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# KUPFFER CELL- MEDIATED CYTOTOXICITY INDUCED BY LIPOPOLYSACCHARIDE0111:B4 IS GREATER IN DOGS THAN IN RATS AND MONKEYS

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**ABSTRACT** — To clarify the mechanism of sensitivity to an endotoxin lipopolysaccharide LPS0111:B4, which causes severe liver injury in a variety of animals, we have developed an *in vitro* assay to measure Kupffer cell-mediated cytotoxicity in the human liver cell line, WRL68. This assay could detect the decrease in Kupffer cell activity induced by gadolinium chloride ($GdCl_3$), which is an inhibitor in Kupffer cells. Among Kupffer cells derived from dogs, rats, and monkeys, LPS-activated canine Kupffer cells exhibited remarkably high cytotoxicity against WRL68 cells. This species difference is correlated with a species difference in the lethality of LPS0111:B4. Additionally, the conditioned medium of LPS-activated canine Kupffer cells was also cytotoxic to WRL68 cells. To identify the mediators of this cytotoxicity, we measured the accelerated release of interleukin-1$\beta$, and interleukin-6 from Kupffer cells on stimulation with LPS0111:B4. From the correlation of the response to LPS0111:B4, interleukin-1$\beta$ and interleukin-6 are considered to be responsible for the canine Kupffer cell-mediated cytotoxicity of LPS0111:B4.

**KEY WORDS:** Kupffer cells, Lipopolysaccharide, Cytokine, Cytotoxicity

## INTRODUCTION

Kupffer cells are resident macrophages of the liver and represent the largest population of macrophages in the mammalian body. Kupffer cells can be activated with chemical agents such as acetaminophen (Laskin *et al.*, 1986), or by biologic agents such as lipopolysaccharides (LPSs; Brown *et al.*, 1997). Activated Kupffer cells release a variety of mediators such as proteases (Decker, 1985), reactive nitrogen intermediates (Aono *et al.*, 1994), tumor necrosis factor-$\alpha$(TNF-$\alpha$; Karck *et al.*, 1988; Yoshioka *et al.*, 1998), interleukin-1 (IL-1; Leser *et al.*, 1989), interleukin-6 (IL-6; Feder *et al.*, 1993;), and prostaglandins (Decker, 1985).

Gadolinium chloride ($GdCl_3$) is known to inhibit the function and reduce the number of Kupffer cells in the liver (Husztik *et al.*, 1980). The extent of hepatic damage induced by LPSs (Brown *et al.*, 1997), acetaminophen (Laskin *et al.*, 1986), alcohol (Adachi *et al.*, 1994), diethyldithiocarbamate (Ishiyama *et al.*, 1995), and carbon tetrachloride (Edward *et al.*, 1993) is reduced by pre-administration of $GdCl_3$ to animals. Thus, Kupffer cells seem to play a key role in the hepatic damage induced by these agents.

LPS, an endotoxin and a component of the cell wall of Gram-negative bacteria (Rictschel *et al.*, 1994), induces a serious hepatotoxicity which appears to be mediated, at least in part, by Kupffer cells. Administration of LPSs to rats has been used as a model of liver injury (Endoh *et al.*, 1993). Sequela of sepsis induced by LPSs is a major cause of morbidity and mortality (Parrillo, 1993). Species differences in toxic responses have been reported for LPS0111:B4. Dogs die after an intravenous administration of 0.04 mg/kg of LPS0111:B4, while doses above 4 mg/kg are needed for morbidity in rats (LeMay *et al.*, 1990; McCuskey *et al.*, 1984). Monkeys show only an acute phase response on intravenous administration of 0.3 mg/kg of LPS0111:B4

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(Auerbach and Parks, 1989).

Of the mediators released from activated macrophages, IL-1$\beta$ has been implicated in LPS toxicity (Alexander *et al.*, 1992; Boermeester *et al.*, 1995). Khoruts *et al.* (1991) and Sun *et al.* (1992) reported that the activities of IL-1$\beta$ and IL-6 in sera are correlated with liver injury.

