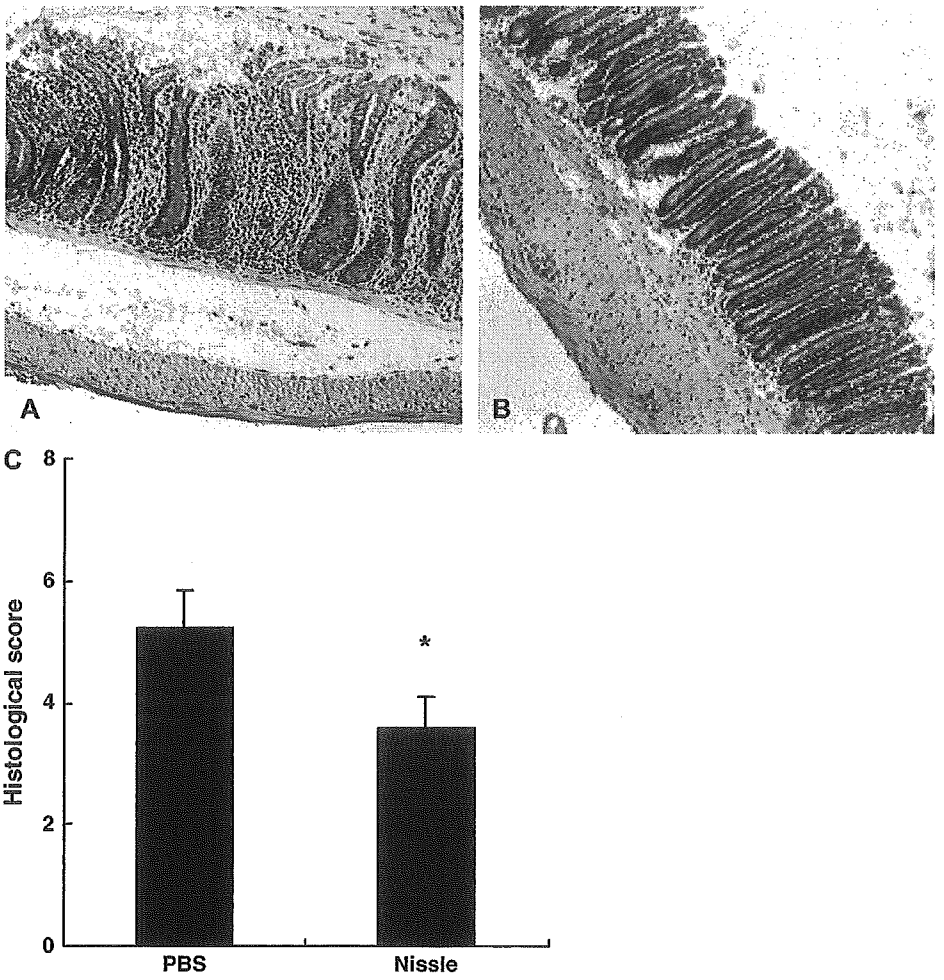

FIGURE 5. Histologic findings of the colonic tissues from IL-10$^{-/-}$ mice treated with Nissle1917. A, microscopic findings of IL-10$^{-/-}$ murine colon (16 wk old). B, Nissle1917-treated IL-10$^{-/-}$ mice (16 wk old). C, histologic scores of IL-10$^{-/-}$ mice (n = 18, PBS group; n = 17, Nissle1917 group). Results are expressed as mean ± SEM of the data from 3 independent experiments. *, $P < 0.05$ compared with PBS groups.

of 4 strains of *Lactobacillus*, 3 strains of *Bifidobacterium,* and strain of *Streptococcus*) treatment showed a therapeutic effect on pouchitis,[19,20] and *E. coli* strain Nissle1917 also has been shown effective in ulcerative colitis[10,11] and Crohn's disease.[12]

In animal experimental colitis models, there are several reports that probiotic bacteria could prevent the development of colitis.[21,22] For instance, administration of probiotic bacteria such as VSL#3, *Clostridium butyricum,* and *Bifidobacterium* suppressed inflammation of DSS-induced murine colitis.[23–25] VSL#3,[26] *Lactobacillus plantarum 299V,*[27] and other *Lactobacillus* spp.[17,28] have been shown to improve chronic inflammation of IL-10$^{-/-}$ mice. Although the precise mechanisms of probiotics remain unclear, several mechanisms have been proposed: competitive exclusion of pathogenic bacterial adherence, translocation, or both[13,29]; production of antibacterial or anti-inflammatory factor(s)[25,30,31]; an enhancement of intestinal barrier function[26]; and others. These protective functions are considered to be derived from bacterial components, cellular protein or bacterial genomic DNA, and/or their secreted factor(s).[23,32]

Although the therapeutic effects of Nissle1917 have been reported on human IBD, there have been only a few reports testing its efficacy on animal models to clarify its mechanisms of action. In this study, we showed that Nissle1917 has therapeutic effects on murine acute and chronic colitis without its colonization. Interestingly, Schultz et al[33] reported that Nissle1917 did not have any therapeutic effect on DSS-induced acute colitis, although it improved chronic colitis in an adaptive transfer model. In their report, Nissle1917 did not improve loss of body weight and histologic inflammation at day 7 after administration of DSS. Consistent with their report, our experiments also showed no significant differences in loss of body weight between Nissle1917 and control groups at day 7. However, our results clearly showed that loss of body weight is significantly reduced in the Nissle1917 group after day 8 and that histologic inflammation is reduced at day 10. We speculate that this discrepancy came from the different concentration of DSS used in these studies. The concentration used in this study was lower (1.3%) than the study of Schultz et al (2.0%). This difference may have resulted in our data showing the therapeutic effect of Nissle1917 in this colitis model.

In IL-10$^{-/-}$ mice, immunocompetent cells remain complete expect for their secretory function of IL-10.

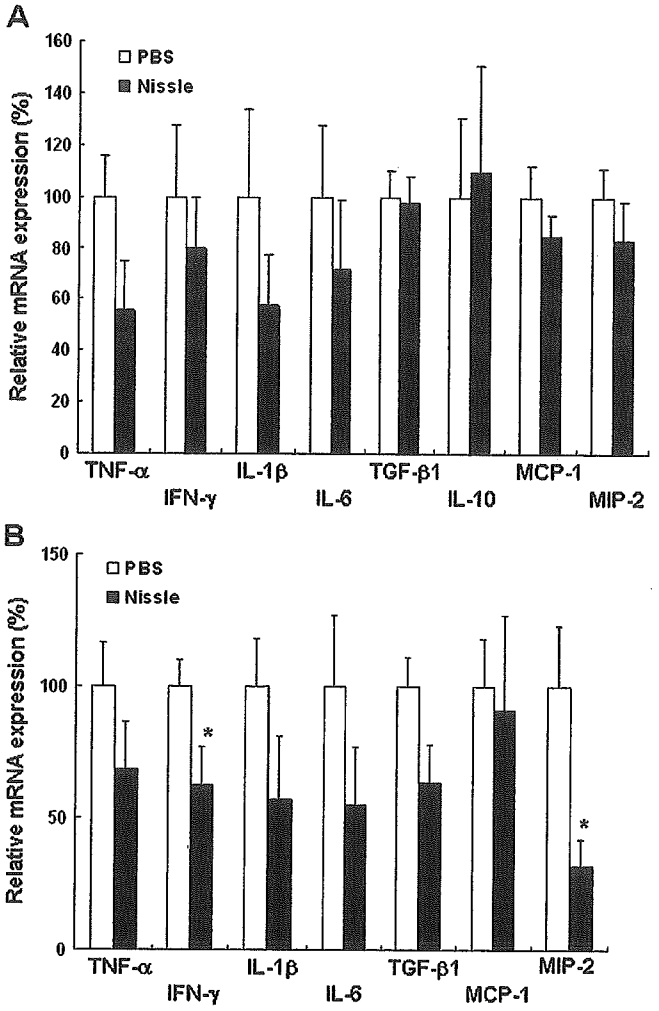

FIGURE 6. Nissle1917 altered the expressions of proinflammatory cytokines and chemokines in LPMCs from DSS colitis and IL-$10^{-/-}$ mice. Total RNA was isolated from LPMC of DSS colitis (A) and IL-$10^{-/-}$ mice (B) treated with PBS or Nissle1917 (n = 8). The expression level of each cytokine was divided by the amount of its own β-actin transcript and expressed as a relative percentage of the PBS-treated group. Results are expressed as mean ± SEM of the data from 3 independent experiments. *, $P < 0.05$ compared with the PBS groups.

Consequently, the IL-$10^{-/-}$ mice model is regarded as a suitable colitis model to comprehend the immunomodulating function of Nissle1917. Hence, in this report, we used IL-$10^{-/-}$ mice for our chronic colitis model. In this model, we first showed that Nissle1917 treatment prevents the onset of rectal prolapse and improves clinical and histologic signs of colonic inflammation. These results suggest that Nissle1917 is also effective on chronic colonic mucosal inflammation. The analysis of cytokines and chemokines expression suggested that Nissle1917 can modulate the expression of proinflammatory cytokine IFN-γ and chemokine MIP-2 in LPMCs from

TABLE 2. Efeect of *E. coli* Nissle1917 Components on DSS-induced Colitis

Treatment	N	DAI (day 7)	Colon Weight (mg/cm)	Histological Score
PBS	19	2.35 ± 0.17	51.68 ± 0.20	10.26 ± 0.55
Live	18	1.61 ± 0.18*	45.43 ± 1.01†	8.89 ± 0.37†
Heat-killed	16	1.83 ± 0.12	46.40 ± 1.25*	8.63 ± 0.43†
DNA	17	1.94 ± 0.20	45.26 ± 1.16†	8.53 ± 0.32†

Data are mean ± SEM.
*$P < 0.05$, and †$P < 0.01$, significant difference from PBS group.

IL-$10^{-/-}$ mice. However, in the acute DSS colitis model, Nissle1917 administration failed to show statistical significance in any cytokine tested, although there seemed to be a similar trend as in the IL-$10^{-/-}$ colitis model. Consistent with our in vivo study, Shultz et al[33] also reported that Nissle1917 modified proinflammatory cytokine production from mesenteric lymph nodes as its therapeutic mechanism. Furthermore, the data presented here indicate that the anti-inflammatory effect of Nissle1917 on murine experimental colitis is preserved even in the heat-killed bacteria and genomic DNA alone. This is the first report to show the therapeutic effect of nonviable Nissle1917 and its DNA. Consistent with our data, the anti-inflammatory effect of DNA from another probiotic, VSL#3, has been reported by another group.[23] Moreover, in their experiments, orally administrated VSL#3 could be detected from lymphoid organs, and its therapeutic effect was lost when it was intrarectally administered. Interestingly, Schultz et al[33] showed in their report that orally administered *E. coli* Nissle1917 could be detected from mesenteric lymph nodes. Taken together, these data indicate that most of the probiotic bacteria, including *E. coli* Nissle1917 and its components, are taken in from the small intestine. After transported to lymphoid organs, they may exert immunosuppressive effects at those sites. In addition, probiotic bacteria are mainly composed of gram-positive bacteria such as *Lactobacillus* or *Bifidobacterium,* but Nissle1917 is a gram-negative nonpathogenic *E. coli.* Because of the unique biologic characteristic of Nissle1917 in that the cell wall components are different from those of other probiotics, there is a possibility that Nissle1917 has a distinct mode of action from other previously reported probiotics.[34–36]

In summary, we have shown for the first time that nonpathogenic *E. coli* strain Nissle1917 prevents the aggravation of murine acute and chronic colitis not only in viable form but also in heat-killed or genomic DNA forms. Our study provides new insights into the efficacy of *E. coli* Nissle1917 in suppressing acute and chronic intestinal mucosal inflammation.

