

ROLE OF CpG ODN IN CONCAVALIN A-INDUCED HEPATITIS IN MICE

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Abstract: Objective: To investigate the effects of an intradermal injection of oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs on concanavalin A (Con A)-induced hepatitis, an experimental model of immune-mediated hepatitis.

Methods: Con A was injected intravenously into Balb/c mice. Twelve hours after Con A challenge, blood and liver samples were obtained. CpG ODN was injected intradermally 48 hours before Con A challenge. The extent of liver injury was assessed by determining serum alanine transaminase (ALT) and by liver histology. Serum levels of cytokines, including interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-4 and IL-5, were measured by enzyme-linked immunosorbent assay.

Results: Co-administration of Con A and CpG ODN significantly increased serum ALT in mice compared with that in the case of administration of Con A alone ($10,268 \pm 4,654$ and $1,140 \pm 832$ IU/l, respectively, $p < 0.05$). In liver histology, mice treated with CpG ODN and Con A showed more extensive midzonal necrosis than did mice treated with Con A alone. These mice also showed significant increases in serum TNF- α and IFN- γ and decrease in serum IL-5.

Conclusions: The results indicate that CpG ODNs aggravate Con A-induced hepatitis by stimulating the production of T-helper-1 (Th1) cytokines, TNF- α and IFN- γ , suggesting that bacterial DNA that contains unmethylated CpG motifs may contribute to the exacerbation of immune-mediated liver injury.

Key words: bacterial DNA, autoimmune hepatitis, interferon- γ , tumor necrosis factor- α , interleukin-5

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INTRODUCTION

Concanavalin A (Con A)-induced hepatitis is an experimental model of immune-mediated liver injury¹⁾. Con A activates T cells and causes release of proinflammatory cytokines such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α , and these cytokines contribute to the development of hepatitis²⁻⁴⁾. IFN- γ has been suggested to play a central role in Con A-induced hepatitis by activating Fas-induced apoptosis of liver cells⁵⁾.

On the other hand, it has been reported that bacterial DNA containing unmethylated CpG motifs activates a T-helper-1 (Th1)-type immune response^{6,7)}. Unmethylated CpG motif-containing DNA activates secretion of a variety of inflammatory cytokines including IFN- α/β , interleukin (IL)-6, IL-12 and TNF- α , by B cells, natural killer cells, and dendritic cells and functions as a Th1-promoting adjuvant⁸⁻¹²⁾. In a D-galactosamine-induced hepatitis model, unmethylated CpG-containing oligodeoxynucleotides (ODNs) induced acute liver failure similar to that induced by lipopolysaccharide¹³⁾. Bacterial translocation is often observed in liver injury, and bacterial DNA is detected in blood and ascites in patients with liver cirrhosis¹⁴⁾. Since CpG DNA derived from bacteria is expected to flow easily into the liver via the portal vein after bacterial translocation and to aggravate immune-mediated liver injury, we examined the effects of CpG ODN on Con A-induced hepatitis in mice.

MATERIALS AND METHODS

This study was carried out under the control of the Animal Research Committee in accordance with the Guideline for Animal Experiments in Fukushima Medical University of School of Medicine, the Japanese Government Animal Protection and Management Law (No. 105), and the Japanese Government Notification on Feeding and Safekeeping of Animals (no. 6).

Preparation of ODN

The following nuclease-resistant phosphorothioate-modified ODNs⁷⁾ were synthesized using an automated DNA synthesizer (model 394; Applied Biosystems, Foster City, CA): CpG ODN 1668, 5'-TCC-ATG-ACG-TTC-CTG-ATG-CT-3', and non-CpG ODN 1720, 5'-TCC-ATG-AGC-TTC-CTG-ATG-CT-3' (analogous to CpG ODN 1668 but with replacement of CpG by GpC as indicated by the underline). Endotoxin levels in these ODNs were <5 ng/ml in the *Limulus* amoebocyte assay (Association of Cape Cod, Woods Hole, MA). These ODNs were dissolved in phosphate-buffered saline (PBS) before use in the experiments.

Animals

Eight-week-old female Balb/c mice were obtained from Kumagai Farm (Sea, Japan). The mice were housed in filtertop cages, and water and food were provided *ad libitum*.

