Protection from T Cell-Mediated Murine Liver Failure by Phosphodiesterase Inhibitors

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ABSTRACT

Injection of the T cell mitogens concanavalin A (Con A) into nonsensitized or of staphylococcal enterotoxin B (SEB) into α-galactosamine (GalN)-sensitized mice is known to cause fulminant liver failure via a cytokine response syndrome with tumor necrosis factor-α (TNF) as the pivotal mediator. We examined in vivo whether the phosphodiesterase (PDE) inhibitors motapizone (PDE3-selective) and rolipram (PDE4-selective) affected cytokine release and hepatic injury after T cell activation. Both motapizone as well as rolipram dose-dependently (0.1–10 mg/kg) attenuated the systemic release of TNF and interferon-γ as initiated by injection of Con A (25 mg/kg) or SEB (2 mg/kg). Although interleukin-4 production was not affected by motapizone or decreased by rolipram, circulating levels of interleukin-10, however, were significantly increased in PDE inhibitor-treated mice compared with controls. Associated with the suppression of the central mediator TNF, motapizone and rolipram protected mice from liver injury in the Con A as well as in the SEB model. Moreover, the combined administration of motapizone plus rolipram at doses which were ineffective when given alone completely protected mice from GalN/SEB toxicity. These data demonstrate that PDE inhibitors effectively attenuate an inflammatory T cell response in vivo and strongly suggest a therapeutic potential as anti-inflammatory drugs in T cell-related disorders. We conclude that CAMP-elevating drugs shift the balance of T cell-derived cytokines from a proinflammatory to an enhanced anti-inflammatory factor release, thus protecting mice from TNF-mediated hepatic failure.

T lymphocytes are causally involved as effector cells evoking liver disease caused by viral infection (i.e., viral hepatitis) or autoimmune disorders such as chronic active hepatitis, autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis (reviewed in Mayer zum Büschenfelde et al., 1993). As a consequence of the activation of these cells, proinflammatory cytokines are systemically released, which possibly accounts for the pathophysiological outcome and clinical manifestation of liver disease. The most potent proinflammatory cytokine in this context is TNF. TNF causes hepatic failure in humans (for review, see Jones and Selby, 1989; Waage, 1993), a phenomenon that has been widely studied in various experimental animal models, mostly in rodents. Pretreatment of mice with inhibitors of hepatic transcription such as GalN renders them extremely sensitive towards TNF-inducible liver failure (Tiegs et al., 1989, 1990; Wendel, 1990) and lethality (Galanos et al., 1979; Lehmann et al., 1987). In these experimental models either TNF itself, or more commonly, LPS, a potent inducer of endogenous TNF production by activation of monocytes and macrophages, is injected into mice. The use of superantigens such as SEB (Miethke et al., 1992; Nagaki et al., 1994; Gantner et al., 1995a) or of agonistic T cell receptor antibodies (Gantner et al., 1995a) as T cell activators in GalN-sensitized animals or of the T cell mitogen Con A in nonsensitized mice (Tiegs et al., 1992) expanded these experimental settings to a variety of different T cell-specific models. In any of them, TNF has been identified as the distal hepatotoxic mediator because passive immunization against TNF completely protected mice from liver injury independent of the T cell activator used (Miethke et al., 1992; Nagaki et al., 1994; Mizuhara et al., 1994; Gantner et al., 1995a,b).

These facts suggest that TNF suppression might be a promising pharmacological approach to prevent liver damage induced by T cell activation. Cyclic AMP-elevating drugs such as PDE inhibitors are known to inhibit monocyte- and macrophage-derived TNF production after LPS stimulation in vitro (Semmler et al., 1993; Fischer et al., 1993; Reinstein et al., 1993).
etal., 1994; Prabhakar et al., 1994; Seldon et al., 1995; Jilg et al., 1996) and in vivo (Fischer et al., 1993; Jilg et al., 1996). In human peripheral blood mononuclear cell cultures (Essayan et al., 1994; Gantner et al., 1995c) or in the murine T[H2 cell line D10G4.1 (Schmidt et al., 1995), the combined inhibition of PDE3 and PDE4 was most effective in blocking T cell proliferation and in modulating cytokine production. Recent reports gave a first hint that PDE4-selective inhibitors such as the antidepressant rolipram might be beneficial in vivo in chronic T cell-mediated disorders such as experimental allergic encephalomyelitis, thought to be a model for multiple sclerosis, in which TNF also plays a significant role (Sommer et al., 1995; Genain et al., 1995). We investigated the in vivo potential of PDE inhibitors selective for PDE3 (motapizone) or PDE4 (rolipram), respectively, in our acute models of T cell-mediated hepatic failure. With these inhibitors, we studied their modulatory effects on the T cell activation-induced cytokine pattern released and on the outcome of liver failure after T cell activation in naive mice (Con A model) and in animals sensitized by transcriptional inhibition (GalN/SEB model).

