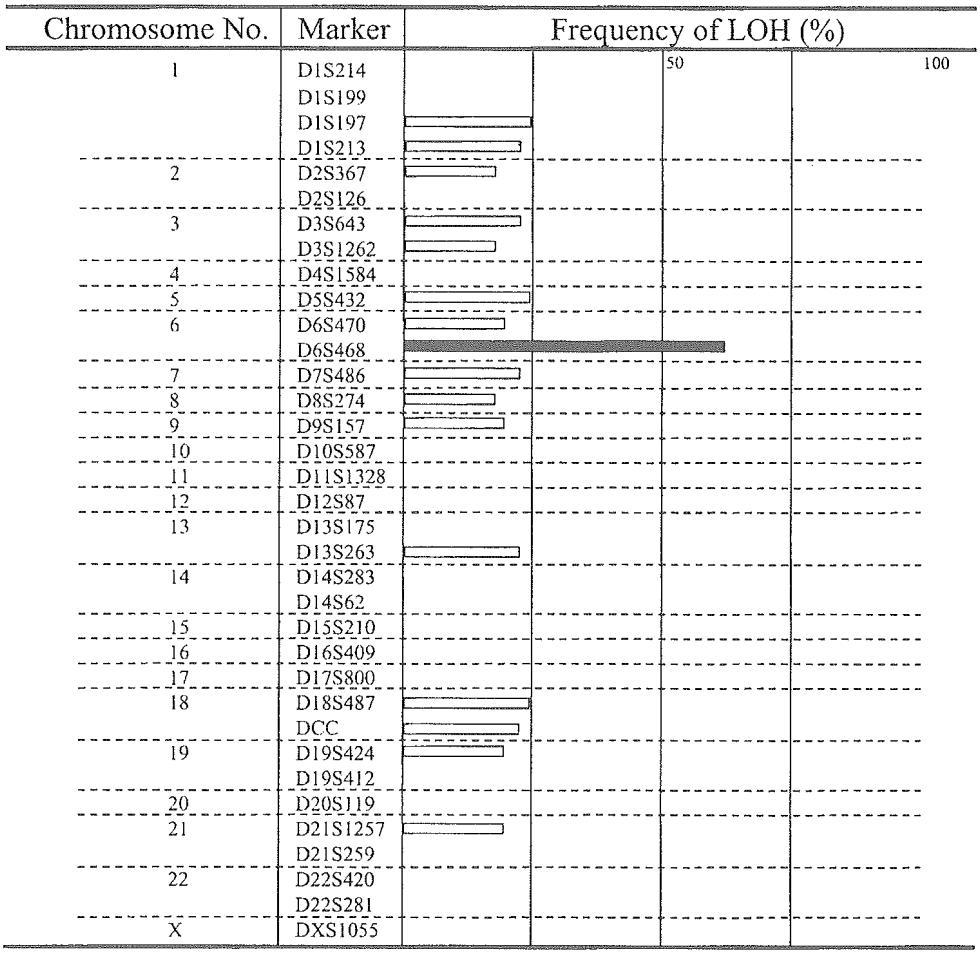

Fig. 1. Allelotype study in UC-associated CRCs and dysplastic lesions. *The left column*, the chromosome number; *the next column*, the microsatellite markers. Chromosomes are separated by *broken lines*. *Horizontal bars*, frequency of LOH. *Solid bar*, high frequency of LOH (62.5%) observed on *D6S468* locus.

LOH analysis at the *D6S283* locus in inflammatory lesions of 20 extensive and longstanding UCs in patients who belonged to the high-risk group of CRCs but had not developed CRCs. All 20 patients showed severe inflammatory lesions of UC in the study. Among the 20 cases, 8 of them were a chronic continuous type. Six patients had suffered from UC for less than 5 years, eight for 5–10 years and six

A

Microsatellite Marker	Sample No. 15	16	12	13	22	18	19	7	14	17	20	21	Frequency of LOH (%)
D6S1606	○	○	○	○	○	○	○	U	○	○	○	○	0/11 (0)
D6S1543	●	●	U	○	○	○	●	U	U	○	○	U	3/8 (37.5)
D6S468	U	●	●	●	●	●	U	RER	RER	○	○	○	5/8 (62.5)
D6S283	●	●	●	●	●	U	U	U	○	U	○	○	5/8 (62.5)
D6S1580	U	○	○	○	○	○	U	U	○	○	○	○	0/9 (0)

B

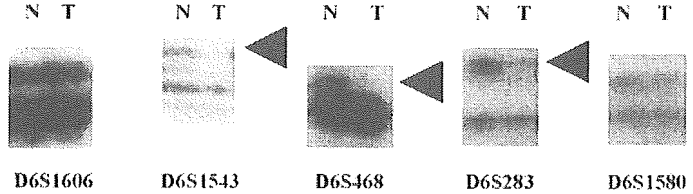

Fig. 2. Deletion mapping on chromosome 6 in 12 UC-associated CRCs and dysplastic lesions. *A*, LOH (●) and retention (○). *U*, uninformative case. *RER*, replication error. *B*, allelic loss in case 16 demonstrating typical patterns of LOH. LOH (*arrowhead*) is detected in the tumor at the loci of *D6S1543*, *D6S468*, and *D6S283*, but not at the loci of *D6S1606* and *D6S1580*. *N*, DNA from noncancerous tissues; *T*, DNA from cancerous tissues.

Table 1 *LOH study in 20 sporadic CRCs*

LOH study was performed using *D6S283* marker located in the commonly deleted region of chromosome 6. No LOH was detected in cancerous lesions.

Histology	Stage[a]	Frequency of LOH
Well differentiated	0	0/7
	I	0/4
	II	0/5
	III	0/1
Moderately or poorly differentiated	II	0/1
	III	0/2

[a] AJCC (American Joint Committee on Cancer) cancer staging.

for >10 years. In none of the patients, was there a genetic alteration at the *D6S283* locus (Table 2). Even in three cases of the chronic continuous type that had continued for more than 10 years, no LOH was detected.

DISCUSSION

We performed allelotype study of human chromosome 1 to 22 and X for UC-associated CRCs or dysplastic lesions, and we defined the commonly deleted lesion between *D6S1543* and *D6S1580* on chromosome 6. To clarify the alteration specific for UC-associated cancers, we investigated LOH analysis for sporadic CRCs by *D6S283*. No LOH was detected in any sporadic CRCs. This result is consistent with a previous report (16). These findings, therefore, indicated that LOH on chromosome 6 is specific in the carcinogenesis of UC-associated CRCs but not with sporadic CRCs. In addition, we carried out LOH analysis by *D6S283* in 20 severe inflammatory lesions of UC

Table 2 *LOH study in 20 severely inflamed UCs without CRC*

LOH study was performed using *D6S283* marker located in the commonly deleted region of chromosome 6. In all of the cases, there was no genetic alteration at the *D6S283* locus.

Type	Duration (yr)	Frequency of LOH
Total[a]	<5	0/4
	5–10	0/6
	>10	0/2
Left side[b]	<5	0/2
	5–10	0/2
	>10	0/4

[a] Total, total colitis.
[b] Left side, left-sided colitis.

without CRCs. They were different in duration of disease and extent of inflammation. However, LOH was not recognized at all. This evidence suggests that LOH on chromosome 6 is a specific event in UC-associated cancerous mucosa and is not involved in sequential events in the inflammatory process of UC.

This region contains at least two genes, and more than 20 expressed sequence tags. The genes are *glutamate receptor-ionotropic-kinase 2* (*GRIK2*) and *angiopoietin-1*. *GRIK2* is a candidate gene that implicates the common idiopathic generalized epilepsies (17) and angiopoietins are angiogenic factors and ligands for the tyrosine kinase receptor TIE2 (18, 19). In some tumors, it has been reported that expression of angiopoietins is detected immunohistochemically (20, 21). Because samples used in this study were fixed in formalin and embedded in paraffin, we could not extract mRNA from tissues and demonstrate whether these genes and sequence tags were expressed or not. According to functions of these genes, however, we consider that these are not suitable for tumor suppressor genes and that novel tumor suppressor genes may exist in this region.

Inactivation of p53 is important for carcinogenesis of sporadic CRCs and UC-associated CRCs,; it appears in late event in sporadic CRCs and in early events in UC-associated CRCs (3, 5). High activity of c-src, which correlates with progressive stages of sporadic CRCs, is also detected in premalignant epithelia of UC. This means that the activation of c-src is an early event in the carcinogenesis of UC-associated CRCs (22). Alteration of *APC*, which is recognized as an early event in the genesis of sporadic CRCs, is not the main molecular mechanism in UC-associated CRCs (4). However, highly frequent mutations of the *APC* gene have been reported in mice deficient in β_2-microglobulin and interleukin 2 (the animal model in the study of UC-associated CRCs; Ref. 23). Although the molecular mechanism for carcinogenesis of UC-associated CRCs is still confused and controversial, it is certain that almost all of the reported genetic alterations are not specific for UC-associated CRCs. The studies reporting new and/or unique genetic alterations in UC-associated CRCs are very few (24). In contrast, LOH on chromosome 6 is not detected in sporadic CRCs nor in severely inflamed mucosa in UC, suggesting that it is specific for the process of UC-associated CRCs. These results indicate that the presence of novel tumor suppressor genes on chromosome 6 are related to the carcinogenesis of UC but not to the carcinogenesis of sporadic CRCs.

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Hyperexpression of Inducible Costimulator and Its Contribution on Lamina Propria T Cells in Inflammatory Bowel Disease

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Background & Aims: To investigate the role of inducible costimulator (ICOS), a new member of the CD28 family involved in regulation of T-cell activation and chronic intestinal inflammation, we assessed its expression and functional role in patients with inflammatory bowel disease (IBD). *Methods:* Expression of ICOS, CD28, and cytotoxic T-lymphocyte antigen (CTLA) 4 on intestinal lamina propria mononuclear cells (LPMC) from patients with ulcerative colitis (UC), Crohn's disease (CD), and normal controls was determined using flow cytometry and immunohistochemistry. Expressions of the ICOS ligand, B7h, on lamina propria B cells, macrophages, and epithelial cells (EC) in the intestinal mucosa were also determined using flow cytometry. The functional costimulatory effect of ICOS on LPMC was assessed by the proliferative response and cytokine production. *Results:* $CD4^+$ LPMC expressing ICOS was significantly increased in the inflamed mucosa of IBD patients but not in inflammatory or normal controls. B7h was also significantly up-regulated on B cells, macrophages, and EC in inflamed mucosa of IBD patients. Proliferative responses of anti-CD3/ICOS costimulation were significantly higher compared with those of anti-CD3 monoclonal antibody (mAb) alone. Anti-CD3/ICOS-stimulated-LPMC from UC secreted significantly increased amounts of interleukin (IL)-5 among the 3 groups. In contrast, anti-CD3/ICOS-stimulated-LPMC from CD secreted significantly increased amounts of interferon (IFN)-γ in the presence of IL-12. *Conclusions:* Highly expressed ICOS in activated $CD4^+$ LPMC of IBD patients contributes to the dysregulated immune responses in IBD. Because ICOS hyperexpression was limited to inflammatory sites in IBD patients, ICOS would be a feasible therapeutic target for the treatment of IBD.

Crohn's disease (CD) and ulcerative colitis (UC) are the 2 major forms of chronic inflammatory bowel disease (IBD). Although their etiopathology remains unknown, increasing evidence has outlined that immune mechanisms play an important role in their pathogenesis.[1–3] These include increased T-cell infiltration in inflamed mucosa and abnormal cytokine production by lamina propria (LP) T cells. In both UC and CD, a cytokine imbalance has been postulated as playing an important role in the initiation and/or perpetuation of intestinal inflammation.[4] T helper (Th)-1 cytokines (interferon [IFN]-γ, interleukin [IL]-12, IL-18) predominate in CD, Th2 cytokines (IL-5) tend to predominate in UC, and activated effector T cells in inflamed mucosa from both diseases have been implicated in their pathogenesis.[5–8]

In the activation of naïve T cells, 2 signals are required from antigen-presenting cells (APC) for optimal activation of antigen-specific T cells.[9,10] The first signal is provided by the specific antigen recognition through the interaction of major histocompatibility complexes (MHC) and the T-cell receptor (TCR)-CD3 complex. The second signal (called a costimulatory signal) is delivered to T cells by costimulatory molecules expressed on APC. Even if T cells receive an adequate TCR signal, they fail to respond effectively and are rendered anergic or undergo apoptosis in the absence of costimulation.[11] The most studied costimulatory signal is that of CD28, which resides on the T cell and responds to its counter receptors, B7-1 (CD80) and B7-2 (CD86), on APC.[12,13]

Abbreviations used in this paper: APC, antigen-presenting cell; CD, Crohn's disease; CTLA, cytotoxic T-lymphocyte antigen; EC, epithelial cells; IBD, inflammatory bowel disease; ICOS, inducible costimulator; LPMC, lamina propria mononuclear cells; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; NL, normal control; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor; Th, T helper; UC, ulcerative colitis.

