

## Distinct Elevation of Levels of Anti-*Caenorhabditis elegans* Antibody in Sera of Patients with Inflammatory Bowel Disease

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**Dysregulation of immune responses to intestinal exogenous antigens contributes to the pathogenesis of inflammatory bowel disease, but the specific antigen responsible for the pathogenesis of inflammatory bowel disease is unknown. We measured serum antibody titers against *Caenorhabditis elegans* antigens. Immunoglobulin G (IgG) and IgG subclass anti-*C. elegans* antibodies in serum samples from 29 patients with ulcerative colitis, 30 patients with Crohn's disease, 7 patients with intestinal Behçet's disease, and 11 healthy controls were measured by enzyme-linked immunosorbent assay. Serum IgG and IgG2 antibody titers against *C. elegans* were significantly higher in patients with inflammatory bowel disease than in controls. Antibody levels were not affected by age, gender, disease activity, extent of disease, or small bowel involvement. The anti-*C. elegans* antibody titer was significantly lower in patients with Crohn's disease taking mesalazine or sulfasalazine than in patients not taking these drugs. The increased immune responses to *C. elegans* found in patients with inflammatory bowel disease reflect dysregulated immune responses to enteric antigens, which might play a role in the pathogenesis of inflammatory bowel disease.**

Inflammatory bowel disease (IBD) is characterized by chronic inflammatory changes of the intestine of unknown origin. One current hypothesis concerning the pathogenesis of IBD is that this disease arises in genetically susceptible individuals as a result of a dysregulated immune response to enteric flora (6). Association of genetic mutations in the *NOD2* gene in patients with Crohn's disease (CD) may affect innate immunity to bacterial lipopolysaccharides in patients with CD, which may contribute to dysregulation of local immune responses (28). However, the antigens responsible for this dysregulation have not been identified. Luminal bacteria are thought to initiate and perpetuate intestinal inflammation (21). Mice with genetically disrupted T-cell receptors develop spontaneous chronic colitis, and this condition is associated with autoantibodies and oligoclonal immune response to luminal bacterial antigens (4). Interestingly, T-cell receptor-deficient mice do not develop colitis under germfree conditions (4), similar to the colitis generated in genetically disrupted cytokine mouse models, which was attenuated or extinguished when animals were kept in germfree conditions (25, 23). Although oligoclonal expansion of T-cell clones in patients with CD (9–11, 16) may reflect presentation of specific antigens, including those of intestinal flora, to these T cells, identification of disease-specific antigens that are responsible for the activation of T cells has not been performed.

Immunological responses to bacteria and yeast have been observed in patients with IBD. Involvement of tissue-adhesive *Escherichia coli* in patients with IBD has been reported (5, 22), and the diagnostic significance of antibody to *Saccharomyces*

*cerevisiae* in CD has been documented (15, 18). We have recently identified intestinal class II major histocompatibility molecule (HLA-DR)-bound peptides and found that most antigenic peptides bound in the HLA-DR antigen-presenting cleft were from exogenous intraluminal proteins, especially food antigens and microorganisms, e.g., *E. coli*, *S. cerevisiae*, and *Caenorhabditis elegans* (20). However, humoral immune responses to nematodes such as *C. elegans*, which are widespread in nature not only in soil but also in seawater and freshwater, have not been reported. Therefore, we examined the titers of immunoglobulin G (IgG) and IgG subclass anti-*C. elegans* antibody (ACEA) in sera from patients with IBD.

