0145-6008/03/2708-0007$03.00/0
ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

Vol. 27, No. 8
August Supplement 2003

# Dalteparin Sodium Prevents Liver Injury Due to Lipopolysaccharide in Rat Through Suppression of Tumor Necrosis Factor-$\alpha$ Production by Kupffer Cells

Shigeki Tsukada, Nobuyuki Enomoto, Yoshiyuki Takei, Miyoko Hirose, Kenichi Ikejima, Tsuneo Kitamura, and Nobuhiro Sato

**Background:** Sensitization of Kupffer cells (KC) to lipopolysaccharide (LPS) and overproduction of tumor necrosis factor (TNF)-$\alpha$ play important roles in the pathogenesis of alcoholic liver damage and sepsis-associated organ injury. Therefore, suppression of TNF-$\alpha$ should prove useful for treatment of LPS-induced liver injury. Recently, heparin has been reported to diminish TNF-$\alpha$ production from macrophages in response to LPS. Dalteparin sodium (DS) is a low-molecular-weight heparin with a mean molecular weight of 5000. DS elicits an antithrombotic effect through a mechanism depending on anti–factor Xa activity but not on the antithrombin activity. DS is thus suitable for treatment of disseminated intravascular coagulation because it has a much smaller prohemorrhagic property. In this study, we evaluated whether DS could prevent LPS-induced liver injury.

**Methods:** Female Wistar rats were administered DS (50 IU/kg intraperitoneally) followed by challenge with LPS (5 mg/kg intravenously) 2 hr later. Livers and sera were collected 24 hr later. KC from rats were isolated and cultured in RPMI 1640 supplemented with 10% fetal bovine serum. After the addition of LPS (10 $\mu$g/ml) to the culture media, intracellular $Ca^{2+}$ was measured by using a fluorescent indicator, fura-2.

**Results:** LPS (5 mg/kg intravenously) caused focal necrosis and neutrophil infiltration in the control liver. The histological changes and increased alanine aminotransferase levels caused by LPS injection were diminished by treatment with DS. LPS increased intracellular $Ca^{2+}$ of KC in control rats from the basal level (26 $\pm$ 6 nmol/liter) to 280 $\pm$ 18 nmol/liter. This increase was blunted by DS (126 $\pm$ 28 nmol/liter). The DS treatment decreased the LPS-induced TNF-$\alpha$ production by KC from 911 $\pm$ 78 pg/ml to 309 $\pm$ 45 pg/ml ($p < 0.05$).

**Conclusions:** These results indicate that DS reduces the LPS-induced liver injury through suppression of TNF-$\alpha$ production.

**Key Words:** TNF-$\alpha$, Kupffer cell, LPS.

ENDOTOXIN [LIPOPOLYSACCHARIDE (LPS)] is a component of the outer wall of Gram-negative bacteria that causes many biological effects. Kupffer cells (KC), resident macrophages in the liver, not only remove gut-derived endotoxin, but also are activated during the phagocytotic process (Nolan, 1981) to produce chemical mediators [i.e., eicosanoids, interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-$\alpha$, superoxide, and nitric oxide]. Sensitization of KC to LPS and overproduction of TNF-$\alpha$ play important roles in the pathogenesis of alcoholic liver damage (Martines et al., 1992; Stahnke et al., 1991) and sepsis-associated organ injury (Parrillo, 1993). Therefore, suppression of TNF-$\alpha$ should prove useful for treatment of LPS-induced liver injury.

Recently, heparin has been reported to diminish TNF-$\alpha$ production from monocytes in response to LPS (Chelmonska-Soyta et al., 2001). But heparin has a potent prohemorrhagic property that may exacerbate septic shock or severe liver injury. Dalteparin sodium (DS) is a low-molecular-weight heparin with a mean molecular weight of 5000 (de Jouge et al., 1998). DS elicits an antithrombotic effect through a mechanism depending on anti–factor Xa activity but not on the antithrombin activity. DS is thus suitable for treatment of sepsis-induced disseminated intravascular coagulation because it has a much smaller prohemorrhagic property. In this study, we evaluated whether DS could prevent LPS-induced liver injury.

