T Cell Activation-associated Hepatic Injury:
Mediation by Tumor Necrosis Factors and Protection
by Interleukin 6

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Summary
This study investigates the molecular mechanisms underlying the induction of and protection
from T cell activation–associated hepatic injury. When BALB/c mice were given a single intravenous
injection of concanavalin A (Con A) (>10.3 mg/mouse), they developed acute hepatic injury as
assessed by a striking increase in plasma transaminase levels within 24 h. Histopathologically,
only the liver was injured while moderate infiltration of T cells and polymorphonuclear cells
occurred in the portal areas and around the central veins. The induction of hepatic injury was
dependent on the existence as well as the activation of T cells, as untreated BALB/c nu/nu mice
or BALB/c mice pretreated with a T cell-specific immunosuppressive drug, FK506, failed to
develop disease. Significant increases in the levels of various cytokines in the plasma were detected
before an increase in plasma transaminase levels. Within 1 h after Con A injection, tumor necrosis
factor (TNF) levels peaked, this being followed by production of two other inflammatory cytokines,
interleukin 6 (IL-6) and IL-1. Passive immunization with anti-TNF but not with anti-IL-1 or
anti-IL-6 antibody, conferred significant levels of protection. Moreover, administration of rIL-6
before Con A injection resulted in an IL-6 dose-dependent protection. A single administration
of a given dose of rIL-6 completely inhibited the release of transaminases, whereas the same
regimen induced only 40–50% inhibition of TNF production. More than 80% inhibition of
TNF production required four consecutive rIL-6 injections. These results indicate that: (a) TNFs
are critical cytokines for inducing T cell activation–associated (Con A-induced) hepatitis; (b)
the induction of hepatitis is almost completely controlled by rIL-6; and (c) rIL-6 exerts its protective
effect through multiple mechanisms including the reduction of TNF production.

Hepatitis represents a worldwide health problem in humans
for which pharmacological treatments have yet to be
discovered. Development of new drugs, however, depends pri-
marily on the availability of animal models relevant to human
hepatitis or hepatocellular damage. For instance, active chronic
hepatitis leading to liver cirrhosis is a disease state in which
liver parenchymal cells are progressively destroyed by activ-
ated T cells (1). The commonly used model of liver injury
induced by chemicals (2), however, does not accurately rep-
resent the clinical situation.

It has been reported that the administration of endotoxin
from gram negative bacteria (LPS) to rodents leads to endo-
toxic shock (3, 4). The injection of LPS at subtoxic amounts
into D-galactosamine–treated animals results in hepatic in-
jury under conditions in which other organs are not affected
by LPS (5, 6). Since the hepatic lesions induced in this model
resemble those of human viral hepatitis (7), this has been
regarded as an appropriate human hepatitis model. Although
this type of hepatitis is likely to be based on the production
of endogenous mediators and their biological actions (8–10),
pretreatment with D-galactosamine is an absolute requirement,
and this substance is known to be a specific hepatotoxic agent
(11). In contrast, another hepatitis model was recently devel-
oped in which liver-specific inflammatory lesion is induced
by injection of Con A without pretreatment with D-galacto-
samine (12). Con A–induced murine hepatitis has provided
a novel model in that hepatitis is induced as a consequence
of T cell activation. However, molecular mechanisms of end-
genous mediators underlying the Con A–induced hepatic
lesions have not been analyzed.

The present study was undertaken to investigate the roles
of various cytokines in the induction of and protection from
Con A-induced hepatitis. The results demonstrate that Con A-induced hepatitis is T cell activation dependent and that the induction of hepatitis is associated with the production of various cytokines. TNF is the first cytokine produced after Con A injection, and is the most critical in inducing hepatitis as anti-TNF antibody confers protection against disease. Hepatitis was also prevented by administration of rIL-6. Importantly, rIL-6 was more effective in the prevention of transaminase release than in the inhibition of cytokine (TNF) production, implying that IL-6 functions to enhance host resistance as well. Thus, these results indicate a role for various cytokines in the induction vs regulation of T cell-dependent hepatic injury.

