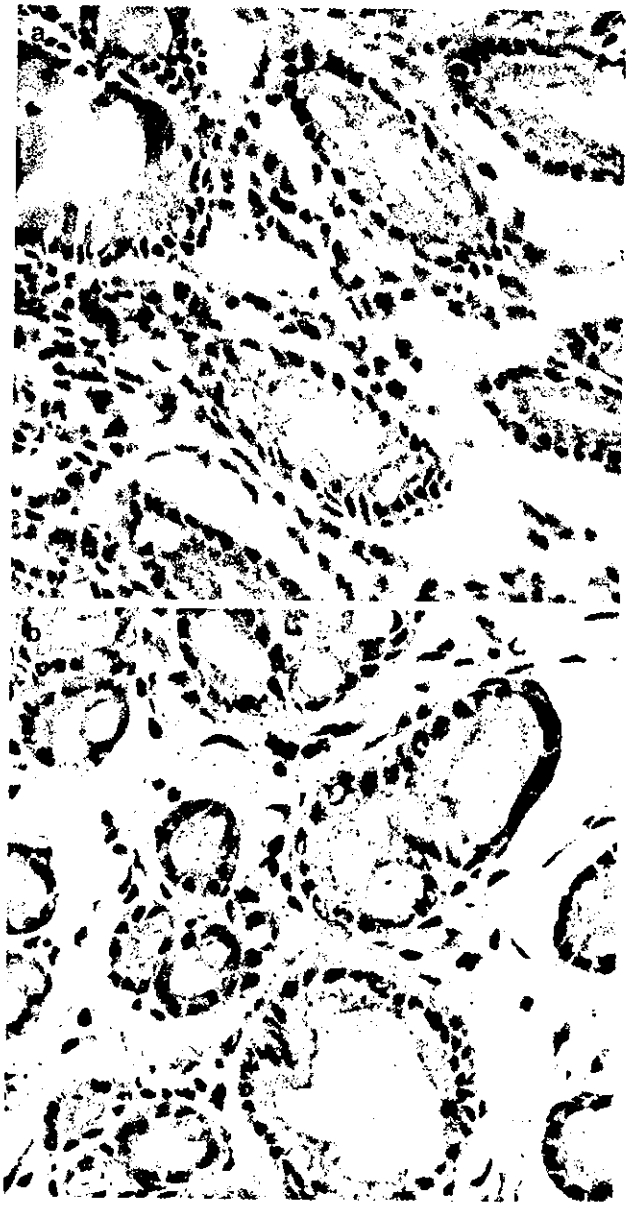


**Figure 6** Detection of IL-12 in the colonic tissues of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-administered C57BL/6 wild-type or IL-12 $p40^{-/-}$ mice. Expression of IL-12 was examined in the colonic tissues of TNBS-administered C57BL/6 wild-type (a) IL-12 $p40^{-/-}$; (b) mice by immunohistochemistry with anti-IL-12 mAb. Original magnification ×400.

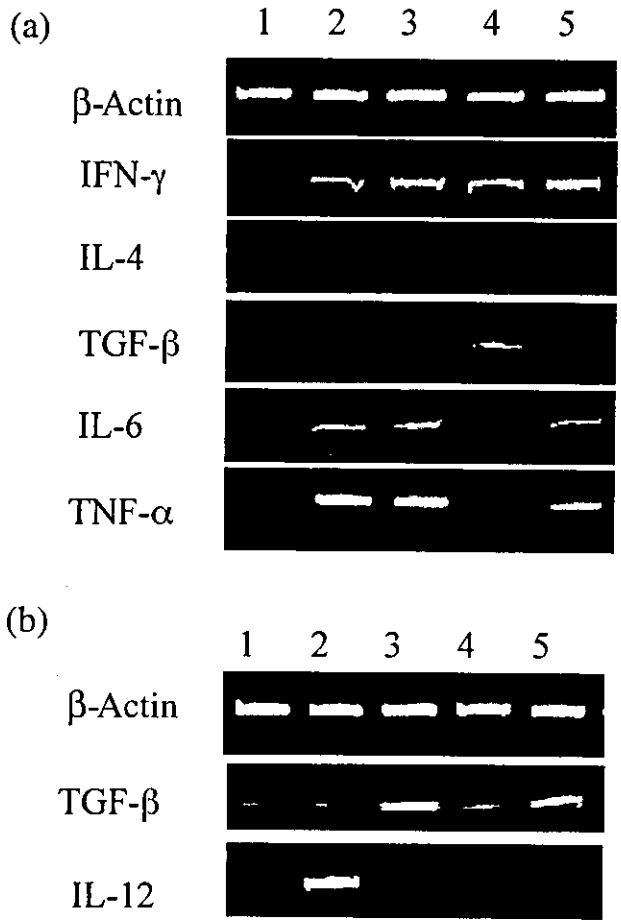


**Figure 7** Detection of cytokine mRNAs in the colon of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-administered mice by reverse transcription-polymerase chain reaction (RT-PCR). (a) Colon specimens from 129Sv/Ev wild-type, anti-IFN-γ mAb-pretreated wild-type, or IFN-$\gamma R^{-/-}$ mice treated with either 50% ethanol alone or TNBS were served for RT-PCR analysis for IFN-γ, IL-4, TGF-β, IL-6, and TNF-α. Lane 1, wild-type mice treated with ethanol alone; lane 2, wild-type mice treated with TNBS; lane 3, anti-IFN-γ mAb-pretreated wild-type mice treated with TNBS, lane 4, IFN-$\gamma R^{-/-}$ mice treated with ethanol alone; lane 5, IFN-$\gamma R^{-/-}$ mice treated with TNBS. β-actin mRNA expression were also examined as a control. (b) Colon specimens from C57BL/6 wild-type, anti-IL-12 p40 mAb-pretreated wild-type or IL-12 $p40^{-/-}$ mice treated with either 50% ethanol alone or TNBS were served for RT-PCR analysis for TGF-β and IL-12. Lane 1, wild-type mice treated with ethanol alone; lane 2, wild-type mice treated with TNBS; lane 3, anti-IL-12 p40 mAb-pretreated wild-type mice treated with TNBS; lane 4, IL-12 $p40^{-/-}$ mice treated with ethanol alone; lane 5, IL-12 $p40^{-/-}$ mice treated with TNBS. β-actin mRNA expression were also examined as a control.

sation for IFN-γ. Therefore, we employed anti-IFN-γ mAb to confirm our observations in IFN-$\gamma R^{-/-}$ mice and found again that the neutralization of IFN-γ failed to prevent the colitis induced by TNBS in 129/Sv/Ev wild-type mice. It is possible that the neutralizing anti-IFN-γ mAb may allow the biological significant level of IFN-γ to escape neutralization. However, this seems unlikely because mice treated with the neutralizing anti-IFN-γ mAb in the same manner were extremely susceptible to IFN-γ-sensitive *Listeria monocytogenes* infection.[27] Taken together, our data indicate that IFN-γ is not required for the development of TNBS-induced colitis.

We observed increased expression of TNF-α and IL-6 mRNA after a TNBS enema in 129/Sv/Ev wild-type,

anti IFN-γ mAb-treated, and IFN-γR$^{-/-}$ mice (Fig. 7a). Neurath *et al.*[28] demonstrated a predominant pathogenic role of TNF-α in experimental colitis in mice employing TNF-α knockout mice, and mice administered with anti-TNF-α mAb. Their results suggest that TNF-α, instead of IFN-γ, may be more important in the establishment of colitis. The increased expression of TNF-α mRNA may be pivotally involved in TNBS-induced colitis in IFN-γR$^{-/-}$ mice, as well as wild-type mice. Interestingly, expression of IFN-γ mRNA was also detected in colonic tissues from control ethanol-treated IFN-γR$^{-/-}$ mice (Fig. 7a). Lack of negative feedback mechanisms in IFN-γR$^{-/-}$ mice may induce the background level of IFN-γ mRNA expression.

We observed that IL-12 p40$^{-/-}$ mice showed a transient weight loss upon TNBS treatment, but failed to develop chronic intestinal inflammation. These findings indicate that IL-12 plays an important role in TNBS-induced wasting disease and severe colitis. In support of our results, Neurath *et al.*[6] reported that administration of anti-IL12 mAb to TNBS-treated BALB/c mice both early (day 5) and late (day 20) after induction of colitis led to a striking improvement in both the clinical and histopathological aspects of the diseases and frequently abrogated the established colitis completely. Recently, Dohi *et al.*[25] described that C57BL/6 IL-12 p40$^{-/-}$ mice developed TNBS-induced colitis characterized by distortion of crypts, loss of goblet cells and mononuclear cell infiltration with fibrosis of the mucosal layer. They administered a TNBS enema twice (on days 0 and 7) and examined tissues and cells from the mice on day 10. In contrast, we administered TNBS once and examined the tissues and cells on day 7 or 8. These differences in the experimental protocol may be the cause of the conflicting results.

The results of immunohistochemical and immunofluorescence staining of CD4 and Mac-1 indicates strong infiltration of CD4$^+$ T cells and colonic macrophages in the TNBS-induced colitis lesion. We observed enhanced expression of IL-12 protein and mRNA in the lesion of TNBS-induced colitis by immunohistochemical and RT-PCR analyses (Figs 6 and 7b). These results suggest that TNBS directly or indirectly activates colonic macrophages and enables them to produce IL-12. IL-12 then activates CD4$^+$ Th1 cells which produce inflammatory cytokines such as TNF-α and IL-6, as well as IFN-γ. Activated macrophages themselves may also produce TNF-α and IL-6. The mechanisms by which IL-12 leads to the development of TNBS-induced colitis seems to be IFN-γ independent. IL-12-induced inflammatory cytokines other than IFN-γ may be involved in the mechanisms. TNF-α is definitely a candidate cytokine involved in the mechanisms, as mentioned previously. In addition, IL-6 and GM-CSF may be also involved in the mechanisms as they have been reported to be induced by IL-12.[29] Interestingly, we observed that TNBS administration to anti-IL-12 p40 mAb-pretreated C57BL/6 wild-type and IL-12 p40$^{-/-}$ mice induced TGF-β message (Fig. 7b). It is possible that blocking IL-12 protects mice from developing colitis by inducing TGF-β from macrophages. In contrast, blocking IFN-γ may not have such an effect (Fig. 7a).

Another IFN-γ-independent mechanism could be the direct effects of IL-12 on hematopoietic cells and other tissues. Fuss *et al.*[21] reported that anti-IL12 mAb treatment lead to alleviation of TNBS-induced colitis by apoptosis-mediated elimination of Th1 cells. They also demonstrated that the observed apoptosis is mediated by the Fas pathway employing MRL/MpJ-lpr$^{fas}$ mice and blocking Fas-Fc molecules. Furthermore, Davidson *et al.*[30] found that anti-IL-12 mAb greatly diminished the ability of CD4$^+$CD45RB$^{high}$ T cells from diseased IL-10$^{-/-}$ mice to expand and cause colitis in RAG-2$^{-/-}$ recipients. These studies support the idea that IL-12 plays an important role in sustaining activated pathogenic Th1 cells by preventing apoptosis.

In summary, we were able to draw the conclusion that IL-12, but not IFN-γ, plays an important role in TNBS-induced wasting disease and severe colitis. Further investigation is necessary to clarify the detailed mechanisms by which IL-12 leads to colitis.

## ACKNOWLEDGMENTS

We thank Dr Michel Aguet (University of Zurich) for IFN-γR$^{-/-}$ mice and their wild-type littermates on the 129/Sv/Ev background, and Dr Giorgio Trinchieri (The Wister Institute) for anti-mouse IL-12 mAb. We are also very grateful to Dr Atsushi Yoshida (University of the Ryukyus) for his excellent technical advice in the early stages of the present work.

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# Induction of Protective Immunity to *Listeria monocytogenes* with Dendritic Cells Retrovirally Transduced with a Cytotoxic T Lymphocyte Epitope Minigene

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**In the present study, we developed a cytotoxic T lymphocyte (CTL) epitope minigene-transduced dendritic cell (DC)-based vaccine against *Listeria monocytogenes*. Murine bone marrow-derived DCs were retrovirally transduced with a minigene for listeriolysin O (LLO) 91-99, a dominant CTL epitope of *L. monocytogenes*, and were injected into BALB/c mice intravenously. We found that the DC vaccine was capable of generating peptide-specific CD8$^+$ T cells exhibiting LLO 91-99-specific cytotoxic activity and gamma interferon production, leading to induction of protective immunity to the bacterium. Furthermore, we demonstrated that the retrovirally transduced DC vaccine was more effective than a CTL epitope peptide-pulsed DC vaccine and a minigene DNA vaccine for eliciting antilisterial immunity. These results provide an alternative strategy in which retrovirally transduced DCs are used to design vaccines against intracellular pathogens.**

Dendritic cells (DCs) are the most potent antigen-presenting cells that patrol all tissues of the body with the possible exceptions of the brain and testes. DCs capture bacteria and other pathogens. Then they migrate to regional lymphoid organs, where they present antigens (Ags) to naïve T cells (3). DCs have a distinct ability to prime naïve helper T lymphocytes and cytotoxic T lymphocytes (CTLs); thus, there has been much interest in the use of these cells for immune modulation of diseases. A number of investigators have demonstrated that DC-based vaccines, such as those pulsed with tumor-associated Ag, can generate specific antitumor immunity in vivo in murine tumor models (6, 21). A DC vaccine genetically engineered to express tumor-associated Ags is one of the most promising methods for tumor immunotherapy as it allows constitutive expression of the Ag, leading to prolonged Ag presentation in vivo (29). In the field of infectious diseases, however, there have been a few studies exploring the efficacy of DC-based vaccines (1, 19, 20, 26, 28).

