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Inhibition of neutrophil elastase prevents the development of murine dextran sulfate sodium-induced colitis

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Editorial on page 395

Background. Neutrophil elastase (NE) is a major secretory product from activated neutrophils and a major contributor to tissue destruction. However, little is known about the pathogenic contribution of NE to ulcerative colitis (UC). This study was designed to investigate the contribution of NE by measuring NE activity in plasma and colonic mucosal tissue from UC patients and a murine acute colitis model, and to elucidate the therapeutic effect of the NE-specific inhibitor ONO-5046. ***Methods.*** The NE enzyme activities in plasma and colonic mucosal tissue from UC patients were directly measured using an enzyme–substrate reaction. Acute colitis was induced in mice by administration of 1.5% dextran sulfate sodium (DSS) for 5 days. DSS-induced colitis mice were then treated with ONO-5046 (50 mg/kg body weight) intraperitoneally twice a day. ***Results.*** In UC patients, the NE enzyme activity was significantly elevated in both the plasma and colonic mucosal tissue compared with healthy controls. In DSS-induced colitis mice, the NE enzyme activity increased in parallel with the disease development. ONO-5046 showed therapeutic effects in DSS-treated mice by significantly reducing weight loss and histological score. ONO-5046 suppressed the NE enzyme activities in both plasma and culture supernatant of colonic mucosa from DSS-induced colitis mice. ***Conclusions.*** ONO-5046, a specific NE inhibitor, prevented the development of DSS-induced colitis in mice. NE therefore represents a promising target for the treatment of UC patients.

Key words: ulcerative colitis, neutrophil elastase, dextran sulfate sodium-induced colitis, ONO-5046

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Introduction

Although the etiology of ulcerative colitis (UC) has not been clarified, increasing evidence indicates that abnormal immune responses are involved in its pathogenesis.[1,2] While recent studies have focused mainly on lymphocytes or antigen-presenting cells such as dendritic cells, little is known about the pathogenic role of neutrophils in UC. Indeed, dense neutrophil infiltration and crypt abscess formation are characteristic pathological findings in the inflamed mucosa of UC patients.[3,4] Moreover, in Japan, granulocyte adsorption apheresis therapy has been reported to show a remarkable therapeutic effect in active UC patients.[5] Taken together, neutrophils almost certainly play an important role in the pathogenesis of UC.

Neutrophil elastase (NE) is a major secretory product from activated neutrophils and a major contributor to tissue destruction in inflammatory diseases such as acute respiratory distress syndrome (ARDS), lung emphysema, glomerulonephritis, and rheumatoid arthritis.[6,7] Ninety percent of the NE circulating in the blood is bound to α_1-anti-trypsin (α_1-AT), an endogenous NE inhibitor, resulting in the formation of NE-α_1-AT complexes. The remaining 10% of the NE is bound to α_2-macroglobulin, another endogenous NE inhibitor. Therefore, NE is systemically and tightly inactivated by endogenous protease inhibitors. However, at inflammatory sites, these protease inhibitors are inactivated by neutrophil-derived reactive oxygen species (ROS).[8] Thus, NE might become capable of displaying its strong protease activity and degradeing the main structural elements of connective tissue, such as elastin, collagen, and proteoglycans, at inflammatory sites.

It has previously been reported that NE is elevated in the plasma, colonic mucosal tissues, and feces of active UC patients.[9–11] It has also been demonstrated that the plasma NE level is correlated with the clinical activity of UC.[9,12] However, a definitive conclusion cannot be

drawn regarding the pathological contribution of NE to UC, since the NE levels measured by using an enzyme-linked immunosorbent assay in the previous studies included NE–α_1-AT complexes, an inactive form of NE. Since only free NE shows protease activity, it is critical to measure the NE enzyme activity directly to clarify the pathogenic contribution of NE to UC.

ONO-5046 is a specific synthetic inhibitor of NE. In contrast to endogenous protease inhibitors, ONO-5046 can even effectively inhibit NE at inflammatory sites, since it is not structurally inactivated by ROS.[8] In Japan, ONO-5046 has already been used clinically in the treatment of patients with ARDS, which is characterized by the accumulation of numerous neutrophils in the lungs. Furthermore, ONO-5046 has shown a protective effect against neutrophil-mediated tissue injury in some animal models, including lung injury, neurologic damage after spinal cord injury, and collagen-induced arthritis.[13–16]

In this study, we first measured NE enzyme activity in plasma and colonic mucosal tissues from UC patients using an enzyme–substrate reaction. We next measured NE enzyme activity in a dextran sulfate sodium (DSS)-induced colitis model. Furthermore, we evaluated the therapeutic effect of ONO-5046 in colonic inflammation.

Materials and methods

Reagent

ONO-5046 was purchased from Ono Pharmaceutical (Osaka, Japan). It was dissolved in phosphate-buffered saline (PBS) and administered intraperitoneally twice a day at a dose of 50 mg/kg. The administration of ONO 5046 began 1 day prior to DSS administration and continued until the end of the experiment. Nontreated control mice were administered the same amount of PBS without ONO-5046.

Patients and samples

UC was diagnosed on the basis of clinical, endoscopic, and histological findings using established criteria.[17,18] Plasma samples were obtained from UC patients with moderate to severe activity (Table 1), defined by a clinical activity index (CAI) >8 points.[19] Patients treated with steroids or immunosuppressants were excluded from the study. Colonic mucosal samples were obtained from biopsy specimens of the inflamed mucosa of UC patients. Control samples of noninflamed colonic mucosa were obtained from macroscopically unaffected areas of patients with colon polyps. The tissues were weighed, gently homogenized in 500 µl of PBS, and then centrifuged (1500 *g*, 15 min, 4°C). The supernatants were collected and immediately stored at –80°C until use. The study using human material was approved by the ethical committee of Keio University and written informed consent was obtained from all patients.

Table 1. Clinical profiles of the ulcerative colitis patients

Number of patients	10
Sex (female/male)	4/6
Age (years), mean (range)	28.7 (21–38)
Clinical Activity Index, mean (range)	11.3 (9–15)
Disease location, pan/left-sided colitis (no.)	9/1
Disease duration (years), mean (range)	4.2 (0.1–11.5)

Murine DSS-induced colitis model

Female 8-week-old C57BL/6 mice weighing around 20 g were used. The mice were housed under specific pathogen-free conditions. Colitis was induced by giving 1.5% DSS (molecular weight 50 kDa; BioResearch, Yokohama, Japan) dissolved in sterile distilled water ad libitum for 5 days followed by regular drinking water for the rest of the experimental period. Body weight was measured every day during the experiments. All experiments were performed in accordance with the institutional animal care guidelines of Keio University.

Histological score

Histological evaluation was performed on day 4. The total colon was fixed in 20% formalin and sectioned with a sagittal aspect. Tissues were embedded in paraffin and stained with hematoxylin-eosin. Histological analysis was performed in a blinded fashion. The histological score was estimated by the combined score of inflammatory cell infiltration (score, 0–3) and tissue damage (score, 0–3) as previously reported.[20,21] Briefly, the infiltration scoring was as follows: 0, no infiltration; 1, presence of occasional inflammatory cells in the lamina propria; 2, increased numbers of inflammatory cells in the lamina propria; and 3, confluent inflammatory cells extending into the submucosa. The tissue damage scoring was as follows: 0, no mucosal damage; 1, discrete lymphoepithelial lesions; 2, surface mucosal erosion or focal ulceration; and 3, extensive mucosal damage and extension into deeper structures of the bowel wall. The combined histological score therefore ranged from 0 to 6.

Colon organ culture

After the induction of colitis, each murine colon sample was weighed, cut into 2–3 pieces, and then cultured in a 6-well dish (Falcon, Franklin Lakes, NJ, USA) in serum-free RPMI-1640 medium supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin (Invitrogen,

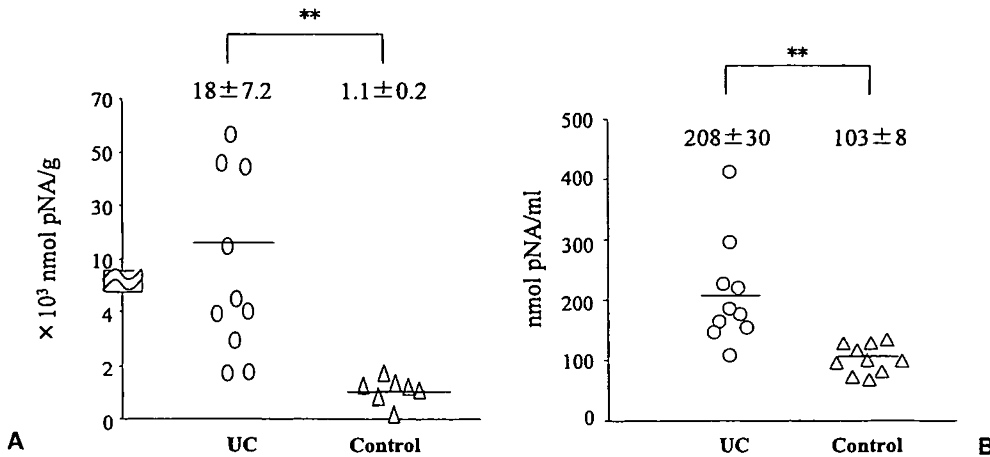


Fig. 1A,B. Neutrophil elastase (NE) enzyme activity is increased in UC patients. NE enzyme activity was directly measured using a NE-specific substrate as described in Materials and methods. The NE enzyme activities in colonic mucosal tissues (**A**) and plasma (**B**) from patients with active ulcerative colitis (*UC*) (*circles*) or controls (*triangles*) are shown (UC, $n = 10$; controls, $n = 7$). The means ± SEM are shown at the top. *pNA*, *p*-nitroanilide; $^{**}P < 0.01$

Grand Island, NY, USA) in a 5% CO_2 incubator for 24 h. The tissues were carefully positioned so that the mucosal surface was uppermost on the insert.[10] The culture supernatant was collected from the well, filter-sterilized (0.22 μm), and stored at −80°C until use.