Materials and Methods

Mice. Female BALB/c (+/+) mice were purchased from the Shizuoka Experimental Animal Center (Shizuoka, Japan), and were used at 7–10 wk of age. BALB/c (nu/nu) mice were obtained from Charles River Japan Inc. (Kanagawa, Japan).

Reagents. Con A was purchased from Vector Laboratories, Inc. (Burlingame, CA). FK506 was from our company (Fujisawa Pharmaceutical Co. Ltd.). Recombinant human IL-6 was expressed on CHO cells and purified in our company. Anti-TNF mAb capable of neutralizing TNF-α and TNF-β, and anti-IL-1α antisera were obtained from Genzyme Corp. (Cambridge, MA) and Advanced Magnetics, Inc. (Cambridge, MA), respectively. Hybridoma-producing antimurine IL-6 mAb (6B4) (13) was a generous gift from Dr. J. Van Snick (Ludwig Cancer Institute for Cancer Research, Brussels, Belgium). Purified mAb from ascitic fluid was used. The following reagents were purchased to perform immunohistochemical examination: purified rat anti-mouse CD4 and CD8 mAb (Pharmingen, San Diego, CA); normal rat IgG as control (Biomeda, Foster City, CA); peroxidase AffiniPure mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA); and 3-amino-9-ethylcarbazole (AEC) (Aldrich Chemical Co., Milwaukee, WI).

Treatment of Mice. Con A was dissolved in pyrogen-free saline and administered to mice via the tail vein. rIL-6 was diluted with pyrogen-free saline and administered to mice subcutaneously. FK506 was administered to mice orally in a dose of 32 mg/kg. Anti-TNF, anti-IL-1, and anti-IL-6 antibodies were injected into mice intravenously.

Assay for Plasma Transaminase Activities. Plasma from individual mice was obtained at various time intervals after Con A injection. Plasma transaminase activity was measured by the standard photometric method (14) using a bichromatic analyzer (model 100; Abbott Laboratories, North Chicago, IL).

Assays for Cytokine Levels in Plasma. TNF, IL-1, IL-2 (Genzyme Corp.), and IL-6 (Endogen, Inc., Boston, MA) amounts in plasma were assayed by ELISA kits. The assays were performed exactly as described by the manufacturer. Each sample was determined in duplicate.

Histological Examination. Various organs including the liver from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination.

Staining Procedure of Immunohistochemical Examination. Cryostat sections (5 μm) were cut, air-dried at room temperature for 2 h, fixed in acetone for 10 min, and air dried. Before staining, the sections were washed in PBS. The tissues were overlayed with the appropriately diluted nonlabeled primary antibody, and incubated for 1 h in a humidified chamber at room temperature. The sections were then washed in three changes of PBS and dipped in methyl alcohol with 0.3% hydrogen peroxide (10 min) for blocking of endogenous peroxidase activity. The sections were then incubated with peroxidase-labeled mouse anti-rat IgG for 1 h. After another wash in PBS, the sections were stained with AEC for several minutes to develop the color and washed in water. The sections were counterstained with hematoxylin for 30 s. 20 sections were prepared from each liver. Sequential parallel sections exhibiting a typical cellular infiltration are presented in each figure.

Statistical Analysis. Results are expressed as mean ± SE. Statistical analysis was performed using Student's t test.