In this article, we describe an *in vitro* assay for Kupffer cell-mediated cytotoxicity using 5-bromo-2'-deoxyuridine (BrdU)-labeled human liver embryo line cells, and compare Kupffer cell responses to LPS0111:B4 among rats, monkeys, and dogs. Finally, we identify the key mediators contributing to the species difference in the Kupffer cell-mediated cytotoxicity induced by LPS0111:B4.

## MATERIALS AND METHODS

### Animals

Fifteen male Sprague-Dawley rats (8-16 weeks old), 9 male beagle dogs (12-24 months old), and 6 male monkeys (*Macaca fascicularis*; 25-26 months old) were obtained from Charles River Japan (Kanagawa, Japan), Ridglan Farms (Mt. Hoseb, Wisconsin, WI, USA), and Clea Japan Inc. (Tokyo, Japan), respectively. The rats were given a standard rodent diet (MF, Oriental Yeast Co., Ltd. Tokyo, Japan) and sterilized tap water *ad libitum*. The dogs were provided with approximately 300 g of canine standard diet daily (DS-5, Oriental Yeast Co., Ltd.) and allowed free access to tap water. The monkeys were provided with approximately 108 g of pellet food (Herlan Teklad, Harlan Sprague Dawley, Inc. Indianapolis, IN, USA) and allowed free access to tap water. All animals were reared in semi-barrier rooms maintained at 22±3°C with 55±20% humidity on a 12-hr light/dark cycle with humane care in compliance with our institutional guidelines based on the recommendations of the animal care and use committee (American Association for Laboratory Animal Science, 1987). After a period of acclimation, all animals were checked for general status, weighed and subjected to a hematological examination (except the rats). Only animals confirmed to be healthy were used in experiments. Three animals were used in each experiment.

### Reagents

The lipopolysaccharide LPS0111:B4 (Lot. # 39H4103) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). It was dissolved in phosphate-buffered saline (PBS).

### Administration of gadolinium (III) chloride hexahydrate

Gadolinium (III) chloride hexahydrate ($GdCl_3 \cdot (H_2O)_6$; Sigma) was dissolved in PBS. Twenty-four hr before the collection of rat Kupffer cells, 0 (vehicle only), 5 or 10 mg/kg of $GdCl_3$ was administered intravenously to groups of 3 rats each.

### Cell culture

The WRL68 (human liver embryo) cell line was purchased from the American-type culture collection (Manassas, VA, USA) and cultured in $\alpha$-Minimum Essential Medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 $\mu$g/ml of streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$.

### Isolation of Kupffer cells

Kupffer cells were isolated by a modified method (Aiken *et al.*, 1990), originally described by Seglen (1973) and Smedsrod and Pertoft (1985). Rats (50 mg/kg, i.p.), dogs (15 mg/kg, i.v.), and monkeys (25 mg/kg, i.v.) were anesthetized with sodium pentobarbital. The livers were perfused with $O_2$-saturated $Ca^{2+}$-free Hanks buffer (Sigma) containing EGTA (0.19 g/l), HEPES (2.38 g/l), and $NaHCO_3$ (0.35 g/l) at a rate of 20 ml/min at 37°C. After about 15 to 20 min, the perfusion solution was switched to Hanks buffer (Sigma) containing *Clostridium* collagenase (0.5 g/l, Wako Pure Chemical Industrial, Ltd., Osaka, Japan), EGTA (0.19 g/l), HEPES (2.38 g/l), and $NaHCO_3$ (0.35 g/l). After collagenase perfusion for 15 to 20 min, each liver was carefully transferred to a dish and gently dispersed with a glass stick in Hanks buffer (Sigma) supplemented with EGTA (0.19 g/l), HEPES (2.38 g/l), and $NaHCO_3$ (0.35 g/l). These suspensions were separately filtered first through sterile cotton gauze and then through 250-$\mu$m mesh filters. The suspensions were centrifuged at 50×g for 2 min. Each supernatant was collected, and the Kupffer cells were isolated by the Percoll centrifugation technique described by Smedsrod and Pertoft (1985). Kupffer cells are known to be peroxidase-positive (Bodenheimer and Charland,1985); therefore, the peroxidase activity in the Kupffer cell fractions was assessed with a diaminobenzidine stain kit for peroxidase (Mutou Chemical, Tokyo, Japan). More than 80% of the cells were stained peroxidase-positive. The viability of Kupffer cells was assessed by trypan blue exclusion. The viability of Kupffer cells isolated from rats was 92.8

±6.4%, that from dogs 99.2±0.7%, and that from monkeys 86.9±6.8%; the source of the cells had no significant effect on their viability. However, the viability of Kupffer cells isolated from rats treated with 10 mg/kg of $GdCl_3$ (74.8±12.3%) was significantly lower than that of cells isolated from control rats.

