Fig. 1 Enteroscopic and radiographic findings of a case of circular ulcers in the ileum. A 78-year-old female who had been diagnosed as rheumatoid arthritis and had been under celecoxib was examined because of anemia. Under VCE (a) and oral BAE (b), multiple circular ulcers were found. Double-contrast barium enteroclysis depicted the ulcers as concentric and multiple stenoses in the ileum (c). The findings are compatible with "pseudo-folds"
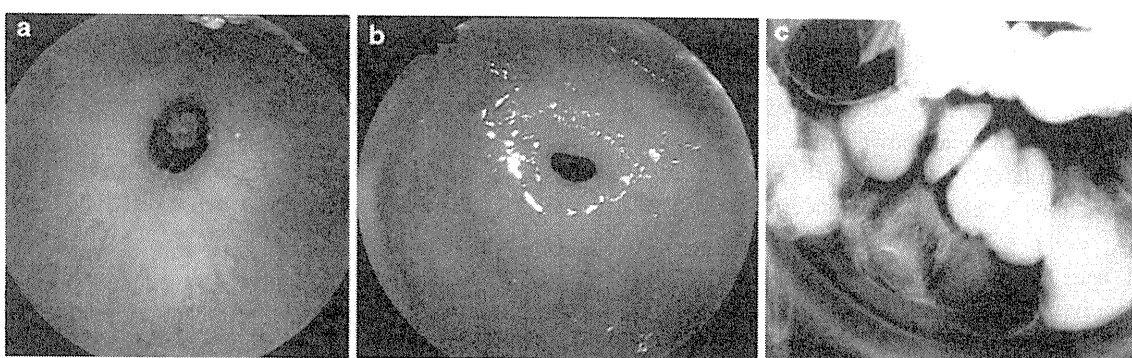

Fig. 2 Enteroscopic and radiographic findings of a case of diaphragms in the ileum. A 72-year-old female had been taking diclofenac for rheumatoid arthritis for 24 months, during which repeated abdominal pain occurred. VCE (a) and anal BAE (b) detected concentric stenosis in the ileum. Double-contrast barium enteroclysis showed concentric stenosis in the lower part of the ileum (c). The findings are compatible with condensed "pseudo-folds." This case experienced capsule retention

where appropriate. Probabilities less than 0.05 were considered to be significant.

Results

Enteroscopic Findings

The subjects were examined by 13 anal BAEs, 8 oral BAEs, and 10 VCEs. Eleven subjects were examined by BAE alone, and 3 patients by VCE alone. The remaining 7 subjects were examined by both BAE and VCE.

Circular ulcers (Fig. 1a, b), linear ulcers and small mucosal defects were found in 12, 3 and 12 patients, respectively. The predominant site of involvement within the small bowel was the jejunum in 3 subjects, the ileum in 6 and both the jejunum and the ileum in 8. Three subjects had both circular ulcer and small mucosal defects. The other 3 subjects had both linear ulcer and small mucosal defects, while 6 subjects had small mucosal defects alone.

Six of 12 subjects with circular ulcers had diaphragms (Fig. 2a, b). Among 10 subjects examined by VCE, 2 experienced capsule retention, which was treated by endoscopic balloon dilatation under BAE.

Table 1 compares clinical features between patients with circular ulcers and those without. While neither gender, age at the time of diagnosis of NSAID enteropathy, predominant site of involvement nor duration of NSAID use was different between the two groups, overt OGIB was less frequent in patients with circular ulcers than those without the ulcers (11 vs. 42%, $P = 0.037$). There was also a trend towards less frequent cardiovascular disease in patients with circular ulcer as the indication of NSAID use. Four of 12 patients with circular ulcers had been taking two NSAID species while patients without circular ulcer had been taking a single species of NSAID (33 vs. 0%, $P = 0.08$). Laboratory data including serum protein, hemoglobin and C-reactive protein values were not different between the two groups.

Table 1 Comparison of clinical features between patients with and without circular ulcers

Clinical features	Patients with circular ulcers (n = 12)	Patients without circular ulcers (n = 9)	Probability
Sex (female/male)	4/8	3/6	1.0
Age (range, median) (years)	46–88, 68.5	47–87, 80	0.26
Indication of NSAID use			
Arthropathy/CVD	12/0	6/3	0.06
Indication for enteroscopy			
Overt OGIB/others	5/7	8/1	0.037
Predominant site			0.81
Jejunum	2	1	
Jejunum and ileum	5	3	
Ileum	5	5	
Laboratory data (range, median)			
Hemoglobin (g/dl)	4.9–13.7, 8.5	5.6–13.5, 8.8	0.36
Serum protein (g/dl)	3.9–7.8, 9.2	4.3–7.9, 8.8	0.52
C-reactive protein (mg/dl)	0.01–11.6, 0.12	0.10–3.88, 0.73	0.10
Use of two NSAIDs species	4	0	0.08
Duration of NSAID use (range, median) (months)	0.2–240, 30	1–192, 12	0.55
NSAID species			
Loxioprofen	4	3	
Diclofenac	4	2	
Aspirin	1	3	
Indomethacin	2	0	
Others	5	1	

OGIB Obscure gastrointestinal bleeding, *CVD* cardiovascular diseases

Table 2 indicates a comparison between subjects with small mucosal defects and those without. As summarized in the table, subjects with small mucosal defects were taking multiple NSAID less frequently than those without (0 vs. 44%, $P = 0.02$). In addition, hemoglobin value was higher in subjects with small mucosal defect than in those without. There was also a trend towards higher serum protein value in the former than in the latter.

Radiography

Review of the radiographic images in 12 subjects with circular ulcers revealed that concentric narrowing with extremely short width was depicted in 10 patients (Figs. 1c and 2c). The narrowing apparently mimicked normal-appearing folds of the jejunum. Such "pseudo-folds" was found in multiplicity. However, the longitudinal alignment of the pseudo-folds was irregular, and the distance inbetween the folds was not uniform. The proximal part of the bowel was not dilated in any subject with circular ulcers. In 2 of 3 subjects with linear ulcers, radiography revealed eccentric rigid area with converging folds.

Radiography failed to depict any significant findings in 10 of 12 subjects with small mucosal defects. In 2 subjects,

compression images showed tiny barium flecks in the lower part of the ileum. The depiction rate of NSAID enteropathy was thus 83% for circular ulcers, 67% for linear ulcers and 17% for small mucosal defects.

Among 10 patients examined by VCE, 2 of 5 subjects with pseudo-folds experienced capsule retention. The capsule was not retained in the other 5 subjects without the radiographic signs.

Discussion

Our retrospective investigation revealed that DCBE could depict circular ulcers in NSAID enteropathy, while it failed to depict small mucosal defects. We could also show that patients with circular ulcers complained of overt OGIB less frequently than those without, and they tended to have been under two species of NSAID. These observations suggest that radiography together with clinical manifestations may be a tool for patency in patients suspected of having NSAID enteropathy.

In the 1980s and 1990s, diaphragms of the small bowel drew much attention as the characteristic lesion of NSAID enteropathy. The diaphragms were histologically characterized by

Table 2 Comparison of clinical features between patients with and without small mucosal defects

Clinical features	Patients with mucosal defects ($n = 12$)	Patients without mucosal defects ($n = 9$)	Probability
Sex (female/male)	5/7	2/7	0.11
Age (range, median) (years)	47–87, 75	46–88, 67	0.30
Indication of NSAID use			
Arthropathy/CVD	9/3	9/0	0.23
Indication for enteroscopy			
Overt OGIB/others	9/3	4/5	0.17
Predominant site			0.48
Jejunum	1	2	
Jejunum and ileum	4	4	
Ileum	7	3	
Laboratory data (range, median)			
Hemoglobin (g/dl)	5.6–13.7, 9.7	4.9–10.1, 8.8	0.036
Serum protein (g/dl)	4.3–7.9, 6.2	3.9–7.2, 5.4	0.051
C-reactive protein (mg/dl)	0.01–3.9, 0.72	0.01–11.6, 0.06	0.12
Use of two NSAID species	0	4	0.02
Duration of NSAID use (range, median) (months)	1–240, 18	0.2–240, 24	0.97
NSAID species			
Loxioprofen	3	4	
Diclofenac	3	3	
Aspirin	3	1	
Indomethacin	0	2	
Others	3	3	

OGIB Obscure gastrointestinal bleeding, *CVD* cardiovascular diseases

marked fibrosis with shallow ulcers at the tip of the Kerckring's folds [10, 11]. While 6 of our 21 subjects actually had diaphragms, we also found circular ulcers in those 6 patients as well as in another 6 patients without apparent diaphragms. It is thus suggested that circular ulcers are the most typical and representative mucosal lesions in NSAID enteropathy. However, it is a fact that there have been cases of diaphragms, which were missed by small bowel radiography and diagnosed by enteroscopy [26–28]. In those reports, small bowel radiography probably failed to depict extremely thin narrowing without dilatation of the proximal bowel.

In the literature, the radiographic features of NSAID-induced diaphragms have been discussed in symptomatic patients who were examined by barium follow-through study [19, 21, 22]. Zalev et al. [22] analyzed four symptomatic cases of diaphragms and found that lifesaver-like stricture and babel-like configuration were depited by small bowel radiography. Although those descriptions were heterogeneous with regards to the width of the narrow septae, they are in concert with respect to concentric and multiple stenoses [19–22]. Our results indicated that the circular ulcers, as well as diaphragms, were depicted as apparently normal folds when the affected small bowel was insufflated with an appropriate amount of air under DCBE. It thus

seems likely that the radiologic finding referred to as "pseudo-folds" sign may be applicable and specific to the diagnosis of NSAID enteropathy.

It has been reported that VCE detected small mucosal defects even in healthy subjects after short-term NSAID use [29–31]. However, DCBE failed to depict most of small mucosal defects in our subjects with NSAID enteropathy. With this regard, VCE or BAE is the procedure of choice for the diagnosis of the disease. Even though VCE has advantages as to safety and convenience, it has also been shown that NSAID enteropathy is one of the major conditions associated with capsule retention [14, 15]. A conspicuously high incidence of capsule retention in NSAID enteropathy seems to be a consequence of undiagnosed diaphragms. Small bowel radiography thus seems to have a role as a patency tool for VCE in patients suspected of having NSAID enteropathy. In fact, capsule retention did occur in 2 of our 5 patients with pseudo-folds sign, while none of the subjects without the sign experienced the adverse event.