Measurement of NE enzyme activity

The NE enzyme activity of each material (plasma, supernatant of colon organ culture, and homogenized tissue) was determined by an enzyme–substrate reaction method using *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma, St. Louis, MO), a specific synthetic substrate of NE.[22] This substrate was specifically cleaved to *p*-nitroanilide (pNA) by NE, and the NE enzyme activity was quantified by measuring the quantity of pNA spectrophotometrically. Each sample was incubated with 0.2 M Tris-HCl buffer (pH 8.0) containing 2.5 M NaCl and 50 mM substrate at 37°C for 24 h, and the amount of pNA was measured by the absorbance at 405 nm.

Statistical analysis

The data were expressed as means ± SEM. Comparisons of the data were performed using a nonparametric Mann-Whitney *U* test. A *P* value below 0.05 was accepted as statistically significant.

Results

NE enzyme activity is elevated not only locally but also systemically patients with active UC

First, we measured the NE enzyme activity in colonic mucosal tissues from UC patients ($n = 10$) to investigate the local production of NE enzyme activity. As shown in Fig. 1A, NE enzyme activity in colonic mucosal tissues from UC patients ($n = 10$) was approximately 16 times that in controls ($n = 7$) (UC, 18 ± 7.2 vs. control, 1.1 ± 0.2 × 10^3 nmol pNA/g, $P < 0.01$). Furthermore, the NE enzyme activity in plasma samples from UC patients ($n = 10$) was approximately twice that in controls ($n = 10$) (Fig. 1B; UC, 208 ± 30.2 vs. control, 103 ± 8.0 nmol pNA/ml, $P < 0.01$). Thus, the NE enzyme activity was elevated not only locally but also systemically in UC patients. These results prompted us to evaluate the therapeutic effect of a NE-specific inhibitor, ONO-5046, in a murine DSS-induced colitis model, which resembles UC in many pathological features, such as dense neutrophil infiltration and crypt abscess formation.

NE increases in DSS-induced colitis in parallel with disease development

Before evaluating the therapeutic effect of the NE inhibitor, we confirmed that the NE enzyme activity was elevated in the murine DSS-induced colitis model. We first examined three concentrations of DSS, because the severity of DSS colitis depends on its molecular weight. In our preliminary results, administration of 1% DSS caused mild inflammation. In contrast, one-third of mice died by administration of 2% DSS. With administration of 1.5% DSS, body weight loss reached about 20% and severe inflammation was histologically confirmed without any mortality. Therefore, we chose 1.5% DSS in the following experiments. Colitis was induced by giving 1.5% DSS for 5 days followed by regular drinking water for the rest of the experimental period. The body weight began to decrease from day 4 and reached its minimum, 23% weight loss on days 8–9 (Fig. 2A). In colon culture supernatants, NE enzyme activity was elevated on day 4, when the body weight loss had just begun (Fig. 2A, B; culture supernatant, 360 ± 16 nmol pNA/ml on day 4). In contrast, the plasma NE enzyme activity was elevated on day 8 (300 ± 100 nmol pNA/ml), when the body weight loss reached its peak (Fig. 2D).

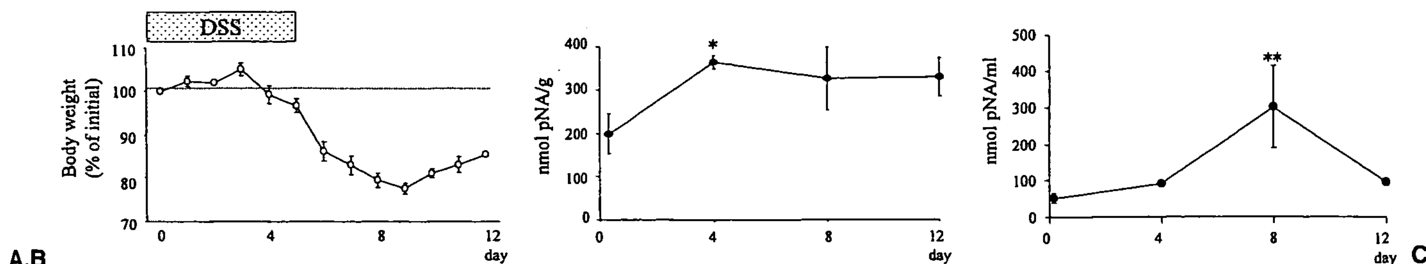


Fig. 2A–C. NE enzyme activity is elevated in a dextran sulfate sodium (*DSS*)-induced colitis model. **A** Body weight loss of DSS-induced colitis mice. The NE enzyme activities in supernatants from colon cultures (**B**) and plasma (**C**) from DSS-induced colitis mice ($n = 6$) are shown. The data represent an means ± SEM. $^{**}P < 0.01$; $^{*}P < 0.05$

ONO-5046 ameliorates DSS-induced colitis

We next assessed the therapeutic effect of ONO-5046. To determine an adequate dose of ONO-5046, we first administered four different doses of ONO-5046 (1, 5, 25, and 50 mg/kg) in DSS-induced colitis. The therapeutic effects were seen in a dose-dependent manner (data not shown), and the administration of 50 mg/kg showed the strongest effect. Therefore, we decided to use this dose through all of our experiments. ONO-5046 was administered intraperitoneally twice a day at the dose of 50 mg/kg in 200 µl PBS. Control mice were given the same dose of PBS without ONO-5046. ONO-5046 was administered 1 day prior to DSS administration and continued until the end of the experiment (Fig. 3A). Figure 3B shows that the body weight loss of mice treated with ONO-5046 was significantly reduced compared with control mice (control, 98.6 ± 0.9% vs. ONO-5046, 101.8 ± 0.9% on day 4, $P < 0.01$; control, 90.6 ± 1.8% vs. ONO-5046, 96.2 ± 1.5% on day 5, $P < 0.05$; control, 81.6 ± 1.6% vs. ONO-5046, 86.6 ± 1.1% on day 6, $P < 0.05$). The macroscopic findings on day 4 are shown in Fig. 3C. Colon length in the control group had a tendency to be shorter than that in the ONO-5046 treated group. However, the difference was not statistically significant (control, 61.3 ± 1.8 vs. ONO-5046, 66.3 ± 2.9 mm, $P = 0.11$). Histologically, the colons from control mice revealed severe ulceration and inflammatory cell infiltration (Fig. 3D, E). In contrast, the colons from DSS-induced colitis mice treated with ONO-5046 showed reduced ulceration and inflammation (Fig. 3F, G). The histological scores on day 4 were significantly reduced in the ONO-5046-treated group compared with the control group (Fig. 3H, control, 2.9 ± 1.2 vs. ONO-5046, 4.0 ± 1.6, $P < 0.05$).

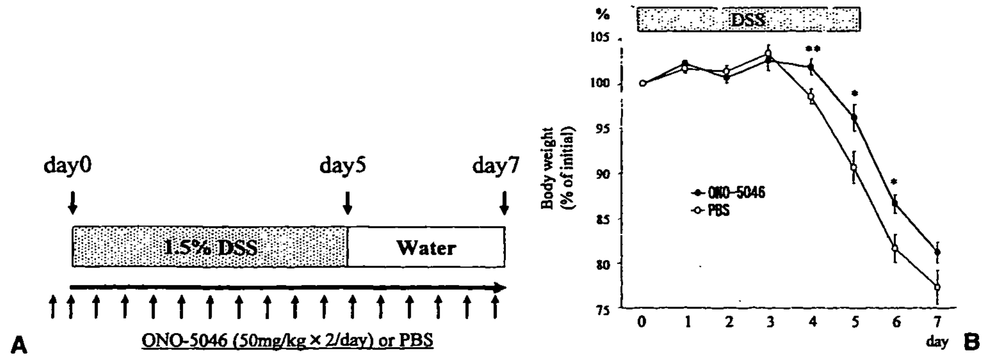
ONO-5046 reduces NE enzyme activity in DSS-induced colitis

To confirm that NE enzyme activity was inhibited by ONO-5046 in the DSS-induced colitis model, the effect of ONO-5046 on the plasma enzyme activity was measured on day 8, when the activity was found to be at its highest level (Fig. 2C). As shown in Fig. 4A, ONO-5046 completely suppressed the increase in NE enzyme activity (DSS-induced colitis mice treated with PBS, 340 ± 270 vs. DSS-induced colitis mice treated with ONO-5046, 93 ± 3 nmol pNA/ml, $P < 0.01$). Furthermore, to investigate the inhibition of local NE enzyme activity, colons from DSS-treated mice were cultured, and then ONO-5046 (1 mg/ml) was added to the culture medium 2 h before the end of the culture. As shown in Fig. 4B, the NE enzyme activity was inhibited in the supernatants from ONO-5046-treated specimens (PBS, 690 ± 110 vs. ONO-5046, 430 ± 57 nmol pNA/ml, $P < 0.05$). These results confirmed that NE enzyme activity was inhibited by ONO-5046 in the DSS-induced colitis model.

Discussion

The most important finding in the present study is that a specific NE inhibitor could prevent the development of DSS-induced colitis in mice. Given that murine DSS-induced colitis possesses certain pathophysiological features of UC, the data indicate that NE may represent a new target for the treatment of UC patients.