Induction of Con A-induced hepatitis and injection of ODNs

Forty female Balb/c mice were randomly assigned to 5 groups of 8 mice each. Group 1 mice as controls were each injected intradermally at the base of the tail with 50 μ l of PBS. Group 2 mice were each injected intravenously with 0.25 mg of Con A (Sigma-Chemical Co., St. Louis, MO) in 50 μ l of PBS. Group 3 mice were each injected intradermally at the base of the tail with 50 μ g of CpG ODN in 50 μ l of PBS. Group 4 mice were each injected intradermally at the base of the tail with 50 μ g of CpG ODN in 50 μ l of PBS 48 hours before Con A (0.25 mg/mouse) injection. Group 5 mice were each injected intradermally at the base of the tail with 50 μ g of non-CpG ODN in 50 μ l of PBS 48 hours before Con A (0.25 mg/mouse) injection.

In other experiments, mice were injected intradermally with the indicated amounts of CpG ODN 12 hours before Con A (0.25 mg/mouse) injection.

Serum alanine transaminase levels and liver histology

The extent of liver injury was assessed by serum alanine transaminase (ALT) level and liver histology. Blood and liver samples were obtained at 12 hours after Con A injection since preliminary experiments showed that serum ALT reached a maximum level in mice 12 hours after Con A injection (data not shown). The sera were stored at -20°C until use. For histopathological evaluation, the liver samples were fixed in 10% buffered formalin and embedded in paraffin, sectioned and stained with hematoxylin and eosin (Sakura Finetechnical, Tokyo, Japan).

Measurements of serum cytokines and transaminase

Serum levels of IFN- γ , TNF- α , IL-4 and IL-5 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Pierce Chemical Com., Rockford, USA) according to the manufacturer's instructions. ALT was measured at SRL Inc. (Tokyo, Japan) by commercially available kits (Azwell Co., Osaka, Japan).

Statistical analysis

Statistical analysis was carried out using the Stat View J-5.0 software package (SAS Institute, Cary, NC, USA) for Windows. Data are expressed as means \pm SD and were analyzed by one-way analysis of variance. *P* values less than 0.05 were considered statistically significant.

RESULTS

Effect of CpG ODN on serum ALT levels in Con A-induced hepatitis

Serum ALT levels in mice treated with or not treated with ODN or Con A are shown in Figure 1. Serum ALT level in mice treated with CpG ODN alone was significantly higher than that in control mice (220 ± 78 and 44 ± 22 IU/l, respectively, $p < 0.05$). Serum ALT level in mice treated with CpG ODN and Con A was significantly higher than that in mice treated with Con A alone and that in mice treated with non-CpG ODN and Con A ($10,268 \pm 4,654$, $1,140 \pm 832$ and $1,476 \pm 908$ IU/l, respectively, $p < 0.05$). There was no significant difference between serum ALT levels in mice treated with Con A alone and mice treated with non-CpG ODN and Con A. In addition, prior injection of CpG ODN significantly increased serum ALT levels in mice treated with Con A in a dose-dependent manner (Figure 2).

Effect of CpG ODN on serum cytokine levels in Con A-induced hepatitis

Serum cytokine levels in mice 12 hours after Con A injection (0.25 mg/mouse) with or without prior injection of 50 μ g of CpG ODN are shown in Figure 3. Serum levels of IFN- γ and TNF- α in mice treated with CpG ODN and Con A were significantly higher than those in mice treated with Con A alone (Figures 3a and b,

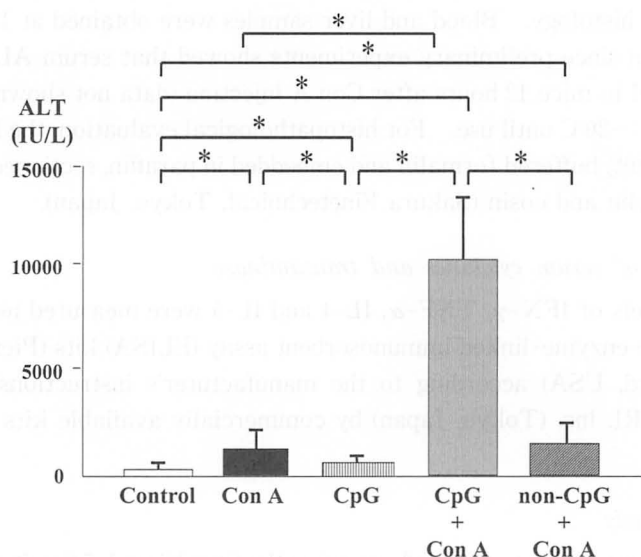


Fig. 1. Serum ALT levels in 5 groups of mice. Group 1 mice as controls were injected intradermally with PBS. Group 2 mice were injected intravenously with Con A. Group 3 mice were injected intradermally with CpG ODN. Group 4 mice were injected intradermally with CpG ODN 48 hours before Con A injection. Group 5 mice were injected intradermally with non-CpG ODN 48 hours before Con A injection. Data are shown as means \pm SD for 5 groups of 8 mice each. Representative results of 3 experiments are shown. * $P < 0.05$.

