doi:10.1111/j.1440-1746.2007.05086.x

EDUCATION AND IMAGING

Gastrointestinal: Ileal ulcers induced by non-steroidal anti-inflammatory drugs

A woman, aged 28 years, was admitted to hospital after a major episode of bright rectal bleeding. For the preceding 7 years, she had been taking prednisolone, 10–25 mg per day, because of Takayasu's arteritis. She had also been taking loxoprofen sodium, a non-steroidal anti-inflammatory drug (NSAID), each day for arthralgia. Blood tests on admission revealed severe anemia. No abnormalities were detected at colonoscopy. Emergency double-balloon enteroscopy was then performed and revealed multiple discrete ulcers in the distal ileum, approximately 20 cm from the ileocecal valve. The ulcers were highlighted by the use of an indigo carmine dye spray (Fig. 1). Possible diagnoses included ulcers induced by arteritis, ulcers induced by NSAIDs and cytomegalovirus enteritis. Biopsies revealed non-specific inflammation without histological features of cytomegalovirus infection. Ileal ulcers induced by NSAIDs was considered to be the most likely diagnosis and treatment with loxoprofen sodium was discontinued. Gastrointestinal bleeding settled spontaneously. Double-balloon enteroscopy with indigo carmine dye spraying was repeated after 3 weeks and showed that the ulcers had largely healed (Fig. 2).

NSAIDs have been associated with a variety of adverse effects on the small bowel distal to the duodenum. The most common appears to be a NSAID enteropathy characterized by occult blood loss and anemia and sometimes associated with malabsorption and a protein-losing enteropathy. These effects have been attributed to disruption of the mucosal barrier in the small intestine that results in the development of mild mucosal inflammation. Another complication is the development of small intestinal ulcers. In one autopsy study, ulcers in the small intestine were identified in 8.4% of patients taking NSAIDs compared with 0.6% in a control group. Small intestinal ulcers have not been associated with the presence of gastric or duodenal ulcers but may be more common with slow-release NSAID preparations. Complications of small intestinal ulcers include bleeding (as in the above case), stricture formation and perforation. Stricture formation appears to be uncommon although the presence of focal strictures called intestinal diaphragms may be pathognomonic of NSAID damage. A notable feature in the above case was the demonstration of ulcers in the distal ileum using double-balloon enteroscopy.

Contributed by
M Matsuura, H Nakase, F Nakamura, Y Ueda, S Mikami, T Yoshino, S Ueno, N Uza and T Chiba
Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

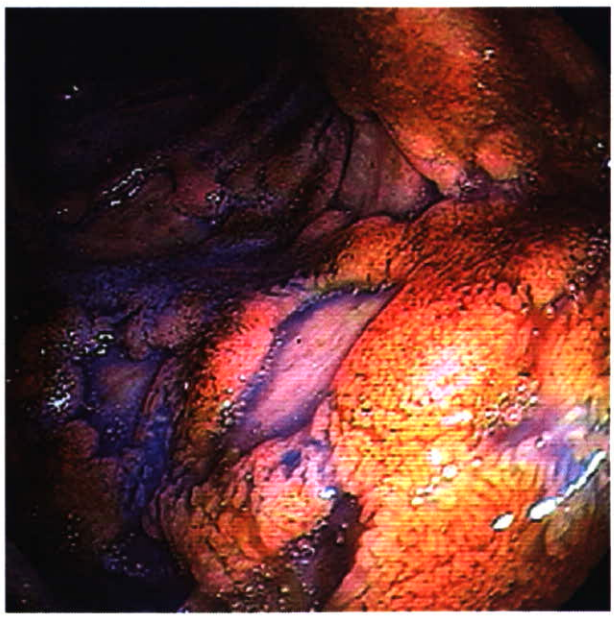


Figure 1

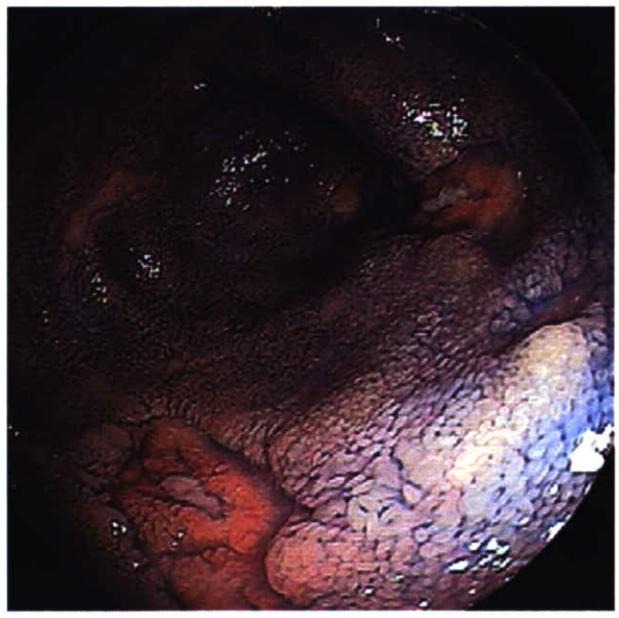


Figure 2

Journal of Gastroenterology and Hepatology **22** (2007) 1346

Involvement of Cytomegalovirus Infection in the Ileal Lesions of the Patient with Behçet's Disease

To the Editor:

Considerable attention has been paid to cytomegalovirus (CMV) infection as one of the exacerbating factors of ulcerative colitis and Crohn's diseases refractory to conventional therapies.[1-4] However, the involvement of CMV infection in the exacerbation of intestinal Behçet's disease (BD) has not been reported to date. We herein reported the first case of Behçet's ileocolitis associated with CMV infection, which was successfully treated with antiviral therapy. This case suggests that the importance of concomitant CMV infection should be kept in mind in patients of Behçet's ileocolitis treated with immunosuppressant as well as patients with inflammatory bowel diseases.

Case

A 43-year-old man with an 8-year history of refractory ocular involvement of BD was admitted to our hospital for anemia and hematochezia. He fulfilled the international study group criteria for diagnosis of BD. He had been treated with prednisolone (PSL) and oral cyclosporine (CyA). Physical examination demonstrated severe tenderness in the right lower abdomen. Laboratory data showed that hemoglobin was 7.1 g/dL. Upper endoscopic examination did not detect any significant lesion. Colonoscopic examination demonstrated deep and discrete ulcers with exposed vessels at the terminal ileum (Fig. 1a). Histological examination of biopsy specimen from ileal ulcerated lesion showed nonspecific inflammation with granulocyte infiltration and mild fibrous changes, but there was no granuloma and obvious inclusion body. Based on these findings, we initially diagnosed ileocecal involvement of BD. However, since endoscopic findings of ileocecal ulcers are so deep and large in addition to the immunosuppressed condition, we speculated on the involvement of CMV infection in the ulcerated lesion of this patient. CMV DNA was detected in the biopsy sample of ileal lesions by real-time polymerase chain reaction (PCR). Moreover, the immunohistochemistry of the biopsy specimen demonstrated positive staining of CMV antigen in endothelial cells. These findings suggested that these ileal lesions were Behçet's ileocolitis with concomitant CMV infection. We discontinued the administration of CyA and started a 10 mg/kg dose of gancyclovir for 2 weeks. After starting antiviral therapy, right lower abdominal tenderness subsided. Colonoscopic findings demonstrated that ileocecal ulcerations were improved (Fig. 1b). CMV DNA in the biopsy sample also became negative following antiviral therapy. In April, 2006, he was doing well, and colonoscopic examination demonstrated no recurrence of ileal ulcerations.

BD is characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions.[5] Generally, gastrointestinal involvement seems to be rare, but ileocecal involvement is relatively more common in Japanese patients with BD.[6] Intestinal BD is intractable and medical treatment for intestinal BD has not been fully established.[5,7] However, drugs such as PSL or immunosuppressants are administered to many patients with BD. Therefore, these patients, who have been treated by PSL or immunosuppressants, are usually in an immunosuppressed condition. Generally, CMV is a clinically important pathogen in immunocompromised hosts because the involvement of CMV infection in intestinal mucosa leads to severe gastrointestinal lesions. Various endoscopic findings have been reported in the intestinal CMV infection but there is no typical endoscopic appearance. To prove whether CMV infection is involved in intestinal lesions it is necessary to investigate the existence of CMV in the gastrointestinal tract by PCR or immunohistochemistry. However, it is difficult to judge whether CMV infection is involved in ileocolitis of BD or CMV enterocolitis in a patient with ocular BD. Finally, the former diagnosis was done because endoscopic findings demonstrated scar formations in the ileum, suggesting the existence of previous ileal lesions associated with BD.

When abdominal symptoms in patients with intestinal BD deteriorate, we consider a dose increase of PSL or adding immunosuppressants. However, if CMV infection is involved in the exacerbation of intestinal lesions in patients with BD, such as we encountered, additional immunosuppressive therapies make intestinal lesions worse. Therefore, we should investigate whether CMV infection is present in the intestinal lesion of patients with BD prior to making a decision regarding additional immunosuppressive therapies.

Sakae Mikami, MD
Hiroshi Nakase, MD
Satoru Ueno, MD
Minoru Matsuura, MD
Takaki Sakurai, MD†
Tsutomu Chiba, MD
*Department of Gastroenterology and Hepatology
Graduate School of Medicine
Kyoto University
Kyoto, Japan
†Laboratory of Diagnostic Pathology
Kyoto University Hospital
Kyoto, Japan

DOI 10.1002/ibd.20105
Published online 25 January 2007 in Wiley InterScience (www.interscience.wiley.com).

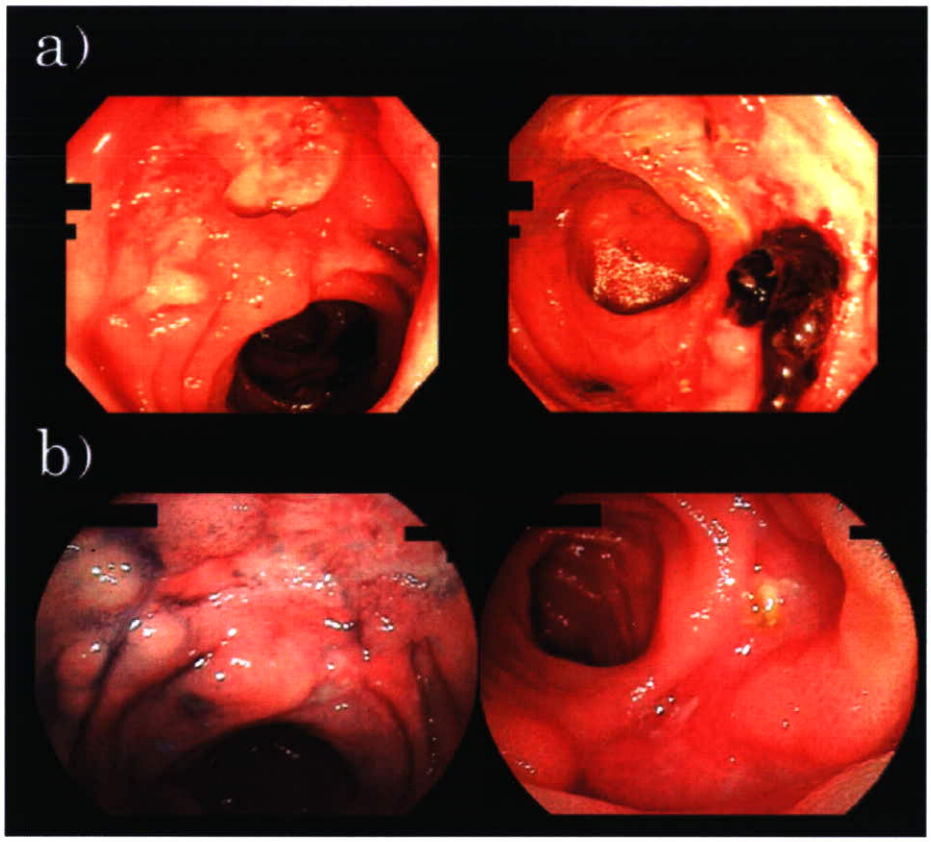


FIGURE 1. a: Endoscopic findings of the terminal ileum showed deep and large ulcers with exposed vessels on the deformed ileal mucosa accompanied by ulcer scars. b: Endoscopic picture of terminal ileum 3 weeks after antiviral treatment showed the improvement of ileal ulcerations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