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REFERENCES

1. Podolsky DK. Inflammatory bowel disease. *N Engl J Med.* 2002;347:417–429.
2. Sutherland L, Singleton J, Sessions J, et al. Double blind, placebo controlled trial of metronidazole in Crohn's disease. *Gut.* 1991;32:1071–1075.
3. Mombaerts P, Mizoguchi E, Grusby MJ, et al. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell.* 1993;75:274–282.
4. Dianda L, Hanby AM, Wright NA, et al. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol.* 1997;150:91–97.
5. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun.* 1998;66:5224–5231.
6. Schultz M, Tonkonogy SL, Sellon RK, et al. IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation. *Am J Physiol.* 1999;276:G1461–G1472.
7. Taurog JD, Richardson JA, Croft JT, et al. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med.* 1994;180:2359–2364.
8. Gionchetti P, Rizzello F, Venturi A, et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology.* 2000;119:305–309.
9. Venturi A, Gionchetti P, Rizzello F, et al. Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment Pharmacol Ther.* 1999;13:1103–1108.
10. Kruis W, Schutz E, Fric P, et al. Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther.* 1997;11:853–858.
11. Rembacken BJ, Snelling AM, Hawkey PM, et al. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet.* 1999;354:635–639.
12. Malchow HA. Crohn's disease and Escherichia coli. A new approach in therapy to maintain remission of colonic Crohn's disease? *J Clin Gastroenterol.* 1997;25:653–658.
13. Boudeau J, Glasser AL, Julien S, et al. Inhibitory effect of probiotic Escherichia coli strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. *Aliment Pharmacol Ther.* 2003;18:45–56.
14. Stentebjerg-Olesen B, Chakraborty T, Klemm P. Type 1 fimbriation and phase switching in a natural Escherichia coli fimB null strain, Nissle 1917. *J Bacteriol.* 1999;181:7470–7478.
15. Cooper HS, Murthy SN, Shah RS, et al. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest.* 1993;69:238–249.
16. Dieleman LA, Ridwan BU, Tennyson GS, et al. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology.* 1994;107:1643–1652.
17. Madsen KL, Doyle JS, Jewell LD, et al. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology.* 1999;116:1107–1114.
18. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell.* 1993;75:263–274.
19. Mimura T, Rizzello F, Helwig U, et al. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut.* 2004;53:108–114.
20. Gionchetti P, Rizzello F, Helwig U, et al. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology.* 2003;124:1202–1209.
21. Dieleman LA, Goerres MS, Arends A, et al. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. *Gut.* 2003;52:370–376.
22. Matsumoto S, Watanabe N, Imaoka A, et al. Preventive effects of Bifidobacterium- and Lactobacillus-fermented milk on the development of inflammatory bowel disease in senescence-accelerated mouse P1/Yit strain mice. *Digestion.* 2001;64:92–99.
23. Rachmilewitz D, Katakura K, Karmeli F, et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology.* 2004;126:520–528.
24. Setoyama H, Imaoka A, Ishikawa H, et al. Prevention of gut inflammation by Bifidobacterium in dextran sulfate-treated gnotobiotic mice associated with Bacteroides strains isolated from ulcerative colitis patients. *Microbes Infect.* 2003;5:115–122.
25. Araki Y, Fujiyama Y, Andoh A, et al. The dietary combination of germinated barley foodstuff plus Clostridium butyricum suppresses the dextran sulfate sodium-induced experimental colitis in rats. *Scand J Gastroenterol.* 2000;35:1060–1067.
26. Madsen K, Cornish A, Soper P, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology.* 2001;121:580–591.
27. Schultz M, Veltkamp C, Dieleman LA, et al. Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm Bowel Dis.* 2002;8:71–80.
28. McCarthy J, O'Mahony L, O'Callaghan L, et al. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut.* 2003;52:975–980.
29. Marteau PR, de Vrese M, Cellier CJ, et al. Protection from gastrointestinal diseases with the use of probiotics. *Am J Clin Nutr.* 2001;73:430S–436S.
30. Pena JA, Versalovic J. Lactobacillus rhamnosus GG decreases TNF-alpha production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. *Cell Microbiol.* 2003;5:277–285.
31. Mattar AF, Teitelbaum DH, Drongowski RA, et al. Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. *Pediatr Surg Int.* 2002;18:586–590.
32. Rachmilewitz D, Karmeli F, Takabayashi K, et al. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology.* 2002;122:1428–1441.
33. Schultz M, Strauch UG, Linde HJ, et al. Preventive Effects of Escherichia coli Strain Nissle 1917 on Acute and Chronic Intestinal Inflammation in Two Different Murine Models of Colitis. *Clin Diagn Lab Immunol.* 2004;11:372–378.
34. Grozdanov L, Zahringer U, Blum-Oehler G, et al. A single nucleotide exchange in the wzy gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of Escherichia coli strain Nissle 1917. *J Bacteriol.* 2002;184:5912–5925.
35. Lammers KM, Helwig U, Swennen E, et al. Effect of probiotic strains on interleukin 8 production by HT29/19A cells. *Am J Gastroenterol.* 2002;97:1182–1186.
36. Otte JM, Podolsky DK. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. *Am J Physiol Gastrointest Liver Physiol.* 2004;286:G613–G626.

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GASTROENTEROLOGY

Clinical significance of microsatellite instability in the inflamed mucosa for the prediction of colonic neoplasms in patients with ulcerative colitis

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Abstract

Background and Aim: Although molecular.mechanisms underlying ulcerative colitis (UC)-associated neoplasms have been studied for years, understanding of these mechanisms remains incomplete and no good predictable marker for development of colonic neoplasms in patients with UC has been established. The aim of this study was to assess if microsatellite instability (MSI) contributes to the development of colonic neoplasms in patients with UC.

Methods: We have examined MSI in chronic inflamed and neoplastic colonic mucosa of UC patients. We have also obtained serial biopsied colonic tissues retrospectively 2–12 years before the final diagnosis from patients with high level MSI (MSI-H+) UC-associated neoplasms, and analyzed MSI using them at different periods.

Results: Eight of 12 UC-associated colon cancers (67%), four of six UC-associated high grade dysplasias (67%), and two of six UC-associated low grade dysplasias (33%) revealed MSI-H+ phenotypes. In contrast, 15 of 59 lesions (25%) in inflamed UC mucosa without colonic neoplasm revealed MSI-H +. Interestingly, all four patients with MSI-H+ phenotypes at the final diagnosis of UC-associated colon cancer or dysplasia had already had MSI-H+ at the stage of chronic colitis, 2–12 years before the final diagnosis.

Conclusion: These results support the notion that MSI contributes to the carcinogenesis of UC-associated neoplasms, and indicate that this analysis in inflamed colonic mucosa at surveillance colonoscopy is useful for identifying UC patients who have high risk for neoplastic progression.

Key words: colon cancer, microsatellite instability, ulcerative colitis.

INTRODUCTION

The cumulative colon cancer rate is 7% at 20 years and 17% at 30 years following the onset of ulcerative colitis (UC).[1] Because of this high colon cancer incidence, regular surveillance colonoscopy is recommended for patients with long-standing extensive UC. However, a colonic surveillance program has led to the detection of only a small portion of colitis-associated cancers, with over 40% of these being Duke's C or more advanced.[2–5] Dysplasia is sometimes very focal and can hardly be detected endoscopically in some cases. Therefore, random colonic mucosal biopsies have been recommended for UC patients, however, the number of biopsy specimens required is large and the cost of this procedure is very high.[6,7] Histological diagnosis is difficult in some cases for distinguishing dyplasias from regenerative changes. In Japan, it was reported that 55 of 192 UC patients with colorectal cancer (29%) were with less than 10 years duration of UC.[8] Because

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various problems are raised in the present surveillance colonoscopy program, as described above, it is crucial to establish an effective and simple surveillance program for UC patients. We have attempted to apply molecular biological techniques to colorectal cancer surveillance for UC patients.

We have previously reported that MSI in biopsy specimens at the stage of chronic gastritis is a predictor for gastric neoplastic progression in patients with chronic gastritis.[9] Well-differentiated gastric adenocarcinoma and adenoma arise from intestinal metaplasia-associated gastritis mucosa. With regard to this point, UC associated neoplasms arise from colonic inflamed mucosa as well. Recent studies have reported that replication error (RER) phenotypes are detected in UC related neoplasms and UC non-neoplastic mucosa.[10–16] However, serial analysis of MSI from the same UC patient has not been reported in the development of UC associated neoplasms. It still remains unclear if this MSI detection has any advantages in surveillance for colon cancer in UC patients. In the present study, we first analyzed mutations of microsatellite loci in biopsied paraffin-embedded tissues from patients retrospectively, to elucidate if MSI leads to the progression from colonic inflamed mucosa to dysplasia and adenocarcinoma in UC patients. We also investigated the relationship between MSI-H+ phenotypes and clinicopathological features in patients with UC.

METHODS

Patients and tissue preparation

We obtained colonic premalignant or malignant tissues from 12 patients with UC, composed of 12 UC associated adenocarcinomas and 12 UC associated dysplasias. Also, 59 colonic inflammatory tissues from 20 patients with UC without neoplasms were obtained. Serial biopsied colonic tissues were obtained from four patients with colitis associated neoplasms to examine MSI phenotypes, endoscopic and histological findings retrospectively 2–12 years before the final diagnosis. Severity of the inflammation of UC inflamed mucosa was evaluated according to a previous report.[17] All tissues were biopsy specimens or surgically resected specimens. Corresponding normal tissues or peripheral-blood lymphocytes were used as a control in the analysis of microsatellite alterations. All tissues were fixed in formalin and embedded in paraffin. All human studies were approved by the ethical review board of our institution.

Histological evaluation

Degree of dysplasia was diagnosed according to previously published criteria.[18] UC associated colorectal cancers were staged according to the fifth edition of the American Joint Committee on Cancer (AJCC) staging system.[19]

Analysis of microsatellite alterations

Genomic DNA was extracted by a standard proteinase K digestion and phenol/chloroform extraction procedure. Analysis of microsatellite alterations was performed with a set of five microsatellite markers (D2S119, D2S123, D3S1265, D5S409, BAT26). The extracted DNA was amplified by polymerase chain reaction (PCR) with an appropriate pair of biotinylated primers for each locus. The sequences of these primers were described previously.[20] Thirty-five cycles of PCR were performed using a denaturation step of 94°C for 0.5 min, an annealing step of 54°C for 1 min, and an elongation step of 72°C for 1 min in a total volume of 50 μL in 1 × PCR buffer containing 20 pM of each primer, 1 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 units AmpliTaq DNA polymerase (Sawaday, Tokyo, Japan), and 100 ng genomic DNA. Forty-five μL of the PCR product was added with 5 μL of a 10 × gel loading buffer, and heated at 96°C for 3 min. Electrophoresis was performed on a 6% polyacrylamide gel containing 7 M urea at 30 W for 3.5–4 h. The gel was transferred to membrane, treated with a biotin-streptavidin system[21] and exposed to X-ray film for 30 min. Bitoinylated DNA blotted onto a positively charged nylon membrane was detected with a chemiluminescence method according to the manufacturer's procedure (Imaging high, Chemilumi, Toyobo, Osaka, Japan). Biotin was linked to alkaine phosphatase (ALP) with conjugated streptavidin and then ALP activity was detected with a chemiluminogenic substrate. Samples with microsatellite alterations in two or more loci were defined as high-level MSI (MSI-H+) phenotypes.

