Development of an Oral Drug Delivery System Targeting Immune-Regulating Cells in Experimental Inflammatory Bowel Disease: A New Therapeutic Strategy

HIROSHI NAKASE, KAZUICHI OKAZAKI, YASUHIKO TABATA, SUGURU UOSE, MASAYA OHANA, KAZUSHIGE UCHIDA, YUMI MATSUMISA, CHIHARU KAWANAMI, CHIKASHI OSHIMA, YOSHITO IKADA, and TSUTOMU CHIBA

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

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ABSTRACT

Several studies have indicated the involvement of macrophages and dendritic cells in active inflammatory bowel disease (IBD). Manipulation of these cells is considered a very important therapeutic strategy for patients with IBD. We evaluated the effect of a new drug delivery system targeting microfold cells and macrophages with poly(DL-lactic acid) microspheres containing dexamethasone (Dx). Colitis was induced in BALB/c mice by 5% dextran sodium sulfate. Dx microspheres (n = 10) and only Dx (n = 10) were orally administered to dextran sodium sulfate-treated mice. Thereafter, serum levels and tissue distributions of Dx were investigated. To estimate the efficacy of this drug delivery system, we measured the histological score, myeloperoxidase activity and nitric oxide production, and gene expressions of tumor necrosis factor-α, interleukin-1β, and interferon-γ in the colonic tissue. Serum Dx levels were not increased after oral administration of Dx microspheres. The tissue distribution of microspheres containing 125I-labeled Dx in inflamed colon was significantly higher than in other organs. The histological score, myeloperoxidase activity, and nitric oxide production of the group treated with Dx microspheres were significantly lower than of those treated with Dx alone. Gene expressions of tumor necrosis factor-α, interleukin-1β, and interferon-γ were down-regulated in mice treated with Dx microspheres. Microspheres containing glucocorticoids such as Dx, which target microfold cells and macrophages, can facilitate mucosal repair in experimental colitis and could be an ideal agent for treatment of human IBD.

Many patients with ulcerative colitis (UC) have been successfully treated with steroid drugs and immunosuppressants. Among these drugs, glucocorticosteroid is known to be very effective in the treatment of UC (Hanauer and Kirsner, 1988). However, because of various systemic side effects, the administration of glucocorticosteroids by oral and i.v. routes is often restricted to patients with severe or acute UC (Elson, 1988). Therefore, to circumvent such side effects, topical administration of glucocorticosteroids (Patterson, 1958; Lee et al., 1980) and its alternative regimens (Swartz and Dhuy, 1978) have been used for UC patients. Recently, newer corticosteroids, such as budesonide, have been widely used because of their low systemic availability (Keller et al., 1997; Friend, 1998). However, some patients are still resistant to these treatments (Elson, 1988; Keller et al., 1997).

It is well known that macrophages and dendritic cells play important roles in the regulation of immunoresponses in the gastrointestinal tract as antigen-presenting cells (Wilders et al., 1984; Allison et al., 1988; Seldenrijk et al., 1989). Microfold (M) cells, which exist in the follicle-associated epithelium overlying the lymphoid follicles of Peyer’s patches, take up various macromolecules, bacteria, viruses, and protozoa and transport them to the follicular areas for uptake by macrophages (Owen et al., 1981; Inman and Cantey, 1983; Wolf et al., 1983; Wassef et al., 1989; Amerongen et al., 1994; Owen, 1997). The existence of colonic mucosal lymphoid organs with M cells was reported, and antigen can be taken up in these organs as in Peyer’s patch in the small intestine (Perry and Sharp, 1988; Owen et al., 1991). Several studies have indicated involvement of macrophages and dendritic cells in active inflammatory bowel disease (IBD) (Wilders et al., 1984; Allison et al., 1988; Seldenrijk et al., 1989). Moreover, CD4+...
T cells also have the important role of pathophysiology of IBD (Okazaki et al., 1993; Probert et al., 1996). Therefore, the regulation of these immune cells is thought to be an important therapeutic strategy for patients with IBD.