Materials and Methods

Animals. Male BALB/c mice weighing 25 ± 3 g were purchased from the internal breeding stock of the animal house of University of Konstanz, Germany. All mice were kept at 22°C and 55% relative humidity in a 12-h day/night rhythm with free access to food (Altromin 1313) and water. Sixteen hours before the experiment started food was withdrawn, and the mice were not fed overnight. All animals received humane care in compliance with National Institutes of Health guidelines and according to legal requirements in Germany.

Treatment schedules. Con A was purchased from Sigma Chemical Co. (St. Louis, MO) and 25 mg/kg were administered i.v. in a volume of 300 μl pyrogen-free saline into the tail vein. GalN (Roth Chemicals, Karlsruhe, Germany) was given intraperitoneally at a dose of 700 mg/kg in 200 μl saline 15 min before injection of SEB (Sigma; 2 mg/kg i.p. in 200 μl saline). Rolipram (4-(3’-cyclopentyloxy-4’-methoxyphenyl)-2-pyrrolidone) and motapizone (4,5-dihydro-6-[4-(11-imidazol-1-yl)-2-thienyl]-5-methyl-3-pyridazinone) were administered i.p. 30 min before challenge in a volume of 200 μl solvent (Sandimmune placebo, Sandoz, Basel, Switzerland). The same volume of solution was injected into control animals. For determination of time courses of cytokine release into the circulation, animals were sacrificed at various time points after challenge by injection of heparinized pentobarbital (150 mg/kg), and blood was withdrawn by heart puncture into heparinized syringes. Blood sampling from the tail vein for determination of plasma cytokine peak concentrations was performed at the time points indicated. Plasma samples were kept frozen at −70°C until further use.

Cytokine assays. All enzyme-linked immunosorbent assays were performed on flat-bottomed, high-binding polystyrene microtiter plates (Greiner, Nürtlingen, Germany) with use of specific rat anti-mouse monoclonal antibody pairs purchased from Pharmingen (San Diego, CA). A polyclonal monospecific ovine anti-mouse TNF antibody prepared by S. Jilg in the laboratory of Dr. A. Wendel (immunoglobulin G fraction; protein content, 20 mg/ml) after immunization...
of a ram with recombinant muTNF-α was used for coating instead of the Pharmingen antibody. Streptavidin-peroxidase was from Jackson Immuno Research (West Grove, PA) and the peroxidase substrate BM blue was from Boehringer (Mannheim, Germany). IL-10 was determined with the INTERTEST-10×TM ELISA kit (Genzyme, Cambridge, MA). The detection limits of the assays were 15 pg/ml for IL-10, 10 pg/ml for TNF and IFNγ and 5 pg/ml for IL-4, respectively.

Assessment of liver failure. Eight hours after Con A or SEB injection animals were sacrificed by cervical dislocation, and blood was collected by heart puncture. Plasma enzyme activities of ALT, AST and SDH, respectively, were assessed according to Horder and Reij (1984).

Statistics. Unless otherwise stated, data are expressed as means ± S.D. of experiments carried out in triplicate. Data were analyzed by nonparametric analysis of variance (Kruskal-Wallis), and where there were differences among the groups (P > .05) data were subjected to one-sided nonparametric multiple comparisons of the disease control group against all other groups (Zar, 1984). P < .05 was considered to be significant.