Statistical analysis

Fisher's exact tests were used to analyze statistical correlation between MSI-H+ and other variables. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features

Eight UC patients had a solitary neoplastic lesion and the remaining four patients had multiple neoplastic lesions. Nine of 12 UC associated adenocarcinomas (75%) were in the recto-sigmoid. Histologically, seven of 12 UC associated cancers were well differentiated adenocarcinomas and the remaining five UC associated cancers were moderately differentiated or poorly differentiated adenocarcinomas. One patient died of carcinomatous peritonitis (had already had it when diagnosed).

Microsatellite instability

Microsatellite alterations at five loci were examined in 12 UC associated colon cancers and in 12 UC associated dysplasias. Eight of 12 cancers (67%) showed

MSI-H+ phenotypes (Fig. 1). The correlations between clinicopathological features and MSI-H+ phenotypes are summarized in Table 1. MSI-H+ phenotypes were detected in six of eight UC associated adenocarcinomas in the rectum and in two of six UC associated adenocarcinomas in the colon. MSI-H+ phenotypes were detected in five of seven well differentiated adenocarcinomas, in two of four moderately differentiated adenocarcinomas, and in one poorly differentiated adenocarcinoma. MSI-H+ phenotypes were detected in six of seven adenocarcinomas with lymph node metastasis and in two of five of those without lymph node metastasis. Four of six UC associated high grade dysplasia (67%) and two of six UC associated low grade dysplasia (33%) showed MSI-H+ (Table 2).

We then examined MSI-H+ phenotypes in inflamed colonic mucosa of 20 patients with UC without cancer (Fig. 2). MSI-H+ phenotypes were demonstrated in 15 of 59 samples (25%). They are significantly less frequent than those of UC associated adenocarcinomas ($P = 0.008$). Among 20 patients, nine patients had at least one inflammatory lesion with MSI-H+ phenotypes. The other 11 patients had no inflammatory lesions with MSI-H+ phenotypes. MSI-H+ phenotypes tended to be more frequently detected in colonic inflammatory mucosa from patients of chronic, continuous type than those of relapsing-remitting type ($P = 0.13$) (Table 3). Interestingly, MSI-H+ phenotypes were not related to disease duration of UC ($P = 0.5$).

Among 12 patients with UC associated neoplasms, inflamed colonic mucosa at the stage of chronic colitis

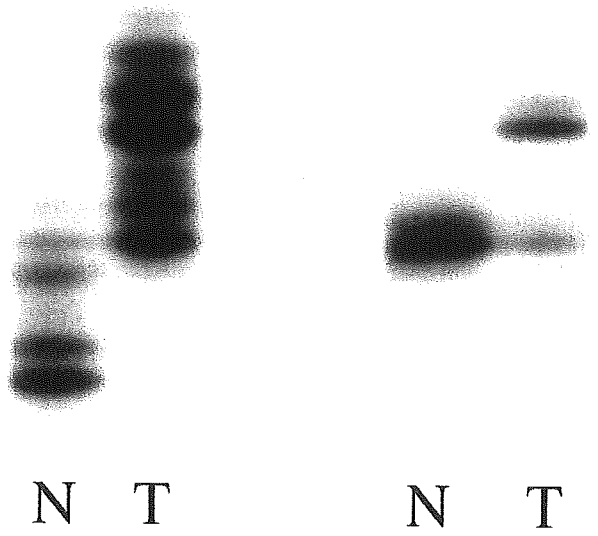

Figure 1 Microsatellite instability detected in ulcerative colitis (UC) associated colon cancers. N, normal; T, UC associated colon cancer. Primers used in the left and right panels were D2S123 and D2S119, respectively.

Table 1 Clinicopathological features and microsatellite instability in ulcerative colitis associated colorectal cancers

Category	No. of samples	MSI-H+
Tumor location		
Rectum	8	6
Sigmoid colon	1	1
Descending colon	2	1
Transverse colon	1	0
Histological differentiation		
Well differentiated	7	5
Moderately differentiated	4	2
Poorly differentiated	1	1
Depth of invasion		
Tis	4	3
T1	3	2
T2	1	1
T3 and T4	2	2
Lymph node metastasis		
Negative	7	6
Positive	3	2

Table 2 Histology and microsatellite instability in ulcerative colitis

Histology	No. of samples	MSI-H+ (%)	*P*-value
Adenocarcinoma	12	8 (67)	0.008*
High grade dysplasia	6	4 (67)	0.06**
Low grade dysplasia	6	2 (33)	NS***
Inflamed mucosa	59	15 (25)	–

*Adenocarcinoma *vs* inflamed mucosa; **high grade dysplasia *vs* inflamed mucosa; ***low grade dysplasia *vs* inflamed mucosa. NS, not significant (by Fisher's exact tests).

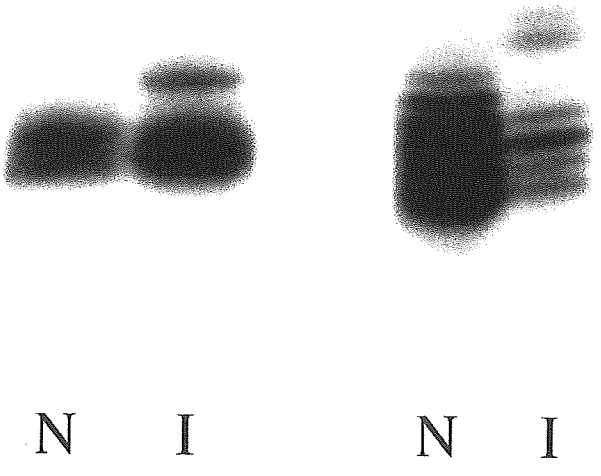

Figure 2 Microsatellite instability detected in the colonic inflamed mucosa from patients with ulcerative colitis (UC). N, normal; I, inflamed colonic mucosa. Primers used in the left and right panels were D2S119 and D2S123, respectively.

Table 3 Clinical features of ulcerative colitis patients without neoplasms

Category	No. of patients	MSI-H+	Male/female	Age range (mean)	*P*-value
Clinical type					
Chronic continuous	7	5	3/4	29–67 (44.7)	0.13*
Relapsing-remitting	12	4	5/7	19–78 (45.2)	
Duration (years)					
<10	9	4	3/6	15–63 (33.0)	0.5**
≥10	11	5	4/7	31–78 (52.1)	
Extension					
Total colitis	12	6	5/7	19–78 (45.7)	NS***
Left sided	7	3	2/5	27–63 (44.0)	
Proctitis	1	0	0/1	15 (15.0)	

*Chronic continuous *vs* relapsing-remitting; **<10 years *vs* ≥10 years; ***total colitis *vs* left sided. NS, not significant (by Fisher's exact tests).

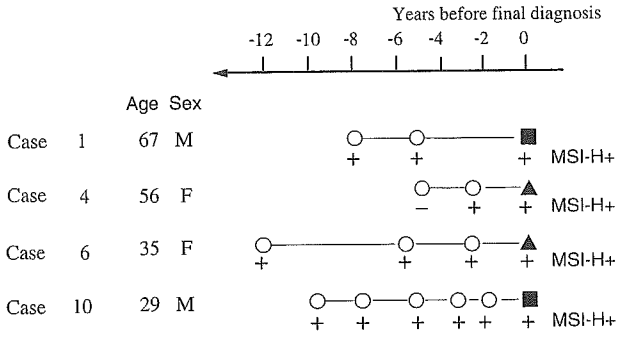

Figure 3 Microsatellite instability and histological findings in serial biopsy specimens from the same patient. Mutations of microsatellite loci in four patients with ulcerative colitis (UC) associated colon cancer or dysplasia, who showed MSI-H+ phenotypes in final diagnosis, were analyzed retrospectively for 2–12 years before final diagnosis at different time points. All four patients had already revealed MSI-H+ at the stage of chronic colitis 2–12 years before the final diagnosis. (○) Chronic colitis; (▲) dysplasia; (■) adenocarcinoma.

could be obtained 2–12 years before the development of neoplasms in four patients. We then analyzed MSI-H+ phenotypes at the stage of chronic colitis in these cases. Interestingly, all four patients who showed MSI-H+ at the diagnosis of neoplasm had already showed MSI-H+ phenotypes at the stage of chronic colitis (Fig. 3). In case 1, 6 and 10, MSI-H+ phenotypes were detected at any stages of chronic colitis. In case 1, colonic adenocarcinoma developed 8 years after the diagnosis of UC, and MSI-H+ was already demonstrated at the initial diagnosis. Interestingly, in case 4, MSI-H+ phenotypes were detected 2 years before the final diagnosis of dysplasia, although MSI-H+ phenotypes had not been detected 5 years before.

DISCUSSION

MSI-H+ is detected in approximately 15% of sporadic colon cancer, and is more frequently seen in proximal colon cancer, associated with improved patient survival.[22–25] There have been a few reports in UC associated neoplasms and UC non-neoplastic mucosa.[10–16] Brentnall *et al.* detected MSI in 46% of high grade dysplasias, in 40% of carcinomas, and in 50% of non-neoplastic mucosa.[10] Ishitsuka *et al.* found MSI in 8% of dysplasias, in 50% of carcinomas, and in 9% of non-neoplastic mucosa from UC patients, who had a long duration with neoplasms.[12] In the present study, we detected MSI in 67% of high grade dysplasias, in 67% of carcinomas, and in 25% of non-neoplastic mucosa. MSI in our study was more frequent than those in previously reported data, especially in UC associated neoplasms. We speculate that the difference in the results might be due to the differences in the criteria of MSI, PCR primers used, race or patients' background. Our results are consistent with the fact that multiple tumors tend to develop simultaneously in UC associated cancer, as it is generally accepted that MSI may play a role in the development of multiple primary cancers of the gastrointestinal tract.[26] With regard to the mechanism responsible for MSI, Fleisher *et al.* reported that hMLH1 hypermethylation causes MSI in at least a subset of UC associated neoplasms.[14] Furthermore, Cravo *et al.* have reported that a defect in DNA repair associated with a low folate status may be one additional cause for patients with UC exhibiting MSI in non-neoplastic mucosa.[16] Importantly, chronic inflammation in UC patients probably leads DNA damage to exceed the capacity of DNA repair mechanisms.[10]

Consistent with this hypothesis, we found that three of four patients already had MSI-H+ more than 7 years before the development of neoplasms, and that MSI-H+ was detected at all stages of chronic colitis. Interestingly, MSI-H+ had been detected at the onset of UC in case 1. These results indicate that MSI may be an early event in the progression of UC associated carcinogenesis. Therefore, MSI in colonic mucosa at the stage of chronic colitis in UC patients may predict high risk for neoplastic progression. Interestingly, these data were similar to our previous reports of microsatellite alteration in the progression of gastric adenoma and well-differentiated adenocarcinoma.[9] These results support the notion that MSI may be a significant and early event in the carcinogenesis from chronic inflammation in a

subset of the primary cancers of the gastrointestinal tract.

