

FIGURE 3. Histologic findings of the colonic tissues from DSS colitis mice treated with Nissle1917. A, normal colon. B, colon of mice treated with Nissle1917. C, DSS-induced colitis treated with PBS (day 10). D, DSS-induced colitis treated with Nissle1917. E, histologic scores of DSS-induced colitis mice (n = 11, PBS group; n = 10, Nissle group). Results are expressed as mean \pm SEM of the data from 3 independent experiments. **, $P < 0.01$ compared with PBS groups.

treatment decreased this inflammatory findings (Fig. 4A and B; control: 52.3 ± 2.4 versus Nissle: 50.0 ± 1.5 mg/cm; $P < 0.05$). The number of mice with rectal prolapse, 1 of the characteristic symptoms of severe rectal inflammation, was significantly decreased in the Nissle1917 group (Fig. 4C; control: 5.6% at 3 wk after administration and 16.7% at 7 wk after administration, Nissle: 0% in the experimental periods).

The SAA protein concentrations, which can be a useful marker of inflammation, were significantly lower in Nissle1917-treated mice (229.5 ± 93.7 μ g/mL) than in control mice (47.0 ± 17.8 μ g/mL; Fig. 4D; $P < 0.01$). Microscopically, marked infiltrations of mononuclear cells and polymorphonuclear cells were observed in colonic mucosa of control IL-10^{-/-} mice (Fig. 5A). In contrast, Nissle1917-treated mice

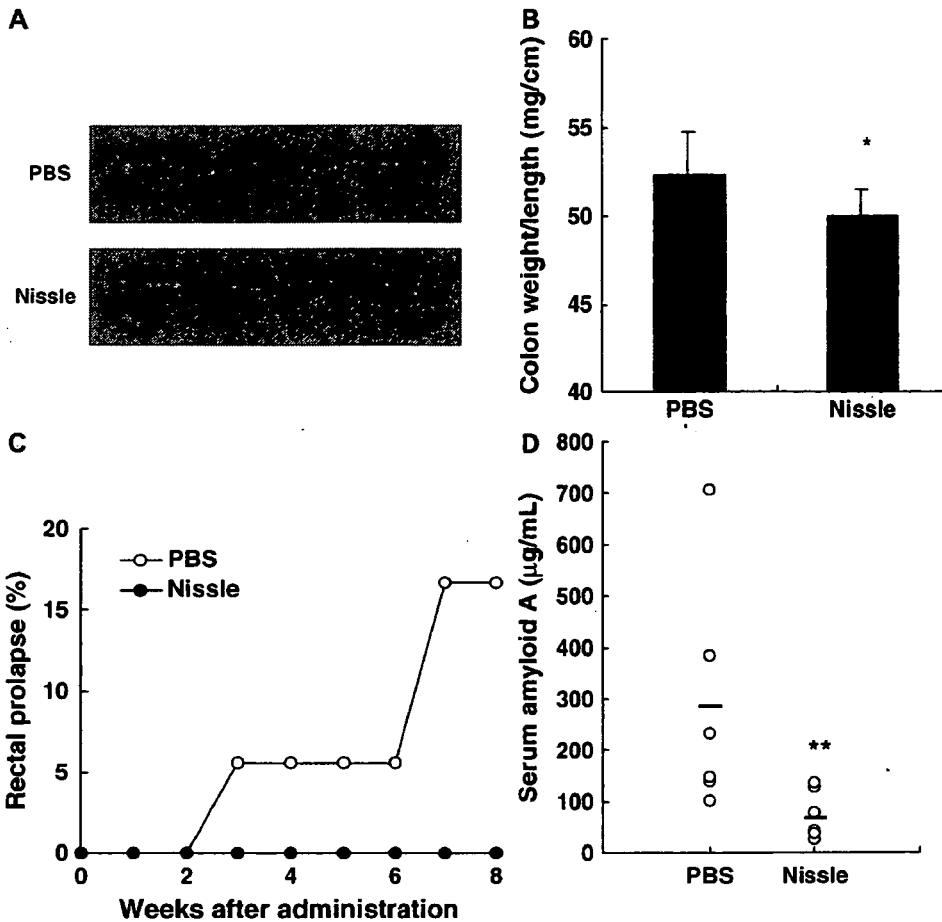


FIGURE 4. Effect of Nissle1917 on IL-10^{-/-} mice. A, macroscopic findings of IL-10^{-/-} murine colon treated with PBS (top) or Nissle1917 (bottom). B, colon weights of IL-10^{-/-} mice treated with PBS (n = 18) or Nissle1917 (n = 17). C, rectal prolapse of IL-10^{-/-} mice treated with PBS (○, n = 18) or Nissle1917 (●, n = 17). D, SAA protein levels of IL-10^{-/-} mice treated with PBS (n = 6) or Nissle1917 (n = 7). Results are expressed as mean ± SEM of the data from 3 independent experiments. *, P < 0.05; **, P < 0.01 compared with PBS groups.

