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Aminoguanidine prevents concanavalin A-induced hepatitis in mice

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Abstract

Aminoguanidine is an inhibitor of the inducible form of nitric oxide synthase (iNOS). In the present study, the effect of aminoguanidine on concanavalin A-induced hepatitis was examined. Treatment of mice with concanavalin A (10 mg/kg, i.v.) induced interferon-γ and iNOS mRNA expression in the liver before the elevation of plasma alanine aminotransferase activity. Immunohistochemical study showed the induction of iNOS protein expression in the area of necrosis. Aminoguanidine (1, 3 and 10 mg/kg, i.p.) inhibited the concanavalin A-induced elevation of alanine aminotransferase activity. Aminoguanidine (10 mg/kg, i.p.) did not inhibit concanavalin A-induced interleukin-2, interferon-γ, tumor necrosis factor-α or iNOS mRNA expression in the liver. The plasma nitrite/nitrate level was elevated at 6 and 24 h after concanavalin A treatment. The elevation of nitrite/nitrate was inhibited by aminoguanidine (10 mg/kg, i.p.). From these results, we conclude that nitric oxide formed by iNOS may be involved in the development of concanavalin A-induced hepatitis.

Keywords: Concanavalin A; Aminoguanidine; Hepatitis

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine through NO synthase (NOS) (Kwon et al., 1990). The constitutively expressed NOS isoform is found in the absence of immunostimulation and this activity maintains the normal physiology (Moncada et al., 1991; Garthwaite, 1991). In contrast, the inflammatory effect of NO has been ascribed to the activity of the inducible NO synthase (iNOS), which is expressed in response to cytokine (Mulligan et al., 1991).

A mouse concanavalin A-induced hepatitis model is dependent on an immunological reaction, and interferon-γ plays a critical role in the development of the hepatitis (Gantner et al., 1995; Mizuhara et al., 1996). The liver is a rich source of iNOS (Curran et al., 1991), and the iNOS gene is responsive to interferon-γ (Schmidt and Walter, 1994). Thus, concanavalin A-induced interferon-γ might activate the iNOS system. However, it is not known whether activation of the iNOS system is involved in the development of concanavalin A-induced hepatitis or not.

In the present study, we examined the possible involvement of the iNOS system in concanavalin A-induced hepatitis by using aminoguanidine, a selective inhibitor of iNOS (Misko et al., 1993).

2. Materials and methods

2.1. Animals and treatment

Female BALB/c mice obtained from Charles River Japan (Atsugi, Japan) were used at 7–10 weeks of age. The animals were kept in an air-conditioned room and given water and food ad libitum. Concanavalin A was purchased from Sigma (St. Louis, MO, USA). Animal experiments were performed under the approval of the experimental protocols by the Institutional Ethics Committee. Mice were anesthetized with ether before killing. Concanavalin A dissolved in pyrogen-free saline was administered to the mice (10 mg/kg) via a tail vein (in the volume of 100 μl). Aminoguanidine was obtained from Research Biochemical International (Natick, MA, USA) and injected intraperitoneally. Plasma transaminase activity, i.e., that of alanine aminotransferase activity, was

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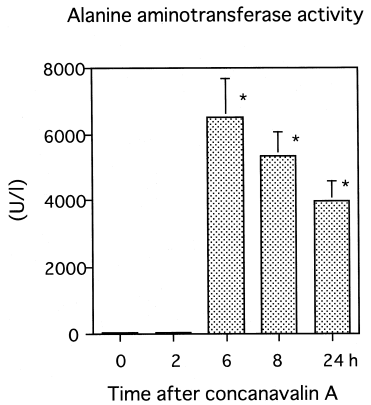

Fig. 1. Time course of the increase in the plasma transaminase level after concanavalin A injection. For each time point, four mice were injected intravenously with 10 mg/kg concanavalin A. Plasma from each mouse was obtained at various times after concanavalin A injection. Alanine aminotransferase activity (U/l) at 0 and 2 h after concanavalin A were 35 ± 3 and 35 ± 6, respectively. Data points represent the means $\pm$ S.E. of alanine aminotransferase activity in plasma obtained at the indicated time points. $^{*}P < 0.01$ vs. normal control.

measured by the standard photometric method with an automatic analyzer (Okamoto et al., 1996).

