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D-Galactosamine Causes Liver Injury Synergistically with Lipopolysaccharide but not Zymosan in Chicks

Maki Takahashi[1], Akira Senga[1], Kei Teraoka[1], Sakirul Khan[2], Ryosuke Makino[1], Mark A. Cline[3] and Tetsuya Tachibana[1]

[1] Department of Agrobiological Science, Faculty of Agriculture, Ehime University, Matsuyama 790-8566, Ehime, Japan
[2] Department of Microbiology, Faculty of Medicine, Oita University, Yufu, Oita 879-5593, Japan
[3] School of Neuroscience, Virginia Polytechnic Institute and State University, 24061, Blacksburg, Virginia, United States

The pathogen-associated molecular patterns (PAMPs) lipopolysaccharide (LPS) and zymosan, derived from gram-negative bacteria and fungi, respectively, activate the innate immune system and cause injury to multiple organs, including the liver and intestine, in mammals. In rodents, PAMP-induced injury has been demonstrated to be potentiated by co-administration of D-galactosamine (D-GalN) in rodents. However, whether PAMPs and D-GalN collectively cause organ injury in birds remains unclear. The present study aimed to measure the effects of intraperitoneal injection of D-GalN with LPS or zymosan on parameters related to hepatic injury in chicks (*Gallus gallus*). Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) activities were not affected by intraperitoneal injection of D-GalN alone. Although these activities were not affected by LPS injection alone, they were increased by combining LPS with D-GalN. In contrast, plasma AST, ALT, and LDH activities were not affected by zymosan, both alone and with D-GalN. The expression of mRNAs for interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) in the liver was significantly increased by the combination of LPS and D-GalN. In contrast, combining zymosan with D-GalN significantly increased iNOS mRNA expression, irrespective of hepatic injury. These results suggest that IL-6 may be the cause and/or result of hepatic injury in chicks. Additionally, chicks are tolerant to the hepatic effects of D-GalN, LPS, or zymosan alone.

Key words: chick, D-galactosamine, injury, lipopolysaccharide, liver, zymosan

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Introduction

Pathogenic microorganisms activate the innate immune system and induce non-specific symptoms, including anorexia, changes in body temperature, and reduced food passage rate in the mammalian digestive tract. These nonspecific symptoms are caused in part by pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall. LPS decreased food intake, induced hyperthermia, and inhibited gastric emptying[1–3]. LPS causes hepatic injury, characterized by the efflux of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in the blood[4]. Hepatic injury is also caused by zymosan, a fungus-derived PAMP[5]. Therefore, rodent models of multiple organ failure have been developed using these PAMPs. Additionally, D-galactosamine (D-GalN), a well-known hepatotoxic agent, has been frequently used to induce multiple organ failure, because the hepatic injury inducing effects of LPS and zymosan are enhanced by it[6–9].

PAMP-induced hepatic injury is likely mediated by inflammation-associated molecules such as tumor necrosis factor (TNF) and nitric oxide (NO) in rodents. Volman et al.[10] demonstrated that TNF-deficient mice exhibited less zymosan-induced lethality and organ damage, including damage to the liver. Similarly, Cuzzocrea et al.[11] demonstrated that zymosan did not induce severe liver injury in inducible NO synthase (iNOS)-deficient mice compared to that in wild-type mice. Additionally, oxidative stress may mediate PAMP-induced organ injury, because lipid peroxidation is increased by treatment with PAMPs[4,9,12].

Similar to mammals, PAMPs activate the innate immune

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Correspondence: Tetsuya Tachibana, Laboratory of Animal Production, Department of Agrobiological Science, Faculty of Agriculture, Ehime University, Matsuyama 790-8566, Ehime, Japan (E-mail: tetsu@agr.ehime-u.ac.jp)

system and induce non-specific symptoms in chickens. Intraperitoneal (IP) injections of LPS and zymosan induce anorexia, change the cloacal temperature, and inhibit crop emptying in chickens[13–16]. As these effects are similar to those observed in mammalian studies, PAMPs may also induce hepatic injury in birds. Indeed, LPS induces hepatic injury in young chickens[17,18]. The liver is a major metabolic organ in vertebrates; therefore, PAMP-induced hepatic injury leads to a loss of productivity in poultry production. Information regarding PAMP-induced hepatic injury in chickens would provide useful information to prevent the loss of productivity in poultry production. However, the information regarding the effects of PAMPs, other than LPS, on chickens have not been well investigated. Although chickens are infected with microorganisms during their juvenile period, the effects of PAMPs on juvenile chicks have not yet been thoroughly examined. In addition, the cooperative effects of PAMPs and D-GalN in birds have not been well documented.