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CD28-mediated costimulation plays a critical role in T-cell activation, as evidenced by studies in animal models of experimental allergic encephalomyelitis (EAE), collagen-induced arthritis, asthma, and experimental colitis, in which the severity of disease was markedly reduced by blocking the CD28/B7 pathway.[14–17] Cytotoxic T lymphocyte antigen 4 (CTLA4), the second member of CD28 family, is another receptor for B7-1/B7-2, and its expression is rapidly up-regulated following T-cell activation. CTLA4 has a higher affinity for B7-1/B7-2 than CD28, and its engagement delivers a negative signal.[18,19] Thus, CTLA4 might inhibit T-cell responses by out-competing CD28 for binding to B7-1/B7-2, by inducing immunosuppressive cytokines, or by directly antagonizing CD28-mediated signaling.[20]

Recently, a third member of the CD28 family, the inducible costimulator (ICOS,[21] also known as AILIM[22]) has been identified. Similar to CTLA4, ICOS is also induced rapidly on T cells after T-cell engagement with APC. Conversely, ICOS enhances T-cell proliferation and characteristic cytokine secretion, high levels of IL-4, IL-10, and IFN-γ and a low level of IL-2. The function of ICOS at the effector phase is more dominant than that of CD28. This is probably because of the induction of its antagonist, CTLA-4, on activated T cells. ICOS is expressed at high levels by Th2 cells and at low levels by Th1 cells in mice.[23] In addition, ICOS is expressed on T cells in germinal centers. These studies indicate a role for ICOS in T-cell help for B cells and functional studies have confirmed this.[23,24] The ligand for ICOS, B7h[25] (also known as ICOSL,[26] B7RP-1,[27] LICOS,[28] and B7-H2[29]) has been identified and is constitutively expressed on B cells and monocytes and induced on nonlymphoid cells by the inflammatory cytokine tumor necrosis factor (TNF)-α. Furthermore, it is known that GL50, a cytoplasmic variant of B7h, exhibits binding to ICOS; however, it has been detected only in lymph nodes, and its functional differences have not yet been determined.[30]

In this study, we demonstrate (1) the hyperexpression of ICOS in LP $CD4^+$ T cells from the inflamed mucosa of IBD patients, (2) a correlation between ICOS expression and disease activities, and (3) a functional role of ICOS in IBD, including proliferative responses and cytokine production by anti-ICOS costimulation.

Materials and Methods

Patients and Samples

Mucosal samples were obtained from inflamed and uninflamed areas of intestinal mucosa of 54 patients with CD (31 surgical and 23 biopsy specimens; 40 inflamed and 14 noninflamed specimens), 46 patients with UC (28 surgical and 18 biopsy specimens; 39 inflamed and 19 noninflamed specimens). The ileum was the primary site of CD involvement in 12 patients, ileocolonic in 27, and colon in 15. As a normal control (NL), mucosal samples were obtained from macroscopically and microscopically unaffected areas of 43 colonic and ileal specimens from colon cancer patients who underwent surgery and in which histopathologic examination revealed no malignancy or inflammation. As a disease control, mucosal samples were obtained from the inflamed areas of intestinal mucosa of 10 patients with acute colitis (1 *Salmonella enteritis*, 4 amebic colitis, 2 diverticulitis, and 3 ischemic colitis). The mucosa was prepared immediately after stripping away the underlying submucosa by blunt dissection. Informed consent was obtained from all patients before obtaining samples.

Disease activity in each patient with CD was analyzed according to Crohn's Disease Activity Index (CDAI) and from endoscopic and histopathologic data. Extraintestinal manifestations had been diagnosed in 6 patients. When the experimental study was performed in patients with CD, 7 patients were receiving only steroids, 24 were receiving steroids and sulfasalazine, and 17 were receiving only sulfasalazine; 6 patients had been undergoing nonspecific therapy for the previous 3 months.

In the UC group, disease activity was defined by the Truelove–Witts criteria and endoscopic (Matts grade) and histopathologic data. When the experimental study was performed, 3 patients were receiving only steroids, 23 were receiving both steroids and sulfasalazine, and 15 were receiving only sulfasalazine; 5 patients had been undergoing nonspecific treatment for at least 3 months.

Cytokine and Antibodies

Purified phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated anti-human ICOS mAb (F44, mouse IgG_1) were generated as described.[21] Purified anti-human B7h mAb (2D3, mouse IgG_{2b}) was purchased from Lab Vision (Fremont, CA). Control mouse IgG_1 mAb (MOPC-21), control mouse IgG_{2b} (G155-178), purified anti-human CD3 mAb (UCHT1, mouse IgG_1), purified anti-human CD28 mAb (CD28.2, mouse IgG_1), FITC-conjugated CD19 (HIB19, mouse IgG_1), FITC-conjugated CD33 (HIM3-4, mouse IgG_1), FITC-conjugated CD45 (HI30, mouse IgG_1), PE-conjugated anti-mouse IgG_2a+b (X57), PE-conjugated anti-human CTLA4 mAb (BNI3, mouse IgG_{2a}), PE-conjugated anti-human CD45RA mAb (HI100, mouse IgG_{2b}), PE-conjugated anti-human CD45RO mAb (UCHL1, mouse IgG_{2a}), FITC and PerCP-conjugated anti-human CD4 mAb (RPA-T4, mouse IgG_1), and FITC and PerCP-conjugated anti-human CD8 mAb (HIT8a, mouse IgG_1) were purchased from BD Pharmingen (San Diego, CA).

Immunohistochemistry

Tissue sections were stained by a well-described method.[7] Bowel specimens were placed in cold calcium- and magnesium-free phosphate-buffered saline (CMF-PBS; GIBCO-BRL, Gaithersburg, MD) at the time of surgery or colonoscopy

examination and transported immediately to the laboratory. Samples were embedded in Tissue-Tek (OCT compound; Sakura Finetechnical, Tokyo, Japan), and snap frozen in liquid nitrogen. Cryostat sections (8 μm) were fixed by microwave (500 W, 15 seconds) with distilled water containing 0.1 mol/L sodium cacodylate and 0.025% calcium chloride. Sections were incubated for 30 minutes with a blocking solution containing normal goat serum (ICN, Aurora, OH) diluted 1:10 in PBS. Control mouse IgG (4 μg/mL), mouse anti-human ICOS mAb (F44) (4 μg/mL), or mouse anti-human B7h (2D3) (4 μg/mL) was incubated for 2 hours at room temperature, followed by 2 hours incubation at room temperature with goat anti-mouse IgG coupled with FITC (1:50 dilution, ICN). The antibodies were diluted in PBS containing normal goat serum. All steps were followed by a wash in 3 changes of PBS, pH 7.4, for 5 minutes. Sections were mounted with glycerol/PBS with paraphenylenediamine, and colocalization of FITC was examined using a confocal fluorescence microscope (LSM 410 inverted laser scan microscope; Carl Zeiss, Jena, Germany).

Isolation of Lamina Propria Mononuclear Cells and Epithelial Cells From Intestinal Mucosa

Lamina propria mononuclear cells (LPMC) were isolated using enzymatic techniques as previously described.[7] Briefly, the dissected mucosa was incubated in calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) containing 2.5% fetal bovine serum and 1 mmol/L dithiothreitol (Sigma-Aldrich, St. Louis, MO) to remove mucus. The mucosa was then incubated in a medium containing 0.75 mmol/L EDTA (Sigma-Aldrich) for 60 minutes at 37°C. During this procedure, intraepithelial lymphocytes and epithelial cells (EC) were released from the tissue, and tissues containing LPMC were collected and incubated in a medium containing 1% collagenase type III (Worthington Biochemical Corp., Freehold, NJ) for 60 minute at 37°C. The fraction was pelleted twice and resuspended in 3 mL of 40% Percoll (Pharmacia Biotech, Piscataway, NJ), which was then layered over 60% Percoll before centrifugation at 1500 rpm for 30 minutes at 18°C. Cells in the top 40%–60% layer interface contained >95% pure viable LPMC. For isolation of EC, supernatant after EDTA treatment were washed twice, pelleted, and resuspended in 3 mL of CMF-HBSS, which was then layered over 30% Percoll before centrifugation at 1500 rpm for 30 minutes at 18°C. Cells in the top CMF-HBSS/30% Percoll layer interface contained >95% pure viable EC. The purity of the resulting EC and LPMC was confirmed by flow cytometry. To isolate mucosal naïve ($CD45RA^+$) $CD4^+$ T cells and memory ($CD45RO^+$) $CD4^+$ T cells, $CD4^+$ T cells were separated from LPMC by positive selection by a magnetic cell sorting system (MACS; CD4 multi-sort kit; Myltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD45RA^+$ naïve T cells or $CD45RO^+$ memory T cells were then separated from LP $CD4^+$ T cells by positive selection using the MACS.

Flow Cytometry

Flow cytometric analysis was performed as previously described.[7] Viable lymphocyte populations were gated using forward scatter/side scatter and negative staining with propidium iodide. For staining, 1×10^6 freshly isolated or cultured cells were incubated with 20 μL of 1 μg/mL PE-conjugated anti-human ICOS mAb (F44) and FITC-conjugated anti-CD4 mAb, anti-CD8 mAb, anti-CD45RA mAb, anti-CD45RO mAb, or isotype-matched mouse IgG for 20 minutes on ice. After washing, the fluorescence intensity on the cell surfaces was analyzed using a FACScan (Becton Dickinson, Mountain View, CA). To analyze the expression of B7h protein, cells were incubated with either purified anti-human B7h mAb (2D3) or control mouse IgG_2, followed by secondary staining with PE-conjugated rat anti-mouse IgG_{2a+b} (BD Pharmingen). Freshly isolated EC were gated using negative staining with propidium iodide and FITC-conjugated CD45 mAb. LP B cells were stained with FITC-conjugated CD19 mAb. LP macrophages were stained with FITC-conjugated CD33 mAb. Cells were analyzed with a FACScan as indicated.

Reverse-Transcription Polymerase Chain Reaction Analysis for Human B7h mRNA

Total RNA was isolated from 1×10^7 freshly purified epithelial cells using RNAzol (Biotex Laboratories, Houston, TX). First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA with oligo (dT) primer and 400 U/mL murine Moloney leukemia virus (MMLV) reverse transcriptase (Perkin Elmer, Norwalk, CT) using the Superscript Preamplification System (GIBCO BRL, Gaithersburg, MD) in 20 μL of the reaction mixture. The mixture was incubated at 42°C for 50 minutes, heated at 94°C for 5 minutes, and then quick chilled on ice. Polymerase chain reaction (PCR) was performed on equal amounts of cDNA to amplify the cDNA of B7h. The PCR reaction mixture contained 5 μL of cDNA, 5 μL of 10X PCR buffer, 1 μL of 1.25 mmol/L deoxynucleoside triphosphate, 34.5 μL of diethyl pyrocarbonate (DEPC)-water, 2 μL of 20 μmol/L 5′ and 3′ primers, and 0.5 U of Taq DNA polymerase (Perkin Elmer, Norwalk, CT). To amplify the cDNA of B7h, the amplification was conducted at 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes. To determine the optimal number of cycles, a range of 21, 24, 27, and 30 PCR cycles were performed. Aliquots of each PCR were transferred on a 1.2% agarose/ethidium bromide gel. Optimal numbers of PCR cycles were determined as 1 or 2 cycles less than needed to overcycle the cDNA. The primers were designed to distinguish B7h and GL50, another variant of the human ICOS ligand. Human B7h specific primers were 5′ primer, CGT GTA CTG GAT CAA TAA GAC GG and 3′ primer, TGA GCT CCG GTC AAA CGT GGC C. The human GL50 specific primers were 5′ primer, CGT GTA CTG GAT CAA TAA GAC GG and 3′ primer, TCA CGA GAG CAG AAG GAG CAG GTT CC. Human glyceraldehyde 3-phos-

phate dehydrogenase (GAPDH, as a housekeeping gene) primers were 5′ primer, TGA AGG TCG GAG TCA ACG GAT TTG GT, and 3′ primer, CAT GTG GGC CAT GAG GTC CAC CAC (Mapping Amplimers; Clonetech, Palo Alto, CA). All specific primer, synthesized by the phosphoramine method using a DNA synthesizer (model 392 PCR-MATA; Applied Biosystems, Inc., Foster City, CA), were purchased from Sawady Technology (Tokyo, Japan). A 100-bp DNA ladder (GIBCO BRL) was used as a marker. The PCR products amplified by B7h and GL50 primers were size fractioned by gel electrophoresis in 2% low-melting point agarose gels and purified by phenol-chloroform extraction. The purified PCR products were then directly sequenced by a modification of the dideoxynucleotide chain-termination method and through cycle sequencing using DNA polymerase (Sequencing High Cycle; Toyobo Co., Osaka, Japan). B7h and GL50 sequences were analyzed by aligning homologous gene regions in reference to advanced BLAST searches.