### MATERIALS AND METHODS

**Subjects.** Serum samples were obtained from 11 controls (5 females and 6 males; mean age, 32 years [range, 25 to 51]), 29 patients with ulcerative colitis

TABLE 1. Clinical features of patients with UC and CD

Characteristic	Value for group	
	UC	CD
No. of patients	29	30
Male/female	12/17	15/15
Mean age; range (yr)	35; 20–55	31; 18–67
Active/quiescent	10/19	15/15
Disease location		
Small bowel		6
Large bowel		10
Small and large bowel		14
Left-sided colitis	29	
Total colitis	9	
Medical treatment		
Sulfasalazine or mesalazine	23	19
Corticosteroids	9	8
Dietary therapy	0	16

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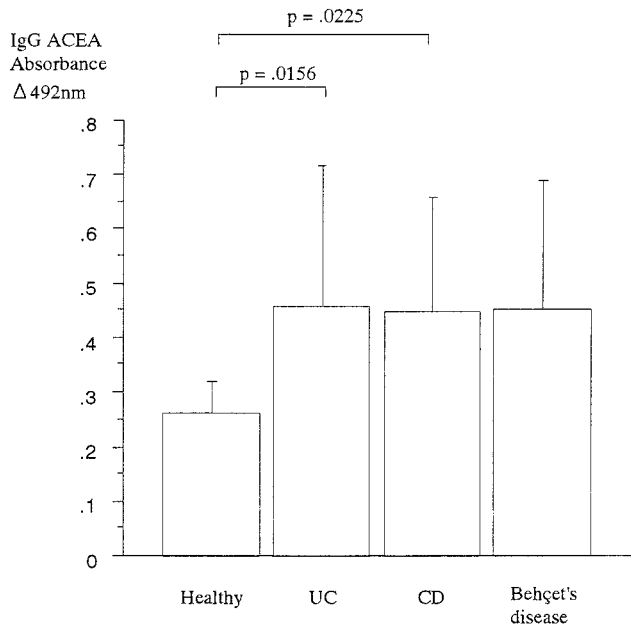


FIG. 1. IgG ACEA titers in healthy controls, patients with IBD, and patients with intestinal Behçet's disease. IgG ACEA titers were significantly higher in patients with IBD than in controls. Error bars indicate standard deviations.

(UC) (17 females and 12 males; mean age, 35 years [range, 20 to 55]), 30 patients with CD (15 females and 15 males; mean age, 31 years [range, 18 to 67]), and 7 patients with intestinal Behçet's disease (2 females and 5 males; mean age, 36 years [range, 16 to 53]). All controls and patients were from the urban area of Osaka, Japan. The diagnosis of IBD was made based on radiological, endoscopic, and histopathological findings. The diagnosis of intestinal Behçet's disease was made based on radiological and endoscopic findings, using the criteria of the International Study Group for Behçet Disease (13). Test sera were stored at  $-80^{\circ}\text{C}$  until enzyme-linked immunosorbent assay (ELISA), and all samples were tested simultaneously. Clinical data for the patients with IBD are summarized in Table 1. Twenty-three patients with UC had been treated with either sulfasalazine (3 to 4.5g/day) or mesalazine (1.5 to 3 g/day) for at least 3 months. Nine patients with UC were treated with prednisolone (5 to 15 mg/day, orally), the dose of which had been gradually reduced from acute-phase treatment. Nineteen patients with CD had been treated with mesalazine (2.25 to 3 g/day) for at least 3 months. Eight patients with CD were being treated with prednisolone (5 to 10 mg/day, orally). Informed consent was obtained from all patients and healthy volunteers before blood samples were taken. Patients with UC who had a daily stool frequency of more than four times or who had rectal bleeding were considered to have active disease. Activity in patients with CD was evaluated by International Organization of Inflammatory Bowel Disease score (8).

**Antigen preparation.** *C. elegans* was a kind gift from M. Ogata, Osaka University. *C. elegans* was extensively washed with physiological saline and homogenized with 20 mM Tris-150 mM NaCl buffer containing 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 2 mM phenylmethylsulfonyl fluoride, 25 mM iodoacetamide, 30 ng of EDTA per ml, and 0.2% sodium azide. The supernatant was obtained after centrifugation and filtered through a 0.22- $\mu\text{m}$ -pore-size Millipore filter. The extract was dialyzed against 0.05 M carbonate-bicarbonate buffer and stored at  $-20^{\circ}\text{C}$  until use.