## MATERIALS AND METHODS

### *Animals and Treatments*

Female Wistar rats weighing 200 to 250 g were used for this study. All animals were given humane care in compliance with institutional guidelines. Twenty rats were administered DS [50 IU/kg intraperitoneally (ip), Kissei Pharmaceutical, Tokyo, Japan] followed by challenge with LPS (5

*From the Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan.*

*Received for publication May 9, 2003; accepted May 9, 2003.*

*Reprint requests: Nobuhiro Sato, MD, PhD, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan; Fax: 81-3-3813-8862; E-mail: nsato@med.juntendo.ac.jp.*



**DOI: 10.1097/01.ALC.0000078610.78390.2A**

mg/kg; *Escherichia coli* serotype O111:B4, Sigma Chemical, St. Louis, MO) 2 hr later. Liver specimens were taken 24 hr after administration of LPS (5 mg/kg), fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin to assess inflammation and necrosis. Blood samples were collected before, 90 and 180 min after, and 24 hr after injection of LPS. Sera were stored at −20°C in microtubes for subsequent assay of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by standardized enzymatic procedures (Bergmeyer, 1988). TNF-$\alpha$ levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillio, CA).

### *KC Preparation and Culture*

KC from rats were isolated by collagenase digestion and differential centrifugation by using Percoll (Pharmacia, Uppsala, Sweden). Briefly, the liver was perfused with Hanks' balanced salt solution containing 0.025% collagenase IV (Sigma Chemical) at 37°C for 5 min. After the liver was digested, cell suspension was filtered through nylon gauze, and the filtrate was centrifuged at 450 × *g* for 10 min at 4°C. Cell pellets were resuspended in buffer, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 1000 × *g* for 15 min, and the KC fraction was collected. Cells were seeded onto 25-mm glass coverslips and cultured in RPMI 1640 (GIBCO Laboratories/Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (100 units/ml of penicillin G and 100 $\mu$g/ml of streptomycin sulfate) at 37°C with 5% $CO_2$. Nonadherent cells were removed after 1 hr by replacing the culture medium. Cells were cultured for 24 hr before experimentation.

### *Measurement of Intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$)*

The ($[Ca^{2+}]_i$ measurement was described in detail elsewhere (Enomoto et al., 1998). Briefly, after loading with fura-2/AM solution (4 mM) at 37°C for 30 min, KC on coverslips were installed in a fluorescence microscope (Diaphot, Nikon, Tokyo, Japan) with a 100-W xenon arc lamp as a light source. Wavelengths of 340 and 380 nm for excitation and 520 nm for emission were used. $[Ca^{2+}]_i$ was determined by the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d[(R_0 - R_{min})/(R_{max} - R_0)]B$$

$K_d$, the $Ca^{2+}$ dissociation constant for fura-2, was confirmed as 224 nM. $R$ represents fluorescence intensity at 340 nm excitation divided by that at 380 nm excitation [$R_0$, experimental data; $R_{min}$, $R$ in 2 mM ethylene glycol-bis ($\beta$-aminoethyl ether) *N*,*N*,*N*', *N*'-tetraacetic acid and 1 mM ionomycin; $R_{max}$ is $R$ in 10 mM $Ca^{2+}$ and 1 mM ionomycin]. $B$ is the ratio of fluorescence intensity at 380 nm in the absence of $Ca^{2+}$ versus a saturating concentration of $Ca^{2+}$.

### *Measurement of TNF-$\alpha$ Concentration of Culture Media*

Isolated KC were cultured in 24-well culture plates at a density of 5 × $10^5$ cells per well for 24 hr before experimentation. The TNF-$\alpha$ concentration in the culture media was determined with an ELISA kit.

For in vivo experiments, KC were isolated 2 hr after DS administration (50 IU/kg ip), and after 24 hr, LPS (10 ng/ml) was added. After that, KC were incubated for 4 hr, and TNF-$\alpha$ in culture media was measured. For in vitro experiments, KC were isolated, and after 24 hr, DS (200 $\mu$g/ml) was added into the culture media. After 2 hr, LPS (10 ng/ml) was added, KC were incubated for 4 hr, and TNF-$\alpha$ was measured.

