may occur early in the course of adalimumab therapy in patients with RA. Careful monitoring, early diagnosis, and proper management are mandatory to secure a good prognosis for these patients.

Keywords Adalimumab · *Pneumocystis jirovecii* pneumonia · Rheumatoid arthritis · TNF antagonist

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by persistent synovitis and structural damage to multiple joints. Tumor necrosis factor (TNF) is abundantly produced in the inflamed synovium and contributes to the imuunopathogenesis of the disease. Adalimumab is the first fully human monoclonal antibody against TNF; treatment with this biologic agent has been well established in patients with RA in multiple clinical trials [1–3]. On the other hand, treatment with adalimumab, as well as infliximab and etanercept, has been associated with increased risk for opportunistic and serious infections in cohort studies using RA patient registries [4–7]. In Japan, strict post-marketing surveillance (PMS) programs have been conducted for patients with RA given TNF antagonists. The numbers of RA patients with *Pneumocystis jirovecii* (*P. jirovecii*) pneumonia (PCP) who were treated with infliximab, etanercept, or adalimumab were 22 (0.4 %) out of 5,000 patients, 25 (0.18 %) out of 13,894 patients, and 25 (0.33 %) out of 7,469 patients, respectively, in these PMS programs [6–8]. Note that these incidence rates of PCP in Japan are apparently higher than the corresponding figure (0.01 %) reported from the United States [9].

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We have previously described the clinical characteristics and risk factors for PCP in RA patients treated with infliximab [10, 11] and etanercept [12]. These risk factors included older age and presence of coexisting lung diseases for both TNF antagonists, a higher daily dose of prednisolone (PSL) for infliximab, and a higher weekly dose of methotrexate (MTX) for etanercept. Considering the similar incidence of PCP in the PMS programs among the three TNF antagonists, it is clinically important and intriguing to characterize PCP in RA patients given adalimumab and to compare the results with those obtained for RA patients treated with other TNF antagonists.

In this paper, we report detailed clinical, laboratory, and radiographic features of PCP that developed in RA patients during treatment with adalimumab. Furthermore, we compared 17 RA patients receiving adalimumab who developed PCP with 89 RA patients who did not develop PCP during treatment, and identified risk factors for PCP in patients with RA treated with adalimumab.

Materials and methods

Patients

Patients included in the present study fulfilled the 1987 American College of Rheumatology (formerly the American Rheumatism Association) criteria for RA [13] and received adalimumab (40 mg every two weeks) with or without concomitant MTX. Between April 2008 and April 2010, 17 patients with PCP (PCP group) were collected from 16 hospitals through either the PMS program for adalimumab ($n = 16$) or through a voluntary case report by attending physicians at a scientific meeting ($n = 1$). We convened a face-to-face meeting in March 2011 to discuss diagnosis and treatment for the collected cases among the investigators of this study. RA patients who did not develop PCP during adalimumab therapy for at least one year from the first dose of adalimumab (non-PCP group, $n = 89$) were randomly collected from the participating hospitals of this study. Other eligibility criteria for the non-PCP group were registration in the PMS program of adalimumab and the use of adalimumab five times or more. The median (range) observation period for the non-PCP group treated with adalimumab was 365 (63–365) days. To increase the statistical power of this case–control study, the number of patients in the non-PCP group was designed to be about five times as many as that in the PCP group [14].

Diagnostic criteria for PCP

Previously established diagnostic criteria for PCP [15, 16] were used in the present study [10]. A diagnosis of PCP was considered definitive if a patient fulfilled the following four conditions: clinical manifestations (fever, dry cough, or dyspnea), hypoxemia, interstitial infiltrates on chest radiographs, and microscopic detection of *P. jirovecii* in induced sputum or bronchoalveolar lavage fluid. The diagnosis of PCP was considered presumptive if a patient fulfilled all of these conditions except for the microscopic detection of *P. jirovecii* in the absence of other infectious diseases and the presence of either a positive polymerase chain reaction (PCR) test for *P. jirovecii* DNA or increased serum 1,3-β-D-glucan (BDG) levels (Fungitec G test MK; Seikagaku, Tokyo, Japan or Wako β-D-glucan test; Wako Pure Chemical Industries, Tokyo, Japan) [17, 18] along with a response to standard treatments for PCP. Both the PCR test for *P. jirovecii* DNA and that for serum BDG are commercially available, validated, and officially approved as clinical laboratory tests by the Ministry of Health, Labour, and Welfare in Japan.