We previously reported that interferon inducible gene family 1–8 U expression was significantly higher in UC associated cancer tissues than in mucosa with mild inflammation in the same UC patients.[27] This gene was also expressed in inflamed colonic mucosa of UC without cancer. This gene expression was not related to the duration of the disease, but the disease activity.

Interestingly, MSI-H+ in inflamed mucosa in UC patients without neoplasms were not correlated with disease duration of UC as well. Taken together, it is suggested that genetic events precede histological progression of UC associated cancer and dysplasia. MSI-H+ phenotypes were more frequently detected in colonic inflammatory mucosa from patients of chronic, continuous type than those of relapsing-remitting type, although statistically not significant. Our analysis of MSI and 1–8 U indicates that the development of UC associated neoplasms may not be dependent only on duration of the inflammation, but on both duration and severity of the inflammation. These findings may explain the fact that UC patients with chronic continuously severe inflammation developed UC associated neoplasms in shorter periods of time than previously reported.[27] The analysis of MSI may be useful for UC patients with a short duration of the disease. We therefore propose that the analysis of MSI at surveillance colonoscopy could improve a conventional surveillance program targeted only at long-standing extensive UC patients.

Even if endoscopists fail to detect the presence of UC associated dysplasia or cancer, analysis of microsatellite alteration may provide us with information such as the risk of cancer development, and may be helpful in deciding the appropriate period and interval of surveillance colonoscopy. The detection of MSI-H+ in the rectal inflamed mucosa is slightly more frequent than at another sites, although statistically not significant. These findings may explain the fact that the rectum is typically the most inflamed site where the epithelium receives most cellular damage by inflammation, and that two-thirds of UC associated neoplasms develop in the recto-sigmoid.[4] The analysis of rectal mucosa is useful for patients whose total colonoscopy is difficult or impossible, and for patients who refuse surveillance colonoscopy because of physical discomfort of total colonoscopy.

Thus, our results suggest that the analysis of microsatellite alteration in inflamed mucosa has potential as a complementary tool for screening for colorectal cancer in UC. Prospective studies are warranted to establish the most efficient surveillance program for UC patients with the analysis of MSI.

In conclusion, the analysis of microsatellite alteration in inflamed mucosa at surveillance colonoscopy might be an effective method for detecting UC patients at high risk for developing colonic neoplasms.

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REFERENCES

1 Lynch DAF, Lobo AJ, Sobala GM, Dixon MF, Axon ATR. Failure of colonoscopic surveillance in ulcerative colitis. *Gut* 1993; **34**: 1075–80.
2 Gyde S. Screening for colorectal cancer in ulcerative colitis. *Gut* 1990; **31**: 1089–92.
3 Bernstein CN, Shanahan F, Weinstein WM. Are we telling patients the truth about surveillance colonoscopy in ulcerative colitis? *Lancet* 1994; **343**: 71–4.
4 Lennard-Jones JE. Is clonoscopic cancer surveillance in ulcerative colitis essential for every patient? *Eur. J. Cancer* 1995; **31**: 1178–82.
5 Axon ATR. Colonic cancer surveillance in ulcerative colitis is not essential for every patient. *Eur. J. Cancer* 1995; **31**: 1183–6.
6 Taylor BA, Pemberton JH, Carpenter HA *et al.* Dysplasia in chronic ulcerative colitis: implications for colonoscopic surveillance. *Dis. Colon Rectum* 1992; **35**: 950–6.
7 Lashner BA, Hanauer SB, Silverstein MD. Optimal timing of colonoscopy to screen for cancer in ulcerative colitis. *Ann. Int. Med.* 1988; **108**: 274–8.
8 Saito S, Nagamine K, Komatsu S *et al.* Five cases of colorectal cancer complicated with ulcerative colitis. *Jap. J. Gastroenterol.* 1998; **95**: 539–46.
9 Kashiwagi K, Watanabe M, Ezaki T *et al.* Clinical usefulness of microsatellite instability for the prediction of gastric adenoma or adenocarcinoma in patients with chronic gastritis. *Br. J. Cancer* 2000; **82**: 1814–8.
10 Brentnall TA, Cripspin DA, Bronner MP *et al.* Microsatellite instability in non-neoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res.* 1996; **56**: 1237–40.
11 Suzuki H, Harpaz N, Tarmin L *et al.* Microsatellite instability in ulcerative colitis-associated colorectal dysplasias and cancers. *Cancer Res.* 1994; **54**: 4841–4.
12 Ishitsuka T, Kashiwagi H, Konishi F. Microsatellite instability in inflamed and neoplastic epithelium in ulcerative colitis. *J. Clin. Pathol.* 2001; **54**: 526–32.
13 Umetani N, Sasaki S, Watanabe T *et al.* Genetic alterations in ulcerative colitis-associated neoplasia focusing on APC, K-ras gene and microsatellite instability. *Jpn. J. Cancer Res.* 1999; **90**: 1081–7.
14 Fleisher AS, Esteller M, Harpaz N *et al.* Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1. *Cancer Res.* 2000; **60**: 4864–8.
15 Noffsinger AE, Belli JM, Fogt F, Fischer J, Goldman H, Fenoglio-Preiser CM. A germline hMSH2 alteration is unrelated to colonic microsatellite instability in patients with ulcerative colitis. *Hum. Pathol.* 1999; **30**: 8–12.
16 Cravo ML, Albuquerque CM, Sousa LS *et al.* Microsatellite instability in non-neoplastic mucosa of patients with ulcerative colitis: effect of folate supplementation. *Am. J. Gastroenterol.* 1998; **93**: 2060–4.

17 D'Haens G, Lemmens L, Geboes K *et al.* Intravenous cyclosporine versus intravenous corticosteroids as single therapy for severe attacks of ulcerative colitis. *Gastroenterology* 2001; **120**: 1323–9.
18 Riddell RH, Goldman H, Ransohoff DF *et al.* Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum. Pathol.* 1983; **14**: 931–68.
19 Fleming ID, Cooper JS, Henson DE *et al. American Joint Committee on Cancer Staging Manual*, 5th edn. Philadelphia: Lipponcott-Raven, 1997.
20 Tamura G, Maesawa C *et al.* Microsatellite alterations in adenoma and differentiated adenocarcinoma of the stomach. *Cancer Res.* 1995; **55**: 1933–6.
21 Beck S, O'keeffe T, Coull JM, Koster H. Chemiluminescent detection of DNA: application for DNA sequencing and hybridization. *Nucl. Acids Res.* 1989; **17**: 5115–23.
22 Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993; **363**: 558–61.
23 Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993; **260**: 816–9.
24 Bubb VJ, Curtis LJ, Cunningham C *et al.* Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. *Oncogene* 1996; **12**: 2641–9.
25 Jernvall P, Makinen MJ, Karttunen TJ, Makela J, Vihko P. Microsatellite instability: impact on cancer progression in proximal and distal colorectal cancers. *Eur. J. Cancer* 1999; **35**: 197–201.
26 Yamashita K, Arimura Y, Kurosawa S *et al.* Microsatellite instability in patients with multiple primary cancers of the gastrointestinal tract. *Gut* 2000; **46**: 790–4.
27 Hisamatsu T, Watanabe M, Ogata H *et al.* IFN-inducible gene family 1–8U expression in colitis-associated colon cancer and severely inflamed mucosa in ulcerative colitis. *Cancer. Res.* 1999; **59**: 5927–31.

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Leukocytapheresis in Ulcerative Colitis: Results of a Multicenter Double-Blind Prospective Case-Control Study with Sham Apheresis as Placebo Treatment

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OBJECTIVE: Leukocytapheresis (LCAP) is a method of therapeutic apheresis that removes peripheral leukocytes. Previous studies showed that in patients with ulcerative colitis (UC), LCAP was more effective than high-dose steroid therapy, and it had few adverse effects. We investigated LCAP in a multicenter study using active and sham devices in a double-blind study in order to elucidate the placebo effect of extracorporeal treatment including anticoagulant medication.

METHODS: Twenty-five patients with active UC of severe or moderately severe grade were enrolled and assigned to the active group or the sham group. Six patients were excluded from the study and 19 (10 in the active group and nine in the sham group) were evaluated. LCAP (treatment using an active device or a sham device) was performed once a week for 5 wk, followed by two additional sessions during the next 4 wk at 2-wk intervals. Steroids and other medications were continued at the same dosage for 4 wk, which included a 2-wk pre-observation period and the first 2 wk after the start of the LCAP treatment. New medications or increase in the dosage of previous medication were prohibited until evaluation was conducted.

RESULTS: The clinical activity index (CAI) value of UC, indicated that the active group showed a significantly greater improvement (80%, 8/10) than the sham group (33%, 3/9; $p < 0.05$). Adverse effects were observed in five patients (one in the active group and four in the sham group). None of these effects was severe and none of the sessions was terminated as a consequence of the adverse effects.

CONCLUSION: The results confirmed that LCAP is a safe and effective therapeutic option for patients with active UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) of the colonic mucosa. Knowledge of the etiology and underlying immunoinflammatory pathomechanisms of UC is inadequate. In general, acute or long-term medical therapy aims at inducing and maintaining the disease activity in the remission stage (1, 2). With regard to medical therapies, 5-aminosalicylate and related compounds, such as sulfasalazine, have been the standard treatments for mild to moderate UC (1–4). However, the efficacy of these medications is limited due to the presence of considerable number of nonresponders. In the 1950s, corticosteroids were also introduced in UC therapy (5) and these have been commonly used in patients who show an inadequate response to treatment with 5-aminosalicylate based compounds, and/or the symptoms of UC are moderate to severe (1, 2). Nevertheless, the nonresponders still account for up to 40% of the patients. Further, the medical treatment has a high frequency of side effects such as osteoporosis; therefore, drugs have to be administered with careful management of dosage and duration.

In addition, medication with immunomodulators such as azathioprine (6), 6-mercaptopurine (7), and cyclosporine (8) has been employed in the treatment of steroid dependent and steroid-resistant UC. Anti-tumor necrosis factor (TNF)-α antibody (Infliximab) and other antibodies have also entered

clinical practice in the treatment of UC (9, 10). The additional use of such newly developed drugs helps to reduce the steroid requirements and to achieve stable remission of UC. The spectrum still remains unsatisfactory for several UC patients, in spite of the medical therapy options that exist.

Surgical procedure of colectomy may be more appropriate for such drug therapy-resistant patients. However, postoperative complications and poor functional results were observed in some cases (11). Therefore, alternative therapies with high efficacy and fewer adverse effects are still desirable.