Infection with intracellular pathogens, such as *Mycobacterium tuberculosis*, poses serious health problems worldwide. Efficient protection against intracellular bacteria critically depends on induction of cellular immune responses. Administration of soluble proteins may be insufficient to stimulate such responses. So far, only live attenuated vaccines are considered to be satisfactory. However, because of the low safety level of live vaccines in immunocompromised individuals and because of the variable effectiveness of these vaccines, development of new, improved vaccines which induce cellular immunity to intracellular pathogens has become a research priority (15, 27).

*Listeria monocytogenes* is a gram-positive facultative intracellular bacterium that causes life-threatening infections during pregnancy and in immunocompromised individuals (13). *L. monocytogenes* enters eukaryotic cells in membrane-bound vesicles, and then it escapes from the vesicles, multiplies within the cell cytoplasm, and spreads directly to adjacent cells. A well-characterized mouse model of *L. monocytogenes* infection has yielded significant insight into the nature of innate and acquired cell-mediated immunity primarily associated with specific CD8$^+$ CTLs (11, 31). Murine *L. monocytogenes* infection can, therefore, serve as a useful model for studying protective immunity to intracellular bacteria. Among the Ags recognized by the CTLs, four different epitopes are presented to CTLs by H2-K$^d$ molecules (4, 24, 32). These epitopes are derived from bacterial virulence factors, including a sulfhydryl-activated pore-forming exotoxin, listeriolysin O (LLO), murein hydrolase p60, and metalloprotease (Mpl). Indeed, infection of BALB/c mice with a sublethal dose of *L. monocytogenes* induces dominant CTL responses against LLO 91-99 and p60 217-225 and subdominant responses against p60 449-457 and Mpl 84-92 (4).

Previously, Harty and Bevan (14) showed that adoptive transfer of CD8$^+$ CTLs specific for LLO 91-99 confers protection against *L. monocytogenes* infection. Consistent with this observation, previous studies demonstrated that DNA immunization with a minigene plasmid encoding a single dominant CTL epitope, LLO 91-99, induces strong CTL activity and confers partial protection against murine *L. monocytogenes* infection (30). Although the mechanisms by which the DNA vaccine achieves immunogenicity have not been fully determined so far, many studies have clearly indicated that DCs are the principal cells initiating the immune responses after DNA vaccination. DCs may be directly transfected with plasmid

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DNA and present Ags to $CD8^+$ and $CD4^+$ T cells through major histocompatibility complex (MHC) class I and II molecules. Alternatively, proteins exogenously produced by transfected somatic cells allow DCs to take up, process, and present Ags not only to $CD4^+$ T cells but also to $CD8^+$ T cells via additional mechanisms, such as cross-priming (2, 5, 17, 25). Theoretically, vaccination with DCs harboring genes encoding Ags of interest would be more effective for induction of specific immunity than naked DNA vaccination. DCs engineered genetically to express the immunodominant T-cell epitope may be a promising vaccine against intracellular bacteria. In the present study, we developed a retrovirally transduced DC-based vaccine expressing LLO 91-99 and determined the ability of this vaccine to generate specific CTLs and to elicit protective immunity to murine *L. monocytogenes* infection. We also compared the efficacy of this vaccine for eliciting antilisterial immunity with the efficacy of a CTL epitope peptide-pulsed DC vaccine and the efficacy of a naked minigene DNA vaccine.

## MATERIALS AND METHODS

**Recombinant retroviral vectors.** The double-stranded oligonucleotide encoding LLO 91-99, adapted to the most frequently used codons in murine genes (22, 30), was subcloned into the *Sma*I site of pIRES2-EGFP (Clontech, Palo Alto, Calif.), resulting in pLLO91-IRES-EGFP. A pMX-based (23) retroviral vector, pMX-LLO91-IRES-EGFP, was constructed by using an *Eco*RI-*Not*I DNA fragment of pLLO91-IRES-EGFP. The nucleotide sequences of the plasmids were confirmed by DNA sequencing by using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). Large-scale purification of expression vectors was conducted by using a Qiagen Plasmid Mega kit system (Qiagen, Chatsworth, Calif.), and endotoxin was removed by Triton X-114 phase separation. Retroviral supernatant was generated by transfecting pMX-LLO91-IRES-EGFP proviral constructs into the Phoenix ecotropic packaging cell line (purchased from the American Type Culture Collection, Manassas, Va., and used with the permission of G. P. Nolan, Stanford University School of Medicine, Stanford, Calif.). A control retroviral vector carried only a DNA fragment containing only the internal ribosome entry site (IRES) and the enhanced green fluorescent protein (EGFP)-encoding region without the LLO 91-99-encoding region.

**Mice.** BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). These mice were maintained under specific-pathogen-free conditions at the Institute for Experimental Animals, Hamamatsu University School of Medicine. All mice used in this study were between 8 and 14 weeks old. All animal experiments were performed according to the animal care guidelines of Hamamatsu University School of Medicine.

**Culture of BM-DCs and transduction with retrovirus.** Bone marrow (BM)-derived DCs (BM-DCs) were cultured by using methods described by Inaba et al. (16), with some modifications. Briefly, murine BM cells were harvested from femurs and tibias of sacrificed mice. Contaminating erythrocytes were lysed with 0.83 M $NH_4Cl$ buffer, and lymphocytes were depleted with a mixture of monoclonal antibodies (GK1.5, anti-CD4 [TIB207]; HO2.2, anti-CD8 [TIB150]; B21-2, anti-H2-$A^{b,d}$ [TIB229]; and RA3-3A1/6.1, anti-B-cell surface glycoprotein [TIB146]; all obtained from the American Type Culture Collection) and rabbit complement (Cedarlane, Hornby, Canada). Cells ($1 \times 10^6$ cells/ml) were placed in 24-well plates in RPMI 1640 supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 1,000 U of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (kindly provided by Kirin Co. Ltd., Tokyo, Japan) per ml, and 1,000 U of recombinant murine interleukin-4 (IL-4) (R&D Systems, Minneapolis, Minn.) per ml (complete RPMI medium) at zero time. The cells were harvested on day 6. To determine the phenotype of cultured DCs, we stained them with phosphatidylethanolamine- or fluorescein isothiocyanate-conjugated monoclonal antibodies against cell surface molecules (CD40, CD80, CD86, and H2-$A^b$; all obtained from Pharmingen, San Diego, Calif.) and analyzed them with an EPICS Profile-II (Beckman Coulter, Fullerton, Calif.). For retroviral transduction, $1 \times 10^6$ BM cells were cultured in complete RPMI medium for 48 h and resuspended in 1 ml of the retroviral supernatant supplemented with 8 μg of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per ml, 1,000 U of recombinant murine GM-CSF per ml, and 1,000 U of recombinant murine IL-4 per ml. These cells were centrifuged at 2,500 × *g* at 32°C for 2 h. After centrifugation, cells were cultured in complete RPMI medium. The transduction processes were repeated on days 3 and 4. The transduction efficiency of the BM-DCs was evaluated by measuring the expression of EGFP by flow cytometry.

**Preparation of LLO 91-99 peptide-pulsed DCs (LLO91-pulsed DCs).** The LLO 91-99 peptide, GYKDGNEYI, representing an H2-$K^d$-restricted immunodominant CTL epitope spanning amino acid residues 91 to 99 of LLO, was synthesized by BEX (Tokyo, Japan). BM-DCs from BALB/c mice after 6 days of culture were resuspended in RPMI 1640 at a concentration of $2 \times 10^6$ cells/ml and pulsed with 5 μM LLO 91-99 peptide and human β2-microglobulin (Sigma Chemical Co.) (21) for 2 h at room temperature with gentle mixing.

**Immunization with DCs.** After two washes in phosphate-buffered saline, $10^5$ retrovirus-transduced or LLO91-pulsed DCs in 0.5 ml of phosphate-buffered saline were injected intravenously into mice twice with a 1-week interval between the injections.

**Plasmid DNA immunization with gene gun bombardment.** Construction of p91mam has been described previously (30). For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, Calif.), the cartridge containing DNA-coated gold particles was prepared according to the manufacturer's instructions. Then 0.5 mg of gold particles was coated with 2 μg of plasmid DNA, and injection was carried out by using 0.5 mg of gold/shot. To immunize mice, the shaved abdominal skin was wiped with 70% ethanol, the spacer of the gene gun was held directly against the abdominal skin, and the device was discharged at a helium discharge pressure of 400 $lb/in^2$. Mice were injected with 2 μg of plasmid twice with a 1-week interval between the injections.

**Preparation of splenocyte culture supernatants for evaluation of IFN-γ production.** Pools of spleen cell suspensions ($2 \times 10^6$ cells/ml) from groups of mice immunized with DCs were cultured in RPMI 1640 supplemented with 10% fetal calf serum in 24-well plates in the presence of 5 μM LLO 91-99 peptide at 37°C in 5% $CO_2$. Supernatants were harvested 5 days later and stored at −20°C until they were assayed for gamma interferon (IFN-γ). The IFN-γ concentration was measured by a sandwich enzyme-linked immunosorbent assay as described previously (34).

**CTL assay.** Eight weeks after the last immunization, immune spleen cells were cocultured in 12-well plates at a density of $2 \times 10^7$ cells/well for 5 days with $2 \times 10^7$ syngeneic splenocytes per ml; the splenocytes had been treated with 100 μg of mitomycin C (Kyowa Hakko, Tokyo, Japan) per ml and pulsed with 5 μM LLO 91-99 peptide for 2 h at 37°C. Each well also received 10 U of human recombinant IL-2 (Hoffmann-La Roche, Nutley, N.J.) per ml. Cell-mediated cytotoxicity was measured by using a conventional $^{51}Cr$ release assay as described previously (30). The target cells used in this study were J774 murine macrophage-like cells ($H2^d$) pulsed with the peptide at a concentration of 5 μM for 1.5 h at 37°C. Target cells at a concentration of $10^4$ cells/well were incubated for 5 h in triplicate at 37°C with serial dilutions of effector cells, and the level of specific lysis of the target cells was determined by using the following equation: percentage of specific lysis = [(experimental counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)] × 100.

**Bacterial infection and evaluation of antilisterial immunity.** *L. monocytogenes* strain EGD, kindly provided by M. Mitsuyama (Kyoto University), was kept virulent by in vivo passage. For inoculation, a seed culture of *L. monocytogenes* was grown overnight in Trypticase soy broth (Becton Dickinson, Sparks, Md.) at 37°C in a bacterial shaker and suitably diluted with phosphate-buffered saline. The exact infection dose was assessed retrospectively by plating. Eight weeks after the last immunization, immunized mice were challenged with $3 \times 10^4$ CFU of *L. monocytogenes*. Seventy-two hours after the challenge infection, the numbers of bacteria in spleens were determined by plating 10-fold dilutions of tissue homogenates on Trypticase soy agar plates.

**Statistical analysis.** Statistical evaluation of differences between means for experimental groups was performed by using Student's *t* test. A *P* value <0.05 was considered significant.

## RESULTS

**Retroviral transduction of BM-DCs and their phenotype.** DCs were generated from murine BM by culturing with GM-CSF plus IL-4 as previously described (16). More than 70% of the cultured cells were determined to be DCs (data not shown).

