

Antiviral Effects of Cyclosporin A in Neonatal Mice With Rotavirus-Induced Diarrhea

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See “Re-CYCLing Rotavirus” by Fuchs on page 1.

ABSTRACT

Objectives: Because rotavirus gastroenteritis is associated with high morbidity and mortality especially in developing countries, it is necessary to develop antirotavirus drugs for the treatment of rotavirus infection. Previous studies have demonstrated that cyclosporin A (CsA) has antiviral properties against rotavirus. Its effect has not yet been evaluated against rotavirus diarrheal disease. The aim of this study was to assess the antirotavirus efficacy of CsA in neonatal mice after induction of rotavirus diarrhea.

Methods: Suckling mice were inoculated with murine rotavirus. On the onset of diarrhea, mice were given different concentrations of CsA. To evaluate the effects of CsA on reduction of rotavirus diarrhea, diarrhea score, fecal virus shedding, and pathological lesion change in the small intestine, messenger RNA (mRNA) expression levels in the small intestine and spleen of mice were measured for type I interferon (IFN- α and IFN- β), inflammation-related cytokines (interleukin [IL]-8, IL-10, IFN- γ , and tumor necrosis factor- α), and inflammatory signaling pathways (p38, c-Jun N-terminal kinase, activator protein-1, and nuclear factor-kappa B).

Results: Among virus-inoculated and CsA-treated groups, a dose of 5 mg \cdot kg⁻¹ \cdot day⁻¹ of CsA inhibited diarrhea and improved fecal virus shedding and intestinal lesion changes. IFN- β mRNA expression was significantly increased in rotavirus-induced diarrhea mice treated with 5 mg \cdot kg⁻¹ \cdot day⁻¹ of CsA, whereas the mRNA expression levels of inflammation-related cytokines (IL-8, IL-10, IFN- γ , and tumor necrosis factor- α) and inflammatory signaling pathways (p38, c-Jun N-terminal kinase, activator protein-1, and nuclear factor-kappa B) were markedly decreased. Antiviral effects of CsA were dose dependent.

Conclusions: CsA can inhibit rotavirus infection in neonatal mice through its antiviral properties. The mechanism for this may be through CsA suppression of inflammation by viral inhibition in animal models.

Key Words: cyclosporin A, diarrhea, gastroenteritis, rotavirus

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Rotavirus is a leading cause of gastroenteritis, malnutrition, and diarrhea in infants and young children, leading to approximately 500,000 deaths worldwide per year in children <5 years old, mostly in developing countries (1,2). The availability of 2 vaccines (Rotarix; GlaxoSmithKline Biologicals, London, and RotaTeq; Merck, Kenilworth, NJ) has reduced the global burden of dehydrating gastroenteritis caused by rotavirus (3,4); however, these vaccines are not yet been distributed globally, nor are they highly effective in some impoverished settings (5). Therefore, there is still a need for improved vaccines and other treatments to prevent the severe disease caused by rotavirus.

Cyclosporin A (CsA) is known as a strong immunosuppressant agent used for the treatment of kidney, liver, heart, and other organ transplantation (6). CsA indirectly elicits an immunosuppressive response via an interaction with peptidyl prolyl *cis/trans*-isomerase (PPIase) cyclophilins (CyPs) (7). The CsA/CyP complex interacts with calcineurin to inhibit phosphatase activity. This calcineurin inhibition results in the suppression of immune responses (8). In the last decade, CsA has been shown to suppress the replication of a variety of viruses such as hepatitis C virus, hepatitis B virus, mouse cytomegalovirus, and HIV, with antiviral activity mediated by the inhibition of CyPs (9–12). In addition, CsA has been shown to suppress the influenza viral replication through CyP A (CyPA)-dependent and -independent pathways (13). Similarly, we also demonstrated that CsA inhibits rotavirus replication in vitro and cures rotavirus-induced diarrhea in neonatal mice through a CYPA PPLase-independent pathway (14); however, there is little evidence whether CsA has an effect on the early overall responses and the development and regulation of lymphocytes in animals or humans with rotavirus infection.

A host typically responds to viral infection through both innate and specific arms of the immune response. Innate immunity occurs in the early phase of infection and provides the first line of host defense against pathogen invasion, with activation of proinflammatory signaling pathways and the rapid production of cytokines and chemokines. In contrast, specific adaptive immunity appears in the later period of infection and builds up the full spectrum of immune defenses, including cellular and humoral immunity. Rotavirus infection induces innate and adaptive immune responses, including production of cytokines and chemokines and virus-specific antibodies (15,16). Studies have shown that the levels of several cytokines, such as interferon- α (IFN- α), IFN- β , IFN- γ , interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α), can become elevated in vitro and in vivo (17–21). Activation of nuclear factor-kappa B (NF- κ B), activator protein (AP)-1, p38, and c-Jun N-terminal kinase (JNK) has been associated with rotavirus replication and rotavirus-induced proinflammatory expression (22–24). These symptoms are similar to inflammatory bowel disease. We know that CsA has been used for the treatment of inflammatory bowel disease and is considered abroad to be an effective drug for the past 20 years. CsA can inhibit the aggregation of regulatory T cells to plays an important anti-inflammatory role (25). It is as yet unknown whether CsA modulates a proinflammatory reaction and whether NF- κ B, AP-1, p38, and JNK signaling pathways are