In compliance with this requirement, LCAP was introduced as a new therapy for UC patients who showed limited response to medical therapies (12–14). An open-label multicenter study showed that the efficacy of LCAP was higher (29/39, 74%) than that of high-dose steroid treatment (14/37, 38%) with statistical significance ($p = 0.005$) (15). In these studies, LCAP was performed using Cellsorba, which is a column device consisting of a non-woven fabric-based leukocyte removal filter. Granulocyte and monocyte adsorption apheresis (GCAP) was also reported to be effective for active UC (12, 16–19). GCAP was performed using Adacolumn, which is filled with cellulose acetate beads. In addition, Ayabe *et al.* (20, 21) reported that centrifugal LCAP was effective in the treatment of patients with severe and corticosteroid-resistant UC.

However, there was a concern that the efficacy of these therapies was due to the placebo effect of extracorporeal circulation therapy along with the administration of an anticoagulant. In this paper, we describe the results of a double-blind case-controlled clinical trial that was conducted to determine the efficacy of LCAP in the treatment of UC, in comparison with the placebo treatment using a sham device.

MATERIALS AND METHODS

Study Design, Patients, and Group Allocation

The study was conducted at six institutions represented by the coauthors between June 1998 and March 2001. The study protocol was approved by the institutional review board of each institution. The inclusion criteria were essentially identical to those reported in our previous study (15) and are as follows: active-stage, moderately severe, or severe UC with the Rachmilewitz's CAI value of 6 or higher (22); extent of the lesion being pancolitis or left-sided colitis; incidence of bloody stool greater than 6 times/day and requiring the administration of IV steroid infusion under fasting conditions; and no response to steroids during the pre-observation period of 2 wk prior to the start of LCAP treatment. The activity of UC was confirmed by endoscopy performed during the pre-observation period. The exclusion criteria were as follows: drug treatment with immunosuppressive agents within 4 wk prior to the commencement of the study and previous experience with LCAP or another leukocyte apheresis.

The assignment of the enrolled patients to the active group or the sham group was performed by a controller who was independent of the other staff, patients, and relatives. The patients who had registered via facsimile to the controller were assigned to groups in order to match their background based on the minimization method reported by Pocock (23).

To ensure proper blinding within the clinical evaluation, the medical staffs of each institution were separated into two independent groups. One group consisted of doctors and nurses in charge of patients' medication or evaluation, and the other consisted of doctors and technicians in charge of the extracorporeal therapy only. For the endoscopy evaluation, the endoscopic images of each case were collected at a clinical center and evaluated by two independent endoscopists.

The patients enrolled in the study were hospitalized during the entire study, including the pre-observation period. All patients provided their written informed consents for participation.

Treatment Procedure

The LCAP treatment procedure was carried out using a leukocyte removal column, Cellsorba (Asahi Medical Co. Ltd., Tokyo, Japan). The column contains a polyester nonwoven fabric, which can trap leukocytes from whole blood (12, 15, 24). The filter mainly traps monocytes and granulocytes (>95%), partially traps lymphocytes and platelets (30–90%), and it does not trap erythrocytes (<10%).

The placebo treatment was carried out using a sham column that consisted of the same materials as the active column, but without the fabric filter inside it. Both columns were covered with an opaque material so that they could not be distinguished by the patients. Other conditions, including anticoagulant medication, were equivalent within both groups.

Extracorporeal circulation therapy was performed as follows: The patient's whole blood from the cubital vein was pumped out at approximately 50 ml/min and introduced into the column. Fifty milligrams of nafamostat mesilate (an anticoagulant; Torii Pharmaceutical Co., Tokyo, Japan) (25) was continuously introduced into the inlet side of the column. The processed blood was subsequently returned to the patient via the cubital vein of the contralateral arm. Generally, 3.0 $\pm$ 0.5 L of blood was processed within approximately 1 h.

As indicated in Figure 1, this session was performed once a week for 5 wk (weeks 1–5), followed by two further sessions during the next 4 wk (weeks 6–9). In patients with severe UC, two sessions were allowed during the first week.

Medication and Nutrition

Since enrollment in the protocol, except for the steroid dosages, other medications remained unchanged. New drug administration and an increase in the dosages of the previous drugs were prohibited during the entire study period. Steroid medications were controlled as follows: The patients in both groups were administered prednisolone at a dose of 15 to 20 mg/day (0.3 $\pm$ 0.05 mg/kg/day) for moderately severe

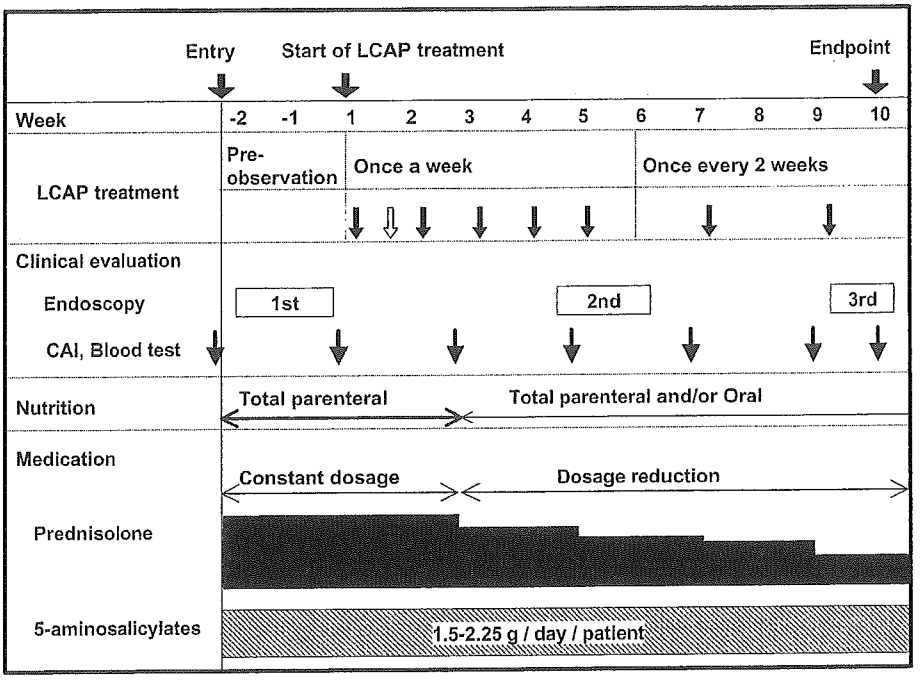

Figure 1. Schematic drawing of the study protocol. Closed arrows in the session and clinical evaluation rows indicate the time of the corresponding events. The open arrow in the session row indicates the optional session, which was performed for a severe-category patient. In the medication row, the closed and hatched boxes indicate the medication periods and dosages of prednisolone and 5-aminosalicylates, respectively.

patients and 30 to 40 mg/day (0.6 ± 0.1 mg/kg/day) for severe patients. The steroid medication was continued at the same dose for 4 wk, which included a 2-wk pre-observation period and the first 2 wk after the start of the LCAP treatment. After week 2, the dosage was reduced based on the improvement in the CAI value, which was evaluated once every 2 wk during the study. The reduction rate was 10 mg/day when the initial dose was more than 30 mg/day, and 5 mg/day in cases where the initial dose was equal to or less than 30 mg/day. The steroid was administered orally; however, intravenous injection was permitted in cases where the dosage was equal to or more than 30 mg/day or when the patient could not tolerate oral administration. All enrolled patients were kept under total parenteral nutrition (TPN). Oral food intake was prohibited until week 2.

Evaluation of CAI and Efficacy

As shown in Figure 1, CAI evaluations were performed at the following time points: before the entry into the study; at week 1 before the start of the LCAP treatment; at weeks 2, 4, 6, and 8; and at the endpoint of week 10. The treatment efficacy was evaluated by comparing the CAI values, at the start and at the endpoint of the LCAP treatment. The criteria are as follows: excellent improvement, CAI fell to 0 point; moderate improvement, CAI improved more than 3 points; negligible, CAI improvement was 0–3 points; deterioration, CAI value decreased.

Endoscopic Analysis

Endoscopic analyses were also performed to evaluate the efficacy of the study. Flexible sigmoidoscopic examinations were performed twice for each patient as indicated in Figure 1; the first examination was performed in the pre-observation period within 2 wks prior to the start of the LCAP treatment and the second examination was performed between weeks 4 and 6. Patients who responded to the therapy underwent a third endoscopic evaluation in week 10. Rachmilewitz's endoscopic index (22), which is based on the observation of granulation scattering reflected light, vascular pattern, vulnerability of mucosa, and mucosal damage were utilized for the evaluation. In addition, a number of biopsy samples were taken at each endoscopic examination from the most damaged mucosa and then evaluated for cell infiltration.

All endoscopic images were collected in one institute and evaluated by two independent endoscopists who were blinded from the patients and doctors. The biopsy samples were also collected in one institute and evaluated in a laboratory by two independent pathologists who were also blinded.

Adverse Effects

The classification of adverse effects was as follows: None, no sign of adverse effects; Mild, occurrence of adverse effects requiring continuing observation but no specific therapy; Moderately severe, occurrence of adverse effects requiring therapeutic intervention but no temporary cessation or discontinuation of session; Severe, occurrence of adverse

Table 1. Patient Data at the Time of Entry into the Study

	Active Group	Sham Group
Sample size	10	9
Age (yr)[a]	26.8 ± 17.3	31.0 ± 11.7
Sex (male/female)	6 (60.0%)/4 (40.0%)	8 (88.9%)/1 (11.1%)
Weight (kg)[a]	49.1 ± 5.2	53.7 ± 9.3
Disease duration (month)[a]	52.8 ± 46.7	62.2 ± 79.6
Steroid dosage till date (mg)[a]	16,970 ± 35,210	19,617 ± 38,230
Previous illness (presence/absence)	8 (80.0%)/2 (20.0%)	8 (88.9%)/1 (11.1%)
Complications (presence/absence)	8 (80.0%)/2 (20.0%)	8 (88.9%)/1 (11.1%)
Severity (severe/moderately severe)	0 (0.0%)/10 (100%)	2 (22.2%)/7 (77.7%)
Extent of lesion (total/left-sided)	10 (100%)/0 (0.0%)	7 (77.7%)/2 (22.2%)
CAI [a,b]	11.4 ± 3.2	10.2 ± 2.6
Endoscopic index [a,b]	10.5 ± 1.8	10.6 ± 1.4

[a] Values are expressed as mean ± standard deviation.
[b] CAI value and endoscopic index were estimated by the method reported by Rachmilewitz (22).

effects with sequelae or threat of mortality. In case the session was discontinued because of an adverse effect related to the use of device, it was classified as severe. Conventional blood tests were performed to monitor the patients' conditions and assess the adverse effects as indicated in Figure 1.

Statistical Analysis

The comparison between the CAI value and the endoscopic index was assessed by the Wilcoxon single rank test and the Student's *t*-test. The efficacy and adverse effects were evaluated by the Mann-Whitney U test. For intergroup comparisons of backgrounds of patients, the Mann-Whitney U test was used for age, disease duration, and the total steroid dosage; the χ^2 test was used for other factors. A p-value of 0.05 or less was considered statistically significant.

RESULTS

Enrollment and Patient Background

Twenty-five patients were initially enrolled in the study, and 13 were assigned to the active group and 12 to the sham group. Three patients from each group were excluded during the pre-observation period, because two patients from each group had improved, and the others had worsened to fulminant cases. These were contrary to the inclusion criteria. Therefore, 10 patients in the active group and nine in the sham group were evaluated for the final study results. The details of the backgrounds of patients from each group are shown in Table 1. As shown in this table, imbalances between the groups were present to a certain extent with regard to gender, number of severe cases, extent of the lesion, and CAI values. In the active group, all patients had total colitis and the mean CAI value of this group was 1.2 grades higher than that of the sham group. On the contrary, two severe cases existed in the sham group and none in the active group.

