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A novel role of alkaline phosphatase in protection from immunological liver injury in mice

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Abstract: *Aims/Background:* Little is known about the role of alkaline phosphatase (AP) in liver diseases, except for its elevation in jaundice or cholestasis. Its substrate, endotoxin, is usually elevated in patients as well as animals with liver damage. This study aimed to provide evidence for its new role as protection against immunological liver damage. *Methods:* Liver injury was induced in mice by delayed-type hypersensitivity to picryl chloride. AP activity was measured using a commercial kit. *Results:* In acute liver injury, a significant decrease in AP activity in serum was observed but there was an increase in liver tissue. Single administration of cyclophosphamide before sensitization with picryl chloride exacerbated the liver injury, with more serious AP changes, while consecutive use after the sensitization alleviated the injury with a recovery from the changes. When liver injury proceeded for 1 week, both serum and liver showed decreased AP activity. Lipopolysaccharide facilitated alanine transaminase release from levamisole-pretreated but not non-treated hepatocytes from naive mice. However, the release was confirmed from liver slices of mice with liver injury proceeding for 1 week, even without levamisole pretreatment. *Conclusion:* The development of liver injury may lead to a dysfunction in AP synthesis and release. Levamisole may make normal hepatocytes, like the hepatocytes from liver-injured mice, highly sensitive to lipopolysaccharide through inhibiting AP synthesis. The findings obtained in this study suggest that AP may contribute to protection from injury by a mechanism involving neutralization of endotoxin.

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Key words: alkaline phosphatase – cyclophosphamide – delayed-type hypersensitivity – levamisole – lipopolysaccharide – liver injury – picryl chloride

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Hepatocyte damage is a key process in the pathogenesis of various liver diseases. In clinical practice, serum contents of substances that are changed by hepatocyte damage are usually measured to assess liver function. These substances include alanine and aspartate transaminases, lactic dehydrogenase, globulin, and albumin. However, not all the substances synthesized by hepatocytes are used to assess the development of liver damage. For example, little has been known about the possible involvement of alkaline phosphatase (AP) in the development of liver diseases, except as an important indicator of jaundice when elevated.

On the other hand, a cellular immunological mechanism has been widely reported in the pathogenesis of various hepatic diseases. To mimic the pathogenesis, we established a novel model of liver injury induced in mice by a delayed-type hypersensitivity to picryl chloride (PCl-DTH) (1). We also demonstrated that lymphocyte function-associated antigen 1 and intercellular adhesion molecule 1 interaction between liver-infiltrating T-lymphocytes and hepatocytes are essential for the production of liver injury, and that several kinds of cytokines, including IFN-γ, are involved in the liver injury (2, 3). These results suggest that the cell–cell interaction that occurred in the injured liver may lead to a change in the liver microenvironment. Furthermore, our previous data (4) have revealed the relationship between the elevation in endotoxin and the exacerbation of the liver injury, implicating endotoxemia in the liver injury, as seen in most patients with hepatitis.

Interestingly, among the changes in various biochemical and pathological parameters, a unique change in AP activity was found in the liver injury

induced by PCl-DTH (5): the activity of this enzyme was significantly decreased in serum during the acute phase or the sustaining process of the liver injury. This finding goes beyond our common knowledge about the role of the enzyme, since almost no clinical and experimental data have dealt with the implication of low AP activity. It suggests the possibility that AP may play some role in the development of liver damage. Since our previous studies have found that this immunological liver injury showed similar changes in various parameters to those in patients with hepatitis (1–6), the changes in AP activity, as another parameter, might imply clinical significance. Therefore, the purpose of this study was to confirm the changes in serum and hepatic AP activities and to clarify the possible involvement of AP in the development of liver injury.

Materials and methods

Animals

Female ICR and Kunming strains of mice, aged 5–6 weeks, were used. They were obtained from the Experimental Animal House of China Pharmaceutical University (Nanjing, China). They were kept in plastic cages at 21±2°C with free access to pellet food and water, and were on a 12-h light/dark cycle. This study complied with the University's current ethical regulations on animal research, and the mice used were treated humanely.