induced by rotavirus infection in animals or humans. To further our understanding of the influence of CsA on rotavirus-induced diarrhea, as well as the effect on type I IFNs and inflammatory cytokines, we investigated diarrhea, fecal virus shedding, histological lesion changes, type I IFNs and cytokine expression, and cellular signaling events in mice. In this study, we report that CsA cures rotaviral enteritis by antiviral effects, and that CsA also increases IFN- β messenger RNA (mRNA) expression, which may counteract inflammation.

METHODS

Cell and Virus

Murine rotavirus, which was originally obtained from Harry Greenberg (Stanford University Medical School, Palo Alto, CA), was cultured in MA-104 monkey kidney cells. Virus pellets were suspended in TNC buffer and stored in aliquots at -80°C . Virus titer was determined by a fluorescent-focus assay in triplicate in 96-well flat bottom plates (Corning Incorporated, Corning, NY), and expressed as fluorescence focus-forming units per milliliter (FFU/mL).

Animals and Experimental Design

Three-day-old suckling specific pathogen-free BALB/c mice with their mothers were obtained from the Animal Research Centre of the Third Military Medical University (Chongqing, China). They were maintained in filter-topped cages as previously described (14). They were randomly divided into 5 groups: mock inoculated and mock treated, virus inoculated and mock treated, virus inoculated and treated with $1\text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of CsA, virus inoculated and treated with $2.5\text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of CsA, and virus inoculated and treated with $5\text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of CsA. Each group contained 10 suckling mice. At 3 days of age, mice from groups 2 to 4 were inoculated with $30\text{ }\mu\text{L}$ of the murine rotavirus containing a virus titer of 10^7 FFU/mL through oral gavages. After day postinoculation (DPI) 1, the mice showed signs of diarrhea, and the severity of diarrheal illness was assessed by the evaluation of fecal material as described (26). Among these groups, 3 groups were treated 3 times daily with 1, 2.5, or 5 mg/kg of CsA for 3 days, respectively. The experiments were performed according to national regulations and approved by the local animal ethics committee.

Diarrhea Score

After the inoculation of murine rotavirus, the antiviral effect of CsA was assessed daily by the examination of fecal material. A 4-point rating system was used to evaluate fecal consistency and color: 1, normal; 2, loose feces; 3, yellow-green feces; 4, watery feces (26). Stools with a score of ≥ 2 were considered to be a symptom of diarrhea. Mice from which no stool sample could be obtained were considered as having no diarrhea. The severity of diarrheal illness was determined by dividing the sum of all scores by the number of total mice.

Detection of Rotavirus in Fecal Specimens

Fecal samples were collected from each individual mouse at 0 to 7 DPI and homogenized in 0.01 mol/L phosphate-buffered saline (pH 7.2) in a 10% (wt/vol) solution or suspension and centrifuged (1200g for 20 minutes). The supernatants were collected and rotavirus shedding was determined through rotavirus antigen detection by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The test was

considered positive if the optical density at 450 nm (OD_{450}) of the well containing stool minus the OD_{450} of control wells was ≥ 0.1 .

Histological Examination and Morphometry

Mice were immediately necropsied after euthanasia, and the small intestines were collected after removing intestine contents. Each part of small and large intestines was dissected and fixed in 10% buffered formalin solution. Serial 5- μm sections from paraffin-embedded blocks were stained by hematoxylin and eosin and examined under the light microscope. The crypt depth and villus height were measured using Image Pro Plus 5.1 software (IPP5.1, Media Cybernetics, Bethesda, MD).

Real-Time Reverse-Transcription Polymerase Chain Reaction Using Lightcycler 480 RNA Master Hydrolysis Probes

Using a primer pair specific to the nonstructural protein 3 (*NSP3*) gene of rotavirus A, a 1-step real-time reverse-transcription polymerase chain reaction (RT-PCR) assay based on Lightcycler 480 RNA Master Hydrolysis Probes (Roche Applied Sciences, Mannheim, Germany) was carried out to quantify rotavirus RNA in the fecal samples, as described previously (27). Briefly, the total RNA extracted from each fecal sample was subjected to reverse transcription at 61°C for 3 minutes; plates were immediately cooled on ice for 2 minutes, followed by 95°C for 5 minutes to activate the DNA polymerase and 45 cycles: 95°C for 5 seconds, 60°C for 45 seconds, and 72°C for 1 minute. The amount of rotavirus RNA in different stool samples was quantified based on the standard curve derived from 10-fold serial dilutions of plasmid DNA, which was used as an external standard for all quantitative 1-step real-time RT-PCR experiments. A standard curve made up by Lightcycler 480 gene scanning software was used to determine the concentration of RNA present in the samples using a linear analysis of the data.