Activation of T Cells by Anti-CD3 Plus Anti-ICOS

For costimulation assay, flat-bottomed 96-well microtiter plates (Iwaki, Tokyo, Japan) were coated with anti-CD3 (1 μg/mL) plus anti-CD28 mAb (10 μg/mL), anti-CD3 (1 μg/mL) plus anti-ICOS mAb (20 μg/mL), or anti-CD3 (1 μg/mL) plus control Ab (10 μg/mL) at 37°C for 3 hours. In addition, as a control, plates were coated with control Ab, anti-CD28, or anti-ICOS alone. Proliferation assays were performed by culturing purified LPMC (2×10^5/well) for 72 hours, as previously described.[7] After incubation, cells were pulsed for 12 hours with [^{3}H]-thymidine (1 μCi/well) (New England Nuclear, Boston, MA), harvested on glass fiber filters, and counted for radioactivity (in counts per minute) in a liquid scintillation system. In another set of experiments, $CD4^+$ T cells were separated from LPMC by positive selection using the MACS. Mixed lymphocyte reaction (MLR) assay was performed by culturing purified LP $CD4^+$ T cells (2×10^5/well) with various concentrations of allogeneic monocytes separated from the same donor's peripheral blood in the presence or absence of anti-B7h mAb (2D3, Lab Vision). After 5 days initiation of MLR, cells were pulsed for 12 hours with [^{3}H]-thymidine (1 μCi/well), harvested on glass fiber filters, and counted for radioactivity (in counts per minute) in a liquid scintillation system.

To detect cytokines, supernatants were collected after 60 hours of culture, and the concentrations of IL-2, IL-4, IL-5, IFN-γ, and IL-10 were determined by sandwich enzyme-linked immunosorbent assay (ELISA; R&D, Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ± SEM. Groups of data were compared using nonparametric Mann–Whitney *U* test. Statistical significance was established at $P < 0.05$.

Results

Expression of ICOS on Lamina Propria Mononuclear Cells

To assess the contribution of ICOS-B7h interactions in IBD, we first analyzed the expression of ICOS on LPMC from inflamed mucosa of IBD patients by flow cytometry. The proportions of LPMC expressing ICOS were significantly increased in inflamed mucosa from patients with UC and CD, compared with those from NL (Figure 1*A*). $ICOS^+$ cells in $CD8^+$ LPMC were detected at very low levels (Figure 1*A*), and there were no significant differences among 3 groups. In contrast, $CD4^+$ $ICOS^+$ or $CD8^+$ $ICOS^+$ cells were minimally detected in PBMC from all 3 groups (Figure 1*B*). Interestingly, surface $CTLA4^+$ $CD4^+$ LPMC were slightly, but significantly, increased in inflamed mucosa from patients with IBD, when compared with those from NL (Figure 1*C*). By contrast, CD28 was constitutively expressed at similar levels on both $CD4^+$ and $CD8^+$ LPMC from all 3 groups (Figure 1*C*). There were no differences of ICOS expression between ileal and colonic samples in CD (data not shown).

Of clinical importance, there was a significant correlation between ICOS expression on $CD4^+$ LPMC and disease activity. The percentages of $CD4^+ICOS^+$ LPMC were significantly higher in inflamed mucosa from patients with UC (49.3% ± 12.3%) and CD (47.9% ± 15.8%) than those in noninflamed mucosa (22.1% ± 3.2%; 24.0% ± 12.1%, respectively) (Figure 2*A*). Interestingly, $CD4^+ICOS^+$ cells were slightly but not significantly increased (23.3% ± 13.1%) on LPMC from patients with acute colitis, such as *Salmonella enteritis,* amebic colitis, colonic diverticulitis, and ischemic colitis when compared with NL (Figure 2*A*). For further confirmation, we next examined expression of ICOS in human IBD by confocal laser scanning microscopy. As shown in Figure 2*B*, $ICOS^+$ mononuclear cells in LP were markedly increased in inflamed mucosa from patients with UC or CD as compared with NL and non-IBD colitis controls.

Expression of B7h Molecules in Intestinal Mucosa

To investigate the surface cell phenotypes of infiltrated mononuclear cells expressing the ICOS ligand B7h, we assessed its expression in inflamed mucosa of IBD patients using confocal laser scanning microscopy. As shown in Figure 3*A*, $B7h^+$ mononuclear cells in LP were markedly increased in inflamed mucosa from patients with UC or CD as compared with NL. To determine which cells express B7h, we examined the expres-

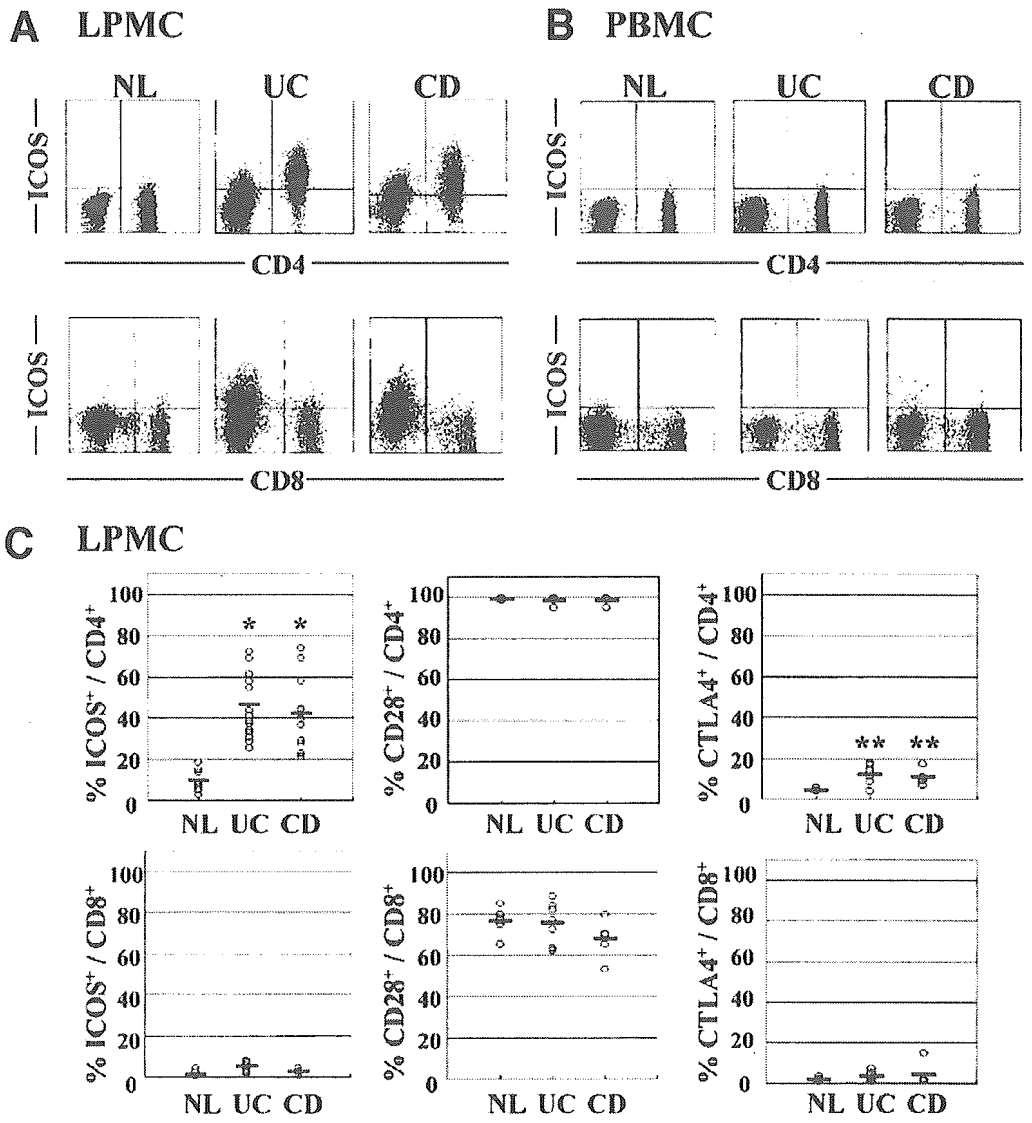

Figure 1. Expression of ICOS molecules on freshly isolated human LPMC and PBMC obtained from NL, UC, and CD patients. (*A*) Representative data showing the increased proportion of $ICOS^+$ cells in $CD4^+$ LPMC (*upper*) but not $CD8^+$ LPMC (*lower*) from UC and CD patients as compared with those from NL. (*B*) Representative data showing no expression of ICOS on $CD4^+$ PBMC (*upper*) but not $CD8^+$ PBMC (*lower*) from NL, UC, and CD patients. (*C*) Proportion of $CD4^+$ or $CD8^+$ LPMC expressing ICOS, CD28, and CTLA4 in NL (16 cases), patients with UC (21 cases), and CD (24 cases) were measured using flow cytometry. *Upper:* Proportion of $CD4^+$ LPMC expressing ICOS is significantly increased in UC and CD (*$P < 0.0005$ vs. NL). Proportion of $CD4^+$ LPMC expressing CTLA4 is slightly, but significantly, increased in UC and CD ($P < 0.005$, UC vs. NL; **$P < 0.005$, CD vs. NL). CD28 is constitutively expressed on $CD4^+$ LPMC from 3 groups. *Lower:* ICOS and CTLA4 are slightly expressed on $CD8^+$ LPMC from 3 groups. CD28 is constitutively expressed on $CD8^+$ LPMC from 3 groups. No differences are detected among the 3 groups.

sion of B7h on LPMC and EC using flow cytometry. $CD19^+B7h^+$ LP B cells and $CD33^+B7h^+$ LP macrophages were significantly increased in both UC and CD as compared with NL (B cells: NL, 41.2% ± 13.2%; UC, 67.4% ± 14.5%; CD, 68.6% ± 16.8%, respectively; NL vs. UC, $P = 0.021$; NL vs. CD, $P = 0.017$; macrophages: NL, 42.4% ± 14.5%; UC, 71.2% ± 13.3%; CD, 72.6% ± 14.7%, respectively; NL vs. UC, $P = 0.013$; NL vs. CD, $P = 0.014$). B7h was slightly but significantly up-regulated on EC from UC and CD as compared with NL (NL, 0.8% ± 1.1%; UC, 6.1% ± 3.1%; CD, 6.4% ± 2.9%; NL vs. UC, $P = 0.022$; NL vs. CD, $P = 0.019$) (Figure 3*B*). To confirm the slight but significant up-regulation of B7h in EC, we examined B7h mRNA transcripts using RT-PCR. Again, B7h mRNA was significantly up-regulated in EC from UC and CD as compared with that from NL. Furthermore, lymph node-specific GL50, a splice variant of the B7h cytoplasmic domain, was not amplified in EC from all 3 groups (Figure 3*C*).