Despite a worldwide, huge population exposed to NSAID, cases of diaphragm are encountered infrequently. In addition, clinical features predisposed to diaphragms are poorly understood except for long-term NSAID use [10–13, 19–22, 26–28]. Although the difference did not

reach statistical significance, our analyses of the subjects' demographics revealed that the simultaneous use of two NSAID species was associated with the occurrence of circular ulcers. In addition, subjects with circular ulcers experienced overt OGIB less frequently than those without. Conversely, subjects with small mucosal defects had less severe anemia and hypoproteinemia, and they had been taking only a single NSAID. These observations strongly suggest that circular ulcers are the consequence of chronic and repeated exposure to NSAID. Accordingly, the history of NSAID intake and clinical manifestations seem to be predictive of circular ulcers, and presumably, of diaphragms.

Recently, patency capsule system for patients at a high risk of capsule retention has become available [16–18]. Although the system has been shown to be a sensitive procedure [16–18], it requires at least 30 h for the final decision. There are also difficulties in determining the localization of the patency capsule under radiographs or by the scanners [17]. Even though Postgate et al. [17] reported that small bowel radiography was normal or minimally abnormal in patients in whom the patency capsule was retained, they did not include any case of NSAID enteropathy. It thus seems possible that DCBE with a special reference to pseudo-folds sign may be a convenient tool for the determination of luminal patency in patients taking NSAID.

Maglinte et al. [32] proposed double-contrast enteroclysis to be equal or superior to VCE for the evaluation of mucosal details such as aphthous lesions and scarred ulcers in Crohn's disease. Our results showed that this was not the case for NSAID enteropathy, probably due to the difference in the number and the depth of diminutive lesions between Crohn's disease and NSAID enteropathy. In addition, CT- and MR-enterographies have become alternative to and more informative than small bowel radiography for the evaluation of transmural or stenotic lesions in Crohn's disease [33–37]. Since CT- and MR-enterographies are also able to depict mucosal alterations, these procedures may be other candidates as a patency tool for NSAID enteropathy.

In conclusion, an analysis of clinical and radiographic features of 21 patients with NSAID enteropathy revealed DCBE to be applicable as a patency tool prior to VCE for subjects suspected of having the disease. On that occasion, the pseudo-folds sign may be suggestive of circular ulcers and the diaphragms. Given such circumstances, BAE with a preparation of endoscopic balloon dilatation would be practically preferred to VCE [9, 38]. The value of small bowel radiography in comparison with patency capsule should be examined in the area of widespread use of NSAID including aspirin.

References

1. Allison MC, Howatson AG, Torrance CJ, Lee FD, Russell RI. Gastrointestinal damage associated with the use of nonsteroidal antiinflammatory drugs. *N Engl J Med.* 1992;327:749–754.
2. Laine L, Smith R, Min K, Chen C, Dubois RW. Systematic review. The lower gastrointestinal adverse effects of non-steroidal anti-inflammatory drugs. *Aliment Pharmacol Ther.* 2006;24:751–767.
3. Graham DY, Opium AR, Willingham FF, Qureshi WA. Visible small-intestinal mucosal injury in chronic NSAID users. *Clin Gastroenterol Hepatol.* 2005;3:55–59.
4. Maiden L, Thjodleifsson B, Seigal A, et al. Long-term effects of nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 selective agents on the small bowel: a crosssectional capsule enteroscopy study. *Clin Gastroenterol Hepatol.* 2007;5:1040–1045.
5. Matsumoto T, Kudo T, Esaki M, et al. Prevalence of nonsteroidal anti-inflammatory drug-induced enteropathy determined by double-balloon endoscopy. A Japanese multicenter study. *Scand J Gastroenterol.* 2008;43:490–496.
6. Sidhu R, Brunt LK, Morley SR, Sanders DS, McAlindon ME. Undisclosed use of nonsteroidal anti-inflammatory drugs may underlie small-bowel injury observed by capsule endoscopy. *Clin Gastroenterol Hepatol.* 2010;8:992–995.
7. Matsumoto T, Nakamura S, Esaki M, et al. Enteroscopic features of chronic nonspecific multiple ulcers of the small intestine. Comparison with nonsteroidal anti-inflammatory drug-induced enteropathy. *Dig Dis Sci.* 2006;51:1357–1363.
8. Hayashi Y, Yamamoto H, Kita H, et al. Non-steroidal anti-inflammatory drug-induced small bowel injuries identified by double-balloon endoscopy. *World J Gastroenterol.* 2005;21:4861–4864.
9. Hayashi Y, Yamamoto H, Taguchi H, et al. Nonsteroidal anti-inflammatory drug-induced small-bowel lesions identified by double-balloon endoscopy. Endoscopic features of the lesions and endoscopic treatments for diaphragm disease. *J Gastroenterol.* 2009;44:57–63.
10. Lang J, Price AB, Levi AJ, Burke M, Gumpel JM, Bjarnason I. Diaphragm disease. Pathology of disease of the small intestine induced by nonsteroidal anti-inflammatory drugs. *J Clin Pathol.* 1988;41:516–526.
11. Bjarnason I, Hayllar J, MacPherson AJ, Russell AS. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine. *Gastroenterology.* 1993;104:1832–1847.
12. Yousfi MM, de Petris G, Leighton JA, et al. Diaphragm disease after use of nonsteroidal anti-inflammatory agents. First report of diagnosis with capsule endoscopy. *J Clin Gastroenterol.* 2004;38:686–691.
13. Manetas M, O'Loughlin C, Kelemen K, Barkin JS. Multiple small-bowel diaphragms. A cause of obscure GI bleeding diagnosed by capsule endoscopy. *Gastrointest Endosc.* 2004;60:848–851.
14. Li F, Gurudu SR, De Petris G, et al. Retention of the capsule endoscope. A single-center experience of 1000 capsule endoscopy procedures. *Gastrointest Endosc.* 2008;68:174–180.
15. Liao Z, Gao R, Xu C, Li ZS. Indications and detection, completion, and retention rates of small-bowel capsule endoscopy. A systematic review. *Gastrointest Endosc.* 2010;71:280–286.
16. Spada C, Shah SK, Riccioni ME, et al. Video capsule endoscopy in patients with known and suspected small bowel stricture previously tested with the dissolving patency capsule. *J Clin Gastroenterol.* 2007;41:576–582.
17. Postgate AJ, Burling D, Gupta A, Fitzpatrick A, Fraser C. Safety, reliability, and limitations of the given patency capsule in patients at risk of capsule retention. A 3-year technical review. *Dig Dis Sci.* 2008;53:2732–2738.

18. Herrerias JM, Leighton JA, Costamagna G, et al. Agile patency system eliminates risk of capsule retention in patients with known intestinal strictures who undergo capsule endoscopy. *Gastrointest Endosc.* 2008;67:902–909.

19. Levi S, de Lacey G, Price AB, Gumpel MJ, Levi AJ, Bjarnason I. "Diaphragm-like" strictures of the small bowel in patients treated with non-steroidal anti-inflammatory drugs. *Br J Radiol.* 1990; 63:186–189.

20. Matsuhashi N, Yamada A, Hiraishi A, et al. Multiple strictures of the small intestine after long-term nonsteroidal anti-inflammatory drug therapy. *Am J Gastroenterol.* 1992;87:1183–1186.

21. Scholz FJ, Heiss FW, Roberts PL, Thomas C. Diaphragm-like strictures of the small bowel associated with use of nonsteroidal anti-inflammatory drugs. *Am J Roentgenol.* 1994;162:49–50.

22. Zalev AH, Gardiner GW, Warren RE. NSAID injury to the small intestine. *Abdom Imaging.* 1998;23:40–44.

23. Esaki M, Matsumoto T, Kudo T, Yanaru-Fujisawa R, Nakamura S, Iida M. Bowel preparations for capsule endoscopy. A comparison between simethicone and magnesium citrate. *Gastrointest Endosc.* 2009;69:94–101.

24. Matsumoto T, Moriyama T, Esaki M, Nakamura S, Iida M. Performance of antegrade double-balloon enteroscopy. Comparison with push-enteroscopy. *Gastrointest Endosc.* 2005;62:392–398.

25. Matsumoto T, Esaki M, Yada S, Jo Y, Moriyama T, Iida M. Is small-bowel radiography necessary before double-balloon endoscopy? *Am J Roentgenol.* 2008;191:175–181.

26. Shumaker DA, Bladen K, Katon RM. NSAID-induced small bowel diaphragms and strictures diagnosed with intraoperative enteroscopy. *Can J Gastroenterol.* 2001;15:619–623.

27. Achanta KK, Petros JG, Cave DR, Zinny M. Use of intraoperateive enteroscopy to diagnose nonsteroidal anti-inflammatory drug injury to the small intestine. *Gastrointest Endosc.* 1999;49: 544–546.

28. Nosho K, Endo T, Yoda Y, et al. Diaphragm disease of small intestine diagnosed by double-balloon enteroscopy. *Gastrointest Endosc.* 2005;62:187–189.

29. Maiden L, Thjodleifsson B, Theodors A, Gonzalez J, Bjarnason I. A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. *Gastroenterology.* 2005;128:1172–1178.

30. Goldstein JL, Eisen GM, Lewis B, et al. Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, and placebo. *Clin Gastroenterol Hepatol.* 2005;3:133–141.

31. Goldstein JL, Eisen GM, Lewis B, Gralnek IM, Zlotnick S, Fort JG. Small bowel mucosal injury is reduced in healthy subjects treated with celecoxib compared with ibuprofen plus omeprazole, as assessed by video capsule endoscopy. *Aliment Pharmacol Ther.* 2007;25:1211–1222.

32. Maglinte DDT, Sandrasegaran K, Chiorean M, Dewitt J, Lappas JC. Radiographic investigations complement and add diagnostic information to capsule endoscopy of small bowel diseases. *Am J Roentgenol.* 2007;189:306–312.

33. Vogel J, Moreira AL, Baker M, et al. CT enterography for Crohn's disease. Accurate preoperative diagnostic imaging. *Dis Colon Rectum.* 2007;50:1761–1769.

34. Wiarda BM, Kuipers EJ, Heitbrink MA, van Oijen J. MR enteroclysis of inflammatory small-bowel diseases. *AJR.* 2006;187: 522–531.

