New Cytokine Delivery System Using Gelatin Microspheres Containing Interleukin-10 for Experimental Inflammatory Bowel Disease

HIROSHI NAKASE, KAZUICHI OKAZAKI, YASUHIKO TABATA, MAKOTO OZEKI, NORIHIKO WATANABE, MASAYA OHANA, SUGURU UOSE, KAZUSHIGE UCHIDA, TOSHIKI NISHI, MINORU MASTUURA, HIROYUKI TAMAKI, TOSHIYUKI ITOH, CHIHARU KAWANAMI, and TSUTOMU CHIBA

Division of Gastroenterology and Endoscopic Medicine, Graduate School of Medicine (H.N., K.O., N.W., M.O., S.U., K.U., T.N., M.M., H.T., T.I., C.K. T.C.), and Institute for Frontier Medicine (Y.T., M.O.), Kyoto University, Kyoto, Japan

Received August 21, 2001; accepted December 12, 2001

This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Interleukin (IL)-10 is an anti-inflammatory cytokine that suppresses the T helper 1 immune response and down-regulates macrophages and monocytes. The therapeutic effect of systemic administration of IL-10 for patients with inflammatory bowel disease, however, has not been satisfactory. We examined whether rectal administration of gelatin microspheres (GM) containing IL-10 (GM-IL-10) prevents colitis in IL-10-deficient (IL-10−/−) mice. GM-IL-10 and IL-10 alone were administered rectally. The colon was examined macroscopically and microscopically. IL-12 mRNA expression and CD40 expression in Mac-1-positive cells were also examined. Macroscopic and microscopic examination revealed marked improvement of colitis in IL-10−/− mice treated with GM-IL-10. mRNA expression of IL-12 in Mac-1-positive cells in GM-IL-10-treated mice was significantly decreased compared with that in the mice treated with IL-10 alone. Additionally, CD40 expression in Mac-1-positive cells in GM-IL-10-treated mice was decreased more prominently than in mice treated with IL-10 alone. The therapeutic effects of GM-IL-10 were associated with decreased expression of IL-12 mRNA and down-regulation of CD40 expression in Mac-1-positive cells. GM-IL-10 might be useful for treatment of patients with inflammatory bowel disease.

Current treatments for patients with inflammatory bowel disease (IBD) are based on recent advances in elucidating the pathophysiology of the disease. Several studies indicate that T helper (Th)1 immune responses have important roles in the development of IBD (Fuss et al., 1996; Sartor, 1997; Simpson et al., 1997). Furthermore, dysregulation of cytokine networks is involved in Th1-dominant immune responses in IBD (Fuss et al., 1996; Sartor, 1997; Simpson et al., 1997). Several murine colitis models with abnormalities of various cytokines or their receptors, which influence Th1-type immune responses, develop colitis (Sandlack et al., 1993; Neurath et al., 1995; Strober et al., 1998).

IL-10, which is a cytokine produced by activated macrophages and Th2-type T cells, has a pivotal inhibitory effect on the Th1-type immune response as well as on the antigen-presenting function of monocytes and macrophages (Fiorentino et al., 1991a,b). In addition, IL-10 induces an antigen-specific anergic state in human CD4-positive T cells (Fiorentino et al., 1991b). Kuhn et al. (1993) reported development of colitis in the IL-10 gene knockout mouse. Thus, IL-10 has an important role in maintaining the normal immune state in the intestine.

In patients with IBD, IL-10 levels in the intestine are abnormal and its kinetics differs from that in the whole body. Serum levels of IL-10 are elevated in patients with active IBD, whereas intestinal tissue concentrations of IL-10 are low or within the normal range in patients with IBD (Kucharzik et al., 1995; Schreiber et al., 1995). Moreover, in situ hybridization and immunohistochemistry studies indicate that local production of IL-10 by mucosal mononuclear cells in IBD is insufficient to down-regulate proinflammatory cytokines such as IL-1β in the lamina propria compartment (Autschbach et al., 1998). Together, these data suggest that impairment of IL-10 function is involved in the pathogenesis of IBD, and therefore, IL-10 is one of the most promising candidates for the treatment of IBD. The clinical efficacy of systemically injected IL-10 for patients with mild to moder-
ately active Crohn’s disease, however, has not been satisfac-
tory (Fedorak et al., 2000; Sands, 2000; Schreiber et al.,
2000). Moreover, several adverse side effects such as head-
ache, high fever, and back pain are inevitable, although they
are reversible (Fedorak et al., 2000; Sands, 2000; Schreiber et
al., 2000). Administration of IL-10 using a more efficient
drug delivery system is required to enhance its effect on the
inflamed colon and to decrease side effects.