Costimulation of T-Cell Proliferation by ICOS

To determine whether ICOS has functional costimulatory activity on LPMC, we stimulated freshly isolated LPMC from the 3 groups with anti-ICOS, anti-CD28, or control IgG in the presence or absence of anti-CD3, and, thereafter, T-cell proliferative response was determined by measuring the incorporation of [^{3}H]-thymidine. As shown in Figure 4*A*, control IgG, anti-ICOS, or anti-CD28 alone in the absence of anti-CD3 stimulation did not enhance proliferation of LPMC from all 3 groups. In contrast, anti-ICOS or anti-CD28 in the presence of anti-CD3 enhanced proliferation of LPMC from the 3 groups, as compared with stimulation by anti-CD3/control IgG (Figure 4). Consistent with a pre-

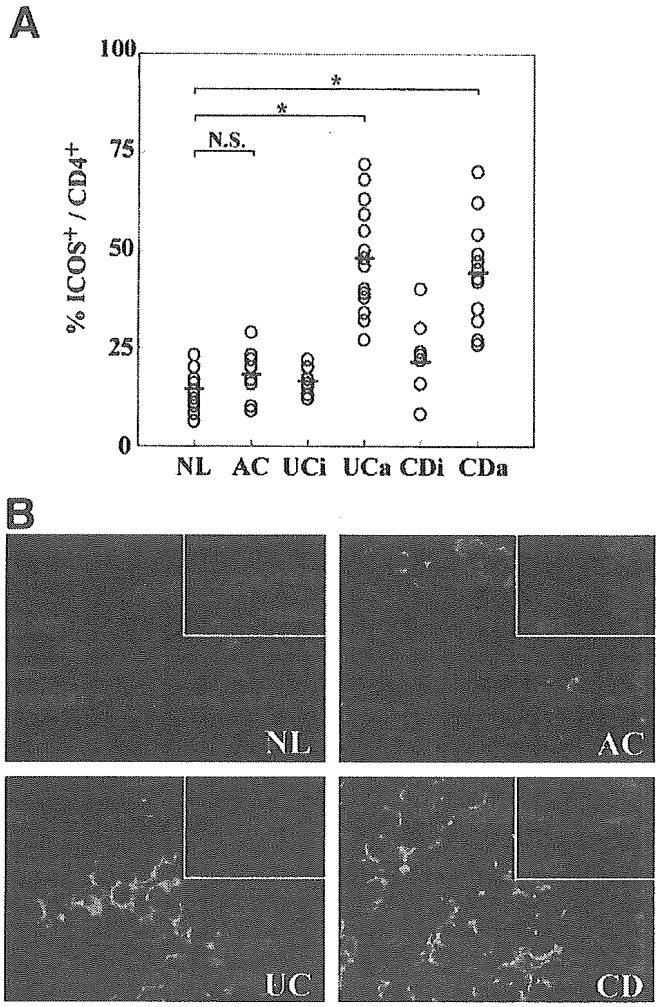

Figure 2. (*A*) Correlation between the proportion of $CD4^+$ LPMC expressing ICOS and disease activity determined by endoscopic criteria (Matts grade) in UC inactive (UCi; Matts grade = 1) and UC active (UCa; Matts grade > 3) or CDAI in CD inactive (CDi; CDAI < 150) and CD active (CDa; CDAI > 150). The proportion of $CD4^+$LPMC expressing ICOS is significantly increased in UCa (*$P < 0.0005$) and CDa (*$P < 0.0005$), compared with UCi and CDi, respectively. As disease controls, the proportion of $CD4^+$LPMC expressing ICOS in acute colitis (AC) is shown. (*B*) Confocal imaging of mucosa from NL, UC, CD, and ischemic colitis as a disease control stained with anti-ICOS mAb. *Insets* show imaging stained by control IgG.

vious report,[6] proliferative responses of LPMC from patients with UC and CD were overall significantly lower than those from NL when stimulated with anti-CD3 in the presence or absence of anti-ICOS or anti-CD28 costimulation. To further determine whether ICOS-B7h interaction affects activation of LPMC, we next conducted MLR assay. As shown in Figure 4*B*, LP $CD4^+$ T cells in the absence of allogenic monocytes (as APC) showed minimal proliferation, which was not inhibited by neutralizing anti-B7h antibody. In contrast, LP $CD4^+$ T cells stimulated by APC proliferated in a dose-dependent manner. The proliferative responses of LP $CD4^+$ T cells were significantly reduced when ICOS-B7h interaction was blocked by anti-B7h antibody, indicating the involvement of ICOS-B7h interaction in MLR. However, a similar inhibitory ability of anti-B7h to MLR was observed in all 3 groups, suggesting that ICOS could be immediately induced on NL LP $CD4^+$ T cells in MLR.

Induction of ICOS Expression on LPMC

To evaluate the kinetics of ICOS expression, LPMC from IBD patients and NL were subsequently activated with anti-CD3, and ICOS expression was determined using flow cytometry. As noted before (Figure 1*A*), ICOS expression was very low before stimuli on LP $CD4^+$ T cells from NL (10.5% ± 3.3% positive on $CD4^+$ cells) but was strongly induced by stimulation with anti-CD3 for 48 hours (84.5% ± 8.3% positive on $CD4^+$ T cells). In contrast, the levels of ICOS expression on LP $CD4^+$ T cells from IBD patients before stimuli were significantly higher than those from NL (UC: 60.3% ± 13.3% [vs. NL, $P < 0.0005$], CD: 50.3% ± 12.7% [vs. NL, $P < 0.0005$] positive on $CD4^+$ T cells). However, ICOS expression on $CD4^+$ LPMC from IBD patients after anti-CD3 stimulation was significantly lower compared with activated $CD4^+$ LPMC from NL after stimuli (UC: 54.3% ± 13.3% [vs. NL, $P < 0.05$], CD: 62.3% ± 12.7% [vs. NL, $P < 0.05$] positive on $CD4^+$ T cells) (Figure 5*A*).

Based on this reversed expression of ICOS after anti-CD3 stimulation between IBD patients and NL, we questioned whether the lower induction of ICOS on LPMC from IBD patients was due to different expressions of naïve/memory T-cell phenotypes. To this end, ICOS induction kinetics were determined using sorted naïve ($CD45RA^+$) or memory ($CD45RO^+$) LP $CD4^+$ T cells. As previously reported, most of the LP $CD4^+$ T cells from all 3 groups have the $CD45RO^+CD45RA^-$ phenotype.[31] Before anti-CD3 stimulation, ICOS was preferentially expressed on parts of $CD45RO^+$, but not $CD45RA^+$ LP $CD4^+$ T cells from all 3 groups, although the level was significantly higher on $CD45RO^+$ LP $CD4^+$ T cells from IBD patients as compared with those from NL (Figure 5*B*). $CD45RA^+$ LP $CD4^+$ T cells from the 3 groups similarly expressed ICOS promptly after anti-CD3 stimulation (Figure 5*C*). Similarly, ICOS was promptly up-regulated on $CD45RO^+$ T cells from NL after anti-CD3 stimulation (Figure 5*C*). In contrast, the expression of ICOS after anti-CD3 stimulation on $CD45RO^+$ LP $CD4^+$ T cells from IBD patients was similar compared with that before anti-CD3 mAb stimulation at any time point (Figure 5*C*).

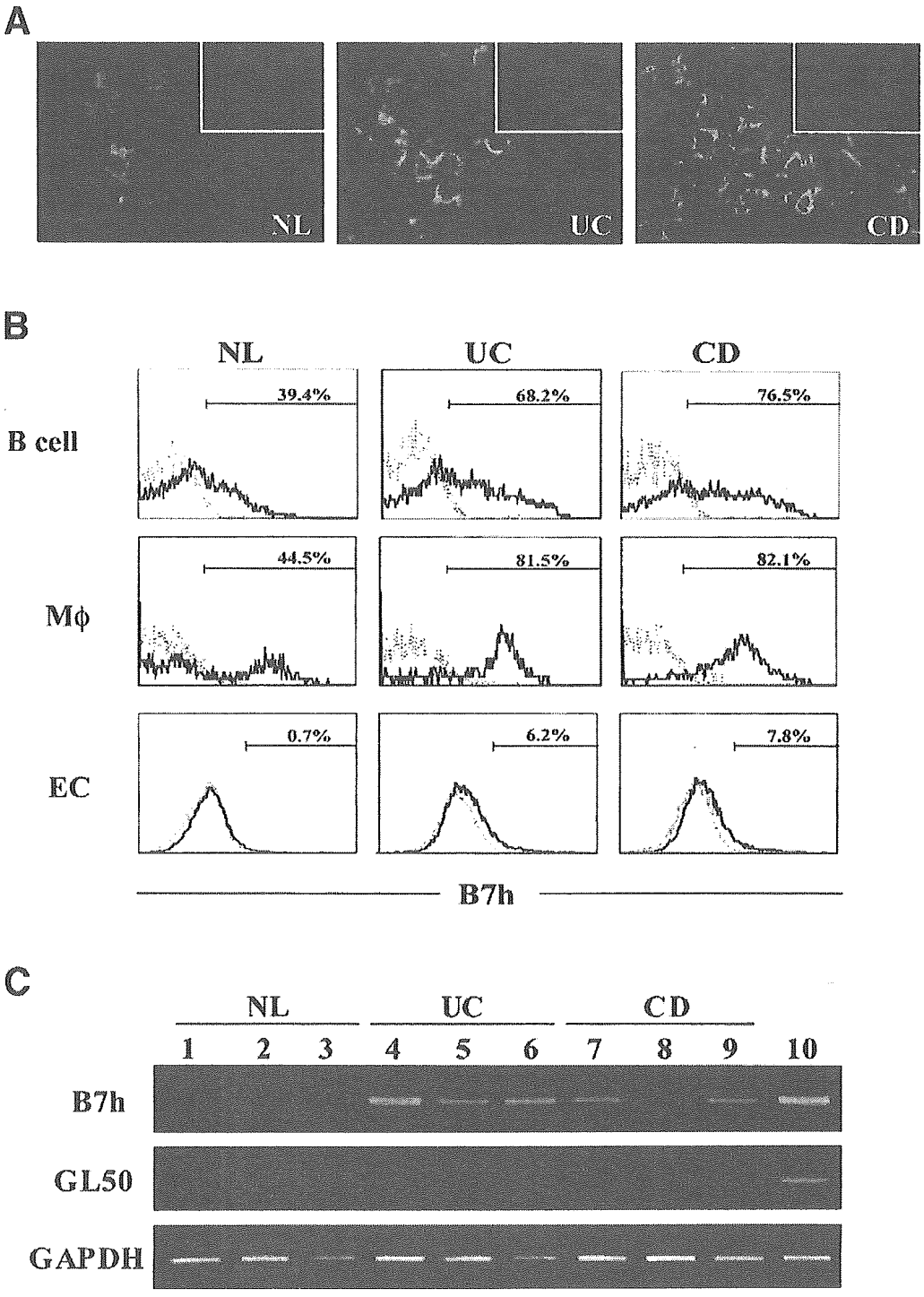

Figure 3. (*A*) Confocal imaging of mucosa from NL, UC, and CD stained with anti-B7h mAb. *Insets* show imaging stained by control IgG. (*B*) Expression of B7h protein on freshly isolated LP B cells, macrophages, and epithelial cells (EC) obtained from NL, UC, and CD (*thick line*; control staining is shown as *thin line*). B7h is down-regulated on LP B cells and LP macrophages from NL, but significantly up-regulated on those from UC and CD. B7h is slightly, but significantly, up-regulated on EC from both UC and CD, as compared with those from NL. (*C*) Transcripts of B7h and GL50 in EC obtained from NL (*lanes 1–3*), UC (*lanes 4–6*), and CD (*lanes 7–9*). As positive control for B7h and GL50, transcripts of LPMC obtained from NL (*lane 10*) are shown. As internal controls, transcripts of GAPDH mRNA are shown.

Cytokine Production

Finally, to address the disease-specific contribution of ICOS, we measured the amounts of IL-2, IFN-γ IL-10, IL-4, and IL-5 produced by PBMC and LPMC from the 3 groups after in vitro stimulation with anti-ICOS, anti-CD28, or control mAb in the presence or absence of anti-CD3. As previously reported, PBMC stimulated by anti-CD3/ICOS produced higher amounts of IL-10 and lower amounts of IL-2 as compared with anti-CD3/CD28. PBMC stimulated by anti-CD28 or anti-ICOS alone produced minimal amounts of cytokines as well as control IgG alone. There was no difference in cytokine amounts among the three groups (data not shown).

