

## Inhibition of the Antibody Production by Acetaminophen Independent of Liver Injury in Mice

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**The causal relationship between the inhibition of antibody production and liver injury induced by single doses of acetaminophen (APAP) was investigated in mice. The liver injury and antibody production were evaluated using the serum transaminase activity and the number of antibody forming cells against sheep red blood cells (SRBC), respectively. The relevance of APAP hepatotoxicity with inhibiting antibody production was elucidated in fasted and fed mice treated with a single oral administration of APAP. In fasted mice, the oral administration of APAP produced serious liver injury, while it was not the case in the fed mice. As the antibody production was measured under these conditions, APAP significantly depressed the antibody production in fed mice as well as in fasted mice. The rate of B220 positive cells in the splenocytes was significantly decreased by APAP administration in both the fasted and fed mice. Splenocytes proliferative responses following mitogenic stimulation with concanavalin A or lipopolysaccharide were inhibited by APAP. Moreover, APAP added directly to the splenocyte culture also inhibited the *in vitro* antibody-producing response to SRBC. These findings indicate that the APAP-induced depression of antibody production may not be a secondary response to APAP-hepatitis, but may be a primary response to APAP.**

**Key words** acetaminophen; immune suppression; hepatotoxicity; antibody production; alanine transaminase (ALT); mice

Acetaminophen (APAP) is one of the most extensively employed analgesic and antipyretic drugs worldwide that is relatively safe at normal therapeutic dose levels. Because of its minimal association with Reye's syndrome during febrile respiratory infections in children and minimal gastric toxicity, it has found increasing use in recent years as a substitute for aspirin.<sup>1)</sup> APAP is recommended for pregnant women as an analgesic and antipyretic because it is safe enough during all phases of pregnancy.<sup>2,3)</sup>

At low dose levels, APAP is mainly detoxified through sulfation and glucuronidation in animals.<sup>4)</sup> APAP hepatotoxicity occurs following overdose as a result of metabolism through a cytochrome P450 system that allows toxic products to accumulate and bind to critical intracellular components.<sup>5,6)</sup> Approximately 5–10% of ingested APAP is metabolized by the cytochrome P450-dependent pathway.<sup>7,8)</sup> The main P450 isoforms, which are currently thought to be responsible for APAP bioactivation and hepatotoxicity, are CYP2E1, CYP1A2 and CYP3A4.<sup>9,10)</sup> CYP2E1 is the major P450 in mice responsible for hepatotoxicity caused by APAP, although other P450 isoforms may also be involved.<sup>11)</sup> The product of oxidation is *N*-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive metabolite that is responsible for the toxicity in many organs, including the liver.<sup>8)</sup> NAPQI is usually rendered non-toxic by conjugation with the intracellular glutathione (GSH). When the GSH levels are low, the metabolite fails to be detoxified by conjugation, it accumulates and causes liver injury.<sup>12)</sup> Hepatotoxicity can be reduced if intracellular GSH is replenished with *N*-acetylcysteine.<sup>13,14)</sup>

It is well known that fasting increases the susceptibility of mice to APAP-induced liver injury.<sup>15–17)</sup> It is also well established that decreases in liver levels of reduced GSH are associated with fasting in these species.<sup>18)</sup> Since the ability of liver microsomes isolated from fasted rats to convert APAP to its reactive metabolite was not different from that in fed

rats, it was suggested that the potentiation of hepatic necrosis was due to the lower levels of GSH available to detoxify the reactive metabolite, rather than to an enhanced formation of the reactive metabolite *in vivo*.<sup>19)</sup>

There exists a significant historical database originating from clinical case reports that describes the accompaniment of immune suppression in patients suffering from hepatic injury or chronic liver disease. It is also reported that some hepatotoxins cause the immune suppression with hepatic injury in experimental animals.<sup>20–22)</sup> It was reported that chemically induced liver injury by exposure to carbon tetrachloride produces a distinct profile in immune suppression characterized by a marked inhibition of T-cell associated responses.<sup>23)</sup> On the other hand, there are a few reports about the effect of APAP on the immune system.<sup>24)</sup>

In this report, we elucidated the causal relationship between APAP-hepatitis and immune suppression by using fasted and fed mice so that we could control the occurrence of liver injury on a basis of the difference in the liver GSH level between both groups of mice receiving the same dose of APAP. Furthermore, we demonstrated the direct effect of APAP on the proliferative responses to mitogen and antibody producibility in the cultured splenocytes.