### *Statistical Analysis*

All results were expressed as means ± SEM. Statistical differences between means were determined by using ANOVA and Bonferroni's post hoc test or Student's *t* test as appropriate. $p < 0.05$ was selected before the study to reflect significance.

## RESULTS

### *Effect of DS on LPS-Induced Liver Injury*

Liver pathology 24 hr after the administration of LPS (5 mg/kg intravenously) is shown in Fig. 1. Histology was normal in control rats (Fig. 1A). As expected, LPS caused focal necrosis and neutrophil infiltration in liver from the LPS-treated, DS-untreated control rats (Fig. 1B). These

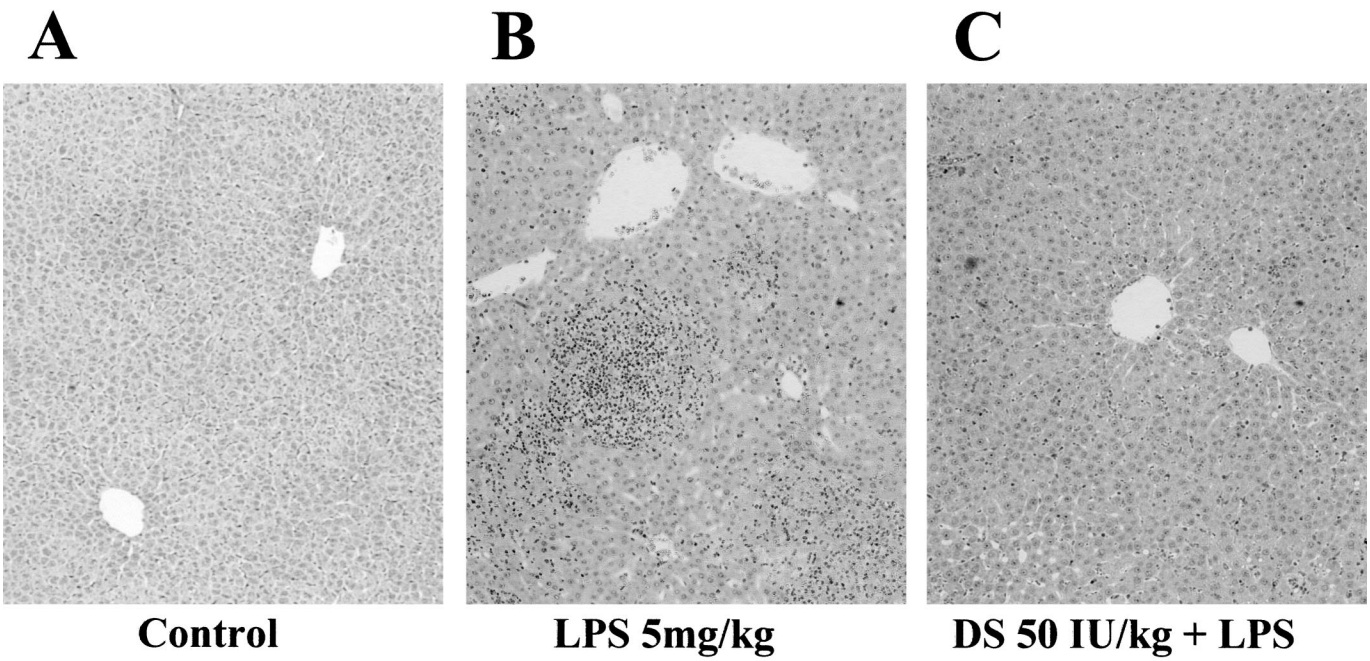


**Fig. 1.** Effect of DS on LPS-induced liver injury. Typical photomicrographs of hematoxylin-eosin sections of livers from rats treated as described in "Materials and Methods." Rats were killed 24 hr after the administration of LPS (5 mg/kg intravenously): (A) no treatment, (B) administration of only LPS, and (C) administration of LPS 2 hr after DS (50 IU/kg) treatment. Original magnification (A–C), × 100.

pathologic parameters were markedly reduced by pretreatment with DS (50 IU/kg ip; Fig. 1C). Whereas mean values of AST and ALT in untreated control rats were 56 ± 2 IU/liter and 22 ± 4 IU/liter, values in the group challenged with LPS (5 mg/kg intravenously) were markedly increased to 1148 ± 77 IU/liter and 736 ± 121 IU/liter, respectively. In the group treated with DS (50 IU/kg ip), AST and ALT values were significantly reduced to 624 ± 133 IU/liter and 365 ± 112 IU/liter, respectively (Fig. 2).