Since neutrophil infiltration and crypt abscess are histological features common to both UC and the murine DSS-induced colitis model, neutrophils may play a critical role in the pathogenesis of both UC and the DSS-induced colitis model. NE is a major secretory product from neutrophils and is capable of hydrolyzing most connective tissue components, leading to tissue injury at inflammatory sites. To clarify the pathological role of NE in intestinal inflammation, we first determined the levels of NE enzyme activity in UC patients. We showed that NE enzyme activity was increased in UC patients, while previous reports have measured total NE, including NE–α1-AT, complexes.[9,11,12] We directly measured the NE enzyme activity by an enzyme–substrate reaction using a specific synthetic substrate of NE. As blood contains abundant physiological NE inhibitors such as


day0
day5
day7
1.5% DSS
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ONO-5046 (50mg/kg × 2/day) or PBS
A
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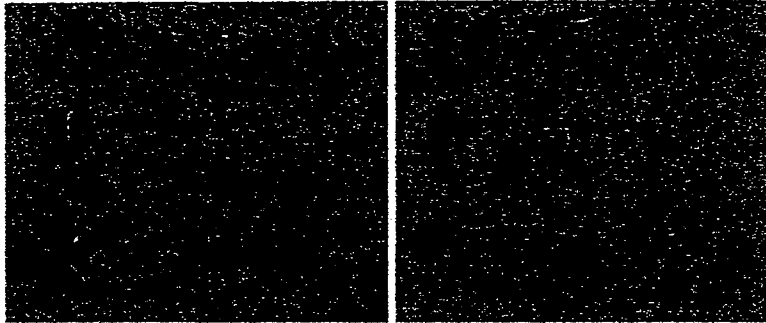
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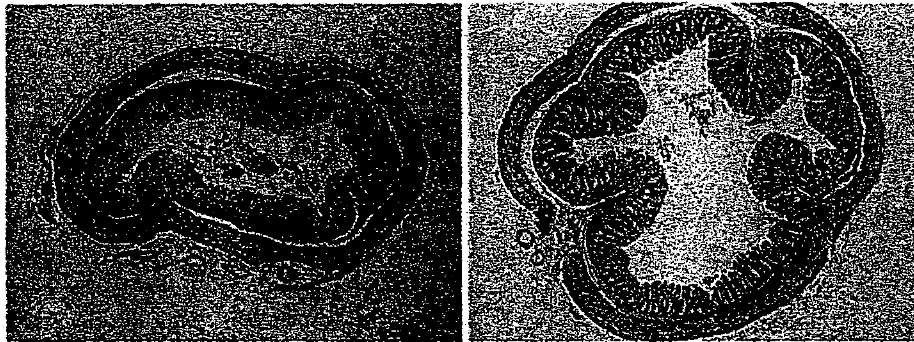
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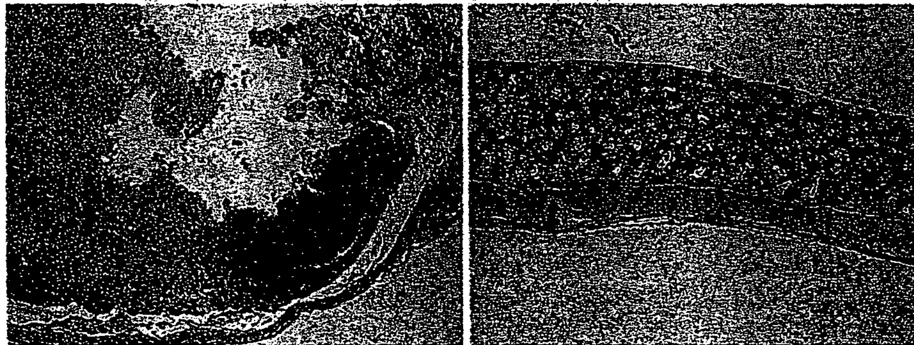
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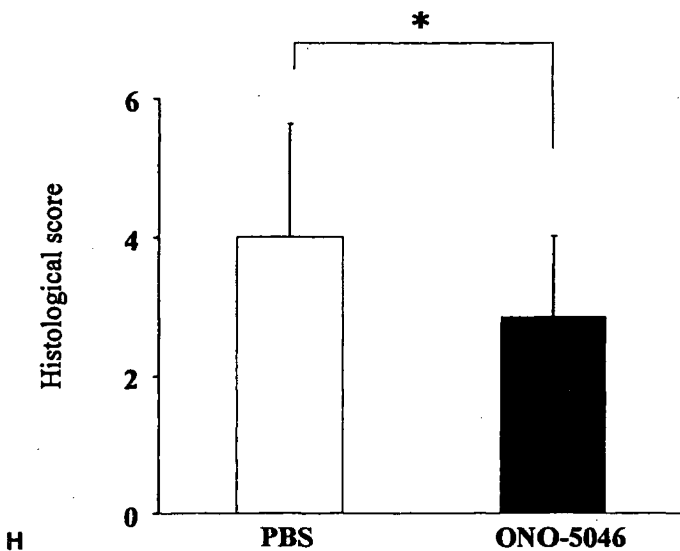

Fig. 3A–H. A NE-specific inhibitor, ONO-5046, suppresses the development of murine DSS-induced colitis. **A** The experimental protocol. ONO-5046 was administered intraperitoneally twice a day at the dose of 50 mg/kg. Nontreated mice were administered the same amount of phosphate-buffered saline (*PBS*) without ONO-5046. **B** Body weight loss of DSS-induced colitis mice treated with ONO-5046 (*closed circles*, n = 12) or PBS (*open circles*, n = 12). The data were collected from three independent experiments. **C** Macroscopic findings of colons from DSS-induced colitis mice treated with PBS (*left*) or ONO-5046 (*right*). Hematoxylin-eosin staining of colons from DSS-induced colitis mice treated with PBS (**D, E**) or ONO-5046 (**F, G**) on day 4. **H** Histological scores of DSS-induced colitis mice treated with ONO-5046 (*black bar*) or PBS (*white bar*) on day 4. The data represent means ± SEM. $^{**}P < 0.01$; $^{*}P < 0.05$

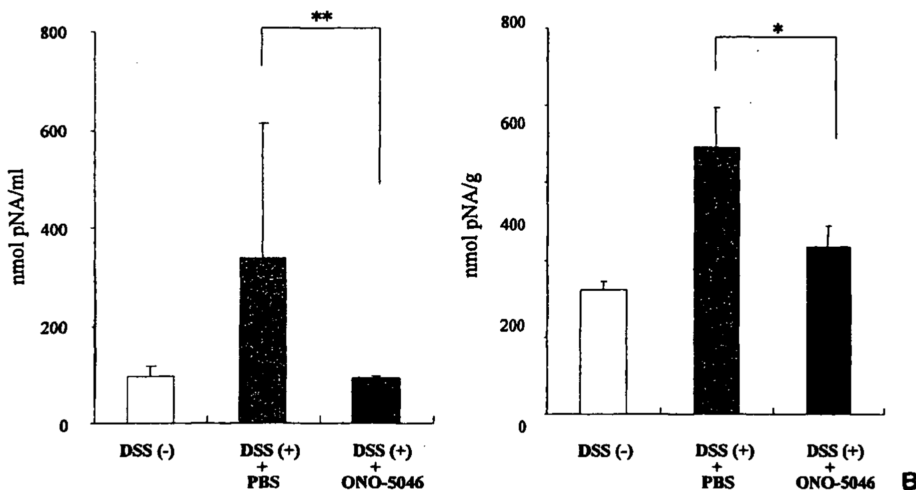

Fig. 4A,B. ONO-5046 reduces NE enzyme activity in DSS-induced colitis. **A** NE enzyme activities in plasma from mice without DSS (*white bar*, n = 6) and DSS-induced colitis mice treated with ONO-5046 (*black bar*, n = 6) or PBS (*hatched bar*, n = 6). Plasma samples were obtained on day 8. **B** Colons were obtained from mice without DSS (*white bar*, n = 6) and DSS-induced colitis mice on day 4. ONO-5046 (1 mg/ml, *black bar*, n = 6) or PBS (*hatched bar*, n = 6) was added into the medium of the colon culture, and the NE enzyme activities of the culture supernatants were measured. The data represent means ± SEM. $^{**}P < 0.01$; $^{*}P < 0.05$

α1-AT, little plasma NE enzyme activity can normally be detected. However, it has been reported that plasma NE enzyme activity can be detected in an ARDS hamster model,[13] possibly because local NE production is so huge that it overcomes the capacity of physiological NE inhibitors. In the same way, we were successful in detecting NE enzyme activity in not only colonic mucosal tissues but also plasma samples of UC patients. These results suggest that a considerable amount of NE is produced in colons of UC patients, which overcomes physiological NE inhibitors in blood. Next, we measured NE enzyme activity in a murine DSS colitis model. We found that local NE enzyme activity was increased from an early stage of the disease and elevated throughout the experimental period and that systemic NE cold be measured following local NE production at the maximal stage of the disease. These results suggest that NE may contribute to both the induction and perpetuation of colitis.

Furthermore, we assessed the therapeutic effect of a specific NE inhibitor. We demonstrated that ONO-5046 could prevent the development of murine DSS-induced colitis. While the results further support the pathophysiological contribution of NE to intestinal inflammation, the precise mechanisms of how the decreased NE enzyme activity led to the amelioration of colitis could

not be determined in this study. NE is considered to be involved in tissue destruction through its protease activity. Moreover, recent studies have revealed some new functions of NE in inflammatin. (1) NE enhances the migration and adhesion of neutrophils;[23] (2) NE stimulates the production of proinflammatory cytokines such as cytokine-induced neutrophil chemoattractant, macrophage inflammatory protein-1, and interleukin-1β;[24] and (3) NE cleaves phosphatidylserine receptors on macrophages and disrupts the phagocytosis of neutrophils, thus enhancing the scattering of NE.[25] Since NE seems to have such various biological activities, further studies are required to identify the exact mechanism of how NE inhibition ameliorates colitis.

ONO-5046 has already been clinically used for the treatment of ARDS associated with systemic inflammatory response syndrome in Japan. It has a few side effects, such as mild liver dysfunction (8.4%) and mild leukopenia (1.6%), but no serious side effects have been reported. Therefore, ONO-5046 might actually have the potential to be a new therapeutic approach for patients with active UC.

In summary, we demonstrated that NE enzyme activity is increased in both UC and a murine DSS-induced colitis model. Furthermore, we showed that ONO-5046, a NE-specific inhibitor, could ameliorate murine DSS-induced colitis. These findings provide evidence that NE contributes to the pathophysiology of mucosal inflammation. NE therefore represents a promising target for the treatment of UC patients.