Results

Induction of Acute Liver Injury After Con A Administration. BALB/c mice were intravenously injected with different doses of Con A, and transaminase activities were measured in plasma from mice at various intervals after Con A injection (Table 1 and Fig. 1). The results demonstrate that a single

Table 1. Dose-dependent Increase in Plasma Transaminase Activities after Con A Injection

<table>
<thead>
<tr>
<th>Dose of Con A (mg/mouse)</th>
<th>No. of mice</th>
<th>Plasma transaminase level</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AST (U/liter)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALT (U/liter)</td>
</tr>
<tr>
<td>0*</td>
<td>5</td>
<td>119 ± 12</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>167 ± 16</td>
</tr>
<tr>
<td>0.3</td>
<td>5</td>
<td>5,458 ± 1,670</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>7,564 ± 2,414</td>
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</table>

* BALB/c mice were injected intravenously with indicated doses of Con A. Plasma from these mice was obtained 24 h after Con A injection.

Figure 1. Time course of increase in plasma transaminase levels after Con A injection. Five BALB/c mice were injected intravenously with 0.3 mg/mouse Con A. Plasma from individual mice was obtained at various hours after Con A injection. Data points represent the mean ± SE of transaminase activity (U/l) in plasma obtained at each time point.
Injection of Con A at a dose of at least 0.3 mg/mouse (approximately equal to 15 mg/kg) elicits a striking increase in plasma transaminase levels. Such an increase was detectable as early as 8 h after Con A injection, and after reaching the peak levels at around 24 h, the levels decreased within 48 h after the injection.

**Histological and Immunohistochemical Examination.** Various organs including the liver, spleen, kidney, lung, and heart were removed various hours after Con A injection. Light microscopic examination revealed that only the liver was severely affected. No significant changes were observed in other organs (data not shown).

Fig. 2 shows the time course of hepatic injury. 8 h after Con A injection, slight degenerative changes in hepatocytes were observed, but necrosis and cellular infiltration were not detected (Fig. 2, A–C). After 24 h, moderate infiltration of mononuclear and polymorphonuclear cells were seen in the portal area within the Glisson's capsules (Fig. 2 F) and around the central vein. Spotty, focal, and massive necroses developed and hepatocytes between these necrotic areas exhibited vacuolar and vesicular cytoplasmic changes (Fig. 2, D and E). These changes continued to exist in the sections 48 h after Con A injection (Fig. 2, G, H, and I), by which time the progress of hepatic injury had ceased. In addition, infiltrated...
inflammatory cells were observed to have accumulated around the necrotic areas (Fig. 2, G and H).

To determine whether mononuclear cells infiltrating the liver represent T cells, we performed immunohistochemical analyses using cryostat sections of the liver 24 h after Con A injection. Fig. 3 (top for the portal area and bottom for the central vein area) shows that a large number of cells are stained with anti-CD4 mAb and a relatively small number of cells exhibit the CD8 phenotype. The infiltration of either CD4+ or CD8+ T cells was not detected in the sections from normal mice (data not shown). Taken collectively, hepatic injury is summarized to consist of (a) the degeneration of parenchymal cells and the existence of necrotic areas, and (b) moderate-to-severe infiltration of polymorphonuclear as well as mononuclear cells, including a large number of CD4+ T cells.

Con A–induced Hepatitis Is Dependent on T Cell Activation. It is known that Con A is a lectin that induces generalized T cell activation. Therefore, we next asked whether the induction of hepatitis induced by Con A administration is based on T cell activation. When athymic nude mice (BALB/c nu/nu) were injected with Con A (0.3 mg/mouse), they did not develop severe hepatic injury (Fig. 4 A). The results in Fig. 4 B demonstrate that the administration of FK506 before Con A injection is capable of inhibiting T cell activation, resulting in protection of euthymic BALB/c mice that would otherwise suffer from severe hepatitis. These results indicate that the development of Con A–induced hepatitis requires both the presence and activation of T cells.