This study aimed to determine the effects of D-GalN, LPS, and zymosan on hepatic injury in chicks (*Gallus gallus*). First, we investigated the effects of D-GalN on plasma AST, ALT, and LDH activity. Second, D-GalN was co-injected with LPS or zymosan to determine whether D-GalN cooperatively affected AST, ALT, and LDH activities. Third, the combined effect of D-GalN and LPS or zymosan on the abundance of hepatic thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) activity was investigated to assess the involvement of oxidative stress. Finally, the combined effects of D-GalN, LPS, and zymosan on the expression of inflammation-associated genes in the liver were measured.

Materials and Methods

Animals

One-day-old male layer chicks (White Leghorn, Julia, purchased from Minami-Iyo Hatchery, Ehime, Japan) were raised in a group cage in a windowless room kept at 30°C with continuous lighting. They were provided with free access to a commercial diet (crude protein: 24%, metabolizable energy: 3050 kcal/kg; Toyohashi Feed Mills Co. Ltd., Aichi, Japan) and water without any vaccinations. The chicks were individually housed in an experimental cage 1 day before each experiment. Body weights were measured, and the chicks were distributed into treatment groups such that their average body weight was as uniform as possible. We allocated 10 chicks for each group for each experiment, and the actual number of chicks used for the analysis has been described in the figure legends. This study was approved by the Committee of Animal Care and Use at Ehime University, Japan (no. 08-o3-10).

Treatments and injections

All experiments were initiated between 05:00 and 10:00. D-GalN hydrochloride, LPS (*Escherichia coli*, O-127:H6), and zymosan (*Saccharomyces cerevisiae*) (all purchased from FUJIFILM Wako Pure Chemical, Osaka, Japan) were dissolved in sterile saline. Vehicle (saline) alone was used as the control. Because zymosan did not completely dissolve in saline solution, a suspension was used. These reagents were used for IP injection at a volume of 0.2 mL per chick, except if noted differently. None of the chicks presented with hemorrhage following injection.

Experiment 1: D-GalN effects on plasma AST, ALT, and LDH

Six-day-old ad libitum-fed chicks (58 ± 1 g) were IP injected with saline or 40 mg of D-GalN. The dose of D-GalN was selected based on a mammalian study[19]. Blood was collected from the jugular vein using heparin-containing microtubes at 6- or 24-h after injection. The blood was centrifuged (9,000 × *g* at 4°C for 5 min), and plasma was collected and stored at −80°C. Plasma AST, ALT, and LDH activities were measured using commercial kits (AST and ALT, FUJIFILM Wako Pure Chemical, Osaka, Japan; LDH, Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

Experiment 2: LPS and D-GalN effects on plasma AST, ALT, and LDH

Six-day-old chicks (57 ± 0 g) were divided into four groups: control, 0.1 mg LPS alone, 40 mg D-GalN alone, and 0.1 mg LPS plus 40 mg D-GalN. The treatment reagents were IP injected into ad libitum-fed chicks. The LPS dose was selected based on a previous study in chicks[14]. Blood was collected and plasma was obtained 6 h after injection. Plasma AST, ALT, and LDH activities were measured as described in Experiment 1.

Experiment 3: zymosan and D-GalN effects on plasma AST, ALT, and LDH

Five-day-old chicks (54 ± 1 g) were divided into four groups: control, zymosan alone (2.5 mg), D-GalN (40 mg), and zymosan (2.5 mg) combined with D-GalN (40 mg). The chicks in the control and D-GalN alone groups were IP-injected with saline first, whereas those in the zymosan alone and zymosan plus D-GalN groups were IP-injected with zymosan (2.5 mg) first. The injection volume was 0.1 mL per chicken in all the groups. The second administration was performed immediately following the first. The chicks in the control and zymosan alone groups received an IP injection of saline, while those in the D-GalN alone and zymosan plus D-GalN groups received 40 mg D-GalN. The second injection volume was 0.2 mL per chick. The zymosan dose was determined based on our previous works[16,20]. Blood was collected and plasma was obtained 6 h after injection. Plasma AST, ALT, and LDH activities were measured as described in Experiment 1.

As these treatments did not affect any parameters, we further investigated the effects of zymosan and D-GalN 24 h after injection. The procedures were the same as those in the 6 h study, except that blood was collected 24 h after injection.

Experiment 4: LPS and D-GalN effects on TBARS, SOD, and inflammation-associated gene expressions

Six-day-old chicks (58 ± 1 g) were divided into four groups: control, 0.1 mg LPS alone, 40 mg D-GalN alone, and 0.1 mg LPS combined with 40 mg D-GalN. The ad-libitum fed chicks were IP injected with each treatment. The chicks were euthanized by decapitation 6 h after injection. Their livers were collected and weighed, snap frozen, and stored at −80°C until analysis.