showed significantly less infiltrations of these cells (Fig. 5B). Histologic scores were also significantly reduced in Nissle1917-treated mice (Fig. 5C; control: 5.2 ± 0.6 versus Nissle: 3.6 ± 0.5; P < 0.05).

Nissle1917 Suppresses the Expression of Proinflammatory Cytokines and Chemokines in LPMCs

Because the mechanisms of the protective effect of Nissle1917 on murine colonic inflammation remain unknown, we next examined the expression of several proinflammatory cytokines and chemokines in LPMCs isolated from DSS colitis mice and IL-10^{-/-} mice with or without Nissle1917 treatment. Quantitative RT-PCR for TNF-α, IFN-γ, IL-1β, IL-6, TGF-β1, IL-10, MCP-1, and MIP-2 were performed. As shown in Figure 6, mRNA expression of most cytokines and chemokines tested were not significantly reduced in DSS colitis mice. However, in IL-10^{-/-} mice, IFN-γ and MIP-2 expressions were significantly lower in Nissle1917-treated mice than those in controls (IFN-γ, control: 100 ± 9.9% versus Nissle: 62.7 ± 14.2% and MIP-2, control: 100 ± 22.8% versus Nissle: 31.8 ± 10.1%; P < 0.05).

Heat-killed and Genomic DNA from Nissle1917 Ameliorates Murine DSS Colitis

To prove the therapeutic mechanism of Nissle1917, the efficacy of nonviable Nissle1917 components on experimental colitis was assessed. In DSS colitis mice, macroscopic findings revealed that the shortening and thickening of the colon were significantly improved in all Nissle groups compared with the PBS control group (Table 2). Histologic findings also showed less inflammation in all Nissle groups, and the histologic scores of these groups were significantly lower than controls (Table 2). In terms of DAI scores at day 7, heat-killed Nissle1917 and genomic DNA treatment diminished the scores, but the difference was not statistically significant against untreated mice. However, in earlier phase of DSS colitis, genomic DNA treatment clearly suppressed the inflammation with reduced DAI score (control: 0.9 ± 0.2 versus DNA: 0.4 ± 0.2; P < 0.05 at day 5, control: 1.8 ± 0.2 versus DNA: 1.0 ± 0.2; P < 0.05 at day 6).

DISCUSSION

Probiotic bacteria have been used on human clinical disorders including IBD. For example, probiotic VSL#3 (comprised