35. Paulsen SR, Huprich JE, Fletcher JG, et al. CT enterography as a diagnostic tool in evaluating small bowel disorders. Review of clinical experience with over 700 cases. *Radiographics.* 2006;26: 641–657.

36. Liu YB, Liang CH, Zhang ZL, et al. Crohn's disease of the small bowel. Multidetector row CT with CT enteroclysis, dynamic contrast enhancement, CT angiography, and 3D imaging. *Abdom Imaging.* 2006;31:668–674.

37. Masselli G, Casciani E, Polettini E, Lanciotti S, Bertini L, Gualdi G. Assessment of Crohn's disease in the small bowel. Prospective comparison of magnetic resonance enteroclysis with conventional enteroclysis. *Eur Radiol.* 2006;16:2817–2827.

38. Kamata Y, Iwamoto M, Nara H, et al. A case of rheumatoid arthritis with protein losing enteropathy induced by multiple diaphragmatic strictures of the small intestine. Successful treatment by bougieing under double-balloon enteroscopy. *Gut.* 2006; 55:1372.

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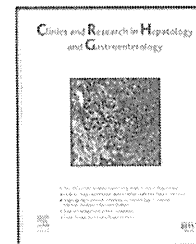
ORIGINAL ARTICLE

Evaluations of capsule endoscopy software in reducing the reading time and the rate of false negatives by inexperienced endoscopists ☆

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Summary

Background and objective: Capsule endoscopy (CE) is a comfortable for the patients; however, CE review is time-consuming. The aim of this study was (1) to evaluate the effectiveness of the CE software in reducing the CE reading time and the number of false negatives by beginners, and (2) to determine the learning curve for reading CE images.

Methods: Capsule endoscopic images were captured by Pillcam SB (Given Imaging Ltd, Tokyo, Japan), and analyzed using the proprietary RAPID 5 software. Comparison of CE reading using different software modes: manual mode, automatic mode, and QuickView (QV) mode. Three trainee endoscopists participated as CE readers. Each participant watched CE videos in which positive findings had been predefined by trained endoscopists. Each participant read the same CE record by using one of three different software modes. These were blinded on clinical history of patients. CE reading time was recorded, and the number of false negatives was counted. Each trainee endoscopist read a total of 45 CE videos, in five steps divided into nine videos per step.

Results: There was no significant reader associated difference between the results for the different modes. The QV software did miss some positive findings. Therefore, the total number

of instances of FN by the software plus the reader in the QV mode was significantly higher than the others. The reading times in the automatic mode and the QV mode were significantly shorter than that in the manual mode. After the second step, the number of instances of false negatives significantly decreased.

Conclusions: CE software is useful for reducing the reading time. Experience of approximately 20 CE readings can be considered as the first step to becoming an expert.

Introduction

Capsule endoscopy (CE) was first reported in 2000 [1] and is a useful method of detecting and diagnosing small-bowel diseases such as obscure gastrointestinal bleeding [2,3], small-bowel tumors [4], and inflammatory bowel disease [5,6]. In the CE procedure, patients are only required to swallow the capsule, which can take two pictures per second while traveling from the mouth to the anus. CE is a comfortable procedure for the patients; however, for the CE reader, it is time-consuming to review more than 50,000 images. This step contributes to the overall cost of CE. Recently, CE systems have been produced by several companies, notably Given Imaging [7—11] and Olympus Medical Systems [12]. These CE providers have introduced several features in the CE software to reduce the time required for analyzing the results of CE and minimize the possibility of missing lesions. These software can reduce the total number of images by combining similar images or by identifying the most unusual image, however, only a few studies [7—9] have assessed the usefulness of these in clinical settings; as a result, the practical usefulness of these features is still undetermined.

The increasing use of CE has led to the demand for training of CE beginners. The utility of computer-aided training for CE reading has been reported [13]. However, there is little information about the actual educational benefit of training and the CE learning-curve [14].

The primary aim of this prospective study was to evaluate the effectiveness of the CE software in reducing the reading time for a trainee endoscopist and in minimizing the probability of missing lesions. The secondary aims were to assess the impact of experience and to determine the learning-curve for CE.

Material and methods

Study design

This study was approved by the ethics committee at Keio University Hospital. Three trainee endoscopists (R.B., R.I., and Y.I.) participated as CE readers. The participants had graduated 5—7 years prior, and all of them had performed CE reading less than five times before participating in this study. All the participants were blinded to the patients' clinical history. Each participant watched the CE video in which positive findings (the reference standard) were predefined by two well-trained endoscopists (N.H., M.N.) who had each performed more than 150 CE examinations. The assignment list and flowchart of CE readings is shown in Fig. 1. Each step consisted of nine CE readings. Each participant read the same CE record by using one of three different software

modes, namely, manual mode, automatic mode, and Quick-View (QV) mode. The CE reading time, which was defined as the interval between the appearance of the first duodenal image and the first cecal image, was recorded. After each step, the CE reading results were judged and the number of false negatives (FN) was counted by trained endoscopists. A FN was defined as an instance where a trainee failed to recognize a lesion that had been identified by the experts. Simultaneously, the number of instances of FN caused by the CE software in the automatic mode and the QV mode was counted by trained endoscopists to evaluate the effectiveness of the CE software. Feedback on the CE readers' performance was shared with them after every step. A total of five steps of nine videos each, comprising 45 CE readings were prospectively conducted.

Capsule endoscopy procedure and software

From July 2008 to August 2009, 95 CE procedures were performed using Pillcam SB (Given Imaging Ltd, Tokyo, Japan) at Keio University Hospital. From these, 45 CE recordings were randomly selected for this study. The indications for performing CE were obscure gastrointestinal bleeding, chronic diarrhea, use of non-steroidal anti-inflammatory drugs,

Case number	Trainee A	Trainee B	Trainee C
STEP I -1	A	Q	M
I -2	M	A	Q
I -3	Q	M	A
I -4	A	Q	M
I -5	M	A	Q
I -6	Q	M	A
I -7	A	Q	M
I -8	M	A	Q
I -9	Q	M	A
STEP II -1	A	Q	M
II -2	M	A	Q
II -3	Q	M	A
II -4	A	Q	M
⋮	⋮	⋮	⋮
STEP V -9			

Step I

Feed back

Step II

Total of 5 steps with 45 cases

Figure 1   Assignment list and flowchart of the CE reading protocol. Each step included nine CE readings. In each step, the participants read the same CE record by using different software modes. For example, in the step I-1, trainee A read the video by using the automatic mode; trainee B, using the Quick-View mode; and trainee C, using the manual mode. After each step, the CE reading time and the number of false negatives were judged by experts; the results of the experts' assessments were shared with the trainees, and the next step was started. M: manual mode; A: automatic mode; Q: QuickView.

Table 1 The predefined findings in the capsule endoscopy video.

Predefined findings	Step 1	Step 2	Step 3	Step 4	Step 5	Total
Erosion	30	31	21	28	18	128
Lymphangiectasia/Lymphangioma	14	7	10	10	2	43
Redness	7	7	12	6	8	40
Ulcer	2	9	6	6	3	26
Petechia	4	3	4	2	1	14
Varix	0	1	2	2	7	12
Bleeding	4	2	4	0	1	11
Scar	1	3	1	2	3	10
Angioectasia	0	2	0	2	6	10
Tattooing	0	1	0	1	7	9
Others	11	5	2	0	1	19
Total	73	71	62	59	57	322

suspected inflammatory bowel disease. Obscure gastrointestinal bleeding was the main indication for performing CE. CE was performed after a 12-hour fasting period. The recorded digital information was downloaded from the recorder into the computer, and the images were analyzed using the proprietary RAPID 5 software. All participants read the CE video using the Quad View viewing format with a frame rate of 25 frames per second. The reader could not slow the frame rate down, could pause the images, review the images. The manual, automatic, and QV (sampling rate: normal) modes of the software were used in the analysis. In the manual mode, CE reading was performed without assistance of the CE software. In the automatic mode, the software reduces the total number of images by combining similar images [8]. In the QV mode, the software reduces the number of recorded images by identifying the most unusual image and presenting the selected images sequentially for review [5,9].

Predefined findings

The gold standard for positive findings was the experts' consensus. The predefined findings in the CE videos are shown in Table 1. Small findings, such as redness, lymphangiectasia, petechia and tattooing, were included as positive findings in this study. The number of predefined findings was not significantly different between each step.

Statistical analysis

Statistical analysis was performed using PASW version 17 software (SPSS Inc., Tokyo, Japan). All data showed a non-normal distribution. The data matched pairs were compared using the Wilcoxon matched pairs signed ranks test to assess statistical significance. Another non-matched data was applied the Mann-Whitney U test. It is recognized that there was multiple testing of outcome data arising from individual CE images reviewed by individual participants. The method of Bonferroni was used to correct P-values to recognize multiple testing of outcome data. However, as this study was meant to highlight differences, each outcome was analyzed separately meaning that the method of Bonferroni was

applied only to multiple considerations of data within each outcome variable. Thus, in order for statistical significance to be associated with a P-value less than 0.05, after Bonferroni correction, we require that the nominal P-values, shown here uncorrected, to take values less than 0.01 to be considered statistically significant after Bonferroni correction for multiple testing.

Results

Few false positives defined as a trainee detected a lesion that had not been identified by the experts were recognized in each step. Therefore, we mainly evaluated lesion detecting ability and false negatives (FN).

The type of lesions and the total number of FN are shown in Table 2. The total number of FN by the three beginners was 179. Distributions of the number of FN for each software mode is shown in Fig. 2. There was no significant reader associated difference between the results for the different modes. There were no instances of FN caused by the automatic mode software. In contrast, the QV software did miss some positive findings. Therefore, the total number of instances of FN by the software plus the reader in the QV mode was significantly higher than the corresponding numbers for the manual and automatic modes.

Table 2 Types of lesions and the total number of false negatives.