**Measurement of serum ACEA titers.** Microtiter plates (Multi Well Plate for ELISA; Sumitomo Bakelite, Co., Ltd., Tokyo, Japan) were coated with 100  $\mu\text{l}$  of

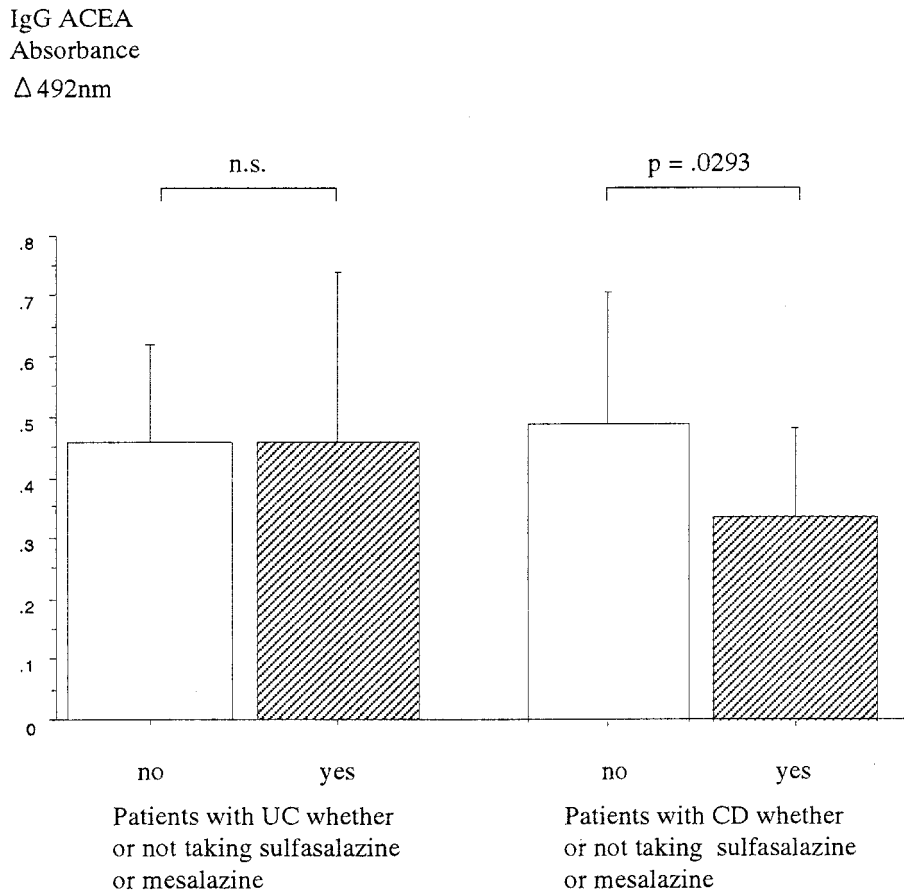


FIG. 2. IgG ACEA and 5-aminosalicylate derivatives. Patients with CD taking sulfasalazine or mesalazine had significantly lower IgG ACEA titers than patients with CD not taking these drugs. n.s., not significant. Error bars indicate standard deviations.