Evaluation of Therapeutic Effects

According to the CAI-based efficacy assessment, the active group showed more improvement than the sham group. As shown in Table 2, the active group showed excellent improvement and moderate improvement at the rates of 20% and 60%, respectively. On the other hand, the sham group showed these improvements at 11.1% and 22.2%, respectively. In addition, no deterioration was observed in the active group, while 33.3% patients deteriorated in the sham group. The rate of 80% improvement (excellent and moderate) for the active group is significantly superior to that of 33.3% for the sham group with statistical significance ($p < 0.05$).

For detailed evaluation, the CAI values of all patients were plotted, as shown in Figure 2. The averages of the CAI values of the active group were significantly improved from 11.4 ± 3.2 at the start of the LCAP treatment to 4.0 ± 2.9 at the endpoint ($p = 0.002$), while those of the sham group changed from 10.2 ± 2.6 to 7.2 ± 5.2 without statistical significance ($p = 0.24$). However, at the endpoint, the value of the active group (4.0 ± 2.9) showed more improvement than that of the sham group value (7.2 ± 5.2); the statistical significance of this result ($p = 0.054$) was just below the cut off established for statistical significance ($p = 0.05$). The responses of the two patients classified as severe UC and assigned to the sham group were as follows: the response in one patient was classified under the category "moderate improvement," while the other patient showed no response and was classified as "negligible" (Table 2). The remaining seven cases in the sham group responded with diverse efficacy from "excellent improvement" to "deterioration." Consequently, the difference in the severity did not indicate a clear difference in the efficacy. Clear differences in the efficacy were not observed with respect to the gender, extent of the lesion, and differences in the background of the patient since the number of samples was limited.

Table 2. Evaluation of Efficacy

Group	Excellent Improvement[a]	Moderate Improvement [a]	Negligible [a]	Deterioration [a]	U test [b]
Active	2	6	2	0	
	20.0%	60.0%	20.0%	0.0%	*
Sham	1	2	3	3	($p < 0.05$)
	11.1%	22.2%	33.3%	33.3%	

[a] Efficacy of each grade was basically evaluated by comparing CAI values as described in the Materials and Methods section.
[b] The rates of excellent or moderate improvement in patients (80% for active group and 33% for sham group) were analyzed by the U test to evaluate their statistical significance.

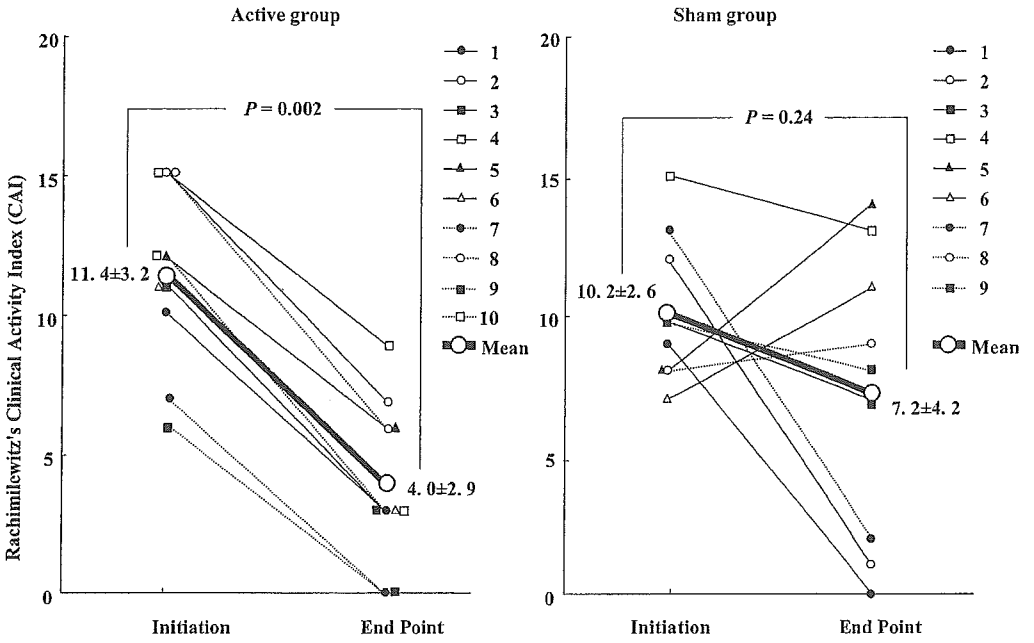

Figure 2. Comparison of CAI between pre- and postsessions in the active group (left) and sham group (right). The thick solid lines with large open circles indicate the average of each group. The thin lines and dotted lines with open or closed circles, squares, and triangles represent the CAI value of each patient.

Endoscopic Findings

Endoscopic evaluation also revealed the efficacy of LCAP. The patients who showed improvement, *i.e.*, eight cases from the active group and three cases from the sham group, underwent the third endoscopy at week 10. The remaining cases were evaluated by the second endoscopy performed between weeks 4 and 6. The Rachmilewitz's endoscopic indices of the active group and the sham group at baseline were 10.5 ± 1.8 and 10.6 ± 1.4, respectively. The evaluation showed that the active group significantly improved to 4.1 ± 3.9 with statistical significance ($p = 0.004$), while the sham group improved to 7.6 ± 4.7 without statistical significance ($p = 0.19$). Statistical significance of the intergroup difference at the evaluation point was $p = 0.066$. The endoscopic results showed that six patients from the active group and two from the sham group showed no signs of bleeding in the region extending from the rectum to the sigmoid colon, thereby showing an improvement in the vulnerability of mucosa. These results indicate that the induction of the remission stage with complete mucosal healing was 60% and 22% for the active group and the sham group, respectively.

The biopsy results support the improvement in the active group. The improvement ratios at the start and the endpoint of LCAP for the active group and the sham group are as follows: cell infiltration, 60.0% and 22.2%, respectively; erosion, 30.0% and 22.2%, respectively; crypt abscess, 40.0% and 22.2%, respectively; and crypt distortion, 30.0% and 11.1%, respectively. The difference is statistically significant ($p < 0.05$).

Steroid Dosage Reduction

As indicated in the Materials and Methods section, the steroid dosage was fixed during the pre-observation period and through weeks 1 and 2; subsequently, it was reduced in accordance with the improvement of the CAI values. The dosages in the pre-observation period were 22.0 ± 3.9 and 27.9 ± 8.3 mg/day for the active group and the sham group, respectively, without statistical significance. In the active group, these dosages were reduced to 11.3 ± 6.4 and 7.8 ± 9.2 mg/day in weeks 6 and 10, respectively. However, in the sham group, the dosage reduction was smaller than in the active group with values of 22.4 ± 9.5 and 19.4 ± 12.8 mg/day in weeks 6 and 10, respectively. The differences between the active group and the sham group in weeks 6 and 10 were statistically significant with values of $p < 0.05$ and $p < 0.01$, respectively. These results tend to substantiate reduction in the steroid dosage; the symptoms in the active group improved more than those in the sham group.

Table 3. Evaluation of Adverse Effects

Group	None	Mild	Moderate	Severe [a]	U test
Active	9	0	1	0	
	90.0%	0%	10.0%	0%	
Sham	5	2	2	0	NS
	55.6%	22.2%	22.2%	0%	

[a] Severe category includes the event when the "session is terminated because of adverse effects."

Adverse Effects

The adverse effects that occurred in both the patient groups in the study are shown in Table 3. One event occurred in the active group and four events occurred in the sham group; there was no statistical significance between the incidence in the active group and the sham group. The adverse effect observed in the active group was probably related to an intestinal infection by methicillin-resistant *Staphylococcus aureus*. Based on the fact that the patient had high fever in the pre-observation period, it is believed that the infection might have been present prior to the commencement of the session. However, this could not be proved since a culture test was not performed during the pre-observation period. The administration of vancomycin subsequently solved this problem. In the sham group, the two adverse effects classified as mild might have been related to the sessions. One patient temporarily exhibited a skin rash; the other had slight fever (37.3°C) during the session. However, the patients were able to complete the sessions, and no additional therapy was required. The other two adverse effects, which were classified as moderately severe, might not have been associated with the session. One was back pain that might have been due to right-sided urinary stone; an abdominal ultrasonography detected the stone, and an anodyne medication relieved the symptom. The second patient had high fever of 38.4°C. The reason for this might have been an infection. Replacement of the catheter used for intravenous high calorie transfusion in TPN and antibiotic therapy controlled the problem.

Follow-Up Study

Two patients in the active group and six patients in the sham group did not improve in the study. At the end of the study, they were treated as follows: One patient in the active group and four patients in the sham group underwent surgery. The other three patients received cyclosporine medication. One of these patients in the sham group responded to the medication, while the rest ultimately had to undergo surgery. All patients who responded to LCAP went into the remission stage during or after the study.

DISCUSSION

The first application of LCAP using Cellsorba for the treatment of UC was reported by our group (14, 15). The results demonstrated the positive effects of LCAP in comparison with steroid therapy. Nevertheless, we could not exclude the concern that the efficacy of LCAP was due to the placebo effect of extracorporeal circulation, and the anticoagulant which was administered to the LCAP group, but not to the steroid group. The present study was conducted in a double-blind, case-controlled fashion with active and sham groups, and the results demonstrate that LCAP therapy has significant efficacy for the UC treatment, while eliminating the placebo effect of extracorporeal circulation therapy with anticoagulant administration. The efficacy of LCAP in this study was consistent with our previous reports (15). The conditions of the patients improved, the administration of steroids was reduced, and in some cases, the improvement was to such an extent that no steroid administration was required.

The incipient application of LCAP for the treatment of IBD was reported by Bicks and Groshart (26, 27) and Lerebours (28). They performed lymphocytapheresis by centrifugal separation, to remove T cells selectively for the treatment of Crohn's disease (CD). However, the results were conflicting; the former study suggested positive effects with increasing remission length, while the latter reported that lymphocytapheresis did not result in a reduction in the relapse rate. Recent reports regarding the treatment of CD by LCAP using Cellsorba (13, 29), or GCAP using Adacolumn (30) supported the positive effects of these therapies, but there are no conclusive results yet. Therefore, in contrast to UC, further studies are required to define the utility of LCAP and GCAP for the treatment of CD.

Previous studies (14, 31) demonstrated fluctuations in the leukocyte count in the peripheral blood during LCAP. The count reduced to approximately 20–40% of the baseline value at approximately 20–30 min after the commencement of the session. However, it recovered to approximately 45–70% at the end of the session. Additionally, it increased to approximately 170–200%, 20 min to 2 h after the end of the session. During the session, Cellsorba itself had a sustained removal performance in excess of 90% of the baseline value for the circulating blood (24). Therefore, the pooled leukocytes in the body, such as those present in the bone marrow, spleen, or vessel walls compensated for the leukocytes removed during the session.