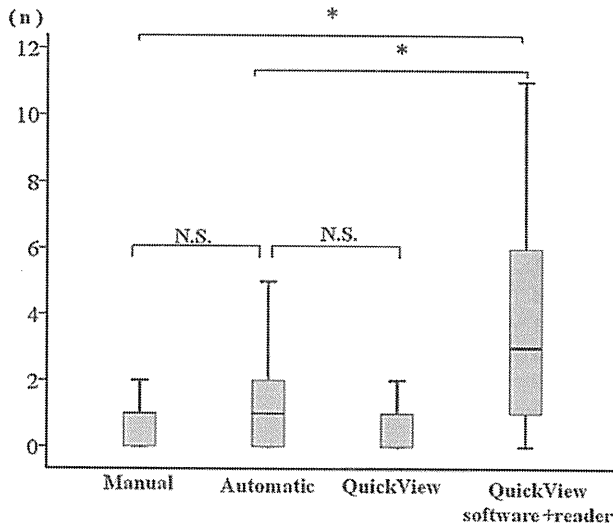| Type of lesion	Number
Erosion	61
Lymphangiectasia/Lymphangioma	39
Petechia	20
Redness	20
Ulcer	14
Angiectasia	9
Scar	8
Stenosis	4
Tatooing	4
Total	179

Figure 2   Distributions of the number of FN in each software mode. The number of FN by the readers was not significantly different across modes. The automatic mode did not cause any instances of FN. However, the QV mode was responsible for some instances of FN; the number of FN by the software plus reader in the QV mode was significantly higher than the corresponding values for the manual and automatic modes. The data were analyzed by multiple-testing analysis. Bonferroni's method was used to recognize the issue of multiple testing of outcome data for this outcome, requiring that only a nominal P-value less than 0.01 could be taken as statistically significant after correction. The box plots show median value, interquartile ranges (IQRs), and 95% ranges (extremes and outliers are not shown). The median number of FN for the manual, automatic, QV, and QV (software + reader) modes were 1, 1, 1, and 3, respectively. The interquartile ranges of FN for the manual, automatic, QV, and QV (software + reader) modes were 1, 2, 1, and 5, respectively. N.S.: not significant.

The reading time of the video for each software mode is shown in Fig. 3. The data represent the time required to read 1 minute of the video. The data for reading time was not normally distributed. The median reading times for the manual, automatic, and QV modes were 7.26, 4.38, and 1.35 s, respectively. The reading times for the automatic and QV modes were significantly shorter than that for the manual mode. The reading time for the QV mode was significantly shorter than that for the automatic mode.

Learning curve

The average number of FN in each step is shown in Fig. 4. The sequential line graph shows more than 1 FN per review in step 1 and step 2. However, in step 3 and later, the average number of FN was less than one per review. The difference between the values for steps 2 and 3 appeared to be substantial, and statistical analysis was performed. The distributions of the number of FN in steps 1—2 and steps 3—5 are shown in Fig. 5. The number of FN in steps 3—5 was significantly lower than the corresponding number in steps 1—2. We also evaluated the reading time in each step. However,
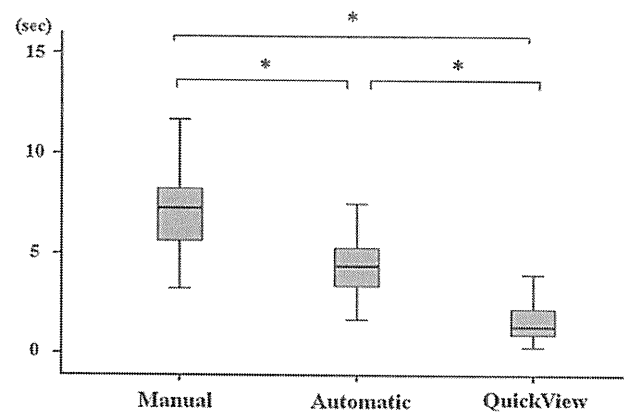

Figure 3   The reading time of the video by using each software mode. The data represent the time required to read 1 minute of the video. This was determined by the following formula: the total reading time divided by the total recording time. The data were analyzed by multiple-testing analysis. Bonferroni's method was used to recognize the issue of multiple testing of outcome data for this outcome, requiring that only a nominal P-value less than 0.01 could be taken as statistically significant after correction. The box plots show median value, interquartile ranges (IQRs), and 95% ranges (extremes and outliers are not shown). The median reading times for the manual, automatic, and QV modes were 7.26, 4.38, and 1.35 s, respectively. The interquartile ranges of FN for the manual, automatic, QV modes were 2.68, 1.97, 1.36 s, respectively.
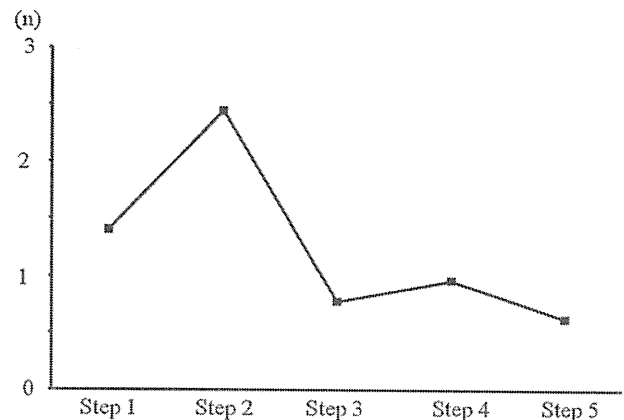

Figure 4   The sequential line graph showing the average number of FN in each step. In step 1 and step 2, more than one FN occurred in each CE reading. However, in step 3 and later, the average number of FN was less than one in each CE reading.

the reading time for each step was not significantly different (data not shown).

Discussion

First, we attempted to evaluate the efficacy of the CE software. We hypothesized that the CE software facilitated CE reading for beginners and compensated for the lack of CE-reading abilities. To our knowledge, only a few reports [9—11] have confirmed the efficacy of CE software. Westerhof et al. [7] suggested that CE software packages are time-saving tools, but they also stated that the QV mode
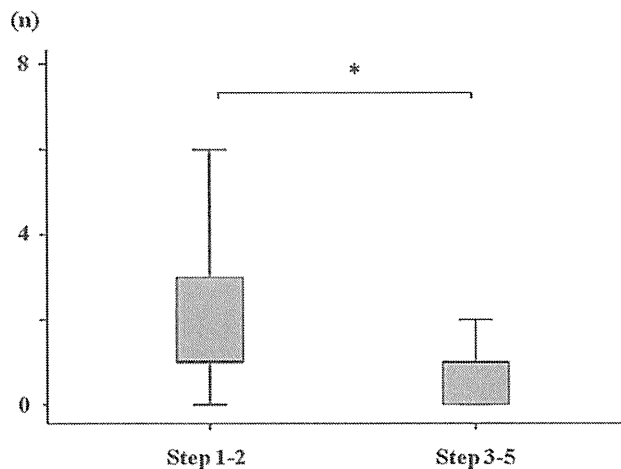
Figure 5 Distributions of the number of FN in steps 1—2 and steps 3—5. The number of errors in recognition in steps 3—5 was significantly lower than that in steps 1—2. Box plots show median value, interquartile range (IQRs), and 95% ranges (extremes and outliers are not shown). The median number of FN in steps 1—2 and steps 3—5 were 1, 1, respectively. The interquartile ranges of FN in steps 1—2 and steps 3—5 were 2, 1, respectively.

is associated with considerably high rates of incorrect diagnosis. Moreover, they concluded that these techniques may be applicable only if diffuse lesions are suspected. In our results, the CE software could reduce the CE reading time but could not reduce the incidence of errors in recognition. The reading time in the automatic mode was significantly shorter than that in the manual mode. However, the number of FN in the automatic mode was not higher than that in the manual mode. Our study was not an equivalence study. These data could not directly confirm the equivalence between the manual and automatic modes. However, these data suggest the possibility that the automatic mode can reduce the reading time without increasing the instances of FN.

The reading time in the QV mode was significantly shorter than that in the manual and automatic modes. However, in the QV mode, the number of FN significantly increased because of an increase in the number of software-associated FN. QV is essentially a technique for previewing video. Thus, QV is not appropriate for identifying small lesions.

The second aim was to assess the educational effect of the CE software and to determine the learning-curve for CE. Postgate et al. [13] reported the efficacy of the computer-aided learning system for CE reading. They showed the significance of CE training by using an E-learning system. Sidhu et al. [14] inferred that 50 CE readings were required to achieve competency in CE reading. Our data suggested the combined efficacy of reading experience and feedback-based education. Experience of approximately 20 CE readings with feedback-based education significantly reduced the possibility of missing lesions. However, the reading time did not decrease after step 2. We believed that this was because the use of the Quad View at 25 frames per second was too fast for a beginner. Therefore, experience and education affected only the number of FN with each step. Our results suggest that the first step in becoming a CE expert is training with approximately 20 CE readings. In

the second step, training with more than 50 CE readings may reduce the reading time, as suggested by Sidhu et al. [14].

In conclusion, CE software was useful for reducing the CE reading time. Experience of approximately 20 CE readings can be considered as the first step to becoming an expert.

Disclosure of interest

Acknowledgements

References

[1] Iddan G, Meron G, Glukhovsky A, et al. Wireless capsule endoscopy. Nature 2000;405(6785):417.

[2] Triester SL, Leighton JA, Leontiadis GI, Fleischer DE, Hara AK, Heigh R, et al. A meta-analysis of the yield of capsule endoscopy compared to other diagnostic modalities in patients with obscure gastrointestinal bleeding. Am J Gastroenterol 2005;100(11):2407—18.

[3] Saperas E, Dot J, Videla S, Alvarez-Castells A, Perez-Lafuente M, Armengol JR, et al. Capsule endoscopy versus computed tomographic or standard angiography for the diagnosis of obscure gastrointestinal bleeding. Am J Gastroenterol 2007;102(4):731—7.

[4] Trifan A, Singeap AM, Cojocariu C, Sfarti C, Stanciu C. Small bowel tumors in patients undergoing capsule endoscopy: a single center experience. J Gastrointestin Liver Dis 2010;19(1):21—5.

[5] Mehdizadeh S, Chen GC, Barkodar L, Enayati PJ, Pirouz S, Yadegari M, et al. Capsule endoscopy in patients with crohn's disease: diagnostic yield and safety. Gastrointest Endosc 2010;71(1):121—7.

[6] Albert JG, Martiny F, Krummenerl A, Stock K, Lesske J, Gobel CM, et al. Diagnosis of small bowel crohn's disease: a prospective comparison of capsule endoscopy with magnetic resonance imaging and fluoroscopic enteroclysis. Gut 2005;54(12):1721—7.

[7] Westerhof J, Koornstra JJ, Weersma RK. Can we reduce capsule endoscopy reading times? Gastrointest Endosc 2009; 69(3 Pt 1):497—502.