### *Effect of DS on Serum TNF-α Levels After LPS Injection*

As depicted in Fig. 3, serum TNF-α was increased markedly from the basal level to 13,242 ± 3,262 pg/ml 90 min after LPS injection, followed by a rapid decrease, returning to the basal level over 180 min. Conversely, the peak increase of TNF-α was suppressed by 80% to 2713 ± 721 pg/ml by DS treatment (Fig. 3).

### *Effect of DS on LPS-Induced TNF-α Production by Cultured KC*

Four rats were treated with DS (50 IU/kg ip), and 2 hr later, KC were isolated. After 24 hr of incubation, LPS was added to the culture media, and KC were cultured for additional 4 hr. TNF-α produced by isolated KC was 911 ± 78 pg/ml in the presence of LPS 10 ng/mL. Pretreatment with DS markedly reduced the LPS-induced TNF-α production to 309 ± 45 pg/ml (Fig. 4).

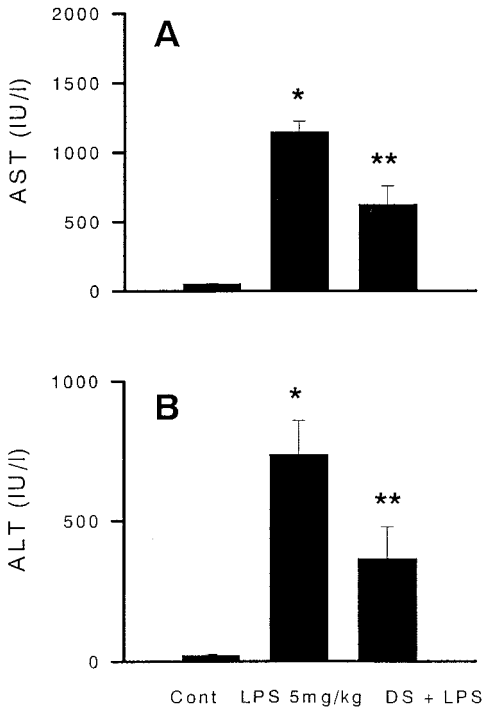


**Fig. 2.** Effect of DS on serum transaminases after LPS injection. Blood samples were collected from the aorta 24 hr after LPS injection (5 mg/kg intravenously). Serum AST and ALT levels were measured as described in "Materials and Methods." Results are mean ± SEM; $n = 4$; $^*p < 0.05$ versus control; $^{**}p < 0.05$ versus LPS by ANOVA and Bonferroni's post hoc test.

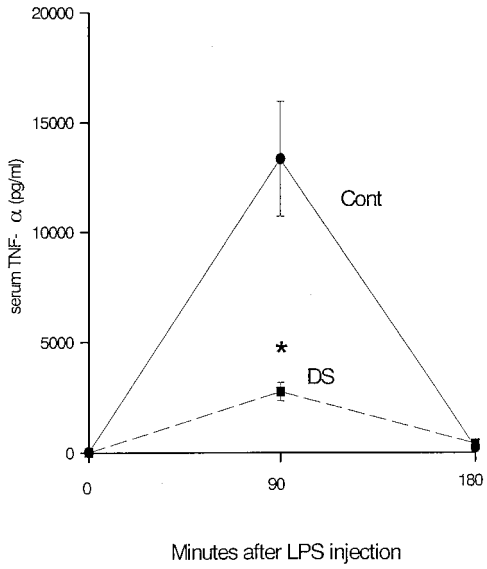


**Fig. 3.** Effect of DS on LPS-induced serum TNF-α concentration. Some rats were treated with DS (50 IU/kg ip). Blood samples were collected from the tail vein. Serum TNF-α was measured by ELISA. Results are mean ± SEM; $n = 4$; $^*p < 0.05$ versus control (Cont) by ANOVA and Bonferroni's post hoc test.