Collection and analysis of clinical data

Clinical information was collected using a standardized format to evaluate demographic information, Steinbrocker's radiographic stage and functional class [19], comorbidities, concomitant drugs, laboratory data, radiographic data, treatment, and outcome. Chest radiographs and computed tomography (CT) scans were evaluated by a pulmonologist (H.S.) and a diagnostic radiologist (F.S.). CT findings were categorized into three patterns, as we did in previous studies [12, 20]: (a) diffuse ground-glass opacity (GGO) distributed in a panlobular manner; that is, GGO was sharply demarcated from the adjacent normal lung by interlobular septa (type A GGO); (b) diffuse GGO that is homogeneous or somewhat inhomogeneous in distribution but without the sharp demarcation caused by interlobular septa (type B GGO); (c) other patterns, such as mixed consolidation and GGO (type C).

Statistical analyses

Demographic data and baseline data were compared between the PCP and non-PCP groups using the χ^2 test for categorical variables and the Mann–Whitney test for continuous variables. To identify risk factors for PCP, the Cox proportional-hazards regression model was used. All analyses were performed using SPSS software, version 16.0 (SPSS Japan, Tokyo, Japan).

Ethics

The guidelines of the Declaration of Helsinki (revised in 2008) and the ethics guidelines for epidemiologic research in Japan were followed. The study protocol was approved

by the Institutional Ethics Committee of the Tokyo Medical and Dental University Hospital (#863 in 2010).

Results

Diagnosis and clinical characteristics of RA patients with PCP

We applied the above diagnostic criteria to the 17 RA patients in the PCP group. Of the 17 cases, three (patients 8, 14, and 17) met the criteria for definitive PCP, and 14 met the criteria for presumptive PCP. The clinical characteristics of each patient are summarized in Table 1. The median age of the 17 patients was 68 years (range 48–78 years), and 12 (71 %) were female. The median duration of RA was eight years. Fourteen patients were at Steinbrocker's stage III or IV. All patients received MTX and 13 (77 %) received corticosteroids from baseline to the onset of PCP. At the onset of PCP, the median dosages of prednisolone and MTX were 5.0 mg/day (range 2.5–9 mg/day) and 8.0 mg/week (range 4–15 mg/week), respectively. One patient was receiving another immunosuppressive drug, tacrolimus, at 3 mg/day. Eight patients had pulmonary comorbidities, including interstitial pneumonia ($n = 4$), chronic obstructive pulmonary disease ($n = 4$), and old pulmonary tuberculosis ($n = 2$). Four patients had diabetes mellitus. None of the patients received chemoprophylaxis for PCP at the time of PCP diagnosis. The median interval between the first injection of adalimumab and the onset of PCP was 12 weeks (range 4–38 weeks). Thirteen patients (76 %) developed PCP within 26 weeks after the first injection. Fever was the most common clinical symptom (it was observed in 15 patients; 88 %), followed by dyspnea on effort (82 %) and dry cough (41 %).

Laboratory and radiographic features of the PCP patients

Laboratory data at the onset of PCP are summarized in Table 2. Fourteen patients either had severe hypoxia (with $PaO_2 < 60$ mm Hg on room air) or required immediate oxygen therapy at the onset of PCP. Peripheral blood lymphocyte (PBL) counts at the onset of PCP were <500 cells/μl in three patients, 500–1,000 cells/μl in five patients, and $>1{,}000$ cells/μl in nine patients. *P. jirovecii* was microscopically identified in three patients. The polymerase chain reaction test for *P. jirovecii* DNA was positive in 13 patients, using either induced sputum (11 patients) or bronchoalveolar lavage fluid (four patients), but three patients were not examined. Serum levels of BDG, one of

Table 1 Characteristics of rheumatoid arthritis patients treated with adalimumab at the onset of PCP