Results

T cell activation-induced cytokine release in vivo. In previous studies we have shown the plasma kinetics of the cytokines TNF, IFNγ, IL-2 and GM-colony-stimulating factor after Con A challenge of mice (Gantner et al., 1995b). Because PDE inhibitors were recently demonstrated to affect murine Th2-type cytokine production in vitro (Schmidt et al., 1995), we first checked whether and when the Th2-type cytokines IL-4 and IL-10 were released in our in vivo models. The T cell activation-induced IL-4 and IL-10 plasma cytokine levels are shown in figure 1 in comparison with the time course of TNF release, i.e., the central mediator of Con A- and GalN/SEB-induced liver failure, and in comparison with the Th1-type cytokine IFNγ. Con A induced detectable amounts of IL-4 as early as 30 min after challenge with a further increase up to a maximum concentration of about 900 pg/ml at 2 to 2.5 h that declined afterward. IL-10 was released in a biphasic manner characterized by a high first peak at 30 min (4600 ± 420 pg/ml). This was followed by complete absence of circulating IL-10 at 4 h, before a second phase of IL-10 production was noted 6 to 8 h after Con A challenge.

When the superantigen SEB was used for T cell activation in vivo in GalN-sensitized mice, similar but somewhat delayed kinetics of TNF (plasma peak at 2.5 h compared with the peak observed on Con A challenge at 2 h), IL-4, IL-10 and IFNγ, respectively, were noted (fig. 1). The maximum circulating IFNγ concentration, however, was severalfold higher than the one observed by Con A injection. IL-4 plasma concentrations reached about 20% of the peak concentrations evoked by Con A. They were maximal 4 h after challenge and then declined continuously. Amounts of IL-10 similar to those noted after Con A administration were measured at 1.5 h, steadily increasing up to 6 h after SEB administration before IL-10 concentrations reached a plateau of about 5000 pg/ml until the end of the experiment. In contrast to Con A, SEB failed to evoke an early IL-10 peak within the first hour after challenge. Notably, GalN treatment did not affect SEB-induced cytokine production (data not shown). Thus both treatment regimens, i.e., injection of Con A or SEB induced the release of substantial amounts of Th2-type cytokines.

Modulation of the cytokine pattern by PDE inhibition. We then examined the pharmacological consequences of PDE inhibition on cytokine production after T cell activation in vivo. For this purpose we treated mice with various doses of either motapizone, a PDE3 inhibitor, or rolipram, a type 4-selective PDE inhibitor, before injection of Con A or GalN/SEB, respectively, and determined the circulating levels of TNF, IL-4 and IL-10 2 h after Con A and 2.5 h after GalN/SEB administration. IFNγ was determined 8 h after challenge, i.e., at the end of the experiment. These time points were chosen, because then all cytokines of interest were present in considerable amounts in the circulation of the animals and could be determined out of one single sample of each individual animal.

Either pretreatment had a drastic effect on the concentrations of cytokines released into the circulation. As shown in figure 2A, motapizone dose-dependently decreased plasma TNF and IFNγ levels induced by Con A (25 mg/kg). In contrast, IL-10 concentrations were significantly increased at the highest dose of motapizone used (10 mg/kg). These remarkable changes observed in Con A-induced cytokine levels...
after PDE3 inhibition were even more pronounced in the SEB model (fig. 2B). Again, TNF and IL-10 levels were inversely modulated by motapizone pretreatment, i.e., an attenuation of TNF concentrations to below 100 pg/ml and a 5-fold increase in IL-10 2.5 h after SEB challenge were observed at doses from 1 to 10 mg/kg motapizone.

In the next set of experiments we wanted to clarify whether the changes in the cytokine pattern were caused by a specific effect of the PDE3 inhibitor or whether rolipram, a commonly used PDE4 inhibitor, would also modulate the in vivo T cell response. Mice were injected with various doses of rolipram before administration of Con A or SEB, respectively, and cytokine release was determined. Like motapizone, rolipram pretreatment resulted in a significant reduction of plasma TNF and IFNγ concentrations, whereas significantly elevated IL-10 levels were found in response to Con A in the circulation of the animals. In contrast to motapizone, however, rolipram also suppressed Con A-induced IL-4 release. Essentially similar rolipram effects were observed in the SEB model, i.e., a dose-dependent inhibition of TNF release and a strongly augmented IL-10 production (fig. 3). In this particular SEB experiment circulating IL-4 concentrations in control animals were relatively low (35 ± 15 pg/ml, i.e., close to the detection limit of our assay, and therefore not shown. However, in PDE inhibitor-pretreated animals, no IL-4 was detectable 2.5 h after GalN/SEB treatment.