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●〈症例〉

大腸全摘術後の回腸嚢炎・多発関節炎に白血球除去療法が奏功した潰瘍性大腸炎の1例

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〔Key Words〕回腸嚢炎，潰瘍性大腸炎関連関節炎

要旨

大腸全摘術後に出現した回腸嚢炎，多発関節炎に対し白血球除去療法(leukocytapheresis：LCAP)が奏功した潰瘍性大腸炎を経験した。また，6-MPによる緩解維持効果もみられ，今後これらが術後の回腸嚢炎や腸管外合併症の緩解導入および維持療法の選択肢のひとつになることが期待された。

症　例

患者：40歳，女性。

主訴：関節痛，腹痛，血便。

既往歴：大腿骨頭壊死(32歳時)。

家族歴：特記すべきことなし。

生活歴：喫煙，アルコールともなし。

現病歴：1999年(34歳)発症の全大腸炎型潰瘍性大腸炎(Ulcerative colitis：UC)で再燃緩解を繰り返し，2000年6月入院時にはステロイド動注療法で緩解導入がえられた。しかし，徐々にステロイド抵抗性となり，難治性と判断され2003年4月大腸全摘・回腸嚢肛門管吻合術を受けた。術後症状は安定していたが，2005年4月より両上下肢などの多発関節炎が出現しエトドラク400mg/日の内服とともにジクロフェナクナトリウムの坐薬を頓用していた。さらに，8月下旬より腹痛，血便の増悪もみられ入院となった。

身体所見：体格中等，体温37.1℃，貧血/黄疸なし。口腔内，表在リンパ節著変なし。腹部右下腹部に圧痛みとめるが反跳痛や筋性防御なし。両側手，足，左顎関節の腫脹あり。

検査成績：血液検査では血沈88mm/1h，白血球数9,180/μl，CRP8.59mg/dlと炎症所見を呈した。Hb11.0 g/dlと軽度貧血がみられたが，TP 7.1g/dl，Alb 3.9g/dl，TC167mg/dl，Ch-E3.76IU/lと栄養状態はよく，肝腎機能も正常であった。RF，ANAとも陰性であった。入院時の内視鏡では回腸嚢粘膜は顆粒状で血管透見性は消失し，膿性粘液を伴った潰瘍がみられ，吻合部でも線状潰瘍がみられた(**Fig. 1-a, b**)。生検組織ではびらんを伴うリンパ球，形質細胞や好中球の著明な浸潤がみられた(**Fig. 2**)。以上よりPDAI score 11点で中等症の回腸嚢炎と診断した[1]。

経過：非ステロイド系抗炎症薬の効果不十分な関節炎に対し，ステロイドの使用も考慮したが，大腿骨頭壊死既往のため不適応と判断した。また回腸嚢炎についてはメシル酸パズフロキサシン1,000mg/日点滴(2週間)を行った。さらにこれらの症状とUCとの関連性を考え第3病日より5-ASA製剤内服2,250mg/日とLCAPを開始しCRPは徐々に低下した。関節痛はLCAP翌日には著明な改善がみられたが施行直後の改善と数日後の増悪を繰り返した。LCAP 5回終了時にはPDAI score 7点となり，第40病日の内視鏡で回腸嚢および吻合線上の潰瘍の縮小，粘膜の炎症の改善が確認された(**Fig. 1-c, d**)。緩解維持目的に9月29日より6-MP 30mg/日内服を開始し2006年7月現在まで再燃をみとめていない。

考　察

潰瘍性大腸炎関連関節症はUCの発症前または経過中に発症する非感染性関節炎である[2]。強直性脊椎炎やReiter症候群などとともにリウマチ因子陰性のseronegative spondyloarthropathyで，発症様式は腸炎先行型，末梢型の関節炎が多い。治療は非ステロイド系抗炎症薬が主で，末梢型では原疾患の治療で軽快することが多いが，大腸摘出術に至る重症例も報告されている。術後に関節炎が消失した例が4例，逆に本例のように術後に関節炎が出現した例も6例みられ原疾患の活動性とは必ずしも一致しない。

最近UC関連関節炎でのLCAPの有用性が報告されているが[3]，術後関節炎を生じた例も含めいずれも本症例と同様腸炎先行型，ステロイド抵抗性症例であった。

一方UCでの回腸嚢炎の発生頻度は20%前後で，診断基準にはMayo Clinicから提唱されたPouchitis disease activity index(PDAI)などがある。臨床症状，内視鏡および組織学的所見から点数化しPDAIでは7点以上が回腸嚢炎と診断される。治療はメトロニダゾールの他，ステロイドの坐薬や注腸，メサラジン注腸の有用性が報告されているが[1]，本症例では大腿骨頭壊死の既往からステロイド不適応と判断した。関節炎症状が主体であったため全身療法としてLCAPをおこな

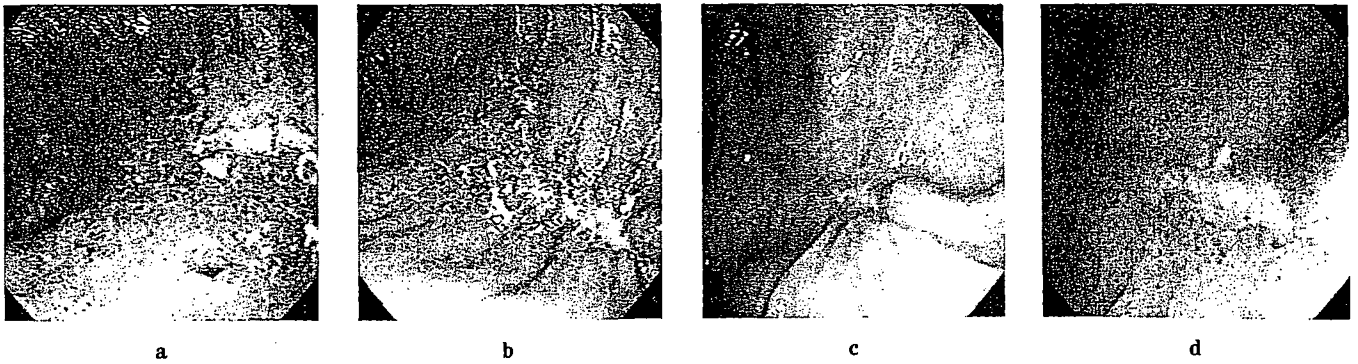

Fig. 1 Colonoscopy on admission. Mucosal inflammation with ulcers in J-pouch(a) and along the staple line(b) were seen. On 40th day remarkable improvement of ulcers and mucosal inflammation was seen both in J-pouch (c) and along the staple line(d).

Fig. 2 Histological finding of biopsy specimen from J-pouch on admission. Remarkable infiltraion of neutrophils, plasma cells and eosinophils with erosive change were seen.

い，局所療法は行わなかったが，抗菌剤終了後も臨床症状，内視鏡所見の改善が維持され回腸嚢炎に対しての有用性も示唆された。

さらに，6-MPはUCの緩解維持に有効とされているが，本症例で術後に発症した回腸嚢炎や多発関節炎の緩解維持にも有用であったことは，免疫異常を主体とするUCにおいては粘膜病変形成のみならず術後発症の合併症の病態を考える上でも興味深い。

本症例は今後術後発症の回腸嚢炎や腸管外合併症にLCAPおよび6-MPが治療法のひとつになることを示唆するものと考えられた。

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A case report of pouchitis and polyarthritis after total colectomy for ulcerative colitis which showed the effect of leukocytapheresis

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●〈症例〉

自然軽快したcap polyposisの1例

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〔Key Words〕cap polyposis

和文要旨

患者：55歳，女性。過敏性腸症候群として加療されていたが，2001年2月頃より粘血便が頻回となり，左下腹部痛が出現したため当科に入院。大腸内視鏡検査にて直腸には平盤状隆起，S状結腸にはタコいぼ状隆起や強い発赤斑を認めた。平盤状隆起の生検で，cap部の化膿性滲出物，炎症性肉芽組織，軽度の線維筋症を認め特異な内視鏡像とあわせcap polyposis(CP)と診断した。排便習慣の是正により症状が軽快したCPの1例を経験したので報告する。

症　例

患者：55歳，女性。

主訴：下痢，粘血便，左下腹部痛。

既往歴：骨髄炎，肺炎。

家族歴：父・母，糖尿病。

生活歴：喫煙歴・飲酒歴なし。

現病歴：元来緊張すると便秘や下痢をきたす傾向があった。1995年，粘血便を認めたため近医で注腸を受けるが異常所見を認めず過敏性腸症候群と診断された。2001年2月頃より粘血便が頻回となり，ポリカルボフィル製剤，整腸剤等の投与を受けるも改善なく，9月頃より左下腹部痛も認めるようになった。近医での大腸内視鏡検査で潰瘍性大腸炎を疑われたため，同年10月に当科を受診，入院となった。

入院時現症：身長157cm，体重53kg，血圧 100/60 mmHg，脈拍66回/分・整，体温36.2度，腹部平坦・軟，腸音低下，左下腹部に圧痛あり，反跳痛なし，筋性防御なし，他異常所見認めず。

検査所見：赤沈 12mm/1st hr，WBC $6.2\times10^3/\mu l$，Hb 13.6g/dl，Plt $25.7\times104/\mu l$，TP 6.2g/dl，Alb 3.7 g/dl，TC 196mg/dl，TB 0.4mg/dl，BUN 8.6mg/dl，Cr 0.6mg/dl，CRP 0.01mg/dl，LDH 177IU/l，AST 24IU/l，ALT 19IU/l。

便潜血反応陽性，軽度の低蛋白血症を認めた。糞便の培養検査は陰性，血清アメーバ抗体も陰性，尿素呼気試験陰性であった。

大腸内視鏡検査所見(**Color 1**)：Rb第一ヒューストン弁上に約半周にわたって横走する平盤状隆起を認め，頂部には黄白色の粘液の付着を伴っていた。Ra, Rsには小型の平盤状隆起，S状結腸には頂部の陥凹が強い発赤を呈するタコいぼ状隆起が多発し，隆起の周囲には小白斑を認めた。口側のS状結腸では隆起は目立たず，強い発赤斑の多発を認めた。なお，下行結腸より口側の結腸には異常所見を認めなかった。

大腸生検病理組織像(**Fig. 1**)：平坦発赤部の生検では炎症性細胞浸潤，腺窩上皮の過形成性変化を認めた。平盤状隆起の頂部からの生検では，いわゆるcap部の化膿性滲出物，炎症性肉芽組織を認め，周囲の隆起部では軽度の炎症細胞浸潤と腺管の延長を認めた。強拡大，およびActin染色(**Color 2**)で，粘膜深層を中心に軽度の線維筋症が確認できた。以上より，特異な内視鏡像とあわせcap polyposis(CP)と診断した。

入院後経過：入院時，排便時のいきみ習慣がみられたため，腸管安静(維持輸液1,000mlと腸炎食)と乳酸菌製剤に加え，排便習慣の改善の指導をしたところ，徐々に症状は改善し，第19病日には腹痛・粘血便ともに消失した。また，低アルブミン血症も改善した。その後，症状の再燃は無く，約1年後に施行した注腸造影，3年後の大腸内視鏡像においても異常所見を認めなかった。