A new drug delivery system using polymer microspheres
was recently developed to obtain sustained release of various
drugs and successful targeting of specific organs (Tabata and
Ikada, 1990). We previously reported a new oral drug deliv-
ery system using poly(DL-lactic acid) microspheres containing
dexamethasone alone by specifically targeting the M cells in
dexamethasone. This drug delivery system has more promi-

nently active Crohn’s disease, however, has not been satisfac-
tory (Fedorak et al., 2000; Sands, 2000; Schreiber et al.,
2000). Moreover, several adverse side effects such as head-
ache, high fever, and back pain are inevitable, although they
are reversible (Fedorak et al., 2000; Sands, 2000; Schreiber et
al., 2000). Administration of IL-10 using a more efficient
drug delivery system is required to enhance its effect on the
inflamed colon and to decrease side effects.

A new drug delivery system using polymer microspheres
was recently developed to obtain sustained release of various
drugs and successful targeting of specific organs (Tabata and
Ikada, 1990). We previously reported a new oral drug deliv-
ery system using poly(DL-lactic acid) microspheres containing
dexamethasone alone by specifically targeting the M cells in
dexamethasone. This drug delivery system has more promi-

Materials and Methods

Animals. IL-10-deficient mice on a C57BL/6 background were purchased
from Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were used
as controls at 4 weeks of age. All mice were housed in specific pathogen-
free conditions in the animal facility of Kyoto University. The studies
were approved by the Animal Protection Committee.

Preparation of Gelatin Microspheres. Gelatin microspheres
were prepared as reported previously (Tabata et al., 1999). Briefly,
10 ml of an aqueous solution of acidic gelatin (10 wt% preheated to
40°C) was added dropwise into 375 ml of olive oil while stirring at
420 rpm at 40°C for 10 min to yield a water-in-oil emulsion. The
emulsion temperature was decreased to 15°C, followed by further
stirring for 30 min to allow for natural gelation of the gelatin aque-
solution. Acetone (100 ml) was added to the emulsion and stir-
ing was continued for 1 h. The resulting microspheres were washed
three times with acetone, recovered by centrifugation (5000 rpm,
4°C, 5 min), passed through sieves with different apertures for size
fractionation, and air-dried. The average size of the microspheres
was adjusted to be less than 12 μm. Gelatin aqueous solution (0.2 ml,
10 wt%) and olive oil (5 ml) were agitated with a vortex mixer for 1
min and then sonicated at 3.0 W cm² for variable time periods. The
prepared emulsion was cooled down, washed with acetone by cen-
trifugation, and air-dried. The noncross-linked and dried gelatin
microspheres (25 mg) were placed in 5 ml of 0.1 wt% Tween 80
aqueous solution containing glutaraldehyde (40 μg/ml) and stirred at
4°C for 15 h to facilitate cross-linking. Following collection by cen-
trifugation (5000 rpm, 4°C, 5 min), the microspheres were agitated in
5 ml of 10 mM aqueous glycine solution at 37°C for 1 h to block the
residual aldehyde groups on unreacted glutaraldehyde. The result-
ing microspheres were washed three times with double-distilled
water by centrifugation and freeze-dried.