Drugs and reagents

Picryl chloride (PCl, Nacalai Tesque, Inc., Kyoto, Japan); cyclophosphamide (Cy, Shanghai Hualian Pharmaceutical Factory, Shanghai, China); lipopolysaccharide (LPS, from *Escherichia coli* Serotype 055:B5, SIGMA); levamisole (LMS, Nanjing 2nd Pharmaceutical Factory, Nanjing, China); kits for determining alanine transaminase (ALT) (Nanjing Jiancheng Biological and Chemical Products Co. Ltd., Nanjing, China); kits for determining AP (Shanghai Rongsheng Biochemical Products Co. Ltd., Shanghai, China).

Liver injury induced by delayed-type hypersensitivity to picryl chloride (PCl-DTH)

Mice were sensitized twice by painting 0.1 ml of 1% PCl in ethanol on the skin of their abdomens with an interval of 5 days. Five days after the 2nd sensitization, their livers were injected with 10 μl of 0.5 % PCl in olive oil. After 18 h (acute phase) or 1 week (sustained phase), they were bled and their livers were removed under ether anesthesia. The serum and liver tissue were used to determine ALT and AP activities. The assays were performed by means of a commercially available kit. Elevated transaminases and histological changes indicated the establishment of liver injury. It was also confirmed that the vehicle, olive oil itself at the dosage used, does not cause the liver damage. Two controls, olive oil challenge to PCl-sensitized mice and PCl challenge to naive mice, were also used and no damage was observed (1).

AP determination as an important indicator in PCl-DTH-induced liver injury

AP activity was determined in serum, liver tissue and culture supernatant of liver cells. Considering the different biochemical and pathological characters in the acute and chronic phases of this liver injury (5), mice with acute liver injury and mice with sustained liver injury of 1 week were used to examine the role of AP in the development of liver injury. Liver nonparenchymal cells (NPC) were also isolated 12 h after PCl challenge, when the liver infiltration of inflammatory cells reaches a peak, in order to assay the AP activities in both supernatant and pellet of NPC after 4 h culture. The pellet was lysed by lysing buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% Triton X-100] followed by centrifuging at 18 000 rpm for 10 min. The supernatant was collected for assaying AP.

Administration of cyclophosphamide during different phases of DTH to change the state of liver injury

Our previous study (6) showed that the degree of PCl-DTH liver injury was related to the extent of immune response in the liver. Based on this, to confirm the relation of AP change to the degree of liver injury, mice were administered Cy intraperitoneally in a single dose of 150 mg/kg 3 days before the first PCl sensitization or a dose of 15 mg/kg for 10 days from the sensitization. The former was to enhance the immune response that causes liver injury, while the latter would improve the injury due to its immunosuppression. The usefulness of Cy for such immunoregulation is based on the concept that damage to lymphocytes by cancer chemotherapeutic agents does not necessarily lead to immunosuppression, but can just as readily result in immunopotentiation. The most important variable determining whether one observes immunosuppression or immunopotentiation is the timing of the administration of Cy and antigen (7). The enhanced immune response is shown to result from the reduced influence on effector cells of Cy-sensitive suppressor cells (8).

Table 1. Changes in ALT and AP activities in serum and liver tissue of mice with PCI-DTH liver injury

Group	No. of mice	Serum ALT (IU/l)	AP	
			Serum (U/l)	Liver tissue (U/g protein)
Normal	8	10.7±2.8	199±39	5.23±0.79
Liver injury	7	233±12**	150±18**	7.56±2.6*

Mice were sensitized twice in an period of 5 days by painting 0.1 ml of 1% PCI in ethanol on the skin of their abdomens. Five days after the second sensitization, their livers were injected with 10 μl of 0.5% PCI in olive oil. Eighteen hours later, blood was collected and the livers were removed and homogenized. Serum was used for the measurement of ALT and AP activities. Liver homogenate was used for measuring AP activity. Each datum represents the mean±SD. *$p<0.05$, ** $p<0.01$ *vs* Normal.