The right lobe of the liver was homogenized (TissueLyser LT,

Table 1. **Primers for semi-quantitative RT-PCR**

Target gene	GenBank accession	Sequence (5' to 3')	PCR product (bp)
IL-1β	NM_204524.1	Forward: gcatcaagggctacaagctc	131
		Reverse: caggcggtagaagatgaagc	
IL-6	NM_204628.1	Forward: aaatccctcctcgccaatct	106
		Reverse: ccctcacggtcttctccataaa	
IFN-γ	NM_205149.1	Forward: ctgacaagtcaaagccgcac	230
		Reverse: gcatctcctctgagactggc	
TL1A	NM_001024578.1	Forward: cctgagttattccagcaacgca	292
		Reverse: atccaccagcttgatgtcactaac	
iNOS	NM_204961.1	Forward: atgggaacagagattggagtgcga	129
		Reverse: tacaacagctcggtccttccacaa	
RPII	NM_001006448	Forward: cagaatttgccgacctcttc	172
		Reverse: ggccagcatcacagtctctt	

Abbreviations: IL-1β, interleukin-1β; IL-8, interleukin-6; IFN-γ, interferon-γ; TL1A, tumor-necrosis factor-like cytokine 1A; iNOS, inducible nitric oxide synthase; RPII, polymerase (RNA) II (DNA directed) polypeptide B.

Qiagen, Netherlands) in phosphate-buffered saline and debris were pelleted. TBARS levels were determined using thiobarbituric acid. The supernatant was mixed with 3.3 M acetate buffer containing 0.9% sodium dodecyl sulfate and 0.06% dibutylhydroxytoluene. A fresh thiobarbituric acid solution (50 mM) was added and the mixture was heated at 110°C for 1 h. After cooling, reactants were extracted using n-butanol. The absorbance of the butanol layer was measured using spectrophotometry at 532 nm. SOD activity was measured using a commercial kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Protein concentrations in supernatants were measured using a Lowry assay kit (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's instructions. TBARS and SOD activity data were expressed as per protein concentration.

The left lobe of the liver was homogenized (TissueLyser LT; Qiagen, Netherlands) in Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) to extract total RNA, according to the manufacturer's instructions. Treatment with DNase I and synthesis of first-strand cDNA with random primers were performed using ReverTra Ace® qPCR RT Master Mix reverse transcription kit with gDNA Remover (Toyobo, Osaka, Japan). cDNA was amplified and quantified by semi-quantitative PCR using primers for genes encoding interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ), TNF-like cytokine 1A (TL1A), and iNOS (Table 1). Semi-quantitative PCR was performed in duplicate using a real-time PCR instrument (LightCycler®Nano; Roche Applied Science, Germany) with the SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan). As an internal control, RNA encoding polymerase (RNA) II (DNA-directed) polypeptide B (RPII) was amplified using specific primers (Table 1). PCR conditions were: denaturation at 95°C for 1 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. Primer accuracy was verified by melting curve analysis and nucleotide sequencing of each PCR product. The PCR efficiencies of IL-1β, IL-6, IFN-γ, TL1A, iNOS, and RPII were 92, 105, 99, 105, 94, and 102%, respectively. The $2^{-\Delta CT}$ values were calculated from real-time quantitative PCR experiments[21], with the threshold cycle (C_T) value calculated using the software of the LightCycler®Nano. For $2^{-\Delta CT}$ values, data were analyzed using the equation $\Delta C_T = (C_{T,\ target} - C_{T,\ internal\ control})$.

Experiment 5: zymosan and D-GalN effects on TBARS, SOD, and inflammation-associated gene expression

Six-day-old chicks (60 ± 1 g) were divided into four groups: control, 2.5 mg zymosan alone, 40 mg D-GalN alone, and 2.5 mg zymosan combined with 40 mg D-GalN. Liver collection, TBARS and SOD activity measurements, and gene expression quantification were performed in the same manner as those in Experiment 4.

Statistical analysis

The data from Experiment 1 were statistically analyzed using Student's *t*-test at each time point. Data from Experiments 2, 3, 4, and 5 were analyzed using one-way analysis of variance (ANOVA), followed by a Tukey–Kramer test to separate the means. Data from Experiment 6 were analyzed using Student's *t*-test. Data are expressed as means ± SEM. Statistical significance was set at $P < 0.05$.

Results

Effect of D-GalN on plasma AST, ALT, and LDH

Plasma AST, ALT, and LDH activities after IP D-GalN injection are shown in Fig. 1. Forty milligrams of D-GalN did not affect plasma AST, ALT, or LDH activities at either time points. However, it tended to increase after 24 h ($P = 0.08$). The plasma ALT and LDH activities were also unaffected by D-GalN.