**Measurement of cytotoxicity mediated by Kupffer cells**

WRL68 cells were incubated with BrdU (10$\mu$M) for 24 hr at 37°C in a humidified 95% air-5% $CO_2$ atmosphere, according to the protocol for a DNA-fragmentation ELISA kit (Boehringer Mannheim, Indianapolis, IN, USA). BrdU-labeled WRL68 cells ($1\times10^4$ /well), isolated Kupffer cells (0-$16\times10^4$/well), and LPS0111:B4 (0-20 $\mu$g/ml) were incubated together at 37°C for 24 hr in 48-well plates containing RPMI-1640 (Gibco BRL) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 $\mu$g/ml of streptomycin. The BrdU-labeled DNA released from the WRL68 cells into the conditioned medium was measured with the DNA-fragmentation ELISA kit (Boehinger Mannheim). In brief, an anti-DNA antibody was fixed to the sides of the wells of a 96-well microtiter plate (Nunc, Roskilde, Denmark). Next, non-specific binding sites on the wall of the plates were blocked with incubation solution that contained bovine serum albumin (BSA), polyoxyethyleneglycol sorbitan monolaurate (Tween 20), and ethylenediaminetetraacetic acid (EDTA). Then a 100 $\mu$l aliquot of the conditioned medium from each Kupffer cell-WRL68 cell-LPS treatment combination was added to individual wells, and the plates were incubated for 1 hr at 37°C. After incubation, the conditioned medium was removed and washed 3 times with a solution that contained Tween 20 and EDTA. The uncovered plates were placed in a microwave oven and irradiated for 5 min at a setting of 1000 W. They were then kept at −20°C for 10 min. One hundred microliters of a solution containing anti-BrdU antibody conjugated to peroxidase was added to each well and the plates were incubated for 90 min at room temperature. After the wells had been washed 3 times with the wash solution, 100 $\mu$l of the substrate solution was added to each well and the plates were incubated for 30 min at room temperature in the dark. The reactions were stopped by adding 1 M sulfuric acid (25 $\mu$l/well). The absorbance was then measured at 450 nm (reference wavelength, 655 nm) by a 96-well microplate reader (Bio Rad model 3550, Bio Rad Laboratories, Hercules, CA, USA). Each treatment combination was assayed in triplicate.

**Kupffer cell-mediated cytotoxicity *in vitro***

Kupffer cell-mediated cytotoxicity in WRL68 cells was assayed *in vitro*. To confirm the availability of the above method, Kupffer cells (0-$16\times10^4$/well) isolated from rats pretreated with $GdCl_3$ or vehicle (n=3) were cultured with WRL68 cells in the presence of 20 $\mu$g/ml of LPS0111:B4. Species difference in Kupffer cell-mediated cytotoxicity was evaluated using Kupffer cells ($1\times10^5$/well) isolated from 3 rats, 3 dogs, and 3 monkeys. These cells were cultured with WRL68 cells ($1\times10^4$/well) in the presence of LPS0111:B4 (0-0.2 $\mu$g/ml). To assess the cytotoxic effects of the soluble factor released from Kupffer cells, those ($1\times10^5$/well) obtained from 3 dogs were incubated with LPS0111:B4 (0-0.2 $\mu$g/ml) for 24 hr. Then these conditioned media were added to BrdU-labeled WRL68 cells ($1\times10^4$/well) and cultured for 24 hr. As a reference, the cytotoxicity of the same Kupffer cells ($1\times10^5$/well) was measured by co-culturing with WRL68 cells ($1\times10^4$/well) for 24 hr in the presence of the same concentration of LPS0111:B4. In each experiment, the BrdU-labeled DNA released from the WRL68 cells into the conditioned medium was measured with the DNA-fragmentation ELISA kit (Boehinger Mannheim) as previously described.