Early and late tolerance to LPS

Endotoxemia is usually seen in patients with hepatitis, as well as in PCl-DTH-induced liver injury (4). A recent study has revealed that AP has an endotoxin-dephosphorylating property as the natural substrate of endotoxin (9). To explore the role of AP as it relates to endotoxin, early and late tolerances to LPS were induced in mice. For early tolerance, mice were injected i.v. with LPS (1 mg/kg). The control mice received saline. Tolerance was confirmed by the challenge of an acute lethal dose (32 mg/kg, i.v.) of LPS 72–96 h later. While all mice in the control group died, all LPS-pretreated mice survived. To induce late tolerance, mice were given LPS intraperitoneally by incremental doses (0.25 mg/kg on days 1 and 2; 0.5 mg/kg on days 3 and 4, and 1.0 mg/kg on days 5 and 6). Forty-eight hours after the last injection, the mice were found to resist a lethal dosage of LPS.

Hepatocyte preparation

Hepatocytes were isolated from normal or liver-injured mice by the modified two-step perfusion method. Briefly, mice received pentobarbital intraperitoneally at a dose of 40 mg/kg of body weight. The liver was first perfused *in situ* via the portal vein with Ca^{2+}- and Mg^{2+}- free Hank's balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (ethylene glycol-bis- (β-amino ethyl)-N, N′-tetracetic acid) (Dojindo Chemical Institute Ltd., Kumamoto, Japan) and 25 mM HEPES (2-[4-(2-hydroxyethyl)-piperazinyl] ethanesulfonic acid, pH 7.4, Dojindo) at 37°C until the blood in the liver was completely removed. Then, the solution was changed to 0.1% collagenase IV (182 units/mg, Wako Pure Chemical Industries Ltd., Osaka, Japan) in HBSS (containing 4 mM $CaCl_2 \cdot 2H_2O$ and 0.8 mM $MgSO_4 \cdot 7H_2O$). After a few minutes perfusion, the liver was excised from the body cavity and dispersed into cold HBSS. The cell suspension generated was filtered though 100-gauze mesh, then centrifuged at 50 × g for 2 min. The pellet was washed twice to remove dead cells and debris and resuspended in RPMI-1640 medium (GIBCO BRL) containing 10% newborn calf serum (Nanjing Weigang Milk Products Co. Ltd., Nanjing, China), 100 U/ml of penicillin and 100 U/ml of streptomycin. The hepatocytes were found to be about 85% viable, as estimated by trypan blue exclusion.

Culture of liver tissue slices *in vitro* and ALT releasing assay

Liver was removed from mice with or without liver injury that had proceeded for 1 week and put into cold medium. The liver tissue was cut with a sharp blade into strips of 20 mm × 10 mm × 5 mm (length × width × height). Then, one strip was cut into slices 1 mm thick. Each slice was cultured with 0.2 ml medium in 24-well plate. The collected supernatant was used for assaying ALT activity.

Statistics

Results were expressed as mean±SD and statistically evaluated using Student's *t*-test. *p*-values <0.05 were considered significant.

Table 2. Change in AP activity in different areas of liver tissue from mice with PCI-DTH liver injury

Group	No. of mice	Serum ALT (IU/l)	AP		
			Serum (U/l)	Liver tissue (U/g protein)	
Normal	8	15.3±7.18	305±71.4	5.12±0.73	
				Necrotic area	*Other area*
Liver injury	8	337±171**	201±36.0**	7.51±1.63**††	5.35±1.46

Liver injury was induced by PCI-DTH. Eighteen hours after the PCI challenge, liver was removed and cut into two parts, necrotic area and other area. Each datum represents the mean ± SD. For other details, see the footnote to Table 1. ** $p<0.01$ *vs* Normal; †† $p<0.05$ *vs* other area.