Quantitation of mRNA

Total RNA was isolated from the spleen and each part of small intestine of suckling mice using TRIzol reagent (Lift Technologies, Westminster, SC), according to the manufacturer's instructions. Isolated RNA was subjected to reverse transcription following the manufacturer's protocol from Toyobo (Osaka, Japan). Real-time PCR primers for mouse IFN- α , IFN- β , p38, JNK, NF- κB , AP-1, IL-10, IL-8, IFN- γ , TNF- α , and β -actin were designed and synthesized by Sangon Biotech (Shanghai, China) (Table 1). The real-time PCR reactions were processed using SensiMix 2-step RT-PCR with SYBR Green (Quantace, London, UK), according to the manufacturer's protocol. The level of β -actin mRNA expression was used as an internal control to normalize the quantities of target mRNA expression.

Statistical Analysis

Wherever appropriate, the data gathered from the test were expressed as mean \pm standard deviation (SD). To compare the significant differences between the groups, data were assessed with the 2-tailed Student *t* test, and *P* values of <0.05 were considered statistically significant. One-way analysis of variance polynomial orthogonal contrast for the proinflammatory markers was

TABLE 1. Sequence of primers for quantitative RT-PCR

Genes	Orientation	Primer sequences
β -Actin	Sense	5'-GAXCATTGCTCCTCTGAGC-3'
	Antisense	5'-A C ATCTG CTGG AAG GTGG AC-3'
IFN- α	Sense	5'-GACTTTGGATTCCCTGGAG-3'
	Antisense	5'-AAGCCTTTGATGTGAAGAGGTTTC-3'
IFN- β	Sense	5'-GTTACACTGCCTTTGCCATCC-3'
	Antisense	5'-CAACAATAGTCTCATTC CAC CCAG-3'
p38	Sense	5'-AGCCAAXTCCAGTGTGGAC-3'
	Antisense	5'-TTCTGGGCTCC AAATGATTC-3'
JNK	Sense	5'-TGCTG ATTGCC AAATCTCAG-3'
	Antisense	5'-GATAAGGCTGACAGGCAAGC-3'
NF κ B	Sense	5'-TCCCAAGCCAGCACCCAGC-3'
	Antisense	5'-GGCCCCAAGTCTTCATCAGC-3'
AP-1	Sense	5'-G AAAACCTTGAAAGCGCAAAA-3'
	Antisense	5'-TAGCATGAGTTGGCACCCAC-3'
IL-8	Sense	5'-CACCTCAAGAAC ATCCAGAGCT-3'
	Antisense	5'-CAAGCAG AACTGAACTACCATCG-3'
IL-10	Sense	5'-AGCCTTATCGGAAATGATCCAGT-3'
	Antisense	5'-GGCCTGTAGACACCTTGGT-3'
IFN- γ	Sense	5'-G GCTGTCCTG AAAGAAA C-3'
	Antisense	5'-AGCGAGTTATTTGTCATTCCGG-3'
TNF- α	Sense	5'-CGTCGTAGCAAACCACC AAGTGG-3'
	Antisense	5'-GGAAGACTCCTCCAGGTATATGGG-3'

AP-1 = activator protein-1; IFN = interferon; IL = interleukin; JNK = c-Jun N-terminal kinase; NF- κ B = nuclear factor-kappa B; p38 = p38-mitogen activated protein kinase; TNF = tumor necrosis factor.

performed using SPSS version 19.0 (IBM SPSS Statistics, Armonk, NY).

RESULTS

Effect of CsA on Rotavirus-Induced Diarrhea in Suckling Mice

To investigate the *in vivo* effects of CsA, neonatal mice were inoculated with murine rotavirus by oral gavages, and showed diarrhea at day DPI 1. Control suckling mice with mock inoculation did not show diarrhea at any time point. To determine the effective dose of CsA on murine rotavirus-induced diarrhea, neonatal mice were orally given 1, 2.5, or 5 mg \cdot kg⁻¹ \cdot day⁻¹ of CsA. Suckling mice with virus inoculation and mock treatment showed diarrhea until the end of the experiment (Fig. 1); however, the neonatal mice treated with 5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA exhibited a decreased diarrhea score at DPI 4, and most diarrhea was inhibited at DPI 5 or DPI 6 (Fig. 1). The suckling mice with 2.5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA also exhibited a decreased diarrhea score at DPI 7, compared with the virus-inoculated and mock-treated mice (Fig. 1).