[8] Liangpunsakul S, Mays L, Rex DK. Performance of given suspected blood indicator. Am J Gastroenterol 2003;98(12): 2676—8.

[9] D'Halluin PN, Delvaux M, Lapalus MG, Sacher-Huvelin S, Ben Soussan E, Heyries L, et al. Does the ''suspected blood indicator'' improve the detection of bleeding lesions by capsule endoscopy? Gastrointest Endosc 2005;61(2):243—9.

[10] Keuchel M, Al-Harthi S, Hagenmüller F. New automatic mode of Rapid 4 software reduces reading time for small bowel Pill-Cam studies [abstract]. Paris: 5th International Conference of Capsule Endoscopy; 2006.

[11] Smelkin I. Rapid 4 Quickview V4 has 100% negative and positive predictive values [abstract]. Paris: 5th International Conference of Capsule Endoscopy; 2006.

[12] Ogata H, Kumai K, Imaeda H, Aiura K, Hisamatsu T, Okamoto S, et al. Clinical impact of a newly developed capsule endoscope: usefulness of a real-time image viewer for gastric transit abnormality. J Gastroenterol 2008;43(3):186–92.

[13] Postgate A, Haycock A, Thomas-Gibson S, Fitzpatrick A, Bassett P, Preston S, et al. Computer-aided learning in capsule endoscopy leads to improvement in lesion recognition ability. Gastrointest Endosc 2009;70(2):310–6.

[14] Sidhu R, Sakellariou P, McAlindon ME, Leeds JS, Shafiq K, Hoeroldt BS, et al. Is formal training necessary for capsule endoscopy? The largest gastroenterology trainee study with controls. Dig Liver Dis 2008;40(4):298–302.

Lemon Grass (*Cymbopogon citratus*) Ameliorates Murine Spontaneous Ileitis by Decreasing Lymphocyte Recruitment to the Inflamed Intestine

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ABSTRACT

Objective: Aberrant leukocyte migration has been implicated in the pathogenesis of inflammatory bowel disease (IBD). Lemon grass is a natural herb that contains citral, which suppresses lymphocyte expression of gut homing molecules by inhibiting retinoic acid formation. We therefore hypothesized that lemon grass intake could ameliorate excess migration of leukocytes to the inflamed intestine in chronic ileitis.

Methods: Migration of fluorescence-labeled T cells to microvessels in the ileal mucosa of SAMP1/Yit mice was monitored using intravital microscopy. In some mice, lemon grass solution was administered for two weeks. For evaluation of the effects on chronic ileitis, mice were treated with lemon grass for 26 weeks.

Results: Surface expression of β7 and CCR9 on T lymphocytes was stronger in SAMP1/Yit mice than in AKR/J mice. Lemon grass treatment attenuated the surface expression of β7-integrin and CCR9. The number of adherent lymphocytes to microvessels in chronic inflamed ileum was significantly few when lymphocytes were isolated from lemon grass treated mice. Long-term lemon grass treatment improved ileitis in SAMP1/Yit mice, which was assessed by body weight, histological changes and the infiltration of β7-positive cells.

Conclusion: Lemon grass ameliorated ileitis through decreasing lymphocyte migration by inhibiting β7-expression, suggesting its therapeutic usefulness for IBD.
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KEY WORDS: retinoic acid, lymphocytes, β7-integrin, CCR9, intestinal microvessels

ABBREVIATIONS: IBD, inflammatory bowel disease; RA, retinoic acid; SAMP/Yit, senescence-accelerated; CCR, chemokine receptor; DC, dendritic cell; TECK, thymus-expressed chemokine; ECAM, endothelial cell adhesion molecule; CD, Crohn's disease; ADH, alcohol dehydrogenase; RALDH, retinal dehydrogenase; MLN, mesenteric lymph node; PP, Peyer's patch; HPLC, high-performance liquid chromatography; ATRA, *all-trans* retinoic acid.

INTRODUCTION

Recirculation of lymphocytes through the body is critical for immune regulation, and the compartmentalization of mucosal and systemic immune responses is mainly based on the selective expression of homing receptors by lymphocytes [3]. For the preferential homing of T cells to the gut, expression of integrinα4β7 [11] and the chemokine receptor CCR9 is induced by antigenic stimulation with dendritic cells (DC) from gut-associated lymphoid organs. They will localize to the small intestinal mucosa, where their appropriate ligands (mucosal addressin cell adhesion molecule-1 [MAdCAM-1] and CCL25 [thymus-expressed chemokine: TECK], respectively) are expressed by postcapillary venules and intestinal mucosa [5,19,21]. It has been shown in both experimental animals and humans

that inflammation is associated with enhanced expression of endothelial cell adhesion molecules (ECAMs) in the intestine [18]. The expression of MAdCAM-1 in Crohn's disease suggests that leukocyte recruitment is strongly involved in CD [2]. Enhanced expression of ECAMs causes aberrant leukocyte migration, leading to extension of inflammatory response. Blocking of leukocyte recruitment as a strategy for treating intestinal inflammation was successful in animal models of Crohn's ileitis [1,8,13]. In clinical studies, therapeutic compounds directed against trafficking of leukocytes have been designed and are being developed as a novel class of drugs for treatment of inflammatory diseases, especially CD [24,29]. Although these biological modifiers have promising effects, there are relatively high risks of side effects such as anaphylaxis known as infusion reaction and formation of human anti-chimeric antibody is responsible for decrease in long-term efficacy [29].

Recently, the mechanisms by which intestinal DCs induce up-regulation of gut-tropic adhesion molecules on lymphocytes have been clarified [11,14]. It is due to their unique expression of retinoid hydrogenase enzymes that convert dietary vitamin A to retinoic acid (RA), which has been shown to directly up-regulate α4β7 and CCR9 expression on T cells [9]. RA is converted from retinol by alcohol dehydrogenases (ADHs) and retinal dehydrogenases (RALDHs). RALDH has three isoforms and the distribution of the three isoforms has organ specificity. Iwata *et al.* found that mesenteric lymph node-dendritic cell expressed ADH class III and RALDH2 and that Peyer's patch-dendritic cell expressed ADH class I, II, III, and RALDH1 protein [9]. RA is widely distributed throughout the body and may have various functions to the body. On the other hand, excess intake of vitamin A has been suggested to increase the risk of inflammatory bowel disease (IBD) [22]. Because of the organ-specific isoform distribution of RALDH, it is possible to control RA concentration locally by using an RALDH isoform-specific inhibitor, leading to decrease in excessive adhesion molecule expression. *Cymbopogon citrates*, known as "lemon grass", is commonly used in Thai and Vietnamese cooking. Lemon grass also has been used for medical purposes, especially in cases of diarrhea, for many years in West Africa and India, though the mechanisms of its actions have not been clarified. Its principal ingredient, citral, has been reported to have two distinct functions, an anti-microbacterial

activity against bacteria or fungi [17] and inhibition of RALDH activity [4]. Therefore, we hypothesized that intake of natural food rich in citral may control aberrant lymphocyte recruitment to the gut and may control intestinal inflammation.

To assess this hypothesis, we chose senescence-accelerated mice (SAMP1/Yit) that develop chronic ileitis spontaneously, especially at the end of the ileum, histologically resembling CD, with virtually 100% penetrance at the age of 30 weeks [12]. In this study, we investigated the effect of lemon grass treatment on lymphocyte recruitment to intestinal microvessels. In addition, the effect of long-term treatment of lemon grass on severity of ileitis in SAMP1/Yit mice was also investigated.

MATERIALS AND METHODS

Animals and Administration of Lemon Grass

SAMP1/Yit mice (kindly provided by Dr. Satoshi Matsumoto, Yakult Central Institute for Microbiological Research, Tokyo, Japan) and control AKR/J (9–35 weeks) mice (Japan Clea Co., Tokyo, Japan) were maintained in an animal colony at National Defense Medical College (NDMC), Saitama, Japan. They were housed in a specific pathogen-free animal facility. Freshly prepared lemon grass solution was added to drinking water. To eliminate difference of citral concentration between batches of lemon grass (imported from Thailand and distributed by MIMURO, Shizuoka, Japan), we prepared 7500 g of lemon grass and mixed it thoroughly and we packed 5 g in each package and stored carefully in cool dark place until just before use. The lemon grass was boiled and added to the drinking water at the concentration of 25 g/500 mL. This concentration was chosen in reference to lemon grass tea and tom yam soup. In a lemon grass tea, one tea spoon of lemon grass (2–3 g) is used in one cup of boiling water (200 mL). In tom yum soup, 25 g of lemon grass is used (for aromatic addition) in about 300 mL of the soup.

For lymphocyte trafficking study, T lymphocytes were isolated from mice which received lemon grass treatment for two weeks ($n = 5$ in each group). To evaluate the effects of long-term treatment on activity of ileitis, SAMP1/Yit mice received lemon grass treatment for 26 weeks from nine weeks of age until 35 weeks of age ($n = 9$ in each group). Because the environment affects the development of ileitis, we

studied control group and treatment study simultaneously. Mice of the same age were divided into two groups. We sacrificed at 26 weeks for the histological evaluation. Observation of body weight was continued until 35 weeks. The experimental protocol was approved by the Animal Research Committee of NDMC (No. 7504), and the care and use of laboratory animals were in accordance with the guideline.

Measurement of Citral Concentration and Evaluation of *All-Trans* Retinoic Acid Formation

The concentration of citral in lemon grass solution and in the serum was measured by high-performance liquid chromatography (HPLC) [30]. *All-trans* retinoic acid (ATRA) formation from *all-trans* retinal was also evaluated in mesenteric lymph nodes (MLNs) by HPLC. In brief, MLNs were sonicated and 500 µg of protein was incubated with 3.0 mg of *all-trans* retinal (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 30 mL dimethyl sulfoxide in 20 mM HEPES buffer, pH 7.5 containing 150 mM KCl and 2 mM DL-dithiothreitol, of which the total volume was 1500 mL. After incubation at 37.8°C for 20 minutes, 500 mL saturated ammonium sulfate was added to 500 mL of reaction mixture to stop reaction. Then, 500 mL ethanol, 2 mL pure water and 4 mL of a solution containing *n*-hexane, acetic acid and dichloromethane (80:9:1), were added to the mixture. In each sample 20 mM arotinoid ethylsulfone (Kindly donated by Hoffmann-La Roche [Basel, Switzerland]) was added as an internal standard. After samples were shaken well for 10 minutes, centrifuged at 2700 *g* for four minutes and the organic layer was collected. After evaporation the extracts were re-dissolved in 200 mL *n*-hexane, and 50 µL of the solution was used for HPLC quantitation. The areas under the curves of various retinoids, including ATRA and arotinoid ethylsulfone, were then calculated using a chromato-integrator.