Considerable attention has been paid to the use of polymer microspheres for the sustained release of various drugs and the targeting of therapeutic or diagnostic agents to their site of action (Tabata and Ikada, 1990a). The use of biodegradable microspheres is particularly preferable from the perspective of avoiding the accumulation of foreign materials in the body (Tabata and Ikada, 1988). It was reported that biodegradable poly(DL-lactic acid) (PDLLA) microspheres can be efficiently taken up by macrophages and M cells (Tabata et al., 1996). Polymer microspheres worked effectively as carriers for biological response modifiers that activate macrophages (Tabata et al., 1989; Tabata and Ikada, 1990a, b, c). Judging from these results, the direct uptake of anti-inflammatory agents by macrophages, achieved with the use of microspheres, appears to have a superior immunosuppressive effect and to be more useful for the treatment of patients with IBD.

We have successfully incorporated dexamethasone (Dx) into microspheres via a solvent double-emulsion method and developed a new therapy with PDLLA microspheres containing Dx (Dx microspheres), which directly target macrophages and M cells. The objectives of this study were to evaluate the pharmacokinetics of Dx microspheres in mice and to examine the therapeutic effects of this drug in treating experimental colitis in mice.

**Materials and Methods**

**Animals.** Female BALB/c mice weighing 20 to 25 g (Japan SLC Inc., Shizuoka, Japan) were used for the experiments. They had access to food and water ad libitum. For induction of colitis, mice (n = 10/group) received three cycles of treatment with dextran sulfate sodium (DSS) (5000 M, 40 kDa; Sigma Chemical Co., St. Louis, MO). Each cycle consisted of 5% DSS in drinking water for 7 days, followed by a 7-day period with normal drinking water (Okayasu et al., 1990).

**Preparation of Dx Microspheres.** PDLLA microspheres were synthesized by the simple polycondensation of DL-lactic acid at 180°C under reduced pressure without any catalyst. Dx phosphate (Decadron) was kindly supplied from Banyu Pharmaceutical Co. (Tokyo, Japan). Dx microspheres were prepared by the solvent-evaporation method with double emulsion, as previously described (Tabata et al., 1996). In brief, 60 μl of a Decadron aqueous solution (W1) was poured into 1 ml of methylene chloride containing 200 mg of PDLLA microspheres (O), followed by emulsifying probe sonication to form a W1/O emulsion. The emulsion was added to 2 ml of a 1 wt% polyvinyl alcohol (PVA; weight-averaged M = 5400; degree of saponification, 79.85 mol%) aqueous solution (W2) that had been saturated with methylene chloride at room temperature and agitated by a vortex mixer to form a double emulsion. The W1/O/W2 double emulsion was stirred by an impeller (200 rpm) at room temperature until the methylene chloride was completely evaporated. The microspheres were collected by centrifugation (5000 rpm, 5 min, 4°C), washed three times with cold distilled water, and finally lyophilized. After hydrolysis of Dx microspheres, the concentration of Dx was measured by HPLC to assess the dosage incorporated in the microspheres (Haeberlin et al., 1993). As a result, 9.6 × 10⁻⁴ mg of Dx could be incorporated into 1 mg of PDLLA microspheres. The prepared Dx microspheres were further fractionated into different sizes by counterflow centrifugal elutriation. The size of the prepared Dx microspheres was assessed with microscopic photographs according to a reference scale. We adjusted the size of the Dx microspheres to within 4 μm because the microspheres with diameters <4.0 μm were phagocytosed by macrophages at the maximum level (Tabata and Ikada, 1990a). A similar microsphere preparation was performed to obtain fluorescent (cumarine 6, laser grade; ACRŚs, Organics, Geel, Belgium)-labeled PDLLA microspheres and 125I-labeled Dx microspheres (Amersham International, Buckinghamshire, UK).

**In Vitro Release of Dx from PDLLA Microspheres.** First, 2.5 mg of PDLLA microspheres containing Dx was suspended in 0.5 ml of normal saline and incubated at 37°C in a shaking bath. After centrifugation, this suspension was serially sampled. The Dx concentration of the sample solution was assessed by HPLC as previously described (Haeberlin et al., 1993).