REFERENCES

1. Vega R, Bretran X, Menacho M, et al. Cytomegalovirus infection in patients with inflammatory bowel disease. *Am J Gastroenterol.* 1999;94:1053–1056.
2. Pfau P, Kochman ML, Furth EE, et al. Cytomegalovirus colitis complicating ulcerative colitis in steroid-naïve patient. *Am J Gastroenterol.* 2001;96:895–899.
3. Kou T, Nakase H, Tamaki H, et al. Cytomegalovirus infection in patients with ulcerative colitis diagnosed by quantitative real-time PCR analysis. *Dig Dis Sci.* 2006;51:1052–1055.
4. Nakase H, Yoshino T, Ueno S, et al. The importance of early detection of cytomegalovirus infection in refractory inflammatory bowel disease. *Inflamm Bowel Dis.* 2007 (in press).
5. Sakane T, Takeno M, Suzuki N, et al. Current concepts Behcet's disease. *N Engl J Med.* 1999; 341:1284–1291.
6. Iida M, Kobayashi H, Matsumoto T, et al. Intestinal Behcet's disease: serial changes at radiography. *Radiology.* 1993;188:65–69.
7. Choi IJ, Kim JS, Cha SD, et al. Long term clinical course and prognostic factors in intestinal Behcet's disease. *Dis Colon Rectum.* 2000;4:692–700.

Crohn's Disease in a Patient With Chronic Renal Failure

To the Editor:

Crohn's disease may present with extraintestinal manifestations [1] but is not usually associated with renal disease. A 47-year-old man from Nigeria presented with a 3-day history of generalized central abdominal pain associated with vomiting and diarrhea. The patient was on renal dialysis three times weekly for the past 3 years for end-stage renal disease caused by hypertension; he was normally anuric.

The patient was clearly in pain but was not septic or toxic. On examination the abdomen was diffusely tender, tense, and distended, but without true peritonitis. On plain radiography, there was no free air under the diaphragm or distended bowel loops. There was a slight neutrophilia with a raised C-reactive protein (CRP) (50 mg/L) increased to 199 mg/L over 4 days and hypoalbuminemia (29 g/L). Computed tomography (CT) revealed markedly thick-walled distal and terminal ileum, diverticular change throughout the large bowel, bilateral atrophic kidneys, and a large amount of ascitic fluid within the abdomen

DOI 10.1002/ibd.20097
Published online 22 January 2007 in Wiley InterScience (www.interscience.wiley.com).

ORIGINAL ARTICLE

Usefulness of Quantitative Real-time PCR Assay for Early Detection of Cytomegalovirus Infection in Patients with Ulcerative Colitis Refractory to Immunosuppressive Therapies

Takuya Yoshino, MD, Hiroshi Nakase, MD, PhD,* Satoru Ueno, MD,* Norimitsu Uza, MD,* Satoko Inoue, MD,* Sakae Mikami, MD,* Minoru Matsuura, MD, PhD,* Katsuyuki Ohmori, MD, PhD,† Takaki Sakurai, MD, PhD,‡ Satoshi Nagayama, MD, PhD,§ Suguru Hasegawa, MD, PhD,§ Yoshiharu Sakai, MD, PhD,§ and Tsutomu Chiba, MD, PhD**

Background: Studies suggest that cytomegalovirus (CMV) infection exacerbates ulcerative colitis (UC) refractory to immunosuppressive therapies. Early and accurate diagnosis of CMV infection is important for the treatment of UC. We evaluated the usefulness of quantitative real-time polymerase chain reaction (PCR) for detecting CMV infection in inflamed colonic mucosa of patients with UC refractory to immunosuppressive therapies.

Methods: From 2003 to 2006, 30 patients (mean age: 41 ± 18 years; 14 men, 16 women) with UC refractory to immunosuppressive therapies were enrolled in the study. We evaluated CMV infection by CMV antigenemia, histologic examination, and quantitative real-time PCR for CMV using colonic mucosa and investigated the clinical outcomes of antiviral therapy.

Results: CMV-DNA was detected only in the inflamed colonic mucosa in 17 (56.7%) of 30 patients. Of the 17 CMV-DNA-positive patients, 4 were positive for CMV antigenemia or inclusion bodies on histologic examination; of the 13 CMV-DNA-negative patients none was positive for CMV antigenemia or inclusion bodies. Of the 17 CMV-DNA-positive patients, 12 (70.6%) were treated with ganciclovir for 2 weeks and 10 patients went into remission. Two other patients required colectomy after antiviral therapy. In contrast, of the 13 CMV-DNA-negative patients 12 (92.3%) achieved remission after intensifying their immunosuppressive therapies.

Conclusions: Quantitative real-time PCR assay for detecting CMV-DNA is useful for early, accurate diagnosis of CMV infection in patients with UC refractory to immunosuppressive therapies, enabling prompt and appropriate treatment.

(Inflamm Bowel Dis 2007;13:1516–1521)

Key Words: ulcerative colitis, cytomegalovirus, real-time PCR

Cytomegalovirus (CMV) infection is an important exacerbating factor in patients with ulcerative colitis (UC).[1–3] Because CMV infection occurs often in immunocompromised hosts,[4] CMV infection in patients with UC refractory to immunosuppressive therapies must always be considered a possibility. If CMV infection is not recognized at an early stage, appropriate treatment is not promptly initiated and the prognosis of patients with UC complicated by CMV infection is generally poor.[5,6] Thus, an accurate and rapid diagnosis of CMV infection is critical in UC patients refractory to immunosuppressive therapies.

Several modalities are currently used for detecting CMV infection.[7–9] For diagnosis of CMV infection in the gastrointestinal tract, combined CMV antigenemia assay and detection of CMV inclusion bodies in biopsy specimens from the gastrointestinal mucosa by either hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) using anti-CMV monoclonal antibodies has been proposed.[1] It is often difficult, however, to accurately diagnose CMV infection in patients with UC,[10] even when using this combined diagnostic method. Several recent studies indicate that the real-time polymerase chain reaction (PCR) assay allows for sensitive and rapid detection of CMV-DNA in clinical samples, and is more useful and beneficial for diagnosing CMV infection than CMV antigenemia assay or histologic examination.[11,12]

From the *Department of Gastroenterology & Hepatology, Graduate School of Medicine, Kyoto University, †Department of Clinical Laboratory, Kyoto University Hospital, ‡Laboratory of Diagnostic Pathology, Kyoto University Hospital, §Department of Surgery, Kyoto University Hospital, Kyoto, Japan.

Supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Culture and Science of Japan to Hisroshi Nakase (grant 18590677), and Grants-in-aid for Scientific Research (16017240, 16017249,17013051, 17659212, and 18012029) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-aid for Scientific Research (15209024 and 18209027) from JSPS, and Grant-in-Aid for Research on Measures for Intractable Diseases, and Research on Advanced Medical Technology (nano005) from the Ministry of Health, Labor, and Welfare, Japan (to T.C.).

Reprints: Hiroshi Nakase, MD, PhD, Department of Gastroenterology & Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan. (e-mail: hiropy-n@kuhp.kyoto-u.ac.jp).

DOI 10.1002/ibd.20253

Published online 7 September 2007 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Endoscopic Index of Rachmilewitz[16]

Items	Scores			
	0	1	2	4
1. Granulation scattering reflected light	No	—	Yes	—
2. Vascular pattern	Normal	Faded/disturbed	Completely absent	—
3. Vulnerability of mucosal	None	—	Slightly increased (contact bleeding)	Greatly increased (spontaneous bleeding)
4. Mucosal damage (mucus, exudates, erosions, ulcer)	None	—	Slight	Pronounced

Total score is sum of the item scores.

A conventional PCR assay, however, might also detect a latent CMV infection that has nothing to do with the deterioration of UC.[11] We recently applied quantitative real-time PCR for detecting CMV in patients with UC refractory to immunosuppressive therapies and found that the CMV-DNA copy number is higher in inflamed colonic mucosa than in noninflamed mucosa in these patients, suggesting the usefulness of this method to accurately diagnose active CMV infection.[13,14]

In the present study we further examined the usefulness of the quantitative real-time PCR assay using colonic mucosa for diagnosing active CMV infection in patients with UC. An accurate diagnosis of CMV infection might enable a more effective treatment for patients with UC refractory to immunosuppressive therapies.

MATERIALS AND METHODS

Patients

Among 93 patients with UC (55 men, 38 women) that visited Kyoto University Hospital from October 2003 to October 2006, 30 patients with UC refractory to immunosuppressive therapies, including steroids and immunomodulators, were studied retrospectively. The diagnosis of UC was based on clinical, endoscopic, radiologic, and histologic parameters. Fecal bacterial culture yielded no specific pathogens in any of the patients. All patients had been treated with immunosuppressive therapies, and had active UC defined as moderate to severe using the disease activity index (DAI) criteria,[15] with a score greater than 6 points.

Assessment of Endoscopic Severity

Endoscopic severity of UC was assessed using the DAI score,[15] Matts grade,[16] and the Endoscopic index of Rachmilewitz.[17] Endoscopic findings were scored from 0 to 3 according to DAI scores as: normal = 0, mild friability = 1, moderate friability = 2, and spontaneous bleeding = 3, and also scored from 1 to 4 according to Matts grade as: normal = 1, mild granularity and edema = 2, marked granularity and edema, and spontaneous bleeding = 3, severe ulceration = 4. The endoscopic index of Rachmilewitz is shown in Table 1.

Histopathology

Colonic biopsies were fixed in formalin, embedded in paraffin, stained with H&E, and IHC was performed using anti-CMV monoclonal antibodies (Dako Cytomation, Kyoto, Japan).[13] These sections were evaluated for characteristic cytomegalic cells and "owl's-eye" nuclear inclusion bodies.

CMV Antigenemia

The antigenemia assay was performed using a monoclonal antibody (C7HRP or C10C11) against a CMV structural protein of the 65 kDa lower-matrix phosphoprotein (pp65).[7,8]

Quantitative Real-time PCR

DNA for real-time PCR assay was extracted from the colonic tissues obtained from patients at endoscopic examination using QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The assay was performed using an ABI Prism 7700 Sequence Detector System (Perkin Elmer Applied Biosystems, Foster City, CA) as described previously.[18] The oligonucleotide primers used for CMV-DNA amplification were constructed to detect the immediate early gene. The upstream primer was 5′-GACTAGTGTGATGCTGGCCAAG-3′ and the downstream primer was 5′-GCTACAATAGCCTCTTCCTCATCTG-3′. The 6-carboxyfluorescein-labeled probe was 5′-AGCCTGAGGTTATCAGTGTAATGAAGCGCC-3′. The PCR conditions were incubation at 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds, followed by incubation at 62°C for 1 minute. Cases in which the CMV-DNA copy number was over 10 copies/μg DNA were defined as positive for CMV infection.