LLO91-EGFP and control EGFP retroviruses were transfected into the DCs. To assess the efficiencies of transduction

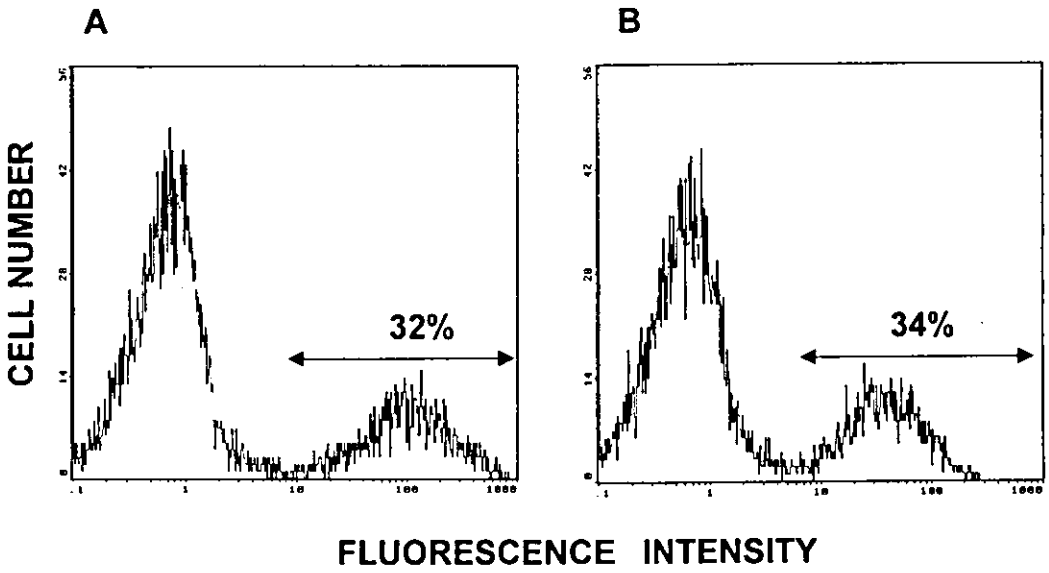


FIG. 1. Expression of EGFP in retrovirally transduced DCs. The transduction efficiencies were evaluated by flow cytometry of the expression of EGFP in LLO91-EGFP-transduced DCs (A) and EGFP-transduced DCs (B). DCs were transduced by the centrifugal enhancement method on days 2, 3, and 4 of culture as described in Materials and Methods. EGFP expression was determined by flow cytometry on day 7. Representative results are shown.

of the LLO91-EGFP and control EGFP retroviruses into DCs, expression of EGFP was evaluated by flow cytometry. As shown in Fig. 1, the levels of expression of EGFP in EGFP- and LLO91-EGFP-transduced DCs were similar (26.2 to 41.2%). Then we examined the patterns of expression of various cell surface molecules by flow cytometry. DCs transduced with an LLO 91-99-encoding retrovirus (LLO91-transduced DCs) and nontransduced DCs expressed similar amounts of CD40, CD80, CD86, and MHC class II molecules (Fig. 2), indicating that retrovirus transduction into DCs does not affect the phenotype of the DCs. Furthermore, we performed CTL assays using spleen cells of mice immunized with a sublethal

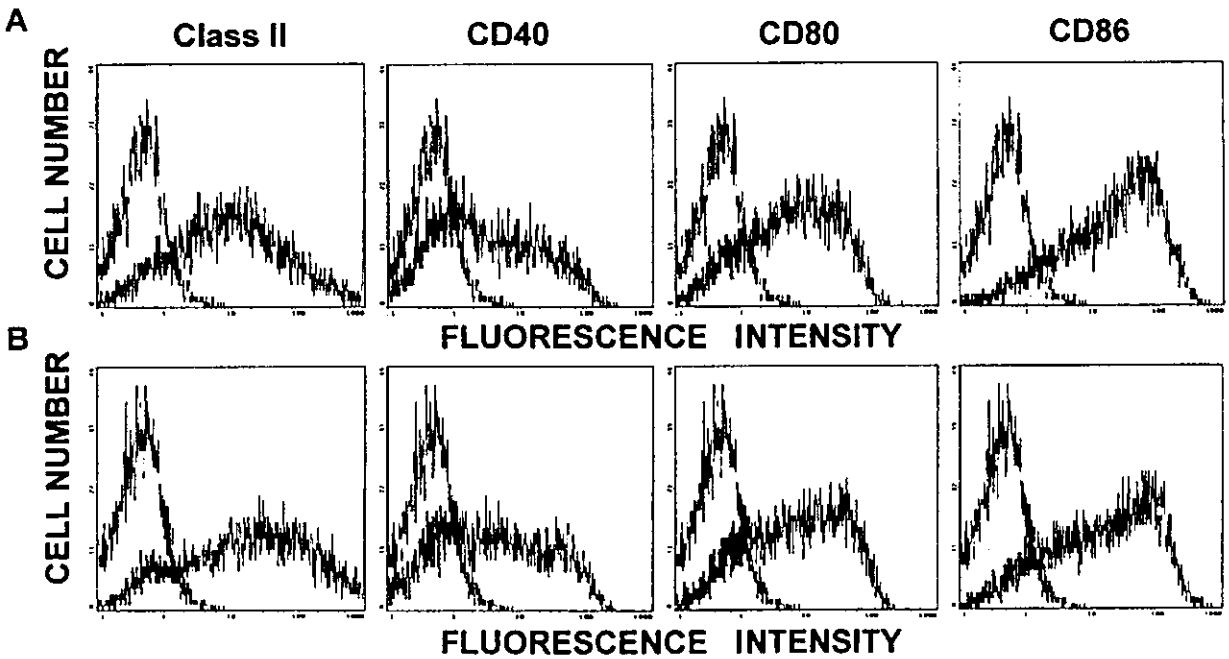


FIG. 2. Phenotypic characteristics of nontransduced DCs (A) and LLO91-EGFP-transduced DCs (B) as determined by flow cytometry. Expression of cell surface molecules on DCs (MHC class II, CD40, CD80, and CD86) was evaluated by flow cytometry. The data are representative data for three independent experiments in which similar results were obtained. The shaded areas indicate binding by isotype-matched control antibodies.

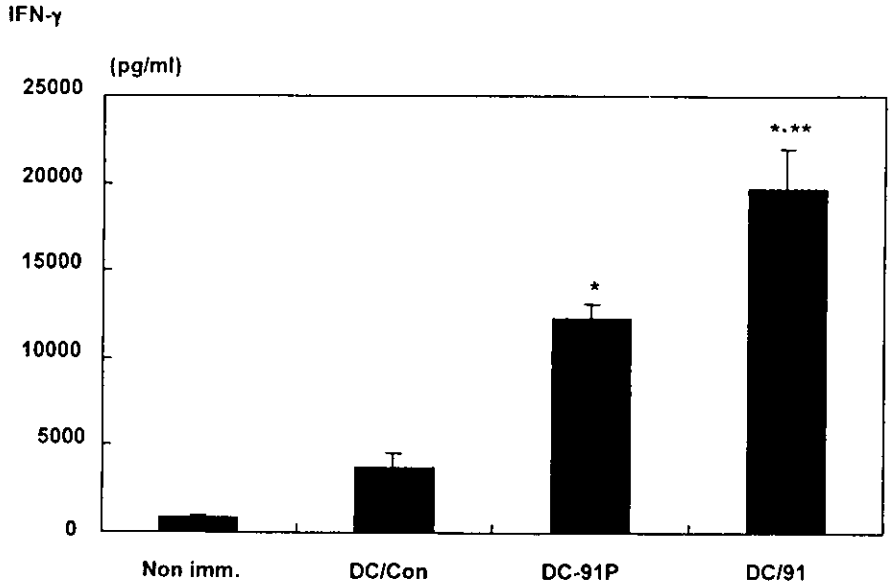


FIG. 3. IFN-γ secretion by LLO 91-99-stimulated splenocytes from mice immunized with EGFP- and LLO91-EGFP-transduced DCs or LLO91-pulsed DCs. BALB/c mice were immunized intravenously with EGFP- and LLO91-EGFP-transduced DCs or LLO91-pulsed DCs twice with a 1-week interval between the immunizations. Spleen cells from mice immunized with EGFP- or LLO91-EGFP-transduced DCs (DC/Con and DC/91, respectively) or LLO91-pulsed DCs (DC-91P) or from naïve mice (Non imm.) were harvested 8 weeks after the last immunization and stimulated in vitro by culturing in the presence of the LLO 91-99 peptide for 5 days. The concentrations of IFN-γ in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay. The data are the means ± standard deviations for five mice for each experimental group. One asterisk indicates statistical significance ($P < 0.001$) compared with DC/Con, and two asterisks indicate statistical significance ($P < 0.01$) compared with DC-91P.

dose of *L. monocytogenes* as the effector cells and LLO91-EGFP-transduced DCs as the target cells. The magnitude of the specific cell lysis of LLO91-EGFP-transduced DCs was much greater than that of control DCs (data not shown), indicating that the LLO 91-99 peptide is efficiently expressed and presented on the surface of LLO91-EGFP-transduced DCs.

**LLO91-transduced DC vaccination generates LLO 91-99-specific IFN-γ-producing cells.** We evaluated LLO 91-99 peptide-specific IFN-γ production by spleen cells from mice immunized with the LLO91-transduced DCs and compared it with that by spleen cells from mice immunized with LLO91-pulsed DCs. Upon stimulation with LLO 91-99 peptide, spleen cells from LLO91-transduced DC-vaccinated mice produced significantly higher levels of IFN-γ than spleen cells from mice immunized with control EGFP-transduced DCs and LLO91-pulsed DCs produced (Fig. 3), suggesting that immunization with LLO91-transduced DCs efficiently generates LLO 91-99-specific IFN-γ-producing cells in vivo. Of interest is the finding that spleen cells from mice immunized with control untreated DCs produced somewhat larger amounts of IFN-γ than spleen cells from naïve mice produced (Fig. 3). We also evaluated the levels of IFN-γ production by spleen cells from mice immunized with LLO91-transduced DCs, LLO91-pulsed DCs, and control EGFP-transduced DCs without LLO 91-99 peptide stimulation. The levels were very low compared with the levels observed with peptide stimulation, but they were higher than the level of production by spleen cells from naïve mice (data not shown).

**LLO91-transduced DC vaccination generates LLO 91-99-specific CTLs.** We next determined whether LLO 91-99-specific CTLs were generated in vivo following LLO91-EGFP-transduced DC or LLO91-pulsed DC vaccination. We observed less than 30% lysis of LLO 91-99-pulsed J774 target cells without in vitro peptide restimulation (data not shown). After in vitro restimulation of immune spleen cells with the LLO 91-99 peptide, the cells from LLO91-EGFP-transduced DC-immunized mice were able to lyse the peptide-pulsed J774 cells more effectively than the cells from control DC-immunized mice were (Fig. 4). The CTL activity of spleen cells from mice immunized with LLO91-EGFP-transduced DCs was similar to or somewhat weaker than that of spleen cells from mice immunized with a sublethal dose ($10^3$ CFU) of *L. monocytogenes* (data not shown). In addition, the CTL activity of spleen cells from mice immunized with LLO91-EGFP-transduced DCs was stronger than that of spleen cells from mice immunized with LLO91-pulsed DCs (Fig. 4). Furthermore, these CTL activities correlated well with the levels of LLO 91-99-specific IFN-γ production observed.

**LLO91-transduced DC vaccination provides protective immunity to a subsequent challenge with viable *L. monocytogenes*.** To ascertain whether immune responses in mice immunized with the DC vaccines were efficiently protective against lethal listerial infection, the vaccinated animals were challenged intravenously with *L. monocytogenes*, and the protection was assessed by quantifying the numbers of *L. monocytogenes* cells recovered from the spleens. The protection was highly significant in mice that received LLO91-transduced DCs compared with the protection in mice that received control DCs and

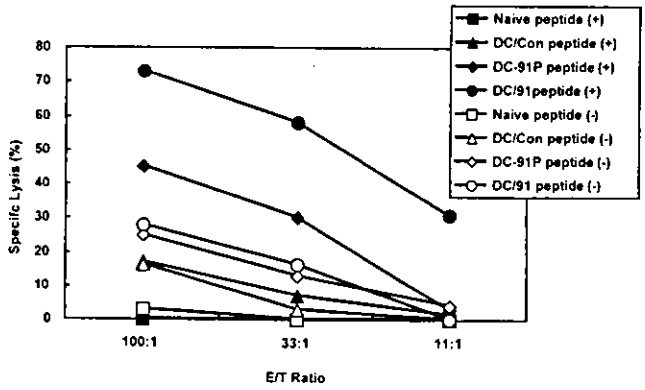


FIG. 4. Induction of CTL activity by LLO91-EGFP-transduced DC immunization. Spleen cells from mice immunized with EGFP-transduced DCs (DC/Con) (▲ and △), LLO91-pulsed DCs (DC-91P) (◆ and ◇), and LLO91-EGFP-transduced DCs (DC/91) (● and ○) were harvested 8 weeks after the last immunization and stimulated in vitro with LLO 91-99 peptide-pulsed spleen cells for 5 days. The percentage of specific lysis was determined by using J774 cells ($H2^d$) pulsed with (solid symbols) or without (open symbols) LLO 91-99 peptide as target cells. Immune spleen cells (effectors) were incubated with target cells in the effector-to-target-cell ratios (E/T Ratio) indicated on the *x* axis. Spleen cells from naïve mice were also harvested, and the same assay was performed simultaneously (■ and □) as a control assay. The data are representative of data obtained in four independent experiments.

LLO91-pulsed DCs and in untreated mice ($P < 0.01$) (Fig. 5). Although the spleens of mice vaccinated with *L. monocytogenes* appeared to contain fewer bacteria than the spleens of mice vaccinated with LLO91-transduced DCs, the difference in the mean bacterial numbers in the spleens of the two groups was not statistically significant.

Vaccination with LLO91-DCs is more effective than vaccination with the minigene DNA vaccine administered with the gene gun system. Previously, it was reported that LLO 91-99 minigene DNA vaccination efficiently evoked the epitope-specific CTL activity and consequently protective immunity to lethal infection by *L. monocytogenes* (30). To compare the efficacies of the genetically modified DC-based vaccine and the minigene DNA vaccine, BALB/c mice were also immunized with plasmid DNA by using the gene gun system. Upon stimulation with the LLO 91-99 peptide, splenocytes from LLO91-DC-vaccinated mice produced significantly higher levels of IFN-γ than splenocytes from mice immunized with the minigene DNA produced (Fig. 6A). In addition, the percentage of specific lysis of LLO 91-99 peptide-pulsed target cells tended to be higher with CTLs from genetically modified DC-vaccinated mice than with CTLs from the DNA-vaccinated mice (Fig. 6B). Finally, the DC vaccine induced significantly stronger protection against lethal infection by *L. monocytogenes* than the minigene DNA vaccine induced (Fig. 6C).