考　察

CPは膿性粘液におおわれた隆起の頂部が帽子様の形態を示す，特異な炎症性疾患として，1985年Williamsら，続いて1993年Campbellらによって報告された比較的新しい疾患概念である。CP提唱前のeroded polypoid hyperplasia(6例)，分類不能腸炎(3例)，直腸腺腫(1例)，多発性炎症性過形成結節(1例)として報告されていた症例をあわせ，Medline，医中誌による検索では自験例を含め68例が該当した。従来より，CPは，病理学的に線維筋症を伴うことや，排便習慣の異常を高率に伴うことから，直腸粘膜脱症候群(以下，MPS)との異同が問題になっており**Table 1**に比較検討の表を示す。年齢・性比に明らかな差は認めなかった。MPSでは排便時のいきみ習慣がほぼ必発であるが，CPでも60%に認めた。MPSでは血液検査所見に異常を認めることはほとんどないがCPでは低蛋白血症を82%と高率に認めた。MPSの好発部位は直腸前壁，歯状線より2cm以内が多く後壁側の発生は稀であるのに対しCPはほとんどがS状結腸や深部結腸

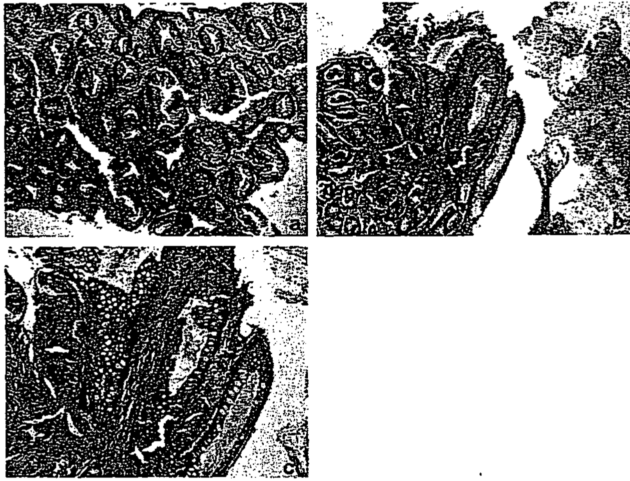

Fig. 1 Microscopic picture of cap polyposis obtained by endoscopic biopsy. a) Early phase of cap polyposis, a small flat lesion with dense superficial and localized inflammation, accompanied by crypt dilatation. b) The surface of sessile polyp which is covered by granulation tissue and fibrinopurulent exudates (arrow head), the so-called "cap". c) Mild fibromuscular obliteration is recognized in the deep lamina propria (hematoxylin & eosin).

にも分布し，腸管の前後左右壁に好発部位はない。CPの個々の病変は大きさ0.5～2 cmで，平盤状，たこいぼ状，芋虫状隆起で，表面は発赤調，易出血性で粘液や白苔付着，びらん形成を伴う。病変の境界は比較的明瞭で介在粘膜は正常ないしは浮腫状で白斑を伴う。病理組織上，MPSでは粘膜表層部の毛細血管の増生と拡張，粘膜固有層にみられる線維筋症および幼若上皮からなる腺管の過形成が特徴的である。一方CPではcapの部分は膿性の線維素性浸潤物と炎症性肉芽組織からなり，隆起部分は腺管の延長，過形成性変化がみられる。線維筋症も高頻度に見られるのが，MPSと異なり隆起深層を中心に軽度のものがほとんどである。

MPSの治療が排便習慣の改善にあるのに対し，CPに対する治療法は多岐にわたるものが報告されている。メトロニダゾールが22例に使用され，約3分の1で有効と報告されていた。ステロイド注腸が有効であった症例は散見されるが，ステロイド内服やサラゾピリンは無効であった。最近では*H. pylori*除菌が行われ6例中5例に有効であり，感染の関与を示唆するもので注目される。本例のように，排便習慣の改善によって軽快したとする報告も11例中6例あった。さらに低蛋白血症の進行や症状により手術を余儀なくされた症例が16例報告されているが，手術が有効とされた症例でも約半数に術後再燃がみられた。その他ポリカルボフィル，EMR，エカベトナトリウム+PSL内服，Infliximab

Table 1 Clinical features-MPS and cap polyposis.

	MPS	cap polyposis (n=68)
年齢・性比	9歳～83歳.やや男性に多い	10歳～77歳で平均50.7歳. 男女比1:2.79
臨床症状	肛門痛. 排便時出血. 排便障害 いきみ習慣あり	粘液下痢 (75%). 粘血便(45.6%). 腹痛(36.8%) いきみ習慣あり(60.5%)
血液検査	特になし	低蛋白血症 (82.2%)
病変部位	下部直腸前壁. 歯状線より 2cm以内が多い	直腸－S状結腸 (58.8%). 直腸 (13.2%). 直腸－下行結腸(5.9%). 直腸－上行結腸(5.9%)
内視鏡像	平坦型. 隆起型. 潰瘍型	平盤状隆起(83.6%). 粗大結節状隆起(38.8%). 表面は発赤調. 易出血性で粘液や白苔付着. びらん形成を伴う.
病理組織	粘膜表層部の毛細血管の増生 拡張. 線維筋症. 腺管過形成.	隆起頂部の肉芽組織. 線維筋症の程度は軽い.
治療法	排便習慣の改善.	メトロニダゾール. PSL注腸. *H.Pylori*除菌. 手術. 排便習慣の改善.
病　因	直腸粘膜脱説	感染説. 免疫説. 腸管運動機能異常説. 直腸粘膜脱説.

1970年から2005年にかけての医中誌、Medlineによる検索で自験例を含めたcap polyposisと考えられた68例を対象にした

などの報告もある。

我々は排便習慣の是正により症状が軽快し，内視鏡的にも正常化したCPの1例を経験した。CPとMPSとの異同は議論のあるところであり，本例においてもその病態に粘膜脱という要因が存在すると考えられた。しかし，文献的検討では，CPはMPSにはみられない，感染・免疫異常・腸管運動機能異常などの様々な要因が発症に関与しており，独立したclinical entityとすべきと考えられた。

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〈カラーは7頁に掲載〉

A case report of cap polyposis with spontaneous remission.

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【炎症性腸疾患の病態と粘膜免疫ー最近の動向ー】

Current Topics in Inflammatory Bowel Disease and Mucosal Immunology

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Key words
inflammatory bowel disease, GALT, innate immunity, cytokine

要 約

腸管は消化吸収・排泄のみでなく常に外来抗原や腸内細菌にさらされながらも複雑な免疫システムを有することによって恒常性を維持している特殊な臓器である。炎症性腸疾患は大きく潰瘍性大腸炎 (UC)とCrohn病(CD)に分類される。現在我が国でも患者数は増加傾向にあり特定疾患登録患者数でみてもUCで7万人を超え、CDも2万人を超えた。いずれも腸管の慢性持続性炎症が特徴で原因究明には至っていない。しかしながら，腸管局所の免疫制御異常が病態に関与しているのは間違いなく，最近では腸内細菌との関係など自然免疫機構が発症メカニズムに関与しているのではないかと注目されている。またいくつかの疾患関連遺伝子も報告され，治療においても抗サイトカイン抗体などの分子標的治療が開発され実用化されつつある。

1. 特殊な免疫装置としての腸管 (Gut Associated Lymphoid Tissue, GALT)

消化管は消化，吸収，排泄を司るだけでなく，複雑なGut Associated Lymphoid Tissue, GALTと呼ばれる免疫担当装置を形成している（図1）。さらに豊富な血管網や神経組織が迷路のように存在し，消化管ホルモンや神経ペプチドなどが生理機能を調節している。全消化管粘膜の表面積はテニスコート1.5面にも及び，そこに10^{14}個以上の腸内細菌が常在している。さらに，病原体や食餌抗原などの外来抗原に曝露される。つまり消化管は体内にありながら常に外界と接している特殊な臓器ということが言える。通常，免疫装置は外来からの侵入者に対して防御的に働き生体を守っている。例えば肺には肺胞マクロファージが存在し侵入してきた病原体に対して速やかに反応，処理し感染を防いでいる。ところが常に食餌抗原や腸内細菌にさらされている腸管粘膜では過剰な免疫応答は好ましくない。むしろ恒常性を保つための抑制的機構が存在すると考えられる。腸管上皮は構造的に微生物や抗原の侵入を防いでおり，さらにムチン，trefoil factorや抗菌ペプチドなどの分泌蛋白を産生し粘膜表面を守っている。しかし，これらの上皮細胞による防御に留まらず，抑制性の免疫学的機序が存在していると思われる。実際，大腸粘膜をポリペクトミーで切除し粘膜を破壊しても我々は腸炎を発症することはない。また一過性に食あたりや感染性腸炎にかかることはあってもほとんどの場合は慢性化せず自然に沈静化する。この腸管の低反応性を説明する機序として腸管の自然免疫を司るマクロファージや樹状細胞の特殊性が明らかとなってきた。我々は，マウスの腸管マクロファージは細菌刺激に対してTNF-αやIL-6などの急性反応性のサイトカインは産生するものの，決してTh1型免疫応答を引き起こすIL-12やIL-23を産生せずむしろ抑制性サイトカインであるIL-10を高産生することを明らかにした。さらに自然発症腸炎モデルマウスであるIL-10 KOマウスではこの腸管マクロファージの抑制性の機能が喪失しており細菌刺激によりTh1反応が誘導されることがわかった[1]。

またSmythiesらはヒトの腸管マクロファージは細菌に対し貪食能を保ったままサイトカイン産生に関しては低応答となっていることを報告している[2]。このように消化管は非常に複雑で精密な仕組みでホ

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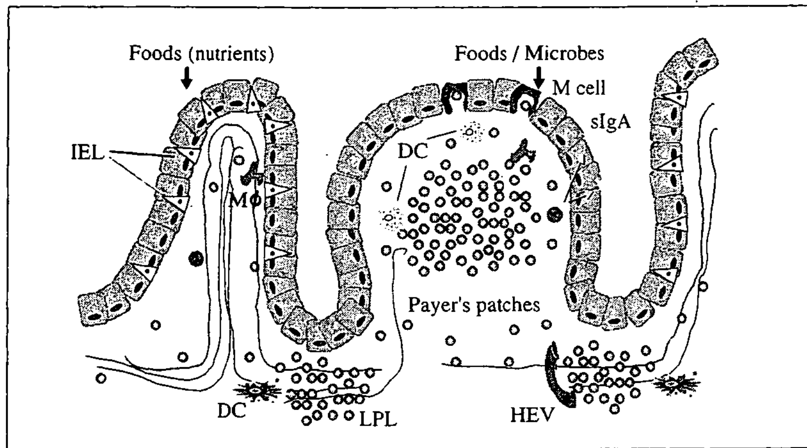