Effect of combined administration of LPS and D-GalN on plasma AST, ALT, and LDH

The effect of combined administration of LPS and D-GalN on plasma AST, ALT, and LDH activities 6 h after injection is shown in Fig. 2. Plasma AST activity was not affected by D-GalN alone, as shown in Fig. 1. LPS alone did not have an effect, but plasma

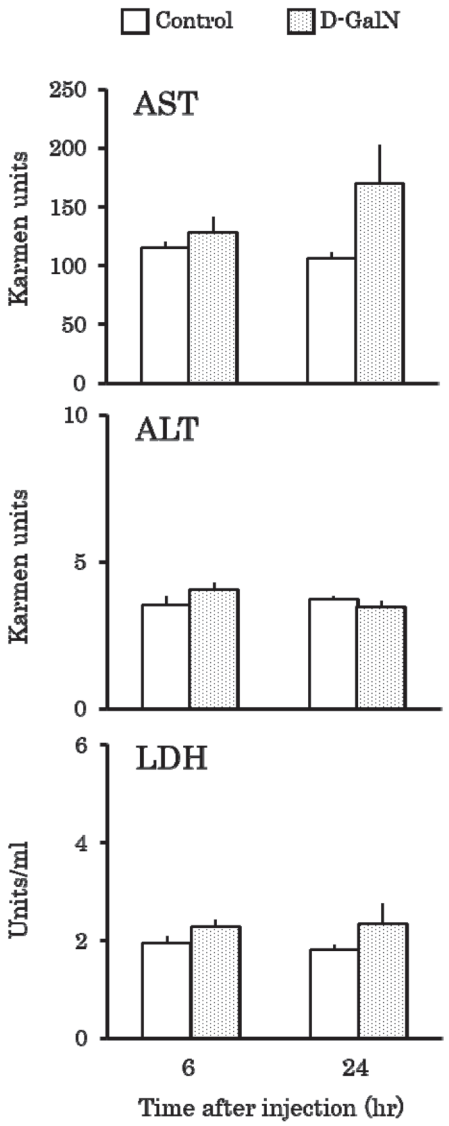

Fig. 1. **Effect of 40 mg D-GalN on the activities of plasma AST, ALT and LDH.** Each group had 9 chicks. Values are means ± SEM. Abbreviations: D-GalN, D-galactosamine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

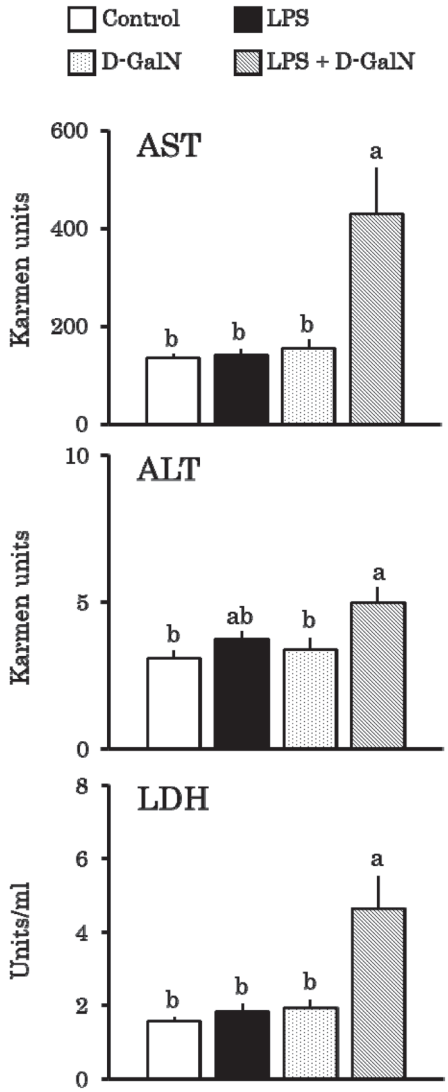

Fig. 2. **Effect of co-administration of 0.1 mg LPS and 40 mg D-GalN on plasma AST, ALT and LDH activities.** Blood was collected at 6 h after the injection. Numbers of chicks in the control, LPS alone, D-GalN alone, and LPS + D-GalN groups were 8, 9, 7, and 9, respectively. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$). Abbreviations: LPS, lipopolysaccharide; D-GalN, D-galactosamine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

AST activity was significantly increased by combined LPS and D-GalN administration. Plasma ALT activity tended to increase following LPS injection alone, but the effect was significant following the subsequent administration of D-GalN. Plasma LDH activity was not affected by LPS alone; however, LDH activity was significantly increased by combined administration of LPS and D-GalN.

Effect of combining zymosan and D-GalN on plasma AST, ALT, and LDH

Plasma AST, ALT, and LDH activities after combined administration of zymosan and D-GalN are shown in Fig. 3. Six hours after injection, zymosan alone decreased plasma AST and LDH activities, but ALT activity was not affected. Enzymatic activity was not affected by the simultaneous injection of zymosan and D-GalN at 6 h. Also, enzymatic activity was not affected by zymosan alone or zymosan and D-GalN combination after 24 h of administration.