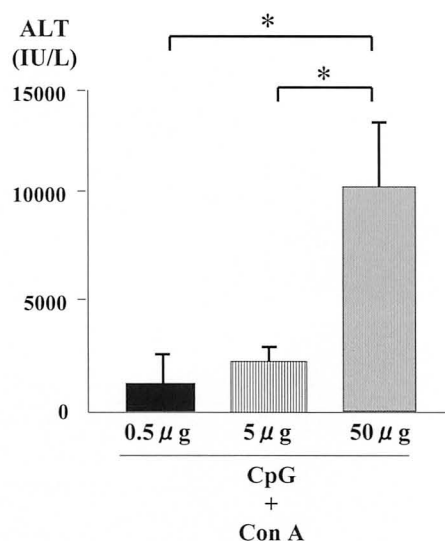


Fig. 2. Serum ALT levels in mice injected with different amounts of CpG ODN before Con A injection. Mice were injected intradermally with 0.5, 5 or 50 μg of CpG ODN 12 hours before Con A injection. Data are shown as means ± SD in 4 mice in each group. Representative results of 3 experiments are shown. * $P < 0.05$.

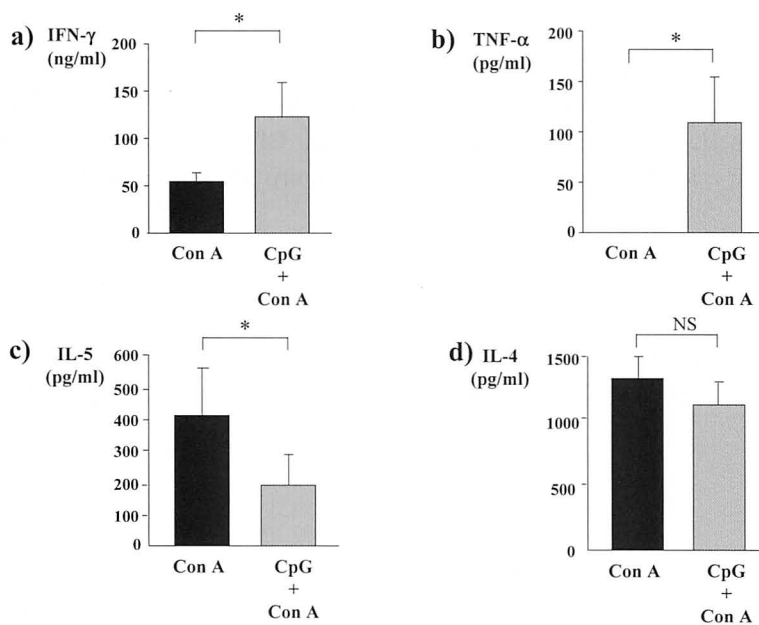


Fig. 3. Serum levels of IFN-γ (a), TNF-α (b), IL-5 (c) and IL-4 (d) in mice 12 hours after Con A injection with or without prior injection of CpG ODN. Representative results of 3 experiments are shown. * $P < 0.05$.

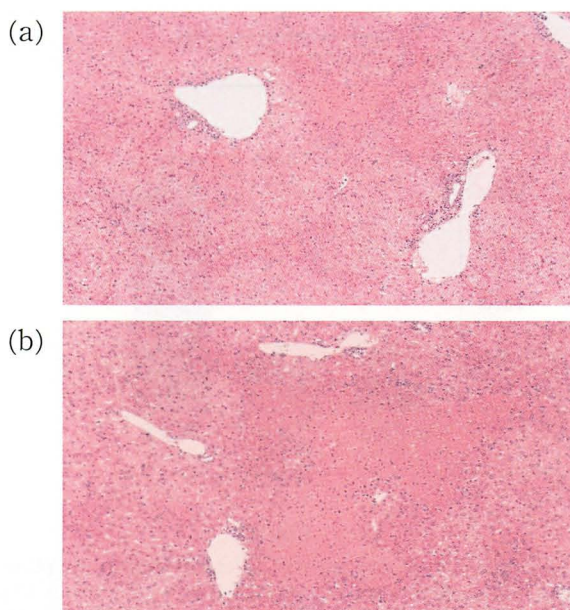


Fig. 4. Liver histology in (a) a mouse treated with Con A alone and (b) a mouse treated with CpG ODN and Con A (hematoxylin and eosin stain; original magnification 100 \times)

respectively). On the other hand, serum IL-5 level in mice treated with CpG ODN and Con A was significantly lower than that in mice treated with Con A alone (Figure 3c). Serum IL-4 level in mice treated with CpG ODN and Con A was also lower than that in mice treated with Con A alone (Figure 3d), but the difference was not significant.