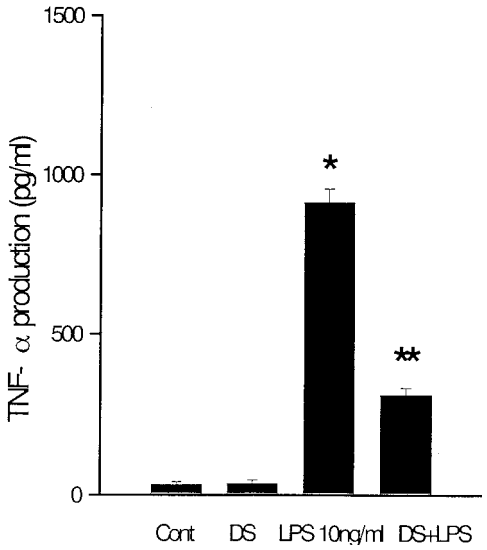


**Fig. 4.** Effect of DS on LPS-induced TNF-α production in culture media. KC were isolated 2 hr after DS (50 IU/kg ip) injection and cultured in 24-well cultured plates at a density of $5 \times 10^5$ cells per well, and after 24 hr, LPS (10 ng/ml) was added. After that, KC were incubated for 4 hr, and TNF-α in culture media was measured. The TNF-α level produced by isolated KC was 911 ± 78 pg/ml in the presence of LPS. DS reduced LPS-induced TNF-α production markedly to 309 ± 45 pg/ml. Results are mean ± SEM; $n = 4$; $p < 0.05$ by ANOVA and Bonferroni's post hoc test. Cont, control. $^*p < 0.05$ versus control; $^{**}p < 0.05$ LPS.

### *Effect of DS on LPS-Induced Increases in $[Ca^{2+}]_i$ in Isolated KC*

To evaluate the effect of DS on KC sensitization to LPS, we measured the LPS-induced increases in $[Ca^{2+}]_i$ of KC. KC were isolated 2 hr after DS (50 IU/kg ip) administration and incubated for 24 hr. After the addition of LPS to culture media, $[Ca^{2+}]_i$ levels of KC in control rats increased quickly, reaching 280 ± 18 nmol/liter in 60 sec, followed by

a gradual decline to basal levels within 4 min (Fig. 5A). The LPS-induced peak increase in $[Ca^{2+}]_i$ was blunted by approximately 50% with DS (Fig. 5).

### *Effect of DS on LPS-Induced TNF-α in Culture Media*

After 24 hr of culture, DS (200 μg/ml) was added to the culture media of KC. Two hours later, LPS (10 ng/ml) was added, and incubation continued for 4 hr. Whereas isolated KC produced large amounts of TNF-α (1291 ± 208 pg/ml) in response to LPS, DS reduced the LPS-induced TNF-α production by 40% to 740 ± 42 pg/ml (Fig. 6).

## DISCUSSION

In this study, increased serum transaminase and massive hepatic necrosis and neutrophil infiltration caused by LPS were suppressed dramatically by DS, low-molecular-weight heparin. One possible explanation for this effect of DS would be the reduced responsiveness of KC to LPS. Previous studies showed that increases in $[Ca^{2+}]_i$ are essential for the release of inflammatory cytokines in response to stimuli including LPS (Enomoto et al., 1998). Our data show that DS markedly blunted TNF-α production and the increase in $[Ca^{2+}]_i$ in KC caused by LPS.

Heparin, produced mainly by activated mast cells present at the site of inflammation, is one of the potent components of the extracellular matrix and may play a role in the modulation of inflammatory response (Wrenshall et al., 1991, 1995). Heparin probably takes part in the process of confinement of the inflammatory response due to its ability to bind IFN-γ (Lortat-Jacob et al., 1995), IL-2 (Najjam et al., 1998), and IL-12 (Hasan et al., 1999). Heparin also affects functions of macrophages. Mouse peritoneal macrophages, when preincubated with heparin, did not respond to LPS stimulation for nitric oxide production (Matsuno et al., 1997). It was also shown that heparin disaccharides inhibit TNF-α production and delayed-type hypersensitivity reaction in mice (Cahalon et al., 1997). Similarly, in humans, cytokine gene expression in LPS- and IFN-γ–stimulated mononuclear cells was inhibited by this polysaccharide (Attanasio et al., 1998). Moreover, Chelmonska-Soyta et al. (2001) reported that heparin reduced TNF-α production from monocytes in response to LPS. Our data suggest that heparin may prevent KC activation in response to LPS through a mechanism dependent on $[Ca^{2+}]_i$ response.