These data clearly demonstrate that PDE inhibitors are potent modulators of a T cell activation-induced systemic inflammatory response. In general, a diminished release of proinflammatory cytokines such as TNF and IFNγ was noted. The production of IL-10, which is considered as a typical anti-inflammatory cytokine, was greatly increased.

**Prevention of T cell-dependent hepatic failure by PDE inhibition.** On the basis of the observation that PDE inhibition in vivo strongly suppressed the release of the central mediator for development of hepatic failure, i.e., TNF, we tested the hypothesis that motapizone and rolipram might prevent liver failure after injection of hepatotoxic amounts of Con A or GalN/SEB. Injection of Con A (25 mg/kg i.v.) in naive mice or of SEB (2 mg/kg i.p.) in GalN-sensitized animals (700 mg/kg) evoked fulminant liver failure within 8 h as assessed by highly increased plasma activities of liver-specific enzymes ALT and SDH, which indicated massive liver cell destruction (cf. figs. 4 and 5). Pretreatment of such mice with either motapizone (fig. 4) or rolipram (fig. 5) dose-dependently prevented development of liver failure in both models. Plasma ALT activities were indistinguishable from untreated control values (35 ± 15 U/l) at the highest drug dose used, and the behavior of the animals was normal. It is important to point out that the dose-response relation of either compound with regard to TNF suppression and protection from liver failure was similar. Thus, protection by the PDE3 inhibitor motapizone as well as by rolipram, a PDE4 inhibitor, was most likely caused by suppression of TNF production.

Finally, we asked whether the combined use of motapizone plus rolipram would result in an additive or synergistic action of these drugs. Therefore, we used dosages of both compounds that were not protective when administered alone, i.e., 0.1 mg/kg each (cf. figs. 4 and 5), and looked for the outcome of liver failure after GalN/SEB injection. A 20% reduction of plasma ALT activity was achieved with the PDE4 but not with the PDE3 inhibitor, and all animals survived after motapizone or rolipram pretreatment, whereas a 33% mortality rate was noted in the GalN/SEB control group. However, combined administration of motapizone plus rolipram fully protected mice from liver failure, i.e., a reduction of plasma liver enzyme activity >95% compared with GalN/SEB-treated mice was noted. In analogy, TNF formation was completely abrogated by the combined use of the PDE3 and PDE4 inhibitors, and IL-10 concentrations were doubled in comparison with the effect of each compound alone (table 1).

These data are in line with the recent observation that both putative cytokine-producing cell populations, i.e., murine T cells as well as murine macrophages, express PDE3 and PDE4 isozymes (Schmidt et al., 1995; Prpic et al., 1993). In addition, our results underline PDE3 and PDE4 as potentially interesting therapeutic targets to prevent T cell-mediated inflammatory disorders.
Discussion

This study investigated the pharmacological influence of PDE inhibitors on T cell activation-induced, cytokine-dependent liver injury in mice. We showed in two different murine models of fulminant hepatic failure, i.e., the Con A model and the GalN/SEB model, that motapizone, a PDE3 inhibitor, as well as rolipram, a PDE4-selective inhibitor, shifted the T cell-dependent cytokine response from proinflammatory cytokine production toward increased release of the anti-inflammatory cytokine IL-10. We conclude that this change in cytokine response conferred protection of mice from T cell-dependent liver damage.

There are two principles by which PDE inhibitors could have exerted their protective effects: 1) either by acting on the effector cell level, i.e., on the cell population(s) producing liver-toxic cytokines such as TNF and IFNγ, or 2) by acting on the target cell level, i.e., by interfering with the toxic signal(s) leading to hepatocyte destruction, or 3) by both principles. Indeed, it has been shown that rolipram protects mice not only from GalN/LPS-induced liver injury by reduction of TNF production but also from GalN/TNF-induced hepatic failure (Fischer, et al., 1993). This observation is in accordance with recent in vitro results demonstrating cytoprotection in murine hepatocyte cultures by mixed PDE inhibitors against actinomycin D/TNF exposure (Jilg et al., 1996).