Liver histology in Con A-induced hepatitis

In liver histology, mice treated with CpG ODN and Con A showed more extensive midzonal necrosis than did mice treated with Con A alone (Figure 4). In mice treated with CpG ODN alone, hepatocyte necrosis was not observed (data not shown).

DISCUSSION

Con A is a lectin that stimulates lymphocytes or monocytes to secrete various cytokines *in vitro*, and it is known to induce liver injury mediated by cellular immunity when injected into mice¹¹. Therefore, Con A-induced hepatitis is used as an experimental model of immune-mediated liver injury. On the other hand, CpG DNA derived from bacteria can potently stimulate a Th1 immune response⁷. Although the actions of CpG DNA resemble those of LPS, CpG DNA stimulates distinct toll-like receptors as well as various cell types¹⁵. In this study, we showed

that Con A-induced hepatitis was aggravated by the injection of CpG ODN, with increases in serum levels of Th1-type cytokines, IFN- γ and TNF- α and a decrease in serum IL-5. In addition, prior injection of CpG ODN significantly increased serum ALT levels in mice treated with Con A in a dose-dependent manner. Thus, CpG DNA is thought to be one of the factors aggravating immune-mediated liver injury via augmentation of a Th1 immune response.

Several cytokines, including TNF- α , IFN- γ and IL-4, have been shown to be involved in the pathogenesis of Con A -induced hepatitis, because neutralization of these cytokines conferred protection against Con A-induced hepatitis²⁻⁴. Tagawa et al. suggested that the induction of Fas antigen expression on hepatocytes after treatment with Con A is mediated by IFN- γ , and that this elevated expression of Fas antigen promotes apoptosis of hepatocytes⁵. In our model, serum IFN- γ level was increased in mice treated with CpG ODN and Con A compared with that in mice treated with Con A alone. Since CpG DNA stimulates dendritic cells to produce IL-12, which promotes the production of IFN- γ by T cells and natural killer cells¹¹, it is possible that exacerbation of Con A-induced hepatitis by CpG DNA is mediated by enhanced production of IFN- γ . Similar to IFN- γ , the enhanced levels of TNF- α were thought to be involved in the exacerbation of Con A-induced hepatitis by CpG DNA. TNF- α is known to be released from macrophages or T cells stimulated by CpG DNA as well as Con A^{16,17}. Heikenwalder et al. reported that livers of repeated CpG-ODN-treated mice showed multifocal infiltrates of B and T cells and marked Kupffer cell hyperplasia expanded the sinusoids¹⁸. These cells are thought to produce Th1-type cytokines in our model. Apoptotic mechanisms are likely to be involved in liver injury related to TNF- α . In this study, 12 hours after Con A challenge, TNF- α was not detected in sera of mice treated with Con A alone because it has been reported that plasma level of TNF- α peaked at 2 hours¹⁷.

Louis et al. reported that IL-5-dependent activation of eosinophils and Fas/Fas ligand interaction constitute the two major effector pathways of liver injury in Con A-induced hepatitis¹⁹. They showed that natural killer T cells are a major source of IL-5, which causes eosinophil-mediated liver damage. However, our study showed increases in serum Th1-type cytokines and a decrease in serum IL-5 in mice treated with CpG ODN and Con A compared with those in mice treated with Con A alone, suggesting that CpG DNA-enhanced production of Th1-type cytokines may suppress the production of IL-5. Since Satoh et al. reported that the infiltrating cells in the liver of Con A-induced hepatitis mice were predominantly T lymphocytes²⁰, Con A-induced hepatitis may be aggravated by T cells activated by enhanced production of Th1-type cytokines by CpG DNA even though the production of IL-5 was reduced in our models. Serum levels of IL-4 were also reduced in mice treated with CpG ODN and Con A compared with those in mice treated with Con A alone, but the difference was not significant. These results suggest that production of IL-4 by various cells may be more resistant to the suppression of Th1-type cytokines than that of IL-5 in our models, but further experiments are neces-

sary to clarify this phenomenon.

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