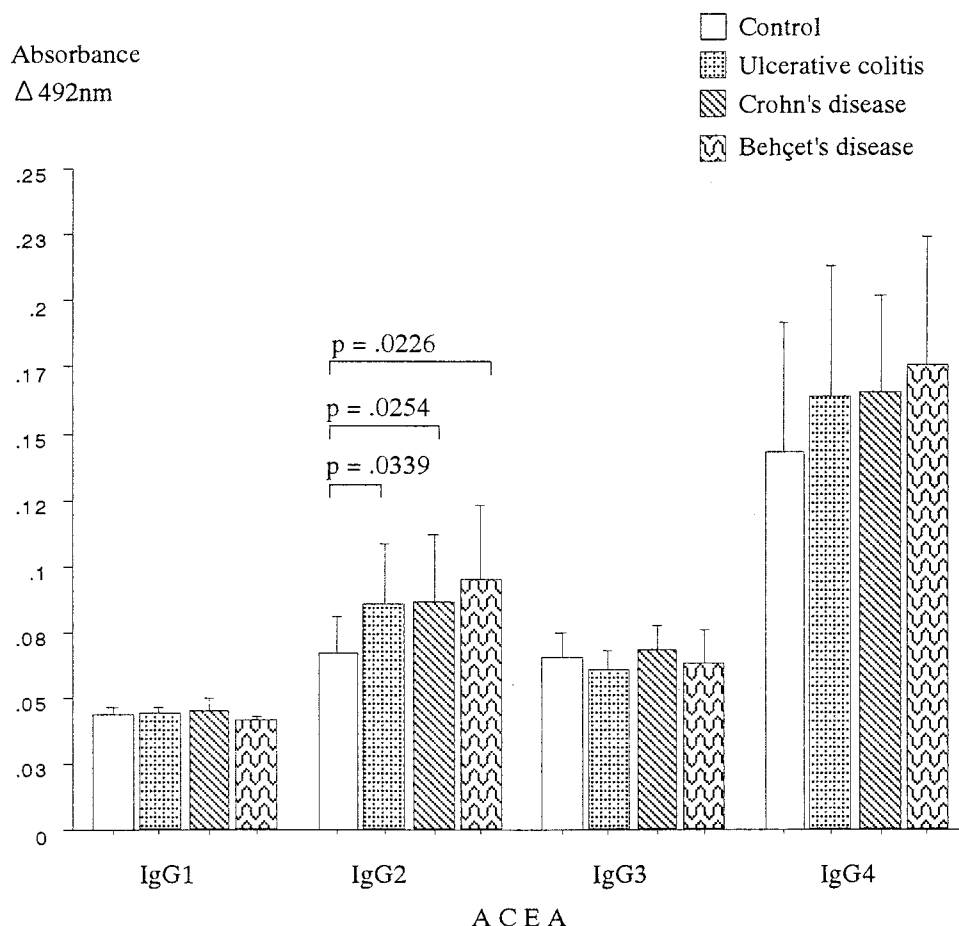


FIG. 3. IgG subclass ACEA titers in healthy controls, patients with IBD, and patients with intestinal Behçet's disease. IgG2 ACEA titers were significantly higher in patients with IBD and intestinal Behçet's disease than in controls. IgG3 ACEA titers were significantly higher in patients with CD than in patients with UC. Error bars indicate standard deviations.

*C. elegans* extract in each well and washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.3). Pooled test sera were diluted 1/50 in 0.01 M PBS containing 0.1% bovine serum albumin. Diluted sera were incubated at 100  $\mu$ l/well at 37°C for 1 h and then washed. A 100- $\mu$ l/well portion of peroxidase-conjugated goat anti-human IgG polyclonal antibody (Caltag Laboratories, Burlingame, Calif.) or anti-human IgG subclass monoclonal antibodies (anti-IgG1, clone HP6070; anti-IgG2, clone HP6014; anti-IgG3, clone HP6047; anti-IgG4, clone HP6023) (Caltag Laboratories) was applied at a dilution of 1:1,000 in 0.01 M PBS containing 0.1% bovine serum albumin at 37°C for 1 h and washed three times with 0.01 M PBS. The peroxidase substrate was developed with *o*-phenylenediamine (400  $\mu$ g/ml) in 0.2 M phosphate-citrate buffer (pH 5.0) containing 0.02% H<sub>2</sub>O<sub>2</sub>. The color reaction was terminated with 100  $\mu$ l of 4 M H<sub>2</sub>SO<sub>4</sub>/well, and the absorbance of each well was measured at 492 nm.

**Statistical analysis.** Values are expressed as means  $\pm$  standard deviations and were analyzed by one-way analysis of variance with Sheffé's correction. Differences were considered significant when *P* values were less than 0.05.

This study was approved by the ethics committee, and informed consent was obtained from all patients.