**Tissue Distribution of PDLLA Microspheres in Mice with DSS-Induced Colitis.** For the determination of the systemic distribution of PDLLA microspheres, microspheres containing 125I-labeled Dx (0.1 mg/g) were orally administered once to normal mice and mice with DSS-induced colitis. The radioactivity of each organ was determined at 6, 12, and 24 h and 3 and 7 days after administration (n = 3 at each time point for each treatment) by gamma counter (ARC-300; Aloka Co., Tokyo, Japan). Other mice with DSS-induced colitis were orally administered, via gastric intubation, 2.5 mg of PDLLA microspheres containing cumarin 6 in 0.3 ml of PBS for the investigation of sites at which the microspheres were taken up in the colon. The mice were sacrificed by cervical dislocation at 12 h after administration of the microspheres. The excised colons were immediately mounted in optimal cutting temperature (OCT) freezing compound (4583; Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. They were cut into 4- to 6-μm serial sections, which were then viewed by confocal fluorescent microscopy.

**Blood Distributions of Dx in Mice with DSS-Induced Colitis Treated with Dx Microspheres or Dx Alone.** In the amount 0.1 mg, Dx microspheres contain about 10⁻⁴ mg of Dx. Therefore, Dx microspheres (0.1 mg·g⁻¹·day⁻¹) or Dx (10⁻⁴ mg·g⁻¹·day⁻¹) in 0.3 ml of PBS was administered by gastric tube to mice with DSS-induced colitis. The mice were anesthetized with ether at 0.5, 1.5, 2.5, 3, and 4 h after drug administration (n = 3 at each time point for each treatment), and 1 to 1.5 ml of blood was then taken before sacrificing the mice. The plasma was separated from the blood by centrifugation and stored at 4°C until assayed.

**Treatments.** Fifty mice with DSS-induced colitis were divided into five groups (10 mice in each; groups A–E) and treated immediately after the above-described three cycles of DSS administration as follows: A, no medication; B, PDLLA microspheres (0.1 mg·g⁻¹·day⁻¹) alone; C, Dx (10⁻⁴ mg·g⁻¹·day⁻¹) alone; D, PDLLA microspheres (0.1 mg·g⁻¹·day⁻¹) + Dx (10⁻⁴ mg·g⁻¹·day⁻¹) (the mixture of Dx and microspheres); E, Dx microspheres (0.1 mg·g⁻¹·day⁻¹). Mice were sacrificed by cervical dislocation after a 2-week administration of these treatments. The colonic tissues were processed according to the procedures described below.

**Determination of the Histological Score of Colitis.** The colon was removed, washed with PBS, and then inside out by cutting longitudinally. The colon was then fixed in 10% formalin in PBS overnight. Tissues from the distal third of the colon were fixed in 3.3% buffered formaldehyde and stained with H&E. Histological analysis was performed in a blind fashion. Microscopically, mucosal damage was quantitated by the scoring system of Kojoharoff et al. (1997). The colitis score of each mouse represented the sum of the different histological subcores.

**Assessment of Colonic Myeloperoxidase (MPO) Activity.** MPO activity was measured according to the method of Bradley et al. (1982). In brief, tissues from the midcolon, were homogenized with a Polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada) three times in hexadecyltrimethylammonium bromide buffer. The homogenate was centrifuged, and MPO activity in the supernatants was measured. One unit of MPO activity was defined as the amount required to degrade 1 mM hydrogen peroxide in 1 min at 25°C.

**Assessment of Nitric Oxide (NO) Production.** Tissues from the proximal third of the colon were homogenized for 15 s in HEPES buffer solution (40 mM, pH 7.4) containing sucrose (320 mM) (Bough-
ton-Smith et al., 1993; Denenberg et al., 1995). The combined concentration of nitrite and nitrate and the degradation products of NO in supernatants (10,000 μg for 20 min at 4°C), were determined by Griess reaction after nitrate reduction, as previously described (Salzman et al., 1995). Total nitrite/nitrate production is described in the text as NO production.

Measurement of Cytokine mRNA Expression in Colonic Tissue. Samples of colonic tissue for mRNA isolation were removed from the distal third of the colon. Total RNA was isolated with the guanidium isothiocyanate method (Khan and Collins, 1994). The concentration of RNA was determined by absorbance at 260 nm in relation to that at 280 nm. The RNA was stored at −70°C until used for reverse-transcription polymerase chain reaction (RT-PCR). One microliter of RT product 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. The mixture underwent PCR amplification for 35 cycles (1 min at 94°C, 1 min at 52°C, and 20 s at 20°C). Negative controls (cDNA-free solution) were included in each reaction. The sequences of primers for the cytokine genes are as follows:

Tumor necrosis factor (TNF)-α forward, 5′-TCTGTCTACTGAACTCGGGGTGATCGGTCC-3′
TNF-α reverse, 5′-GTATGAGATAGCAAATCGGCTGACGGTGTTGG-3′
Interleukin (IL)-1β forward, 5′-ATGGCAACTGTTCCTGAACCTCAACT-3′
IL-1β reverse, 5′-CAGGACAGTGATAAGCGTGCAGGCTCTCCATGTC-3′
Interferon (IFN)-γ forward, 5′-TGCACTTTGGTTGCACTCTTGCC-3′
IFN-γ reverse, 5′-GGTACTGTGGTGTTGACCTGAACACTTGCC-3′

Statistics. Student’s 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 <.05 was considered significant.

Results

Spontaneous Release of Dx from Dx Microspheres In Vitro. Thirty-three ± 3.2, 50 ± 5.5, and 97 ± 3.0% of the Dx incorporated in the microspheres was released into the media within 24, 30, and 48 h, respectively (Fig. 1).

Colonic Lymphoid Follicle Uptake of PDLLA Microspheres. Fluorescent micrographs of cryosections of the colonic mucosa showed that the microspheres were predominantly taken up into the colonic lymphoid hyperplasia of the DSS-treated mice (Fig. 2).

Tissue Levels of Microspheres Containing 125I-Labeled Dx. Tissue distributions of the microspheres containing 125I-Dx at 6, 12, and 24 h, 3 and 7 days after oral administration, are summarized in Table 1. The levels in the colons of the DSS-treated mice at 12 and 24 h were significantly higher than the respective values in the other organs in both the DSS-treated and normal mice. Moreover, no significant difference was observed between the DSS-treated and normal mice in any other organs throughout the experiments.

Plasma Concentrations of Dx after Oral Administration of Dx Microspheres or Dx Alone. After oral administration of Dx alone (10−4 mg/g), the concentration of Dx in the plasma increased markedly and reached the maximum level at 30 min. In contrast, no significant elevation in the plasma Dx level was observed after administration of Dx microspheres (Fig. 3).

Effects of Various Dx Treatments on Body Weights of Mice. During administration of DSS, the body weights of the mice gradually decreased. There were no significant differences in body weight among the groups at the start of treatment. However, after starting the treatments, body weights of mice in groups C, D, and E gradually increased, whereas body weights in groups A and B did not improve. After the 2-week treatment, there were no significant differences in the weight between groups A and B or among groups C, D, and E. Body weights in groups A and B were significantly lower than those in groups C, D, and E (Fig. 4).

Histological Evaluation. In the non-Dx-treated groups (groups A and B), colitis scores were significantly higher than those in the Dx-treated groups (groups C, D, and E) (Fig. 5A). There were no significant differences in histological scores between groups A and B or between groups C and D. However, the histological score in group E was significantly lower than those in groups C and D.

MPO Activity of Colonic Tissue. The MPO activity in colonic tissues from mice in the non-Dx-treated groups was significantly higher than that from mice in the Dx-treated groups (groups C–E). Moreover, the MPO levels in group E were significantly lower than those in both groups C and D. There were no significant differences between groups A and B or groups C and D (Fig. 5B).

NO Production of Colonic Tissue. The levels of NO production in the non-Dx-treated groups (A and B) were significantly higher than those in the Dx-treated groups (C, D, and E). Similar to the MPO activity, NO production in group E was significantly lower than that in either groups C or D. There were no significant differences in NO production between groups A and B or groups C and D (Fig. 5C).

mRNA Expression of Proinflammatory Cytokines Shown by RT-PCR. The gene expressions of TNF-α, IL-1β, and IFN-γ were all up-regulated in the colons from group A and group B mice compared with those from normal mice. The expressions of each cytokine in groups C and D were weaker than those in groups A and B. The expression of each cytokine was nearly undetectable in group E (Fig. 6).

Fig. 1. Spontaneous release of dexamethasone from Dx microspheres in vitro. First, 2.5 mg of Dx microspheres was incubated in 0.5 ml of saline at 37°C, and 97 ± 3.0% of the incorporated Dx had been released at 48 hrs after the start of incubation. Each data point represents the mean ± S.E. (n = 3).
Discussion

Regulation of macrophages, which present antigens for activating T cells and produce various cytokines, is a key issue in the treatment of IBD. The results of our study clearly demonstrate that Dx microspheres, which target macrophages or M cells, have a novel therapeutic effect in a colitis model.