Analysis of RALDH2 mRNA Expression by RT-PCR

DCs were isolated from MLNs by magnetic cell sorting system (MACS; Miltenyi Biotec, Auburn, CA, USA) with Pan DC MicroBeads (130-092-465; Miltenyi Biotec). MicroBeads were conjugated to monoclonal anti-mouse CD11c (N418) antibodies (isotype: hamster IgG) and anti-mRDCA-1 antibodies (isotype: rat IgG1). Total RNA was extracted from isolated DCs using the RNeasy Mini isolation kit (Qiagen, Hilden, Germany). Aliquots

of total RNA were reversely transcribed using SuperScriptII Reverse Transcriptase (Invitrogen Co., Carlsbad, CA, USA). Primers and probes for RALDH2 were performed by using Assays-on-Demand Gene Expression probes (Mm00501306) (Applied Biosystems, Foster City, CA, USA). TaqMan RT-PCR was performed in triplicate for each sample using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). To standardize the quantitation of RALDH2 gene, GAPDH from each sample was quantified on the same plate with the target genes by using TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems).

Isolation of T Lymphocytes and Analysis of Surface Adhesion Molecules by Fluorescence-Activated Cell Sorter

AKR/J and SAMP1/Yit mice spleen were isolated and red blood cells were lysed in ammonium phosphate/chloride lysis buffer. A whole cell population of 2×10^7 lymphocytes in 3 mL RPMI medium with 1% fetal calf serum was incubated in 1 g of nylon wool (Wako Pure Chemical, Osaka, Japan) in a column for one hour at 37°C, and the eluted fraction was designated as the T cell. Purity of T cells was confirmed by CD3 positive cells by flow-cytometry and was at least more than 95%. For immunofluorescence staining, anti-mouse MAbs against mouse β7-integrin (FIB27) (PharMingen, San Diego, CA, USA) and CCR9 (#242503) (R&D Systems, Minneapolis, MN, USA) were used. Flow cytometric analysis was performed using FACScalibar (Becton Dickinson, Mountain View, CA, USA).

Lymphocyte Labelling with Carboxyfluorescein Diacetate Succinimidyl Ester and Intravital Observation of Lymphocyte Migration in Murine Small Intestinal Mucosa

Carboxyfluorescein diacetate succinimidyl ester (CFDSE; Molecular Probes, Eugene, OR, USA) was used for lymphocyte labeling as previously described [5]. After an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the five-cm ileal segment was gently placed onto the plate. A longitudinal incision was made by microcautery along its anti-mesenteric border. The intestine was kept warm and moist by continuous superfusion with warmed physiological saline. Lymphocytes (2×10^7 dissolved in 0.2 mL RPMI) were injected into the jugular vein of the recipient mice for three minutes. The cell kinetics of infused labeled lymphocytes in

villus microcirculation was observed from the mucosal surface by an inverted fluorescence microscope (Diaphot TMD-2S; Nikon, Tokyo, Japan) equipped with a silicon-intensified target camera with a contrast-enhancing unit (C-2400-08; Hamamatsu Photonics, Shizuoka, Japan) [5,13] and recorded at ten-minute intervals for 60 minutes. We defined the sticking lymphocytes as the lymphocytes that adhered to the wall at the same position in the microvessels without moving away for more than 30 seconds [5,13]. We measured the number of sticking lymphocytes to the ileal microvessels per mm^2.

Effect of Long-Term Lemon Grass Treatment on Spontaneous Ileitis of SAMP1/Yit Mice

After long-term lemon grass treatment, terminal ileum was removed at 26 weeks of age, and histological changes were evaluated in H&E stained sections in a blinded fashion.

The MAdCAM-1 expression in vascular endothelium and β7-integrin positive cell infiltration were assessed immunohistochemically by using the labeled streptavidin biotin method. Small intestine of mice were removed and fixed in periodate lysine paraformaldehyde. Cryostat sections were incubated with mAb against MAdCAM-1 (MECA-367; 0.5 mg/mL) (PharMingen) or β7-integrin (FIB27; 0.5 mg/mL) (PharMingen) diluted 50 times with PBS overnight at 4°C. Sections were visualized by FITC-conjugated streptavidin (Amersham) for 30 minutes at room temperature. These sections were observed under a confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

Statistics

All results were expressed as means ± SD. The data were analyzed using an one-way ANOVA with Fisher's *post hoc* test. Statistical significance was set at $p < 0.05$. Student's *t*-test was used when only two groups were compared. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of Oral Intake of Lemon Grass Solution on Citral Concentration and Formation of ATRA in AKR/J Mice

We first examined whether oral intake of lemon grass solution affects the physical condition of con-

trol AKR/J mice. There was no difference in solution consumption between lemon grass treated AKR/J mice group and control water AKR/J mice group (each 7.5 mL/day). There was no difference in diet consumption and there was no difference in weight gain and general appearance between two groups during observation period for two weeks. The concentration of citral in the blood of lemon grass-treated AKR/J mice was 24.8 ± 13 ng/mL by HPLC assay (Figure 1A).

RALDH2 mRNA is expressed in DC of MLNs in AKR/J mice as well as in SAMP1/Yit mice. The level of RALDH2 mRNA expression in DC from MLNs was not changed by lemon grass treatment (data not shown). RALDH2 catalyzed the irreversible pathway from retinal to *all-trans* RA and *9-cis*-RA. Both *all-trans* RA and *9-cis*-RA are physiologic RA, although physiologic activity of *9-cis*-RA is much less than that of *all-trans* RA. We also determined by *in vitro* experiments whether conversion of retinal into RA occurs in MLNs of AKR/J mice. *All-trans* retinal was converted into ATRA by the addition of protein extract from MLNs (Figure 1B). Conversion of ATRA was significantly attenuated by adding citral to them.
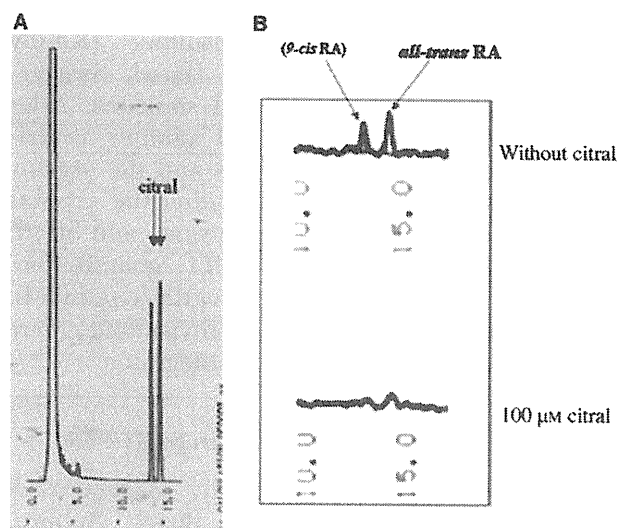

Figure 1. (A) Peak curve of citral in murine serum detected by using high-performance liquid chromatography (HPLC). (B) Formation of *all-trans* retinoic acid from *all-trans* retinal by incubation with protein extract from mesenteric lymph nodes of AKR/J mice using HPLC and the effect of citral. Representative data from four individual measurements are shown.

Effect of Oral Intake of Lemon Grass Solution on Expression of Gut-Homing Molecules on T cells by Fluorescence-Activated Cell Sorter

Citral has been shown to attenuate the expression of β7-integrin and CCR9 on T lymphocytes co-cultured with DC *in vitro* [14]. We therefore investigate whether oral administration of lemon grass rich in citral to mice affects the expression of gut-homing molecules on lymphocytes *in vivo*. Figure 2A shows the expression of β7-integrin on T lymphocytes isolated from AKR/J mice and SAMP1/Yit mice. Percentage of β7-integrin^{high} expression positive cells of AKR/J mice was

decreased by lemon grass treatment for two weeks compared with that in the controls, suggesting that a decrease in β7-integrin^{high} expression level occurred even in AKR/J mice. The expression level of β7-integrin^{high} in non-treated SAMP1/Yit mice (diseased controls) was significantly higher than that in AKR/J mice. In SAMP1/Yit mice treated with lemon grass, however, β7-integrin^{high} expression was successfully attenuated to a level almost the same as that in AKR/J mice. Figure 2B shows the effect of lemon grass treatment on expression of CCR9 levels. Similar to the observation of β7-integrin^{high} expression, lemon grass treatment decreased the expression level of CCR9 in AKR/J
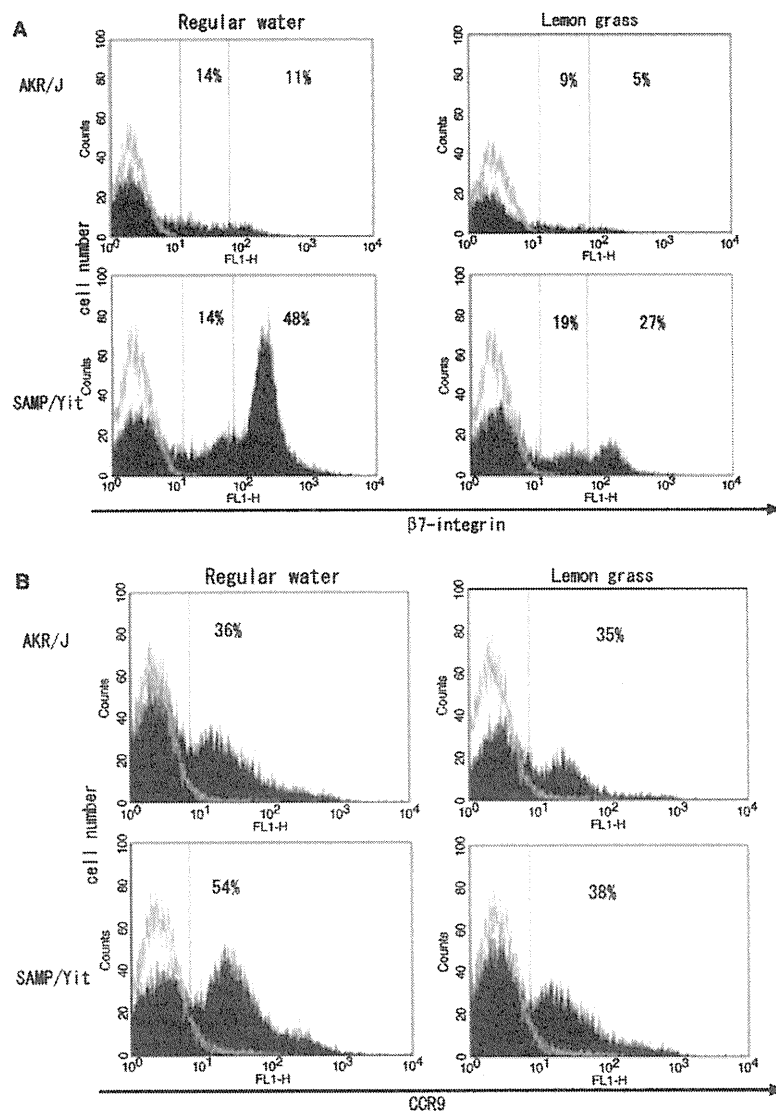

Figure 2. Expression of β7-integrin (A) and CCR9 (B) on T lymphocytes determined by flow cytometric analysis. Representative data from four individual measurements are shown.

mice, although the degree of decrease was less observed than that of $\beta7$-integrin^{high}. The increased level of CCR9 expression in SAMP1/Yit mice (diseased control) was also significantly reduced to a level comparable to that in AKR/J mice.