### MATERIALS AND METHODS

**Reagents** APAP, concanavalin A (Con A), antibiotic antimycotic solution, phenazine methosulfate and the carboxymethylcellulose sodium salt (CMC) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A transaminase assay kit was purchased from Iatron Co. (Tokyo, Japan). Sheep red blood cells (SRBC) were obtained from the Nippon Seibutsu Zairyo Center (Tokyo, Japan). RPMI-1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Complement was obtained from Denka

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Seiken Co. (Tokyo, Japan). Anti-CD4-PE, anti-CD8-FITC, anti-CD3-FITC and anti-B220-PE were purchased from Beckman Coulter Inc. (Miami, FL, U.S.A.). Lipopolysaccharide (LPS) was purchased from Difco (Detroit, MI, U.S.A.). 2-Mercaptoethanol was from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, U.S.A.). Trypan blue stain 0.4% was purchased from Gibco BRL (Grand Island, NY, U.S.A.). Tissue culture plates were from Costar (Cambridge, MA, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, U.S.A.).

**Animals** Female, SPF-grade BALB/c mice, 7 to 8 weeks of age, were purchased from Charles River Japan Co. (Atsugi, Japan). The mice were fed commercial rodent chow and water *ad libitum*. The room in which the mice were kept was maintained at 22–24 °C and with a 12 h light/dark cycle. Animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by The Japanese Pharmacological Society.

**Dosing Regimen** The BALB/c mice were orally administered 0.1 ml/10 g body weight of a vehicle (0.5% CMC) or APAP at a concentration of 300 mg/kg to fasted and non-fasted (fed) mice for the regulation of hepatotoxicity. Food, but not water, was removed from a cage for the fasted animals at 7:00 p.m., 18 h prior to the administration of APAP, and was resumed 6 h after the administration of APAP.

**Serum Transaminase** The serum transaminase activity was determined according to the method described by Lippi and Guidi.<sup>25)</sup> Briefly, mice were anesthetized using ether, and a blood sample was withdrawn from the orbital sinus of each animal with a heparinized capillary tube. The activities of the serum alanine transaminase (ALT) were measured using a commercial transaminase assay kit (Iatron).

**In Vivo Antibody-Producing Responses** For sensitization, the mice were injected i.v. into the tail vein with  $1 \times 10^8$  SRBC in 0.2 ml of saline for the T-dependent response 1 d after the treatment with the vehicle or APAP. The sensitization interval for the response to SRBC was 4 d, and then the animals were sacrificed and their spleens removed. Single-splenocyte suspensions were prepared from each spleen in 5 ml of RPMI-1640 and counted. Enumeration of the antibody-forming cells (AFC) was performed using a modified Jerne's plaque assay.<sup>26)</sup> The results were calculated as AFC/ $10^6$  splenocytes.

**In Vitro Antibody-Producing Responses** Single-splenocyte suspensions prepared from non-treated mice were adjusted to  $1.3 \times 10^6$  cells/ml in RPMI 1640 supplemented with 10% FBS,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10 ml/l antibiotic antimycotic solution (Sigma) and transferred in 1.5 ml aliquot to a 24-well culture plate. Each plate was incubated with APAP (1, 10, 100, 1000, 10000  $\mu$ M) and SRBC ( $1 \times 10^6$  SRBC/ml) for 4 d at 37 °C and 5% humidity. Enumeration of the AFC was performed using a modified Jerne's plaque assay. The results were calculated as AFC/ $10^6$  recovered splenocytes.