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Okamoto et al., 1996). cDNA was amplified by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1.5 min. Gene-specific primers were as follows:

Interleukin-2 (Genbank Acc#K02292)
(sense) 5′-ATGTACAGCATGCAGCTCGCATC-CTGTGTC-3′
(antisense) 5′-AGTCAAATCCAGAACATGC-CGCAGAGGTCC-3′
Interferon-γ (Genbank Acc#K00083)
(sense) 5′-ATCAGCAGCGACTCCTTTTCCGCTT-3′
(antisense) 5′-GAAAGCCTAGAAAGTCT-GAATAACT-3′
Tumor necrosis factor-α (TNF-α) (Genbank Acc#M11731)
(sense) 5′-AGCCCACGTCGTAGCAAACCACCAA-3′
(antisense) 5′-ACACCCATTCCCTTCACA-GAGCAAT-3′
iNOS (Genbank Acc#L09126)
(sense) 5′-TGGGAATGGAGACTGTCCCAG-3′
(antisense) 5′-GGGATCTGAATGTGATGTTTG-3′
GAPDH (Genbank Acc#X02231)
(sense) 5′-ATGGTGAAGGTCGGTGTGAACG-3′
(antisense) 5′-GTTGTCATGGATGACCTTGGCC-3′

2.3. Histology and immunohistochemistry

For morphological study, the livers were fixed with 10% phosphate-buffered neutral formalin. Paraffin sections (4 μm) were stained with hematoxylin and eosin for microscopic examination. For immunohistochemistry, sections were incubated for 1 h with a 1:500 dilution of anti-mouse iNOS antibody (Wako, Osaka, Japan). Antibody binding was visualized with biotinylated rabbit anti-goat IgG, avidin–biotin-complex and 3.3′-diaminobenzidine (DAKO, Glostrup, Denmark).

2.4. Measurement of nitrite / nitrate production

Plasma was filtered with Centricon 10 (Millipore, MA, USA) by centrifugation at 7500 rpm for 30 min and plasma nitrite/nitrate concentration was measured using the Griess method with a NO_2/NO_3 assay kit (Wako, Osaka, Japan).

2.5. Statistical analysis

The results were analyzed by means of the Dunnett multiple comparison test.

3. Results

3.1. Effects of concanavalin A on interferon-γ and iNOS mRNA expression in the liver

Mice were treated with concanavalin A (10 mg/kg), and killed at 2, 6, 8 and 24 h after the treatment, when plasma was sampled for measurement of alanine aminotransferase activity, and liver samples obtained at 2, 6 and 8 h were used for RT-PCR analysis with interferon-γ and iNOS gene-specific primers. The plasma alanine aminotransferase activity level was increased at 6 h (Fig. 1). Expression of the interferon-γ and iNOS genes was induced at 2 h after concanavalin A administration (Fig. 2). GAPDH gene was evenly expressed, indicating there was no tube-to-tube variation.

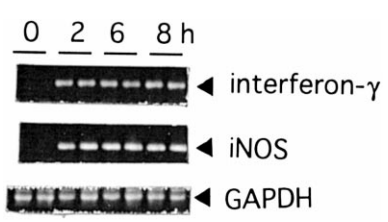

Fig. 2. RT-PCR analysis of the induction of interferon-γ and iNOS mRNA expression in the liver. Liver samples obtained in Fig. 1 at 2, 6 and 8 h after concanavalin A treatment were subjected to RT-PCR analysis with interferon-γ, iNOS and GAPDH gene-specific primers.

3.2. Effect of concanavalin A on iNOS protein expression in the liver

Mice were treated with concanavalin A (10 mg/kg), and killed at 24 h after the treatment, when liver was sampled for immunohistochemical staining of iNOS protein. Treatment of mice with concanavalin A-induced necrosis in the liver (Fig. 3, Top). A liver sample from the same part of the liver as in Fig. 3 (Top) was stained with iNOS antibody. Incubation of the liver sample with anti-mouse iNOS antibody showed staining of iNOS protein, specifically on the hepatocytes in the same region where necrosis was induced (Fig. 3, Bottom).