図1　GALT (gut associated lymphoid tissue)

メオスターシスを保っており，その破綻が炎症性腸疾患という特殊な慢性持続炎症を引き起こすものと考えられる。

2．炎症性腸疾患(Inflammatory Bowel Disease, IBD)の病態

IBDは大きく潰瘍性大腸炎 (UC)とCrohn病 (CD)の二大疾患に区別される。これら二つの疾患は基本的に独立した疾患概念と考えられている。UCでは標的臓器は大腸のみであるのに対し，CDでは小腸，大腸を含めた全消化管が標的となり，しばしば瘻孔を形成する。内視鏡所見や病理像も大きく異なり，UCでは直腸からびまん性，連続性の病変分布を呈し炎症の主座は大腸粘膜表層にある。一方，CDの病変はskip lesionと呼ばれ非連続性で縦走潰瘍と呼ばれる特徴的な所見を呈する。病理所見では炎症は全層性で非乾酪性肉芽腫が特徴的な所見とされる。CDでは腸管局所の免疫応答はTh1型にシフトしていることが分かっており，エフェクター細胞は腸管局所のCD4陽性T細胞である。一方，UCでの局所の免疫応答の状態は報告により異なっておりTh2型にシフトしていると報告しているグループもあるがコンセンサスは得られていない。

IBDが純然たる自己免疫疾患であるというエビデンスはなく，むしろ遺伝素因，環境因子，免疫応答の異常が複雑に関与した多因子疾患であると考えられている（図2）。しかしながら，IBD患者ではしばしば腸管外に虹彩炎，皮膚症状，関節炎などの多彩な全身症状を合併することが知られており，また逆にSLEやシェーグレン症候群などの膠原病患者にIBD様の慢性腸炎が認められることもある。さらにUCでは大腸上皮やムチンに対する抗体産生が亢進していることやpANCA陽性例が多いことが知られている。一方CDではASCAs (anti-Saccharomyces cerevisiae antibodies)，Omp C, I2, flagellinに対する抗体CBir1など酵母や腸内細菌に対する抗体価の上昇が認められる[3,4]。これらのことから食餌や腸内細菌などの何らかの外来抗原に対する異常な免疫応答が背景にあり，時として腸管局所のみならず全身の免疫系が活性化し自己抗原とも交差反応することで自己免疫疾患に類似した症状を呈するのではないかとも考えられる。

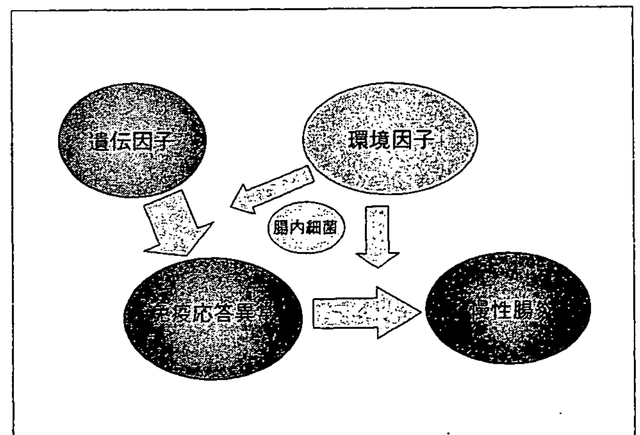

図2　IBDの病因

3．遺伝的素因

先に述べたように，以前より遺伝的素因が発症に重要な要素であることは疫学的研究から強く疑われていた。IBDの発症率は人種間で大きく異なっており，例えば居住地域が異なっていてもユダヤ系白人での発症が非ユダヤ系白人よりも高いことが報告されている。また家族内発症例が以前より報告されており，一卵性双生児間の発症率は二卵性双生児間の発症率より高いことが判明している。特に遺伝因子の関与はUCと比較しCDでより高いと考えられている。Linkage解析によりいくつかのIBDのsusceptibility locusが同定されていたが，ヒトゲノム解析が進むのと平行していくつかの疾患関連遺伝子が同定された。

1）NOD2/CARD15

NOD2/CARD15は第16染色体の*IBD1 locus*に位置するCD疾患関連遺伝子として2001年に二つのグループから報告された[5,6]。NOD2/CARD15は細菌の菌体構成成分であるペプチドグリカンのムラミルジペプチド (MDP) 部分を認識する細胞質内受容体であると考えられている。*In vitro*の研究ではNOD2/CARD15のC末端側に存在するleucine rich repeat (LRR)がMDPの結合部位でN末端側のCARDドメインを介して細胞内シグナルを活性化し最終的にNF-κBを活性化することがわかっている。白人のCD患者ではこのLRRおよびその近傍の3箇所の遺伝子多型が発症に相関を示している。これらの変異型ではMDPによるNF-κBの活性化能が低下していることが*in vitro*で示されている。同遺伝子のCDとの相関は人種によって大きく異なっており日本人を含めたアジア人種では相関が確認されていない[7]。また白人CD患者においても変異の保有率は約20～30%であり同遺伝子だけですべてのCDを説明することはできない。

さらにNOD2/CARD15がいかに病態に関与しているかはまだ解明されてはいないが，NOD2/CARD15は単球系細胞であるマクロファージや樹状細胞，腸管上皮細胞，特にPaneth細胞に発現しており，おそらくは侵入してきた細菌の認識と生体防御に働いていると考えられている。一方でMDP-NOD2のシグナルがペプチドグリカン-TLR2のシグナルに対し抑制的に働いている可能性も報告されている[8]。

2）OCTN (SLC22A4), OCTN2 (SLC22A5)

OCTN (organic cation/carnitine transporter)は第5染色体の*IBD 5 locus*に位置する疾患関連遺伝子として2004年に報告された[9]。しかしながら，日本人でのOCTNとCDとの相関は確認されていない。

3）DLG5

第10染色体に位置するDLG5のsingle nucleotide polymorphism (SNP)がIBDおよびCDと相関を示すことが報告された[10]。DLG5は腸管上皮細胞のintegrityの維持に働くscaffolding proteinをcodeしており，SNP多型によりこの機能が変化するのではないかと考えられているが病態への関与は解明されていない。またDLG5も日本人のCDやIBDでの相関は明らかとなっていない。

4）TNFSF15

これまで述べてきたようにNOD2/CARD15, OCTN, DLG5など疾患関連遺伝子として同定された報告はいずれも欧米を中心としたデータで，これまで日本人におけるCDでは相関が確認されていなかった。一方，Yamazakiらは第9染色体上に位置する炎症性サイトカインであるTNFSF 15 (Tumor necrosis factor family member 15)のSNPが日本人CD感受性と相関していることを報告した[11]。今後の機能解析の結果が待たれる。

4．免疫関与分子をターゲットとした新たな治療

近年の研究により詳細な病態が解明され，それに伴い特定の分子を標的とした治療の開発が進んでいる。

1）TNF-αをターゲットとした治療薬

キメラ型TNF-α抗体Infliximab（商品名：レミケード）は1998年に米国で承認され，我が国でも2002年から使用可能となっている。現在我が国では活動性CDに対する単回投与もしくは外瘻を有する症例に対する3回投与が認められている。すでに欧米ではACCENT I試験の結果からレミケード継続投与による緩解維持治療の有効性が報告されており[12]，さらに早期からレミケードによる治療介入を行うべきであるというTop-down therapyを推奨する意見もあり今後CD治療の中心にレミケードが位置

してくるのは間違いない。またレミケードの有効性が報告されて以来，様々なTNF-α阻害剤が開発されており，その中でcertolizumab pegol (CDP870)はPEG化された抗TNF-α抗体のFab fragmentであり，Schreiberらはcertolizumab pegol (CDP870)のCDに対するrandomized, placebo-controlled studyの結果を報告している[13]。

2）抗IL-12抗体

完全ヒト型抗IL-12p40抗体の活動性CDに対する有効性が2004年に報告された[14]。IL-12はTh1型免疫応答を誘導するkeyサイトカインであり，CDの病態形成に重要な役割を果たしていると考えられている。さらに，活性型IL-12はp35とp40のヘテロダイマーからなっており，新たに発見されたTh1誘導サイトカインであるIL-23はp40とp19のヘテロダイマーの形をとる。したがってp40に対する抗体はこの両者を抑えている可能性がある。

3）抗IL-6抗体

ヒト型抗IL-6抗体 (MRA)の活動性CDに対するプラセボ対照多施設二重盲検試験が我が国で行われ有効性が報告された[15]。特にレミケードに見られる抗核抗体や中和抗体の出現が認められず今後が期待されている。

4）接着因子を標的とした治療薬

リンパ球の腸管へのホーミングには接着因子であるα4β7インテグリンが必要であるが，α4インテグリンに対するヒト化モノクローナル抗体であるNatalizumabのCDに対する大規模試験の結果が報告された[16]。Natalizumabは緩解導入についてはそれほど高い効果は期待できないものの有効例では維持療法としての効果は期待できる可能性がある。しかしながら投与については重篤な副作用である進行性多発性白質脳症 (progressive multifocal leukoencephalopathy, PML) の発症のリスクを考慮しなければならず汎用化には課題が残されている。

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<細　胞ニュース>

第40回　日本成人病（生活習慣病）学会

日本成人病（生活習慣病）学会では下記の日程にて第40回学術集会を開催いたします。

会　期：2006年1月14日（土）～15日（日）
会　場：日本都市センター会館（東京 千代田区）
会　長：堀　正二（大阪大学大学院医学系研究科循環器内科学）
テーマ：個人に還元される医療のあり方を探る
ーテーラーメイド医療をめざしてー

ープログラムー
プレナリーセッション　テーラーメイド医療の実現に向けて：心疾患とテーラーメイド医療，肥満治療成功の秘訣ーテーラーメイド型食事指導の実践，他／教育講演：生活習慣と腎移植成績，医療情報と個人情報保護／ランチョンセミナー：糖尿病発症早期の治療戦略，他／シンポジウム：メタボリックシンドロームの徹底解明ー職域から防ぐメタボリックシンドロームー，メタボリックシンドロームと高血圧ー職場高血圧を含めて，糖代謝異常とメタボリックシンドローム，他／特別講演：大腸癌治療ガイドラインの解説

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Nonpathogenic *Escherichia coli* Strain Nissle1917 Prevents Murine Acute and Chronic Colitis

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Background: Nonpathogenic *Escherichia coli* strain Nissle1917 has been used as a probiotics in human inflammatory bowel disease; however, there are few reports examining its therapeutic effect on animal colitis models, and its therapeutic mechanisms remain unknown. The aim of this study was to elucidate the therapeutic effect and mechanism of Nissle1917 using murine acute and chronic colitis models.