Pt	Age/sex	Stage/class	Number of injections[a]	Treatment duration (days)[b]	MTX (mg/w)	PSL (mg/d)	Lung disease	DM	Clinical manifestations
1	48/F	III/I	7	105	8	2.5	–	–	Fever/DOE
2	69/M	IV/III	4	62	10	0	E	–	Cough/DOE
3	74/F	IV/II	9	131	8	5	IP E	–	DOE
4	52/M	III/II	5	59	4	8	IP	–	Fever/cough/DOE
5	61/F	IV/II	3	45	8	9	–	–	Fever
6	67/F	III/III	3	28	8	8	IP	–	Fever/cough/DOE
7	61/F	IV/II	4	59	6	0	Old TB	–	Fever/DOE
8	77/F	IV/II	6	129	6	5	–	+	Fever/DOE
9	52/F	III/I	3	55	8	5	–	–	Fever/DOE
10	78/M	III/III	6	86	8	0	IP	+	Fever/DOE
11	66/F	I/III	6	106	8	3	–	–	Fever/cough
12	70/F	II/II	2	23	8	5	Old TB	–	Fever/cough/DOE
13	68/M	I/II	3	28	8	0	E	+	Fever/DOE
14	71/F	III/II	15	214	8	7.5	–	–	Fever/DOE
15	73/M	III/II	18	268	15	3	–	+	Fever/cough/DOE
16	65/F	III/II	16	227	8	2	–	–	Fever/DOE
17	78/F	IV/II	16	252	4	4	–	–	Fever/cough

PCP Pneumocystis jirovecii pneumonia, *Pt* patient, *w* week, *d* day, *M* male, *F* female, *MTX* methotrexate, *PSL* prednisolone, *E* emphysema, *IP* interstitial pneumonia, *old TB* old tuberculosis, *DM* diabetes mellitus, *DOE* dyspnea on effort, *cough* dry cough

[a] Number of injections of ADA prior to the diagnosis of PCP

[b] Treatment duration with ADA before the onset of PCP

Table 2 Laboratory data of rheumatoid arthritis patients treated with adalimumab at the onset of PCP

Pt	WBC (/μl)	Lymphocytes (/μl)	SpO_2 or PaO_2 (Torr) [O_2, l/min][a]	Serum β-D-glucan (μg/ml) [normal range at the institute]	*Pneumocystis jirovecii* PCR
1	7,870	912	SpO_2 96 % [0]	289 [<11]	+
2	5,100	1,989	SpO_2 92 % [0]	30.5 [<11]	+
3	6,300	252	55.1 [0]	1041 [<11]	NA
4	6,200	874	68.0 [0]	25.76 [<11]	+
5	8,050	1,110	60.4 [0]	50.3 [<20]	NA
6	6,400	716	58.9 [0]	37.8 [<6]	+
7	5,660	1,041	71.8 [0]	22.1 [<11]	+
8	6,800	279	31.3 [0]	29 [<11]	+[b]
9	15,900	832	85.7 [3]	79.5 [<20]	+
10	7,500	1,350	65.4 [0]	22.3 [<20]	+
11	8,400	3,696	69.5 [0]	16.4 [<11]	+
12	11,700	1,029	26.1 [0]	21.06 [3.5]	+
13	7,950	1,761	SpO_2 85 % [2]	160 [<5]	+
14	9,580	34	56.7 [0]	13.0 [<11]	NA[b]
15	5,700	1,140	55.1 [0]	13.0 [<11]	–
16	7,000	1,330	56.1 [10]	21.38 [<11]	+
17	3,200	704	52.5 [0]	419 [<11]	+[b]
Median (IQR)	7,000 (5950–8225)	1,029 (710–1340)	Not applicable	Not applicable	Not applicable

PCP Pneumocystis jirovecii pneumonia, *Pt* patient, *WBC* white blood cell, *PCR* polymerase chain reaction, *NA* not assessed, *SpO2* oxygen saturation measured using a pulse oximeter, *IQR* interquartile range

[a] Oxygen therapy during the measurement of PaO_2

[b] *Pneumocystis jirovecii* microscopically detected in bronchoalveolar-lavage fluid

the major components of the cell walls of fungi and a serum maker for PCP [17, 18], were elevated in all patients. Results of sputum culture performed in 14 patients revealed no causative bacteria or fungi.

Chest radiographs and thoracic CT scans were analyzed for all 17 patients. The most common CT finding was ground-glass opacity (GGO) (in 17 patients), either with sharp demarcation by interlobular septa in one patient (type A GGO) (Fig. 1a) or without interlobular septal boundaries in 14 patients (type B GGO) (Fig. 1b). Two patients demonstrated mixed patterns (type C).