Table 3. Effects of Cy on serum ALT and AP activities and hepatic AP activity in mice with PCI-DTH liver injury

Group	No. of mice	Dose (mg/kg)	Serum ALT (IU/l)	AP Serum (U/l)	AP Liver tissue (U/g protein)
Naive	8	–	18.6±5.40	228±79.0	3.49±1.37
Liver injury	9	–	238±56.9**	140±13.0**	5.48±2.30*
Cy pre	10	150	279±96.9**	108±32.3**††	6.08±2.84**
Cy post	9	15	179±54.5**††	163±31.7*†	4.97±1.59

Cy was given i.p. at a dose of 150 mg/kg once 3 days before the first sensitization (Cy pre) or for 10 days from the first PCl sensitization at a dose of 15 mg/kg (Cy post). Each datum represents the mean ± SD. For other details, see the footnote to Table 1. * $p<0.05$, ** $p<0.01$ *vs* Naive; † $p<0.1$,†† $p<0.05$, *vs* Liver injury control.

Table 4. Change in AP activity in different areas of liver tissues from mice with PCI-DTH liver injury that had proceeded for 1 week

Group	No. of mice	Serum ALT (IU/l)	AP Serum (U/l)	AP Liver tissue (U/g protein)	
Normal	8	15.5±9.69	394±48.3	5.30±0.61	
				Necrotic area	*Other area*
Liver injury	7	110±39.5**	314±74.4*	3.94±0.54**†	5.00±1.46

Liver injury was induced by PCl-DTH. One week after the elicitation, the serum and liver were collected for assaying ALT or AP. For other details, see the footnote to Tables 1 and 2. * $p<0.05$, ** $p<0.01$, *vs* Normal; † $p<0.1$, *vs* other area.

Results

Characteristic changes in AP activity in serum and liver tissue of mice with acute liver injury induced by PCl-DTH

In the acute phase of PCl-DTH liver injury, serum ALT levels rose significantly. In comparison with the elevation of transaminases, AP activity changed dramatically: the level in serum fell significantly while that in liver tissue increased significantly (Table 1).

There were several areas of necrosis in the liver of mice with PCl-DTH liver injury. AP activity was significantly higher in necrotic areas than in other areas. AP in other areas was almost normal (Table 2).

The main histological changes in this kind of liver injury have been well reported (1, 2, 5, 10–12). Hepatocellular necrosis, inflammatory infiltration and other changes were observed and some drugs improved the changes. We counted the neutrophil population contained in total NPC at 12 h of liver injury by Giemsa exclusion and measured AP production by the NPC. The result was that only about 0.4% of neutrophil counts in total NPC were found. Also, AP activity was detected only at a very low level in both culture supernatant (11.3±0.2 against normal 10.8±0.3) and lysate (13.8±0.6 against normal 8.2±0.3 IU/2×10^6 cells/0.2 ml) of NPC isolated at 12 h of liver injury.

Dependence of AP change on the state of liver injury changed by cyclophosphamide, given during different phases of DTH

Compared with the liver injury control, the administration of Cy 3 days before the first PCl sensitization caused a more elevated ALT activity and significantly exacerbated the reduction in serum AP level. A tendency to increased elevation in hepatic AP was also observed. In contrast, consecutive injection for 10 days from the sensitization significantly reduced serum ALT and serum AP recovered from its reduced status. A tendency to reduction in hepatic AP was also observed (Table 3).

Reduction in AP activity in both serum and liver tissue of mice with liver injury that proceeded for 1 week

When the liver injury was allowed to proceed for 1 week, AP levels in both serum and the necrotic areas of liver tissue decreased significantly as compared with normal. The AP in other areas of the liver was almost normal (Table 4).

Inhibition by levamisole of the synthesis and release of AP from normal hepatocytes *in vitro*

Twenty thousand hepatocytes from normal mice were pre-cultured in a 96-well microplate for 4 h.