Effect of CsA on Fecal Murine Rotavirus Shedding

To determine the effects of cyclosporine on rotavirus shedding, stool samples were collected from each mouse throughout the experimental period, and rotavirus antigen and rotavirus RNA were detected by ELISA and real-time RT-PCR in fecal samples, respectively. Suckling mice treated with 1 and 2.5 mg \cdot kg⁻¹ \cdot day⁻¹ of CsA did not show any improvement of rotavirus shedding, which remained high until the termination of the experiment, compared with the virus-inoculated and mock-treated suckling mice (Fig. 2A). Mice treated with 5 mg \cdot kg⁻¹ \cdot day⁻¹ of CsA

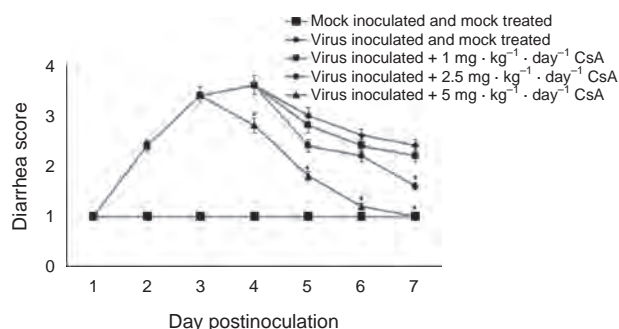


FIGURE 1. The diarrhea score for neonatal mice administered different concentrations of cyclosporin A (CsA) after induction of rotavirus diarrhea. The mean diarrheal score was determined by dividing the sum of all diarrhea scores by the number of mice in each group each day. A score of ≥ 2 was considered diarrhea, whereas a score of ≤ 2 was considered normal. Values are mean \pm standard deviation ($^*P < 0.05$).

significantly reduced rotavirus antigen in the stool samples compared with virus-inoculated and mock-treated mice (Fig. 2A). Similarly, suckling mice treated with 1 and 2.5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA did not show any improvement in viral copy numbers, whereas mice treated with 5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA revealed a rapid decrease in viral copy numbers, compared with the virus-inoculated and mock-treated mice (Fig. 2B).

Effect of CsA on Rotavirus-Induced Histopathological Changes of the Small Intestine

To determine the effects of CsA on rotavirus-induced changes in the pathology of the whole intestine (duodenum, jejunum and ileum), we collected duodenum, jejunum, and ileum samples from experimental suckling mice. In mock-inoculated mice, duodenum, jejunum, and ileum enterocytes were clearly polarized, and the nuclei were localized at the base of the enterocytes. Moreover, mock-inoculated mice showed long and slender villi with short, small intestinal crypts (Fig. 3A–C). Rotavirus-inoculated mice had significantly swollen villus tips with enterocytes containing large vacuoles, villi atrophy, epithelium defluxion, and crypt hyperplasia (Fig. 3D–F). In many villi, lesions seemed to be present at the tips. Furthermore, in virus-inoculated mice, nuclei were enlarged and irregularly positioned within the cells (Fig. 3D–F). Moreover, virus-inoculated mice showed a thickening of the lamina propria and substantial mononuclear cells infiltrate (Fig. 3D–F). Mice treated with 1 or 2.5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA showed gradual improvement of lesion changes compared with severe histopathological changes of small intestine from suckling mice treated with murine rotavirus alone (Fig. 3G–L). Mice given 5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA dramatically exhibited better improvement of lesion changes (Fig. 3M–O). Furthermore, mice appeared to show thickening of the lamina propria and a substantial mononuclear cell infiltrate. This infiltrate appeared most dense in the animals treated with 5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA (Fig. 3M–O). These results are shown in Table 2. Mice alone inoculated with virus showed severe vacuolar degeneration (Fig. 3D–F) whereas mice given 1 and 2.5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA showed gradual improvement in vacuolar degeneration (Fig. 3G–L), and mice given 5 mg \cdot kg⁻¹ \cdot day⁻¹ CsA showed marked improvement in vacuolar degeneration (Fig. 3M–O).