This finding led to the concept and investigation of LCAP as a therapy for UC through the removal of leukocytes from the peripheral blood, which includes activated granulocytes and lymphocytes that serve as "primed reserve cells" for the continuous attack on the mucosa. By this removal, communication between the activated peripheral blood cells and the cells attacking the mucosa would be interrupted, resulting in the alleviation or elimination of mucosal inflammation. In fact, the etiology of IBD is generally recognized to involve the ready infiltration of activated leukocytes into the intestinal mucosa, a high density of leukocytes in the inflamed mucosa (32, 33), and the production of proteinase and superoxides by these leukocytes resulting in strong and repeated damage to the mucosa.

In addition, LCAP affected the concentration of cytokines (14). The levels of proinflammatory cytokines TNF-α, interleukin (IL) 1β, IL-2, IL-8, and interferon-γ were high in the responding patients at the baseline and significantly reduced by LCAP (14). These cytokines are mainly secreted from activated leukocytes circulating in the peripheral blood (34, 35). In contrast, levels of IL-4, an immunoregulatory cytokine, increased after LCAP. These results suggest that the LCAP efficacy is achieved by the remedial action on the cytokine network in the diseased state, returning it to a normal level via the inhibition of several pro-inflammatory cytokines and by

stimulation of an immunoregulatory cytokine. Furthermore, a platelet-related mechanism should also be considered in the future because Cellsorba removes not only leukocytes but also platelets from the blood (14, 31), and some reports suggest that platelet activation is related to UC (36, 37).

LCAP using Cellsorba is very practical and applicable because of its low level of adverse effects. The treatment time is approximately 1 h per session, which is considerably shorter than hemodialysis or other apheresis therapies. In 2001, the LCAP therapy using Cellsorba for the treatment of UC was approved by the public health system in Japan. Since then, LCAP using Cellsorba is used in the therapy of UC for both inpatients and outpatients. In fact, at present, it is used as an outpatient procedure in approximately half of the cases, without increasing the incidence of side effects. In addition, it has been applied to both IBD and autoimmune diseases such as rheumatoid arthritis (38) and rapidly progressive glomerulonephritis (39) with outstanding efficacy.

In the future, further studies are required to elucidate the most effective frequency of LCAP treatments and their optimal combination with medication. In addition, these studies should be applied to the active stage of UC, and should also address the extent of time that should be considered as the remission phase. In conclusion, the present study shows that LCAP, which is classified between drug therapy and surgical intervention, should be a reasonable therapeutic option for the treatment of active UC.

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REFERENCES

1. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417–29.
2. Stein RB, Hanauer SB. Medical therapy for inflammatory bowel disease. Gastroenterol Clin North Am 1999;28:297–321.
3. Sandborn WJ, Hanauer SB. Systematic review: The pharmacokinetic profiles of oral mesalazine formulations and mesalazine pro-drugs used in the management of ulcerative colitis. Aliment Pharmacol Ther 2003;17:29–42.
4. Greenfield SM, Punchard NA, Teare JP, et al. Review article: The mode of action of the aminosalicylates in inflammatory bowel disease. Aliment Pharmacol Ther 1993;7:369–83.
5. Truelove SC, Witts LJ. Cortisone in ulcerative colitis; Preliminary report on a therapeutic trial. BMJ 1954;4884:375–8.
6. Bowen GE, Irons GV Jr, Rhodes JB, et al. Early experiences with azathioprine in ulcerative colitis; A note of caution. JAMA 1966;195:460–4.
7. Bean RH. The treatment of chronic ulcerative colitis with 6-mercaptopurine. Med J Aust 1962;49:592–3.
8. Lichtiger S, Present DH, Kornbluth A, et al. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. N Engl J Med 1994;330:1841–5.
9. Gornet JM, Couve S, Hassani Z, et al. Infliximab for refractory ulcerative colitis or indeterminate colitis: An open-label multicentre study. Aliment Pharmacol Ther 2003;18:175–81.
10. Sandborn WJ, Targan SR. Biologic therapy of inflammatory bowel disease. Gastroenterology 2002;122:1592–608.
11. Binderow SR, Wexner SD. Current surgical therapy for mucosal ulcerative colitis. Dis Colon Rectum 1994;37:610–24.
12. Sawada K. Leukocytapheresis as an adjunct to conventional medication for inflammatory bowel disease. Dis Colon Rectum 2003;46(Suppl):S66–77.
13. Sawada K, Ohnishi K, Kosaka T, et al. Leukocytapheresis therapy, performed with leukocyte removal filter, for inflammatory bowel disease. J Gastroenterol 1995;30:322–9.
14. Sawada K, Ohnishi K, Kosaka T, et al. Leukocytapheresis with leukocyte removal filter as new therapy for ulcerative colitis. Ther Apher 1997;1:207–11.
15. Sawada K, Muto T, Shimoyama T, et al. Multicenter randomized controlled study for the treatment of ulcerative colitis with a leukocytapheresis column. Curr Pharma Des 2003;9:307–21.
16. Shimoyama T, Sawada K, Hiwatashi N, et al. Safety and efficacy of granulocyte and monocyte adsorption apheresis in patients with active ulcerative colitis: A multicenter study. J Clin Apheresis 2001;16:1–9.
17. Hanai H, Watanabe F, Saniabadi A, et al. Therapeutic efficacy of granulocyte and monocyte adsorption apheresis in severe active ulcerative colitis. Dig Dis Sci 2002;47:2349–53.
18. Tomomasa T, Kobayashi A, Kaneko H, et al. Granulocyte adsorptive apheresis for pediatric patients with ulcerative colitis. Dig Dis Sci 2003;48:750–4.
19. Hanai H, Watanabe F, Takeuchi K, et al. Leucocyto adsorptive apheresis for the treatment of active ulcerative colitis: A prospective, uncontrolled, pilot study. Clin Gastroenterol Hepatol 2003;1:28–35.
20. Ayabe T, Ashida T, Taniguchi M, et al. A pilot study of centrifugal leukocyte apheresis for corticosteroid-resistant active ulcerative colitis. Intern Med 1997;36:322–6.
21. Ayabe T, Ashida T, Kohgo Y. Centrifugal leukocyte apheresis for ulcerative colitis. Ther Apher 1998;2:125–8.
22. Rachmilewitz D. Coated mesalazine (5-aminosalicylic acid) *versus* sulphasalazine in the treatment of active ulcerative colitis: a randomized study. BMJ 1989;298:82–6.
23. Pocock SJ. Clinical trials, a practical approach. Wiley: Chichester, 1983, 66–89.
24. Shibata H, Kuriyama T, Yamawaki N. Cellsorba. Therap Apher Dial 2003;7:44–7.
25. Aoyama T, Ino Y, Ozeki M, et al. Pharmacological studies of FUT-175, nafamostat mesilate. I. Inhibition of protease activity in vitro and in vivo experiments. Jpn J Pharmacol 1984;35:203–27.

26. Bicks RO, Groshart KD. The current status of T-lymphocyte apheresis (TLA) treatment of Crohn's disease. J Clin Gastroenterol 1989;11:136–8.
27. Bicks RO, Groshart KD. Tempest about T-lymphocyte apheresis in Crohn's disease. Gastroenterology 1995;108: 952–3.
28. Lerebours E, Bussel A, Modigliani R, et al. Treatment of Crohn's disease by lymphocyte apheresis: A randomized controlled trial. Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives. Gastroenterology 1994;107:357–61.
29. Kosaka T, Sawada K, Ohnishi K, et al. Effect of leukocytapheresis therapy using a leukocyte removal filter in Crohn's disease. Intern Med 1999;38:102–11.
30. Matsui T, Nishimura T, Matake H, et al. Granulocytapheresis for Crohn's disease: A report on seven refractory patients. Am J Gastroenterol 2003;98:511–2.
31. Yamaji K, Yang K, Tsuda H, Hashimoto H. Fluctuations in the peripheral blood leukocyte and platelet counts in leukocytapheresis in healthy volunteers. Ther Apher 2002;6:402–12.
32. Allison MC, Dhillon AP, Lewis WG, Pounder RE. eds. Inflammatory Bowel Disease. London: Mosby, 1998, 91–5.
33. Fiocchi C. Inflammatory bowel disease: Etiology and pathogenesis. Gastroenterology 1998;115:182–205.
34. Nikolaus S, Bauditz J, Gionchetti P. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin-10 during intestinal inflammation. Gut 1998;42:470–6.
35. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol Today 1995;16:21–6.
36. Nakamura T, Sakaguchi T, Unno N, et al. Relationship between the platelet activating factor acetylhydrolase gene and intractability of ulcerative colitis. Dis Colon Rectum 2002;45:389–93.
37. Suzuki K, Sugimura K, Hasegawa K, et al. Activated platelets in ulcerative colitis enhance the production of reactive oxygen species by polymorphonuclear leukocytes. Scand J Gastroenterol 2001;36:1301–6.
38. Hidaka T, Suzuki K, Matsuki Y, et al. Filtration leukocytapheresis therapy in rheumatoid arthritis: A randomized, double-blind, placebo-controlled trial. Arthritis Rheum 1999;42:431–7.
39. Furuta T, Hotta O, Yusa N, et al. Lymphocytapheresis to treat rapidly progressive glomerulonephritis: A randomised comparison with steroid-pulse treatment. Lancet 1998;352: 203–4.

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Identification and characterization of novel gut-associated lymphoid tissues in rat small intestine

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Background. The crypt lamina propria of the mouse small intestine has been shown to harbor multiple tiny clusters filled with c-kit- and interleukin 7 receptor (IL-7R)-positive lympho-hemopoietic cells (cryptopatches; CPs). However, it has remained an open question whether similar lymphoid tissue are present in the gastrointesitinal tract in other animals. In the present study, we investigated whether the small intestine of rats harbored lymphoid tissues similar to mouse CPs. ***Methods.*** Immunohistochemical and flow cytometric analyses were carried out using various antibodies, including those to c-kit and IL-7R molecules. ***Results.*** Lymphocyte-filled villi (LFVs), populated predominantly with c-kit- and IL-7 receptor (IL-7R)-positive cells and less with T cell receptor (TCR)-αβ T cells were found throughout the small intestine of young adult rats. Although LFVs were absent from fetal rat intestine, they were first detected at around 2 weeks after birth. Notably, in most LFVs that settled in the antimesenteric wall of the small intestine in young adult rats, immunoglobulin M-positive B cells were also detectable at the bottom of the LFVs. In aged rats, lymphocytes in some LFVs displayed a different phenotype, comprising a large B-cell area that included a germinal center. Thus, these clusters represent the first description of isolated lymphoid follicles (ILFs) in the rat small intestine. ***Conclusions.*** The present study provides the first evidence for c-kit- and IL-7R-positive lymphocyte clusters in the rat small intestine. Our data also indicating that LFVs and ILFs may constitute novel organized gut-associated lymphoid tissues in lamina propria of the rat small intestine.

Key words: lymphocyte-filled villi, isolated lymphoid follicle, cryptopatch, gut-associated lymphoid tissue

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Introduction

Skin and mucous membranes, which constitute the interface between the outside world and the inside of our bodies, protect us from various pathogens, allergenic substances, and toxins. For instance, infection occurs when a pathogen enters the body after circumventing these barriers. It is evident that numerous pathogens enter the body through mucous membranes, rather than through skin. Thus, to cope with these potentially dangerous antigenic loads, mucous membranes display various barrier functions, and among various mucous membranes, the largest is that covering the gastrointestinal tract (GIT).