## DISCUSSION

As DCs are the most powerful antigen-presenting cells that initiate the primary immune responses, they have been attractive targets for developing vaccines against tumors (6, 21, 27). However, the potential of DCs for development of vaccines against intracellular bacteria has not been explored very much. Here, we evaluated DC vaccination against intracellular bacteria with a murine *L. monocytogenes* infection system. We analyzed a vaccination whose target is a single immunodominant CTL epitope of *L. monocytogenes* in order to make evaluation of the results clear.

There are several strategies for using DCs as vaccines, including ex vivo pulses with pathogen-derived peptides or Ags and transfer of genes encoding Ags to DCs. Viral and nonviral vector systems have been developed to obtain efficient gene transfer and stable gene expression in DCs. Among these systems, the retroviral transduction system is the most advantageous for long-term Ag presentation in vivo, as this system lets the transgene integrate into the chromosome, leading to gene expression throughout the life of the cell and its progeny (12, 18). In the present study, therefore, we used the retroviral transduction system to deliver Ags to DCs. We evaluated the immunizing effects of retrovirally transduced DC vaccination and compared them with those of peptide-pulsed DC vaccination. Since the transduction efficiency with retroviral vectors is relatively low, we employed the centrifugal enhancement method. Our data showed that BM-DCs were successfully transduced by recombinant retroviruses with 26.2 to 41.2% transduction efficiencies and that the transduced DCs still expressed the LLO 91-99 peptide. We found that the retrovirally transduced DC vaccine was more effective than the peptide-pulsed DC vaccine for eliciting antilisterial immunity. The stable expression of CTL epitope peptides on DCs is necessary for generating potent CTLs possessing lytic activity against infected cells. However, peptides pulsed onto DCs may stay

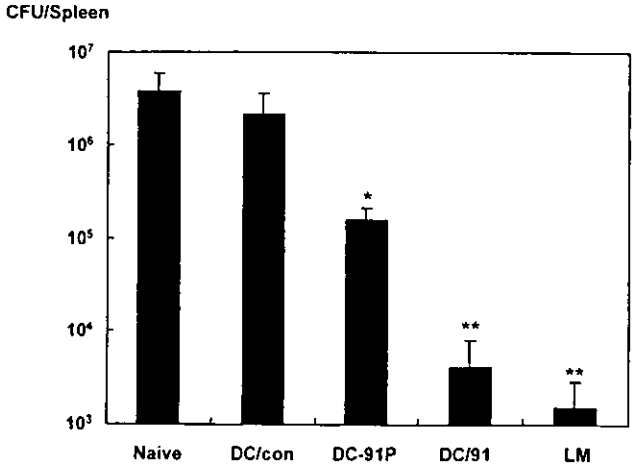


FIG. 5. Induction of protective immunity by immunization with LLO91-transduced DCs. Mice were immunized with EGFP-transduced DCs (DC/Con), LLO91-EGFP-transduced DCs (DC/91), or LLO91-pulsed DCs (DC-91P) twice with a 1-week interval between the immunizations or with a single sublethal dose ($10^3$ CFU) of *L. monocytogenes* (LM). Eight weeks after the last immunization, the mice were challenged with $3 \times 10^4$ CFU of *L. monocytogenes*. The numbers of bacteria in spleens from the immunized mice and naïve mice (Naive) were determined 72 h after challenge infection by plating 10-fold dilutions of tissue homogenates on plates. The data are the means ± standard deviations for five mice for each experimental group. One asterisk indicates statistical significance ($P < 0.01$) compared with Naive and DC/Con. Two asterisks indicate statistical significance ($P < 0.01$) compared with Naive, DC/Con, and DC-91P.

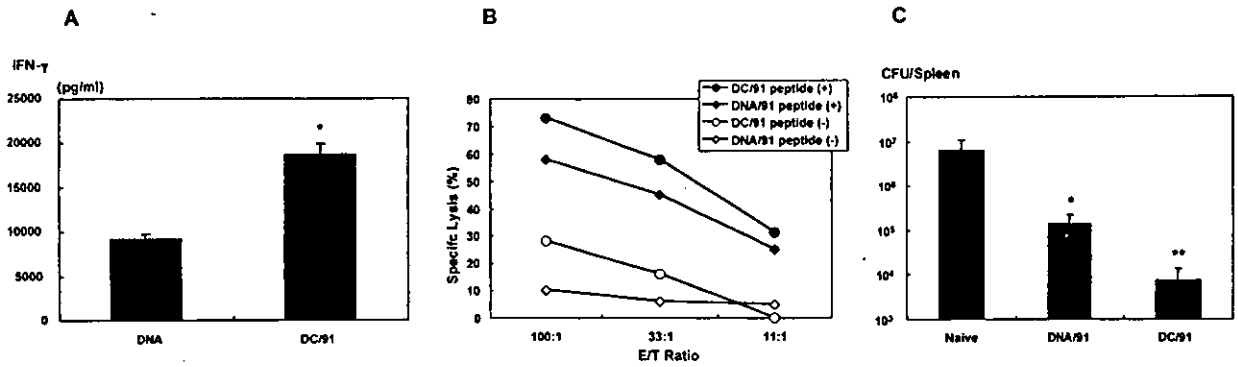


FIG. 6. Retrovirally transduced DC vaccination is more effective than naked DNA vaccination. (A) IFN-γ secretion by LLO 91-99 peptide-stimulated splenocytes from mice immunized with the LLO 91-99 minigene DNA vaccine (DNA) or the LLO91-EGFP-transduced DC vaccine (DC/91). IFN-γ secretion by spleen cells from the immunized mice was analyzed as described in the legend to Fig. 3. The data are means ± standard deviations for five mice for each experimental group. An asterisk indicates statistical significance ($P < 0.01$). (B) CTL activity of spleen cells from mice immunized with the LLO 91-99 minigene DNA vaccine (DNA/91) or the LLO91-EGFP-transduced DC vaccine (DC/91). Spleen cells from mice immunized with LLO91-transduced DCs (● and ○) or with LLO 91-99 minigene plasmid DNA (◆ and ◇) were harvested 8 weeks after the last immunization and stimulated in vitro with LLO91-pulsed splenocytes for 5 days. Then the cells were used for the CTL assay as described in the legend to Fig. 4. The percentage of specific lysis was determined by using J774 cells ($H2^d$) pulsed with the LLO 91-99 peptide (solid symbols) or medium alone (open symbols). Immune spleen cells (effectors) were incubated with target cells in the effector-to-target-cell ratios (E/T Ratio) indicated on the *x* axis. The data are representative of data obtained in four independent experiments. (C) Induction of protective immunity in mice immunized with the LLO 91-99 minigene DNA vaccine (DNA/91) or the LLO91-EGFP-transduced DC vaccine (DC/91). Mice immunized with the vaccines were examined by the in vivo *L. monocytogenes* protection assay as described in the legend to Fig. 5. Naïve mice (Naive) were also examined as controls. The data are means ± standard deviations for three independent experiments. One asterisk indicates statistical significance ($P < 0.01$) compared with Naive, and two asterisks indicate statistical significance ($P < 0.01$) compared with Naive and DNA/91.

bound to MHC molecules only transiently, and the peptide may be bound to MHC molecules in an unnatural way. In contrast, endogenous Ag synthesis within retrovirally transduced DCs ensures direct access of the Ag to the MHC class I Ag processing pathway in a natural way, efficiently stimulating Ag-specific CTLs (7, 9). In this context, retrovirally transduced DC vaccination may have important advantages over antigenic peptide-pulsed DC vaccination (29). Our data clearly demonstrate that the DC vaccine retrovirally transduced with DNA encoding a single immunodominant CTL epitope, LLO 91-99 of *L. monocytogenes*, protects against lethal challenge by the bacterium, and this protection is much more potent than that induced by peptide-pulsed DC vaccination.

Furthermore, we compared the efficacy of the retrovirally transduced DC vaccine with that of a naked DNA vaccine delivered with the gene gun system. Consistent with previous studies (30, 35), the present data showed that gene gun-mediated inoculation of plasmid DNA encoding LLO 91-99 induced the peptide-specific CTLs, as well as protective immunity to lethal infection by *L. monocytogenes*. After lethal listerial challenge, however, mice immunized with LLO91-transduced DCs had 1 log fewer CFU of *L. monocytogenes* in their spleens than mice immunized with the minigene DNA vaccine had. These data indicate that the antilisterial immunity induced by the genetically modified DC vaccine is much more effective than that induced by the naked DNA vaccine. The precise mechanisms responsible for the difference between the immunizing effects of the DC vaccine and the naked DNA vaccine are not clear. However, the genetically modified DC vaccine generated more CTL activity, as well as higher levels of IFN-γ secretion from splenocytes, than the naked DNA vaccine generated, which may have contributed to the enhanced protection observed in mice immunized with the DC vaccine. DCs have been shown to act as the principal cells initiating the immune responses after DNA vaccination by direct transfection and/or cross-priming (5, 8, 10, 17, 25). Therefore, it is reasonable to assume that introduction of genes encoding Ags directly into DCs is superior to naked DNA vaccination into the skin for developing protective immunity. In support of our data, a recent study demonstrated that immunization with murine DCs transfected with human gp100, a human melanoma-associated Ag, elicited more potent antitumor immunity than immunization with naked DNA encoding gp100 (33). Collectively, although DNA vaccines are more manageable in clinical applications than DC-based vaccines, genetically modified DC vaccines may be more effective than naked DNA vaccines for inducing protective immunity to intracellular bacteria.

This is the first report describing a highly efficacious vaccine against intracellular bacteria in which DCs retrovirally transduced with a minigene encoding a single immunodominant CTL epitope are used. Our findings should lead to a new strategy for creating vaccines against intracellular bacteria in the future.

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# Curcumin Prevents and Ameliorates Trinitrobenzene Sulfonic Acid–Induced Colitis in Mice

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*Background & Aims:* Curcumin is known to have a variety of pharmacologic effects, including antitumor, anti-inflammatory, and anti-infectious activities. The pleiotropic effects of curcumin are attributable at least in part to inhibition of transcriptional factor nuclear factor κB (NF-κB). However, the effect of curcumin on intestinal inflammation has hitherto not been evaluated. The aim of this study was to determine whether treatment with curcumin prevents and ameliorates colonic inflammation in a mouse model of inflammatory bowel disease. *Methods:* Mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis were treated with 0.5%, 2.0%, or 5.0% curcumin in the diet, and changes in body weight together with histologic scores were evaluated. Colonic T-cell subsets were characterized, and NF-κB in colonic mucosa was detected by immunohistochemistry. NF-κB activity in the colonic mucosa was evaluated using electrophoretic mobility shift assay. Cytokine messenger RNA expression in colonic tissue was assessed by semiquantitative reverse-transcription polymerase chain reaction. *Results:* Treatment of mice with curcumin prevented and improved both wasting and histopathologic signs of TNBS-induced colonic inflammation. Consistent with these findings, $CD4^+$ T-cell infiltration and NF-κB activation in colonic mucosa were suppressed in the curcumin-treated group. Suppression of proinflammatory cytokine messenger RNA expression in colonic mucosa was also observed. *Conclusions:* This study has shown for the first time that treatment with curcumin can prevent and improve murine experimental colitis. This finding suggests that curcumin could be a potential therapeutic agent for the treatment of patients with inflammatory bowel disease.

One major component of the imbalance of mucosal homeostasis in the pathogenesis of inflammatory bowel disease (IBD) is characterized by changes in cytokine production by macrophages and lymphocytes.[1] In various animal models of IBD, including the trinitrobenzene sulfonic acid (TNBS)-induced colitis model, increases in mucosal proinflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, IL-12, and interferon (IFN)-γ have been shown to play an important role in sustained inflammatory responses.[2–4] Furthermore, elevation of expression levels of IL-1β, IL-6, TNF-α, and IFN-γ in the colonic tissue from patients with IBD has been shown.[5] The expression of these proinflammatory cytokine genes is mainly regulated by the transcription factor nuclear factor κB (NF-κB).[6] In its inactive state, NF-κB predominantly exists as a heterodimer of p65 (RelA) and p50 subunits and resides in the cytoplasm associated with an inhibitory protein, IκB. On exposure to various stimuli, IκB undergoes rapid degradation. Free NF-κB translocates to the nucleus, where it binds to the promoter region of the target genes and induces the transcription.[6,7]

Two lines of evidence suggest that NF-κB may play a pivotal role in the development of IBD. First, previous studies have shown that NF-κB activation is strongly elevated in ulcerative colitis and Crohn's disease.[8–10] Second, it was shown in mouse models that p65 NF-κB antisense oligonucleotides efficiently prevent increased NF-κB activation and cytokine production and abrogate clinical and histologic signs of mucosal inflammation.[11,12] Thus, inhibition of NF-κB activation may be a promising target for the treatment of patients with IBD.