In vivo Observation of Lymphocyte Migration to Ileal Microvessels and the Effect of Lemon Grass Treatment

To investigate the functional significance of changes in gut-homing molecules, adhesive interaction of labeled T lymphocytes with ileal microvessels was observed. Lymphocytes were isolated from the spleens of AKR/J mice. In order to examine the effect of oral lemon grass solution intake, AKR/J mice were treated for two weeks before isolation of lymphocytes. In these experiments, the numbers of lymphocytes entering ileal microvessels were not significantly different among the treatment groups at 10 minutes after infusion (data not shown). The number of lymphocytes adhering to intestinal microvessels increased in a time-dependent manner and reached almost a saturated condition at 40–60 minutes. Figure 3A–C shows representative microscopic images 60 minutes after cell infusion. Consistent with significant ileitis, a remarkable enhancement of lymphocyte adherence was observed in the ileal mucosa of SAMP1/Yit mice (Figure 3B) compared with that in AKR/J animals (Figure 3A). However, interestingly, when we used lymphocytes isolated from lemon grass treated animals, the increased lymphocyte recruitment to microvessels was abrogated in SAMP1/Yit mice (Figure 3C) and the number of adhering lymphocytes remained small as was comparable to that in control AKR/J mice (Figure 3D). Although we showed that lemon grass treatment decreased T cell recruitment to inflamed intestinal mucosa by using T cells from AKR/J mice in this study, we observed in our preliminary study that lemon grass treatment decreased T cell recruitment to inflamed intestinal mucosa by using T cells from SAMP1/Yit mice also (data not shown).

Preventative Effect of Long-Term Lemon Grass Treatments Against Development of Ileitis in SAMP1/Yit Mice

Next we investigated the preventative effect of long-term lemon grass treatment against development of ileitis. At the time of weaning, there was
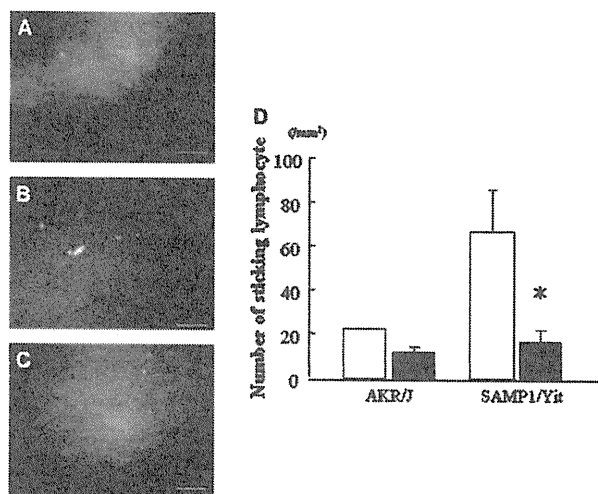

Figure 3. Representative images of distribution of fluorescence-labeled lymphocytes in the ileal microvessels after infusion of T lymphocytes isolated from spleen of AKR/J mice with or without lemon grass treatment for two weeks. (A) Adherent lymphocytes in AKR/J mice (Bar: 100 μm). (B) Increased adherent lymphocytes in SAMP1/Yit mice. (C) Decreased number of adherent lymphocytes from lemon grass treated AKR/J mice to SAMP1/Yit mice. (D) Number of lymphocytes sticking in the 1 mm^2 observation field in AKR/J mice and SAMP1/Yit mice. T lymphocytes isolated from AKR/J mice without lemon grass treatment: (open bar). T lymphocytes isolated from AKR/J mice with lemon grass treatment : (closed bar). Values are expressed as means ± SD for five animals. *$p < 0.05$ vs. without lemon grass treatment.

no histological evidence of ileitis in SAMP1/Yit mice. As previously reported [19], mild to moderate ileitis was first found in SAMP1/Yit mice at 20 weeks of age and reached 100% penetration by 30 weeks. As shown in Figure 4, body weight of SAMP1/Yit mice without receiving treatment (diseased controls) stopped increasing at about 20 weeks of age and gradually decreased after 25 weeks of age. However, body weights of SAMP1/Yit mice treated with lemon grass continued to increase after 25 weeks.

Histological changes in the ileum are shown in Figure 5. SAMP1/Yit mice at 26 weeks showed characteristic features in the ileum, decreased villus height (Figure 5B), submucosal thickening (Figure 5E) and increased size of muscular layer (Figure 5H) compared with those in control AKR/J mice (Figure 5A,D,G). On the other hand, these histological alterations were remarkably attenuated in the ileal mucosa of SAMP1/Yit mice
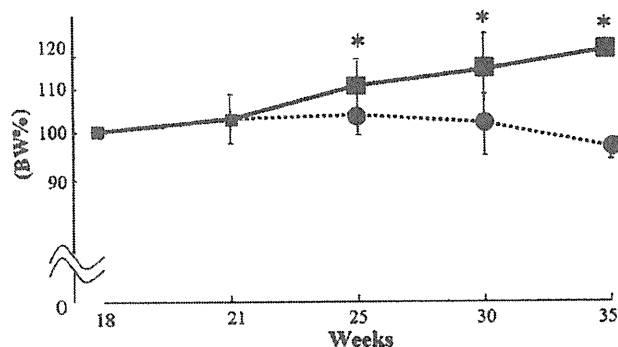
Figure 4. Time course of changes in body weight of SAMP1/Yit mice receiving regular water or lemon grass treatment. Body weight was expressed as percentage of weight at 18 weeks. Solid line represents lemon grass drinking, and dotted line for regular water drinking. Data are means ± SD for nine animals. *$p < 0.05$ vs. SAMP1/Yit mice receiving regular water.

that received long-term lemon grass treatment (Figure 5C,F,I). The level of histological changes remained small as was comparable to that in control AKR/J mice (Figure 5J,K,L). Figure 5J shows villus height. SAMP1/Yit mice showed significantly shortened villus height than those in AKR/J mice. Lemon grass treatment in SAMP1/Yit mice significantly attenuated the shortness of the villus height. Figure 5K,L showed the thickness of submucosal and muscular layer. SAMP1/Yit mice showed significantly greater thickness than those in AKR/J mice. Lemon grass treatment also effectively attenuated those parameters in SAMP1/Yit mice.

Next we investigated how lemon grass treatment affected the infiltration of β7-integrin-positive cells and the expression of MAdCAM-1, a counter ligand for β7-integrin, in the ileal mucosa of SAMP1/Yit mice. In control AKR/J mice, some β7-integrin-positive cells were found in the lamina propria, and MAdCAM-1 was also weakly expressed constitutively in the small venules. In SAMP1/Yit mice, on the other hand, a significant increase in the number of β7-integrin-positive cells (Figure 6B,F) with increased expression of MAdCAM-1 (Figure 6C,G) were demonstrated by immunohistochemistry. With or without lemon grass treatment, no difference was observed in the expression level of MAdCAM-1 in SAMP1/Yit mice (Figure 6C,D,G). However, lemon grass treatment significantly inhibited the increase in β7-integrin-positive cells in the ileum of SAMP1/Yit mice (Figure 6B,E) to levels almost comparable to those in AKR/J mice (Figure 6F).

DISCUSSION

In this study, we first demonstrated that oral administration of lemon grass, which contains citral, a RALDH2-specific inhibitor, successfully attenuated lymphocyte recruitment to the inflamed small intestinal mucosa by attenuating the expression of β7-integrin and CCR9 on T cells. We further demonstrated that long-term lemon grass treatment significantly attenuated the development of ileitis in SAMP1/Yit mice, a murine model for CD. Among the vitamin A metabolites, RA is the most powerful substance for activating β7-integrin expression on T cells [10]. In addition, the concentration of RA is controlled by RALDH locally. Thus it is possible to control tissue RA concentration by the use of an RALDH inhibitor. It is well known that citral is a natural RALDH2 inhibitor [4], and it has recently been reported that citral has the ability to attenuate the expression of gut-tropic homing molecules on lymphocytes *in vitro* by decreasing RA [9]. Although citral blocked RALDH2 activity, the expression of RALDH2 mRNA itself was not inhibited by citral treatment in this study. As RALDH2 is distributed specifically in MLNs [9], local control of the concentration of RA in MLNs is thought to be an effective strategy for attenuation of T cell recruitment to the inflamed gut. However, in this study, we also observed decreased expression of gut-homing molecules in splenic T lymphocytes after lemon grass treatment for two weeks. As the spleen is a secondary lymphoid tissue through which trafficking of many lymphocytes occurs, we speculate that a significant amount of lymphocytes migrated from MLNs to the spleen via systemic circulation after expression of homing molecules or receptors.