Elevation of Plasma Cytokine Levels in Mice After Con A Injection. To investigate which types of endogenous mediators are produced to induce liver injury after Con A injection, we examined the plasma levels of various cytokines/lymphokines. IL-2 is, in general, the most representative of lymphokines produced by antigen- or lectin (Con A)-activated T cells. In this model, a significant increase in the plasma level of this lymphokine was observed at early time points after Con A injection with the peak response around 2–4 h after the injection (Fig. 5 A). We also examined the plasma levels of inflammatory cytokines including TNFs, IL-1, and IL-6. The results are summarized in Fig. 5, B–D. A striking increase in the levels of all three cytokines was observed earlier than an increase in plasma transaminase levels. Notably, TNF levels were elevated as early as 1 h after Con A injection. Fig. 5, B–D also illustrates that the strikingly elevated levels of these three cytokines decreased sharply although the levels after 24 h remained slightly increased compared with those from normal untreated animals.

Anti-TNF Antibody Prevents Con A–induced Hepatitis. To investigate how cytokines induced in vivo by Con A contribute to the development of acute liver injury, we examined the protective effect of passive immunization with anti-cytokine antibodies. The results in Table 2 demonstrate that passive immunization with anti-TNF antibody conferred protection, whereas pretreatment with anti-IL-1 or anti-IL-6 antibody failed to prevent disease development. These results indicate that TNF which is produced earliest after Con A injection plays a critical role in the development of Con A–induced hepatitis.

rIL-6 Confers Protection of Con A–induced Hepatitis. As mentioned previously, the production of TNF was followed by the production of other cytokines, IL-6 and IL-1. We there-
fore investigated whether these secondary cytokines augment the role of TNF in inducing hepatitis or function to stimu-
late host defense. Various doses of rIL-6 were administered to mice 6 h before Con A injection, and plasma transaminase (ALT) lev-
els were determined 24 h after Con A injection. The results of Fig. 6 illustrate that pretreatment of mice with rIL-6 produces IL-6 dose-dependent protection of liver injury. Additional experiments were performed to determine whether the protective effect of rIL-6 is related to the time of administration of this cytokine. rIL-6 was administered at a given dose (20 μg/mouse) at various times before or after Con A injection (Fig. 7). The rIL-6 administration before Con A injection (24–3 h) conferred almost complete protection, whereas administration of the same rIL-6 sample failed to produce a protective effect when administered at the same time as or after Con A injection. It should be noted that

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**Table 2. Inhibition of Con A–induced Hepatitis by Anti-TNF but Not Anti-IL-1 or Anti-IL-6 Antibodies**

<table>
<thead>
<tr>
<th>Treatment with Ab*</th>
<th>No. of mice</th>
<th>Percent inhibition of transaminase release†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11</td>
<td>AST – –</td>
</tr>
<tr>
<td>Anti-TNF</td>
<td>4</td>
<td>82 84</td>
</tr>
<tr>
<td>Anti-IL-1</td>
<td>4</td>
<td>0 0</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>3</td>
<td>0 0</td>
</tr>
</tbody>
</table>

* Various antibodies against cytokines were administered to mice at the same time as Con A (0.3 mg/mouse) injection; anti-TNF mAb, 250 μg/mouse; anti-IL-1 Ab, 250 μg/mouse; and anti-IL-6 mAb, 850 μg/mouse.
† Plasma transaminase levels were determined 24 h after Con A injection. Percent inhibition of transaminase release was calculated as follows: % inhibition = 100 \times \left[1 - \frac{\text{transaminase units in Ab-treated mice}}{\text{transaminase units in untreated mice}}\right].
IL-6 functions as a negative modulator of TNF production in vivo, but that this modulatory effect is only partial, given that it almost completely protects against hepatitis.

Discussion

The development of hepatitis or hepatocellular destruction has not been studied extensively at the cellular or molecular level mainly because of the lack of experimental systems. Thus, an appropriate animal model was required for investigating the pathophysiology of immunologically mediated hepatic disorders such as autoimmune hepatitis, as well as for establishing an attempt to prevent and/or protect from the diseases.