Diagnosis of CMV Infection

Cases that were detected as CMV infection by at least one of these methods (histopathology, CMV antigenemia, and quan-

titative real-time PCR) were defined as positive for CMV infection.

Statistical Analysis

Categorical and continuous data were compared using a 2-tailed Fisher exact test and Mann–Whitney *U*-test. CMV-positive patients were compared with CMV-negative patients for different parameters (age, DAI score, extent of disease, endoscopic score of DAI, Matts grade, endoscopic index of Rachmilewitz, ratio of patients undergoing colectomy, and treatment). A *P*-value <0.05 was considered statistically significant.

RESULTS

The clinical characteristics of the 30 patients are summarized in Table 2. The mean age of the 30 patients was 40.8 ± 17.6 years (range 16–73 years), and the mean DAI score was 9.5 ± 1.4. The extent of the disease was proctitis (3.3%), left-sided colitis (23.3%), and pancolitis (73.3%). The mean endoscopic DAI score was 2.3 ± 0.7, the mean Matts grade was 3.0 ± 0.8, and the mean endoscopic index score was 9.2 ± 2.4 (Table 2).

Of the 30 patients, 23 (76.7%) had been treated with corticosteroids, 6 (20.0%) with azathioprine, 7 (23.3%) with tacrolimus, and 2 (6.7%) with leukocytapheresis when visiting our institution. Six patients (20.0%) received colectomy during the observation period (Table 2, Fig. 1).

CMV-DNA was detected in the colonic tissues of 17 patients (56.7%) (4 with left-sided colitis, 13 with pancolitis) (Table 3). Notably, in all positive cases CMV-DNA was detected only in the inflamed colonic mucosa and not in the noninflamed mucosa. As a control, we examined CMV-DNA in the inflamed mucosa of 4 patients with UC who were in clinical remission with immunosuppressive therapies. CMV-DNA was not detected in the inflamed mucosa of any of these patients (data not shown). On the other hand, CMV antigenemia and histologic examination were positive in only 3 (17.6%) and 1 (5.9%) of the 17 patients positive for CMV-DNA in the colonic mucosa, respectively, and none of the patients negative for CMV-DNA in the colonic mucosa was positive for either CMV antigenemia or histologic examination.

A comparison of differences in age, DAI score, disease extent, ratio of patients undergoing colectomy, and the endoscopic score between CMV-DNA-positive and -negative patients revealed no significant differences between the 2 groups, although the number of patients who received colectomy tended to be greater in CMV-DNA-positive patients than in -negative patients (Table 4).

Moreover, a comparison of difference in treatment between CMV-DNA-positive and -negative patients also revealed no significance difference between the 2 groups. However, the number of patients treated with corticosteroid tended to be greater than in CMV-DNA-positive patients than in -negative patients (Table 5).

Of the 17 CMV-DNA-positive patients, 12 (70.6%) were treated with ganciclovir daily for 2 weeks (Fig. 1). CMV-DNA in the colonic mucosa became negative in all patients that received antiviral therapy. Four patients (33.3%) went into remission following antiviral therapy only. Although 7 patients (58.3%) were improved after antiviral therapy, the underlying UC remained active. These patients were treated with additional granulocytapheresis (GCAP) using an Adacolumn (Japan Immunoresearch Laboratories, Takasaki, Japan) or additional tacrolimus after their CMV-DNA became negative. Six (50.0%) of them went into remission, but 1 (8.3%) patient did not and therefore received a colectomy.

TABLE 2. Clinical Characteristics of 30 Patients with UC Refractory to Immunosuppressive Therapies

Age (mean ± SD)		40.8±17.6
Sex (M/F)		14/16
DAI score		9.5 ± 1.4
Extent of disease	Proctitis	1 (3.3)[a]
	Left-sided	7 (23.3)[a]
	Pancolitis	22 (73.3)[a]
Endoscopic score of DAI		2.3 ± 0.7
Matts grade		3.0 ± 0.8
Endoscopic index of Rachmilewitz		9.2 ± 2.4
Treatment on admission	Corticosteroid (CS)	23 (76.7)[a]
	Corticosteroid alone	14 (60.9)[b]
	With azathioprine	5 (21.7)[b]
	With tacrolimus	1 (4.3)[b]
	With LCAP	2 (9.1)[b]
	With GCAP	1 (4.3)[b]
	Azathioprine (AZA)	6 (20.0)[a]
	Azathioprine alone	1 (16.7)[c]
	With corticosteroid	5 (83.3)[c]
	Tacrolimus	7 (23.3)[a]
	Tacrolimus alone	5 (71.4)[d]
	With corticosteroid	1 (14.3)[d]
	With infliximab	1 (14.3)[d]
	LCAP	2 (6.7)[a]
	With corticosteroid	2 (100.0)[e]
Ratio undergoing colectomy		6 (20.0)[a]

Number of patients is shown. Age, DAI Score, Endoscopic Score of DAI, Matts Grade, and Endoscopic Index of Rachmilewitz are presented as mean ± SD. LCAP, leukocytapheresis

[a] Values in parentheses are percentages of all 30 patients.
[b] Values in parentheses are percentages of patients treated with CS.
[c] Values in parentheses are percentages of patients treated with AZA.
[d] Values in parentheses are percentages of patients treated with tacrolimus.
[e] Values in parentheses are percentages of patients treated with LCAP.

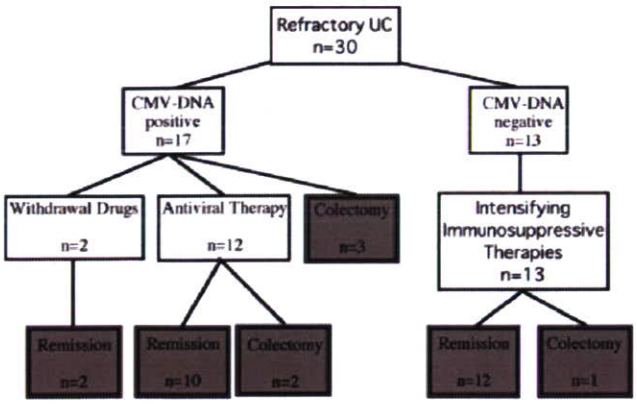


FIGURE 1. Clinical course of 30 patients with UC refractory to immunosuppressive therapy. CMV-DNA in inflamed mucosa was positive in 56.7% (17/30) of patients with UC, and negative in 43.3% (13/30) of patients with UC. Shaded rectangular columns with double lines show final outcomes.

Eventually, 10 (83.3%) of the 12 patients that received antiviral therapy went into remission and the remaining 2 patients (16.7%) received a colectomy. Three (17.6%) of the remaining 5 patients positive for CMV-DNA required urgent colectomy without receiving antiviral therapy, and 2 (11.8%) achieved remission by withdrawal of the immunosuppressive drugs.

Of the 13 CMV-DNA-negative UC patients, 12 (92.3%) went into remission after treatment with more intense immunosuppressive therapies. Only 1 patient (7.7%) was refractory to additional immunosuppressive therapies and finally required a colectomy.

DISCUSSION

In the present study, we applied quantitative real-time PCR for the diagnosis of active CMV infection in the colonic mucosa of 30 patients with UC refractory to immunosuppressive therapies. Using this method the detection rate of CMV

TABLE 3. Detection Rate of CMV-DNA in the Colonic Mucosa of UC Patients Refractory to Immunosuppressive Therapies

	CMV-DNA in Inflamed Mucosa	CMV-DNA in Noninflamed Mucosa	Antigenemia	IHC
CMV infection				
Postitive	17 (56.7%)	0	3 (17.6%)	1 (5.9%)
Negative	0	0	0	0
Total	17	0	3	1

Number of patients is shown. Values in parentheses are percentages of the total number of patients (n = 30).

TABLE 4. Comparison of Clinical Parameters between UC Patients with and without Detectable CMV-DNA in the Inflamed Mucosa

	CMV-DNA-positive N = 17	CMV-DNA-negative N = 13	P-value
Age	44.1 ± 16.3	36.5 ± 18.9	0.247
DAI score	9.8 ± 1.2	9.2 ± 1.6	0.206
Extent of disease			
Proctitis	0 (0.0)	1 (7.7)	0.245
Left-sided	4 (23.5)	3 (23.1)	0.977
Pancolitis	13 (76.5)	9 (69.2)	0.657
Endoscopic score of DAI	2.4 ± 0.7	2.1 ± 0.6	0.194
Matts grade	3.1 ± 0.8	2.9 ± 0.8	0.687
Endoscopic index of Rachmilewitz	9.5 ± 2.4	8.8 ± 2.4	0.444
Ratio undergoing colectomy	5 (29.4)	1 (7.7)	0.196

Number of patients is shown. Age, DAI Score, Endoscopic Score of DAI, Matts Grade, and Endoscopic Index of Rachmilewitz are presented as mean ± SD. Values in parentheses are percentages of the total number of patients either positive or negative for CMV-DNA in the inflamed mucosa.

TABLE 5. Comparison of Treatment between UC Patients with and without Detectable CMV-DNA in the Inflamed Mucosa

Treatment	CMV-positive n = 17	CMV-negative n = 13
Corticosteroid (CS)	15 (65.2)[a]	8 (34.8)[a]
Corticosteroid only	10	4
With azathioprine	3	2
With tacrolimus	1	0
With LCAP	1	1
With GCAP	0	1
Azathioprine	3 (50.0)[b]	3 (50.0)[b]
Azathioprine alone	0	1
With corticosteroid	3	2
Tacrolimus	3 (42.9)[c]	4 (57.1)[c]
Tacrolimus alone	1	4
With corticosteroid	1	0
With infliximab	1	0
LCAP	1 (50.0)[d]	1 (50.0)[d]
With corticosteroid	1	1

Number of patients is shown. There is no significant difference in treatment between CMV-DNA-positive and -negative patients.
[a] Values in parentheses are percentages of patients treated with CS.
[b] Values in parentheses are percentages of patients treated with AZA.
[c] Values in parentheses are percentages of patients treated with tacrolimus.
[d] Values in parentheses are percentages of patients treated with LCAP.

infection tended to be higher than when using other conventional methods such as CMV antigenemia and histologic examination. Moreover, a high remission rate was achieved in UC patients refractory to immunosuppressive therapies by applying either antiviral therapy or modulating immunosuppressive therapies according to the results of the quantitative real-time PCR for CMV-DNA in the inflamed colonic mucosa. Thus, our real-time PCR method for detecting CMV-DNA appears to be more useful than conventional modalities for diagnosing active CMV infection in patients with UC refractory to immunosuppressive therapies.