In LPMC, the patterns of cytokine secretion were mostly similar to those of PBMC (higher IL-10 production and lower IL-2 production). However, in contrast to PBMC, LPMC stimulated by anti-ICOS in the absence of

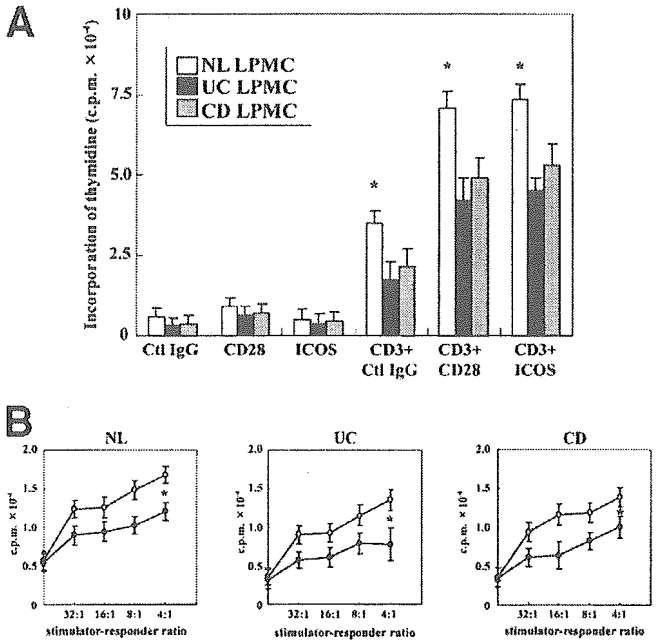

Figure 4. (*A*) Proliferative responses of LPMC obtained from NL (n = 5), CD (n = 5), and UC (n = 5) induced by plate-bound control IgG (10 μg/mL), anti-CD28 mAb (10 μg/mL), or anti-ICOS mAb (20 μg/mL) in the presence or absence of plate-bound anti-CD3 mAb (1 μg/mL). Proliferative activity was measured after 72-hour culture. *Error bars* represent SEM from studies of 5 experiments for NL, UC, and CD. ICOS costimulation is comparable with CD28 costimulation. The proliferative response of LPMC from patients with UC and CD is significantly lower than that from NL ($P < 0.05$). (*B*) Mixed lymphocyte reaction (MLR) assay between responder (LP $CD4^+$ T cells) and stimulator (allogenic peripheral blood monocytes) in the presence of control IgG (*open circle*) or anti-B7h mAb (*solid circle*). The responder proliferates in a dose-dependent manner, and the blockade of ICOS-B7h interaction inhibits MLR. There are no significant differences among the 3 groups (*$P < 0.05$).

anti-CD3 from all 3 groups secreted small but significantly higher amounts of IL-10 than anti-CD28 alone or control IgG alone. NL LPMC stimulated with anti-CD3 in the presence or absence of costimulation (anti-CD28 or anti-ICOS) secreted significantly higher amounts of IL-2, IL-4, and IL-10 but not IL-5 and IFN-γ when compared with those of UC and CD LPMC. IL-5 production by anti-ICOS alone or anti-CD3/ICOS from UC LPMC was significantly higher than that from CD or NL LPMC. In addition, IFN-γ production was significantly lower in UC LPMC as compared with CD or NL LPMC when stimulated with anti-CD3/ICOS. To further elucidate potential Th1 activity in CD, we measured IFN-γ production when stimulated with anti-CD3/ICOS in the presence of IL-12. As expected, anti-CD3/ICOS/IL-12-stimulated LPMC from CD produced significantly higher amounts of IFN-γ as compared with that from NL or UC. Similar results were obtained when LPMC were stimulated with anti-CD3/IL-12 or anti-CD3/anti-CD28/IL-12 (Figure 6).

Discussion

In this study, we have demonstrated for the first time that a novel costimulatory molecule, ICOS, was markedly up-regulated in LP $CD4^+$ T cells from patients with active but not inactive UC and CD. Furthermore, ICOS in LP $CD4^+$ T cells from acute colitis, such as infectious and ischemic colitis, was not increased regardless of the disease activity. In addition, we showed that ICOS was not increased in PB $CD4^+$ T cells in either NL or in patients with active or inactive IBD. Most importantly, we also demonstrated that anti-ICOS stimulation enhanced production of a Th2 cytokine, IL-5, in UC and production of a Th1 cytokine, IFN-γ, in CD. Our present results suggest that ICOS up-regulation is limited to the inflammatory sites of IBD and plays a major role in chronic intestinal inflammation.

Consistent with a previous study showing that anti-ICOS antibody could enhance T-cell proliferation

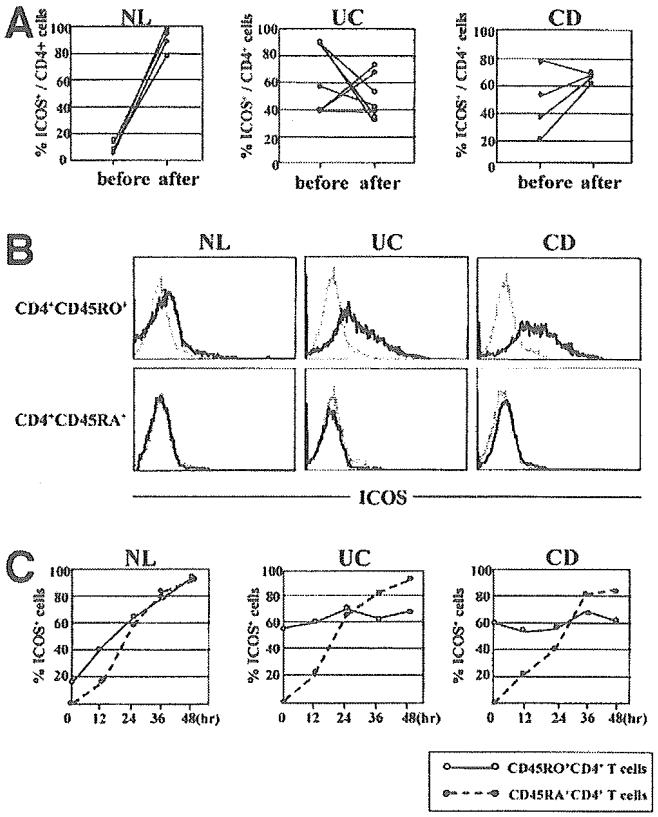

Figure 5. (*A*) Induction of ICOS on $CD4^+$ LPMC by anti-CD3 stimulation for 36 hours in vitro. LPMC from 3 groups were stimulated by plate-bound anti-CD3mAb, and the expression of ICOS was measured by flow cytometry using PE-anti-CD4 mAb and FITC-anti-ICOS mAb. (*B*) Expression of ICOS on naïve ($CD45RA^+$) or memory ($CD45RO^+$) LP T cells was measured using flow cytometry (*thick line;* control staining is shown as *thin line*). (*C*) Induction of ICOS on $CD4^+$ LP T cells was determined after separation of $CD45RA^+$ or $CD45RO^+$ T cells. ICOS expression is significantly up-regulated on $CD45RA^+$ T cells from all 3 groups and on CD45 RO^+ T cells from NL. Up-regulation of ICOS is dull on CD45 RO^+ T cells from UC and CD.

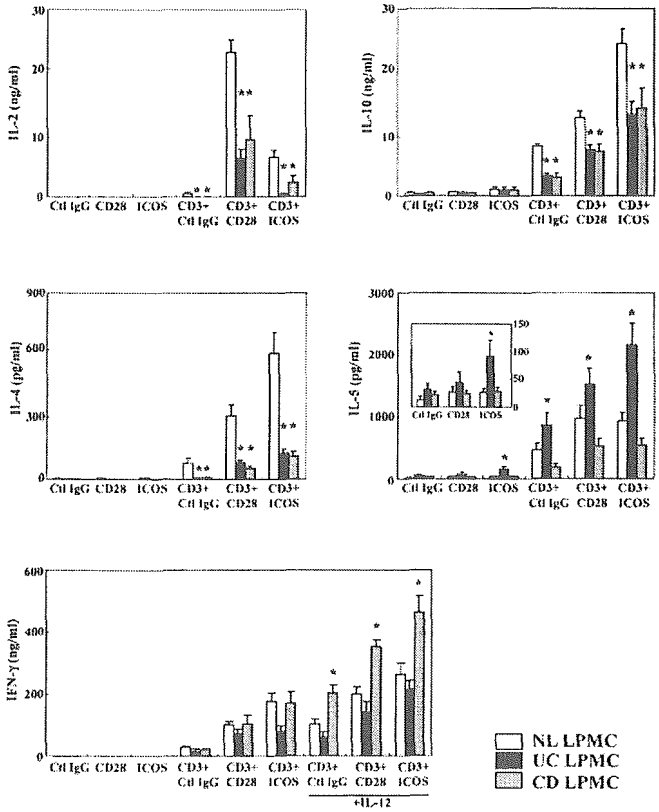

Figure 6. Cytokine productions of LPMC obtained from NL (n = 5), UC (n = 10), and CD (n = 10) induced by plate-bound control IgG (10 μg/mL), anti-CD28 mAb (10 μg/mL), or anti-ICOS mAb (20 μg/mL) in the presence or absence of plate-bound anti-CD3 mAb (1 μg/mL). In the study of IFN-γ, LPMC are also stimulated in the presence of IL-12 (10 ng/mL). *Error bars* represent SEM from studies of 5 experiments for NL and 10 experiments for UC and CD. *Inset* shows magnified figure of IL-5 productions stimulated by control IgG, anti-CD28, and anti-ICOS alone ($*P < 0.05$).

through TCR signal,[21] anti-CD3/ICOS stimulation enhanced LPMC proliferation comparable with anti-CD3/CD28 stimulation. However, NL LPMC proliferated more potently than IBD LPMC with anti-CD3 stimulation, with or without costimulation. This paradoxical result of LPMC proliferation via the CD3/CD28 pathway has already been observed by Fuss et al.[6] They concluded that LPMC from IBD patients were less responsive to anti-CD3 stimulation because of chronic exposure to inflammatory environments in the intestine as compared with NL LPMC. This is in line with a recent study showing that chronic inflammation down-regulates the T-cell receptor ζ chain and thereby induces resistance against anti-CD3 stimulation in T cells.[32] Although ICOS expression in NL LPMC was significantly lower than that of IBD LPMC, the proliferative responses of NL LPMC by anti-CD3/ICOS stimulation were much higher compared with those of IBD patients. To address the discrepancy, we next compared ICOS expression on LPMC before and after in vitro anti-CD3 stimulation. Although ICOS expression was rapidly induced and most $CD4^+$ T cells (>80%) expressed ICOS in NL, its expression level increased moderately (70%) in CD and decreased (50%) in UC after activation. Furthermore, we separately examined ICOS expression in $CD45RO^+$ (memory) and $CD45RA^+$ (naïve) subpopulations. Consistent with previous reports, $CD45RA^+$ $CD4^+$ LP cells did not express ICOS, whereas $CD45RO^+$ $CD4^+$ LP cells did, especially from UC or CD patients. Before anti-CD3 stimulation, ICOS expression on a $CD45RO^+$ subset was higher on UC or CD LP $CD4^+$ T cells than on NL LP $CD4^+$ T cells, which could be explained by the observation that memory cells were activated in IBD lamina propria. Intriguingly, whereas ICOS expression was remarkably enhanced in $CD45RO^+CD4^+$ NL LP cells when stimulated with anti-CD3, it remained at similar levels in the same population from UC or CD. This might be due to ICOS gene regulation specific to activated memory $CD4^+$ cells, or again due to the hyporesponsiveness of IBD LPMC to anti-CD3 antibody. Alternatively, it is also possible that ICOS is crucially involved in the early phase of $CD4^+CD45RA^+$ naïve T-cell activation rather than reactivation of $CD4^+CD45RO^+$ memory T cells. Therefore, total ICOS expression on NL LP $CD4^+$ cells could rapidly overtake that of IBD LP $CD4^+$ cells after CD3 stimulation, which in part explains their stronger proliferation with anti-ICOS costimulation than with IBD LP cells.