**Cytokine assay**

Kupffer cells were isolated from 3 rats, 3 dogs, and 3 monkeys as previously described. LPS0111:B4 (0-0.2 $\mu$g/ml) was added to isolated Kupffer cells ($1\times10^5$/well) grown in 48-well plates that contained RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 $\mu$g/ml of streptomycin. After 24 hr of incubation at 37°C, an aliquot (200 $\mu$l/well) of the conditioned medium was collected for both IL-1$\beta$ and IL-6 assays and stored at −20°C. The cytokines in these samples were measured by bioassay. Briefly, IL-1$\beta$ was assayed by lysis of A375 subclone 1 cells (Nakai *et al.*, 1988, Yamashita *et al.*, 1994). IL-6 was assayed by measuring the cell proliferation of 7TD-1 cells (Nachbaur *et al.*, 1991). Human recombinant IL-6 and IL-1$\beta$ (Gibco BRL) were used as standards. For each assay, the number of cells was measured from the mitochondrial activity using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1, Dojindo, Kumamoto, Japan), as a substrate (Ishiyama *et al.*, 1993; Hamasaki *et al.*, 1996). Briefly, cells were incubated with 0.5 mM WST-1 and 0.02 mM 1-Methoxy-5-methylphenazinium methylsulfate in each culture medium. After 2 hr

incubation, absorbance was measured at 450 nm (reference wavelength, 655 nm) by the 96-well microplate reader (Bio Rad model 3550, Bio Rad Laboratories). Each treatment combination was assayed in triplicate.

### Statistical analysis

The data of experiments were analyzed by using Student's *t*-test (Steel and Torrie, 1980) or Tukey-Kramer's test (Tukey, 1949; Kramer, 1956). In the cytotoxicity results of the conditioned medium of canine Kupffer cells, homogenicity of variance was confirmed by F-test, and then Student's *t*-test was conducted. Other results were analyzed for homogeneity of variance using Bartlett's test (Bartlett, 1937). For homogeneity, Tukey-Kramer's test (parametric type) was conducted. Since variances in heterogeneity were found in the data of IL-1$\beta$ and IL-6 released from LPS-treated Kupffer cells, Tukey-Kramer's test (non-parametric type) was applied. Differences were considered significant when the p value was $<0.05$. All data are expressed as the mean ± standard deviation (SD). All statistical analyses were performed using the SAS® system (Ver. 6.12, SAS Institute Inc., Cary, NC, USA).

## RESULTS

### Development of an *in vitro* assay of Kupffer cell-mediated cytotoxicity

To assess Kupffer cell-mediated cytotoxicity that might cause hepatic damage *in vivo*, we designed an *in vitro* assay using BrdU-labeled WRL68 line cells. To confirm the availability of this method, Kupffer cells isolated from rats which had been administered either saline or $GdCl_3$ for 24 hr at doses of 5 or 10 mg/kg (n=3) were co-cultured with WRL68 cells in the presence of 20 $\mu$g/ml of LPS0111:B4. The cytotoxicity of Kupffer cells from saline-administered rats increased almost linearly with the number of Kupffer cells up to $16\times10^4$ cells (Fig. 1). This Kupffer cell-mediated cytotoxicity was inhibited by pre-administration with $GdCl_3$ in a dose-dependent manner; 60% inhibition at 5 mg/kg of $GdCl_3$ and 90% inhibition at 10 mg/kg of $GdCl_3$ at 8 and $16\times10^4$ Kupffer cells (Fig. 1). These results are considered to represent the hepatic damage by LPS0111:B4 *in vivo* that is mediated by Kupffer cells, and indicate that this *in vitro* assay is useful for measuring Kupffer cell-mediated cytotoxicity.