Confocal laser microscopic examination of colonic tissue from the DSS-treated mice confirmed that the fluorescence-labeled microspheres were taken up into the colonic lymphoid tissue and Peyer’s patches (Tabata et al., 1996). Moreover, analysis of the tissue distribution of microspheres containing 125I-labeled Dx revealed that the Dx microspheres were predominantly distributed in the inflamed colon. These results suggest that the PDLLA microspheres have been taken up predominantly by the inflamed colonic lymphoid tissue, which is thought to be the initiation site of immune responses. Several reasons may be considered for the difference in the distribution of Dx microspheres between inflamed and normal colon. First, there are more activated macrophages in the inflamed colon than in the normal colon.

![Image](a) Fluorescence microscopy findings. The fluorescent-labeled PDLLA microspheres were predominantly present in the peripheral portion of the colonic lymphoid tissue of the DSS-treated mice 12 h after oral administration of microspheres. (b) H&E staining at the same section (×200).

### Table 1

| Tissue distribution of PDLLA microspheres containing 125I-labeled Dx in normal and DSS-treated mice |
|----------------|---------------------------------|
|                | 6 h    | 12 h    | 24 h    | 3 Days  | 7 Days  |
| Blood          |        |        |        |        |        |
| DSS            | 14 ± 7.4 | 26 ± 21 | 17 ± 1.7 | 8.6 ± 8.5 | 4.7 ± 2.6 |
| Normal         | 18 ± 4.2 | 20 ± 3.1 | 18 ± 0.8 | 3.0 ± 2.5 | 2.8 ± 2.5 |
| Heart          |        |        |        |        |        |
| DSS            | 6.0 ± 3.8 | 1.3 ± 0.9 | 2.0 ± 0.1 | 2.7 ± 0.7 | 2.5 ± 0.8 |
| Normal         | 4.0 ± 2.0 | 1.5 ± 0.5 | 1.0 ± 0.3 | 1.7 ± 0.5 | 2.0 ± 0.5 |
| Lung           |        |        |        |        |        |
| DSS            | 0.3 ± 0.2 | 9.3 ± 5.5 | 8.7 ± 5.5 | 4.0 ± 3.0 | 0.4 ± 0.2 |
| Normal         | 0.3 ± 0.2 | 6.5 ± 4.2 | 5.0 ± 2.1 | 2.0 ± 0.3 | 1.0 ± 0.5 |
| Liver          |        |        |        |        |        |
| DSS            | 11 ± 1.1 | 6.0 ± 3.7 | 7.0 ± 2.3 | 0.3 ± 0.1 | 0.6 ± 0.3 |
| Normal         | 8.0 ± 3.2 | 4.5 ± 2.2 | 4.9 ± 2.0 | 0.4 ± 0.1 | 0.8 ± 1.3 |
| Spleen         |        |        |        |        |        |
| DSS            | 2.0 ± 1.5 | 3.7 ± 2.7 | 4.7 ± 4.2 | 2.0 ± 0.1 | 0.7 ± 0.5 |
| Normal         | 1.8 ± 0.9 | 2.0 ± 0.5 | 3.4 ± 2.1 | 1.8 ± 0.2 | 0.2 ± 0.1 |
| Kidney         |        |        |        |        |        |
| DSS            | 1.7 ± 0.9 | 1.7 ± 1.7 | 2.0 ± 1.8 | 2.7 ± 2.4 | 3.3 ± 2.0 |
| Normal         | 1.5 ± 0.3 | 1.8 ± 1.5 | 1.8 ± 0.2 | 1.7 ± 0.5 | 2.3 ± 1.0 |
| Small intestine|        |        |        |        |        |
| DSS            | 45 ± 6.0 | 74 ± 36 | 134 ± 90 | 8.6 ± 4.6 | 2.3 ± 1.3 |
| Normal         | 40 ± 6.0 | 55 ± 28 | 45 ± 20  | 9.0 ± 3.0 | 3.0 ± 1.0 |
| Colon          |        |        |        |        |        |
| DSS            | 55 ± 19 | 956 ± 455* | 343 ± 223* | 30 ± 3.5 | 2.0 ± 1.5 |
| Normal         | 83 ± 28 | 46 ± 18 | 16 ± 8  | 8.6 ± 1.0 | 3.3 ± 1.5 |