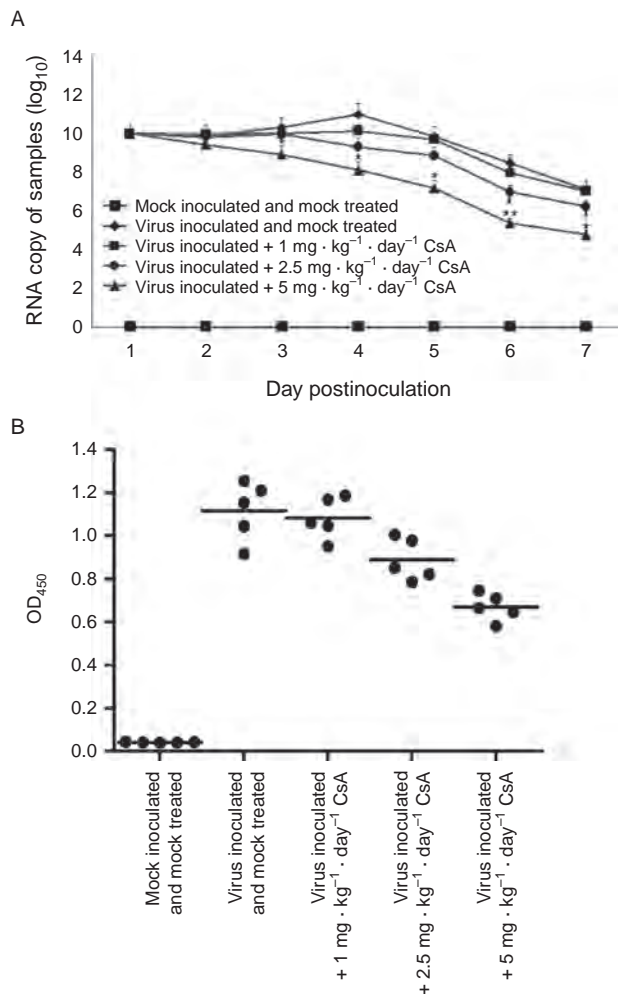


FIGURE 2. Viral antigens shedding of neonatal mice administered different concentrations of cyclosporin A (CsA) after induction of rotavirus diarrhea. A, Quantification of viral RNA copy numbers in the fecal samples of mock-treated mice and mice treated with different concentrations of CsA after induction of rotavirus diarrhea. Values are mean \pm standard deviation (* $P < 0.05$, ** $P < 0.01$). B, Virus antigen in the fecal samples of mock-treated mice and mice treated with different concentration of CsA after induction of rotavirus diarrhea. Fecal samples ($n = 5$) from 4 days postinoculation were assayed for rotavirus shedding by enzyme-linked immunosorbent assay. Data are expressed as net and mean OD₄₅₀ and represent individual values obtained for fecal samples.

Effect of CsA on mRNA Expression Levels of IFN- α and IFN- β

We previously demonstrated that CsA can restore IFN- β mRNA expression, but not IFN- α in vitro (14). We therefore hypothesized that CsA can increase IFN- β mRNA expression in rotavirus-induced diarrhea mice. To determine the effects of CsA on mRNA expression of IFN- α and IFN- β , we collected intestine and spleen samples from each group. Mice inoculated with virus alone showed higher mRNA expression levels of type I IFN, especially IFN- β , compared with mock inoculated mice (Fig. 4). Although

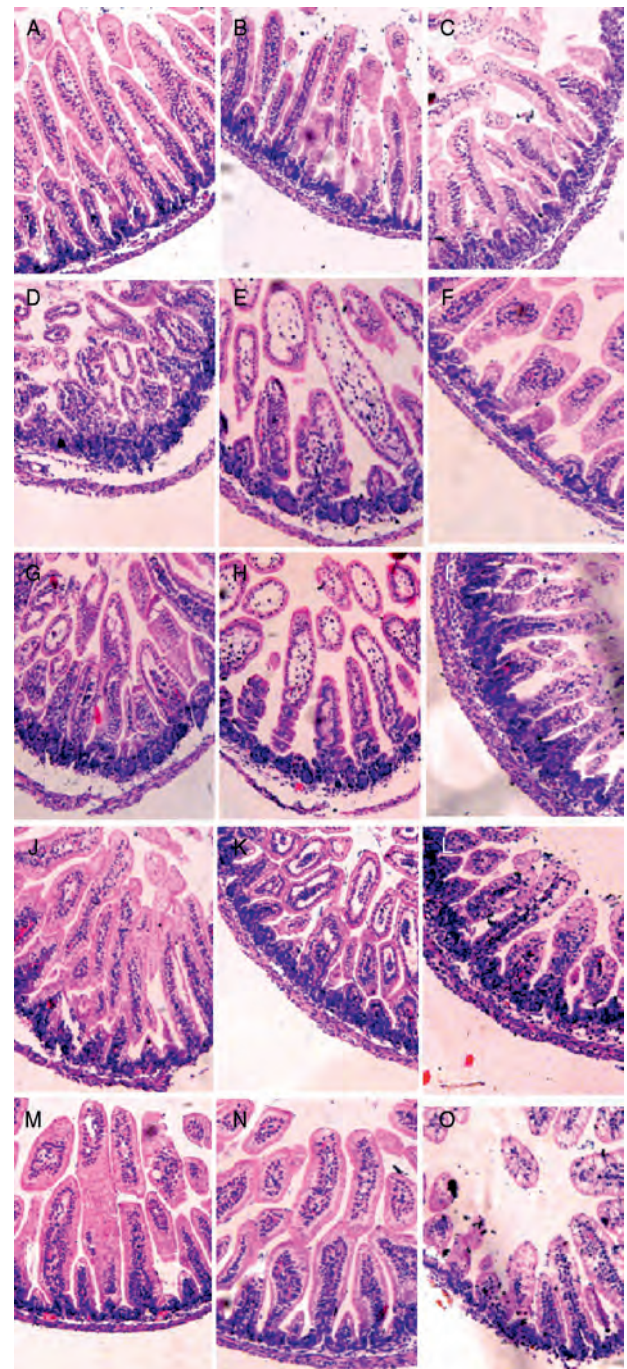


FIGURE 3. Small intestine changes as assessed by histology using a light microscope. (A–C) Control suckling mice with mock inoculation show unaltered duodenum (A), jejunum (B), and ileum (C) with long and slender villi with short, small intestinal crypts. D–F, Mice inoculated with murine rotavirus show severe villi atrophy, crypt hyperplasia, and vacuolar degeneration in the duodenum (D), jejunum (E), and ileum (F). G–I, Mice treated with 1 mg · kg⁻¹ · day⁻¹ Cyclosporin A (CsA) a slight improvement in lesions in the duodenum (G), jejunum (H), and ileum (I). J–L, Mice treated with 2.5 mg · kg⁻¹ · day⁻¹ CsA show a moderate improvement in lesions in the duodenum (J), jejunum (K), and ileum (L). M–O, Mice treated with 5 mg · kg⁻¹ · day⁻¹ CsA show a better improvement in lesions in the duodenum (M), jejunum (N), and ileum (O).