RadioIodelabelling of IL-10. Recombinant mouse IL-10 (rmIL-10)
was purchased from Genzyme Corporation (Cambridge, MA). Recom-
binant rmIL-10 was labeled with 125I by radioiodination using chlo-
ramine-T essentially as described (Vaisman et al., 1990; Cohen et al.,
1995). Briefly, 2.0 μg of rmIL-10 were labeled with about 100 mCi
sodium (Amersham International UK, Ltd., Little Chalfont, Buck-
inghamshire, UK) and 10 μg of chloramine-T in 20 mM sodium
phosphate buffer (pH 7.4) in a final volume of 120 μl. After 1 min, the
iodination was quenched by the addition of 50 μl of sodium met-
abisulphite (2 mg/ml). 125I-Labeled rmIL-10 was separated from free
iodine using a heparin-Sepharose column. The specific activity of
125I-labeled rmIL-10 was 0.5 to 1.5 × 10⁸ cpm/μg.

Incorporation of Protein into GM. Incorporation of rmIL-10
into GM was performed by allowing the freeze-dried microspheres to
swell in the aqueous solution of the protein. Briefly, 10 μl of IL-10
solution (5 μg/ml) was dropped onto 2.5 mg of freeze-dried glutaral-
dehyde cross-linked microspheres, and allowed to sit at 37°C for 1 h.
125I-Labeled and unleaded GM were prepared in the same way.

Release Test and Blood Distribution of IL-10. The in vitro
release test of rmIL-10 from the microspheres was conducted at
37°C. GM containing 125I-labeled rmIL-10 were immersed under
shaking in collagenase solution (1 ml, 0.8 mg/ml) prepared from
phosphate-buffered saline (PBS). After centrifugation, the radioac-
tivity of 125I-labeled rmIL-10 in supernatants was determined using
a gamma counter (ARC-300; Aloka Co., Tokyo, Japan).

125I-Labeled rmIL-10 (0.05 μg) or GM containing 125I-
labeled rmIL-10 (2.5 mg of GM that contained 0.05 μg of IL-10) were suspended in
200 μl of PBS, allowing the solution to reach the entire colon. The
mice were anesthetized with diethyl ether. After anesthesia, a catheter
was carefully inserted into the colon with the tip 4 cm proximal to the
anus. The solution was administered and then mice were kept in a
vertical position for 120 s before being returned to their cages.

The in vivo retention ratio of rmIL-10 (percentage of 125I-
labeled rmIL-10 remaining in the colon) and the blood distribution of rmIL-10
(percentage of 125I-labeled rmIL-10 remaining in the blood) were deter-
mmed using a gamma counter at 15, 30, and 45 min, and 1, 12, 24, and
48 h by measuring radioactivity of the removed colon and blood.

Treatments. Female IL-10-deficient mice at 4 weeks of age were divided
into five groups (five mice each; groups A–E) and treated as follows:

- Group A, no treatment; group B, GM (2.5 mg/body) alone; group C,
rmIL-10 (0.05 μg) alone; group D, GM containing rmIL-10 (GM-IL-10; 2.5 μg/body that contained 0.05 μg of rmIL-10). C57BL/6 mice were

Fig. 1. In vitro release of rmIL-10 from gelatin microspheres. The 2.5 mg of
gelatin microspheres containing 125I-labeled rmIL-10 were immersed under
shaking in 1 ml of PBS with collagenase (0.8 mg/ml). Each point represents the
mean ± S.E. of the percentage of the released radioactivity (n = 3).
used as controls (group E). GM, rmIL-10, and GM-IL-10 were sus-
pended in 200 μl of PBS. Each mouse received rectal administrations three times per week as mentioned above and were sacrificed after 1 month. The spleen and colonic tissues were removed from each mouse and examined in further studies as described below.

Assessment of the Severity of Colitis. The colon was opened by longitudinal incision, washed in PBS, and subsequently excised for microscopic observation of damage and isolation of Mac-1-positive cells. Microscopic damage was assessed after fixation in 10% formalin, followed by hematoxylin and eosin staining. Histologic analysis was performed in a blind manner as reported previously (Fuss et al., 1999).