3.3. Effect of aminoguanidine on concanavalin A-induced elevation of plasma alanine aminotransferase activity

Mice were treated with concanavalin A (10 mg/kg), and killed at 24 h after the treatment, when plasma and livers were sampled for measurement of alanine aminotransferase activity and a morphological study, respectively. Aminoguanidine has been given at the dose of 100

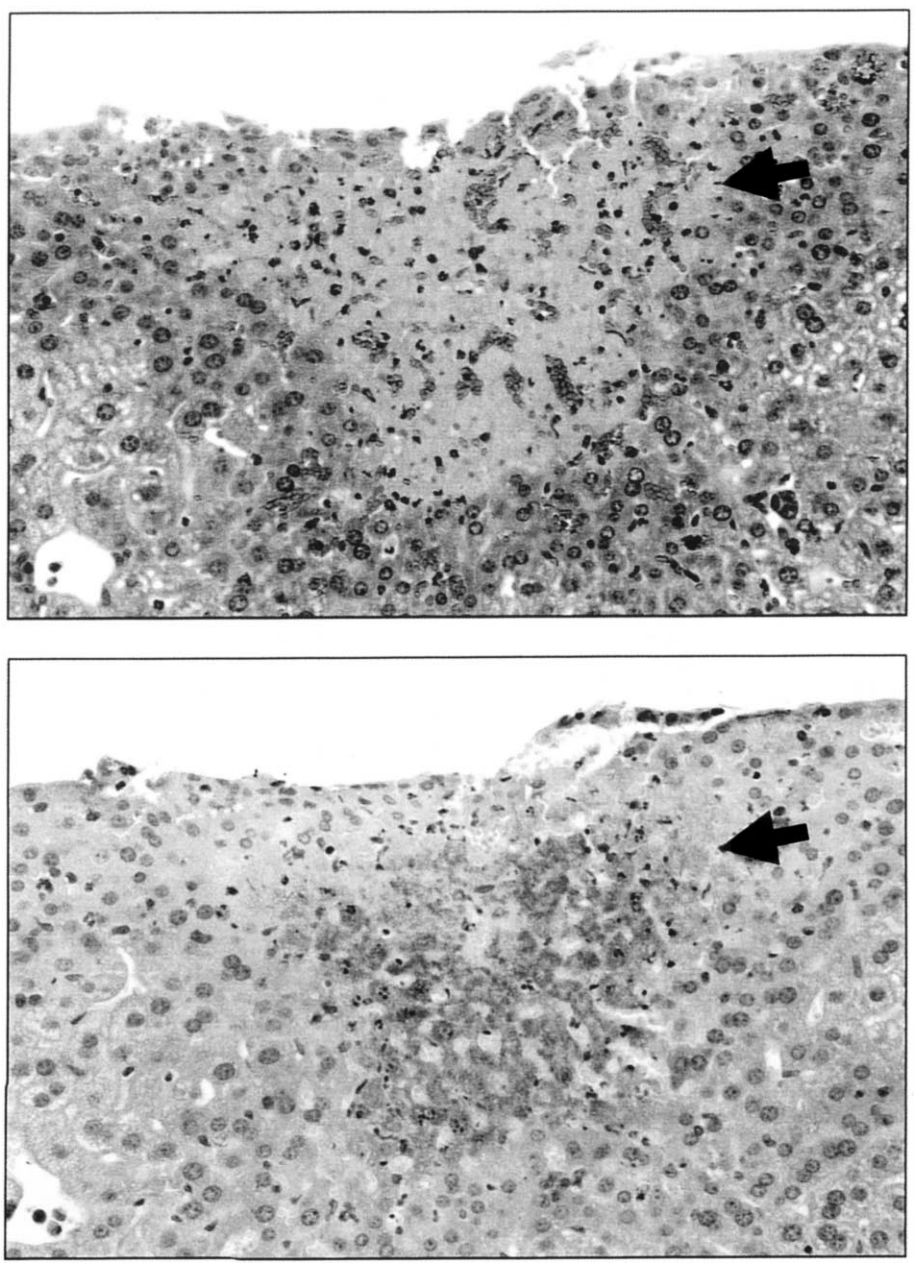

Fig. 3. Effect of concanavalin A on iNOS protein expression. The livers were removed at 24 h after concanavalin A (10 mg/kg) administration. Top: Hepatocyte necrosis was shown in the areas indicated (hematoxylin and eosin staining × 300). Bottom: Expression of iNOS protein, brown-stained with anti-mouse iNOS antibody, shown in the hepatocytes where necrosis was induced.