A new hepatitis model was recently developed in which a single injection of Con A resulted in liver injury (12). Using this model, the present study investigated the molecular mechanisms (endogenous mediators) underlying the development of and protection from hepatitis. The results demonstrated that (a) Con A–induced hepatitis is T cell activation dependent as massive infiltration of lymphoid cells, including CD4+ T cells, was detected at the site of injury and FK506-pretreated or nude mice failed to develop disease; (b) in addition

**Figure 6.** Protective effect of IL-6 administration on the development of Con A–induced hepatitis. Various doses (indicated) of rIL-6 were administered to BALB/c mice (five mice per group) 6 h before Con A (0.3 mg/mouse) injection. Plasma ALT levels of normal untreated mice were 41 ± 2.

with rIL-6 influences the production of TNF by Con A injection. rIL-6 was administered once (at 6 h) or four times (at 26, 22, 6, and 1 h) before Con A injection (Fig. 8). A single injection of rIL-6 significantly reduced the production of TNFs; a 40–50% reduction was observed in plasma obtained 1 h after Con A injection. This was also the case with the suppression of IL-1 production when determined in plasma 3 h after Con A injection. More profound suppression was obtained by four rounds of IL-6 injection. This is in contrast to almost complete prevention of transaminase release by a single rIL-6 administration. Thus, these results indicate that

**Figure 7.** rIL-6–induced protection depends on its administration before Con A injection. rIL-6 (20 μg/mouse) was administered to BALB/c mice (five mice per group) at various hours (indicated) before or after Con A (0.3 mg/mouse) injection (time 0). Negative and positive controls represent untreated normal mice and mice given Con A injection without IL-6 treatment, respectively. Plasma was obtained 24 h after Con A injection. (∗) p <0.05 vs. positive control; (∗∗) p <0.01 vs. positive control.

**Figure 8.** Effects of rIL-6 administration on the production of TNFs and IL-1 by Con A injection. rIL-6 (20 μg/mouse) was administered to BALB/c mice (more than four mice per group) once (at 6 h) or four times (at 26, 22, 6, and 1 h) before Con A (0.3 mg/mouse) injection. Plasma was obtained at various hours after Con A injection, and assayed for TNF and IL-1 activity. (∗) p <0.05 vs. control; (∗∗) p <0.01 vs. control.
to a representative T cell–derived lymphokine, IL-2, various inflammatory cytokines (TNFs, IL-1, and IL-6) are overproduced sharply even after Con A injection, and elevated levels decreased sharply at later time points; (c) TNFs have a critical role in inducing hepatitis, as passive immunization with anti-TNF but not with anti-IL-1 or anti-IL-6 antibody conferred protection; and (d) IL-6 also conferred almost complete protection, but only when administered at least 3 h before Con A injection. The protective effect of this cytokine is mediated by various mechanisms including its function as a negative modulator for TNF production.

The pathophysiology of immunologically mediated hepatic disorders has been investigated to date using models for systemic endotoxic shock (3, 4, 15) and its related hepatitis (5–10). Administration of endotoxin to animals can prompt severe metabolic and physiological disturbances leading to death (3, 4, 16). A common observation in endotoxic shock in rodents is acute liver failure. It has been established that macrophages or their production of TNF-α are central in causing endotoxic reactions (16–18). D-galactosamine, which impairs protein biosynthesis only in the liver (11), sensitizes the liver by several orders of magnitude toward subtoxic amounts of LPS (6). D-galactosamine/LPS–induced liver injury was also demonstrated to be mediated by TNF-α (9, 10) and induced under conditions in which other organs are not affected. Thus, this has represented a hepatitis model induced by endogenous mediators. Nevertheless, this model may be prone to the limitations inherent to the experimental approach. These include presensitization with D-galactosamine (a specific hepatotoxic agent) as an absolute requirement, and disease is induced by macrophages or their products without critical involvement of activated T cells as required for autoimmune-type liver cell destruction (1). In this context, the hepatitis induced by Con A (termed Con A–induced hepatitis) may represent a more suitable model for hepatitis whose development is associated with T cell activation and inducible without involvement of metabolically active/toxic substances.