Initial studies have shown that ICOS-mediated costimulation was more effective than CD28 in the production of IL-10, and it also enhanced the production of other cytokines such as IL-4, IL-5, and IFN-γ with 50%–70% of the levels achieved with CD28.[21] Coyle et al. reported that Th2 cells express higher amounts of ICOS mRNA and protein[23] and Gonzalo et al. demonstrated that ICOS is critical for the Th2 lung mucosal injury model.[24] However, other studies have recently shown that ICOS blockade was effective for the Th1-mediated disease, EAE,[33] and cardiac allograft rejection.[34] To clarify the cytokine profile induced by anti-ICOS stimulation in IBD, we stimulated LPMC under various kinds of stimulating conditions. Fuss et al. previously reported that CD LP T cells produced increased IFN-γ production via the CD2/CD28 pathway compared with NL LP T cells, whereas IL-5 secretion was increased in UC LP T cells even without in vitro stimulation.[6] Consistent with this, we found that UC LPMC produced significantly increased amounts of IL-5 as compared with NL or CD LPMC when stimulated with anti-ICOS antibody alone or anti-CD3/ICOS. However, IL-4 and IL-10, 2 other Th2 cytokines, were secreted most promi-

nently from NL LPMC in any stimulatory condition tested in this study as Fuss et al.[6] demonstrated with similar data about IL-4. It remains to be clarified why UC LPMC enhance IL-5 but not IL-4, despite both cytokines being located in close proximity on human chromosome 5 and regulated by similar transcription factors.[35] Next, we examined Th1 cytokine production in CD LPMC. When LPMC were stimulated via CD3, costimulation with either anti-CD28 or anti-ICOS enhanced IFN-γ production from LPMC in all groups. However, we could not detect any differences between the NL and CD LPMC. Fuss et al. demonstrated similar data and implied the involvement of hyporesponsiveness to anti-CD3.[6] Again, chronic inflammation and exposure to intestinal antigen may confer resistance against anti-CD3 stimulation and thereby modify cytokine production. In particular, the T-cell receptor ζ chain was down-regulated by chronic inflammation in the setting of a Th1 but not a Th2 response.[32] Based on these findings, we added IL-12 to the anti-CD3/CD28 or ICOS stimulatory system to emphasize the microenvironment in CD because IL-12 augments IFN-γ production and has been shown to be increased in the lamina propria of CD patients.[36] In this condition, anti-ICOS, as well as anti-CD28, increased IFN-γ production in both groups, but the costimulatory effect was more effective in CD LPMC. Thus, IL-12 has a dispensable role on CD LPMC to augment the Th1 response, and anti-ICOS costimulation synergistically enhanced IL-12 stimulation. As discussed previously, the association of ICOS costimulation and the T-cell cytokine pattern is still a matter of debate. First, we could not detect any differences of ICOS expression between UC (Th2-like disease) and CD (Th1-mediated disease). Second, anti-ICOS antibody enhanced the secretion of all cytokines tested, irrespective of Th1 or Th2, from anti-CD3 stimulated LPMC. These data suggest that the ICOS signal is neither restricted to Th1 nor Th2 responses, at least in the lamina propria.

To further reinforce the possibility of the contribution of ICOS to the pathogenesis of IBD, we examined the expression of the ICOS ligand B7h in intestinal mucosa. Flow cytometry and immunohistochemistry revealed significantly increased expression of B7h on B cells and macrophages in the inflamed mucosa. Its expression was also very faintly detected on epithelial cells from IBD patients. The distribution suggests that the interaction between T cells and professional APC via the ICOS/B7h system plays an important role in chronic intestinal inflammation.

Recently Akbari et al. showed that regulatory T cells inhibit inflammation through ICOS-B7h engagement.[31] In their study, when naïve T cells interacted with IL-10-producing dendritic cells, they differentiated into ICOS-expressing memory T cells and produced significant amounts of IL-10 but no IL-4 and IFN-γ through ICOS-B7h interaction. It has also been reported that $ICOS^+$ T cells could be divided into 3 fractions by their cytokine production pattern: $IL\text{-}10^{high}/IL\text{-}4^{low}/IFN\text{-}\gamma^{low}$, $IL\text{-}10^{high}/IL\text{-}4^{high}/IFN\text{-}g^{low}$, and $IL\text{-}10^{high}/IL\text{-}4^{low}/IFN\text{-}g^{high}$ fraction.[37] In contrast to ICOS-expressing regulatory T cells predominantly producing IL-10, LPMC stimulated by anti-ICOS produced not only IL-10 but also IL-4 and IFN-γ. In addition, the proliferation of LPMC was enhanced by anti-ICOS costimulation and was inhibited by blockade of the ICOS-B7h interaction. Furthermore, we demonstrated the ameliorating effect of an anti-ICOS monoclonal antibody in a murine model of chronic colitis.[38] Taken together, IL-10 production from anti-ICOS stimulated LPMC may function in a counter-regulatory role rather than an active regulatory one; however, there may be a small fraction of ICOS-expressing regulatory T cells. Further studies will be needed to address this issue.

In conclusion, ICOS and B7h are highly expressed in inflamed mucosa and are involved in a distinct cytokine production of LPMC in UC and CD; thus, ICOS might be an ideal therapeutic target without systemic immunosuppression because its expression is exclusively limited to activated $CD4^+$ LPMC in inflammatory sites.

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A Pilot Randomized Trial of a Human Anti-Interleukin-6 Receptor Monoclonal Antibody in Active Crohn's Disease

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Background & Aims: Interleukin-6 (IL-6) regulates immune response and inflammation. We carried out a pilot placebo-controlled study to investigate the efficacy, pharmacokinetics, and safety of MRA, a humanized monoclonal antibody to IL-6 receptor, in patients with active Crohn's disease. *Methods:* Thirty-six patients with active Crohn's disease (Crohn's Disease Activity Index [CDAI] ≥150) were randomly assigned to receive biweekly intravenous infusion of either placebo, MRA, or MRA/placebo alternately for 12 weeks at a dose of 8 mg/kg. The study's primary end point was a clinical response rate that was defined as a reduction of CDAI ≥70. *Results:* At the final evaluation, 80% of the patients (8 of 10) given biweekly MRA had a clinical response as compared with 31% of the placebo-treated patients (4 of 13; $P = 0.019$). Twenty percent of the patients (2 of 10) on this regimen went into remission (CDAI <150), as compared with 0% of the placebo-treated patients (0 of 13). The clinical response rate of the every-4-week regimen was 42% (5 of 12). The serum concentrations of MRA were detected at 2 weeks after every infusion, at which time acute phase responses were completely suppressed; however, they were not suppressed at 4 weeks. Endoscopic and histologic examination showed no difference between MRA and placebo groups. The incidence of adverse events was similar in all the groups. *Conclusions:* This is the first clinical trial of humanized anti-IL-6 receptor monoclonal antibody in Crohn's disease. A biweekly 8 mg/kg infusion of MRA was well tolerated, normalized the acute-phase responses, and suggests a clinical effect in active Crohn's disease.

Crohn's disease (CD) is a chronic granulomatous inflammation of the gastrointestinal tract. The incidence of CD is the greatest in early adult life, and increases year by year; therefore, effective therapy with long-term safety is earnestly desired. Although the exact cause of CD remains unclear, overproduction of proinflammatory cytokines has been repeatedly emphasized,[1] and these cytokines may be potential targets for the treatment.

Interleukin-6 (IL-6) is a pleiotropic cytokine with central roles in immune regulation and inflammation.[2] IL-6 can transduce its signal into the cells lacking IL-6 receptors (IL-6R) when it forms a complex with soluble IL-6R (sIL-6R).[3] The importance of IL-6 and sIL-6R in the physiopathology of CD has been well documented. Serum concentrations of sIL-6R were increased in patients with active CD, and serum IL-6 and sIL-6R concentrations correlated with C-reactive protein (CRP) levels.[4] The levels of IL-6 and sIL-6R in colonic organ cultures were elevated in patients with CD, especially in those with active inflammation.[5]

In the T-cell transfer murine colitis model, administration of rat anti-mouse IL-6R monoclonal antibody suppressed the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the intestinal vascular endothelium. The treatment also reduced colonic expression of tumor ne-

Abbreviations used in this paper: CDEIS, Crohn's disease endoscopic index of severity; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBDQ, Inflammatory Bowel Disease Questionnaire; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; mAb, monoclonal antibody; SAA, serum amyloid A; SAE, serious adverse event; sIL-6R, soluble interleukin-6 receptor; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule-1.

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crosis factor α (TNF-α), IL-1β, and interferon-γ mRNA without affecting the production of transforming growth factor β (TGF-β), IL-4, and IL-10.[6] Furthermore, anti-IL-6R mAb displayed therapeutic efficacy against established colitis through the induction of lamina propria T-cell apoptosis.[7] Therefore, blocking the IL-6 signaling pathway is considered a new therapeutic strategy for CD, and we carried out a pilot clinical trial of humanized anti-IL-6R mAb MRA for active CD.

Materials and Methods

Patients

Patients with CD, diagnosed in terms of history and radiologic or endoscopic intestinal appearance, who were at least 20 years of age were eligible for the study. Patients were to have a score on the Crohn's Disease Activity Index (CDAI)[8] ≥150, which indicates active CD, and abnormal serum levels of CRP. Patients were screened for eligibility at least 2 weeks before treatment. A total of 36 patients were screened, and all patients underwent randomization at 7 study centers in Japan between May 2001 and December 2001. Each center enrolled from 1 to 10 patients. The study was approved by the Institutional Review Board for each participating center, and all patients gave written informed consent. Patients were required to have previous treatments with mercaptopurine or azathioprine for at least 6 months before screening or corticosteroids (a maximum dosage of 60 mg/day of prednisone), mesalazine, salazosulfapyridine, metronidazole, or elemental diet for at least 4 weeks before screening. In addition, increasing the dose of mercaptopurine or azathioprine was prohibited from 8 weeks before screening; corticosteroids, mesalazine, salazosulfapyridine, or metronidazole from 2 weeks; and elemental diet from 4 weeks. Increasing the dose of those drugs was also prohibited throughout the study. Moreover, eligible patients had to have a white blood cell count ≥3500/mm^3 and platelet count ≥100,000/mm^3 at enrollment. Discontinuation of cyclosporin, methotrexate, or tacrolimus treatment before screening required a 12-week washout; surgical operation for CD or total parenteral nutrition before screening required a 4-week washout. Women were required to have a negative pregnancy test and to practice adequate birth control for the study duration. Breast-feeding women were also excluded. Patients with a history of the following were excluded from the study: medical history of serious allergic reaction; serious infections; significant cardiac, blood, respiratory system, neurologic, endocrine, renal, and hepatic diseases; and psychiatric disorders.

Study Design and Randomization

The study was a randomized, double-masked, placebo-controlled study. The central enrollment center assigned randomly eligible patients to 1 of 3 treatment groups according to a computer-generated randomization schedule. Each group received 6 intravenous infusions 2 weeks apart; MRA at a dose of 8 mg/kg (M2W), alternating infusions of 8 mg/kg of MRA and placebo (M4W), and placebo. The regimen was determined according to the previous study of rheumatoid arthritis.[9] Patients were given intravenously the study drug over a 1-hour period and were kept under careful observation for 1 hour after administration. Individual randomization concealment codes were held by the center for emergency use. Investigators, patients, and the trial's sponsor remained masked to the randomization codes until data analysis was completed.

The clinical response rate, defined as a decrease in the CDAI scores of 70 points or more, hypothesized for the placebo group was between 10% and 20% and that for the MRA groups was between 40% and 60%. Based on this hypothesis, we estimated that a sample size of 10 patients in each group would be needed to detect a significant difference in clinical response rates between the placebo and MRA groups (M2W and M4W) at a power of 80% and a 5% level of significance.

MRA (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) is a humanized anti-human IL-6R mAb that binds to both the membrane-bound form and the soluble form of human IL-6R with high affinity and specificity. It was constructed by grafting the complementarity-determining regions of the mouse anti-human IL-6R mAb into human IgG1 to re-create a properly functioning antigen-binding site in a reshaped human antibody.[10]

Study Procedures and End Points

Every 2 weeks from initial treatment to final visit at 12 weeks, the patient's CDAI scores and samples for clinical laboratory measurements were collected. The clinical response was defined by a decrease in the score ≥70 points from baseline, and a clinical remission was defined by the score <150. The health-related quality of life, as measured by the Inflammatory Bowel Disease Questionnaire (IBDQ),[11] was evaluated at baseline, 6, and 12 weeks.

A primary end point was the clinical response rate at the final evaluation. The secondary end points were the remission rates, changes from baseline of the IBDQ, erythrocyte sedimentation rate (ESR), CRP, serum amyloid A protein (SSA), and fibrinogen at each assessment time. An endoscopic examination was performed at the baseline and the last observation and CDEIS (Crohn's disease endoscopic index of severity)[12] was measured by using a 10-cm visual analog scale.