Treatment and clinical course of PCP in patients with RA receiving adalimumab

All patients were hospitalized on the same day that PCP was suspected. Fourteen patients (all except for patients 2, 5, and 11) received oxygen therapy on admission. MTX and adalimumab were immediately discontinued in all patients. All patients received therapeutic doses of trimethoprim/sulfamethoxazole (TMP/SMX). Because of adverse drug reactions that included skin eruptions, liver dysfunction, thrombocytopenia, and hyperpotassemia, TMP/SMX was reduced or stopped in eight patients. One patient was changed to pentamidine isethionate. Sixteen patients were concomitantly treated with high-dose corticosteroids within a few days after admission. Eleven patients were empirically treated with antibiotics and four with antifungal agents. Three patients (patients 1, 3, and 8) were intubated on the day of admission because of progressive respiratory failure; two of these patients responded to treatment and were successfully weaned from artificial ventilation. One patient (patient 17) died because of PCP with progressive respiratory failure. Two patients died because of multiple organ failure (patient 12) and gastrointestinal bleeding, cytomegalovirus infection, and multiple organ failure (patient 3) after improvement of PCP.

Case–control study

In order to characterize the PCP group more precisely, we compared demographic information, comorbidities, treatments, and laboratory data at baseline (i.e., at the initiation of treatment with adalimumab) between the PCP and non-PCP groups using a univariate analysis (Table 3). The PCP group was significantly older ($p = 0.003$) and had a more advanced radiographic stage (Steinbrocker's stage III or IV) ($p = 0.010$) than the non-PCP group. Although the rates of patients with preexisting pulmonary diseases and diabetes mellitus in the PCP group were numerically higher, these differences were not statistically significant. There were no differences in disease duration and the dosages of prednisolone and methotrexate between the two groups. None of the patients in the PCP group and fourteen patients in the non-PCP group received prophylaxis for

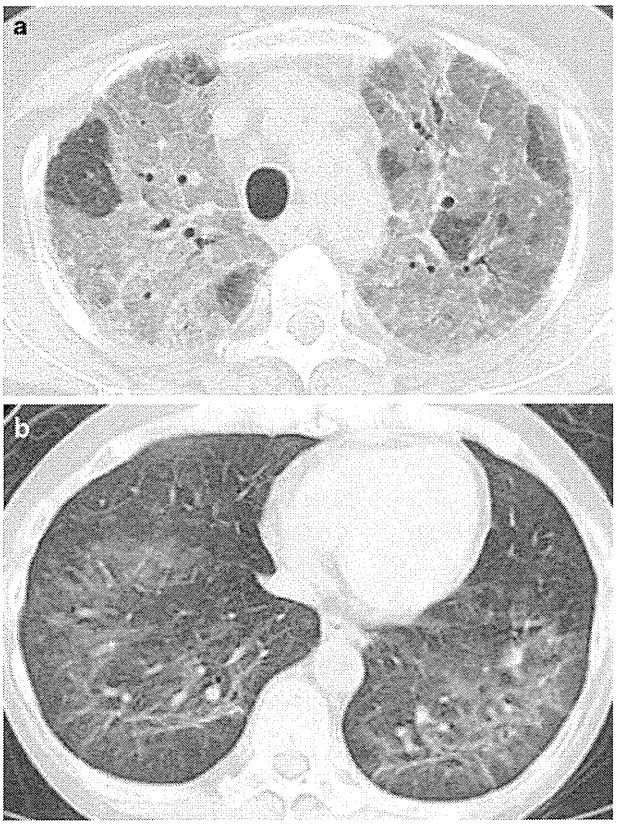

Fig. 1 Representative thoracic computed tomography findings of rheumatoid arthritis patients who developed *Pneumocystis jirovecii* pneumonia while receiving adalimumab. **a** Ground-glass opacity (GGO) with sharp demarcation by interlobular septa (type A) (patient 12). **b** Inhomogeneous GGO without obvious demarcation by interlobular septa (type B) (patient 1)

PCP for at least three months during the observation period. Twelve patients used TMP/SMX and two used aerosolized pentamidine.

Based on the results of the univariate analysis, age, sex, pulmonary comorbidities and Steinbrocker's stage of RA were analyzed as candidate predictors for the development of PCP. The Cox proportional-hazards regression analysis revealed a significant association between advanced radiographic stage (stage III or IV) and development of PCP (hazard ratio (HR) 3.76, 95 % confidence interval (CI) 1.03–7.30, $p = 0.045$). While the hazard ratios of older age and preexisting pulmonary diseases tended to be higher, they did not reach statistical significance (Table 4).

Because 14 patients in the non-PCP group received prophylaxis for PCP, we performed the multivariate analysis after excluding these 14 patients, and found a significant association between older age and development of PCP (HR 3.31, 95 % CI 1.09–10.0, $p = 0.034$). The HR of the radiographic stage did not reach statistical significance (HR 2.82, 95 % CI 0.74–10.7) in this model.