Quantitative real-time PCR revealed that CMV-DNA was positive in the inflamed colonic mucosa of 56.7% (17/30) of our UC patients, whereas CMV antigenemia and histologic examination were positive in only 17.6% (3/17) and 5.9% (1/17) of the patients positive for CMV-DNA, respectively. Thus, the detection rate of CMV infection by quantitative real-time PCR far exceeded that by CMV antigenemia and histologic examination. Several methods are used to diagnose CMV infection, including histologic examination, CMV antigenemia, and PCR assay.[7–9] Among them, CMV antigenemia and PCR assay using whole blood potentially reflect the reactivation of CMV in the whole body, but does not necessarily indicate CMV infection in the colonic mucosa. Indeed, there are several reports of reactivation of CMV in the plasma of patients with collagen disease and AIDS without gastrointestinal involvement of CMV infection.[7,19] Reactivation of CMV in the plasma does not reflect the involvement of CMV infection in UC.[20] Histologic examination is often considered the "gold standard" for diagnosing CMV infection in the gastrointestinal tract.[21] Its sensitivity for diagnosis, however, ranges from 10%–87%, and moreover, 37.5% of patients with gastrointestinal CMV disease fail to demonstrate any inclusions.[21] To overcome such low sensitivity, IHC with monoclonal antibodies was developed. The sensitivity for detecting CMV infection with IHC ranges from 78%–93%.[21] The sensitivity and specificity of CMV antigenemia for detecting CMV infection are 60%–100% and 83%–100%, respectively.[21] The present data, however, indicate that the detection rates of CMV infection by those established methods are lower than previously reported. In contrast, we found a significantly higher detection rate of CMV-DNA in the inflamed colonic mucosa by our quantitative real-time PCR system than by conventional modalities such as histologic examination and CMV antigenemia. These data strongly suggest that our quantitative real-time PCR for detecting CMV-DNA in the inflamed mucosa is very useful for diagnosing active CMV infection in patients with UC refractory to immunosuppressive therapies. It should be emphasized that none of the patients that were positive for CMV-DNA in the inflamed colonic mucosa were positive for CMV-DNA in the noninflamed mucosa. Thus, the high sensitivity of our method for detecting CMV infection is likely to be due to the sampling of the inflamed mucosa for the assay.

CMV is present in its latent form in most healthy subjects.[18] Therefore, we might expect low specificity for diagnosing active CMV infection when using a sensitive PCR method, because it is possible that sensitive PCR will detect CMV-DNA in subjects with a latent CMV infection. In this respect, we observed that CMV-DNA was detected only in the inflamed colonic mucosa and not in the noninflamed colonic mucosa. Moreover, by using biopsy specimens from the inflamed colonic mucosa, 12 of our 17 CMV-DNA-positive patients achieved remission by either antiviral therapy or withdrawing immunosuppressive therapies, whereas 12 of 13 CMV-DNA-negative patients achieved remission by intensifying the immunosuppressive therapies. In addition, none of the patients negative for CMV-DNA were positive based on either histologic examination or CMV antigenemia. Taken together, these findings suggest that both the sensitivity and specificity of our quantitative real-time PCR for diagnosing active CMV infection are high, and indeed, the findings were useful for making an appropriate decision regarding whether the immunosuppressive therapies should be intensified or tapered.

Endoscopy is a useful modality for diagnosing CMV infection when the characteristic findings such as deep ulceration are observed.[22] The endoscopic findings in CMV-positive colitis, however, vary,[21,23] and thus it might be difficult to distinguish CMV infection from severe UC. Sakamoto et al[24] reported that no specific endoscopic findings were observed in UC patients with concomitant CMV infection. We also evaluated whether endoscopic findings was useful for early detection of CMV infection using endoscopic score in the present study. Our data revealed no significant difference in endoscopic score according to 3 different indexes between the CMV-DNA-positive and -negative patients. Based on both the previous reports and the present report, the significance of endoscopic findings for diagnosing CMV in patients with UC remains unclear. Hommes et al[25] proposed a mechanism of CMV replication and activation in the intestinal tissue during active inflammatory bowel disease and classified the findings into 3 stages (initiation, reactivation, and consolidation). According to their proposal, the stage at which we detected CMV-DNA in the inflamed colonic mucosa of patients with UC might correspond to the initiation or reactivation stage prior to the occurrence of characteristic endoscopic findings in CMV colitis. Thus, one reason for the lack of a significant difference in the endoscopic score between the CMV-DNA-positive and -negative patients in our study might be due to the detection of CMV infection at an early stage.

An interesting observation in our study is that, as noted above, CMV-DNA was detected only in the inflamed colonic mucosa, and not in the noninflamed mucosa by quantitative

real-time PCR. Hahn et al[26] reported that proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α induce the reactivation of CMV. Hommes et al[25] also reported that those proinflammatory cytokines induce the migration of monocytes to the inflammatory sites of the colonic mucosa and promote their differentiation into macrophages, which have a role in supporting active replication of CMV as CMV reservoir cells. Thus, it might be that, in patients with UC, CMV is more easily reactivated in the inflamed mucosa than in the noninflamed mucosa.

The therapeutic strategy for UC patients with concomitant CMV infection is a very important issue. In this study, 10 (83.3%) of the 12 CMV-DNA-positive patients went into remission after applying antiviral therapy and modulating immunosuppressive therapies. Of the 13 CMV-DNA-negative UC patients, moreover, 12 (92.3%) went into remission after treatment with more intense immunosuppressive therapies. At present, in UC patients refractory to immunosuppressive therapies we first perform quantitative real-time PCR using inflamed mucosa. In CMV-DNA-positive cases, antiviral therapy should be applied promptly and immunosuppressive therapies should be tapered. After CMV-DNA became negative, immunosuppressive therapies could be intensified. On the other hand, in CMV-DNA-negative cases immunosuppressive therapies could be intensified. Thus, clinical outcome in this study revealed that our quantitative real-time PCR using inflamed mucosa was useful for making a decision of treatment for patients with UC refractory to immunosuppressive therapies.

In conclusion, our use of quantitative real-time PCR for detecting CMV-DNA in inflamed mucosa was very useful for the early and accurate diagnosis of active CMV infection in patients with UC refractory to immunosuppressive therapies, enabling prompt and appropriate treatment. Further studies are required to determine whether this method will contribute to improving the prognosis of UC complicated by active CMV infection.

REFERENCES

1. Vega R, Bertran X, Menacho M, et al. Cytomegalovirus infection in patients with inflammatory bowel disease. *Am J Gastroenterol.* 1999; 94:1053–1056.
2. Loftus EV Jr, Alexander GL, Carpenter HA. Cytomegalovirus as an exacerbating factor in ulcerative colitis. *J Clin Gastroenterol.* 1994;19: 306–309.
3. Cottone M, Pietrosi G, Martorana G, et al. Prevalence of cytomegalovirus infection in severe refractory ulcerative and Crohn's colitis. *Am J Gastroenterol.* 2001;96:773–775.
4. Rowshani AT, Bemelman FJ, van Leeuwen EM, et al. Clinical and immunologic aspects of cytomegalovirus infection in solid organ transplant recipients. *Transplantation.* 2005;79:381–386.
5. Kaufman HS, Kahn AC, Iacobuzio-Donahue C, et al. Cytomegaloviral enterocolitis: clinical associations and outcome. *Dis Colon Rectum.* 1999;42:24–30.
6. Kotanagi H, Fukuoka T, Shibata Y, et al. A case of toxic megacolon in ulcerative colitis associated with cytomegalovirus infection. *J Gastroenterol.* 1994;29:501–505.
7. Yoda Y, Hanaoka R, Ide H, et al. Clinical evaluation of patients with inflammatory connective tissue diseases complicated by cytomegalovirus antigenemia. *Mod Rheumatol.* 2006;16:137–142.
8. Eizuru Y, Minematsu T, Minamishima Y, et al. Rapid diagnosis of cytomegalovirus infections by direct immunoperoxidase staining with human monoclonal antibody against an immediate-early antigen. *Microbiol Immunol.* 1991;35:1015–1022.
9. Storch GA, Buller RS, Bailey TC, et al. Comparison of PCR and pp65 antigenemia assay with quantitative shell vial culture for detection of cytomegalovirus in blood leukocytes from solid-organ transplant recipients. *J Clin Microbiol.* 1994;32:997–1003.
10. Maconi G, Colombo E, Zerbi P, et al. Prevalence, detection rate and outcome of cytomegalovirus infection in ulcerative colitis patients requiring colonic resection. *Dig Liver Dis.* 2005;37:418–423.
11. Machida U, Kami M, Fukui T, et al. Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. *J Clin Microbiol.* 2000;38:2536–2542.
12. Kishore J, Ghoshal U, Ghoshal UC, et al. Infection with cytomegalovirus in patients with inflammatory bowel disease: prevalence, clinical significance and outcome. *J Med Microbiol.* 2004;53:1155–1160.
13. Kou T, Nakase H, Tamaki H, et al. Cytomegalovirus infection in patients with ulcerative colitis diagnosed by quantitative real-time PCR analysis. *Dig Dis Sci.* 2006;51:1052–1055.
14. Nakase H, Yoshino T, Ueno S, et al. Importance of early detection of cytomegalovirus infection in refractory inflammatory bowel disease. *Inflamm Bowel Dis.* 2007;13:364.
15. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med.* 1987;317:1625–1629.
16. Matts SG. The value of rectal biopsy in the diagnosis of ulcerative colitis. *Q J Med.* 1961;30:393–407.
17. Rachmilewitz D. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. *BMJ.* 1989;298:82–86.
18. Tanaka N, Kimura H, Iida K, et al. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J Med Virol.* 2000;60:455–462.
19. Steininger C, Puchhammer-Stockl E, Popow-Kraupp T. Cytomegalovirus disease in the era of highly active antiretroviral therapy (HAART). *J Clin Virol.* 2006;37:1–9.
20. Matsuoka K, Iwao Y, Mori T, et al. Cytomegalovirus is frequently reactivated and disappears without antiviral agents in ulcerative colitis patients. *Am J Gastroenterol.* 2007;102:331–337.
21. Kandiel A, Lashner B. Cytomegalovirus colitis complicating inflammatory bowel disease. *Am J Gastroenterol.* 2006;101:2857–2865.
22. Goodgame RW. Gastrointestinal cytomegalovirus disease. *Ann Intern Med.* 1993;119:924–935.
23. Korkmaz M, Kunefeci G, Selcuk H, et al. The role of early colonoscopy in CMV colitis of transplant recipients. *Transplant Proc.* 2005;37:3059–3060.
24. Sakamoto I, Shirai T, Kamide T, et al. Cytomegalovirus enterocolitis in an immunocompetent individual. *J Clin Gastroenterol.* 2002;34:243–246.
25. Hommes DW, Sterringa G, van Deventer SJ, et al. The pathogenicity of cytomegalovirus in inflammatory bowel disease: a systematic review and evidence-based recommendations for future research. *Inflamm Bowel Dis.* 2004;10:245–250.
26. Hahn G, Jores R, Mocarski ES. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc Natl Acad Sci U S A.* 1998;95:3937–3942.