TABLE 2. Summary of the histopathological lesion changes in small intestine samples from each experimental group

Experimental groups	Lesion score		
	Duodenum	Jejunum	Ileum
Mock inoculated and mock treated	6.38 ± 0.26	8.75 ± 0.64	7.21 ± 0.37
Virus inoculated + mock treated	5.05 ± 0.57	6.33 ± 0.87	5.11 ± 0.25
Virus inoculated + 1 mg · kg ⁻¹ · day ⁻¹ CsA	5.23 ± 0.42	7.05 ± 0.41	5.49 ± 0.33
Virus inoculated + 2.5 mg · kg ⁻¹ · day ⁻¹ CsA	5.67 ± 0.38	7.94 ± 0.53*	6.34 ± 0.64*
Virus inoculated + 5 mg · kg ⁻¹ · day ⁻¹ CsA	6.07 ± 0.53*	8.55 ± 0.68**	7.02 ± 0.42**

The intestinal changes are scored according to the average villi height/crypt depth (V/C) ratio. Data represent the mean ± standard deviation (SD). The asterisk indicates significant differences (* $P < 0.05$, ** $P < 0.01$) compared with the positive control. CsA = cyclosporin A.

CsA had little effect on mRNA expression levels of IFN- α in the duodenum, jejunum, ileum, and splenocytes sampled from each experimental group (Fig. 4A), IFN- β mRNA expression was markedly increased (Fig. 4B).

Effect of CsA on Activation of p38, JNK, AP-1, and NF- κ B

To determine the effects of CsA on mRNA expression of p38, JNK, AP-1, and NF- κ B, we collected intestinal and spleen samples from each group. Mice inoculated with virus alone showed higher mRNA expression levels of p38, JNK, AP-1, and NF- κ B in the duodenum, jejunum, ileum, and splenocytes compared with control mice with mock inoculation (Fig. 5A–D). CsA treatment reduced p38, JNK, AP-1, and NF- κ B expression levels in the duodenum, jejunum, ileum, and splenocytes, and significantly decreased these expression levels in the 5 mg · kg⁻¹ · day⁻¹ CsA treatment group (Fig. 5A–D).

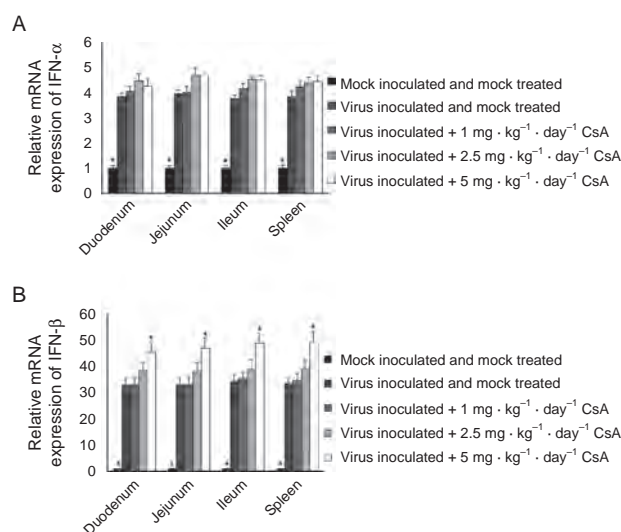


FIGURE 4. Influence of cyclosporin A (CsA) on rotavirus-induced expression of type I interferon (IFN) in neonatal mice. Comparison of messenger RNA (mRNA) expression levels of IFN- α (A) and IFN- β (B) with β -actin in each experimental group was determined by real-time reverse-transcription polymerase chain reaction. Data shown are expressed as mean ± standard deviation of triplicate cultures, and 3 independent experiments were carried out (* $P < 0.05$).

Effect of CsA on mRNA Expression Levels of IL-8, IL-10, IFN- γ , and TNF- α

To determine the effects of CsA on mRNA expression of IL-8, IL-10, IFN- γ , and TNF- α , we collected intestinal and spleen samples from each group. Virus-inoculated and mock-treated mice exhibited higher mRNA expression levels of IL-8, IL-10, IFN- γ , and TNF- α in the duodenum, jejunum, ileum, and splenocytes compared with mock inoculation (Fig. 6A–D). Interestingly, CsA treatment reduced IL-8, IL-10, IFN- γ , and TNF- α expression levels in the duodenum, jejunum, ileum, and splenocytes, and significant down-regulation was observed in the 5 mg · kg⁻¹ · day⁻¹ CsA treatment group (Fig. 6A–D). Taken together, we found that the CsA-treated murine rotavirus diarrhea group exhibited a gradual decrease in these cytokines in a dose-dependent manner in these tissues.