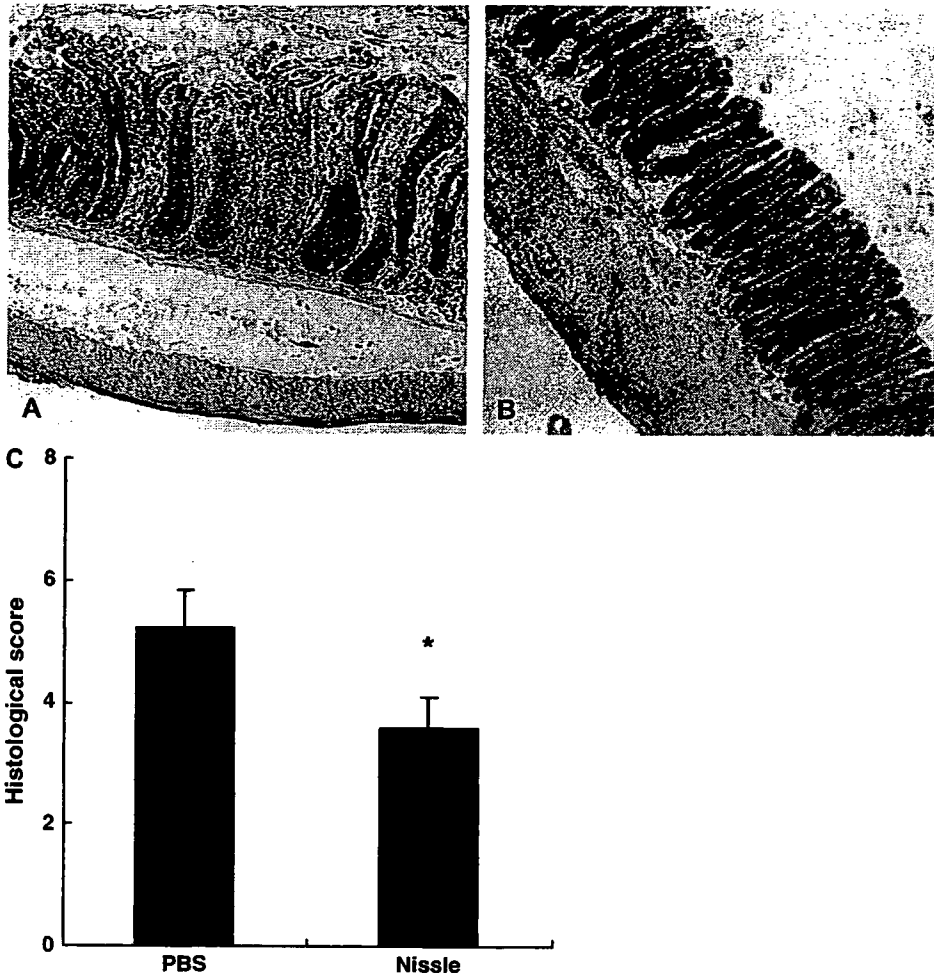


FIGURE 5. Histologic findings of the colonic tissues from IL-10^{-/-} mice treated with Nissle1917. A, microscopic findings of IL-10^{-/-} murine colon (16 wk old). B, Nissle1917-treated IL-10^{-/-} mice (16 wk old). C, histologic scores of IL-10^{-/-} mice (n = 18, PBS group; n = 17, Nissle1917 group). Results are expressed as mean \pm 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 function²⁶; 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 al³³ 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.

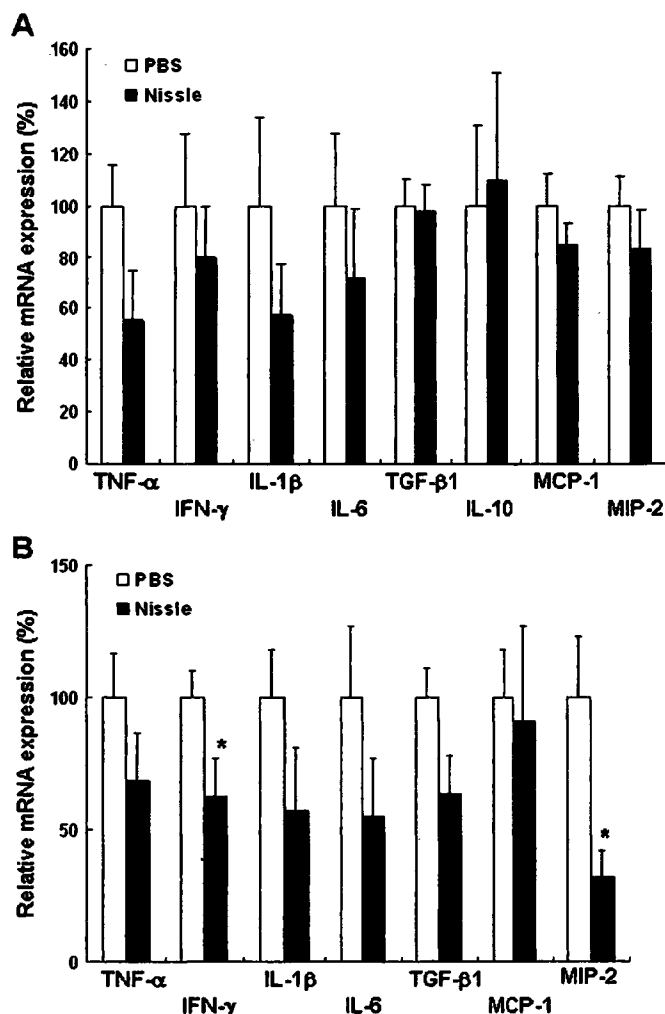


FIGURE 6. Nissle1917 altered the expressions of proinflammatory cytokines and chemokines in LPMCs from DSS colitis and IL-10^{-/-} mice. Total RNA was isolated from LPMC of DSS colitis (A) and IL-10^{-/-} mice (B) treated with PBS or Nissle1917 (n = 8). The expression level of each cytokine was divided 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. Effect 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 al³³ 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 administered VSL#3 could be detected from lymphoid organs, and its therapeutic effect was lost when it was intrarectally administered. Interestingly, Schultz et al³³ 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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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.