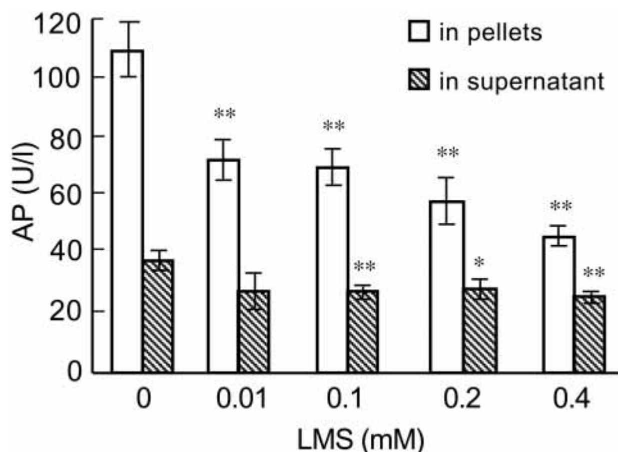

Fig. 1. Dose-dependent inhibition of levamisole (LMS) on the synthesis and release of AP from normal hepatocytes *in vitro*. Twenty thousand hepatocytes in 0.2 ml RPMI-1640 medium were cultured in a 96-well microplate for 4 h. After washing twice with the medium, they were co-cultured with 0.01–0.4 mM of levamisole for 30 min. Then, the supernatant and pellets were collected for assaying ALT and AP activities. Each column indicates the mean±SD of 3 experiments and each experiment included triplicate sets. * $p<0.05$, ** $p<0.01$ *vs* control (LMS free).

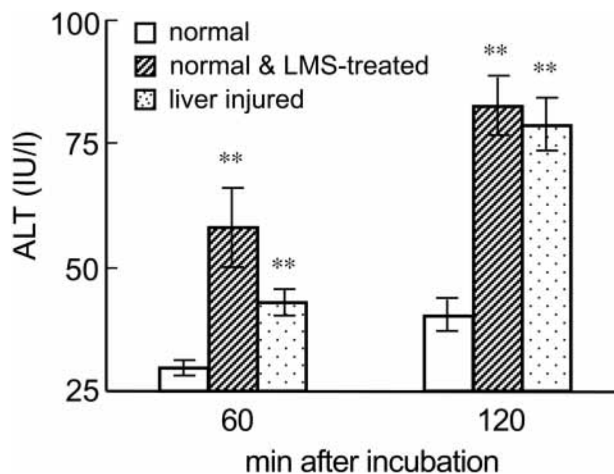

Fig. 3. Effect of LPS and/or levamisole on the normal liver pieces from normal mice and mice with liver injury that proceeded for 1 week. Liver slices from naive mice were pre-treated with 0.2 mM levamisole for 30 min. Then, these liver slices and those from mice with liver injury were cultured in RPMI-1640 medium. Thirty and ninety minutes later, the medium was changed to that containing 1×10^{-5} g/ml of LPS and cultured for a further 30 min. The supernatants collected after 60 and 120 min were used for assaying ALT activity. Each column indicates the mean±SD of 3 experiments and each experiment included triplicate sets. ** $p<0.01$ *vs* normal.

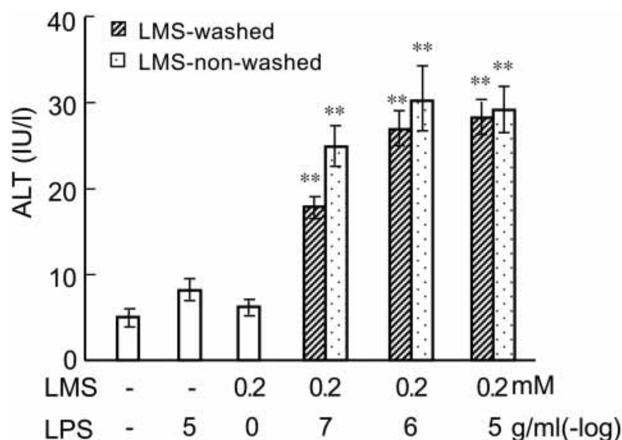

Fig. 2. LPS-induced release of ALT from normal hepatocytes by the pretreatment of levamisole. Twenty thousand hepatocytes were pretreated with 0.2 mM levamisole or medium for 30 min. After no washing or washing with the medium once, they were co-cultured with various concentrations of LPS for a further 3 h. Then the supernatant was collected for assaying the activity of ALT. Each column indicates the mean±SD of 3 experiments and each experiment included triplicate sets. ** $p<0.01$ *vs* controls (LMS and LPS free, LMS alone and LPS alone).