DISCUSSION

The rotavirus is the most frequent etiological agent of severe dehydrating gastroenteritis in infants and young children worldwide. Because of the high morbidity and mortality, rotaviral diarrhea represents a major health problem, particularly in developing countries (28). We previously demonstrated that CsA inhibits rotavirus replication in vitro and in vivo (14). Nevertheless, there is little evidence that there is an association between CsA dose and disease severity, and CsA affects the early overall responses and the development and regulation of lymphocytes in neonatal mice. Therefore, the antiviral activity of CsA in a mouse model needs to be evaluated.

Rotavirus infection induces the cellular and humoral responses that are crucial for controlling viral infection. Several rotavirus strains, including the monkey rotavirus, bovine rotavirus B641, and human Wa rotaviruses, have, however, evolved highly sophisticated gene-silencing mechanisms to evade the host-immune response (29–31). In this study, we selected the murine model of homologous rotavirus infection because of the host range restriction. This model showed diarrhea at DPI 1 after oral inoculation of a murine rotavirus. Among virus-inoculated and treated groups, 5 mg · kg⁻¹ · day⁻¹ of CsA administered to the test mice inhibited rotavirus diarrhea; however, CsA 2.5 mg · kg⁻¹ · day⁻¹ was reported to clear rotavirus from stools in our previous study (14). In fact, this phenomenon is the result of using different types of rotavirus strains in 2 articles. In our previous study, suckling mice were inoculated with simian rotavirus SA11 (14). In this study, suckling mice were inoculated with murine rotavirus. The efficiency of CsA in clearing different strains of rotavirus from the stools may differ. The antiviral effect of CsA was confirmed by a significant improvement in fecal virus shedding and histopathological changes of the small intestine, during rotavirus

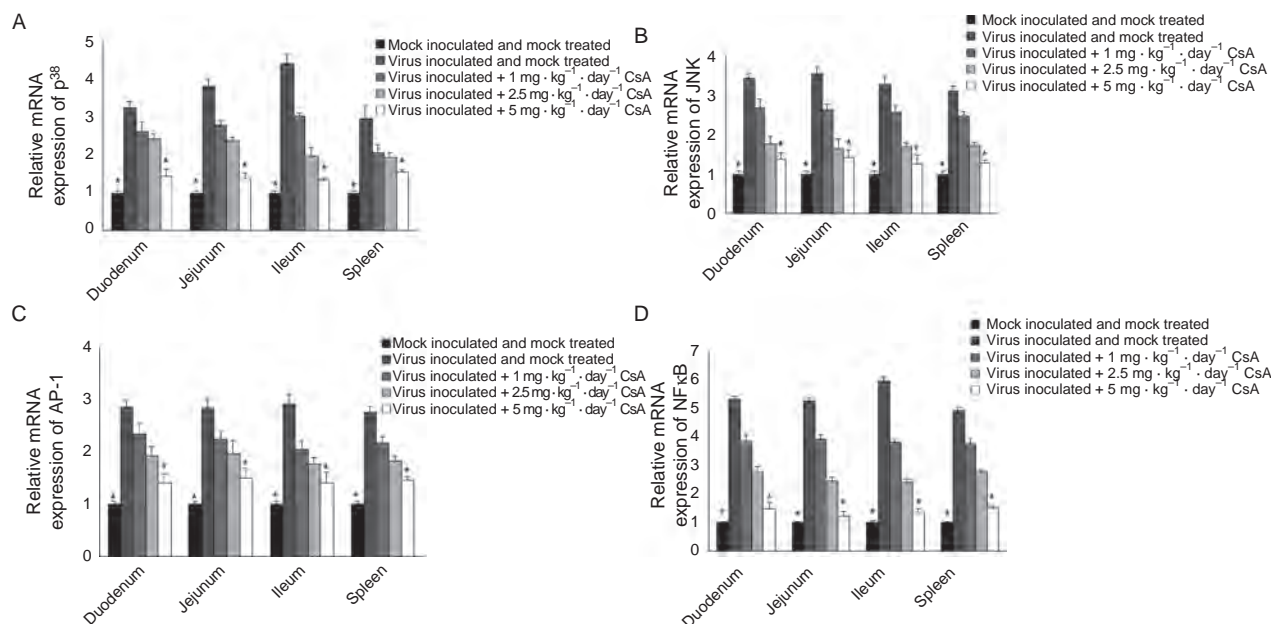


FIGURE 5. Influence of cyclosporin A (CsA) on rotavirus-induced expression of p38, c-Jun N-terminal kinase (JNK), nuclear factor-kappa B (NF- κ B), and activator protein-1 (AP-1) in neonatal mice. Comparison of messenger RNA expression levels of p38 (A), JNK (B), AP-1 (C), and NF κ B (D) with β -actin in each experimental group was determined by real-time reverse-transcriptase polymerase chain reaction. Data shown are expressed as mean \pm standard deviation of triplicate cultures, and 3 independent experiments were carried out (* $P < 0.05$).

infection of infant mice. Virus-inoculated mice appeared to show thickening of the lamina propria and a substantial mononuclear cell infiltrate, which appears most dense in the animals with $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ CsA. These monocytes and macrophages can generate and release a variety of cytotoxins, interferons, and interleukins, which can participate in clearing rotavirus from the body. Mononuclear cells can undergo cell division and surround the foreign substances, which are located around the inflammatory tissue.