Methods: Two models were used. (1) Acute model: colitis was induced by administration of 1.3% dextran sodium sulfate for 7 days. Nissle1917 or phosphate-buffered saline were orally administered for 10 days. Mice were killed at day 10, and the colonic lesions were assessed macro- and microscopically. (2) Chronic model: IL-$10^{-/-}$ mice were treated with Nissle1917 or phosphate-buffered saline for 8 weeks. After 8 weeks of treatment, mice were killed to assess the colonic lesions macro- and microscopically. In the acute dextran sodium sulfate colitis model, viable, heat-killed, or genomic DNA of Nissle1917 was orally administered for 10 days, and the therapeutic effect was assessed.

Results: In the acute model, Nissle1917 ameliorated body weight loss, disease activity index, and macro- and microscopic damage. In the chronic model, it also suppressed the mucosal inflammatory findings and histologic damages. Moreover, heat-killed Nissle1917 or its genomic DNA alone also ameliorated the acute DSS colitis and viable bacteria macro- and microscopically.

Conclusions: Nonpathogenic *E. coli* strain Nissle1917 prevents both acute and chronic colitis, and its anti-inflammatory effect is exhibited not only by viable bacteria but also by heat-killed bacteria or its DNA.

Key Words: dextran sodium sulfate colitis, IL-10–deficient mouse, inflammatory bowel disease, Nissle1917, probiotics

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Although the etiology of inflammatory bowel disease (IBD) remains unclear, a role for gut flora in the initiation and the perpetuation of IBD has been proposed. There is a possibility that an agent that is normally nonpathogenic, including a component of the normal gut flora, may be pathogenic in the susceptible host.[1] This hypothesis is supported by the observation that IBD is often improved by administration of antibiotics.[2] Studies of experimental colitis in various animal models have also shown the importance of the resident luminal flora in the initiation and the perpetuation of intestinal inflammation. The spontaneous colitis model, such as *T-cell receptor α-chain* gene–deficient mouse,[3,4] *interleukin-10* (*IL-10*) gene–deficient mouse,[5] *IL-2* gene–deficient mouse,[6] and *HLA-B27* gene transgenic rat,[7] requires the luminal bacteria for development of colitis. These genetically engineered rodents do not develop intestinal inflammation under germ-free conditions.

Probiotics are defined as living nonpathogenic organisms that confer health benefits by improving the microbial balance. Recently, it has been reported that probiotics are effective for treatment of IBD.[8–13] Nonpathogenic *Escherichia coli* strain Nissle1917 is one of the probiotic bacteria. This strain has no pathogenic potential besides the ability to colonize within the intestine. The results of a recent clinical trial showed that treatment with Nissle1917 is equivalent to mesalazine in maintaining remission of ulcerative colitis.[10,11] However, in animal experimental colitis models, there are few reports about the therapeutic effect of Nissle1917, and its mechanism of action remains unknown.

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This study aimed to estimate the therapeutic effect of Nissle1917 on murine acute and chronic colitis models and to elucidate its mechanisms of action.

MATERIALS AND METHODS

Animals

Specific pathogen-free female C57BL/6 mice, 6 to 7 weeks of age, were obtained from Clea Japan (Tokyo, Japan). Mice were housed less than 3 per cage at the animal center of Kitasato Institute Hospital (Tokyo, Japan) for 1 week before initiating the study. *IL-10* gene–deficient mice, generated on a C57BL/6 background, were obtained from Dr. Hiroshi Kiyono (Osaka University, Osaka, Japan) and were housed under conventional conditions at Keio University (Tokyo, Japan).

Preparation of Bacteria

The *E. coli* strain Nissle1917 (Mutaflor; DMS6601, serotype O6:K5:H1) was kindly supplied by Ardeypharm (Herdecke, Germany). It was expanded from freeze stock to a Luria-Bertani (LB) agar plate and grown for 24 hours at 37 °C. Colonies on LB agar plates were collected and suspended in sterile phosphate-buffered saline (PBS, pH 7.4). Concentration was determined by photometric comparison with a previously established growth curve. Bacterial suspension was prepared in 5×10^8 colony forming units (CFUs) per milliliter. Heat-killed bacteria were prepared by resuspending viable bacteria in PBS followed by incubation for 30 minutes at 60 °C. Killed bacteria were washed and resuspended in PBS. The complete killing was confirmed with 24-hour incubation at 37 °C on an LB agar plate. Genomic DNA was isolated from Nissle1917 using the Genomic DNA isolation kit (Qiagen Sciences, Valencia, Calif.).

Genomic Polymerase Chain Reaction of Nissle1917

Female C57BL/6 mice (8 wk of age) were orally administered *E. coli* Nissle1917. One to 10 days after administration, stools were collected and cultured in LB medium overnight at 37 °C. Bacterial genomic DNA was extracted from the cultured medium using QIA amp DNA Stool Mini Kit (Qiagen Sciences). Genomic polymerase chain reaction (PCR) for Nissle1917 was performed as previously described.[14] Briefly, *E. coli fimB* gene primers were forward, 5′-GGCGTCGACTAACCCAGCACAG-CTA-3′, and reverse, 5′-GCGCGGATCCGTAAGAATAATGT-AGT-3′. For genomic PCR, equivalent amounts of genomic DNA (1 μL), 10× PCR buffer, 1.25 mM dNTPs, 0.5 U recombinant Taq polymerase (Takara, Tokyo, Japan), and 0.5 μM of the forward and the reverse primers were used. Cycling conditions for PCR amplification were 94 °C for 5 minutes and 40 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, followed by a final extension at 72 °C for 7 minutes.

Nissle1917 Treatment in a Murine Acute Colitis Model

Each group of mice was fed 1.3% dextran sulfate sodium (DSS; molecular weight 50,000; BioResearch Corp., Yokohama, Japan) for 7 days. All groups of mice were orally administered with Nissle1917 (1×10^8 CFU/mouse/d) or PBS for 10 days. In other experiments, heat-killed Nissle1917 (1×10^8 CFU/mouse/d) and Nissle1917 genomic DNA (10 μg/mouse/d) were administered in the same manner. In all mice, body weight and disease activity index (DAI) were assessed on a daily basis. DAI was determined by a scoring system as previously described.[15] Briefly, the index is as follows: loss of body weight—0 = none, 1 = 1% to 5%, 2 = 5% to 10%, 3 = 10% to 20%, 4 = >20%; stool consistency—0 = normal stool, 2 = loose stool, 4 = diarrhea; hemoccult—0 = normal, 2 = hemoccult positive, 4 = gross blood. DAI is the sum of the 3 scores divided by 3. Mice were killed at day 10, and the resected colonic tissue was examined macroscopically and microscopically. For cytokine analysis, mice were killed at day 7 and purified lamina propria mononuclear cells (LPMCs) were examined.

Nissle1917 Treatment in a Murine Chronic Colitis Model

Eight-week-old IL-$10^{-/-}$ mice were orally administered Nissle1917 (1×10^8 CFU/mouse/d) or PBS for 8 weeks. The disease activity of colitis was determined in the same way as the experiments for the acute colitis model.

Histologic Score

Mice were killed at each period. Colons were harvested and fixed in 10% phosphate-buffered formalin. These samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Tissues were reviewed in a blinded fashion and assessed with a previously validated gastrointestinal histologic inflammatory score. (1) For DSS colitis mice,[16] histologic scores ranged from 0 to 12. Total score was assessed as the sum of the following 4 parameters: extent, 0 to 3 [0, none; 1, focal; 2, limited to 1 segment (proximal, middle, distal); 3, involving more than 1 segment]; inflammation, 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe); damage/necrosis, 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe); and regeneration, 0 to 3 (0, complete re-epithelialization; 1, broad or multifocal re-epithelialization; 2, focal migration and mitotic features; 3, none). (2) For IL-$10^{-/-}$ mice,[17] histologic scores ranged from 0 to 10. Total score was assessed as the sum of the following 4 parameters: mucosal ulceration, 0 to 3 (0, normal; 1, surface epithelial inflammation; 2, erosions; 3, ulcerations); epithelial hyperplasia, 0 to 3 (0, normal; 1, mild; 2, moderate; 3, pseudopolyps); lamina propria mononuclear infiltrate, 0 to 2 (0, normal; 1, slightly increased; 2, markedly increased); and lamina propria neutrophil infiltrate, 0 to 2 (0, normal; 1, slightly increased; 2, markedly increased).

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Enzyme-linked Immunosorbent Assays for Serum Amyloid A

Blood samples were collected, and the serum fraction was separated. Serum was analyzed for serum amyloid A (SAA) protein by enzyme-linked immunosorbent assay kit (BioSource International, Calif.). Basal SAA protein levels were obtained from age-matched wild-type control mice. The cut-off value was calculated as mean + 2 SD of wild-type control SAA concentrations.

Isolation of LPMCs

Mice were killed, and colonic tissues were removed. Removed colons were washed with calcium- and magnesium-free Hanks balanced salt solution (HBSS; Sigma, St. Louis, Mo.) and dissected into small pieces. The tissues were incubated in HBSS containing 2.5% fetal bovine serum (BioSource) and 1 mM dithiothreitol (Sigma) to remove mucus. They were incubated twice in HBSS containing 1 mM EDTA (Sigma) for 20 minutes at 37 °C. Then they were washed with HBSS for 3 times and incubated in HBSS with 1 mM collagenase type IV (Sigma) for 2 hours at 37 °C. The digested tissues were filtered and washed twice with HBSS. Cells were resuspended in 40% Percoll (Pharmacia Biotech, Piscataway, NJ) and layered on the 75% Percoll before centrifugation at 2000 rpm for 20 minutes. Cells recovered from the interphase were washed twice and suspended in HBSS. Cell viability was >95%, as determined by trypan blue exclusion dye.