### Canine Kupffer cells have stronger cytotoxicity than rat and monkey Kupffer cells

LPS0111:B4 increased Kupffer cell-mediated cytotoxicity remarkably in dogs and moderately in monkeys at 0. 2 $\mu$g/ml of LPS, but had no effect in rats (Fig. 2). These results indicate that canine Kupffer cells were the most sensitive to LPS0111:B4 in terms of cytotoxicity in WRL68 cells. This species difference in the Kupffer cell-mediated cytotoxicity was not specific for WRL68 cells. We confirmed that in other cell lines, such as L929 mouse connective tissue line cells, the cytotoxicity caused by Kupffer cells of dogs was more severe than that of rats in this system (data not shown). The conditioned medium of canine Kupffer cells treated with LPS0111:B4 for 24 hr was also cytotoxic to the WRL68 cells in a dose-dependent manner; however, the effect was only 70 - 80% of that obtained on co-culture with Kupffer cells (Fig. 3). This result

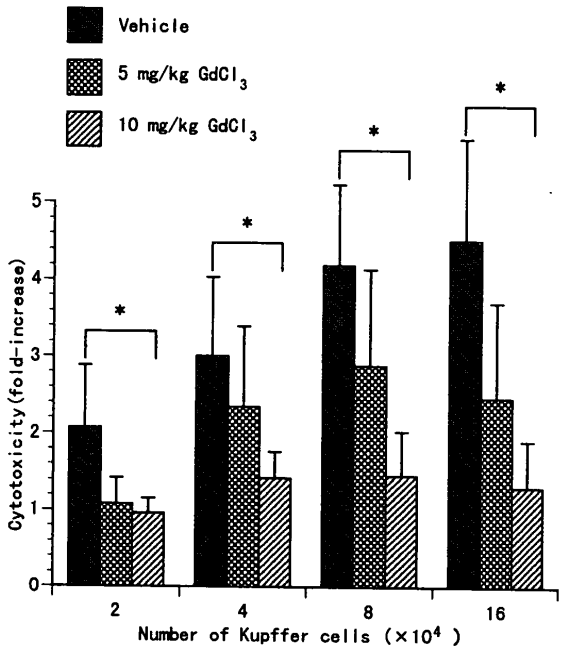


**Fig. 1** Response of Kupffer cell-mediated cytotoxicity in WRL68 cells and the effect of $GdCl_3$. Kupffer cells were isolated from PBS-treated rats (■), 5 mg/kg $GdCl_3$-treated rats (▩), and 10 mg/kg $GdCl_3$-treated rats (▨). BrdU-labeled WRL68 cells ($1\times10^4$/well) were co-cultured with isolated Kupffer cells (0-$16\times10^4$/well) in the presence of LPS0111: B4 (20 $\mu$g/ml) in 48-well plates. Cytotoxicity is expressed as the amount of BrdU released from WRL68 cells compared with WRL68 cells grown without Kupffer cells. Bars indicate the SD. The mean and SD were obtained from 3 animals. *, Significantly different from the vehicle control at the same number of Kupffer cells ($p<0.05$, Tukey-Kramer's test).

suggests that the soluble factors released from Kupffer cells by LPS0111:B4 treatment caused the cytotoxicity in WRL68 cells.

**Release of IL-1β and IL-6 from Kupffer cells of rats, dogs, and monkeys**

Activities of IL-1β and IL-6 in conditioned media of rat, monkey, and dog Kupffer cells treated with saline were compared. There were no significant differences in the baseline activities of the IL-1β released from Kupffer cells among these species in the absence of LPS0111:B4. The activities of IL-6 could not be determined in any of the species in the absence of LPS0111:B4 (Table 1).

The amount of IL-1β released from Kupffer cells following stimulation with LPS0111:B4 was 2- to 6-fold and 8-fold greater in dogs than in monkeys and rats, respectively (Fig. 4). Similarly, the amount of IL-6 was 6-fold and 4- to 14-fold greater in dogs than in monkeys and rats, respectively (Fig. 5). These results indicate that IL-1β and IL-6 are mediators of the high sensitivity to LPS-activated Kupffer cell-mediated cytotoxicity in dogs.