Isolation of Mac-1-Positive Cells from Lamina Propria and Spleen. Lamina propria and spleen mononuclear cells were isolated as described previously (Neurath et al., 1995), and the cell suspension was plated on a monocyte-seperating plate (Nihon-Koutai Laboratory, Gunma, Japan) for 60 min at 37°C. After incubation, nonadherent cells were washed three times with PBS and removed. Adherent cells containing Mac-1-positive cells were incubated with 0.2% EDTA in PBS for 30 min at 4°C. After washing twice with PBS, adherent cells were analyzed using flow cytometry, and more than 90% of the cells were determined to be Mac-1-positive using a CD11b antibody (Serotec, Oxford, UK).

mRNA Expression of IL-12 in Mac-1-Positive Cells in the Lamina Propria of the Colon. Total RNA from 106 Mac-1-positive cells in the lamina propria of the colon was isolated using the guanidium isothiocyanate method as described previously (Khan and Collins, 1994). The concentration of RNA was determined by absorbance at 260 nm relative to that at 280 nm. The RNA was stored at −70°C until use. RNA (1 μl) was transcribed to cDNA using a Superscript preamplification system (Invitrogen, Carlsbad, CA). The reverse transcription prod-
uct (1 μl) was added to 1 mM of each primer and a solution of 1 U of Taq DNA polymerase (Takara, Biochemicals, Ohtsu, Japan) in a final volume of 20 μl. Polymerase chain reaction (PCR) amplification was performed for 35 cycles (1 min at 94°C, 1 min at 52°C, and 20 s at 20°C). cDNA-free solution served as a negative control for each reaction. The sequences of primers for each cytokine are as follows: IL-12p35 forward 5'-GAGGACTTGAAGATGTACCAG-3'; IL-12p35 reverse 5'-TTC-TATCTGTGTGAGGAGGGC-3'; IL-12p40 forward 5'-GACCCTGCCATTGGAAGAATGGC-3'; IL-12p40 reverse 5'-CAACGTTGCATCCTAGGGATCG-3'; β-actin forward 5'-TCTGTTACACCTCGAGGATGATG-3'; and β-actin reverse 5'-GATCTTGATCTCCATGGGTCTAGG-3'.

For semiquantitative reverse transcription-PCR, serially diluted cDNA were amplified with increasing numbers of cycles. After gel electrophoresis, PCR products were visualized using FOTODYNE FOTO/Analyst Archiver Eclipse (FOTODYNE Inc., Hartland, WI). Bands densities were measured using the computer software program, 1D Advanced (Advanced American Biotechnology, Fullerton, CA). The IL-12 p35 and p40 signals were standardized against the β-actin signal for each sample, and results were expressed as IL-12 p35 and p40/β-actin ratios.

**Flow Cytometric Cell Sorting (FACS) Analysis.** Mac-1-positive cells of lamina propria and spleen from IL-10−/− mice were resuspended in FACS buffer (1× PBS, 0.2% bovine serum albumin fraction V; Sigma-Aldrich, St. Louis, MO) to a final concentration of 10^6 cells/ml. Cells (1×
10⁶) were preincubated with mouse serum for 20 min on ice and stained with fluorescein isothiocyanate-conjugated antibodies against CD40 (BD PharMingen, San Diego, CA). Cells were washed with FACS buffer and analyzed using a FACS flow cytometer (EPICS XL; Beckman Coulter, Inc., Fullerton, CA). Results were analyzed using SYSTEM II software (Beckman Coulter, Inc.).

Statistical Analysis. The generalized Wilcoxon t test and the Mann-Whitney test were used where appropriate for statistical analysis. The data were presented as means ± S.E. A two-tailed P value of less than 0.05 was considered to be statistically significant.

Results

In Vitro Release of rmIL-10 from GM-IL-10. Recombinant mIL-10 was released from GM-IL-10 in a time-depen-
dent manner: 38 ± 5% (2 h), 66 ± 4% (6 h), 83 ± 3% (12 h), and 90 ± 5% (48 h) (Fig. 1).