Analysis of Proinflammatory Cytokines Expression by Quantitative Reverse Transcription-PCR

Total RNA was isolated from LPMCs using RNeasy Mini kit (Qiagen Sciences). cDNA was synthesized from 1 μg total RNA with Omniscript reverse transcriptase (RT; Qiagen Sciences). For quantitative RT-PCR, equivalent amounts of cDNA (2 μL), 0.5 μM of the forward and reverse primers, and DyNAmo SYBR Green qPCR Kit (MJ Research, Waltham, Mass.) were used. The PCR reactions were carried out in a thermocycler DNA engine, OPTICON2 (MJ Research). Cycling conditions for PCR amplification were 95 °C for 10 minutes, and 40 cycles of 95 °C for 10 seconds and 58 °C for 50 seconds. Murine-specific primers for tumor necrosis factor (TNF)-α were forward, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, and reverse, 5′-TGGGAGTAGACAAGGTACAACCC-3′. Interferon (IFN)-γ primers were forward, 5′-TCAAGTGGCATAGATGTGGAAGAA-3′, and reverse, 5′-TCACCATCCTTTTGCCAGTTCCTCCAG-3′. IL-1β primers were forward, 5′-CAACCAACAAGTGATATTCTCCATG-3′, and reverse, 5′-GATCCACACTCTCCAGCTGCA-3′. IL-6 primers were forward, 5′-GAGGATACCACTCCCAACAGACC-3′, and reverse, 5′-AAGTGCATCATCGTTGTTCATACA-3′. Transforming growth factor (TGF)-β1 primers were forward, 5′-TGACGTCACTGGAGTTGTACGG-3′, and reverse, 5′-GGTTCATGTCATGGATGGTGC-3′. Monocyte chemoattractant protein (MCP)-1 primers were forward, 5′-CTTCTGGGCCTGCTGTTCA-3′, and reverse, 5′-CAAGCCTACTCATTGGGATCA-3′. Macrophage inflammatory protein (MIP)-2 primers were forward, 5′-ATCCAGAGCTTGAGTGTGACGC-3′, and reverse, 5′-AAGGCAAACTTTTTGACCGCC-3′. IL-10 primers were forward, 5′-GGTTGCCAAGCCTTATCGGA-3′, and reverse, 5′-ACCTGCTCCACTGCCTTGCT-3′. β-actin primers were forward, 5′-AGAGGGAAATCGTGCGTGAC-3′, and reverse, 5′-CAATAGTGATGACCTGGCCGT-3′.

Statistical Analysis

All data are expressed as mean ± SEM. Differences between means were evaluated using Mann-Whitney *U* test for analysis.

RESULTS

Nissle1917 Ameliorates Murine DSS Colitis

To identify the mechanisms of action of Nissle1917 in murine acute colitis models, we first examined the effect of Nissle1917 in the DSS colitis model. To detect the *Nissle1917* gene from murine fecal samples, we generated primers to amplify a specific insert in the *fimB* gene in Nissle1917 (Fig. 1A). As shown in Figure 1B, specific gene amplification of Nissle1917 in feces of mice could be detected only on day 1 after the single administration, which suggested the absence of its colonization. Because, in this detection system, primers could amplify the other *E. coli fimB* gene in a competitive manner, the smaller product from the other *E. coli* may be amplified easier than Nissle1917. Therefore, these data may

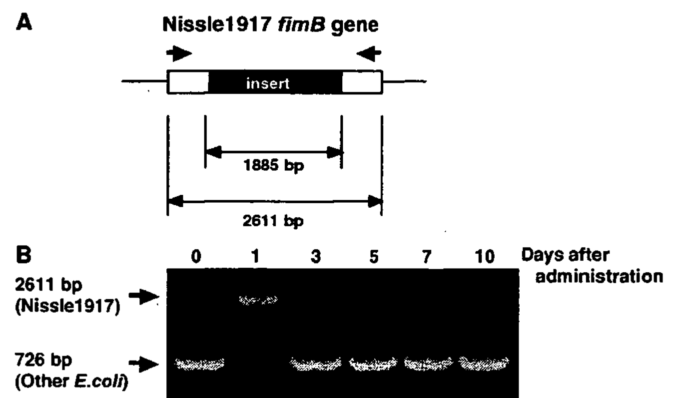

FIGURE 1. Detection of Nissle1917 from murine fecal samples. A, the set of primers was designed to amplify the sequence containing the specific insert element located in the *fimB* gene of Nissle1917. B, detection of Nissle1917 from murine fecal samples by PCR. Nissle1917 (1×10^8 CFU/mouse) was orally administrated into C57BL/6 mice. Nissle1917-specific amplified products were detected only 1 day after administration.

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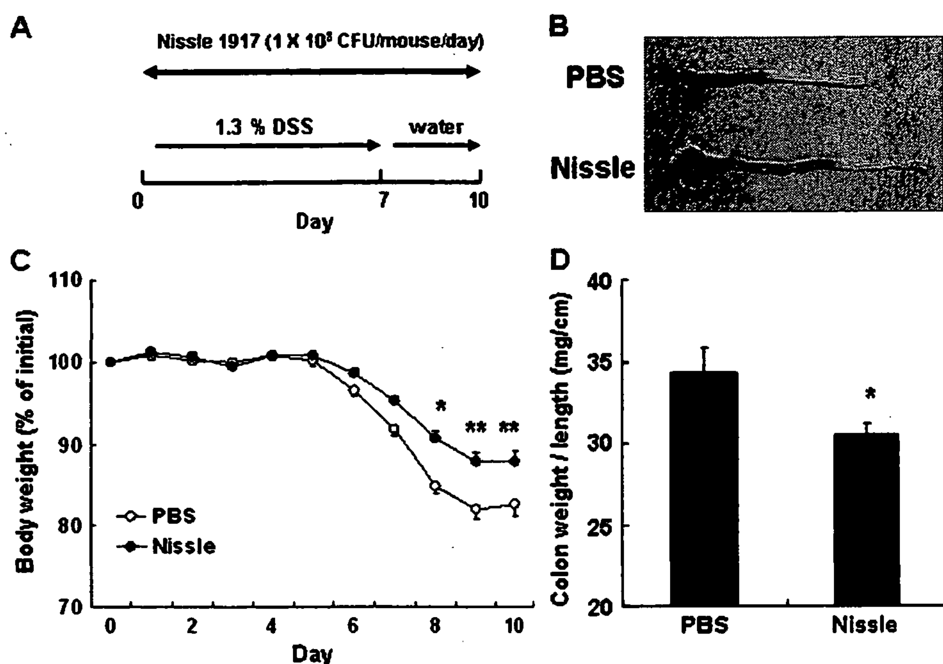

FIGURE 2. Effect of Nissle1917 on DSS-induced colitis. A, protocols for induction of DSS colitis. B, macroscopic findings of the colons from DSS colitis mice treated with PBS (top) or Nissle1917 (bottom). C, body weight losses of mice treated with PBS (○, n = 36) or Nissle1917 (●, n = 34). D, colon weights of DSS colitis mice treated with PBS (n = 10) or Nissle1917 (n = 10). Results are expressed as mean ± SEM *, $P < 0.05$; **, $P < 0.01$ compared with PBS groups.

not show the complete disappearance of orally administrated Nissle1917. In this study, we administered Nissle1917 for 10 consecutive days to make sure of its sufficient presence and examined the therapeutic effect (Fig. 2A). Macroscopic findings revealed that the shortening and thickening of the colon were improved in the Nissle1917 group (Fig. 2B). The body weight started to decline 6 days after the intake of DSS in both control and Nissle1917 groups. However, after day 8, the body weight loss was significantly reduced in the Nissle1917-treated group (Fig. 2C; control: 84.9 ± 1.0% versus Nissle: 90.6 ± 0.9%; $P < 0.05$ at day 8, control: 82.0 ± 1.1% versus Nissle: 87.8 ± 1.1%; $P < 0.01$ at day 9, and control: 82.6 ± 1.3% versus Nissle: 87.8 ± 1.2%; $P < 0.01$ at day 10). Consistent with the results of body weight, DAI scores and colon weight (control: 34.3 ± 1.5 versus 30.5 ± 0.8 mg/cm; $P < 0.05$ at day 10) were also lower in the Nissle1917 group (Fig. 2D; Table 1). Neither healthy controls nor the Nissle1917 group without DSS administration showed any body weight loss or macroscopic sign of colitis (data not shown). Colons from DSS-induced colitis mice treated with PBS contained severe ulceration and inflammatory cell infiltration over the proximal and distal region (Fig. 3C). In contrast, DSS colitis mice treated with Nissle1917 showed significantly less inflammation and ulceration (Fig. 3D). On the other hand, Nissle1917 itself did not affect normal colonic mucosa (Fig. 3B). Histologic scores were also reduced in the Nissle1917 group (Fig. 3E; control: 10.7 ± 0.3 versus Nissle: 8.2 ± 0.5; $P < 0.05$). Thus, we considered that Nissle1917 provided protective effect on colonic mucosa in murine acute colitis.

Nissle1917 Reduces Chronic Colonic Inflammation in IL-10$^{-/-}$ Mice

We next examined whether Nissle1917 was also effective against chronic colonic inflammation. We used IL-10$^{-/-}$ mice as a model of chronic colonic inflammation, as previously described.[18] IL-10$^{-/-}$ mice developed spontaneous enterocolitis associated with body weight loss, passage of mucus, rectal prolapse, and diarrhea. In this experimental period, there was no significant alteration in body weight in each group (data not shown). Colon weights of IL-10$^{-/-}$ mice were increased after the development of inflammation, but Nissle1917

TABLE 1. DAI Scores of DSS-induced Colitis Mice

	PBS (N = 36)	Nissle (N = 34)
Day 1	0.05 ± 0.02	0.04 ± 0.02
Day 2	0.15 ± 0.04	0.09 ± 0.03
Day 3	0.22 ± 0.04	0.15 ± 0.03
Day 4	0.42 ± 0.06	0.31 ± 0.07
Day 5	0.79 ± 0.10	0.49 ± 0.06
Day 6	1.47 ± 0.13	0.87 ± 0.11†
Day 7	2.32 ± 0.13	1.31 ± 0.14‡
Day 8	2.62 ± 0.10	1.73 ± 0.12‡
Day 9	2.47 ± 0.08	1.81 ± 0.15†
Day 10	2.23 ± 0.12	1.69 ± 0.13*

Data are mean ± SEM.
*$P < 0.05$, *$P < 0.01$, and ‡$P < 0.0001$, significant difference from PBS group.

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