**Flow Cytometric Analyses** Splenocytes were stained in phosphate buffer saline (PBS) containing 2% fetal bovine serum (FBS) and incubated for 30 min with appropriate dilutions of various mAbs coupled to PE or fluorescein.

Dead cells were excluded on the basis of forward and side

scatter. At least  $10^5$  live lymphoid cells were acquired in each run. Samples were analyzed using flow cytometry (EPICS ELITE; Beckman Coulter, Miami, FL, U.S.A.).

**Mitogenicity** Spleens were isolated from non-treated mice and single-splenocyte suspensions were prepared and adjusted to  $1 \times 10^6$  cells/ml in RPMI 1640 supplemented with 10% FBS,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10 ml/l antibiotic antimycotic solution (Sigma). Cell suspensions were transferred in 0.2 ml aliquot to 96-well flat-bottom culture plates in the presence of optimal concentrations of Con A (3  $\mu$ g/ml) and LPS (30  $\mu$ g/ml) as mitogens and APAP (3–10000  $\mu$ M). To assay the mitogen-stimulated cell proliferation, we used the thiol-sensitive tetrazolium salt assay.<sup>27)</sup> Each culture was maintained for 48 h at 37 °C and 5% humidity, pulsed with 40  $\mu$ l of a freshly prepared solution of MTS plus phenazine methosulfate. After incubation for 4 h at 37 °C, the absorbance at 490 nm was recorded by using a micro plate reader MPR A4 (Tosoh). At the same time, cell viabilities were performed with trypan blue stain. Aliquots of cultures were mixed with an equal volume of 0.4% of trypan blue stain and staining cells and non-staining cells were counted within 15 min as died and viable cells, respectively.

**Statistics** A statistical analysis by 1-way ANOVA using Dunnett's test was used for multiple groups and the Student's *t*-test was used for two groups to assess the statistical significance of differences.

## RESULTS

**Serum Transaminase after Oral Administration of APAP to Fed and Fasted Mice** The serum ALT levels in oral APAP (300 mg/kg)-treated fasted mice, where fasting is supposed to exhaust the liver GSH level,<sup>17,28)</sup> were significantly increased over the control by 1600%. In contrast, the serum ALT levels in fed mice treated orally with 300 mg/kg of APAP were not significantly different from those of the control at all times (Fig. 1).

**In Vivo Antibody-Producing Responses of Fasted and Fed Mice Treated with APAP** The *in vivo* antibody-producing responses were measured to evaluate the humoral immunity after a single oral administration of APAP to the fasted or fed mice. In both the fasted and fed mice, an oral dose of 300 mg/kg of APAP produced a significant suppression of AFC by 29 and 25%, respectively (Fig. 2).

**Flow Cytometric Analyses of Splenocytes of APAP-Treated Fasted and Fed Mice** Flow cytometric analyses were used to determine the relative number of B220<sup>+</sup> and

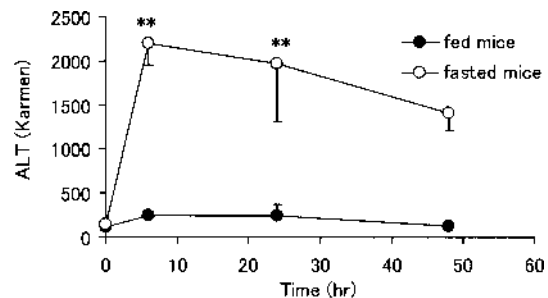


Fig. 1. Effect of Oral APAP on Serum ALT Levels in Fed and Fasted Mice

Serum ALT levels were measured at various times after the administration of APAP (300 mg/kg, *p.o.*). The mean and S.E.M. were obtained from five animals. Significantly different from group before treatment: \*\* $p < 0.01$ .