Discussion

We accumulated the largest possible number of patients with RA who developed PCP during treatment with adalimumab, and described the clinical and radiologic characteristics of the 17 patients that we found.

Adalimumab is the third TNF antagonist to be approved in Japan. We have already reported the clinical characteristics and risk factors for PCP in RA patients treated with infliximab or etanercept [10–12]. The median interval (range) between the first dose of TNF antagonists and the onset of PCP was 12 weeks (range 4–38) for adalimumab, nine weeks (range 2–90) for infliximab [11], and 14 weeks (range 3–43) for etanercept [12]. PCP developed within six months in the majority of RA patients after the initiation of each TNF antagonist: 90 % for infliximab, 80 % for etanercept, and 76 % for adalimumab.

Previous studies have revealed that patients without HIV infection develop PCP abruptly and progress to fulminating pneumonia with acute respiratory failure [21, 22]. We also reported that RA patients treated with infliximab or etanercept developed PCP rapidly and progressed to severe respiratory failure [10–12]: 18 out of 21 PCP patients using infliximab, all 15 PCP patients using etanercept, and 14 of 17 PCP patients in this study showed severe hypoxemia and required oxygen therapy. The mortalities of the patients with PCP given infliximab (0 %) or etanercept (6.7 %) are numerically lower than the mortality of this study, in which three patients (17.6 %) died. Walzer etal. [23] identified older age, second or third episode of PCP, low hemoglobin level, low PaO_2 breathing room air at admission, pulmonary Kaposi sarcoma, and presence of medical comorbidity as early predictors of mortality of PCP in HIV-infected patients. Although such prognostic factors in non-HIV PCP patients are unknown, all three patients in our study who died were females over 70 years old, and their PaO_2 on admission was less than 60 Torr. Two of these patients had pulmonary comorbidities. One patient had a quite high serum level of BDG, and one was positive for both microscopic detection and the PCR test for the organism. These data would suggest severe pulmonary injury at presentation and a high burden from *P. jirovecii*.

In our study, all patients received therapeutic doses of TMP/SMX. However, eight patients (47.1 %) were obliged to reduce the dosage or stop using the drug due to adverse drug reactions, such as gastrointestinal symptoms and hematological abnormalities. Kameda et al. [24] also reported that more than one-third of the patients could not complete the standard protocol of the TMP/SMX treatment. These data indicate that the optimal dosage and treatment period of TMP/SMX for PCP should be investigated. The clinical benefit of adjunctive corticosteroid

Table 3 Baseline characteristics of patients with rheumatoid arthritis treated with adalimumab

Characteristic	PCP group ($n = 17$)	Non-PCP group ($n = 89$)	p value
Age (years)[a]	68 (48–78)	60 (24–79)	0.003
Female (%)	70.6	80.9	0.255
Disease duration (years)[a]	8.0 (0.7–36)	9.5 (3–40)	0.491
Chronic pulmonary disease (%)	47.1	22.5	0.107
Diabetes mellitus (%)	23.5	7.9	0.074
Steinbrocker's radiographic stage (III or IV) (%)	82.4	48.3	0.010
Steinbrocker's functional class (III or IV) (%)	17.6	19.1	0.596
MTX (%)	100	86.5	0.108
MTX (mg/week)[a]	8.0 (4–10)	8.0 (4–15)	0.119
MTX ≥ 8 mg/week (%)	11.8	28.1	0.228
PSL (%)	76.5	56.2	0.118
PSL (mg/day)[a]	5.0 (3–12)	5.0 (1–17)	0.529
PSL ≥ 5 mg/day (%)	52.9	33.7	0.131
WBC <4,000/μl (%)	0	2.2	0.731
Serum IgG (mg/dl)[a]	1421 (846–1954)	1316 (827–3165)	0.817

PCP Pneumocystis jirovecii pneumonia, *MTX* methotrexate, *PSL* prednisolone, *Chronic pulmonary disease* = interstitial pneumonia, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma, middle lobe syndrome, old pulmonary tuberculosis

p values were calculated using the Mann–Whitney test for continuous variables or χ^2 test for categorical variables

[a] Median (range)

Table 4 Cox regression analysis of risk factors for the development of PCP in rheumatoid arthritis patients treated with adalimumab

	Hazard ratio (95 % CI)	p value
Age (≥ vs. <65 years old)	2.38 (0.80–7.05)	0.119
Gender (female vs. male)	0.53 (0.18–1.58)	0.258
Chronic pulmonary disease (yes vs. no)	2.14 (0.79–5.76)	0.133
Steinbrocker's radiographic stage (III/IV vs. I/II)	3.76 (1.03–7.30)	0.045