Retention Ratio of IL-10 in the Colon and Blood Distribution of IL-10. Retention ratios of rmIL-10 remaining in the colon were measured at 15, 30, and 45 min, and 1, 6, 12, 24, and 48 h after rectal administration of GM containing 125I-labeled IL-10 or 125I-labeled IL-10 alone (Fig. 2). The retention ratios of rmIL-10 in the colon of GM-IL-10-treated normal mice at 1, 6, and 12 h after administration were significantly higher than the respective values of rmIL-10-treated normal mice. Furthermore, the retention ratios of rmIL-10 in the colon of GM-IL-10-treated IL-10⁻/⁻ mice at 6, 12, and 24 h were significantly higher than the respective values of the GM-IL-10-treated normal mice.

The blood distribution of 125I-labeled rmIL-10 after rectal administration of 125I-labeled IL-10 alone was detected at 15, 30, and 45 min but not at 60 min. In contrast, the blood distribution of 125I-labeled rmIL-10 after rectal administration of GM containing 125I-labeled rmIL-10 was detected from 30 min to 24 h (Fig. 3). The blood distributions of IL-10 in GM-IL-10-treated IL-10⁻/⁻ mice throughout the experiment were significantly higher than the respective values of IL-10-treated normal mice.

Animal Profile. IL-10⁻/⁻ mice generally appeared healthy until 4 weeks of age, when they gradually developed diarrhea and spontaneous colitis. Histological studies in the colon of IL-10⁻/⁻ mice at birth, 2, and 3 weeks of age were normal. However, at 4 weeks of age, the IL-10⁻/⁻ mice displayed various degrees of colonic inflammation characterized by mucosal ulceration and mild to moderate epithelial hyperplasia. IL-10⁻/⁻ mice had a normal small intestine macroscopically and histologically from birth to 4 or 8 weeks of age (data not shown).

Macroscopic Evaluation. Macroscopic examination of the colon from groups A (nontreated), B (GM alone), and C (rmIL-10 alone) revealed marked thickening of the colonic wall. In contrast, macroscopic examination of group D (GM-IL-10) revealed no thickening of the colonic wall. There was no difference in the GM-IL-10 treatment effect with respect to the site within the colon (data not shown).

Histologic Evaluation. Histologic findings revealed epithelial hyperplasia, mucosal ulceration, and remarkable infiltration of mononuclear cells in the colons of groups A (nontreated), B (GM alone), and C (rmIL-10 alone). In contrast, histologic findings in group D (GM-IL-10) were almost normal, except for low levels of infiltrating monocytes (Fig. 4a). The histologic score for group D was significantly lower than those for groups A, B, and C. There were no significant differences in the histologic scores among groups A, B, and C (Fig. 4b). The jejunum and ileum were histologically normal in all groups.

mRNA Expression in Mac-1-Positive Cells from the Lamina Propria of the Colon. The reverse transcription-PCR results demonstrated that mRNA expressions of IL-12 p40 and IL-12 p35 were up-regulated in Mac-1-positive cells in the colon of groups A (nontreated), B (GM alone), and C (rmIL-10 alone). Transcript levels of these cytokines in group D (GM-IL-10), however, were significantly lower than those in groups A, B, and C (Fig. 5).

Expression of CD40 on Mac-1-Positive Cells from the Lamina Propria of the Colon and Spleen. Compared with untreated IL-10⁻/⁻ mice, CD40 expression on Mac-1-positive cells in the lamina propria and spleen were mark-
edly decreased in group D (GM-IL-10) but not in group C (rmIL-10 alone) (Fig. 6).