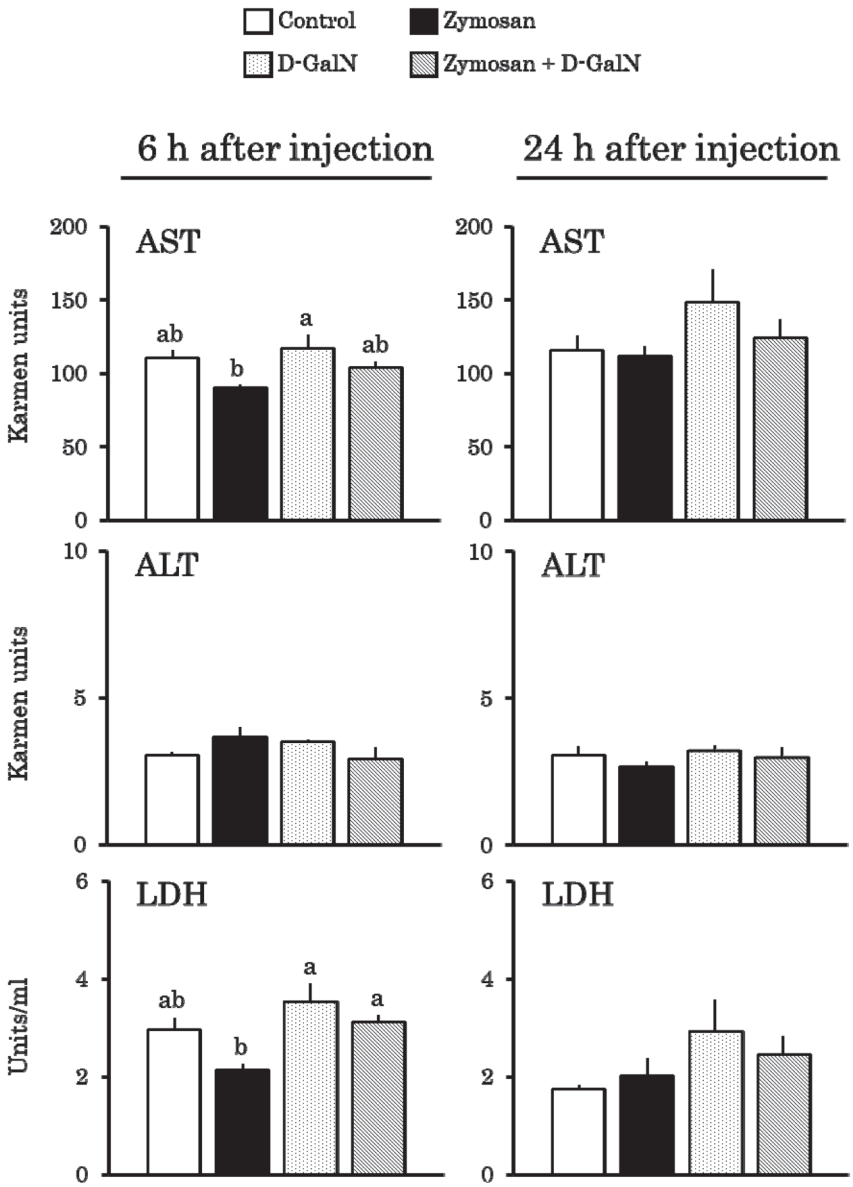

Fig. 3. **Effect of co-administration of 2.5 mg zymosan and 40 mg D-GalN on plasma activities of AST, ALT, and LDH.** Numbers of chicks in the control, zymosan alone, D-GalN alone, and zymosan + D-GalN groups were 9, 8, 7, and 8 for 6 h and 8, 8, 9, and 9 for 24 h, respectively. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$). Abbreviations: D-GalN, D-galactosamine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

Effect of combining LPS and D-GalN combination on liver TBARS, SOD activity, and gene expression

Liver TBARS levels and SOD activity 6 h after combined LPS and D-GalN administration are shown in Fig. 4. The TBARS levels were not affected by any of the treatments. Hepatic SOD activity was significantly increased by D-GalN alone, whereas LPS alone had no effect. SOD activity in the LPS and D-GalN combination groups was similar to that in the control group.

The effect of combined injection of LPS and D-GalN on liver inflammation-associated gene expression is shown in Fig. 5. The mRNA expression levels of IL-6 and iNOS tended to be increased by LPS alone, whereas they were not affected by D-GalN alone. The mRNA levels of IL-6 and iNOS were significantly increased by simultaneous injection of LPS and D-GalN. Injection of LPS alone significantly increased TL1A mRNA expression; however, simultaneous injection with D-GalN abolished this effect. None of the treatments affected hepatic IL-1β or IFN-γ mRNA expression levels.