The following aspects of the Con A–induced hepatitis model should be considered. First, it is obvious that the induction of hepatitis is dependent on T cell activation (12 and this study). Generalized T cell activation leads to the pathological status termed “T cell–mediated cytokine-related syndrome.” Previous studies (19–23) have revealed that the administration of anti-CD3 mAb induces shocklike syndromes through the overproduction of T cell–derived lymphokines. Similar phenomena were observed by injection of the superantigen staphylococcal enterotoxin B (24). Animals suffering from this syndrome exhibited acute inflammatory lesions in various organs (lung and kidney, in addition to liver). We have also performed experiments in which shocklike syndromes are induced by injection of anti-CD3 mAb. The increase in plasma transaminase levels induced by injection of anti-CD3 mAb was one order of magnitude lower than that induced in the Con A model (our unpublished observation). In contrast to the damage to various organs observed in the anti-CD3 mAb model, Con A injection leads to induction of liver-selective injury. This selectivity could be ascribed, in part,
tivities of IL-6 in acute inflammatory responses have not yet been completely uncovered. IL-6 negatively regulates the production of TNFs (36, 37), whereas TNF functions conversely as a positive modulator of IL-6 production (15, 38). Regarding the mechanisms by which IL-6 protects from hepatitis, our results suggest the existence of multiple aspects. One such aspect is the reduction of TNF production as has been reported in other studies (36, 39). However, a single IL-6 injection conferred complete protection against hepatitis whereas TNF production was inhibited by at most 50%. Therefore, it is conceivable that IL-6 exerts its protective effect through additional mechanisms. IL-6 is a major inducer of the acute phase response, mediating the synthesis and release of acute phase proteins such as C-reactive protein, α2-macroglobulin and α1-proteinase inhibitor (40). Furthermore, because TNF cytotoxicity may be partly dependent on protease activity (41), IL-6-induced production of protease (proteinase) inhibitors by the liver could theoretically contribute to moderating TNF cytotoxicity. Thus, IL-6 has the potential to reduce the total inflammatory burden mediated by TNF.

It is important to note that the beneficial effect of IL-6 administration was observed only when this cytokine was given before disease induction. The administration of IL-6 after Con A injection induced significantly enhanced transaminase release. The following possibilities may be raised to explain the opposing effects of IL-6 on disease severity, depending on the relative times of administration. It is known that in addition to negative regulation of TNF production, IL-6 increases the level of TNF-α receptor (TNFR) expression (42-44) on various types of cells including hepatocytes (43). Because TNF is produced early (only 1 h) after Con A injection, IL-6 given 3–6 h after the injection may function to enhance the expression of TNFRs on hepatocytes without inhibiting the TNF production. Therefore, it is conceivable that the already released TNF reacts more effectively with hepatocytes expressing increased levels of TNFRs, resulting in more potent cytotoxic effects on hepatocytes. Although not mutually exclusive, it is also possible that treatment with IL-6 may stimulate the generation of some cytotoxic mediators. In fact, IL-6 was demonstrated to stimulate the release of TNF-α/lymphotoxin by once activated lymphoid cells (42). Thus, IL-6 enhancement of TNF cytotoxicity may involve more than the upregulation of TNFRs (44). irrespective of the mechanism(s) underlying IL-6 enhancement, it is likely that each pathway is upregulated by a large amount of IL-6 endogenously produced after Con A injection. Thus, further studies will be required to investigate how IL-6 influences the production/release of various cytokines and the expression of TNFRs on hepatocytes, and consequently, enhances the progress of inflammatory lesions when given after Con A injection.

Our results illustrate that TNF has a critical role in inducing T cell-mediated hepatitis and that the development of disease is completely prevented by IL-6 through potentially multiple pathways. Thus, the present experimental system could provide a model for investigating the pathophysiology of T cell activation-associated hepatitis in humans such as autoimmune hepatitis, as well as for developing therapeutic approaches based on the observations obtained in such a model.

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