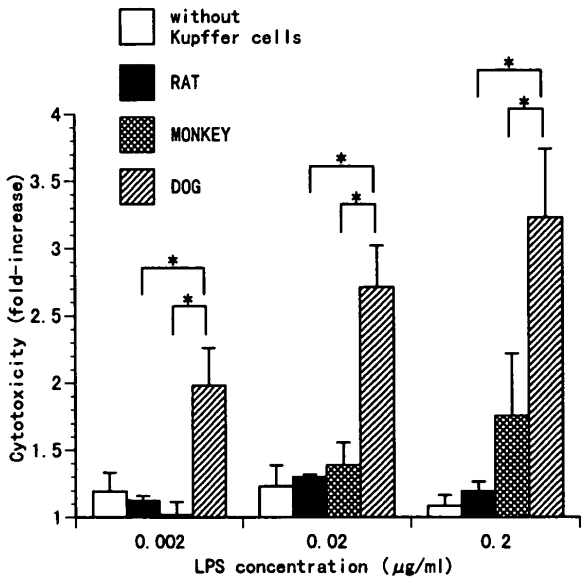


**Fig. 2** Species-specific differences in Kupffer cell-mediated cytotoxicity. Kupffer cells ($1\times10^5$/well) isolated from rats (■), monkeys (▩), and dogs (▨)were cultured with BrdU-labeled WRL68 cells ($1\times10^4$/well) in the presence of LPS0111:B4. Cytotoxicity is measured as the amount of BrdU released from WRL68 cells. The data is expressed as the fold-increase of cytotoxicity in the presence compared with the absence of LPS0111:B4. Bars indicate the SD. The mean and SD were obtained from 3 animals. *, Significantly different at the same LPS concentration ($p<0.05$, Tukey-Kramer's test).

## DISCUSSION

In this study, we used the same number of Kupffer cells for each experiment to control for a possible source of variation between species caused by differences in cell sensitivity to the stimulus. Our results indicated that the Kupffer cell-mediated cytotoxicity induced by LPS0111:B4 was stronger in dogs than in rats or monkeys. Accordingly, there must be a species difference in the cytotoxic effects of isolated

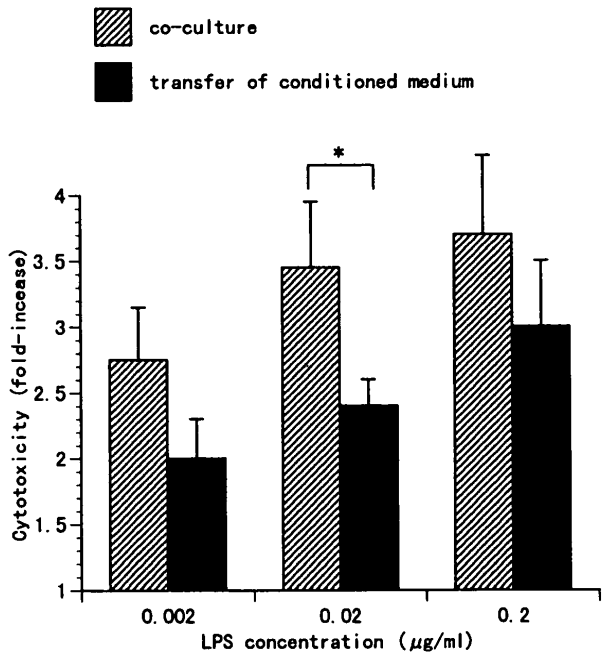


**Fig. 3** The WRL68 cytotoxicity induced by canine Kupffer cells and conditioned media thereof. Dog Kupffer cells ($1\times10^5$/well) were cultured with BrdU-labeled WRL68 cells ($1\times10^4$/well) in the presence of LPS0111:B4 for 24 hr (▨). The conditioned medium of canine Kupffer cells ($1\times10^5$/well) treated with LPS0111:B4 for 24 hr was added to BrdU-labeled WRL68 cells ($1\times10^4$/well) and cultured for 24 hr (■). Cytotoxicity is measured as the amount of BrdU released from WRL68 cells. The data is expressed as the fold of cytotoxicity in the presence compared with the absence of LPS0111:B4. Bars indicate the SD. The mean and SD were obtained from 3 animals. *, Significantly different from cytotoxicity in co-culture at the same LPS concentration ($p<0.05$, Student's *t*-test).