In summary, DS markedly reduced liver injury by LPS. Although the effect of DS is not entirely understood, it most likely acts by suppressing activation of KC, thereby preventing liver injury. Because sensitization of KC to LPS and the resultant overproduction of TNF-α are critical factors for the pathogenesis of alcoholic liver damage (Enomoto et al., 1998, 1999, 2002), DS may hold promise for the treatment of alcoholic liver disease.

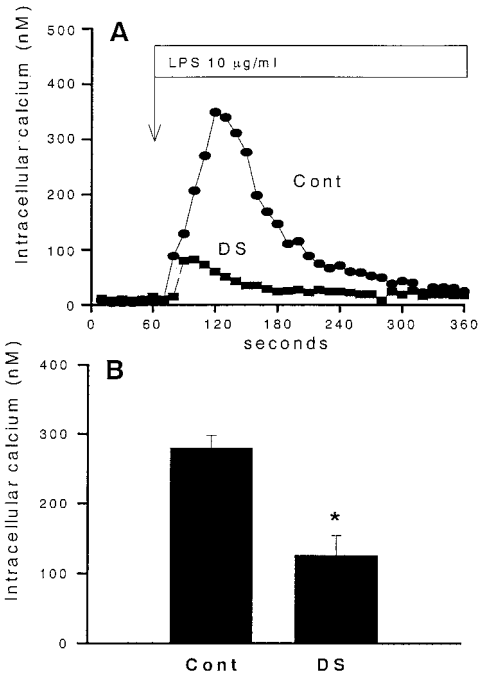


**Fig. 5.** Effect of DS on LPS-induced increases in intracellular $Ca^{2+}$ in isolated KC. Isolated KC were cultured in 35-mm culture dishes at a density of $5 \times 10^5$ cells per dish for $[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ was measured by using a microspectrometer with the fluorescent indicator fura-2. LPS was added to KC from control rats and to KC from rats treated with DS. (A) Typical traces; (B) peak $[Ca^{2+}]_i$ value. Results are mean ± SEM; $n = 4$; $^*p < 0.05$ versus control (Cont) by ANOVA and Bonferroni's post hoc test.

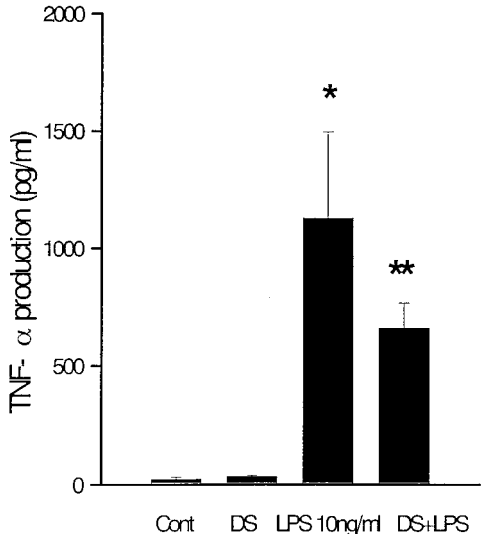


**Fig. 6.** Effect of DS on TNF-α in culture media in vitro. KC were isolated and cultured for 24 hr, and DS (200 μg/ml) was added to the culture media. After 2 hr, LPS (10 ng/ml) was added, incubation was continued for 4 hr, and TNF-α in the culture media was measured. Isolated KC produced large amounts of TNF-α (1291 ± 208 pg/ml) in the presence of LPS, and DS reduced LPS-induced TNF-α production markedly to 740 ± 42 pg/ml. Results are mean ± SEM; $n = 4$; $^*p < 0.05$ versus control (Cont); $^{**}p < 0.05$ versus LPS by ANOVA and Bonferroni's post hoc test.