With respect to this study the reduced circulating TNF levels measured in PDE-inhibitor-pretreated animals are sufficient to explain the protective effect of these drugs against T cell-dependent hepatic failure. Protection from Con A might have been potentiated by simultaneous inhibition of IFNγ generation, a lymphokine that has been identified recently as an additional critical mediator in Con A-induced
liver injury (Küsters et al., 1996). Our results are in line with the known capacity of PDE4 inhibitors to suppress LPS-induced TNF production (Zabel et al., 1991; Semmler et al., 1993; Schade and Schudt, 1993; Fischer et al., 1993; Molnar-Kimber et al., 1993; Prabahkar et al., 1994; Seldon et al., 1993; Leist et al., 1996). Most likely, elevated cAMP levels account for the mechanism of action of these drugs. This nucleotide mediator acts via a PKA pathway that finally can result in negative (IL-2) or positive (IL-6, IL-10) regulation of cytokine gene expression via a so-called CRE sequence on the promoter region (Novak and Rothenberg, 1990; Munoz et al., 1990; Dendorfer et al., 1994; Platzer et al., 1995). The mechanism leading to cAMP-dependent inhibition of TNF production is less clear, because a CRE consensus sequence has been identified neither on the murine nor on the human TNF gene promoter enhancer region. Moreover, rolipram failed to influence TNF mRNA expression in murine peritoneal macrophages stimulated with endotoxin (Kambayashi et al., 1995). Alternatively, cAMP-dependent regulation of TNF production at the posttranscriptional level has been suggested (Sever et al., 1992). Recently, an indirect mode of cAMP-regulated cytokine synthesis involving the PKA pathway has been demonstrated for IL-5 (Lee et al., 1993). Another explanation might be a cAMP/PKA-mediated modulation of the activity of nuclear factors. In activated T cells, cAMP was shown to decrease NF-AT induction and that consequently leads to up-regulation of IL-4 and IL-5 (Lacour et al., 1994). Inhibition by cAMP of NF-κB activation, a molecular event necessary for the induction of the TNF gene expression in B and T cells (Goldfeld et al., 1994), could represent an alternate mechanistic explanation of diminished TNF production observed after PDE inhibition.

In addition to a possible NF-κB effect, two more TNF-regulatory principles should be taken into account: first, the reduced TNF plasma levels noted on PDE inhibition could be the consequence of increased TNF receptor shedding after cAMP elevation. Such a mechanism is believed to account for the protective effect of methylxanthines in murine models of septic liver failure (Jilg et al., 1996). Second, recent reports as well as our present study make endogenous IL-10 a likely candidate to contribute to the sequence of events finally resulting in low plasma concentrations of TNF. IL-10 is known to suppress TNF production in a variety of experimental settings and IL-10 neutralization leads to elevation of LPS-induced TNF (Fiorentino et al., 1991; Bogdan et al., 1991; reviewed in Moore et al., 1993). Moreover, it has been shown that endogenously produced IL-10 (Florquin et al., 1994) or exogenously administered rmuIL-10 (Bean et al., 1993) counteract the toxic effects of TNF induced by GalN/SEB challenge of mice. Sequence analysis studies have demonstrated a CRE on both the murine and human IL-10 promoter region (Kim et al., 1992; Platzer et al., 1995), which indicates cAMP regulation of IL-10 synthesis. Indeed, cAMP-elevating drugs such as mixed-type PDE inhibitors or adenyl cyclase stimulants as well as direct administration of dibutyryl cAMP have been reported to increase LPS-induced IL-10 production (Platzer et al., 1995; Jilg et al., 1996; Araoi et al., 1995). Moreover, anti-TNF antibodies have been shown to result in an increase of LPS-induced IL-10 production in mice (Barsig et al., 1995). Regarding the IL-10 plasma kinetics after in vivo T cell activation in comparison with plasma TNF levels (cf. fig. 1), a role of IL-10 in down-regulation of TNF in our T cell-dependent models seems possible.