Discussion

The present study clearly demonstrated that rectal administration of GM-IL-10 inhibits colonic mucosal inflammation in IL-10^−/− mice more efficiently than treatment with IL-10 alone. Analysis of the retention ratio of 125I-labeled IL-10 in the colon indicated that IL-10 remained in the colon of GM-IL-10-treated mice longer than in IL-10-treated mice. Thus, IL-10 appears to be released from GM-IL-10 gradually and continuously in the colonic mucosa, resulting in prolonged availability of IL-10 to the colon. Moreover, the retention ratio of IL-10 after administration of GM-IL-10 in IL-10^−/− mice was greater at each time point than the respective values in normal mice. The reason that IL-10 remains in the inflamed colon in greater concentrations as well as for a longer period of time than in normal colon is unclear. We previously reported a new oral drug delivery system using poly(DL-lactic acid) microspheres, and demonstrated that poly(DL-lactic acid) microspheres specifically target the inflamed colonic mucosa not only by preferentially adhering to the inflamed mucosa but also by being absorbed by M cells (Nakase et al., 2000b). Whether GM are more adhesive to the tissues than poly(DL-lactic acid) microspheres is unclear in the present study. Similar mechanisms might be considered for prominent targeting of GM for inflamed colon compared with normal colon. Additionally, the number of M cells is increased in inflamed mucosa (Kucharzik et al., 2000), which might also contribute to the targeting of GM to the inflamed colon.

As for blood distribution of IL-10, there was a longer period of distribution in the GM-IL-10-treated group than in the IL-10-treated group. This result is considered to be due to a difference in the colonic retention ratio between IL-10 and GM-IL-10, and reflects the local sustained release in the colon by GM-IL-10. The ratio of blood distribution is extremely low (less than 0.07% of administered 125I-labeled rmIL-10) compared with that of intestinal distribution, and therefore, it is unlikely to induce systemic side effects. In this study, instead of directly measuring the concentration of IL-10 in the blood and colonic tissue, we measured radioactivity of blood and colonic tissue during the experiment. Although the results of radioactivity may partially include a 125I-fragment from 125I-labeled rmIL-10, it is considered to neglect this possibility because of very low levels in the preliminary study (data not shown). In any event, GM-IL-10 appears to exert a more favorable effect on the inflamed colon with fewer systemic side effects. Thus, rectal administration of GM-IL-10 might be an ideal cytokine delivery system for treating IBD.

Spontaneous colonic inflammation in IL-10^−/− mice under specific pathogen-free conditions occurs at approximately 4 weeks of age as reported previously (Madsen et al., 2000). Therefore, in the present study, the treatments were started at 4 weeks, and the therapeutic effects on the colonic inflammation were observed at 8 weeks of age. The results of both macroscopic and histologic studies revealed that GM-IL-10 has more potent inhibitory effects on colitis than IL-10 alone. Furthermore, we previously reported that rectal administration of GM-IL-10 ameliorated established trinitrobenzene sulfonic acid-induced colitis in rats with strong inhibition of nuclear factor κB (Nakase et al., 2000a). These results suggested that GM-IL-10 not only prevents but also improves colonic inflammation.

IL-12 is a cytokine involved in Th1 T-cell differentiation, which promotes the production of interferon-γ (Wenner et al., 1996). Systemic administration of monoclonal antibodies against IL-12 leads to the improvement of colitis in mice by elimination of the Th1 T cells through induction of Fas-mediated apoptosis (Fuss et al., 1999). These data suggest that IL-12 has a key role in the development of Th1 dominant colitis. In the present study, gene expression of IL-12 p40 as well as p35 was enhanced in Mac-1-positive mononuclear cells in the lamina propria of the colonic mucosa of IL-10^−/− mice, and topical administration of GM-IL-10 to the colon reduced the expression of IL-12 p40 and p35 to levels similar to those of normal mice, whereas IL-10 alone had very little effect. Therefore, improvement of colitis in IL-10^−/− mice following rectal administration of GM-IL-10 might be due, at least in part, to decreased mRNA expression of IL-12 p40 and p35. The data also support the idea that administration of GM-IL-10 with its sustained release of IL-10 is more advantageous for treatment of colitis than administration of IL-10 alone.

To further examine the mechanism of therapeutic action of GM-IL-10 on colitis, we investigated CD40 expression on Mac-1-positive mononuclear cells in the lamina propria of the colon and also in the spleen. CD40, a 45-kDa cell surface glycoprotein on B cells, dendritic cells, and activated macrophages, is a member of the tumor necrosis factor receptor superfamily, and CD154, a 39-kDa surface glycoprotein on activated T cells, has been identified as a ligand of CD40...
References