Sulfasalazine, mesalamine, and corticosteroids, which have been shown to inhibit activation of NF-κB, are generally used for the treatment of patients with IBD.[13–17] However, treatment with corticosteroids causes many undesirable side effects, such as impaired glucose tolerance, adrenal suppression, and increased risk of infection. Furthermore, although rare, treatment with sulfasalazine and mesalamine may also cause several severe side effects, such as hepatitis, pancreatitis, and bone marrow suppression.[18] Therefore, medications that cause

*Abbreviations used in this paper:* Ab, antibody; IFN, interferon; IL, interleukin; NF-κB, nuclear factor κB; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

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minimal side effects are desired for the treatment of patients with IBD.

Curcumin, widely used as a spice and responsible for the yellow color of curry, is a natural product of plants obtained from *Curcuma longa* Linn (turmeric).[19] Curcumin has been shown to have a variety of pharmacologic effects, including antitumor, anti-inflammatory, and anti-infectious activities.[20-25] Recently, curcumin was reported to inhibit the activation of NF-κB in different type of cells.[26-29] Jobin et al.[29] showed that curcumin blocks a signal upstream of NF-κB–inducing kinase and IκB kinase using intestinal epithelial cells. These observations suggest that curcumin may be useful in the treatment of patients with IBD.

In the present study, we administered curcumin to mice with TNBS-induced colitis to evaluate the beneficial effects of curcumin on intestinal inflammation. Here, we show that curcumin is able to down-regulate TNBS-induced colitis in a dose-dependent fashion. Furthermore, we show for the first time that administration of curcumin is capable of suppressing NF-κB activity and proinflammatory cytokine gene expression in colonic mucosa in murine TNBS-induced colitis. Our results suggest that curcumin may be of therapeutic value for patients with IBD.

## Materials and Methods

### Animals

In this study, 7- to 8-week-old male C57BL/6 and BALB/c mice (Japan SLC Co., Hamamatsu, Japan) weighing 21–23 g were used. All mice were maintained under specific pathogen-free conditions in our own animal facilities and handled according to institutionally recommended animal care guidelines.

### Diet and Reagents

Curcumin (purity, 99.9%) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). For dietary administration, curcumin was mixed with the diet to concentrations of 0.5% (wt/wt), 2.0%, or 5.0%. Mice were fed with either diet supplemented with curcumin or regular rodent chow as a control diet. All feeds were pelleted to avoid stratification and to ensure uniform feed and curcumin intake in the treated animals.

### Induction of Colitis

To induce colitis in C57BL/6 mice, 2.5 mg of the hapten reagent TNBS (Sigma Chemical Co., St. Louis, MO) in 50% ethanol (to break the intestinal epithelial barrier) was slowly administered into the lumen of the colon via a catheter fitted onto a 1-mL syringe with the animals under pentobarbital anesthesia, and they were then kept in a vertical position for 30 seconds. To induce colitis in BALB/c mice, 2.0 mg TNBS in 50% ethanol was administered. Control mice received 50% ethanol in phosphate-buffered saline (PBS) using the same technique as previously described. The total injection volume was 100 μL in both groups. Mice were killed 7 days after administration of TNBS.

### Treatment Protocols

To investigate the dosage effect, we administered 0.5%, 2.0%, or 5.0% curcumin just after administration of TNBS (0.5% curcumin-D0, 2.0% curcumin-D0, or 5.0% curcumin-D0, respectively) and treatment with curcumin was continued until day 7. To examine preventive and therapeutic effects, the experimental diet containing 2.0% curcumin was administered starting 3 days before (preventive mode 2.0% curcumin-Pre; preventive mode), just after (2.0% curcumin-D0; early therapeutic mode), or 2 days after (2.0% curcumin-D2; late therapeutic mode) administration of TNBS enema.

### Grading of Histologic Changes

Colons were histologically investigated on microscopic cross sections. Histologic changes were graded semiquantitatively from 0 to 4 according to previously described criteria[30] as follows: 0, no signs of inflammation; 1, very low level of leukocyte infiltration; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the colon wall; 4, transmural infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall.

### Histologic and Immunohistochemical Analysis

For histologic analysis, tissues were fixed in 10% formalin, and paraffin-embedded tissue sections were stained with H&E using standard techniques. For immunohistologic analyses, tissues were freshly frozen in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Five-micrometer cryostat sections were fixed in ice-cold acetone for 10 minutes and dried. To inactivate internal peroxidases, sections were incubated in 0.3% $H_2O_2$-PBS for 30 minutes and washed with PBS. To detect $CD4^+$ T cells, the sections were then incubated with biotin-conjugated rat anti-mouse CD4 monoclonal antibody (Ab) (PharMingen, San Diego, CA) in PBS containing 1% bovine serum albumin at 4°C overnight. On the following day, sections were washed in PBS and immunolabeled cells were visualized with streptavidin-peroxidase (Organon Teknika Corp., West Chester, PA) and diaminobenzidine (Sigma Chemical Co.). The sections were lightly counterstained with hematoxylin. Immunostaining for p65 and IκB was also performed after internal peroxidase inactivation. The sections were washed with PBS and then incubated with either of the following primary Abs at 4°C overnight: anti-p65 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-IκB polyclonal Ab (Santa Cruz Biotechnology) in PBS containing 1% bovine serum albumin. After a wash in PBS, secondary biotinylated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology) was applied for 1 hour at room temperature

and washed with PBS. The immunolabeled cells were visualized with streptavidin-peroxidase and diaminobenzidine. Counterstaining was not performed in these sections.

### Double Immunofluorescence Studies

We performed double immunofluorescence staining of anti-IκB Ab and anti–Mac-1 (CD11b) Ab. Sections pretreated with preimmune goat sera were incubated in a moist chamber at 4°C overnight with anti-IκB polyclonal Ab (Santa Cruz Biotechnology). After washing with PBS, secondary rhodamine-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology) and fluorescein isothiocyanate–conjugated anti-mouse Mac-1 monoclonal Ab (PharMingen) were applied for 1 hour at room temperature. After another wash with PBS, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for analysis by fluorescence microscopy. A Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with an HBO 100-W/z mercury lamp was used. For visualization of the fluorescein labeling, we used 2 different filters to detect rhodamine and fluorescein fluorescence.

### Nuclear Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to the method of Yang et al.[31] Briefly, the excised colon was washed with ice-cold PBS and homogenized in ice-cold lysis buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.1% [vol/vol] Igepal CA-630, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL leucine thiol). The homogenates were then incubated on ice for 45 minutes and centrifuged at 5000 rpm for 10 minutes at 4°C. The pellets were washed twice with 1.0 mL ice-cold lysis buffer and incubated on ice for 1 hour with 80 mL nuclear extraction buffer (20 mmol/L HEPES, pH 7.8, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L ethylenediaminetetraacetic acid, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.2% [vol/vol] Igepal CA-630, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL leucine thiol). The resultant homogenates underwent centrifugation (4°C) at 15,000 rpm for 15 minutes. The supernatants were collected and stored at −70°C. Protein concentrations were measured by the BioRad protein assay kit (BioRad Laboratories, Hercules, CA). For electrophoretic mobility shift assay, 8 μg of nuclear extracts was preincubated with 2 pg poly(dI) · (dC) (Pharmacia Fine Chemicals, Piscataway, NJ) in 20 μL reaction buffer (10% glycerol, 2% polyvinyl alcohol, 20 mmol/L HEPES, pH 7.9, 40 mmol/L KCl, 7 mmol/L $MgCl_2$, and 1 mmol/L dithiothreitol) for 10 minutes at room temperature. As a control, a 50-fold molar excess of cold NF-κB competitor oligonucleotide was added during preincubation. After preincubation, 0.5 ng of $^{32}P$ end-labeled NF-κB oligonucleotide probe (5′-CGGGGACTTTCCGCTGGGGACTT-TCCGCTTGAGCT-3′) was added to the reaction mixture and incubated for an additional 30 minutes. The reaction mixture was loaded onto a 4% polyacrylamide gel containing 0.25-fold Tris/borate/ethylenediaminetetraacetic acid buffer, and electrophoresis was performed at 120 V for 3 hours. Gels were dried and exposed to a Fuji imaging board and analyzed by a BAS1000 system (Fuji Photo Film, Tokyo, Japan).

### Reverse-Transcription Polymerase Chain Reaction

This method has been described previously.[32] Briefly, after extraction of total RNA from distal colon tissue by the acid guanidinium thiocyanate-phenol-chloroform method,[33] the RNA was converted to complementary DNA using random hexonucleotides in 20 μL of reverse-transcription reaction solution and then used for polymerase chain reaction. The primers used were as follows: β-actin sense, 5′-GCACCACACCTTCTACAATGAG-3′; β-actin antisense, 5′-AAATAGCACAGCCTGGATAG-CAAC-3′; IFN-γ sense, 5′-TCTGAGACAATGAACGCTAC-3′; IFN-γ antisense, 5′-GAATCAGCAGCGACTCCTTT-3′; IL-4 sense, 5′-ACAGAGCTATTGATGGGTCT-3′; IL-4 antisense, 5′-GTGATGTGGACTTGGACTCA-3′; IL-6 sense, 5′-TATGAAGTTCCTCTCTGCAA-3′; IL-6 antisense, 5′-CTTTGTATCTCTGGAAGTTT-3′; IL-12 (p40) sense, 5′-GGGACATCATCAAACCAGACC-3′; IL-12 (p40) antisense, 5′-GCCAACCAAGCAGAAGACAGC-3′; TNF-α sense, 5′-AGCCCACGTCGTAGCAAACCACCAA-3′; TNF-α antisense, 5′-ACACCCATTCCCTTCACAGAGCAAT-3′.

### Statistics

The results are expressed as means ± SE. Statistical significance of differences was determined by the Mann–Whitney $U$ test, with $P$ values $<0.05$ considered significant.

## Results

### Preventive Curcumin Therapy Improves the Mortality Rate of Mice With TNBS-Induced Colitis

In the present study, the mortality rate of C57BL/6 mice with TNBS-induced colitis was 35.5%, although that of control mice (administration of 50% ethanol without TNBS) was 0% (Figure 1). In early therapeutic mode, the mortality rates of 0.5% and 2.0% curcumin-treated mice with TNBS-induced colitis were 25.9% and 23.1%, respectively, and comparable to that of mice with TNBS-induced colitis. Treatment with 5.0% curcumin in early therapeutic mode decreased the mortality rate to 16.7%, which still was not significantly different from that of control mice. However, it is of particular interest that the mortality rate was 0% in mice treated with 2.0% curcumin in preventive mode ($P < 0.02$ against mice with TNBS-induced colitis), indicating that preventive treatment with curcumin is capable of protecting death of mice after administration of TNBS.

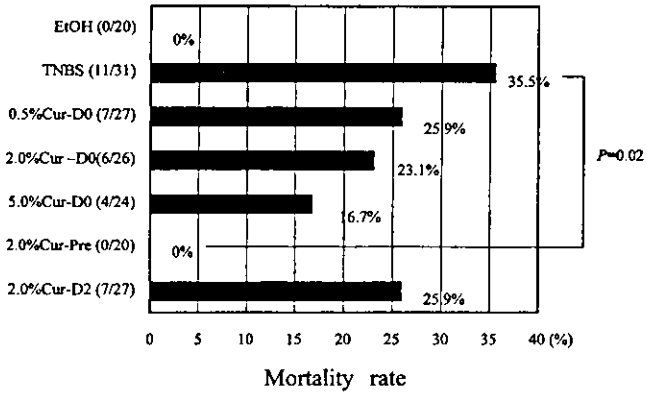



**Figure 1.** Preventive curcumin therapy protects against death after administration of TNBS. Values in parentheses indicate the number of surviving animals compared with the total number of animals 7 days after administration of TNBS. Results are shown as the mean mortality rate for each experimental group.

### Curcumin Therapy Affects Change in Body Weight of Mice With TNBS-Induced Colitis

We investigated whether a difference in curcumin concentration could affect the change in body weight caused by TNBS-induced colitis in C57BL/6 mice. As shown in Figure 2*A*, administration of TNBS caused a dramatic decrease in body weight (more than 15% after 2 days); body weight recovered gradually from day 3 but not fully to the initial weight in 7 days. Control mice receiving 50% ethanol without TNBS showed transient and slight loss of body weight. However, body weight recovered quickly from day 1 and increased to approximately 105% of the initial weight in 7 days. In the group of 2.0% curcumin-D0–treated mice, body weights recovered from day 2 and reached the same level as those of control mice on day 7. Body weights were significantly higher than those of the untreated mice on days 2–7 ($P < 0.01$). However, compared with 2.0% curcumin-D0–treated mice, 5.0% curcumin-D0–treated mice showed slightly less efficient recovery of body weight loss. No statistically significant difference was observed between 2.0% and 5.0% curcumin-D0–treated mice on days 2–7. In the group of 0.5% curcumin-D0–treated mice, body weights recovered from day 3 and increased to the initial body weight on day 7. No statistically significant difference was observed between 0.5% curcumin-D0–treated and untreated mice on days 2–7.