Finally, the question arises of whether motapizone and rolipram, respectively, at their protective doses used, still affected PDE3 or PDE4 isozymes selectively. On the basis of our present in vivo data, this question remains open. However, the fact that zaprinast, a PDE5-selective compound, did not protect mice from GalN/SEB-induced liver failure (data not shown), makes a critical regulatory contribution of PDE families other than PDE3 and PDE4 to T cell cytokine synthesis rather unlikely. This assumption is further supported by the complete protection from GalN/SEB-initiated liver failure that was observed by the combined use of both compounds (cf. table 1) at doses at which these drugs were ineffective with regard to plasma liver enzyme activities when given alone. Whether motapizone and rolipram in vivo act in an additive or in a synergistic manner is difficult to answer for several reasons. First, although these drugs are selective for PDE3 or PDE4, respectively, they loose their selectivity at higher concentrations and might have affected both isoenzymes, probably even at the dose of 0.1 mg/kg. Second, regarding modulation of circulating cytokines, the combined use of motapizone and rolipram was additive, whereas ALT levels were reduced synergistically. This apparent synergism with regard to protection from liver injury might be the consequence of an additive effect on TNF reduction (cf. table 1).

However, our observations argue in favor of an important role of both isoenzymes for the regulation of T cell-mediated inflammatory processes. Accordingly, T cells mainly express PDE3 and PDE4 isozymes (Tenor et al., 1995) and from several in vivo studies in human (Essayan et al., 1994; Gantner et al., 1995c) as well as murine T cells (Schmidt et

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**TABLE 1**

Effects on GalN/SEB-induced inflammatory responses in mice by combined use of motapizone and rolipram(*)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>ALT *</th>
<th>AST *</th>
<th>SDH *</th>
<th>mVL</th>
<th>TNF *</th>
<th>IL-10 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>5561 ± 2863</td>
<td>3125 ± 1340</td>
<td>5249 ± 2327</td>
<td>4/12</td>
<td>341 ± 131</td>
<td>2730 ± 1122</td>
</tr>
<tr>
<td>Motapizone</td>
<td>6651 ± 3425</td>
<td>2938 ± 2037</td>
<td>4078 ± 1623</td>
<td>0/6</td>
<td>164 ± 88*</td>
<td>4235 ± 1412</td>
</tr>
<tr>
<td>Rolipram</td>
<td>4142 ± 1957</td>
<td>1607 ± 646</td>
<td>1593 ± 588</td>
<td>0/6</td>
<td>156 ± 67*</td>
<td>3978 ± 1011</td>
</tr>
<tr>
<td>Motapizone +rolipram</td>
<td>158 ± 98*</td>
<td>134 ± 23*</td>
<td>80 ± 38*</td>
<td>0/6</td>
<td>&lt;10*</td>
<td>8620 ± 372*</td>
</tr>
</tbody>
</table>

* Data are mean values ± S.D. from two independent experiments performed on separate days with six (disease control) or three mice (all other groups). *P < .05 versus solvent control.
* GalN-sensitized (700 mg/kg i.p.) mice were pretreated with the PDE inhibitor(s) (0.1 mg/kg i.p.) or the corresponding volume of solvent 30 min before injection of SEB (2 mg/kg i.p.).
* Plasma enzyme activities were assessed 8 h after challenge.
* Survival was monitored (n = number of animals that died during the experiment, n = number of animals per group).
* Cytokines were determined 2.5 h after SEB injection in blood samples taken from the tail vein.

Gantner, F., Leist, M., Lohe, A. W., Germain, P. G., and Tegos, G.: Cenonaval-

Gantner, F., Schuch, C., Wendel, A. and Hattemm, A.: Synergistic inhibi-

nation in non-human primates by a cAMP-specific phosphodiesterase inhibi-


Kamath, T., Jacob, C. O., Zhou, D., Mazuere, N., Fong, M. and Strassmann, G.: Cyclic nucleotide phosphodiesterase type IV participates in the regula-


Lehrmann, V., Fiedhenn, M. A. and Galanos, C.: Lethal toxicity of lipopoly-

Leist, M., Auers-Bartels, S. and Wendel, A.: Tumor necrosis factor production in the perfused mouse liver and its pharmacological modulation by methylx-


Munoz, E., Zviragia, A. M., Meerio, S., Sauter, N. P. and Herlin, B. T.: Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-

Nagaki, M., Muto, Y., Ohnishi, H., Yamasu, S., Sano, K., Naito, T., Maida, T., Yamada, T. and Moriwaki, H.: Hepatic injury and lethal shock in galac-


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