PCP Pneumocystis jirovecii pneumonia, *CI* confidence interval

Chronic pulmonary disease = interstitial pneumonia, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma, middle lobe syndrome, old pulmonary tuberculosis

therapy for PCP patients without HIV infection has not been established [25]. All patients except for one in this study received adjunctive corticosteroid therapy with various treatment durations and dosages, including intravenous methylprednisolone pulse therapy. Nineteen out of 21 PCP patients who used infliximab and nine out of 15 PCP patients who used etanercept used adjunctive corticosteroid therapy as well [11, 12]. Pareja etal. [26] retrospectively analyzed the clinical courses of 30 cases of severe PCP without HIV infection, among which 16 cases who received high doses of adjunctive corticosteroid therapy presented a good clinical outcome. Considering the intense inflammatory response to the organism in non-HIV PCP patients [25] and the favorable effectiveness of adjunctive corticosteroid therapy in previous studies, it is necessary to consider treatment with corticosteroids for PCP patients with RA who show hypoxemia at presentation or during their clinical courses.

In the present study, using the Cox proportional-hazards analysis, Steinbrocker's radiographic stage III or IV was identified as a statistically significant risk factor for the development of PCP in patients receiving adalimumab. Although there was no significant difference in Steinbrocker's functional class, it is plausible that advanced radiographic stages associated with decreased physical function contributed to the development of PCP. Steinbrocker's functional class may be less sensitive to the detection of such differences in physical function. On the other hand, older age was a significant risk factor in another Cox proportional-hazards regression analysis after excluding those who received TMP/SMX or aerosolized pentamidine for prophylaxis at least three months from the non-PCP group. The different results from the Cox proportional-hazards regression analyses can be explained by the fact that nine out of 14 patients given prophylaxis were aged 65 or older. Pulmonary diseases were not significant risk factors for PCP in either Cox proportional-hazards analysis, perhaps because of the small number of PCP cases enrolled.

None of the 17 patients had received prophylaxis for PCP. Vananuvat etal. [27] conducted a retrospective cohort study for patients with connective tissue diseases (CTD) who were at risk for PCP in order to examine the effectiveness of primary prophylaxis with TMP/SMX and the incidence of adverse drug reactions (ADR) of TMP/SMX. Six patients without and none with prophylaxis developed PCP; the overall incidence rate was 4.3 % and the relative risk reduction was 100 %. Five patients (8.5 %) developed ADR: four had drug eruptions and one had mild hepatitis. These data indicate that TMP/SMX can be used effectively for primary prophylaxis against PCP.

There are definite limitations to our study. First, we included definite and presumptive cases of PCP in our analysis. It has been well documented that the microscopic detection of *P. jirovecii* is difficult in non-HIV PCP [28, 29], as confirmed in this and our previous studies. To increase the specificity of the diagnosis of PCP without detecting the organism microscopically, we utilized composite diagnostic criteria, including clinical symptoms, laboratory tests, radiological findings, and the clinical

course. Kameda etal. found no difference in clinical characteristics of PCP in RA patients between definite PCP (i.e., acute-onset diffuse interstitial lung disease and microscopic positivity for *P. jirovecii* or positivity in both PCR test and BDG) and probable PCP (acute-onset diffuse interstitial lung disease and positivity in either PCR test or BDG) [24]. Their data support the use of composite diagnostic criteria for PCP in patients with RA. Second, we had only 17 RA patients with PCP, which decreased the sensitivity of the Cox proportional-hazards analysis for detecting statistically significant risk factors. Third, a higher incidence of PCP in Japanese RA patients receiving TNF antagonists and their risk factors have gained widespead recognition in the past few years by Japanese rheumatologists who use TNF antagonists; this may have affected the characteristics of the patients who were treated with adalimumab. For example, we found a significant difference in the daily dose of PSL between the PCP and non-PCP groups in our previous two studies, but not in this study.

In summary, the results of this study show that PCP is a serious complication in patients with RA who receive treatment with adalimumab. The majority of the patients developed PCP early in the course of adalimumab treatment and progressed to respiratory failure. Treating physicians should therefore take prophylaxis with TMP/SMX or other agents into consideration in RA patients with a high risk for PCP. Careful monitoring of clinical manifestations and laboratory tests for early diagnosis and treatment of PCP are strongly recommended.

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