They were then co-cultured with various concentrations of levamisole for 30 min. As a result, levamisole inhibited the synthesis of AP by hepatocytes remarkably in a dose-dependent manner and significantly lowered the AP release into the supernatant (Fig. 1). A time-dependent inhibition was also found after treatment for 15, 30, 60 and 120 min (data not shown). However, the drug did not influence the release of ALT from hepatocytes (data not shown).

Increased sensitivity of normal hepatocytes to LPS by levamisole pretreatment

As shown in Fig. 2, LPS dose-dependently induced an increased release of ALT from the hepatocytes that had been pretreated with 0.2 mM of levamisole for 30 min. However, even at a high concentration of 1×10^{-5} g/ml, LPS did not influence the release of ALT from normal hepatocytes without levamisole pretreatment. Levamisole itself had no effect on ALT release.

LPS-induced ALT release from the liver slices of liver-injured mice without the treatment of levamisole

The liver slices from naive or liver injured mice, where the liver injury had proceeded for 1 week, were cultured in RPMI-1640 medium. Thirty or 90 min later, the medium was changed to that containing 1×10^{-5} g/ml of LPS and cultured for a further 30 min. As compared to the release from normal hepatocytes, a remarkably elevated ALT release was observed in LPS-treated supernatant and a higher ALT level was shown in the supernatant collected after 120 min rather than 60 min.

Table 5. Effect of early and late tolerance to LPS on the serum AP activity

Group	No. of mice	AP in serum (U/l)
Normal	5	208.0±15.2
Early tolerance	7	141.0±21.6**
Late tolerance	7	267.3±46.2*

Mice were injected with LPS (1 mg/kg) i.v. to induce an early tolerance. Tolerance to LPS was confirmed by the challenge of an acute lethal dose (32 mg/kg, i.v.) of LPS 72–96 h later. To induce late tolerance, mice were given LPS i.p. by incremental doses (0.25 mg/kg on days 1 and 2; 0.5 mg/kg on days 3 and 4, and 1.0 mg/kg on days 5 and 6). Forty-eight hours after the last injection, the mice were found to resist the lethal attack from LPS. The blood at 72 h (early tolerance) or on the 8th day (late tolerance) was collected for determining AP activity. Each datum represents the mean±SD. * $p<0.05$, ** $p<0.01$, *vs* Normal.

In the case of liver slices from normal mice, the potential of LPS to release ALT was also realized by the 30-min pretreatment of 0.2 mM levamisole (Fig. 3).

Changes in AP activity in serum of mice with early or late-tolerance to LPS

Early or late tolerance to LPS was induced in mice. A lowered and an increased serum AP activity was found, respectively, with early and late tolerance (Table 5).

Discussion

The present study first examined the changes in serum and hepatic AP activities in mice with acute liver injury induced by PCl-DTH. Characteristic changes in AP levels, a decrease in serum and an increase in liver tissue, accompanied serum ALT elevation 18 h after the PCl elicitation (Table 1). The AP increase in liver tissue was located mainly in the necrotic areas of the liver (Table 2). However, the decrease in AP activity was observed in both serum and liver tissue of mice with liver injury that proceeded for 1 week (Table 4). Such unique changes in AP might reflect the degree of liver injury.