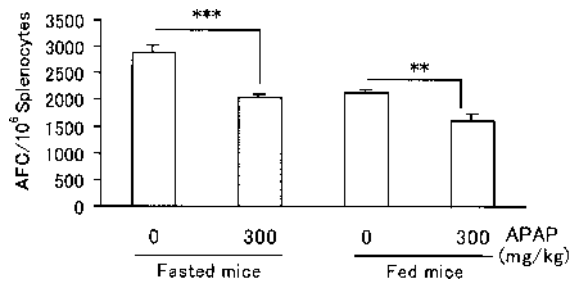


Fig. 2. Effect of Oral APAP on T-Dependent Antibody Response in Fed and Fasted Mice

Mice were sensitized with SRBC one d after the administration of APAP (300 mg/kg, *p.o.*). The mean and S.E.M. were obtained from five animals. Significantly different from the respective control: \*\**p*<0.01 and \*\*\**p*<0.001.

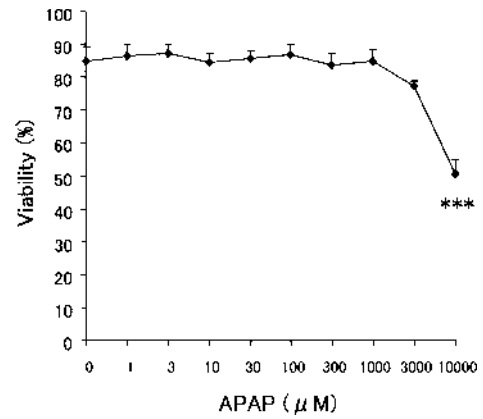


Fig. 3. Effect of APAP on Cell Viability of Splenocytes

Splenocytes were cultured with APAP (from 1 to 10000 μM) for 48 h and cell viabilities were performed with trypan blue stain. The mean and S.E.M. were obtained from sextuplicate cultures. Significantly different from the control: \*\*\**p*<0.001.

Table 1. Effect of Oral Administration of APAP on the Lymphocytes Populations of Splenocytes in Fed and Fasted Mice

| APAP (Mg/kg)              | Fasted mice |            | Fed mice  |            |
|---------------------------|-------------|------------|-----------|------------|
|                           | 0           | 300        | 0         | 300        |
| Cells (×10 <sup>7</sup> ) | 9.8±0.93    | 8.6±0.58   | 7.8±0.50  | 8.1±0.48   |
| B220 (%)                  | 56.6±1.54   | 45.8±4.22* | 50.0±0.64 | 47.5±0.54* |
| CD3 (%)                   | 31.0±2.60   | 37.5±3.26  | 37.9±1.24 | 40.0±0.84  |
| B220/CD3                  | 1.87±0.20   | 1.28±0.22* | 1.32±0.05 | 1.19±0.03* |
| CD4/CD8                   | 2.06±0.09   | 2.16±0.15  | 2.34±0.15 | 2.15±0.11  |

Mice were sacrificed one day after the administration of APAP (300 mg/kg, *p.o.*) and the lymphocyte populations of splenocytes were determined. The mean and S.E.M. were obtained from four animals. Significantly different from the respective control: \**p*<0.05.

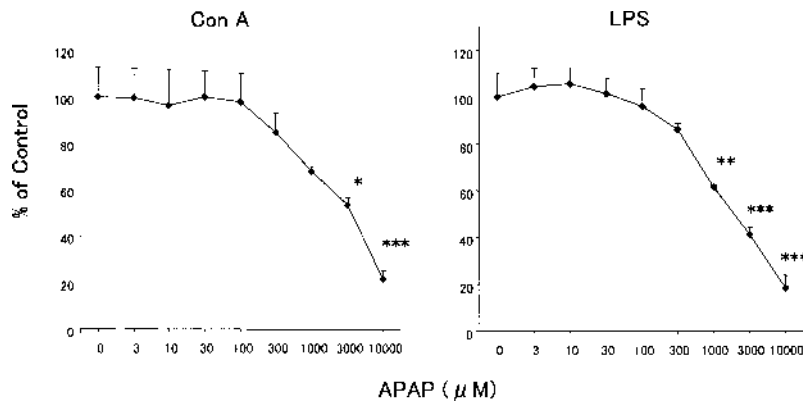


Fig. 4. Effect of APAP on Mitogen-Stimulated Cultures of Mouse Splenocytes

Splenocytes were cultured with APAP (from 3 to 10000 μM) in the presence of Con A (3 μg/ml) or LPS (30 μg/ml) for 48 h. The percent of control and S.E.M. were obtained from three experiments. Significantly different from the control: \**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001.