Kupffer cells. This species-specific cytotoxicity was correlated with the amount of IL-1$\beta$ and IL-6 released from Kupffer cells on stimulation with LPS0111:B4, and is considered to reflect the species-specific difference in the lethality of LPS0111:B4 in these animals (LeMay *et al.*, 1990; McCuskey *et al.*, 1984; Auerbach and Parks, 1989). After endotoxin shock, an important reaction in the body is the activation of macrophages followed by a release of mediators from macrophages (Altavilla *et al.*, 1998). IL-1$\beta$ has been implicated as a mediator of LPS toxicity (Alexander *et al.*, 1992; Boermeester *et al.*, 1995). Khoruts *et al.* (1991) and Sun *et al.* (1992) reported that the activities of IL-1$\beta$ and IL-6 in sera are correlated with liver injury. Yamano *et al.* (2000) reported that Kupffer cell activation is essential for inflammatory liver damage and IL-1$\beta$ is an important mediator of the inflammatory response induced by cadmium. These reports and our data indicate that the effects of Kupffer cell-mediated IL-1$\beta$ and IL-6 release are important to understand the mechanisms underlying the hepatotoxicity.

However, our data also revealed that the cytotoxicity of soluble factors released from Kupffer cells was slightly lower than that obtained on co-culture with Kupffer cells. Therefore, it is considered that not only IL-1$\beta$ and IL-6, but also other factors contribute to Kupffer cell-mediated hepatotoxicity. McCuskey *et al.* (1984) reported that differences in the $LD_{50}$ to LPS0111:B4 among guinea pigs, hamsters, mice and rats correlated well to the differences in number and phagocytic activity of their Kupffer cells. Harashima *et al.* (1996) found that the phagocytic ability of Kupffer cells differs among mice, rats, and rabbits. It is known that IL-1$\beta$ and IL-6 are multifunctional cytokines that induce the production of C-reactive protein (CRP) by hepatocytes (Mazlam and Hodgson, 1994). CRP is an opsonin-like protein that stimulates the phagocytic activity of macrophages, which contributes to liver

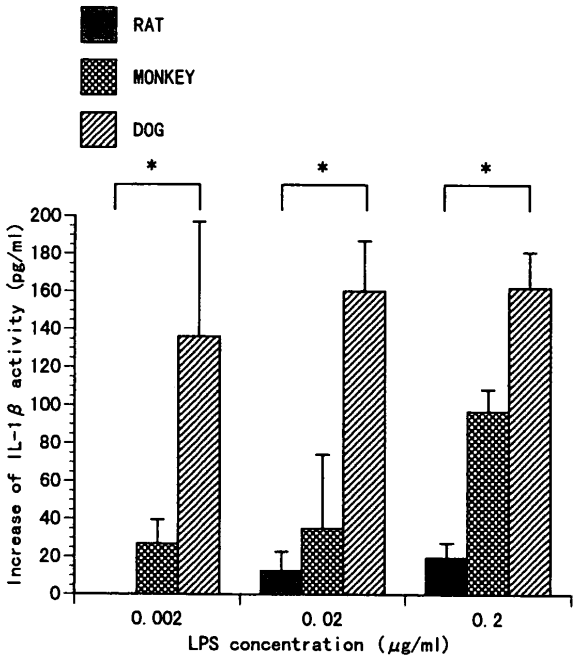


**Fig. 4** The increase of IL-1$\beta$ activity released from LPS-treated Kupffer cells of rats, monkeys, and dogs. Kupffer cells ($1\times10^5$/well) isolated from rats (■), monkeys (▩), and dogs (▨) were cultured in the presence of LPS0111:B4 for 24 hr. A bioassay was used to measure the activity of IL-1$\beta$ in the conditioned medium. Bars indicate the SD. The mean and SD were obtained from 3 animals. *, Significantly different at the same LPS concentration ($p<0.05$, Tukey-Kramer's test).