## RESULTS

**Serum IgG ACEA.** The IgG ACEA titer was significantly higher in patients with UC or CD than in controls (Fig. 1). Significant differences in ACEA titer were not found by age, gender, or disease activity. Neither small bowel involvement in the patients with CD nor extent of disease in the patients with UC affected IgG ACEA titers. The IgG ACEA titer did not

significantly differ between those patients taking corticosteroids and those not doing so. Dietary therapy with an elemental diet and/or polymeric diet did not affect the IgG ACEA titer. Patients with CD taking mesalazine or sulfasalazine had significantly lower IgG ACEA titers than patients with CD not taking these medications (Fig. 2). The IgG ACEA titer did not differ significantly between patients with UC taking mesalazine or sulfasalazine and those not taking them; 23 of 29 patients with UC were taking either of the drugs. Therefore, a correlation between the use of those drugs and ACEA levels was not found.

**IgG subclass ACEA.** IgG subclass ELISA revealed significant increases in IgG2 ACEA titers in patients with UC or CD compared with controls (Fig. 3). The IgG2 ACEA titer was significantly higher in patients with IBD and intestinal Behçet's disease than in controls. The IgG3 ACEA titer was significantly higher in patients with CD than in patients with UC. The IgG3 ACEA titer was significantly higher for CD colitis than for total and left-sided UC and was significantly higher for CD ileitis than for left-sided UC (Fig. 4). The IgG2 ACEA titer was significantly lower in patients with CD taking either sulfasalazine or mesalazine than in patients not taking those medications (Fig. 5).