CD3<sup>+</sup> splenocytes and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> splenocytes 24 h after the administration of APAP to the fasted or fed mice. The relative number of B220<sup>+</sup> splenocytes was slightly but significantly decreased by APAP treatment in each of the fasted and fed mice (Table 1). On the other hand, the CD3<sup>+</sup> splenocyte related number and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> splenocytes showed no statistically significant change after treatment with APAP.

**Effect on Cell Viability of Splenocytes** There was no difference in cell viability between groups treated with 1 to 1000 μM APAP, and their all values of cell viability exceeded 85% as determined in trypan blue viability test (Fig. 3).

However, the high concentrations of 3000 and 10000 μM APAP resulted in a decrease in cell viability by 9 and 40%, respectively.

**Effect on Mitogen-Stimulated Cultures of Mouse Splenocytes** The effect of APAP on splenocytes proliferative responses was evaluated by addition of Con A, a specific mitogen for T cells, or LPS, a specific mitogen for B cells, plus the APAP to splenocytes. The non-cytotoxic dose of 1000 μM APAP inhibited the proliferative responses to Con A or LPS, by approximately 30 or 40%, respectively and the inhibition of the LPS response was significant (Fig. 4).

**Effect on *in Vitro* AFC Response in SRBC-Stimulated**

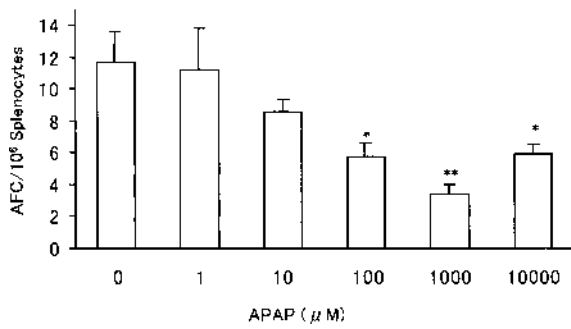


Fig. 5. Inhibition of IgM AFC Response by APAP in SRBC-Stimulated Splenocytes

Splenocytes at  $1.3 \times 10^6$  cells/ml were incubated with APAP (from 1 to 10000  $\mu\text{M}$ ) and SRBC ( $1 \times 10^6$  cells/ml) for 4 d. IgM AFC response is expressed as the mean  $\pm$  S.E.M. of quadruplicate cultures. Significantly different from the control: \* $p < 0.05$  and \*\* $p < 0.01$ .

**Splenocytes** *In vitro* AFC response was tested to explore the direct effect of APAP on antibody producing responses. In the presence of APAP, the development of anti-SRBC AFC induced by sheep erythrocytes was inhibited in a concentration-dependent fashion (Fig. 5). It was notable that the non-cytotoxic concentration of 100 and 1000  $\mu\text{M}$  APAP significantly inhibited the AFC response by approximately 50 and 70%, respectively.

## DISCUSSION

The present study demonstrated that a single oral administration of 300 mg/kg of APAP to fasted mice induces liver injury. In addition, the liver injury did not occur in fed mice receiving the same APAP treatment. Massively administered APAP is metabolized into NAPQI, an active metabolite that is mainly converted by CYP2E1, and NAPQI is known to damage the hepatocyte; the liver glutathione (GSH) is closely associated with detoxification of NAPQI by conjugation. In this study, we did not show the levels of liver GSH, but it has been reported in many species that fasting for more than 18 h produces a significant decrease in the liver GSH level.<sup>17,28,29</sup> Thus, we tried to explore the dose of APAP which could control the hepatic disorder by limiting the feeding in mice. As a result, the oral administration of 300 mg/kg of APAP has been found to be the treatment that does not influence the liver function in the fed mice, but induces liver injury in the fasted mice. The comparison between both the fed and fasted mice may give a suggestion of the influence of APAP-induced liver injury on the immune system.