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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 dysplasias 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 MgCl₂, 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 system²¹ and exposed to X-ray film for 30 min. Biotinylated 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 alkaline 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.

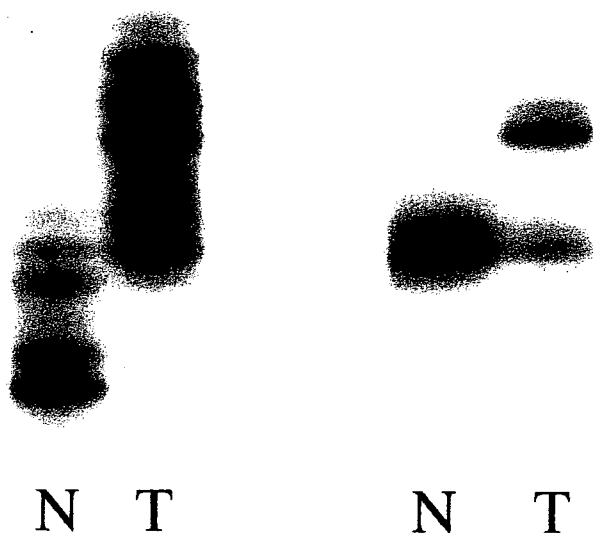


Figure 1 Microsatellite instability detected in ulcerative colitis (UC) associated colon cancers. N, normal; T, UC associated colon cancer. Primers used in the left and right panels were D2S123 and D2S119, respectively.

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

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

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).

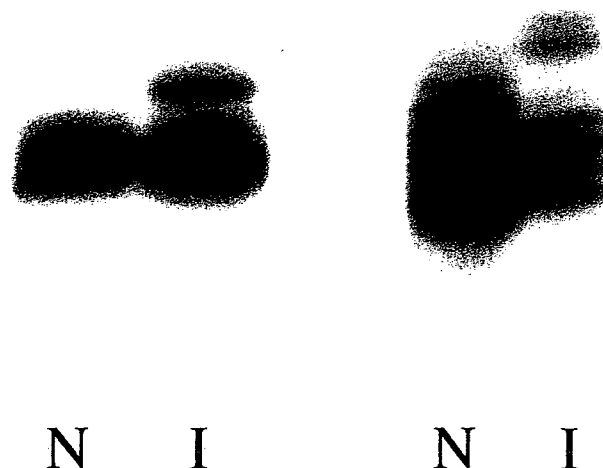


Figure 2 Microsatellite instability detected in the colonic inflamed mucosa from patients with ulcerative colitis (UC). N, normal; I, inflamed colonic mucosa. Primers used in the left and right panels were D2S119 and D2S123, respectively.

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).

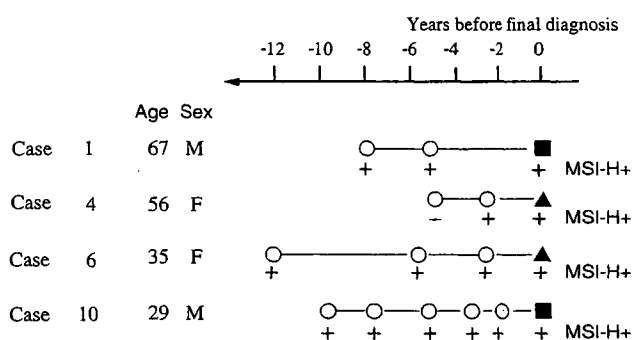


Figure 3 Microsatellite instability and histological findings in serial biopsy specimens from the same patient. Mutations of microsatellite loci in four patients with ulcerative colitis (UC) associated colon cancer or dysplasia, who showed MSI-H+ phenotypes in final diagnosis, were analyzed retrospectively 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 sur-

vival.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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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 in-

roduced 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 ± 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 ± 0.05 mg/kg/day) for moderately severe