It is generally accepted that control of lymphocyte recruitment to the inflamed intestinal mucosa is an effective strategy for treatment of IBDs. Actually, we previously reported that control of T cell recruitment by using an antibody against MAdCAM-1 ameliorated ileitis in the same SAMP1/Yit mice [13]. In a clinical setting, much interest has been shown in the therapeutic benefit of endothelium-leukocyte interaction for patients with IBD, and several agents have been investigated in placebo-controlled trials in IBD patients. The use of anti-α4 integrin antibody therapy for the treatment of CD with the humanized antibody natalizumab, which targets the α4-integrin on leukocytes expressing α4β1 as well as α4β7 has been most
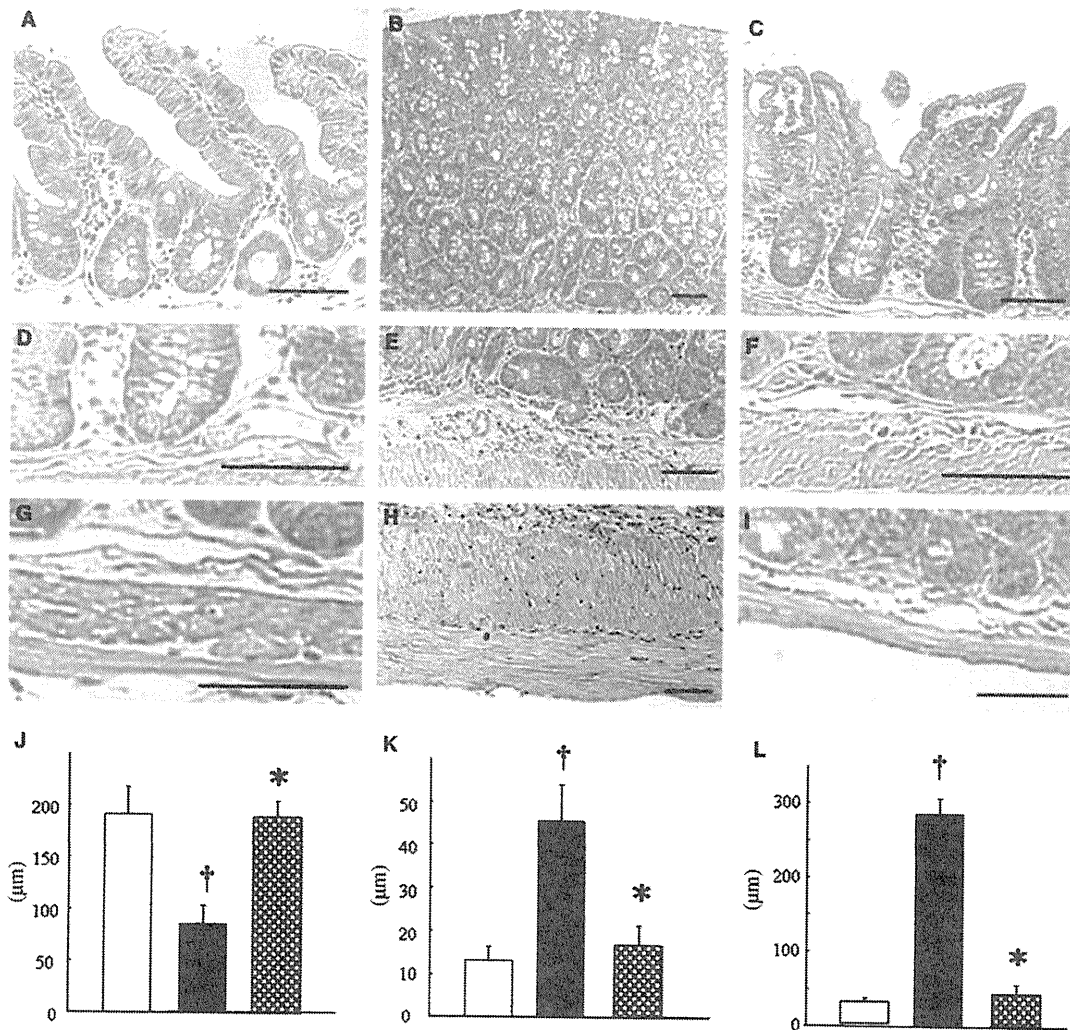
Figure 5. Histological changes of the ileum as determined in hematoxylin and eosin stained sections. (Bar: 100 µm) (A, D & G). Control AKR/J (B, E & H) SAMP1/Yit mice at 26 weeks show discontinuous inflammation, characterized by distorted villous architecture (A and B), expansion of lamina propria (D and E), and thickening of muscularis propria (G and H). (C, F & I) SAMP1/Yit mice at 26 weeks with long-term lemon grass drinking. These histological changes are significantly attenuated. Histological determination of villus height (J), submucosal thickness (K), and thickness of muscular layer (L) in control AKR/J mice (open bar), SAMP1/Yit mice drinking water (diseased control) (closed bar), and lemon grass drinking SAMP1/Yit mice (cross-hatched bar). Values are expressed as means ± SD for four animals. *$p < 0.05$ vs. SAMP1/Yit mice drinking regular water. †$p < 0.05$ vs. AKR/J mice.

extensively investigated. [6,24,29]. Although natalizumab showed benefit in a subset of patients during an induction and maintenance trial in Crohn's disease [23], the emergence of a life-threatening JC viral reactivation encephalitis interrupted pivotal trials in 2005, and the potential development of natalizumab remains in question, largely due to these safety issues [31].

Moreover, the CCL25/CCR9 chemokine ligand/receptor pair has been reported to play an important role in lymphoid cell trafficking in the small

bowel [7]. As CCL25 expression is limited to the small intestine and CCL25 is not expressed in the colon in physiological or inflamed conditions, CCL25/CCR9 play a significant role in small bowel inflammation. Actually, aberrant small bowel expression of CCL25 in patients with CD and increased proportion of CCR9(+) T cells in peripheral blood of patients with small bowel inflammatory diseases such as CD and celiac disease have been reported [20]. Saruta et al. recently demonstrated that CCR9(+) T cells in small bowel CD
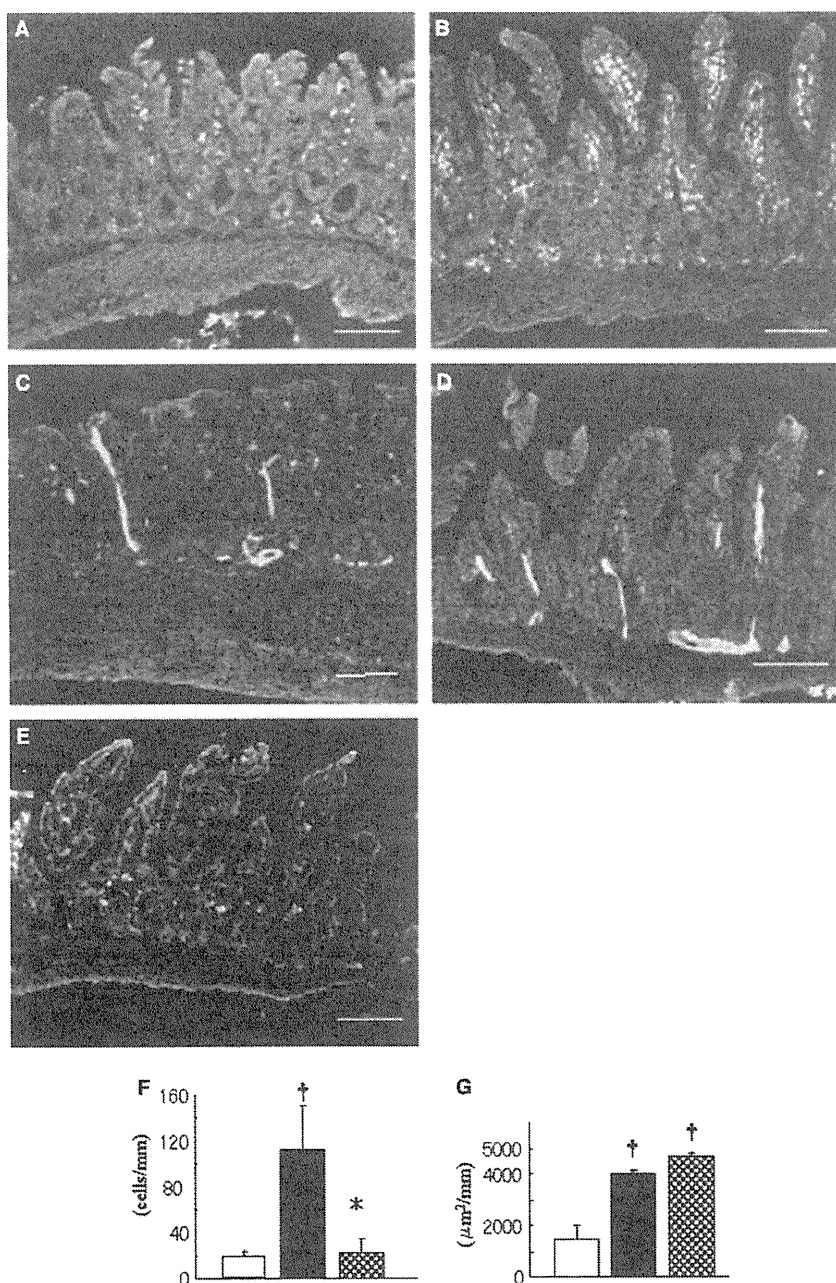
Figure 6. β7-integrin positive cells and MAdCAM-1 expression in the ileum as determined by immohistochemistry. (Bar: 100 μm) As a control, using the IgG staining image in ileal mucosa of SAMP1/Yit mice (A). Immunostaing for β7-integrin (B, E) and MAdCAM-1 (C, D) in SAMP1/Yit ileitis mice with control water treatment (B, C) and SAMP1/Yit mice with lemon grass treatment (D, E) at the age of 26 weeks. The number of β7-positive cells is expressed as cells per millimeter of muscularis mucosa (F) in control AKR/J mice (open bar), SAMP1/Yit mice drinking water (diseased control) (closed bar), and lemon grass drinking SAMP1/Yit mice (cross-hatched bar). The MAdCAM-1 positive vessels are quantified by using an image analyzer, and expressed as area of positively stained vessels per millimeter muscularis mucosa (G). Values are means ± SD for four animals. $^*p < 0.05$ vs. SAMP1/Yit mice drinking regular water. $^†p < 0.05$ vs. AKR/J mice.

are pro-inflammatory, supporting the rationale for the use of CCR9 antagonists for treatment of human small bowel CD [25].

However, we must emphasize that care must be taken in the use of antibodies against adhesion molecules or synthetic antagonists of chemokine