Case Reports in Gastroenterology
Case Rep Gastroenterol 2007;1:157–161
DOI: 10.1159/000112654
Published online: December 21, 2007

ISSN 1662–0631
www.karger.com/crg

Adminstration of PEG-Interferon to a Patient with Ulcerative Colitis and Chronic Hepatitis C Correlated with Reduced Colonic Inflammation and Reversal of Peripheral Th1/Th2 Ratios

Katsuhiro Kasahara Hiroshi Nakase Norimitsu Uza
Satoru Ueno Minoru Matsuura Sakae Mikami
Satoko Inoue Tsutomu Chiba

Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Key Words
Interferon · Ulcerative colitis · Intracellular cytokine · Th1/Th2 ratios

Abstract
A 42-year-old man with chronic hepatitis C and ulcerative colitis (UC) was referred to our hospital in August 2004 because of bloody diarrhea. He was clinically and endoscopically diagnosed with flare of UC. After informed consent had been obtained, he was treated with PEG-IFN-α-2a. Four weeks after initiation of PEG-IFN therapy, his abdominal symptoms gradually subsided. Intracellular cytokine assay revealed that the ratio of T-helper (Th) 1 (IFN-γ)/Th 2 (IL-4) increased after IFN therapy. Three months after starting IFN therapy, colonoscopy revealed a normal mucosal pattern. He was uneventfully treated with PEG-IFN-α-2a for one year. When last seen in November 2006, he was still in remission of UC. Our intracellular cytokine data suggested that alteration of Th1/Th2 cytokine balance by IFN is one possible mechanisms of reducing intestinal inflammation in patients with UC. In this regard, IFN therapy could be useful for some patients with UC refractory to other conventional therapies.

Several clinical trials have been performed to investigate the therapeutic effect and safety of IFN on patients with UC. Bargiggia and colleagues reported that IFN-α can be safely administered to patients with chronic hepatitis C and inflammatory bowel disease

Hiroshi Nakase, MD, PhD
Department of Gastroenterology & Hepatology
Graduate School of Medicine, Kyoto University
54 Shogoin Kawahara-cho, Sakyoku, Kyoto 606-8507 (Japan)
Tel. +81 75 751 4319, Fax +81 75 751 4303, E-Mail hiropy@kuhp.kyoto-u.ac.jp

[1]. Madsen and colleagues also reported that IFN-α 2a treatment resulted in significant depression of the disease activity of ulcerative colitis (UC) [2]. On the other hand, recombinant IFNβ-1a was safe but not significantly effective for steroid-refractory UC [3]. Thus, clinical outcome of treatments with IFN for patients with UC is inconsistent depending on the dose, duration of therapy, and types of IFNs. Therefore, the effects of IFN-α on patients with UC are still controversial. We report here the case of an UC patient with chronic hepatitis C who was successfully treated with PEG-IFN-α-2a.

Case Report

A 42-year-old man with HCV was referred to our hospital in August 2004 because of diarrhea and bloody stool. Ten years before admission, he had been diagnosed with total UC endoscopically and histologically. He had been treated with 3,000 mg mesalazine alone. Physical examination on admission showed mild tenderness in the lower abdomen. Colonoscopic examination revealed reddish and edematous mucosa with multiple erosions through the entire colon (fig. 1a). Disease activity index (Sutherland Index) was scored as 8. Laboratory data showed thrombocytopenia (57,000/mm3), asparate aminotransferase (AST) 124 IU/l, alanine aminotransferase (ALT) 110 IU/l, and hepatitis C virus (HCV) RNA (genotype Ib) 757 kIU/ml. Liver biopsy revealed liver injury associated with HCV (F3/A2). The patient refused both administration of corticosteroid and leukocytapheresis therapy. After informed consent had been obtained, he was treated with PEG-IFN-α-2a (90 μg/week) for improving both colonic and liver inflammation. Four weeks after initiation of PEG-IFN therapy, his abdominal symptoms gradually subsided. We performed intracellular cytokine assay with peripheral CD4 T cells before and after IFN therapy. The ratio of T-helper (Th) 1 (IFN-γ)/Th 2 (interleukin (IL)-4) increased (40.2) at 4 weeks after IFN therapy compared to that before (27.4) (fig. 2a, b). However, there was no significant difference of IL-10 production from CD4 T cells between before and after IFN therapy. Three months after starting IFN therapy, colonoscopy revealed normal mucosal pattern (fig. 1b). DAI was scored as 2. He was uneventfully treated with PEG-IFN-α-2a for one year. When last seen in November 2006, he was still in remission of UC and his laboratory data showed a negative serum HCV-RNA, which was suggestive of sustained virological response.

Discussion

INF-α, through its immunomodulatory function, could have an impact on pathways in the immune system [4]. Therefore, the effect of INF-α on the pathophysiology of IBD has been focused, because patients with IBD are characterized by imbalance of the Th1/Th2 cytokine response.

Previous reports showed that a treatment with INF-α might be a trigger for development of Th1-related intestinal disease such as celiac disease and Crohn's disease, because INF-α plays an important role in T cell differentiation towards a Th1 type of immune response [5, 6]. Generally, the pathophysiology of UC is associated with a Th2 phenotype. In this regard, INF-α is considered to have beneficial effects in the treatment of diseases characterized by excess Th2 cells such as UC. In fact, several clinical trials with IFN seem to be successful in chronic active UC [1, 2]. However, the effect of INF-α on UC remains controversial, because of some reports on the provoking onset and exacerbation of UC by treatment of INF-α [7–9].

In this case, we evaluated the change of Th1/Th2 ratio of CD4 T cells before and after INF-α therapy. The data on intracellular cytokine assay clearly demonstrated that IFN administration increased Th1/Th2 ratio of peripheral CD4 T cells, which was suggestive of shifting cytokine profile toward Th1. In addition, our data showed that administration of PEG-IFN-α-2a did not increase IL-10 production from peripheral CD4 T cells. Many mechanisms of effect of IFN therapy on patients with UC have been speculated. Judging from our clinical data, alteration of Th1/Th2 cytokine balance is possible. Thus, alteration

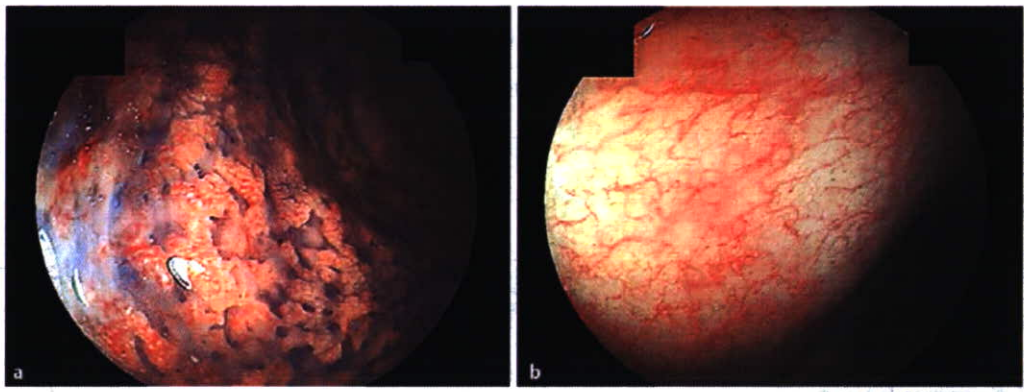
Case Rep Gastroenterol 2007;1:157–161
DOI: 10.1159/000112654
Published online: December 21, 2007

ISSN 1662–0631
www.karger.com/crg

of Th1/Th2 cytokine balance of peripheral CD4 T cells may be a biomarker for type 1 interferon therapy in patients with UC.

In the future, IFN therapy could be useful for patients with UC refractory to other conventional therapies by practical application of this biomarker.

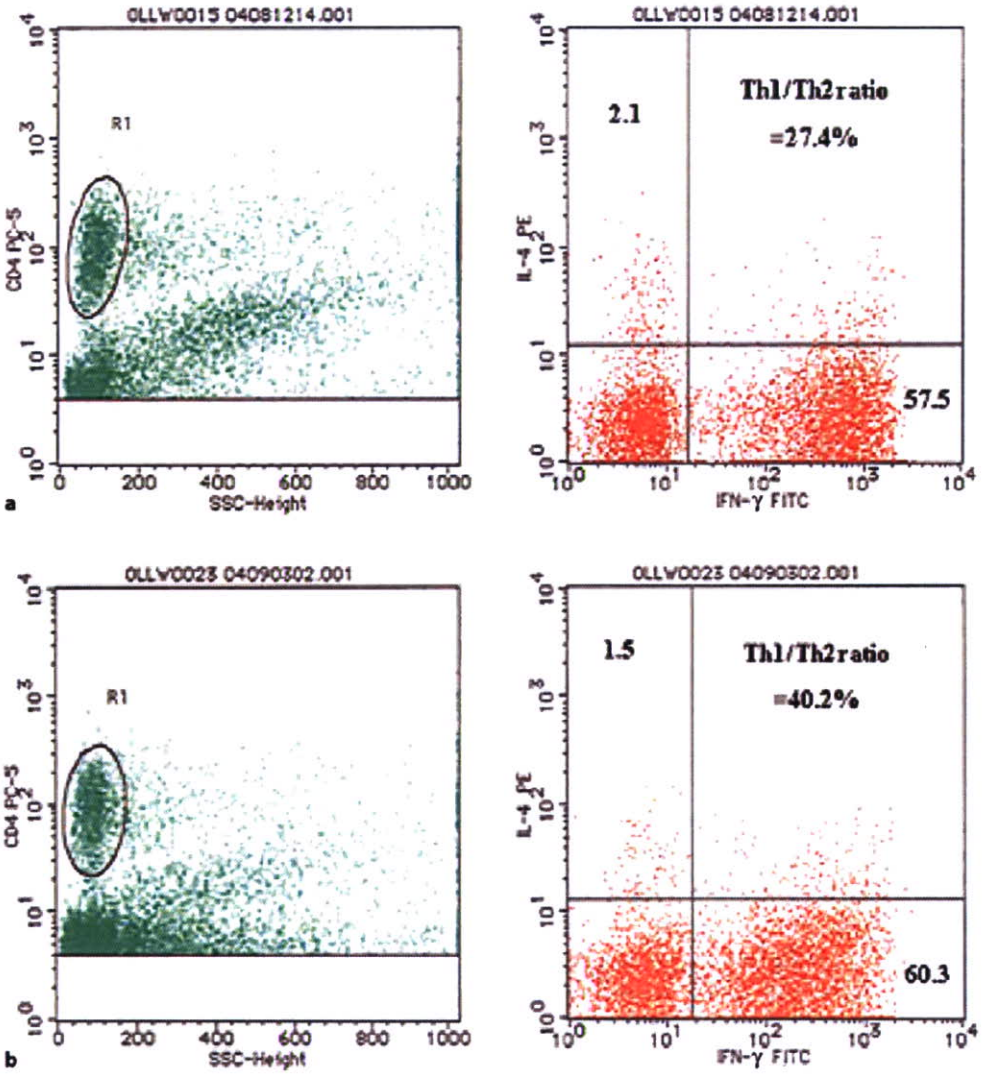
Fig. 1. a Colonoscopic finding before PEG-IFN-α-2a administration showing reddish and edematous mucosa with multiple erosions through the entire colon. **b** Colonoscopic finding three months after PEG-IFN-α-2a administration showing normal colonic appearance.



Case Rep Gastroenterol 2007;1:157–161
DOI: 10.1159/000112654
Published online: December 21, 2007

ISSN 1662–0631
www.karger.com/crg

Fig. 2. Comparison of Th1/Th2 ratio of peripheral CD4+ T cells before (**a**) and after PEG-IFN-α-2a administration (**b**). Flow cytometric analysis showing peripheral IFN-γ and IL-4 CD4 T cells. Plots showed IL-4-PE on the y-axis and IFN-γ on the x-axis.