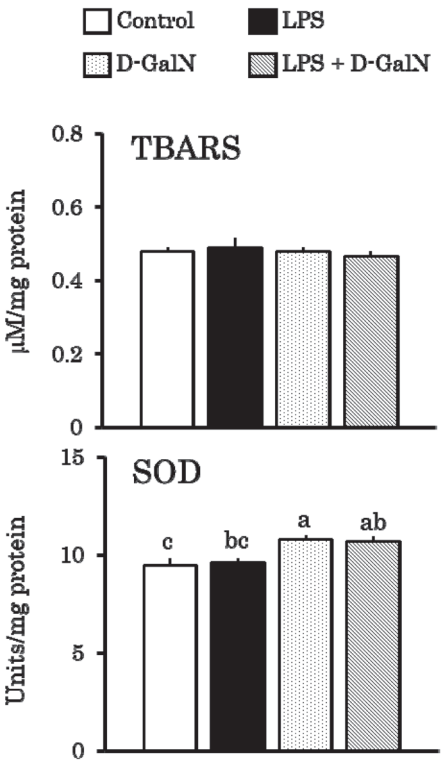

Fig. 4. **Effect of co-administration of 0.1 mg LPS and 40 mg D-GalN on TBARS and SOD activity in the liver (collected 6 h after treatment).** Numbers of chicks in the control, LPS alone, D-GalN alone, and LPS + D-GalN groups were 8, 8, 8, and 6, respectively. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$). Abbreviations: LPS, lipopolysaccharide; D-GalN, D-galactosamine; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase.

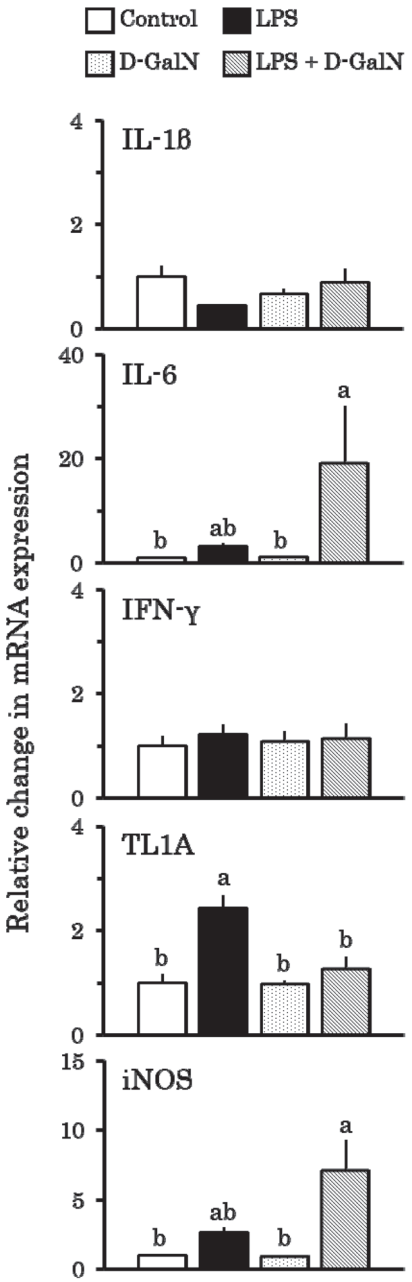

Fig. 5. **Effect of co-administration of 0.1 mg LPS and 40 mg D-GalN on the expression of inflammatory-associated genes in the liver (collected at 6 h following treatment).** Numbers of chicks in the control, LPS alone, D-GalN alone, and LPS + D-GalN groups were 8, 8, 8, and 6, respectively. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$). Abbreviations: LPS, lipopolysaccharide; D-GalN, D-galactosamine; IL-1β, interleukin-1β; IL-8, interleukin-6; IFN-γ, interferon-γ; TL1A, tumor necrosis factor-like cytokine 1A; iNOS, inducible nitric oxide synthase; RPII, polymerase (RNA) II (DNA-directed) polypeptide B.

Effect of combined zymosan and D-GalN on liver TBARS, SOD activity, and gene expression

Hepatic TBARS levels and SOD activity after combined administration of zymosan and D-GalN are shown in Fig. 6. Both parameters were unaffected by zymosan alone, D-GalN alone, or zymosan and D-GalN combination.

Liver inflammation-associated gene expression 6 h after collective administration of zymosan and D-GalN is shown in Fig. 7. IL-1β, IL-6, IFN-γ, and TL1A mRNA levels were not affected by zymosan alone, D-GalN alone, or zymosan and D-GalN combination. Collective administration of zymosan and D-GalN significantly increased iNOS mRNA expression, although neither independently had a significant effect.