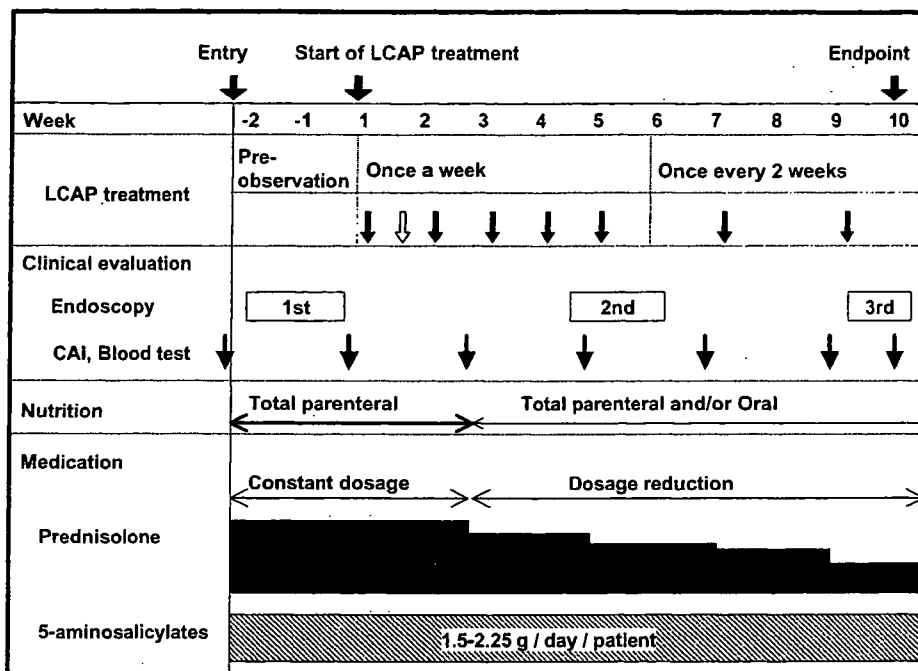


Figure 1. Schematic drawing of the study protocol. Closed arrows in the session and clinical evaluation rows indicate the time of the corresponding events. The open arrow in the session row indicates the optional session, which was performed for a severe-category patient. In the medication row, the closed 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 20.0%	6 60.0%	2 20.0%	0 0.0%	* (<i>p</i> < 0.05)
Sham	1 11.1%	2 22.2%	3 33.3%	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.

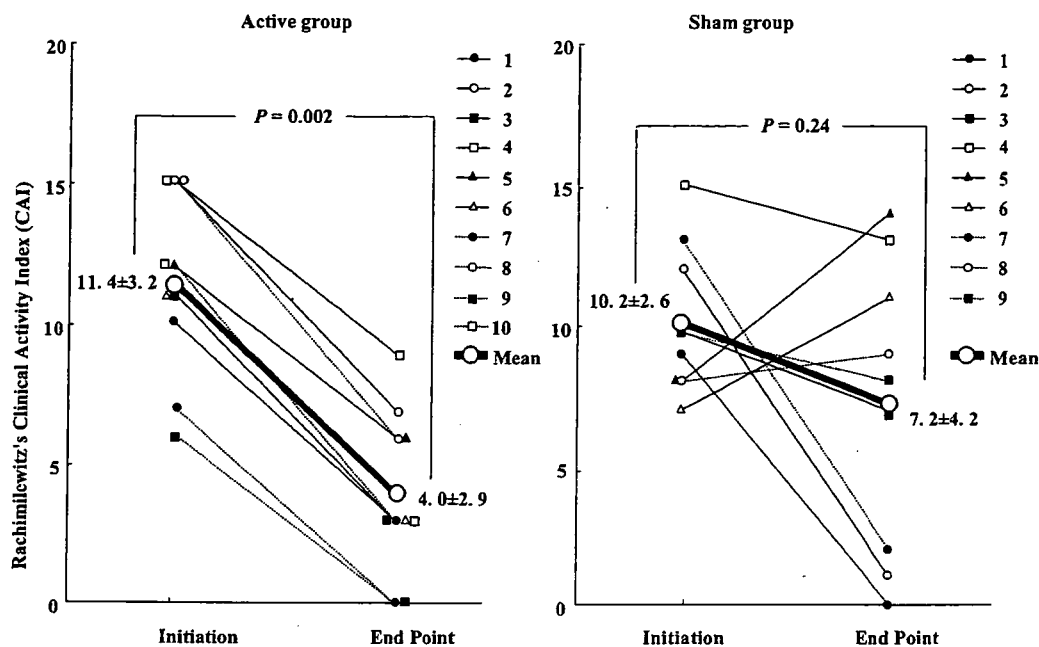


Figure 2. Comparison of CAI between pre- and postsessions in the active group (left) and sham group (right). The thick solid lines with large open circles indicate the average of each group. The thin lines and dotted lines with open or closed circles, squares, and triangles represent the CAI value of 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 90.0%	0 0%	1 10.0%	0 0%	NS
Sham	5 55.6%	2 22.2%	2 22.2%	0 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) 1 β , 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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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 gastrointestinal 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)- $\alpha\beta$ 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

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 $\gamma\delta$ -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)