Case Rep Gastroenterol 2007;1:157–161
DOI: 10.1159/000112654
Published online: December 21, 2007

ISSN 1662–0631
www.karger.com/crg

References

1 Bargiggia S, Thorburn D, Anderloni A, et al: Is interferon-alpha therapy safe and effective for patients with chronic hepatitis C and inflammatory bowel disease? A case- control study. Aliment Pharmacol Ther 2005;22:209–215.

2 Madsen SM, Schlichting P, Davidsen B, et al: An open-labeled, randomized study comparing systemic interferon-a-2A and prednisolone enemas in the treatment of left-sided ulcerative colitis. Am J Gastroenterol 2001;96:1807–1815.

3 Musch E, Andus T, Kruis W, et al: Interferon-β-1a for the treatment of steroid-refractory ulcerative colitis: A randomized, double-blind, placebo controlled trial. Clin Gastroenterol Hepatol 2005;3:581–586.

4 Tilg H: New insights into the mechanisms of interferon alfa: An immunoregulatory and anti-inflammatory cytokine. Gastroenterology 1997;112:1017–1021.

5 Cammarota G, Cuoco L, Cianci R, et al: Onset of celiac disease during treatment with interferon for chronic hepatitis C. Lancet 2000;356:1494–1495.

6 Khalil A, Lucidarme D, Desurmont P, et al : Crohn's disease associated with interferon and ribavirin treatment for chronic hepatitis C. Gastroenterol Clin Biol 2005;29:193–196.

7 Watanabe T, Inoue M, Harada K, et al : A case of exacerbation of ulcerative colitis induced by combination therapy with PEG-interferon alpha-2b and ribavirin. Gut 2006;55:1682–1683.

8 Tursi A: Rapid onset of ulcerative colitis after treatment with PEG-interferon plus ribavirion for chronic hepatitis C. Inflamm Bowel Dis 2007;13:1189–1190.

9 Mavrogiannis C, Papanikolaou IS, Elefsiniotis IS, et al: Ulcerative colitis associated with interferon treatment for chronic hepatitis C. J Hepatol 2001;34:964–965.

A novel M cell–specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses

Tomonori Nochi,[1,3] Yoshikazu Yuki,[1,3] Akiko Matsumura,[1,3] Mio Mejima,[1,3] Kazutaka Terahara,[1,3] Dong-Young Kim,[1,3] Satoshi Fukuyama,[1,3] Kiyoko Iwatsuki-Horimoto,[2,3] Yoshihiro Kawaoka,[2,3] Tomoko Kohda,[4] Shunji Kozaki,[4] Osamu Igarashi,[1,3] and Hiroshi Kiyono[1,3]

[1]Division of Mucosal Immunology, [2]Division of Virology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
[3]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Saitama 332-0012, Japan
[4]Laboratory of Veterinary Epidemiology, Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 599-8531, Japan

Mucosally ingested and inhaled antigens are taken up by membranous or microfold cells (M cells) in the follicle-associated epithelium of Peyer's patches or nasopharynx-associated lymphoid tissue. We established a novel M cell–specific monoclonal antibody (mAb NKM 16-2-4) as a carrier for M cell–targeted mucosal vaccine. mAb NKM 16-2-4 also reacted with the recently discovered villous M cells, but not with epithelial cells or goblet cells. Oral administration of tetanus toxoid (TT)– or botulinum toxoid (BT)–conjugated NKM 16-2-4, together with the mucosal adjuvant cholera toxin, induced high-level, antigen-specific serum immunoglobulin (Ig) G and mucosal IgA responses. In addition, an oral vaccine formulation of BT-conjugated NKM 16-2-4 induced protective immunity against lethal challenge with botulinum toxin. An epitope analysis of NKM 16-2-4 revealed specificity to an α(1,2)-fucose–containing carbohydrate moiety, and reactivity was enhanced under sialic acid–lacking conditions. This suggests that NKM 16-2-4 distinguishes α(1,2)-fucosylated M cells from goblet cells containing abundant sialic acids neighboring the α(1,2) fucose moiety and from non-α(1,2)-fucosylated epithelial cells. The use of NKM 16-2-4 to target vaccine antigens to the M cell–specific carbohydrate moiety is a new strategy for developing highly effective mucosal vaccines.

CORRESPONDENCE
Hiroshi Kiyono:
kiyono@ims.u-tokyo.ac.jp

Membranous or microfold cells (M cells), which are located in the follicle-associated epithelium (FAE) of Peyer's patches (PPs) or nasopharynx-associated lymphoid tissue (NALT), play a pivotal role in the uptake of luminal antigens for induction of antigen-specific immune responses in both systemic and mucosal compartments (1). Unlike their neighboring columnar epithelial cells, M cells are morphologically unique because they have irregular and short microvilli for the effective uptake of ingested or inhaled antigens from luminal sites in the aerodigestive tract; they subsequently transport the sampled antigen to professional antigen-presenting cells (e.g., dendritic cells) to initiate antigen sensitization (2).

The mucosal immune system consists of two types of immunologically important sites, termed "inductive" and "effector" tissues, connected by the common mucosal immune system (3). In general, antigen sensitization occurs at inductive sites, such as PPs, after antigen uptake by M cells. Induction of antigen-specific T helper 2 (Th2) cell–mediated IgA responses and Th1 cell– and CTL-dependent immune responses then occurs at effector sites such as the lamina propria (3). However, our recent study demonstrated that the effector sites are also able to take up antigen, because antigen-sampling cells termed villous M cells are distributed in the intestinal villous epithelium (4), and antigen-specific mucosal immune responses can be induced in PP-deficient mice (5).

Although mucosal vaccination is thought to be an ideal strategy for combating mucosal infectious diseases, only a few mucosal vaccines (e.g., polio vaccine and influenza vaccine) are

The online version of this article contains supplemental material.

JEM © The Rockefeller University Press $30.00
Vol. 204, No. 12, November 26, 2007 2789-2796 www.jem.org/cgi/doi/10.1084/jem.20070607

currently used in humans because they have lower efficacy than the currently used injectable vaccines in inducing antigen-specific immune responses (6). Because M cells possess the ability to take up luminal antigens, it is logical and attractive to develop a system of delivery of vaccine antigen to both PP-associated and villous M cells to create an effective mucosal vaccine (7). In fact, *Ulex europaeus* agglutinin-1 (UEA-1)–conjugated (8, 9) or σ1 protein–conjugated nasal vaccination (10, 11) induce not only strong antigen-specific plasma IgG and mucosal IgA responses but also CTL immunity, because UEA-1 specific for α(1,2) fucose specifically reacts with murine PP–associated and villous M cells (4, 12), and σ1 protein derived from reovirus specifically binds to a carbohydrate structure containing α(2,3)-linked sialic acid on the membranes of M cells (13). However, because UEA-1 also reacts strongly with goblet cells and the mucus layer covering the intestinal epithelium (14), there have been no effective oral vaccines with UEA-1 as an M cell–targeting vehicle. To overcome this obstacle, we established an M cell–specific mAb and developed a novel strategy for oral vaccination with high efficacy.

RESULTS AND DISCUSSION

Establishment of an M cell–specific monoclonal antibody (NKM 16-2-4)

To characterize the antigen-sampling M cells for development of an effective M cell–targeted mucosal vaccine, Sprague-Dawley (SD) rats were immunized 4 times at 2-wk intervals with highly purified (>95%) UEA-1–positive cells isolated from murine PPs to establish an M cell–specific mAb. A total of 1,000 hybridomas were generated and screened by immunohistochemical analysis of intestinal tissue sections containing PPs. On the basis of the initial screening, one clone (NKM 16–2-4; rat IgG2c), which possessed specificity to M cells located in the FAE of PPs (Fig. 1 A), was selected. Half of the hybridomas showed no specificity to tissue sections; ~40% of them showed strong reactivity to goblet cells and their secretions; and 10% showed reactivity to the microvilli in all parts of the intestinal epithelium, including M cells and neighboring columnar epithelial cells (unpublished data). These initial screening data indicated that the goblet cells contained in the immunized UEA-1–positive fraction, and their secretions, were vastly immunodominant compared with M cells. However, importantly, NKM 16–2-4 possessed no reactivity to UEA-1–positive goblet cells located in the intestinal villi (Fig. 1 A), indicating that NKM 16–2-4 is a novel mAb possessing high specificity to murine M cells. This is unlike the already known murine M cell–specific lectin UEA-1, which also reacts with goblet cells and their secretions (14). In addition, NKM 16–2-4 reacted very strongly with the apical surfaces of the M cells (Fig. 1 A), rather than the cytoplasm, suggesting that it might be able to be used as a carrier vehicle of M cell–targeted mucosal vaccine. In support of these results, flow cytometric and immuno- and lectin-cytochemical analyses demonstrated that NKM 16–2-4 specifically reacted with the surfaces of isolated UEA-1–positive M cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070607/DC1), but not of UEA-1–negative epithelial cells. In addition, an electronmicroscopic analysis revealed that NKM 16–2-4 specifically reacted with typical M cells, which have short and irregular microvilli and a pocket structure containing lymphocytes and/or monocytes (Fig. 1 B). Furthermore, whole-mount staining analysis revealed that NKM 16–2-4 specifically reacted with villous M cells, in a manner similar to the reaction with PP-associated M cells (Fig. 1 C).

M cells recognized by UEA-1 in mice are also present in the FAE of NALT, as they are in PPs, and act as antigen-sampling cells for the induction of mucosal immunity (15), although our previous finding demonstrated that the mechanism of NALT organogenesis is distinct from that of PP organogenesis (16, 17). Recently, it was reported that group A streptococcus infects its hosts through M cells (15), meaning that M cells could be defined as a portal cell subset of mucosal infection in both the gastrointestinal and respiratory tracts. A subsequent immunohistochemical analysis of NALT tissue sections revealed that NKM 16–2-4 specifically reacted with UEA-1–positive M cells, but not UEA-1–positive, morphologically typical goblet cells with secretory granules (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20070607/DC1). These results further suggest the possibility of formulating an M cell–targeted nasal vaccine with NKM 16–2-4 for protection against infectious diseases entering through the respiratory tract. Thus, in summary, the novel mAb NKM 16–2-4 specifically reacted with all subsets of M cells, but not epithelial cells or goblet cells, located in PPs, NALT, and the intestinal villi; i.e., in both the gastrointestinal and respiratory tracts (Table I).

Use of NKM 16-2-4 to develop an M cell–targeted mucosal vaccine

Because it had been reported that the use of monoclonal antibodies to target injectable vaccine antigen to dendritic cells expressing endocytic receptor effectively initiated antigen-specific immunity (18, 19), we next addressed the characteristics of NKM 16–2-4 as a carrier vehicle of M cell–targeted mucosal vaccines. When we injected FITC-conjugated NKM 16–2-4 or FITC-conjugated control rat IgG into intestinal loops containing PPs, FITC-conjugated NKM 16–2-4 specifically attached to the apical surfaces of M cells in the dome regions of PPs within 10 min of inoculation, whereas FITC-conjugated control rat IgG did not (Fig. 2 A). Furthermore, FITC-conjugated NKM 16–2-4 was taken into the cytoplasmic regions of M cells within 30 min (Fig. 2 A) and reached the basal membrane of the M cells within 4 h, indicating that NKM 16–2-4 could likely be used as a carrier vehicle of orally administered vaccine antigen to M cells.