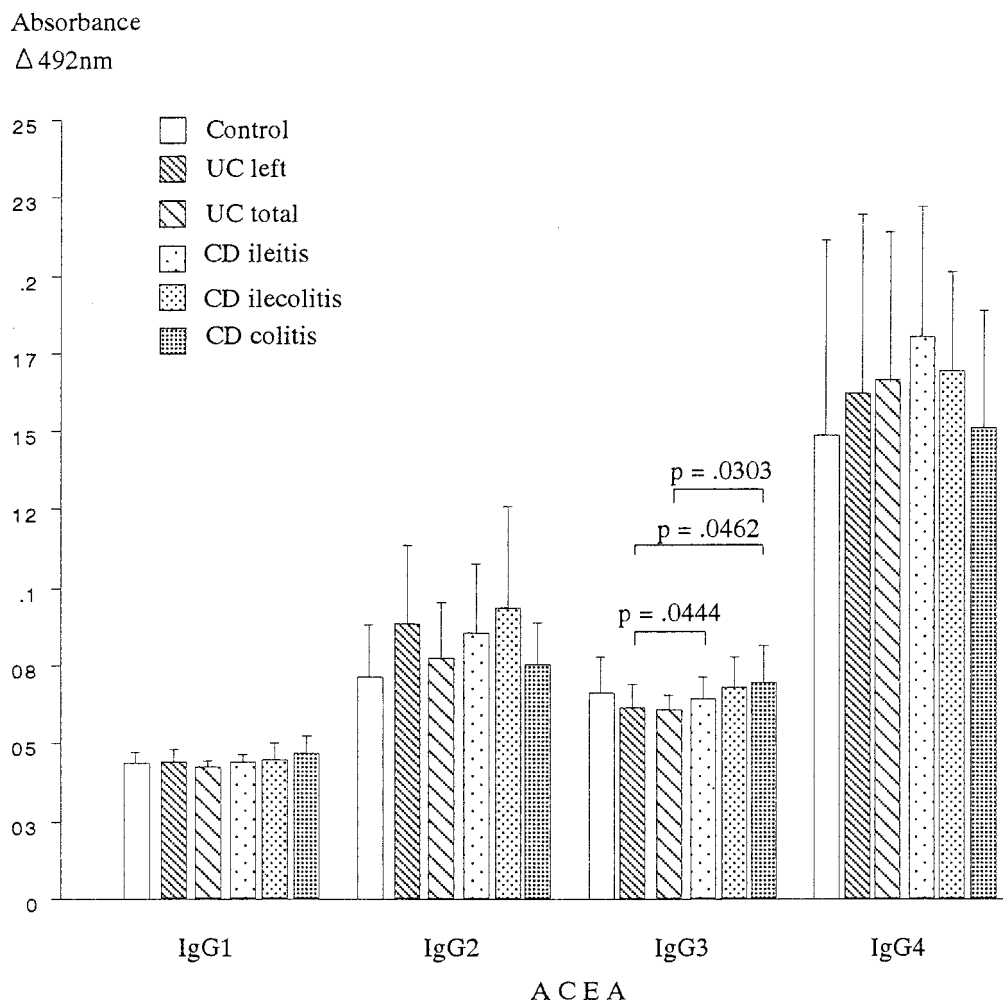


FIG. 4. IgG subclass ACEA titers and disease involvement in patients with IBD. IgG3 ACEA titers were significantly higher for CD colitis than for total and left-sided UC and were significantly higher for CD ileitis than for left-sided UC. Error bars indicate standard deviations.

## DISCUSSION

Involvement of intestinal microflora in the pathogenesis of IBD has been demonstrated. In our recent study sequencing intestinal HLA-DR-bound antigenic peptides, most antigenic peptides bound in the HLA-DR groove were from exogenous intraluminal proteins, especially food antigens and microorganisms, e.g., *E. coli*, *S. cerevisiae*, and *C. elegans* (20). Tabaqchali et al. (27) reported increased antibody titers against *E. coli* in patients with CD. Intestinal *E. coli* strains isolated from patients with IBD are more frequently adhesive than intestinal *E. coli* isolated from controls or patients with infectious colitis (5, 22). Such adhesive *E. coli* disrupts the intestinal barrier by synthesizing an  $\alpha$ -hemolysin (7). Hence, adhesive *E. coli* may cross the intestinal barrier, translocate into mesenteric lymph nodes, and generate disturbed immune responses.

Although increased anti-*S. cerevisiae* antibody (ASCA) is characteristic of patients with CD (15), the etiological role of *S. cerevisiae* in this disease is still unknown. Differences in elevations of IgG subclass ASCA were found between patients with IBD and those with intestinal Behçet's disease (19), which may

reflect differences in pathways of immunological stimulation between those patient groups. Exclusion of yeast from the diet may help maintain quiescence in patients with CD (2), and perhaps it is not the pathogenicity of *S. cerevisiae* but its cross-reactivity with various antigens generated from altered immune regulation in patients that plays a role in the pathogenesis of CD.

The elevation of serum ACEA levels in patients with IBD is reported here for the first time. *C. elegans* is a nematode that is widespread in soil and elsewhere in the natural world, and antigens from this worm can be incidentally ingested orally as native proteins or digested substances. Both ingested *S. cerevisiae* and *C. elegans* antigens may invade the intestinal mucosa through the disrupted intestinal barrier and are phagocytosed by intestinal antigen-processing cells. As a consequence, B cells are sensitized to become plasma cells to secrete IgG antibodies against *S. cerevisiae* and *C. elegans*. Age, sex, disease activity, small bowel involvement in patients with CD, and extent of disease in patients with UC each had no effect on the IgG ACEA titer in the present study.