The GIT, or gut, is essential for nutrient absorption, with most animals displaying a single GIT connecting intake (mouth) and output (anus) openings. The GIT is constantly exposed to innumerable food- and commensal bacterium-derived exogenous antigens, and often to pathogenic microorganisms, and toxins, making this the most dangerous place in the body. As defenses against such threats, physical and chemical barriers such as mucin, digestive enzymes, and peristalsis, in addition to various immunologically competent functions, protect the GIT. In fact, about 60% of all peripheral lymphocytes congregate in our GIT, and 2.5 g per day of secretory immunoglobulin A (S-IgA) is produced on the surface of the intestinal mucosa.[1] Furthermore, the intestinal epithelial layer is enriched with unique T-cell subsets such as γδ–T cell receptor (TCR)$^+$ intestinal intraepithelial T cells (IELs). Overall, the GIT represents the largest lymphoid tissue in the body, and immune responses are generally accepted as being regulated to a great extent by gut-associated lymphoid tissues (GALTs).[2]

GALTs consist of organized lymphoid tissues and immunocompetent cells, diffusely distributed throughout the mucous membrane. In most animals, Peyer's patches (PPs) and mesenteric lymph nodes (MLNs)

form the organized lymphoid structures, while lamina propria lymphocytes (LPLs) and IELs represent diffusely distributed immunocompetent cells. Numerous IELs reside above the basement membrane, together with columnar epithelial cells (IECs) and, due to their anatomical location, IELs are believed to encounter intestinal antigens early, thus allowing these cells to play an important role in activating and regulating intestinal mucosal immunity. However, the details of the physiological function of IELs have remained unclear. The mechanisms and site of IEL production have also remained unknown for a long period of time.

In 1996, small clusters of lymphocytes were first identified in the lamina propria (LP) near crypts throughout the mouse small intestine, and these were named cryptopatches (CPs). There are about 1000–1500 CPs in the mouse small intestine,[3] and immunohistochemical analyses have shown that the majority of these CP lymphocytes (70%–80%) are undifferentiated lymphocytes expressing c-kit, interleukin-7 receptor (IL-7R), Thy-1, and leukocyte function-associated antigen-1 (LFA-1), but not CD3, TCR$\alpha\beta$, TCR$\gamma\delta$, sIgM, or B220. In addition to lymphocytes, dendritic cells that express CD11c/CD18 integrin account for 20%–30% of CP cells, and these dendritic cells are widely distributed in the margin of CPs surrounding lymphocytes.[3] These findings indicate that CPs are more than simple lymphocyte clusters; rather, they consist of legitimate small lymphoid tissues.

In subsequent studies, lymphocytes isolated from CPs, MLNs, or PPs from 4-week-old nude mice were transplanted into severe combined immunodeficiency (SCID) mice. The results showed that IELs had developed only in SCID mice receiving CP lymphocytes, confirming the compartmentalization of undifferentiated IEL progenitor cells in gut CPs.[4] Furthermore, when athymic common cytokine receptor γ-chain mutant mice that lacked thymus, PPs, CPs, and IELs[5] were transplanted with wild-type bone marrow (BM) cells, CPs filled with BM-derived c-kit$^+$ cells were first reconstituted, then IELs appeared to develop and differentiate from the BM-derived CP lymphocytes.[6] Taken together, these results support the notion that CPs are an extrathymic IEL-producing plant in the mouse small intestine.

While CPs[3–6] were described in mice, ascertaining whether similar lymphoid tissues are present in the GIT of other animals, including humans, is very important. Several groups have actively sought for evidence of CPs in the human GIT, and, although lymphocyte-filled villi (LFVs), comprising villi containing mature lymphocytes,[7] have been found, clusters of undifferentiated lymphocytes, similar to mouse CPs, have not.[7,8] After humans and mice, various monoclonal antibodies (mAbs) to lymphocyte cell surface antigens have become available for rats, enabling detailed immunohistochemical analysis of rat lymphoid tissues. In fact, LFVs not resembling PPs have been described in the rat intestinal mucosa.[9–11] According to these studies, LFVs can be detectable from around 10-day-old suckling rats. Although recent evidence supports the extrathymic origin of intestinal $\gamma\delta$ T cells in normal rats[12,13] and lymphoid progenitor cells are believed to develop and differentiate in the rat LFVs, details of the underlying mechanisms remain elusive.

To investigate whether rats possess lymphoid tissues similar to mouse CPs, we carried out an immunohistchemical study on rat small-intestinal tissue. The result showed that rat intestinal mucosa did not possess lymphocyte clusters in crypt lamina propria (LP) that were structurally comparable to the mouse CPs. However, as reported by Mayrhofer and colleagues,[9] about 1600 lymphocyte clusters were found in crypts and villi, thus representing LFVs. Next, we addressed whether the possible progenitor IELs equivalent to those present in the mouse CPs were present in the rat LFVs. As a result, we have identified for the first time that rat LFVs contain abundant c-kit$^+$ and IL-7R$^+$ cells. These findings suggest that these structures may be similar to the mouse CPs, in that they harbor immature lymphocytes expressing c-kit and/or IL7-R molecules and in that they are found to develop after birth. In the present article, all of these newly elucidated aspects of rat LFVs are described, with respect to their transfiguration with age.

Materials and methods

Rats

We purchased Jcl:Wistar (Wistar) rats from Clea Japan (Tokyo, Japan).

Antibodies and peanut agglutinin

The following monoclonal antibodies (mAbs) and polyclonal Abs (pAbs) were used: murine anti-rat TCR-$\alpha\beta$ mAb (R73, 0.625 μg/ml; BD Pharmingen, Franklin Lakes, NJ, USA); murine anti-rat IgM mAb (G53-238, 5 μg/ml; BD Pharmingen), murine anti-rat IgA mAb (A93-3, 2.5 μg/ml; BD Pharmingen), murine anti-rat pan B cell mAb (RLN-9D3, 20 μg/ml; Serotec, Raleigh, NC, USA), goat anti-c-kit pAb (5 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-IL-7R pAb (5 μg/ml; Santa Cruz Biotechnology). Biotinylated peanut agglutinin (PNA, 7.5 μg/ml; Vector Laboratories, Burlingame, CA, USA) was also used in this study. For flow cytometric analysis, 1–3 × 10^5 cells were stained in 50 μl of staining medium containing the above mAbs.

Immunohistochemical procedures

The small intestine was longitudinally opened along the mesenteric wall, and a 10-mm length of intestine that had been either kept flat for horizontal sections or rolled up for vertical sections was embedded in OCT compound (Tissue-Tek; Miles, Elkhalt, IN, USA) at –80°C. Tissue segments were sectioned using a cryostat at 5 μm, and sections were preincubated with Blockace (Dainippon Pharmaceutical, Osaka, Japan) to block nonspecific binding of Abs. Sections were then incubated with mouse, goat, or rabbit Ab for 30 min at 37°C and rinsed three times with phosphate-buffered saline (PBS), followed by incubation with biotin-conjugated goat anti-mouse Ig Ab (1.25 μg/ml; BD Pharmingen), biotin-conjugated donkey anti-goat IgG (10 μg/ml; Jackson ImmunoResearch, West Grove, PA, USA), or biotin-conjugated donkey anti-rabbit IgG (10 μg/ml; Jackson ImmunoResearch). In staining with biotinylated PNA, the second biotin-conjugated anti-IgG Ab was not used. Sections were subsequently washed three times with PBS and then incubated with avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories). Histochemical color development was achieved using a Vectastain DAB (3,3'-diaminobenzidine) substrate kit (Vector Laboratories) according to the manufacturer's instructions. Finally, sections were counterstained with hematoxylin for microscopy. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 and 0.1% NaN_3 in distilled water for 10 min at room temperature. Tissue sections incubated either with isotype-matched normal mouse IgG, normal goat IgG, or normal rabbit IgG showed only minimal background staining.

In vivo labeling and in situ immunohistochemical visualization of proliferating lymphocytes

Rats were administered drinking water containing 1 mg/ml bromodeoxyuridine (BrdU) for 20 h. Small intestines were opened along the mesenteric wall. Next, 10-mm segments of intestine that had been rolled up were embedded in OCT compound at –80°C. Cryostat tissue sections 9-μm-thick were fixed in 4% paraformaldehyde for 15 min at 4°C, washed three times with PBS, and treated with 2 M HCl for 20 min at 37°C, followed by neutralization with 0.1 M sodium tetraborate. Subsequent immunohistochemical color development using the first anti-BrdU mAb (B44; BD Biosciences, San Jose, CA, USA) and the second biotinylated goat anti-mouse Ig Ab (20 μg/ml; Cappel, Aurora, OH, USA) was performed according to the methods described above.

Flow cytometry

A single lymphoid cell suspension was prepared, and nucleated cells were counted using a hemocytometer. LFV cells were isolated using essentially the same technique as that used for the isolation of murine CP and ILF cells.[4–6] In brief, the small intestine was opened longitudinally along the mesenteric wall, and mucus and feces were removed using filter paper. Subsequently, a 10-mm-long segment of intestine was pasted on a plastic culture dish. An 18-gauge needle (inner diameter, 940 μm) was cut off at the proximal end of the tapering tip. The needle was then bent in the middle, and the cross-section was sharpened using a small UA12A electric dental grinder (Urawa Kogyo, Saitama, Japan). This needle was finally fitted onto a 1-ml syringe. LFVs were identified under transillumination stereomicroscopy and a tiny fragment of the small intestine containing one LFV was isolated using the needle described above. Lymphoid cells were incubated first with biotinylated mAb, then with streptavidin phycoerythrin (PE; BD Biosciences)—conjugated second mAb. Stained cells were suspended in staining medium (Hanks' solution without phenol red, 0.02%, NaN_3, and 2% heat-inactivated fetal bovine serum [FBS], containing 0.5 μg/ml propidium iodide [PI]) and analyzed using FACScan with CellQuest software (BD Biosciences). Dead cells were excluded by PI gating.

Results

We have recently described CPs and ILFs in the mouse small intestine and have characterized the histogenetic, cellular, and functional aspects of these newly identified GALTs.[3–6,14,15] Mice display about 1500 CPs throughout the small intestinal mucosa,[3] and 140–180 ILFs mostly on the antimesenteric wall of the small intestine.[15] Several research groups, including our own, have been actively searching for clusters of undifferentiated lymphocytes in the human GIT, but no such clusters have been identified.[7,8] Rat GIT displays LFVs, and, because these structures predominantly comprise $CD3^-$, $\alpha\beta TCR^-$, $CD25^+$, and $CD44^+$ cells, LFVs in rats appear comparable to CPs in mice.[10] We therefore investigated whether clusters of undifferentiated lymphocytes expressing c-kit and IL-7R existed in the rat LFVs. We first prepared and examined hematoxylin-and-eosin (H&E)-stained sections of small-intestinal mucosa from 13-day-old Wistar rats under microscopy. The results showed clusters of lymphoid cells in the small-intestinal mucosa (Fig. 1a, b). While most CPs in mice were localized near crypt LP,[3] lymphocyte clusters on the rat small intestinal mucosa were densely distributed inside villi, and were morphologically classified as LFVs.[9–11] Fur-