To directly confirm that M cell–targeted mucosal vaccination with NKM 16–2-4 is an effective strategy for inducing high-level, antigen-specific immune responses, tetanus toxoid (TT) was selected as a prototypical vaccine antigen, as TT has been extensively used in our previous experiments to elucidate the mechanism of the antigen-specific immune responses induced in both the mucosal and systemic compartments by mucosal immunization (20). A chimeric complex of

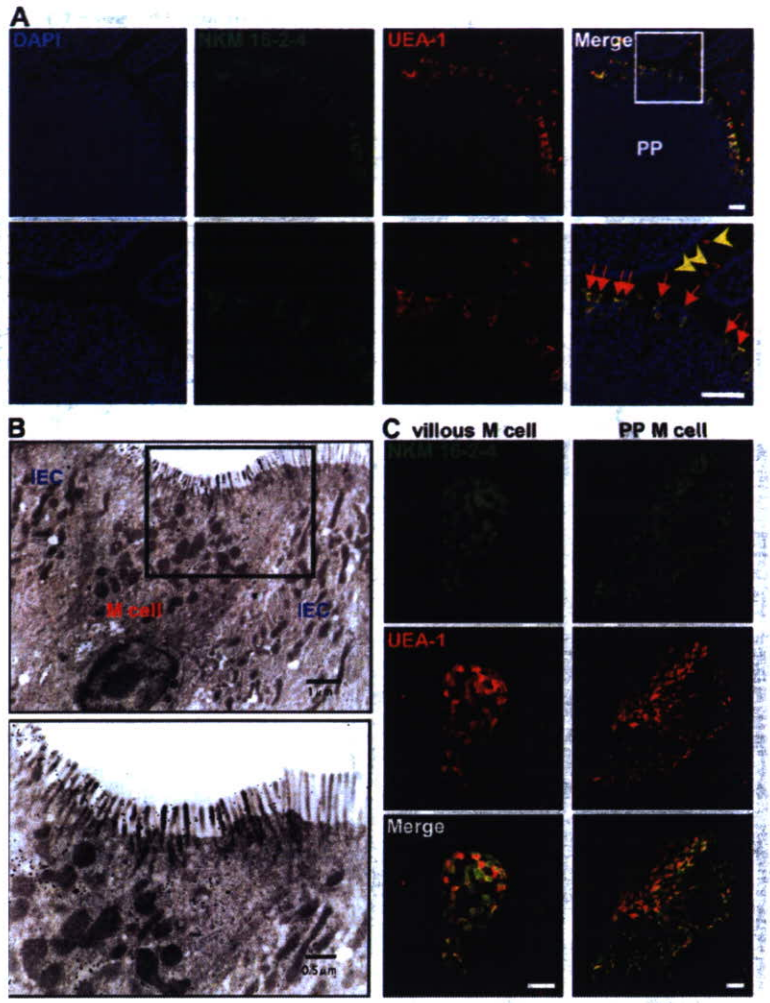


Figure 1. Immunohistochemical analysis for the specificity of NKM 16–2-4. (A) Immunohistochemical analysis of PPs revealed that NKM 16–2-4 specifically reacted with UEA-1–positive M cells (red arrows), but not UEA-1–positive goblet cells (yellow arrowheads). (B) Electronmicroscopic analysis revealed that typical M cells, which had short and irregular microvilli and pocket structures containing lymphocytes and/or monocytes, specifically reacted with NKM 16–2-4. Positive reactions are shown by gold particles (18 nm). IEC, intestinal epithelial cell. (C) Whole-mount staining of PPs and villous epithelium demonstrated that, in addition to PP-associated M cells, UEA-1–positive villous M cells were specifically recognized by NKM 16–2-4. Bars, 50 μm.

TT conjugated with NKM 16–2-4 or control rat IgG (in total, each 200 μg contained 50 μg TT per mouse) was prepared by using avidin–biotin complexes (see Materials and methods). The prepared complexes consisted of TT and NKM 16–2-4 or control rat IgG; these complexes, or noncoupled TT, were orally administered to mice, together with the mucosal adjuvant cholera toxin (CT). In addition, it has been reported that M cell–targeted mucosal vaccine with UEA-1 is effective in inducing antigen-specific humoral and cellular immunity when administered via the nasal route (8, 9); therefore, we prepared an orally administered TT-conjugated UEA-1 as a control for the efficacy of the NKM 16–2-4–based M cell–targeted mucosal vaccines. As expected, brisk TT-specific serum IgG and mucosal IgA responses were induced in mice immunized with TT-conjugated NKM 16–2-4, whereas TT-conjugated control rat IgG or 50 μg noncoupled TT induced, at best, very low TT-specific immune responses (Fig. 2 B). In addition, the level of the TT-specific immune response induced by TT-conjugated UEA-1 was lower than that induced by TT-conjugated NKM 16–2-4. These data suggest that an M cell–targeted mucosal vaccine with UEA-1 might be insufficient for antigen delivery to M cells, because the UEA-1–based vaccine is trapped by goblet cells and their secreting mucus, as well as by M cells. Furthermore, 10 times more noncoupled TT (500 μg) induced a small TT-specific immune response compared with TT-conjugated NKM 16–2-4 containing 50 μg TT (Fig. 2 B), perhaps because of the low efficacy of antigen delivery to M cells for the induction of antigen-specific immune responses. Although the levels of the antigen-specific antibody responses induced here by immunization with noncoupled TT and CT tended to

Table I. Immunological and biochemical characteristics of newly established mAb (NKM 16-2-4) and UEA-1 in M cells

mAb/lectin	Specificity	Cell specificity		
		M cells	Epithelial cells	Goblet cells
NKM 16-2-4	α(1,2) fucose-containing carbohydrate moiety	+	–	–
UEA-1	α(1,2) fucose	+	–	+

be lower than those in a previous study (20), this discrepancy might have been caused by differences in the mouse haplotype or the sources of TT and CT. Despite the discrepancy, our current findings emphasize the effectiveness of the newly established NKM 16–2-4 for the targeting of vaccine antigen to M cells to induce antigen-specific immune responses.

Moreover, when mice were orally immunized with botulinum toxoid (BT) conjugated with NKM 16–2-4 or control rat IgG (in total, each 200 μg contained 50 μg BT per mouse) in the presence of CT, brisk botulinum toxin–specific serum IgG and fecal IgA responses were induced in mice immunized with BT-conjugated NKM 16–2-4, but not in those immunized with BT-conjugated control rat IgG (Fig. 2 C). In addition, the mice immunized with BT-conjugated NKM 16–2-4 survived after challenge with 200 ng (10,000× LD_{50}) of botulinum toxin, whereas the mice immunized with BT-conjugated control rat IgG died within 3 h (Fig. 2 D). These data strongly indicate that the M cell-targeted mucosal vaccine with NKM 16–2-4 can effectively induce protective immunity with the minimum dose of vaccine antigen.

To confirm the mechanism by which the NKM 16–2-4–based M cell–targeted mucosal vaccine induces brisk antigen-specific immune responses in the systemic and mucosal compartments, and its universality, OVA was then chosen as a prototype antigen with low antigenicity. An immunocytochemical analysis revealed that Alexa Fluor 647–labeled OVA conjugated with NKM 16–2-4 and FITC-conjugated avidin specifically reacted with UEA-1–positive isolated M cells in vitro (Fig. 3 A), and intestinal loop assay clearly demonstrated that it specifically attached to the apical surfaces of M cells and was subsequently taken up into the cytoplasmic regions of M cells in vivo (Fig. 3 B). Furthermore, brisk increases in the levels of OVA-specific serum IgG were induced in mice immunized with only 200 μg OVA-conjugated NKM 16–2-4 (containing 50 μg OVA), but not with the same amount of OVA-conjugated control rat IgG (Fig. 3 C). Our previous study showed that amounts of OVA as high as 1 mg were required to induce OVA-specific immune responses (5); now, oral immunization with even small amounts of poorly immunogenic antigens (e.g., OVA) is possible by using the M cell–targeting concept with NKM 16–2-4.

We could not directly compare the efficacy of NKM 16–2-4–based mucosal vaccine with those of already published σ1-based mucosal vaccines (10, 11) because the latter systems have been used for nasal, but not oral, vaccines and no information is currently available on whether σ1 possesses

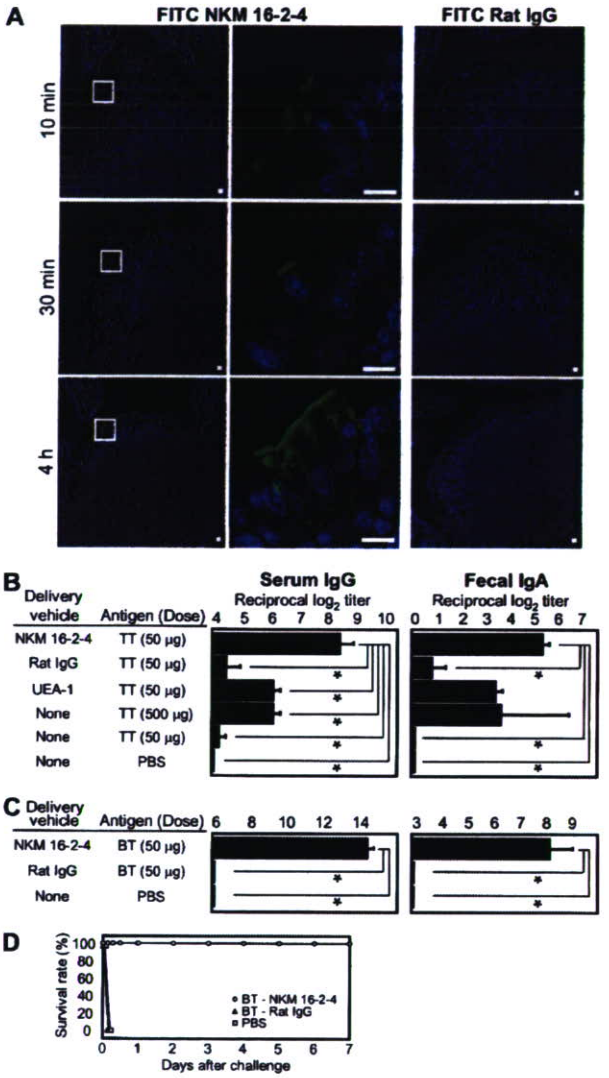


Figure 2. Development of an M cell–targeted mucosal vaccine with NKM 16–2-4. (A) FITC-conjugated NKM 16–2-4, but not FITC-conjugated control rat IgG, was specifically attached to the apical surfaces of M cells in FAE of PPs within 10 min of inoculation in an intestinal loop assay. The NKM 16–2-4 was subsequently taken up into the cytoplasmic regions of M cells within 30 min and reached the basal membrane of M cells within 4 h. Bars, 10 μm. (B) TT conjugated with NKM 16–2-4 effectively induced high-level, TT-specific serum IgG and fecal IgA responses, unlike TT conjugated with control rat IgG or UEA-1. Furthermore, the levels were superior to those in mice immunized with 10 times the amount of noncoupled TT (500 μg). *, $P < 0.01$, Tukey's *t* test. (C) BT-conjugated NKM 16–2-4, but not BT-conjugated control rat IgG, induced brisk botulinum toxin–specific serum IgG and fecal IgA responses. (D) Mice orally immunized with BT-conjugated NKM 16–2-4 were protected from an i.p. challenge with 10,000× LD_{50} type A botulinum toxin. Data are expressed as the mean ± the SD.