Elevation of IgG4 ASCA levels in patients with IBD reflects

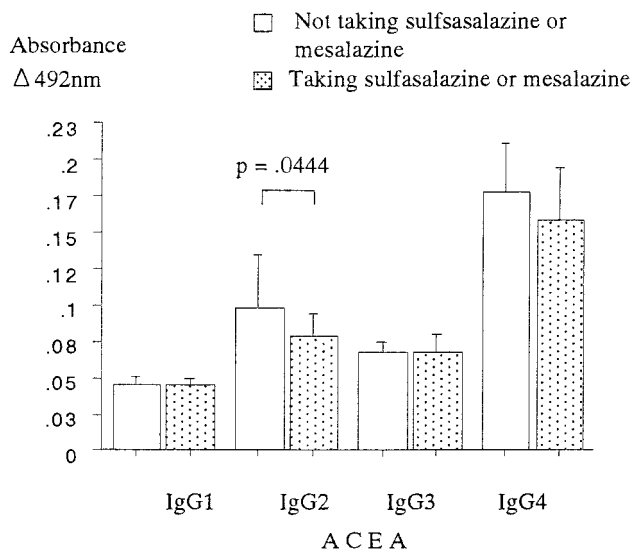


FIG. 5. IgG subclass ACEA titers and 5-aminosalicylate derivatives. IgG2 ACEA titers were significantly lower in patients with CD taking either sulfasalazine or mesalazine than in patients not taking these drugs. Error bars indicate standard deviations.

the presence of chronic yeast stimulation in the pathophysiology of IBD (2), while elevation of IgG2 antibody to *C. elegans* may reflect a Th1 response to this antigen (26). The mechanism of induction of diverse IgG subclass reactivities against different antigens such as *S. cerevisiae* and *C. elegans* is not known, but differences in accessory signals might contribute to such diversity.

The significantly higher IgG3 ACEA titer in patients with CD than in those with UC (Fig. 4) was due to the higher IgG3 ACEA titer in Crohn's colitis than in total and left-sided UC (Fig. 5). Although IgG3 antibody tends to react to protein antigens in a T-cell-dependent manner and possesses strong complement-binding activity, IgG2 antibody tends to react to carbohydrate and polysaccharide antigens in a T-cell-independent manner and has poor or no complement-binding activity (26). Serum IgG4 elevation is often associated with the chronic inflammatory state (26), and IgG4 subclass antibody is predominant among serum anticolon antibodies in patients with UC (17). Although IgG1 and IgG2 are the predominant subclasses of perinuclear-antineutrophil cytoplasmic antibody, IgG4 predominates in vasculitis-associated antineutrophil cytoplasmic antibody (1). IgG2 antipancreatic antibody was found to be the predominant IgG subclass in patients with CD (24). Elevation of IgG4 ASCA in patients with IBD may reflect chronic immunological reactivity to dietary antigens (19), whereas IgG2 elevation in ACEA may reflect a unique humoral immune response against nematodes, perhaps to carbohydrate and polysaccharide antigens.

Dietary therapy and corticosteroid administration did not affect the ACEA titer in patients with IBD, and patients with CD treated with mesalazine or sulfasalazine had significantly lower IgG and IgG2 ACEA titers than patients with CD who were not taking these drugs, a finding also obtained for ASCA titers in patients with CD (18). Significance might not have been obtained for the patients with UC because most patients

with UC were taking either sulfasalazine or mesalazine. Since patients with CD taking mesalazine did not have lower total IgG concentrations in serum than patients with CD not taking mesalazine (18), oral mesalazine or sulfasalazine administration may suppress local IgG production against *C. elegans* at the intestinal level, as observed in the ASCA reaction (18).

Disruption of epithelial barrier may be involved in the pathogenesis of ASCA or ACEA generation in patients with IBD; however, *NOD2* gene mutation is not involved in such dysregulation, because of the absence of the *NOD2* mutation in Japanese patients with CD (12, 30). Alterations in innate mucosal immunological factors such as defensins (14, 29) and Toll-like receptors (6) may be involved in disrupted innate immunity and should be further studied. Polyclonal stimuli by lipopolysaccharide via Toll-like receptor 4 in addition to stimuli by bacterial unmethylated single-stranded DNA motifs via Toll-like receptor 9 or T-cell bystander help may activate memory B cells to produce ACEA (3), perhaps as a result of altered immune responses in IBD.

In conclusion, exogenous antigens are processed by human intestinal antigen presentation to increase the humoral immune reaction, and a variety of dysregulated immune responses to intraluminal antigens may play roles in the pathogenesis of IBD.

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