We next examined whether change in body weight is affected by timing of the start of administration of curcumin (Figure 2*B*). C57BL/6 mice administered 2.0% curcumin 2 days after TNBS enema (2.0% curcumin-D2) had higher body weights than untreated mice with TNBS-induced colitis on days 3 and 4 ($P < 0.05$). However, recovery of body weight loss was less than that of 2.0% curcumin-D0–treated mice. Unexpectedly, the recovery of body weight loss in 2.0% curcumin-Pre–treated mice was worse than that in 2.0% curcumin-D0–treated mice and comparable to that in 2.0% curcumin-D2–treated mice.

The strain of mice used may affect the results. Therefore, we also investigated whether curcumin could improve the body weight loss caused by TNBS-induced colitis in BALB/c mice (Figure 2*C*). In the group of 2.0% curcumin-D0–treated mice, body weights were significantly higher than those of the untreated mice on days 2–7 ($P < 0.01$). In BALB/c mice, 2.0% curcumin-Pre treatment was as effective as 2.0% curcumin-D0 treatment. Furthermore, we investigated whether 5.0% curcumin-D2 treatment could improve body weight loss caused by TNBS-induced colitis in BALB/c mice (Figure 2*D*) to assess whether this drug can be used in the therapeutic modality. Body weight seemed to be significantly higher than that of the untreated mice on days 5–7 ($P < 0.05$).

### Curcumin Therapy Improves Histologic Images of TNBS-Induced Colitis

We characterized the histologic features of colitis in C57BL/6 mice subjected to TNBS enema. In control mice (which received 50% ethanol alone), no signs or only a very low level of leukocyte infiltration in the colon was observed (Figure 3*A*). In mice with TNBS-induced colitis, the entire colonic wall became thick due to edema (macroscopically). The major lesions of colitis were observed in the distal half of the colon, and focal ulcers were detected in 60%–70% of colonic tissues. Distortion of crypts, loss of goblet cells, and infiltration of mononuclear cells were also observed (Figure 3*B*). These inflammatory cells were mainly $CD4^+$ T cells (Figure 4*A*).

We then evaluated the effects of curcumin on TNBS-induced colon lesions by performing the detailed dose-response study. In the first experiments, mice were treated with 0.5% curcumin, 2.0% curcumin, or 5.0% curcumin just after administration of TNBS. As shown in Figure 3*C–E*, histologic images were more improved as the concentration of curcumin administered to mice with TNBS-induced colitis was increased. The histologic scores of 2.0% curcumin-D0–treated mice and 5.0% curcumin-D0–treated mice were significantly lower than that of untreated mice with colitis ($P < 0.05$ and $P < 0.01$, respectively) (Figure 5). In addition, treatment with curcumin reduced $CD4^+$ T-cell infiltration, accompanied by improvement of histologic images (Figure 4*B*). Furthermore, we examined the effect of timing of the start of curcumin treatment on histologic scores. We

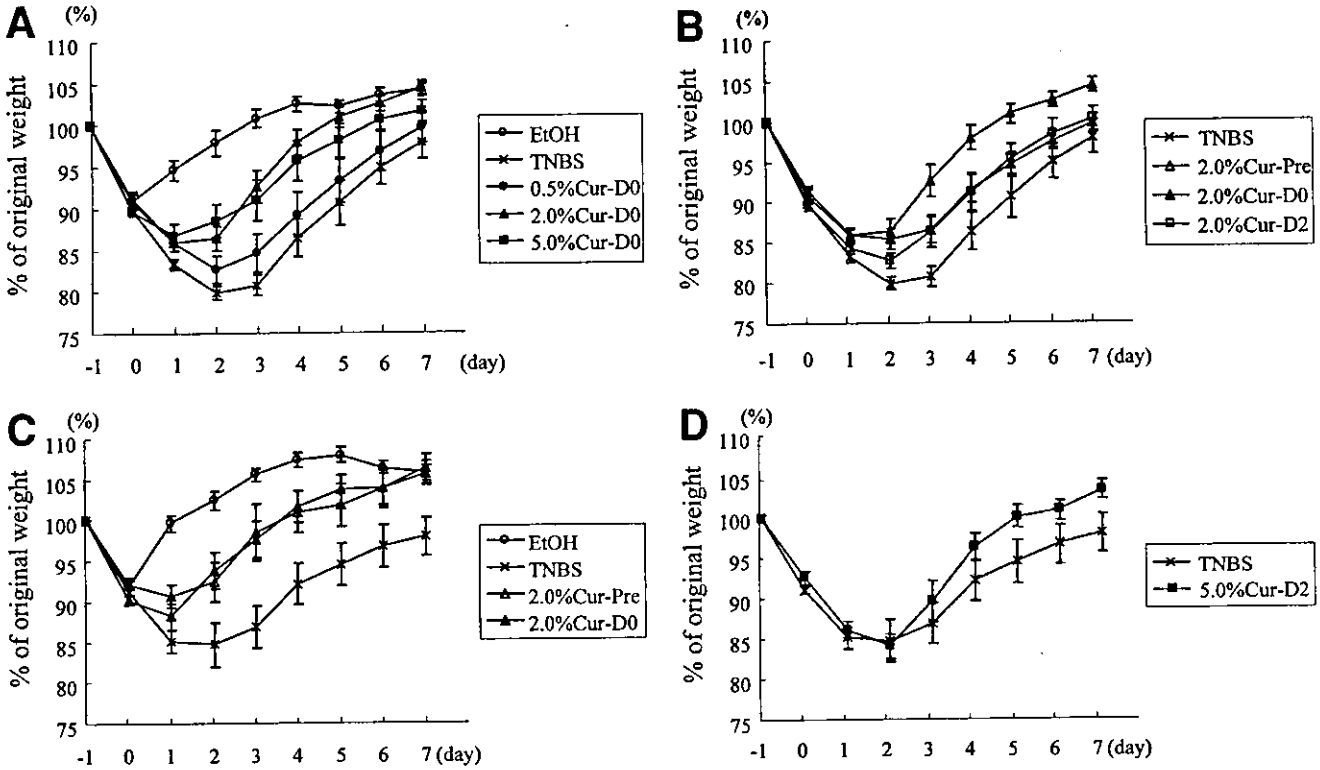



**Figure 2.** Weight changes in mice with TNBS-induced colitis treated with curcumin. (*A*) Effect of the dosage of curcumin on improvement in body weight. C57BL/6 mice were untreated (×) or treated with 0.5% (●), 2.0% (▲), or 5.0% (■) curcumin just after administration of TNBS. Instead of TNBS, 50% ethanol alone was administered to control mice (○). Change in body weight is expressed as a percentage of the original weight on day −1 (D −1). The body weights of mice treated with 2.0% curcumin was significantly higher than those of untreated mice on days 2–7 ($P < 0.01$). The body weights of mice treated with 5.0% curcumin were significantly higher than those of untreated mice on days 2, 3, and 4 ($P < 0.01$). No significant difference in weight was observed between 0.5% curcumin-D0–treated and untreated mice on days 2–7. Each bar represents the mean ± SE from 20 mice. (*B*) Effect of timing of the start of curcumin administration on body weight. C57BL/6 mice were untreated (×) or treated with 2.0% curcumin 3 days before (△; 2.0% curcumin-Pre, preventive mode), just after (▲; 2.0% curcumin-D0, early therapeutic mode), or 2 days after (□; 2.0% curcumin-D2, late therapeutic mode) administration of TNBS. The body weights of mice of early therapeutic mode were significantly higher than those of untreated mice on days 2–7 ($P < 0.01$). The body weights of mice of late therapeutic mode were significantly higher than those of untreated mice on days 3 and 4 ($P < 0.05$). However, no significant difference in weight was observed between mice of preventive mode and untreated mice. Each bar represents the mean ± SE from 20 mice. (*C*) Effect of curcumin on improvement in body weight in BALB/c mice. Mice were untreated (×) or treated with 2.0% curcumin 3 days before (△; 2.0% curcumin-Pre, preventive mode), or just after (▲; 2.0% curcumin-D0, early therapeutic mode) administration of TNBS. Instead of TNBS, 50% ethanol alone was administered in control mice (○). The body weights of mice of preventive mode and early therapeutic mode were significantly higher than those of untreated mice on days 2–7 ($P < 0.01$). Each bar represents the mean ± SE from 6 mice. (*D*) Effect of 5.0% curcumin-D2 treatment in BALB/c mice. The body weights of mice treated with 5.0% curcumin-D2 (■) were significantly higher than those of untreated mice (×) on days 5–7 ($P < 0.05$). Each bar represents the mean ± SE from 6 mice.

observed that as curcumin treatment for TNBS-induced colitis was started earlier, histologic images were more improved (Figure 3*F–H*). The histologic score of 2.0% curcumin-Pre–treated mice was significantly lower than that of untreated mice with colitis ($P < 0.01$) (Figure 5).

### Curcumin Therapy Suppresses NF-κB Activity of Colonic Mucosa in Mice With TNBS-Induced Colitis

Figure 6 shows immunohistochemical staining of NF-κB p65 and IκB in tissue sections from mice with TNBS-induced colitis and 2.0% curcumin–treated mice with TNBS-induced colitis. Mice with TNBS-induced colitis had p65 in the epithelial nuclei (Figure 6*A*, arrows), although IκB staining was unclear (Figure 6*B*). These findings may imply that degradation of IκB results in translocation of activated NF-κB into the nucleus. Treatment with 2.0% curcumin-D0 diminished p65 in the nuclei (Figure 6*C*), and IκB appeared around the epithelial nuclei in ring-shaped configurations (Figure 6*D*). Taken together, these findings suggest that curcumin is able to inhibit both the degradation of IκB and translocation of NF-κB into the epithelial nuclei induced by TNBS in colonic epithelial cells.

Infiltrating macrophages have been reported to be involved in intestinal inflammation by producing IL-18.[34] Curcumin has been reported to inhibit production of IL-12 by macrophages in vitro.[35] Accordingly, cur-

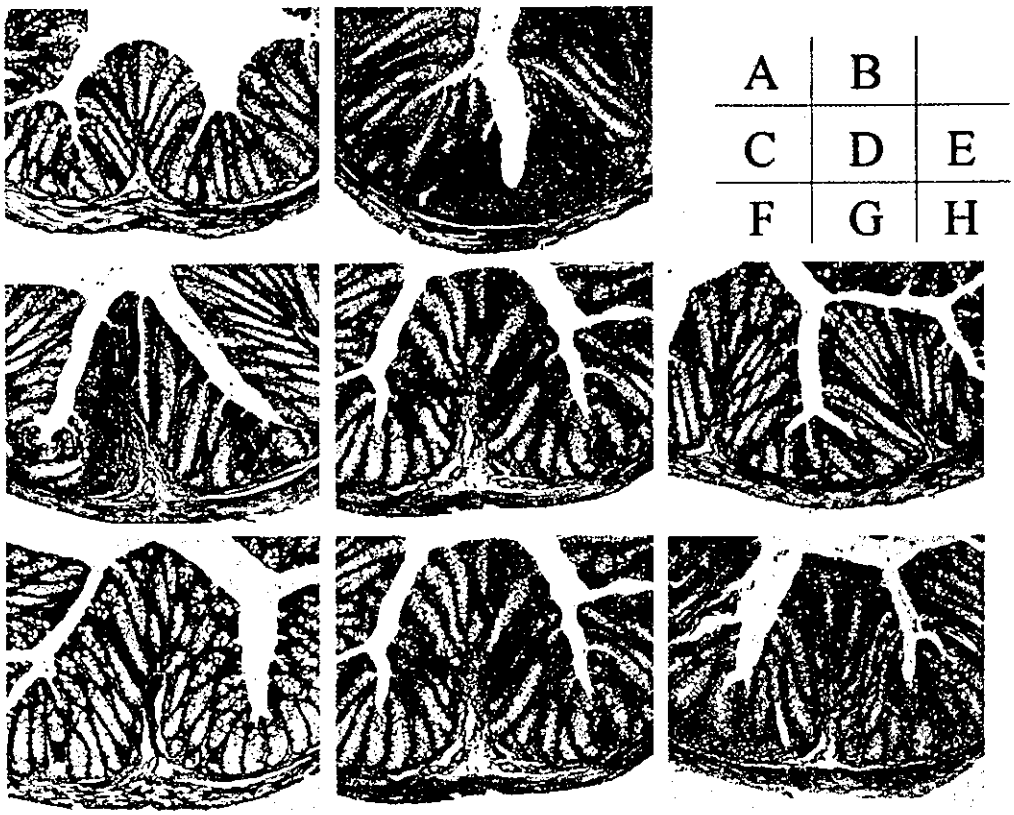



**Figure 3.** Histologic analysis of the colon in C57BL/6 mice. (*A*) Normal architecture of the colonic mucosa from mice treated with 50% ethanol alone. (*B*) Erosions of the epithelium, distortion of crypts, loss of goblet cells, and massive mononuclear cell infiltration in lamina propria in mice after administration of TNBS. (*C–E*) TNBS-induced colitis is dose-dependently improved by curcumin. Mice were treated with (*C*) 0.5%, (*D*) 2.0%, or (*E*) 5.0% curcumin just after administration of TNBS. (*F–H*) Improvement of TNBS-induced colitis is affected by timing of curcumin administration. Mice were treated with 2% curcumin in (*F*) preventive mode, (*G*) early therapeutic mode, or (*H*) late therapeutic mode. (Original magnification 50×.)