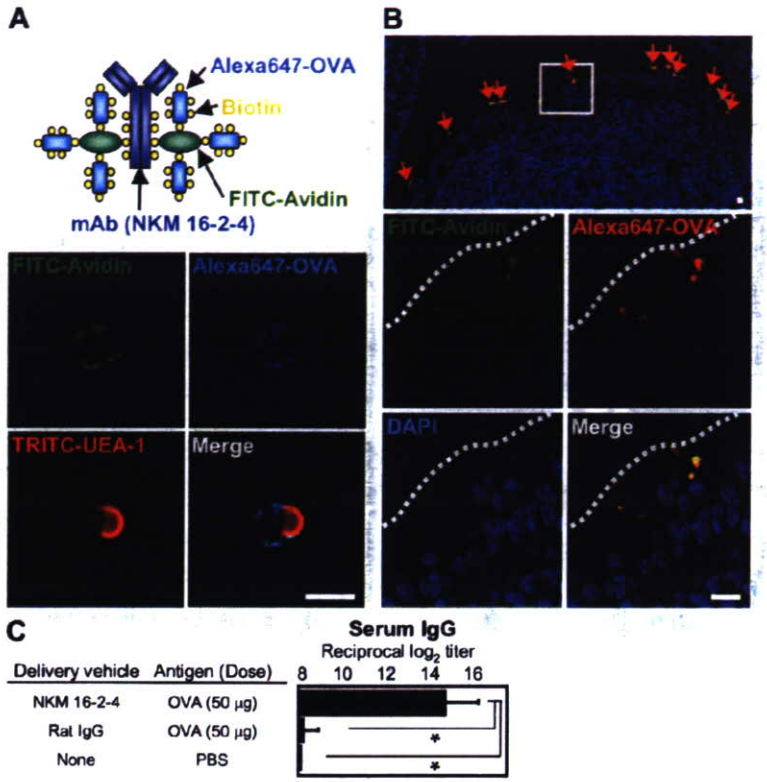


Figure 3. Effective uptake and universality of the M cell–targeted mucosal vaccine. (A) Immunocytochemical analysis showed that an M cell–targeted OVA vaccine composed of Alexa Fluor 647–conjugated OVA, FITC-conjugated avidin, and NKM 16–2-4 specifically reacted with isolated UEA-1–positive M cells. (B) In an intestinal loop assay, the M cell-targeted OVA specifically attached to the apical surfaces of M cells (red arrows) and was immediately taken up into the cytoplasmic regions of M cells. Bars, 10 μm. (C) Orally administered OVA-conjugated NKM 16-2-4 effectively induced an OVA-specific serum IgG response, whereas an OVA-conjugated control rat IgG did not. Data are expressed as the mean ± the SD.

specificity for villous M cells. However, our strategy for using NKM 16–2-4 as an M cell–targeting vehicle might be superior, because NKM 16–2-4 possesses specificity for both villous M cells and PP-associated M cells. In support of our hypothesis, our previous data showed that villous M cells are capable of taking up orally administered antigens for the induction of PP-independent, antigen-specific immune responses (4). However, it should be noted that TT- or OVA-specific immune responses were not effectively induced without the presence of the mucosal adjuvant CT, even if the antigen was targeted to M cells by using NKM 16–2-4. This finding could be explained by the observation that the gastrointestinal immune system generally operates via a sophisticated mucosal regulatory network to avoid unnecessary hyperimmune responses to the numerous orally encountered antigens in the harsh environment of the intestinal tract (3). Therefore, it is essential to use the mucosal adjuvant, which temporarily breaks the mucosal regulatory network system, to activate gastrointestinal immunity. In practical terms, further studies are needed to develop a safe mucosal adjuvant and take advantage of M cell–targeted mucosal vaccines with NKM 16–2-4.

Identification of antigens recognized by NKM 16–2-4

In attempts to elucidate the antigen-sampling mechanism of M cells for the induction of antigen-specific immune responses, a major drawback has been the lack of knowledge of the specific genes and the corresponding molecules expressed by M cells. In addition, no information regarding which murine M cell–specific glycoproteins are recognized by UEA-1 is currently available, although UEA-1 is used extensively as a specific marker of M cells in mice. Therefore, we tried to identify the membrane antigen recognized by NKM 16–2-4 by using a proteomics approach with liquid chromatography–tandem mass spectrometry (LC-MS/MS) after immunoprecipitation of an M cell lysate with NKM 16–2-4. 4 major bands (3 bands >250 kD and 1 band of ~150 kD) were precipitated by NKM 16–2-4 (Fig. 4 A), and these were identified by LC-MS/MS as maltase glucoamylase (top three bands) and alanyl (membrane) aminopeptidase (bottom band). These two molecules, which have been reported as intestinal enzymes of 410, 275, and 260 kD (21), and 150 kD, respectively, (22) under denatured conditions, are distributed at the brush borders of epithelial cells for the final digestion of dietary nutrients (21, 22). Because they were not homologous

with each other, and subsequent in situ hybridization analysis demonstrated that their mRNAs were ubiquitously and abundantly expressed in the intestinal epithelium, including in M cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20070607/DC1), we hypothesized that NKM 16–2-4 possesses specificity for the M cell–specific carbohydrate moiety containing α(1,2) fucose, as the precipitated antigens were commonly recognized by UEA-1 (Fig. 4 B).

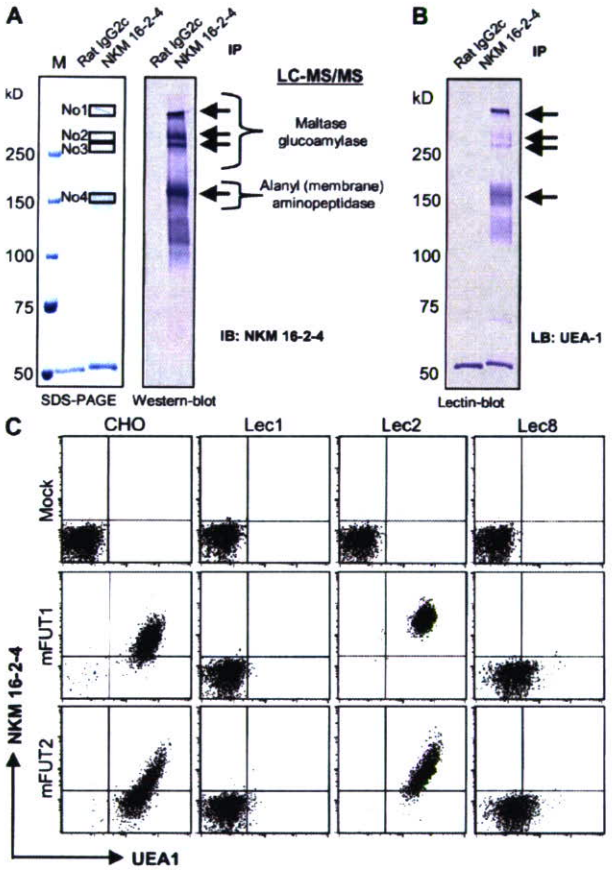


Figure 4. Identification of the antigen recognized by NKM 16–2-4. (A) Immunoprecipitation and Western blot analysis with NKM 16–2-4 were performed with an M cell lysate. 4 major bands (3 bands >250 kD and 1 band of ~150 kD) were precipitated. A subsequent LC-MS/MS analysis identified the three top bands as maltase glucoamylase and the bottom band as alanyl (membrane) aminopeptidase. (B) Lectin blot analysis performed after immunoprecipitation with NKM 16–2-4 showed that the precipitated antigens were all recognized by UEA-1. (C) mFUT1 and mFUT2 genes were transfected into original CHO cells and CHO-derived mutant lines (Lec1, Lec2, and Lec8 cells) with a pIRES2-EGFP expression system, and the specificity of NKM 16–2-4 and UEA-1 for EGFP-expressing transfectants was analyzed. NKM 16–2-4 and UEA-1 specifically reacted with mFUT1- or mFUT2-expressing original CHO cells. The reactivity of NKM 16–2-4 but not UEA-1 to mFUT1- or mFUT2-expressing Lec2 cells was enhanced compared with that to mFUT1- or mFUT2-expressing CHO cells. On the other hand, mFUT1- or mFUT2-expressing Lec1 or Lec8 cells were not recognized at all by NKM 16–2-4.

On the basis of our hypothesis, we transfected Chinese hamster ovary (CHO) cells with the genes encoding murine fucosyl transferase 1 (mFUT1) or mFUT2, which have been identified as α(1,2) fucose transfer enzymes (23). A flow cytometric analysis revealed that both NKM 16–2-4 and UEA-1 specifically reacted with CHO cells expressing mFUT1 or mFUT2, but neither NKM 16–2-4 nor UEA-1 showed specificity for original or empty vector–transfected CHO cells (Fig. 4 C). In addition, a blocking analysis showed that pretreatment of NKM 16–2-4 with α-L-fucose did not completely abolish reactivity to mFUT1- or mFUT2-expressing transfectants, although UEA-1 reactivity to these transfectants was dramatically decreased (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070607/DC1), indicating that the epitope recognized by NKM 16–2-4 is an mFUT1- or mFUT2-mediated carbohydrate complex containing α(1,2) fucose that is different from the UEA-1–reactive portion of α(1,2) fucose.

Because immunohistochemical analysis demonstrated that UEA-1, but not NKM 16–2-4, recognized goblet cells in the intestinal villi (Fig. 1 A), we turned to examining the differences in recognition patterns between NKM 16–2-4 and UEA-1 by using a mutant line of CHO cells to elucidate the importance of the glycosylation of M cells and goblet cells in the mucosal immune system. When the mFUT1 or mFUT2 gene was introduced into a mutant line of CHO cells (Lec2) with an inactivated CMP-sialic acid transporter (24), the reactivity of NKM 16–2-4, but not of UEA-1, was higher in these transfectants than in the mFUT1- or mFUT2-expressing original CHO cells; however, mFUT1- or mFUT2-expressing Lec1 cells with inactivated GlcNAc transferase I (i.e., a lack of *N*-glycans) (25) or Lec8 cells with inactivated UDP-galactose transporter (26) were not recognized at all by NKM 16–2-4. On the other hand, we observed very low reactivity of UEA-1 to mFUT1- or mFUT2-expressing Lec8 cells, although mFUT1- or mFUT2-expressing Lec1 cells were not recognized by UEA-1. This is because UEA-1 might recognize α(1,2) fucose, which is linked to very low levels of galactose on *N* glycans in mFUT1- or mFUT2-expressing Lec8 cells because it has been reported that Lec8 cells retain 10–20% of their galactosylation (26), and no information is currently available on whether α(1,2) fucose links to anything other than galactose. These data suggest that sialic acid might be useful in distinguishing the reactivity of NKM 16–2-4, but not UEA-1 to galactose-binding α(1,2) fucose on *N*-glycans, although the reactivity to α(1,2) fucose regulated by *O*-glycans remains unclear. Thus, our initial immunohistochemical analyses demonstrated that the specificity of NKM 16–2-4 to UEA-1–positive M cells, but not UEA-1–positive goblet cells, is attributable to the existence of abundant sialic acids neighboring the α(1,2) fucose–containing carbohydrate moiety on goblet cells, but not on M cells. With the exception of their expression patterns at the tissue level, there is currently little reliable information available on the glycobiological and molecular biological differences between mFUT1 and mFUT2 as α(1,2) fucosyltransferases (23). Therefore, further studies, especially in terms of in situ expression patterns at a cellular level at inductive