AP, a membrane-bound ectoenzyme, is used as an indicator to reflect hepatobiliary or bone diseases (13). However, except in obstructive jaundice or cholestasis, the role of AP activity remains unknown in a variety of liver diseases such as viral hepatitis and cirrhosis. Although many reports have indicated the relationship between low serum AP level and fulminant hepatic failure caused by Wilson's disease and indicated the usefulness of AP as a marker for diagnosing the disease (14, 15), there is still a lack of clarity on the mechanism of low AP level and its role in the Wilson's disease. For experimental liver injuries, except for the decreased AP activity in some models reported by Nagai et al. (16), fewer publication could be found on the role of AP. Nevertheless, the dramatic changes in AP seen in the model with PCl-DTH liver injury might imply its significant involvement in the development of liver damage.

Since the AP change varied in different tissues and times of liver injury, and since our previous study (6) found a relationship between the extent of liver damage and immune response in PCl-DTH liver injury, we examined the changes in AP by changing the immune state in liver-injured mice using Cy, an immunosuppressor. This was based on the finding that Cy could regulate the degree of PCl-DTH liver injury when given during different phases of DTH (6). The result was that a single administration of Cy before PCl sensitization caused exacerbation of liver injury, with more serious AP changes (Table 3) than those seen in the control group of liver injury. On the other hand, the consecutive use of Cy after sensitization resulted in alleviation of liver injury with recovery of AP from the changes in either serum or liver tissue. These results suggest a relation of AP change to the state of liver function. Although AP release by some kinds of inflammatory cells including neutrophils and macrophages is known, at 12 h of liver injury we found only very low counts of neutrophils contained in total NPC whose dominant compositions were T lymphocytes (2). At the same time, AP activity in the NPC was almost undetectable. These findings suggest that the enrichment of AP in the necrotic liver tissues was not derived from infiltrated inflammatory cells, and AP might be an important factor involved in the mechanisms against liver injury.

In a previous study, we found that elevated endotoxin aggravated PCl-DTH liver injury by enhanced release of inflammatory cytokines (4), suggesting that endotoxin may be one of the important hepatotoxic causes. Recent studies have also indicated that inadequate AP may lead to dysfunction in endotoxin detoxification (17). Moreover, in this study the decrease in AP activity may be considered as the dysfunction in AP synthesis. Thus, we next examined the effect of endotoxin on hepatocytes in different states, using the assay of cultured hepatocytes with LPS. We found that LPS caused a remarkable release of ALT only when hepatocytes isolated from normal mice were pretreated with levamisole, a specific inhibitor of AP synthesis (Fig. 2). Similar results were seen in cultured liver slices from naive mice (Fig. 3). Such increased sensitivity of hepatocytes to LPS may be linked to the fact that levamisole inhibited the AP synthesis by and release from hepatocytes in a dose- (Fig. 1) and time-dependent fashion. Poelstra et

al. reported that inhibition of endogenous AP by levamisole significantly reduced survival of rats intraperitoneally injected with *E. coli* bacteria (9), suggesting the important relevance of sensitivity to LPS of hepatocytes to their AP activity. Interestingly, in this study LPS-elevated ALT release was also found in liver slices from mice with liver injury after 1 week, even without levamisole pretreatment. These results suggest that LPS exerts its hepatotoxicity only at low AP levels, and AP may act as an endogenous protective enzyme against LPS. Levamisole may make normal hepatocytes highly sensitive to lipopolysaccharide by inhibiting AP synthesis. Therefore, endotoxin detoxification by AP may be important to protect the liver from injury.

To further confirm the effect of endotoxin on AP, we measured AP activity in the state of early or late tolerance to LPS. The serum AP fell in the early state but increased in the state of late tolerance (Table 5). Regarding these two kinds of tolerance to LPS, several reports have indicated that early tolerance could result in deficient phagocytosis of Kupffer cells to LPS (18), and in late tolerance antibodies to LPS are usually produced by repeated LPS injection (19). Detailed mechanisms on the AP changes in LPS-tolerant status observed in this study are under examination.

Overall, AP synthesized by hepatocytes may display a protective role during liver damage by neutralizing the endotoxin produced in the process of liver damage.

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