Safety evaluations including all adverse events reported by investigators, clinical laboratory tests, and vital signs were performed throughout the study. Clinical laboratory tests included complete blood count, coagulation test, blood chemistry test, ESR, antinuclear antibodies, anti-DNA antibodies, and urinalysis. The examination of antibodies to MRA and serum concentrations of MRA was performed by a central laboratory (SRL Inc., Tokyo, Japan), and the data were kept masked until code break except for positive results of anti-MRA antibodies that were to be reported for discontinuation of the treatment.

Serum levels of MRA were measured by an enzyme immunoassay using MT18 monoclonal antibody specific for another binding site on IL-6R than that detected by MRA in combi-

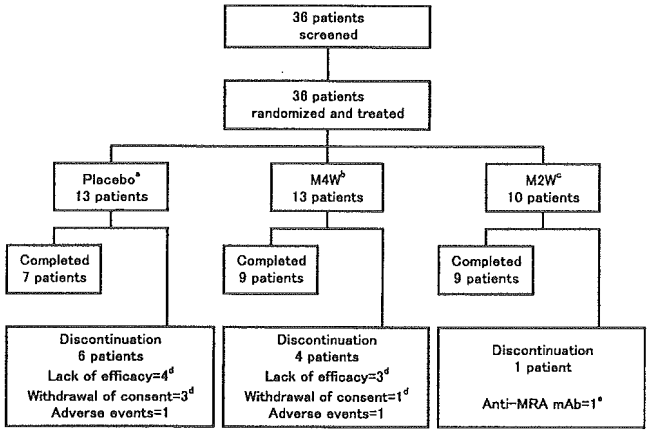

Figure 1. Trial profile of patients with active Crohn's disease who received MRA or placebo by masked randomization. [a]Placebo biweekly; [b]MRA 8 mg/kg/placebo alternately, biweekly; [c]MRA 8 mg/kg biweekly; [d]some patients had more than 1 reason, [e]anti-MRA mAb at baseline was subsequently determined to be a false positive.

nation with the sIL-6R. The captured MRA was detected using a biotinylated monoclonal antibody specific for an epitope in the variable region of MRA, at a dose at the concentration that does not inhibit the binding of IL-6R. The lowest level detected reliably was 1.0 μg/mL.[9]

Statistical Analysis

All patients who received at least 1 dose of the study drug were included in the safety and efficacy assessments. All efficacy comparisons were performed using data from the full analysis set population of patients. The efficacy analysis of an end point was a comparison of the CDAI scores based on the clinical response rates for each MRA regimen with placebo by using the χ^2 test. A P value <0.05 was used to indicate significance.

The χ^2 test was used to test for differences in the remission rates, and the Student t test was used for analyses of parametric data between groups. The final observation for patients discontinued prematurely was carried forward as the final evaluation. All reported P values are 2-sided, and all analyses were performed using SAS (version 8e TS2M0).

Results

Patient Characteristics

Thirty-six patients were randomly assigned to the M2W, M4W, or placebo group (Figure 1). Demographic data for the randomized patients are given in Table 1. There were no significant differences in age, sex, duration of disease, CDAI scores, or laboratory test values including CRP levels at baseline among the groups. All the patients had colonic disease with or without involvement of the small intestine. There were no significant differences in the number of the patients who had undergone previous CD-related surgery among the groups. A similar number of patients in each group had been treated with corticosteroids (4 in placebo, 2.5 to 10 mg/day; 2 in M4W, 5 to 10 mg/day; 2 in M2W, 2.5 to 10 mg/day), mesalamine-derived drugs, metronidazole, and elemental

Table 1. Baseline Characteristics of the Patients

	Placebo[a]	M4W[b]	M2W[c]
Number of patients	13	13	10
Male sex, No. (%)	10 (77)	10 (77)	6 (60)
Age, yr	30.1 ± 7.4	31.0 ± 10.3	32.8 ± 8.2
Weight, kg	53.3 ± 13.7	54.2 ± 5.8	51.2 ± 8.7
Duration of disease, yr	8.6 ± 5.3	7.8 ± 5.7	7.1 ± 5.4
Involved intestinal area, No. (%)			
Small bowel	0	0	0
Small + large bowel	12 (92)	13 (100)	8 (80)
Large Bowel	1 (8)	0	2 (20)
Score of CDAI	294.7 ± 70.2	286.9 ± 65.6	305.7 ± 42.0
CRP, mg/L	31.2 ± 23.2	30.4 ± 22.2	23.4 ± 13.7
Complication, No. (%)	9 (69)	8 (62)	7 (70)
Concurrent infections, No. (%)	0	1 (8)	2 (20)
Previous medications, No. (%)			
Mercaptopurine	0	0	0
Azathioprine	0	1 (8)	0
Corticosteroid	4 (31)	2 (15)	2 (20)
Mesalazine	12 (92)	12 (92)	8 (80)
Salazosulfapyridine	2 (15)	2 (15)	3 (30)
Metronidazole	2 (15)	0	3 (30)
Elemental Diet	10 (77)	10 (77)	8 (80)

NOTE. Plus minus values are means ± SD.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/placebo alternately, biweekly.
[c]MRA, 8 mg/kg biweekly.

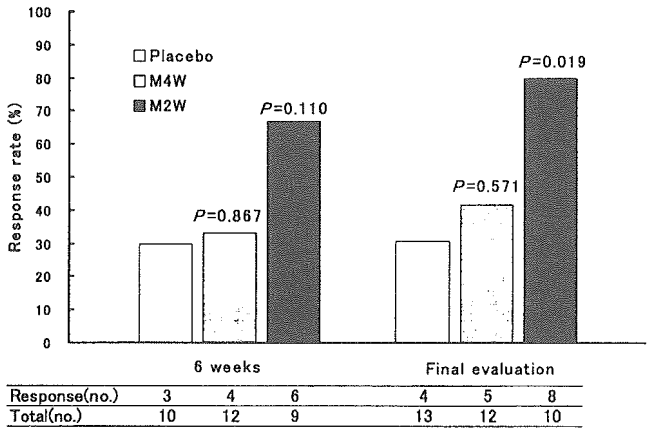

Figure 2. Percentages of patients with Crohn's disease with response (decrease in CDAI score from baseline ≥70 points) according to each treatment group. All significant differences are indicated in the Figure (χ^2 test vs. placebo). *M2W*: MRA 8 mg/kg biweekly; *M4W*: MRA 8 mg/kg/placebo alternately, biweekly; *Placebo*: biweekly.

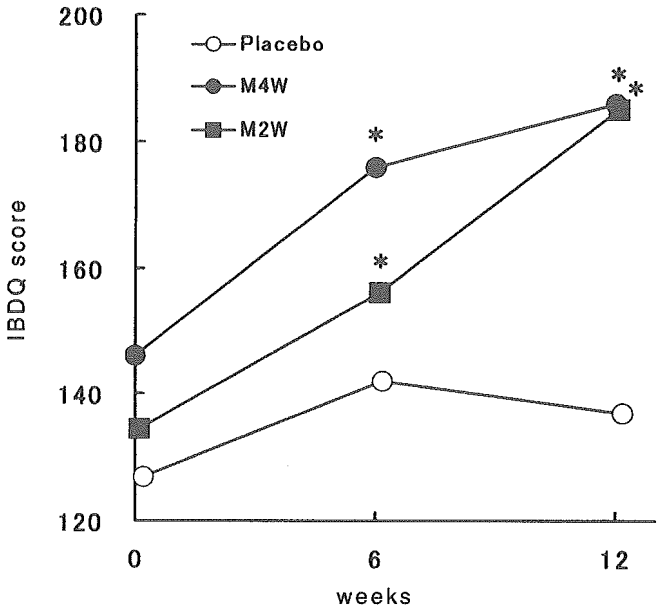

Figure 3. Median IBDQ scores according to each treatment group. *M2W*: MRA 8 mg/kg biweekly; *M4W*: MRA 8 mg/kg/placebo alternately, biweekly; *Placebo*: biweekly. *$P < 0.05$: significantly different from baseline based on paired *t* test.

diet at baseline. Only 1 patient in the M4W group was treated with 60 mg/day of azathioprine; none was treated with mercaptopurine. All treatment groups had a mean CDAI score of approximately 300, despite concomitant medications.

Eleven patients discontinued treatment (6 in placebo, 4 in M4W, and 1 in M2W). The reasons for discontinuation were lack of efficacy or withdrawal of consent (5 in placebo and 3 in M4W) and adverse events (1 each in placebo and M4W). One patient in the M4W group was not assessed for CDAI at 2 weeks because of discontinuation owing to a serious adverse event. One patient in the M2W group was reported positive for anti-MRA antibody at baseline and was discontinued according to the protocol, although it was subsequently determined to be a false positive based on a reassessment of the assay method.

Clinical Response to Treatment

With respect to the primary end point, 80% of the patients in the M2W group had a clinical response at the final evaluation that was statistically significantly higher than 31% of the placebo group (Figure 2). Twenty percent of the patients (2 of 10) on this regimen went into remission, as compared with 0% (0 of 13) of the placebo group ($P = 0.092$). The clinical response rate in the M4W group was 42%. The remission rate in this group (25%; 3 of 12; $P = 0.055$ vs. placebo) was similar to the M2W group.

The mean reduction in the CDAI score in the M2W group was 88 points, from 306 (range, 250–384) to 218 (range, 123–334) points; that in the M4W group was 75 points, from 287 (range, 195–393) to 216 (range, 59–463) points; and that in the placebo group was 41 points, from 295 (range, 183–400) to 254 (range, 164–371).

The quality of life measured by the IBDQ improved in the MRA group (Figure 3). In particular, the MRA groups showed a significant increase in the mean score from baseline at 6 weeks and 12 weeks.

Endoscopic examination was performed in 11 patients and evaluated by using CDEIS. As shown in Table 2, there was no significant difference among the groups. Tissue samples were examined for histology in some patients; however, there was no remarkable improvement either. Patients were on stable doses of corticosteroids

Table 2. Changes in CDEIS From Baseline to Final Evaluation

Group	Patient	Baseline	Final evaluation
Placebo[a]	1	13.6	19.1
	2	13.8	14.0
	3	15.5	24.6
	4	10.6	10.5
M4W[b]	1	19.0	21.7
	2	11.1	20.3
	3	12.2	11.6
	4	5.5	4.6
M2W[c]	1	20.9	18.9
	2	9.7	16.4
	3	19.4	14.6

CDEIS, Crohn's disease endoscopic index of severity.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/placebo alternately, biweekly.
[c]MRA, 8 mg/kg biweekly.

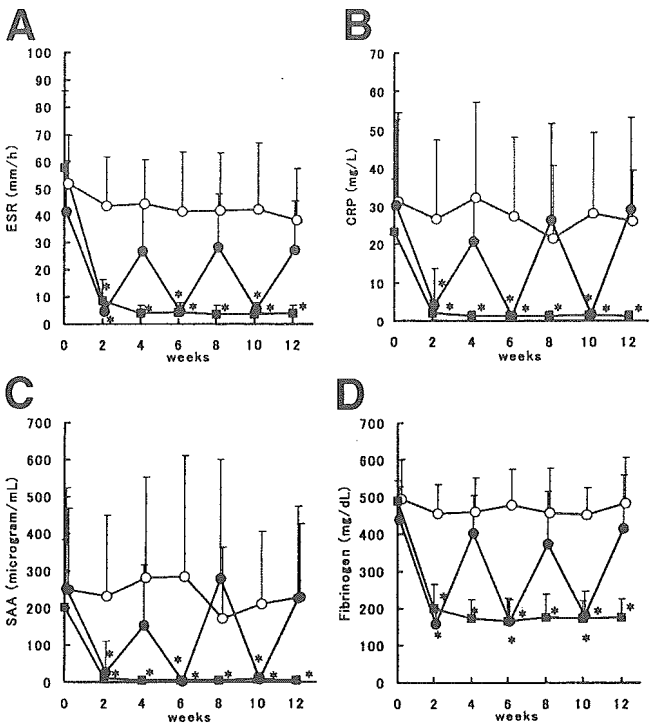

Figure 4. Mean values for (*A*) ESR, (*B*) CRP, (*C*) SAA, and (*D*) fibrinogen concentrations after repeated administration according to each treatment group. ○: Placebo biweekly; ●: MRA 8 mg/kg/placebo alternately, biweekly; ■: MRA 8 mg/kg biweekly. *$P < 0.05$: significantly different from placebo based on Student *t* test. *Bars* indicate SD.

during the study, and steroid-sparing effect was not investigated in this preliminary study.