## REFERENCES

Attanasio M, Gori AM, Giusti B, Pepe G, Comeglio P, Brunelli T, Prisco D, Abbate R, Gensini GF, Neri Serneri GG (1998) Cytokine gene expression in human LPS and IFN-gamma stimulated cells is inhibited by heparin. Thromb Haemost 79:959–962.

Bergmeyer HU (1988) *Methods of Enzymatic Analysis*. Academic Press, New York.

Cahalon L, Lider O, Schor H, Avron A, Gilat D, Hershoviz R, Margalit R, Eshel A, Shoseyev O, Cohen I (1997) Heparin disaccharides inhibit tumor necrosis factor-$\alpha$ production by macrophages and arrest immune inflammation in rodents. Int Immunol 9:1517–1522.

Chelmonska-Soyta A, Zimecki M, Nowacki W, Nikolajczuk M (2001) Differential effect of heparin on NO and tumor necrosis factor alpha production in bovine blood mononuclear cells stimulated with *Salmonella typhimurium* lipopolisaccharide. Comp Immunol Microbiol Infect Dis 24:151–164.

de Jouge E, Levi M, Stoutenbeek OP, van Deventer SJ (1998) Current drug treatment strategies for disseminated intravascular coagulation. Drugs 55:767–777.

Enomoto N, Ikejima K, Bradford BU, Rivera CA, Kono H, Brenner DA, Thurman RG (1998) Alcohol causes both tolerance and sensitization of rat Kupffer cells via mechanisms dependent on endotoxin. Gastroenterology 115:443–451.

Enomoto N, Takei Y, Hirose M, Ikejima K, Miwa H, Kitamura T, Sato N (2002) Thalidomide prevents alcoholic liver injury in rats through suppression of Kupffer cell sensitization and TNF-$\alpha$ production. Gastroenterology 123:291–300.

Enomoto N, Yamashina S, Kono H, Schemmer P, Rivera CA, Enomoto A, Nishiura T, Nishimura T, Brenner DA, Thurman RG (1999) Development of a new, simple rat model of early alcohol-induced liver injury based on sensitization of Kupffer cells. Hepatology 29:1680–1689.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hasan M, Najjam S, Gordon MY, Gibbs RV, Rider CC (1999) IL-12 is a heparin binding cytokine. J Immunol 162:1064–1070.

Lortat-Jacob H, Turnbull JE, Grimaud JA (1995) Molecular organization of the interferon $\gamma$-binding domain in heparan sulfate. Biochem J 310:497–505.

Martines F, Abril ER, Earnest DL, Watson RR (1992) Ethanol and cytokine secretion. Alcohol 9:455–458.

Matsuno R, Aramaki Y, Arima H, Tsuchiya S (1997) Scavenger receptors may regulate nitric oxide production from macrophages stimulated by LPS. Biochem Biophys Res Commun 237:601–605.

Najjam S, Mulloy B, Theze J, Gordon MY, Gibbs R, Rider CC (1998) Further characterization of the binding of human recombinant interleukin 2 to heparin and identification of putative binding sites. Glycobiology 8:509–516.

Nolan JP (1981) Endotoxin, reticuloendothelial function, and liver injury. Hepatology 1:458–465.

Parrillo JE (1993) Pathogenetic mechanism of septic shock. N Engl J Med 328:1471–1477.

Stahnke LL, Hill DB, Allen JI (1991) TNF-$\alpha$ and IL-6 in alcoholic liver disease, in *Cells of the Hepatic Sinusoid* (Wisse E, Knock DL, McCuskey RS eds), vol 3, pp 472–475. Kupffer Cell Foundation, Leiden, The Netherlands.

Wrenshall LE, Cerra FB, Carlson A, Bach FH, Platt JL (1991) Regulation of murine splenocyte responses by heparan sulfate. J Immunol 147:455–459.

Wrenshall LE, Cerra FB, Singh RK, Platt JL (1995) Heparan sulfate initiates signals in murine macrophages leading to divergent biologic outcomes. J Immunol 154:871–880.