Discussion

First, we found that plasma AST, ALT, and LDH activities were not affected by administration of D-GalN (approximately

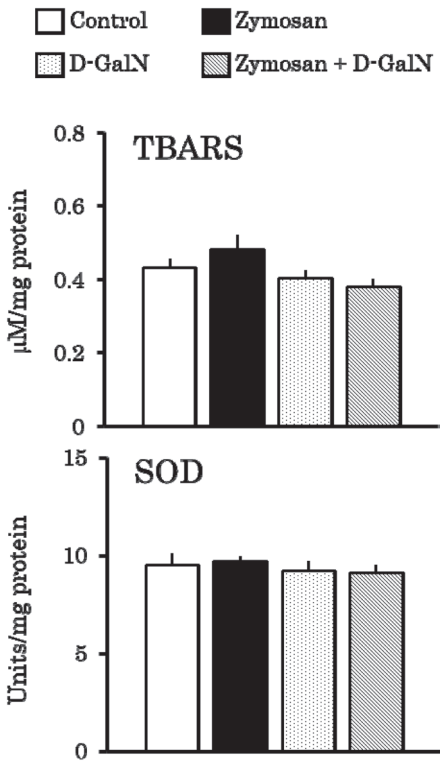

Fig. 6. **Effect of co-administration of 2.5 mg zymosan and 40 mg D-GalN on TBARS and SOD activity in the liver.** The liver was collected at 6 h after the injection. Numbers of chicks in the control, zymosan alone, D-GalN alone, and zymosan + D-GalN groups were 8, 7, 8, and 8, respectively. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$). Abbreviations: D-GalN, D-galactosamine; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase.

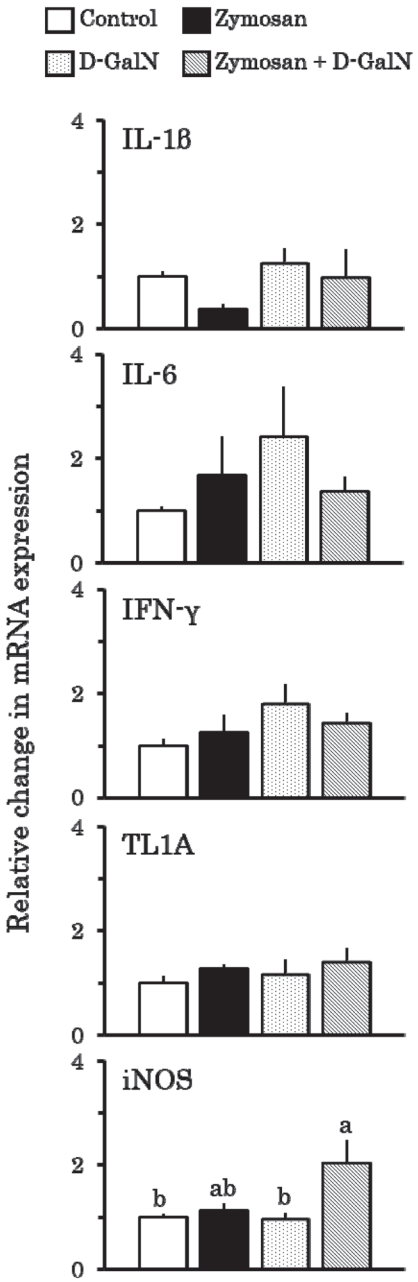

Fig. 7. **Effect of co-administration of 2.5 mg zymosan and 40 mg D-GalN on the expression level of hepatic inflammatory-associated genes in the liver.** The livers were collected 6 h after the injection. Numbers of chicks in the control, zymosan alone, D-GalN alone, and zymosan + D-GalN groups were 8, 7, 8, and 8, respectively. Values are means ± SEM. Groups with different letters are significantly different ($P < 0.05$). Abbreviations: D-GalN, D-galactosamine; IL-1β, interleukin-1β; IL-8, interleukin-6; IFN-γ, interferon-γ; TL1A, tumor necrosis factor-like cytokine 1A; iNOS, inducible nitric oxide synthase; RPII, polymerase (RNA) II (DNA-directed) polypeptide B.

700 mg/kg body weight (BW)) (Fig. 1). Therefore, D-GalN alone is unlikely to cause hepatic injury in chicks. In contrast, in rats serum AST and ALT activities were increased by IP injection of D-GalN (400 or 500 mg/kg BW)[19,22]. The sensitivity to D-GalN may differ among animal species. Iwaki et al.[6] demonstrated that endotoxin-free rats did not experience D-GalN-induced hepatic injury by colectomy. Additionally, D-GalN increases bacterial translocation in rats, corresponding to hepatic injury[23]. Therefore, we sought to measure the effects of combining LPS and D-GalN on liver injury in chicks.

We found that plasma AST and LDH activities were not affected by the injection of LPS alone (Fig. 2). These results were also contrary to those of previous studies in rodents, in which serum AST and LDH activities were increased[4,24]. Interestingly, LPS-induced hepatic injury has also been observed in young chickens[17,18], although the experimental conditions, including age and LPS source, were different from those of our study. In our preliminary histological experiment, LPS injection induced no apparent hepatic injury (characterized by hemorrhage; data

not shown). Therefore, LPS may have less of an effect on hepatic injury in juvenile chicks. However, plasma AST, ALT, and LDH activities were significantly increased when LPS was combined with D-GalN (Fig. 2). These findings agree with the results of murine studies that employed the combination treatment[8,9].