Inflammatory Markers

The levels of inflammatory markers including ESR, CRP, SAA, and fibrinogen normalized within 2 weeks after a single dose of MRA (Figure 4). The M2W group, but not the M4W group, maintained the normal levels during the trial period. Increased platelet counts seen at baseline decreased to a normal range at 12 weeks: from 375 to 289 $\times 10^3/mm^2$ in the M2W group, from 378 to 378 $\times 10^3/mm^2$ in the placebo group, and from 324 to 313 $\times 10^3/mm^2$ in the M4W group.

Tolerability and Adverse Events

The infusion was generally well tolerated. As presented in Table 3, the adverse events observed in at least 20% of the patients in any of the groups throughout the study were common cold, nausea, pharyngolaryngeal pain, headache, retching, vomiting, and insomnia. Overall, 5 serious adverse events (SAE), which required hospitalization, were reported: 1 in the M2W group, 2 in the M4W group, and 2 in the placebo group. There were 2 SAEs that led to discontinuation from the study. One patient in the M4W group discontinued the treatment because of paralytic ileus, which developed 13 days after the initial infusion. The symptom resolved within 5 days without any intensive treatment. The causal relationship was determined as "possibly" by the investigator. Another patient in the placebo group discontinued treatment because of a suspected intraperitoneal abscess. The remaining 3 SAEs were abdominal pain/gastrointestinal bleeding in the M2W group, gastrointestinal bleeding in the M4W group, and relapse of a perianal abscess in the placebo group. The gastrointestinal bleeding in the M4W group was determined as a "possible" causal relationship. It cannot be explained that the observed paralytic ileus and gastrointestinal bleeding might be related to blockade of IL-6 function. No serious infusion reactions, occurring on any of the infusion days, were reported in any of the treatment groups. No significant trends were observed in the routine laboratory values. No clinically significant abnormalities were found in electrocardiograms, and no deaths occurred during the trial.

Immunologic Results and Pharmacokinetics

No patient developed antinuclear or anti-DNA antibody during the trial period. Specific antibodies to MRA could not be found in the serum from the patients in any of the treatment groups.

Although paired biopsy before and after treatment was performed in only 2 patients, TUNEL-positive, apoptotic mononuclear cells increased in an M2W patient, whereas no remarkable difference was observed in a placebo-treated patient (Figure 5).

The serum concentrations of MRA were detected 2 weeks after every infusion; however, they were no longer detectable values at 4 weeks (Figure 6). Pharmacokinetic analyses revealed that mean half-life of MRA was 113.17

Table 3. Adverse Events

Variable	Placebo[a]	M4W[b]	M2W[c]
Number of patients evaluated	13	13	10
Adverse event, No. (%)			
Common cold	3 (23)	3 (23)	2 (20)
Nausea	2 (15)	3 (23)	2 (20)
Pharyngolaryngeal pain	3 (23)	1 (8)	2 (20)
Headache	2 (15)	1 (8)	2 (20)
Retching	1 (8)	0	3 (30)
Vomiting	1 (8)	1 (8)	2 (20)
Insomnia	1 (8)	0	2 (20)

NOTE. Adverse events that occurred in 20% or more of the patients in any of the groups are reported.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/Placebo alternately, biweekly.
[c]MRA 8 mg/kg biweekly.

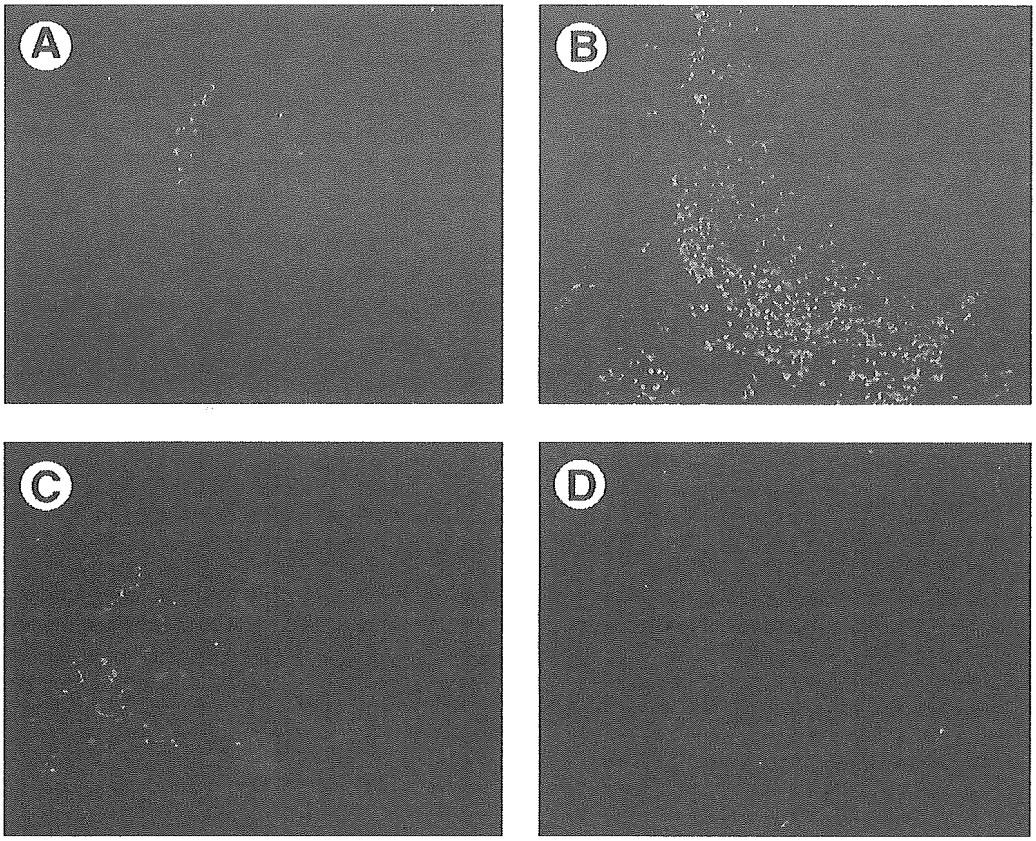

Figure 5. MRA induced apoptosis. Pairs of colonic tissue samples taken from an M2W patient before (*A*) and after treatment (*B*) and from a placebo patient before (*C*) and after treatment (*D*) were stained by using fluorescent TUNEL assay kit (MBL Co., Ltd., Nagoya, Japan; original magnification 100×).

hours in the M2W group and 97.34 hours in the M4W group. The mean volumes of distribution were 63.56 and 64.65 mL/kg in the M2W and M4W groups, respectively. Serum concentration of IL-6 and sIL-6R increased after administration of MRA; however, repetitive infusions of MRA did not induce further increase of these concentrations (Figure 7).

Discussion

Our study is the first randomized placebo-controlled trial of anti-IL-6R mAb MRA in the treatment of patients with active CD. Although this is a preliminary study, the results presented here show that the therapy with MRA for CD is safe and well tolerated and suggests a beneficial effect.

The clinical response rate of the M2W group was higher than that of the M4W group. The different response rate between the MRA groups might be attributable to a continuous suppression of acute-phase reactants such as CRP, ESR, SAA, and fibrinogen for M2W in contrast to M4W, and it is considered that such suppression may require the presence of MRA in the

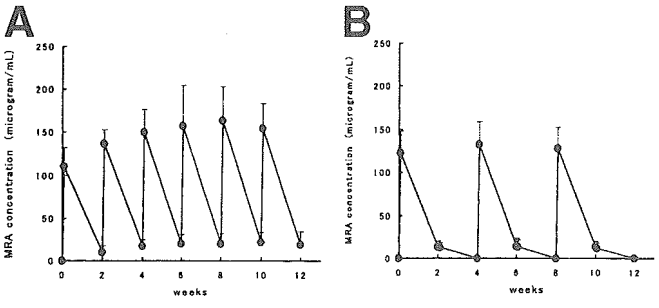

Figure 6. Mean serum MRA concentration (mg/mL) after repeated administration. Serum samples were collected before each infusion and 1 hour after each infusion and at week 12. (*A*) MRA 8 mg/kg were infused biweekly. (*B*) MRA 8 mg/kg or placebo were infused alternately, biweekly. The concentrations were under the limit of detection before each infusion at week 4, 8, and at week 12. *Bars* indicate SD.

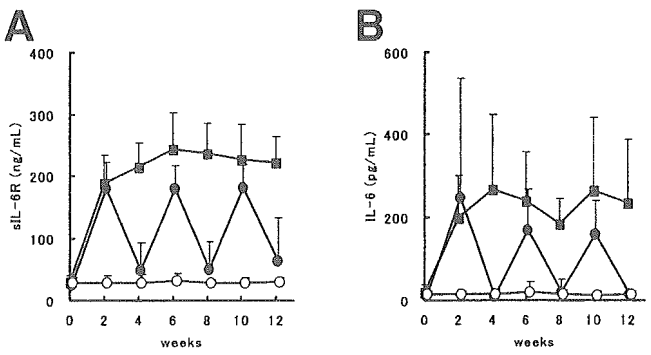

Figure 7. Mean values for (*A*) concentration of IL-6 and (*B*) concentration of sIL-6R after repeated administration according to each treatment group. ○: Placebo biweekly, ●: MRA 8 mg/kg/placebo alternately, biweekly; ■: MRA 8 mg/kg biweekly. *Bars* indicate SD.

serum throughout the treatment period. The levels of acute-phase reactants were completely normalized after only a single dose of MRA. A similar result was observed after administration of MRA for rheumatoid arthritis.[9,13] In studies of other anti-cytokine therapies, such as TNF-α blockers[14–16] and IL-1R antagonist,[17] the acute-phase reactants decreased with the treatment but not to normal levels. Therefore, it is now unquestionable that IL-6 is the principal cytokine responsible for the production of acute-phase reactants in both rheumatoid arthritis and CD. It has been shown that CRP is not a mere serum marker of inflammation but is a promoter of the IL-6R shedding to supply sIL-6R.[18] Therefore, normalization of CRP itself seems to be of benefit in the treatment of CD.

The effect on the anal complications of CD was not included in the principal evaluation of this study; however, 4 out of 6 MRA-treated patients showed disappearance of anal fissure, and 2 out of 10 showed closure of anal fistula, whereas none in the placebo group showed improvement in any of these lesions. Endoscopic and histologic healing was reported after infliximab therapy; however, such healing was not observed in this trial.[19] Although TUNEL staining displayed increased apoptosis of mononuclear cells by MRA treatment, induction of apoptosis was not conclusive because only 1 paired biopsy specimen each from MRA and placebo patient were obtained. Further study is needed to provide definitive results.

It is notable in this pilot study that MRA did not induce autoantibodies or antibodies to MRA itself, although no patients received any immunosuppressive drugs except 1 in the M4W group (azathioprine), in contrast to anti-TNF-α antibody.[14,20] Emergence of autoantibodies such as anti-nuclear antibody and anti-DNA antibody was observed in some patients treated with TNF-α blockers.[20] These antibodies are often seen in patients with systemic lupus, and reduced TNF-α levels were correlated with severe disease in lupus nephritis model,[21] which suggests that TNF-α and IL-6 have different relevance in the autoimmune phenomenon, and blocking the latter might have better safety, although the number of patients was limited in this study. Furthermore, there was no incidence of serious infusion reactions and infections during the treatment period.

This study was performed in Japan, and we have to be circumspect in comparing this study with other therapies because the modalities of the therapy for CD might be different from the Western countries, e.g., the use of steroids, immunosuppressive drugs, and elemental diet. The difference of ethnic background should also be taken into consideration. Therefore, it is desirable that the study of MRA be extended to the countries where other studies were carried out.

In conclusion, this preliminary study shows that a biweekly 8 mg/kg infusion of MRA for 12 weeks is safe and well tolerated by the patients with active CD and showed a significantly higher response rate than placebo, although endoscopic and histologic healing was not observed during the trial period. Striking improvement was observed in the acute-phase reactants, which confirms that IL-6 is the major cytokine responsible for their production in CD. Further work is needed to establish the safety and efficacy of MRA in a larger population of the patients with CD.

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