* P < .05 versus normal colon or other organs in DSS-treated mice.
inflamed colon, microspheres may be coated with various proteins such as immunoglobulins and complement components, which are produced by colonic inflammation. These coating proteins may render the microspheres more recognizable, so that they are phagocytosed by macrophages (Tabata and Ikada, 1990a). Phagocytosis of Dx microspheres by eosinophils in colonic mucosa cannot necessarily be denied (Beeken et al., 1987). However, eosinophils are less active than neutrophils in phagocytosis (Fabian et al., 1992), and particles with diameters <5 mm tend to be ingested by MAC-1+ cells at Peyer’s patches (Eldridge et al., 1990).

Taken together, we considered that Dx microspheres were mainly taken up by M cells and macrophages, although the absorption rates of microspheres by these cells were not examined.

The pharmacokinetics of this drug delivery system showed that the serum levels of Dx in the mice treated with Dx microspheres were not increased, in contrast to the mice treated with Dx alone, which showed a significant increase in Dx level in the serum. It might be considered that microspheres cannot be absorbed by enterocytes because of their size (Tabata and Ikada, 1988, 1990a). Instead, it is likely that they are preferentially taken up by the inflamed colon mucosa. Thus, it is tempting to speculate that M cells in the inflamed colon play a major role in absorption of the microspheres. When considered together with the observed distribution of microspheres containing 125I-labeled Dx, this drug delivery system appears unlikely to induce systemic side effects, including adrenal suppression.

The body-weight loss induced by DSS feeding recovered significantly after oral administration of Dx microspheres,
of the gel.

of IBD patients (Choi and Targan, 1994; Schreiber et al., 1993; Salzman et al., 1995). The Dx-treated groups showed reduction in both the MPO activity and NO production compared with the non-Dx-treated groups. The groups treated with Dx alone, or those treated with the mixture of Dx and PDLLA microspheres. Taken together, these results indicate that oral administration of Dx microspheres is more potent in reducing and repairing lesions of experimental colitis than administration of either Dx alone or a mixture of Dx and PDLLA microspheres.

In conclusion, this study has clearly demonstrated a favorable therapeutic effect of microspheres containing Dx in a model of IBD. Various reports suggest that it is important to inhibit the action of proinflammatory cytokines and up-regulate the levels of immunomodulatory cytokines when treating IBD patients (Choi and Targan, 1994; Schreiber et al., 1995; van Dullemen et al., 1995). Considering the ongoing activation of macrophages in the inflamed intestine, which produces various cytokines, the oral drug delivery system described here appears to be a promising treatment for IBD. The clinical usefulness of microspheres containing immunomodulatory agents such as glucocorticoids remains to be examined.

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References


DN alone, and a mixture of Dx and PDLLA microspheres. The histological score in the group treated with Dx microspheres was significantly lower than the scores in the non-Dx-treated groups, the groups treated with DN alone, or those treated with the mixture of Dx and PDLLA microspheres. In this study, the grade of tissue inflammation was determined by both MPO and NO assays (Bradley et al., 1982; Boughton-Smith et al., 1993; Salzman et al., 1995). The Dx-treated groups showed reduction in both the MPO activity and NO production compared with the non-Dx-treated groups. The Dx microspheres reduced both MPO activity and NO production more significantly than Dx alone or the mixture of Dx and microspheres. With regard to proinflammatory cytokines, no cytokine mRNA was detectable in the colonic tissue of mice treated with Dx microspheres, whereas some was expressed in mice treated with Dx alone or the mixture of Dx and microspheres. Taken together, these results indicate that oral administration of Dx microspheres is more potent in reducing and repairing lesions of experimental colitis than administration of either Dx alone or a mixture of Dx and PDLLA microspheres.


Send reprint requests to: Kazuichi Okazaki, M.D., Ph.D., Division of Gastroenterology and Endoscopic Medicine, Graduate School of Kyoto University, 54 Shogunkawara-cho, Sakyoku, Kyoto, 606-8807, Japan. E-mail: okak@kuhp.kyoto-u.ac.jp