The immune suppressive effect of APAP on the *in vivo* AFC responses for SRBC was demonstrated not only in the fasted but also in the fed mice. These results indicate that immune suppression and APAP-hepatitis noted after the APAP administration may be causally independent. On the other hand, a statistically significant difference was seen in the AFC responses between each control of fasted and fed mice. Several studies have reported that the immune response may be enhanced by the acute fasting in human and animals.<sup>30–32</sup> Accordingly, the augmentation of the AFC responses in the fasted mice is consistent with that noted in the previous reports.

It was reported that absorption of APAP is significantly lowered by food.<sup>33,34</sup> Thus, a blood concentration of APAP in

the fed mice is possibly lower than in the fasted mice. However, we consider that the pharmacokinetic affection of APAP in the AFC production was small if any, because the extent of inhibition of AFC production by APAP in fed mice was equivalent to that seen in fasted mice.

The mechanism responsible for the development of hepatic necrosis after doses of APAP in humans and experimental animals are not fully understood. Recent studies have suggested that tissue injury caused by APAP is mediated in part by soluble products derived from hepatocytes and/or resident and inflammatory macrophages including the tumor necrosis factor  $\alpha$ , interleukin 1, and reactive oxygen intermediates.<sup>35,36</sup> Moreover, nitric oxide is considered to be one of the important mediators of APAP-induced hepatotoxicity, and inhibition of inducible nitric oxide synthase showed a depressive effect on APAP-induced hepatic injury.<sup>37</sup>

In an association with the hepatic disorder, it is possible that immune suppression is caused by the release of several hepatic factors that mediate the immunotoxicity. The transforming growth factor- $\beta 1$  (TGF- $\beta 1$ ) is one of the hepatic factors which are released as part of the liver repair process following hepatic injury.<sup>38,39</sup> Moreover, it was shown that the immune suppression affecting both the helper T-cell function and antibodies which were specific for TGF- $\beta 1$  inhibited the immune suppressive effect of TGF- $\beta 1$ .<sup>40</sup> However, our data have suggested that APAP suppresses the immune system independent of the effect of TGF- $\beta 1$  derived from the liver, because the immune suppressive effect of APAP was not abolished by inhibition of the APAP-hepatitis by feeding.

Flow cytometric analyses have demonstrated that APAP significantly lowered the splenic B220<sup>+</sup> cells ratio, but did not affect the CD3<sup>+</sup> cells ratio. B220 is used widely as B cell specific surface antigen.<sup>41</sup> Recently, it has been reported that B220 was expressed on apoptotic T cells.<sup>42–44</sup> In this study, we consider that B220<sup>+</sup> cell is a B cell, because the CD3<sup>+</sup>B220<sup>+</sup> double positive cell does not exist in the tested splenocytes. Accordingly, the administration of a single dose of APAP is considered to decrease the B cell ratio of splenocytes. In our preliminary study, the strongest immune suppression was observed when mice were sensitized 24 h after the APAP administration, but the flow cytometric analyses at other time points will be necessary for time-course change in the effect of APAP.

Splenocytes proliferation following mitogenic stimulation was also suppressed by APAP. According to the present results, both T- and B-cell proliferation was significantly affected by APAP, although APAP seemed to influence B cells more sensitively. Moreover, APAP added directly to the splenocyte culture also inhibited the *in vitro* antibody-producing response to SRBC without affecting cell viability. In the AFC response, macrophages and T cells are needed as accessory cells to facilitate the activation, proliferation, and differentiation of B cells into antibody-producing plasma cells. Then, further investigation should be done in order to clarify the mechanism for the inhibition of antibody production by APAP, for example, which cell is strongly affected by APAP.

In conclusion, the inhibitory effect of APAP on the antibody formation against SRBC may not be a secondary response to APAP-hepatitis, but may be a primary response to APAP.

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