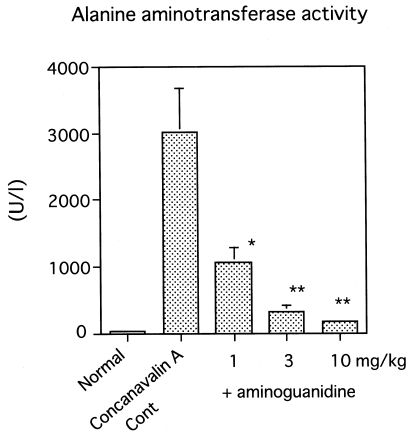

Fig. 4. Effect of aminoguanidine on concanavalin A-induced elevation of plasma alanine aminotransferase activity. Concanavalin A was injected and plasma was obtained from each mouse at 24 h afterwards. Pretreatment with aminoguanidine was performed 1 h before concanavalin A treatment. The data represent the means ± S.E. of alanine aminotransferase activity in plasma obtained for each treatment. Normal: Non-treated ($n = 2$). Concanavalin A Cont: Concanavalin A (10 mg/kg, i.v.)-treated ($n = 4$). + aminoguanidine: Concanavalin A (10 mg/kg, i.v.) + aminoguanidine (1, 3 and 10 mg/kg, i.p.)-treated ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. concanavalin A alone.

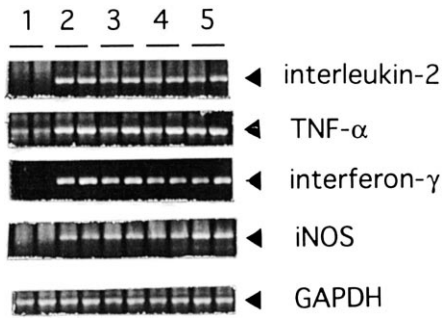

Fig. 6. RT-PCR analysis of the effects of aminoguanidine on concanavalin A-induced cytokine and iNOS mRNA expression in the liver. Concanavalin A (10 mg/kg) was injected, and livers were removed at 2 and 6 h after injection. Aminoguanidine (10 mg/kg) was given at 1 h before concanavalin A administration. RNA was isolated and subjected to RT-PCR analysis with interleukin-2, TNF-α, iNOS and GAPDH gene-specific primers. The contents of the gel lanes were as follows; Lane 1, normal liver samples; Lane 2, concanavalin A-treated for 2 h; Lane 3, concanavalin A + aminoguanidine-treated for 2 h; Lane 4, concanavalin A-treated for 6 h; Lane 5, concanavalin A + aminoguanidine-treated for 6 h.

mg/kg for the inhibition of lipopolysaccharide-induced iNOS activation in vivo (Khatsenko and Kikkawa, 1997). In the present study, aminoguanidine 10 and 100 mg/kg i.p., $n = 2$ for each dose) was administered, and at 24 h plasma was sampled. Aminoguanidine alone, at either dose, did not affect plasma alanine aminotransferase activity (not shown). Treatment of mice with concanavalin A-induced an elevation of plasma alanine aminotransferase activity ($n = 4$) (Fig. 4). When mice were pretreated with aminoguanidine (1, 3 and 10 mg/kg, i.p., $n = 4$) at 1 h before concanavalin A treatment, the concanavalin A-induced elevation of plasma alanine aminotransferase activity was inhibited (Fig. 4).