We previously demonstrated that CsA inhibited rotavirus-induced diarrhea in neonatal mice through a CYPA PPLase-independent pathway (14). There is, however, no information about CsA in association with the cytokine profiles. It is known that rotavirus infection can induce the production of inflammatory cytokines and activation of inflammatory signaling pathways (22,32,33). In this study, we analyzed proinflammatory cytokines (IL-8, IFN- γ , and TNF- α) and a regulatory cytokine (IL-10) and showed that murine

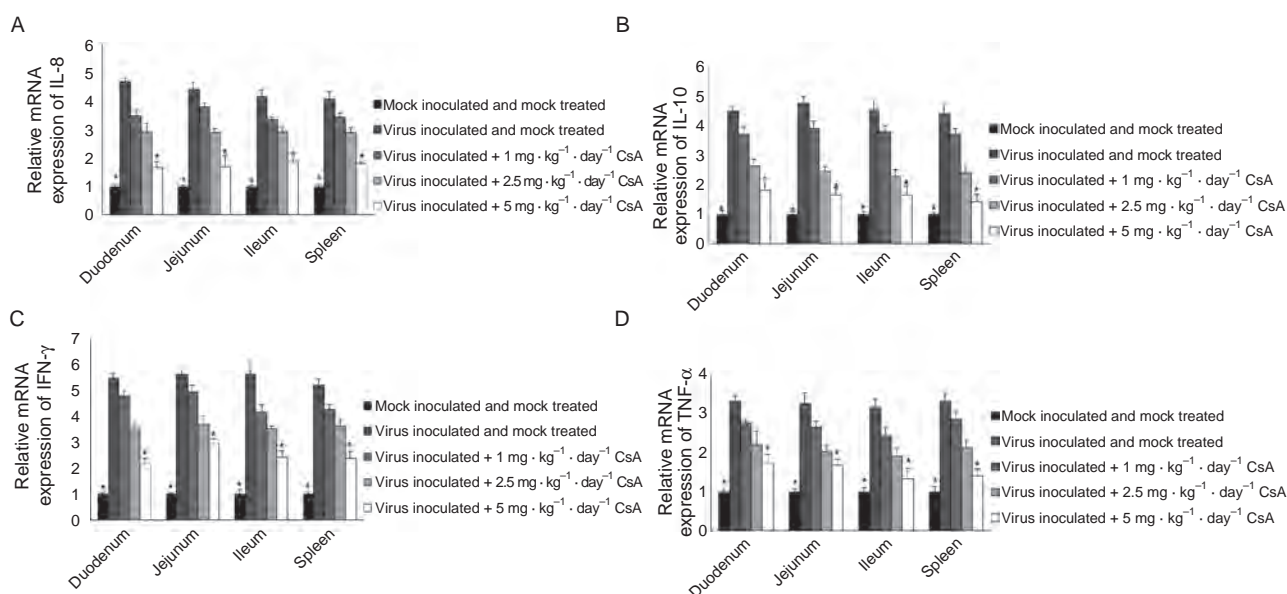


FIGURE 6. Influence of cyclosporin A (CsA) on rotavirus-induced expression of interleukin (IL)-8, IL-10, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α in neonatal mice. Comparison of messenger RNA expression levels of IL-8 (A), IL-10 (B), IFN- γ (C), and TNF- α (D) with β -actin in each experimental group was determined by real-time reverse-transcriptase-polymerase chain reaction. Data shown are expressed as mean \pm standard deviation of triplicate cultures, and 3 independent experiments were carried out (* $P < 0.05$).

rotavirus induced increased mRNA expression of IL-8, IL-10, IFN- β , IFN- γ , and TNF- α in the 4 selected tissues, whereas CsA treatment decreased the mRNA expression levels of these inflammatory cytokines. In addition, rotavirus infection activated *p38*, *JNK*, *AP-1*, and *NF- κ B* genes, but CsA inhibited *p38*, *JNK*, *AP-1*, and *NF- κ B* genes in the rotavirus-infected neonatal mouse model. We found in our previous work that CsA can inhibit rotavirus invasion in the biopsies by transmission electron microscopy (14). Therefore, we believe in the hypothesis that the reduction of local inflammation influences rotavirus invasiveness. These data suggested that CsA treatment modulates an inflammatory reaction alleviating intestinal lesions exacerbated by host inflammation.

In summary, we reveal in this study that CsA can inhibit rotavirus-induced diarrhea in neonatal mice through its antiviral properties. The mechanism for this may be through CsA suppression of inflammation by viral inhibition in animal models.

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