Similar to LPS, plasma AST, ALT, and LDH activities were not affected by zymosan alone in chicks (Fig. 3), unlike murine experiments showing that they increased (1 mg/kg BW, 4 h after IP injection)[5]. In addition, zymosan induces injury in multiple mammalian organs[25]. The ineffectiveness of zymosan and LPS in this study suggests that chicks are tolerant to PAMP-associated organ injury. In rats, zymosan aggravates D-GalN-induced hepatic injury[7]. However, no such potentiation was observed in our study (Fig. 3). Alternatively, the latent ability of zymosan to induce hepatic injury in chicks may be weaker than that of LPS. We administered zymosan as a suspension owing to its low solubility in water, whereas LPS was completely dissolved. The differences in the effects of zymosan and LPS may be this difference in solubility.

In rodents, D-GalN-induced oxidative stress contributes to hepatic injury[26,27]. LPS increases hepatic TBARS levels and decreases SOD activity in rats[4]. In mice, IP injection of zymosan also decreases hepatic SOD activity[12]. Moreover, in mice LPS combined with D-GalN (1 and 300 mg/kg) increased TBARS levels and decreased SOD activity[9]. However, chick hepatic TBARS levels and SOD activity were not affected by either D-GalN, LPS, zymosan, or their combination (Figs. 4 and 6). Therefore, oxidative stress in the chicks was not induced by these treatments. This may explain why they did not cause hepatic injury in this study. Nevertheless, co-injection of LPS and D-GalN increased plasma AST, ALT, and LDH activities (Fig. 2). The changes in the activities of these enzymes induced by LPS and D-GalN (Fig. 2) may be induced by an oxidative stress-independent mechanism.

In mammals, PAMPs cause the excessive expression of bioactive molecules, including inflammatory cytokines and NO, inducing organ injury. Indeed, plasma IL-6, TNF-α, and IFN-γ levels were increased by IP injection of zymosan in mice[5,28]. TNF-deficient mice exhibited less lethality and organ damage, including the liver, in response to zymosan[10]. In rats, zymosan also increased NO production by inducing iNOS expression; NO was associated with zymosan-induced tissue damage in the lungs, liver, and intestines[29]. Mice lacking iNOS showed a reduced response to zymosan-induced plasma ALT and LDH activities and liver injury compared to wild-type controls[29]. Since in chicks, IP injection of LPS and zymosan has been shown to induce changes in cytokine mRNAs in the spleen and digestive tract, and increase plasma NOx levels[15,16,20,30], these bioactive molecules may be involved in the effect of LPS and D-GalN on liver injury. Indeed, IL-6 and iNOS mRNA expression was increased by simultaneous injection of LPS and D-GalN, whereas LPS and D-GalN alone had no effect (Fig. 5). However, an increase in liver iNOS mRNA expression was also observed after co-administration of zymosan and D-GalN (Fig. 7), although this combination did not affect the plasma activities of enzymes associated with hepatic injury (Fig. 3). Excess NO derived from iNOS induces oxidative stress[29]; therefore, combining D-GalN with LPS or zymosan could trigger oxidative stress to induce hepatic injury. However, co-injection of zymosan and D-GalN did not induce hepatic injury (Fig. 3), indicating that increased iNOS expression was not sufficient to induce injury. Indeed, iNOS mRNA expression was approximately increased 7-fold by the combination of D-GalN and LPS (Fig. 5), whereas it was only increased approximately 2-fold by the combination of D-GalN and zymosan (Fig. 7). Therefore, combining D-GalN and LPS may have strongly increased the expression of iNOS and NO production in the liver, inducing injury. In contrast, an increase in IL-6 mRNA was observed when D-GalN was co-administered with LPS but not with zymosan (Figs. 5 and 7). Thus, increased production of IL-6 may be required to induce hepatic injury. Alternatively, increase in IL-6 mRNA may be the result, rather than the cause, of hepatic injury. In mice, IL-6 prevents LPS- and D-GalN-induced hepatic injury[8]. The expression of IL-6 may be used to prevent hepatic injury induced by the combination of D-GalN and LPS. The actual roles of IL-6 and NO in liver injury need to be clarified in future studies.

In conclusion, this study showed that D-GalN enhanced the effect of LPS, but not zymosan, and may induce hepatic injury, whereas administration of LPS and zymosan independently had no effect. Our findings suggest that approximately 1-week-old chicks possess tolerance to hepatic injury induced by D-GalN, LPS, and zymosan alone, in contrast to rodents. The differences between chicks and rodents may be due to their age and species, which should be clarified in the future.

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Author Contributions

Maki Takahashi conducted experiments, analyzed data, and wrote the paper; Akira Sengan and Kei Teraoka conducted experiments and analyzed data; Sakirul Khan designed experiments, discussed results, and checked the paper; Ryosuke Makino discussed results and checked the paper; Mark A. Cline discussed results and checked the paper; and Tetsuya Tachibana conceived the study and experimental design, conducted experiments, analyzed data, and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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