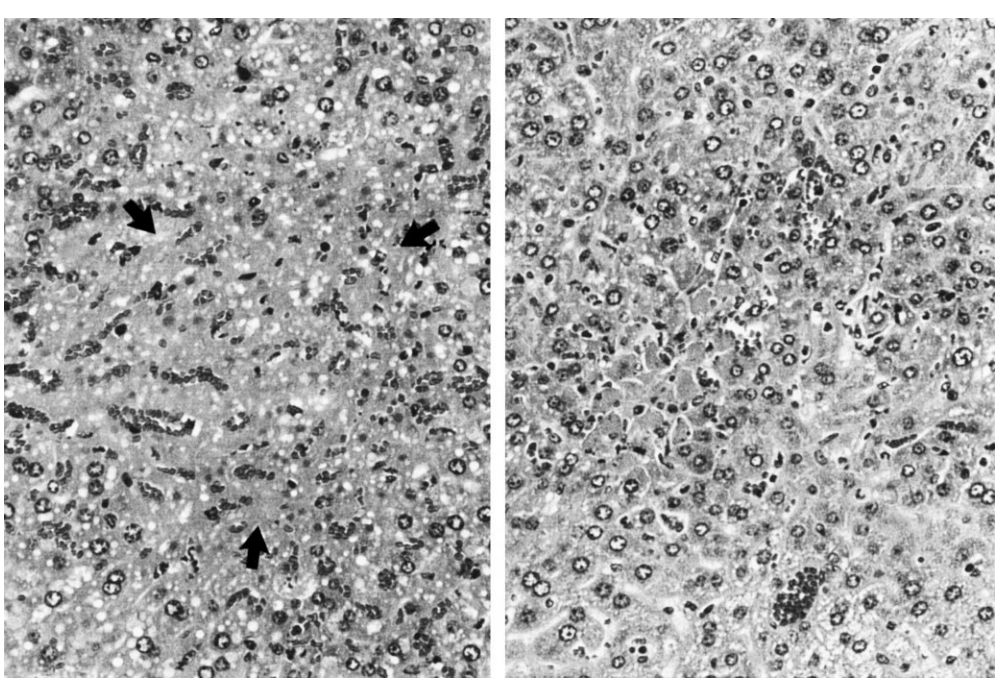

Fig. 5. Effects of aminoguanidine on concanavalin A-induced hepatocyte necrosis. The liver was removed at 24 h after concanavalin A injection. Aminoguanidine treatment was performed at 1 h before concanavalin A administration (hematoxylin and eosin staining ×238). Left: concanavalin A (10 mg/kg,i.v.)-treated. The region of focal necrosis with congestion is surrounded by arrows. Right: concanavalin A (10 mg/kg,i.v.) + aminoguanidine (10 mg/kg,i.p.)-treated. Minimal necrosis and congestion.

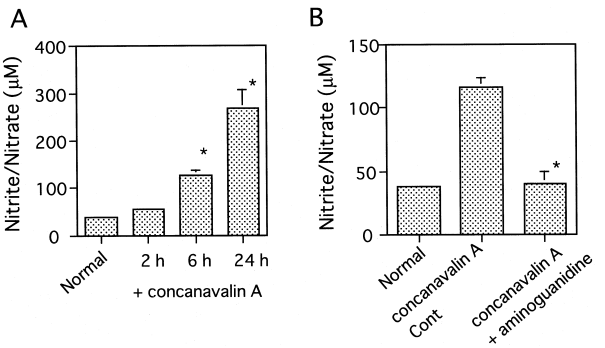

Fig. 7. Effect of concanavalin A on plasma nitrite/nitrate. (A) Concanavalin A-induced plasma nitrite/nitrate. Concanavalin A (10 mg/kg) was administered and plasma was sampled at 2, 6 and 24 h afterwards ($n = 4$). Plasma nitrite/nitrate level was measured with the Griess method. * $P < 0.01$ vs normal control. (B) Effect of aminoguanidine on concanavalin A-induced plasma nitrite. Concanavalin A (10 mg/kg) was administered and at 6 h plasma was sampled for nitrite/nitrate measurement. Aminoguanidine (10 mg/kg) treatment was given 1 h before concanavalin A administration ($n = 4$). * $P < 0.01$ vs. concanavalin A Cont.

3.4. Morphological study of the effect of aminoguanidine

The liver was processed for morphological study. A liver sample same mice as for plasma from a mouse treated with concanavalin A alone showed necrosis in the lobules with congestion (Fig. 5, left). In contrast, a liver sample from a mouse pretreated with concanavalin A + aminoguanidine (10 mg/kg), showed slight congestion and necrosis (Fig. 5, right). Infiltration by T-cells or neutrophils was not affected by aminoguanidine treatment.

3.5. Effect of aminoguanidine on concanavalin A-induced cytokine and iNOS mRNA expression in the liver

Effect of aminoguanidine (10 mg/kg) on concanavalin A-induced interleukin-2, TNF-α, interferon-γ and iNOS mRNA expression was studied. Mice were treated with concanavalin A (10 mg/kg) and the liver was removed at 2 or 6 h after concanavalin A treatment. Liver samples were subjected to RT-PCR analysis. The concanavalin A-induced interleukin-2, TNF-α, interferon-γ or iNOS mRNA expression was not affected by aminoguanidine treatment at either 2 or 6 h (Fig. 6).