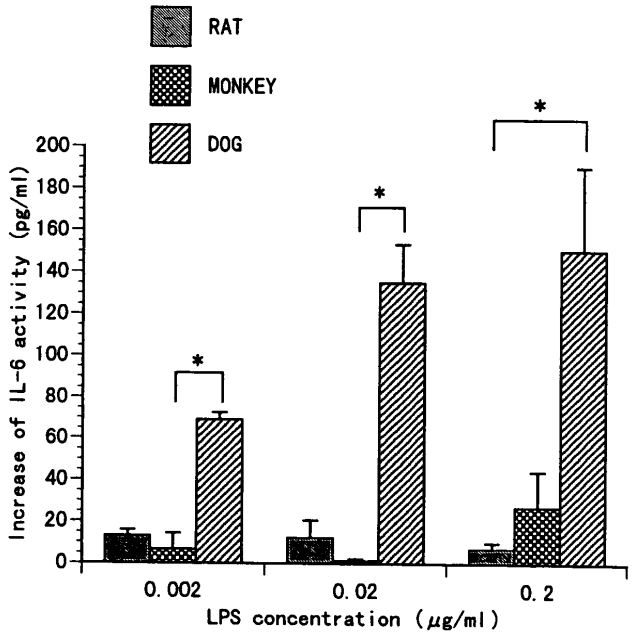


**Fig. 5** The increase of IL-6 activity released from LPS-treated Kupffer cells of rats, monkeys, and dogs. Kupffer cells ($1\times10^5$/well) isolated from rats (■), monkeys (▩), and dogs (▨) were cultured in the presence of LPS0111:B4 for 24 hr. A bioassay was used to measure the activity of IL-6 in the conditioned medium. Bars indicate the SD. The mean and SD were obtained from 3 animals. *, Significantly different at the same LPS concentration ($p<0.05$, Tukey-Kramer's test).

**Table 1.** Baseline of cytokine production by Kupffer cells isolated from rats, monkeys, and dogs.

| | IL-1β (pg/ml) | IL-6 (pg/ml) |
|---|---|---|
| Rat | 598.7 | ND |
| (n = 3) | ± 212.7 | |
| Monkey | 551.6 | ND |
| (n = 3) | ± 139.5 | |
| Dog | 545.6 | ND |
| (n = 3) | ± 137.3 | |

Kupffer cells ($1 \times 10^5$ /well) isolated from rats, monkeys, and dogs were cultured for 24 hr. A bioassay was used to measure the activity of cytokines in each conditioned medium sample. Data are expressed as the mean±SD, with number of animals in parentheses. ND, not detectable. No significant difference was observed among these species by Tukey-Kramer's test.

injury. Anasagasti *et al.* (1996) reported that the addition of IL-1$\beta$ stimulates the release of hydrogen peroxide ($H_2O_2$) from Kupffer cells *in vitro*.

Kupffer cell-mediated hepatotoxicity has been well studied *in vivo*, but not *in vitro*. Recently, Hoebe *et al.* (2000) reported an *in vitro* assay for screening hepatotoxic agents. In the assay, rat primary hepatocytes are co-cultured with Kupffer cells and then mitochondrial enzymatic activities are measured as a parameter of cytotoxicity. Their assay is considered applicable to assessment of the interaction between hepatocytes and Kupffer cells in same species, but not for comparisons of isolated Kupffer cells from different species. In addition, a primary hepatocyte system using enzymatic activity as an endpoint has several problems such as obtaining a stable cell preparation and reproducibility of data. In this study, we designed an *in vitro* assay for Kupffer cell-mediated cytotoxicity, applying the BrdU-labeled cells used for cytotoxicity assays (Ito *et al.*, 1996; Arimilli *et al.*, 1996). Our assay showed a good correlation between the number of co-cultured Kupffer cells and cytotoxicity. With the system, we could detect a decrease of Kupffer cell-mediated cytotoxicity induced by $GdCl_2$, an inhibitor of Kupffer cell function. Therefore, this assay system is considered available to assess Kupffer cell-mediated cytotoxicity induced by agents as well as to compare species differences in Kupffer cells. Our *in vitro* assay using BrdU-labeled cells seems to be efficient in terms of simplicity and the reproducibility of data.

Consequently, further study will be needed to examine the function of Kupffer cells, especially the role of IL-1$\beta$ and IL-6 in Kupffer cell-mediated hepatotoxicity. Our assay design appears to be useful for studying the function of isolated Kupffer cells.

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