cumin may affect infiltration of macrophages in vivo. Therefore, we examined the effect of curcumin on IκB degradation in colonic macrophages by using a double immunofluorescence method. For this purpose, we used anti–Mac-1 Ab to detect the colonic macrophages. As shown in Figure 7*A*, Mac-1–positive cells were significantly increased in colonic mucosa from TNBS-colitis mice. However, most of these Mac-1–positive cells were

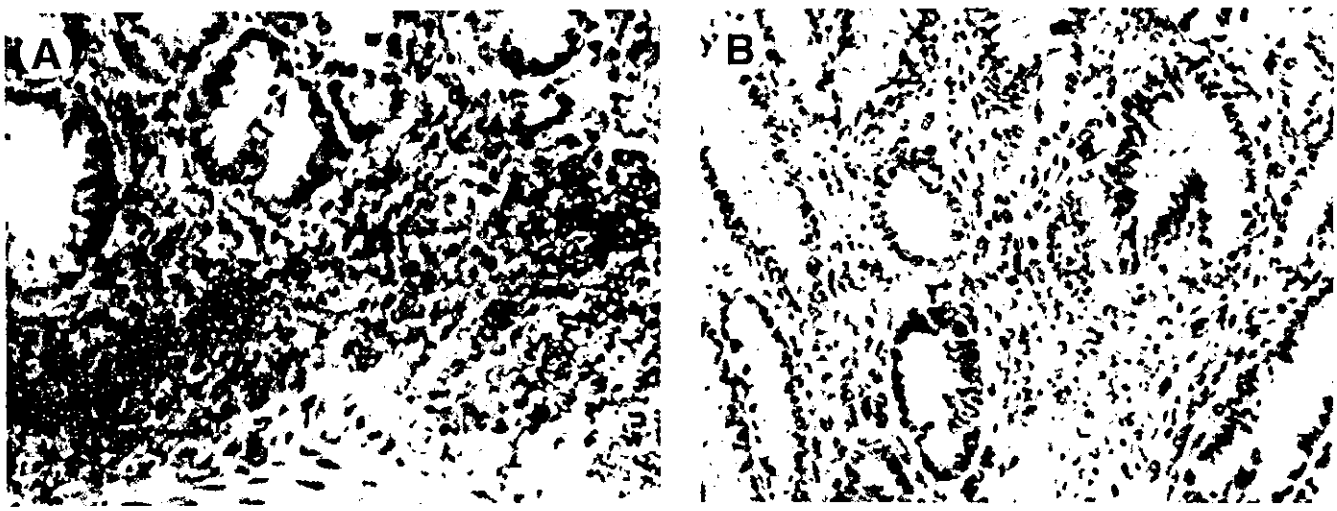
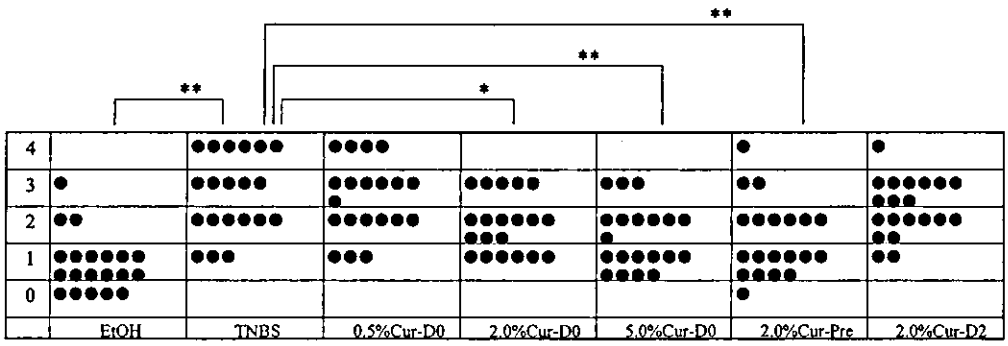



**Figure 4.** Detection of $CD4^+$ T cells in colonic mucosa. $CD4^+$ T cells infiltrated in colonic mucosa of (*A*) untreated mice with TNBS-induced colitis and (*B*) 2.0% curcumin–treated mice with TNBS-induced colitis were stained. $CD4^+$ T cells were markedly decreased in lamina propria of the colon of curcumin-treated mice compared with untreated mice with TNBS-induced colitis. (Original magnification 100×.)




*$P$<0.05 vs TNBS
**$P$<0.01 vs TNBS

**Figure 5.** Differences in inflammatory activity were confirmed by histologic grading of colon sections. Histologic scores for mice receiving 50% ethanol (Control), TNBS, curcumin (0.5%, 2.0%, or 5.0%) just after administration of TNBS (0.5% curcumin-D0, 2.0% curcumin-D0, or 5.0% curcumin-D0, respectively), or 2.0% curcumin 3 days before or 2 days after administration of TNBS (2.0% curcumin-Pre and 2.0% curcumin-D2, respectively). The magnitude of inflammatory changes in the colons was analyzed on H&E-stained cross sections of the colon. The histologic scores of 2.0% curcumin-D0–treated mice, 5.0% curcumin-D0–treated mice, and 2.0% curcumin-Pre–treated mice were significantly lower than that of untreated mice with colitis ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively; Mann–Whitney $U$ test). Twenty mice were examined for each group.

not stained with IκB (Figure 7*B* and *C*). On the contrary, a few Mac-1–positive cells were seen in colonic mucosa from 2.0% curcumin-D0–treated mice (Figure 7*D*), and these Mac-1–positive cells were clearly stained with IκB (Figure 7*E* and *F*). These findings suggest that curcumin is able to inhibit degradation of IκB of colonic macrophages.

Furthermore, we performed electrophoretic mobility shift assay using nuclear extracts of whole colonic tissues from control mice (which received 50% ethanol without TNBS) and TNBS-induced colitis mice with untreated or 2.0% curcumin-D0–treated TNBS-induced colitis (Figure 8). The administration of TNBS alone enhanced NF-κB DNA binding activity of nuclear extracts in the inflamed colonic tissue, which was suppressed by treatment with 2.0% curcumin-D0. Excess unlabeled specific oligonucleotides inhibited NF-κB mobility shift (Figure 8, lane 7), indicating that the DNA-protein complex is specific.

### Curcumin Alters the Expression of Cytokine Genes in the Colonic Mucosa in TNBS-Induced Colitis

Reverse-transcription polymerase chain reaction analysis showed increased messenger RNA levels for proinflammatory and inflammatory cytokines, such as IL-6, IFN-γ, TNF-α, and IL-12, in colon specimens from mice treated with TNBS (Figure 9). On the other hand, messenger RNA expression of these cytokines was dramatically suppressed in colon specimens from curcumin-treated mice with TNBS-induced colitis and comparable to that in control mice (which received 50% ethanol without TNBS). IL-4 messenger RNA expression was not detected in any of the 3 groups in our experimental conditions. These findings suggest that treatment with curcumin can suppress proinflammatory and inflammatory cytokine production in mice with TNBS-induced colitis.

## Discussion

From the findings described in this report, we drew the following conclusions concerning the effect of curcumin on TNBS-induced colitis. (1) Curcumin is able to completely prevent death due to TNBS-induced colitis. (2) Administration of curcumin significantly attenuates both wasting disease and colonic inflammation induced by TNBS. (3) Treatment with curcumin markedly reduces $CD4^+$ T-cell infiltration in the lamina propria. (4) NF-κB activation in colonic mucosa is suppressed by treatment with curcumin. (5) Treatment with curcumin inhibits TNBS-induced expression of proinflammatory and inflammatory cytokine genes in the colonic mucosa.

To investigate whether curcumin has a therapeutic effect on IBD, we administered curcumin to mice with TNBS-induced colitis. In our experimental conditions, intracolonic administration of TNBS induced body weight loss that lasted for 7–10 days and some mice with severe colitis died. TNBS-induced colitis has been reported to be associated with Th1 cell responses and to be immunologically similar to Crohn's disease, which is also characterized by a predominance of Th1 cells.[36,37]

First, we investigated whether curcumin therapy could improve the mortality rate of mice with TNBS-induced colitis. Of particular interest are our findings that preventive administration (2.0% curcumin-Pre) enabled all of the mice with TNBS-induced colitis to survive, whereas early and late therapeutic modes did not

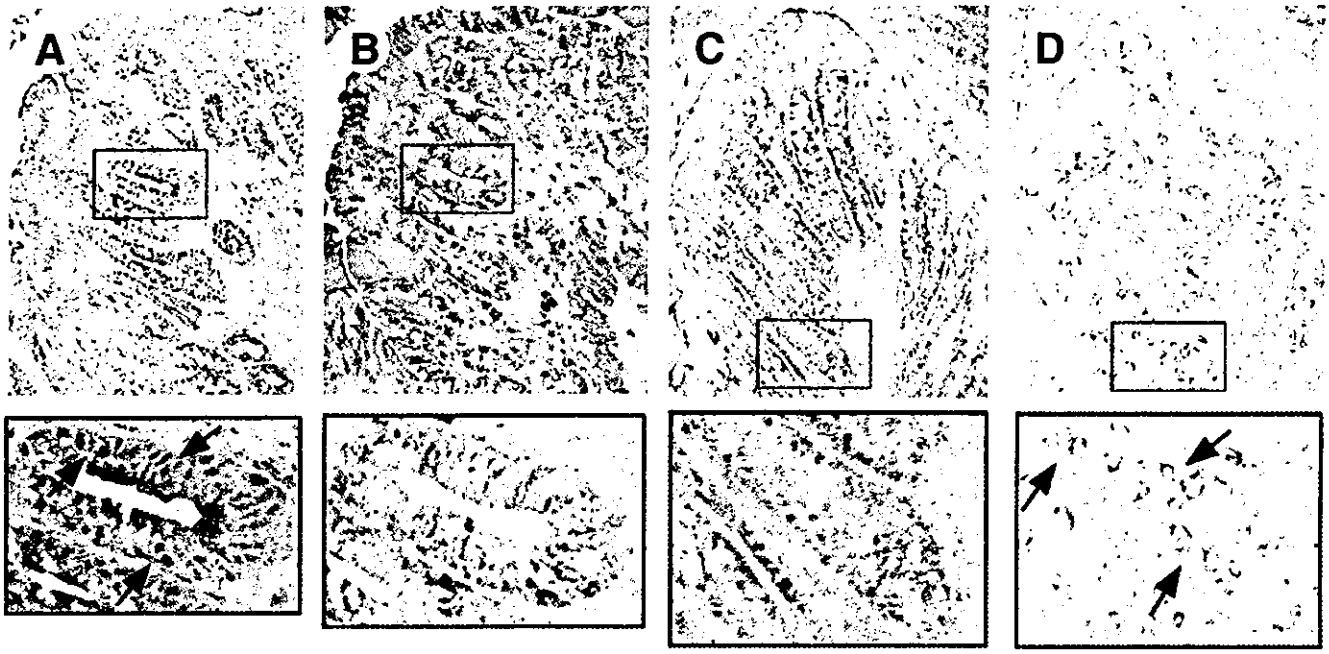



**Figure 6.** Effect of curcumin treatment on NF-κB activation in situ. Tissue sections from (*A* and *B*) untreated mice with TNBS-induced colitis and (*C* and *D*) 2.0% curcumin–treated mice with TNBS-induced colitis. Specimens were stained immunohistologically with (*A* and *C*) anti-p65 and (*B* and *D*) IκB Abs. In untreated mice with TNBS-induced colitis, p65 was detected in epithelial nuclei (*A*; arrows) but not in 2.0% curcumin–treated mice. (*C*) Staining for p65 in nuclei was unclear. In contrast, IκB was stained for around epithelial nuclei in a ring-shaped configuration in 2.0% (*D*; arrows) curcumin–treated mice but not stained for in (*B*) untreated mice with TNBS-induced colitis. (Original magnification 200×.)