3.6. Effect of concanavalin A on plasma nitrite / nitrate

Mice were treated with concanavalin A (10 mg/kg), and killed at 2, 6 and 24 h afterwards when plasma was sampled for measurement of nitrite/nitrate production ($n = 4$). Plasma nitrite/nitrate was significantly induced at 6 and 24 h after concanavalin A treatment (Fig. 7A). The effect of aminoguanidine on concanavalin A-induced elevation of plasma nitrite/nitrate was examined at 6 h ($n = 4$). Aminoguanidine (10 mg/kg) treatment was found to inhibit the concanavalin A-induced elevation of plasma nitrite/nitrate (Fig. 7B).

4. Discussion

Treatment of mice with concanavalin A-induced interferon-γ and iNOS mRNA expression in the liver. iNOS mRNA expression suggests activation of the iNOS system in the liver of this model. Interferon has the potential to trigger the expression of other genes (Samanta et al., 1986). Thus, iNOS mRNA expression might be an effect of interferon-γ. The induction of iNOS mRNA expression by concanavalin A treatment before the elevation of plasma alanine aminotransferase activity suggests the involvement of iNOS activation in the development of hepatitis. Treatment of mice with concanavalin A-induced necrosis in the liver at 24 h after concanavalin A treatment. Immunohistochemical study showed the induction of iNOS protein specifically in the hepatocytes in the same region where necrosis was induced. Thus, iNOS protein which is induced in hepatocytes might play a role in inducing hepatitis. Aminoguanidine is a specific inhibitor of iNOS (Misko et al., 1993), and aminoguanidine alone did not affect the plasma alanine aminotranferase activity level. However, pretreatment of the mice with aminoguanidine inhibited the concanavalin A-induced elevation of plasma alanine aminotransferase activity. Furthermore, a morphological study demonstrated inhibition of the development of concanavalin A-induced necrosis in the liver. In concanavalin A-induced hepatitis, T-cell activation (Tiegs et al., 1992), and T-cell generated TNF-α (Gantner et al., 1995) and interferon-γ (Mizuhara et al., 1996) play an essential role in the development of the hepatitis. Interleukin-2 is commonly measured as a marker of T-cell activation (Paul and Seder, 1994). In the present study, aminoguanidine did not affect concanavalin A-induced interleukin-2 mRNA expression in the liver, indicating that concanavalin A-induced T-cell activation was not inhibited by aminoguanidine. Furthermore, aminoguanidine did not inhibit concanavalin A-induced TNF-α or interferon-γ mRNA expression in the liver. Thus, aminoguanidine does not seem to inhibit T-cell generated inflammatory cytokines in concanavalin A-induced hepatitis. Aminoguanidine is a selective inhibitor of iNOS (Misko et al., 1993) and is reported to inhibit iNOS activity in the hepatocytes (Gardner et al., 1998). In the present study, aminoguanidine did not inhibit concanavalin A-induced iNOS mRNA expression in the liver. Thus, aminoguanidine probably inhibits iNOS protein activity in hepatocytes in concanavalin A-induced hepatitis. Moreover, the elevation of the plasma nitrite/nitrate level by concanavalin A treatment and the inhibition of this nitrite/nitrate elevation by aminoguanidine confirmed iNOS expression. All these results strongly suggest an involvement of the toxic effect of NO formed by iNOS in the development of concanavalin A-induced hepatitis.

NO exerts both cytoprotective and cytotoxic effects (Kamijo et al., 1994). In the experimental cirrhotic liver, NO released from the endothelium of the blood vessels reduces intrahepatic resistance and has a protective effect (Gupta et al., 1998). A role of the hepatoprotective effect of endogenous NO has also been reported in ischemia-reperfusion in the rat liver (Cottart et al., 1999). In the present study, we obtained results which suggested a toxic effect of NO in concanavalin A-induced hepatitis. However, there is still also a possibility of the involvement of the cytoprotective effect of NO in concanavalin A-induced hepatitis.

The livers of patients with hepatitis C virus-infection have shown the expression of interferon-γ and iNOS gene transcripts (Mihm et al., 1997). Although this iNOS gene expression is thought to be due to interferon-γ, the role of iNOS gene expression is not yet fully understood. As found in the present study, interferon-inducible iNOS gene expression in the liver seems to play a role as a toxic factor. From this finding, we speculate that iNOS gene expression in the livers of patients with hepatitis C virus-infection might be possibly involved as a toxic factor.

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