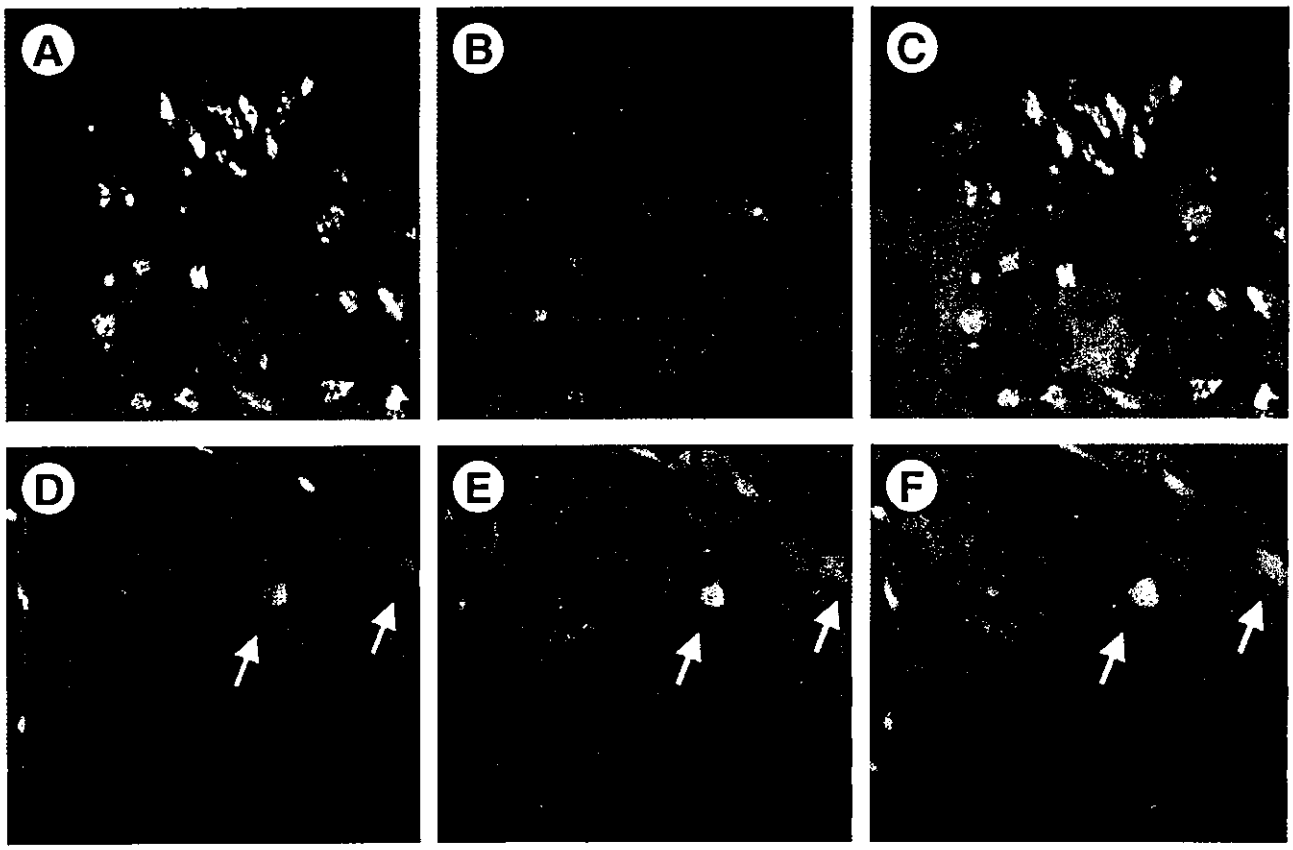



**Figure 7.** Immunofluorescence analysis of Mac-1 and IκB. The colonic mucosa from (*A–C*) untreated mice with TNBS-induced colitis and (*D–F*) 2.0% curcumin–treated mice with TNBS-induced colitis was analyzed with double immunofluorescence staining with anti–Mac-1 (*green*) and anti-IκB (*red*) Abs. (*A*) Mac-1–positive cells were significantly increased in colonic mucosa from mice with TNBS-induced colitis, and in the same section, (*B*) a few IκB-positive cells were seen. (*C*) Double immunofluorescence staining showed that most of these Mac-1–positive cells were not stained with IκB. In mice treated with 2.0% curcumin-DO, a few Mac-1–positive cells were seen (*D*; arrows) and, in the same section, (*E*) many IκB-positive cells were seen. Double immunofluorescence staining showed that these Mac-1–positive cells were also clearly stained with IκB (*F*; arrows). (Original magnification 200×.)

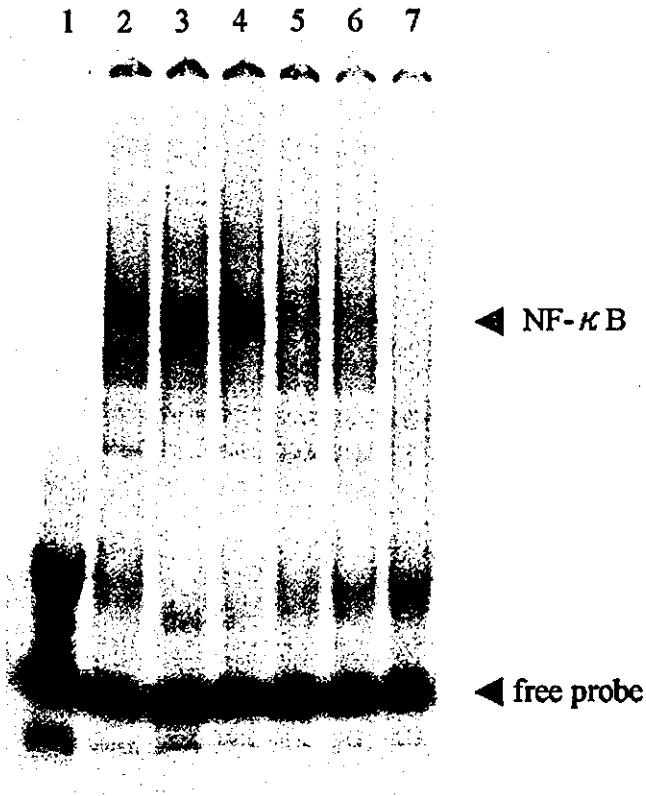



**Figure 8.** Electrophoretic mobility shift assay for NF-κB. NF-κB activity was up-regulated in nuclear extracts from TNBS-induced intestinal inflammation and suppressed by curcumin therapy. Nuclear extracts of the colonic tissues from mice treated with 50% ethanol alone (*lane* 1), untreated mice with TNBS-induced colitis (*lanes* 3 and 4), and 2.0% curcumin-D0–treated mice with TNBS-induced colitis (*lanes* 5 and 6) were analyzed. In *lane* 7, unlabeled probes of 50× molar excess were added to the reaction mixture of *lane* 3. As a control, labeled probes without nuclear extracts were also shown in lane 1.

improve the mortality rate significantly. These findings suggest that curcumin plays an important role, especially in the prevention of the initial event of inflammation.

We next showed that administration of curcumin significantly reduced TNBS-induced body weight loss. It is of particular interest that the late therapeutic mode (administration of curcumin 2 days after TNBS enema) was also effective in recovery of body weight loss. However, the body weight loss of mice treated with curcumin was not always improved dose dependently. Treatment with 5.0% curcumin-D0 was slightly less effective in improving body weight than 2.0% curcumin-D0. Two speculations can be made to reconcile these contradictory results. First, curcumin may have toxicity. Toxicity studies with turmeric or curcumin in animals showed no histopathologic changes when these substances were fed to rats, dogs, guinea pigs, or monkeys (0.5–2 g/kg) for 8–60 weeks.[38] In humans, the pharmacologic safety of curcumin was shown by nontoxic consumption of up to 100 mg/day.[39,40] In our study, 0.5%–5.0% curcumin-treated mice are supposed to take curcumin at doses of 0.75–7.5 $g \cdot kg^{-1} \cdot day^{-1}$. However, no macroscopic abnormalities were observed in such mice, suggesting that curcumin is unlikely to be toxic. A second possibility is insufficient intake of nutrition because 5.0% of the diet was occupied with curcumin, which has no calories, in 5.0% curcumin–treated mice. We also administered 5.0% curcumin to the control mice (which received 50% ethanol without TNBS) and found that the body weight on day 7 was 102% of initial body weight, which was less than that of control mice receiving normal diet (data not shown).

It is also curious that preventive treatment with curcumin (2.0% curcumin-Pre) was less efficient in improving body weight than early therapeutic treatment (2.0% curcumin-D0). This may be due to the difference in mortality rate between preventive and therapeutic treatments. Preventive treatment enabled all mice to survive, even when severe weight loss occurred, thereby resulting in poor recovery of mean body weight. In the case of BALB/c mice, body weights of 2.0% curcumin-D0–treated and 2.0% curcumin-Pre–treated mice were significantly higher than those of untreated mice. There was no difference in body weight change between 2.0% curcumin-D0–treated and 2.0% curcumin-Pre–treated BALB/c mice. This may be due to no difference in the mortality rate among 2.0% curcumin-D0–treated, 2.0% curcumin-Pre–treated, and untreated groups (14.3% in all the groups).

The histologic scores of mice treated with curcumin were dose-dependently improved. Along with histologic improvement, $CD4^+$ T-cell infiltration decreased in the colonic mucosa of mice treated with curcumin.

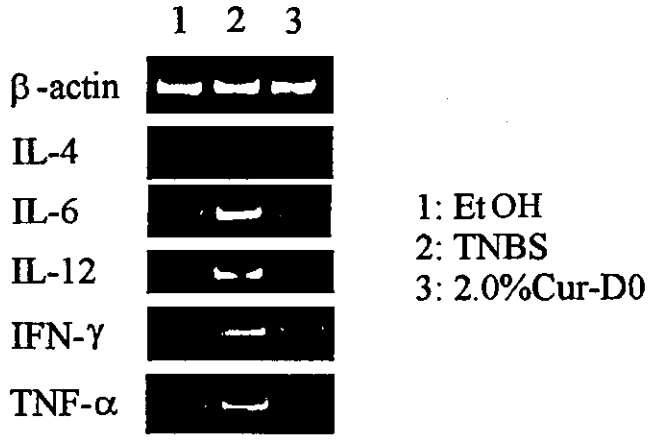



**Figure 9.** Detection of messenger RNAs for cytokines by reverse-transcription polymerase chain reaction. Colon specimens were derived from mice treated with 50% ethanol alone (*lane* 1), TNBS (*lane* 2), or 2.0% curcumin just after administration of TNBS (*lane* 3). Reverse-transcription polymerase chain reaction was used to detect messenger RNAs of IL-4, IL-6, IL-12, IFN-γ, and TNF-α.

To investigate the effect of curcumin on NF-κB activity in vivo, we performed immunohistochemical staining for NF-κB p65 and IκB. Our findings indicate that curcumin is capable of inhibiting degradation of IκB and translocation of NF-κB into the nuclei of the colonic epithelial cells and macrophages. We also showed that curcumin is capable of inhibiting NF-κB activity in electrophoretic mobility shift assay using nuclear extracts of whole cells of the colonic tissue.

The increased proinflammatory cytokines in the intestinal mucosa are believed to be an important factor in the pathophysiology of intestinal inflammation in human IBD.[5] However, the pivotal elements in the regulation of the increased inflammatory activity remain unclear. Important candidates are transcription factors, such as NF-κB, that bind to promoter regions of target genes and are involved in the regulation of proinflammatory gene transcriptions.[6] Indeed, it has been reported that NF-κB may play a pivotal role in the development of human IBD.[8–10] Therefore, inhibition of NF-κB–driven transcriptional control of proinflammatory cytokines may be an important target to treat human IBD.

NF-κB inhibitors, such as curcumin, are known to inhibit expression of an array of κB-inducible genes, such as proinflammatory cytokines including TNF-α, IL-6, and IL-12 as well as adhesion molecules and chemokines such as intercellular adhesion molecule 1, IL-8, and monocyte chemotactic protein 1.[6] Here, we found suppression of proinflammatory cytokine gene expression, including genes for TNF-α, IL-6, and IL-12, in the colon tissue of mice treated with curcumin. By blocking NF-κB activation, curcumin may inhibit early steps of inflammation and modulate up-regulation of multiple proinflammatory genes by interrupting the downstream inflammatory cascade. Therefore, treatment targeting the NF-κB/IκB system seems a conceptually superior approach compared with blocking each single cytokine.

Studies on the metabolism of curcumin have shown that it is rapidly metabolized to glucuronide and sulfate conjugates that are excreted primarily in bile and to a lesser extent in urine.[41–43] Low or undetectable blood levels of unchanged curcumin were observed after oral administration.[43,44] It is unclear if this is due to poor absorption or efficient first-pass metabolism. Ammon and Wahl[39] reported that curcumin was found in the cecum after oral ingestion, a finding believed to be due to poor absorption. However, luminal curcumin may have topical activity on colonic epithelial cells independent of systemic absorption.[29] Oral intake of curcumin seems to readily achieve the therapeutic concentrations in the intestinal mucosa in vivo.

In addition to its anti-inflammatory effect, curcumin has been reported to have chemopreventive activity in animal models of colon cancer.[44–46] Although the mechanism is not well understood, the chemopreventive activity of curcumin has been suggested to be related to inhibition of cyclooxygenase 2 expression via modulation of signaling pathways that regulate stability of NF-κB sequestering protein IκB.[46] The most important clinical issue in the management of patients with long-standing IBD is an increased risk for development of dysplasia and neoplasia. It is conceivable that curcumin not only improves colitis but also may inhibit the development of colorectal cancer in patients with IBD.

At present, sulfasalazine and mesalamine are generally used to treat patients with mild to moderate IBD; for more severe IBD, corticosteroids are generally used in addition to sulfasalazine or mesalamine. However, these drugs are sometimes difficult to administer because of their side effects. Given that curcumin has little toxicity, as previously described, it can be safely given to patients with IBD.

In conclusion, we have shown that curcumin can significantly attenuate the colonic injury and inflammation induced by TNBS in mice. We have also shown that treatment with curcumin suppresses NF-κB activity in the colonic mucosa of mice with TNBS-induced colitis. Our findings suggest that curcumin may be of therapeutic value for the treatment of IBD in humans.

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