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Inhibition of TNF-α Produced by Kupffer Cells Protects Against the Nonspecific Liver Toxicity of Immunotoxin Anti-Tac(Fv)-PE38, LMB-2

Masanori Onda,* Mark Willingham, † Qing-cheng Wang,* Robert J. Kreitman,* Yasuo Tsutsumi,* Satoshi Nagata,* and Ira Pastan†*

LMB-2 (anti-Tac(Fv)-PE38) is a recombinant immunotoxin composed of the Fv fragment of the anti-Tac Ab fused to a 38-kDa form of Pseudomonas exotoxin A. Recent clinical trials showed that LMB-2 is a promising agent for the treatment of patients with Tac-positive leukemia or lymphoma. One major side effect that needs to be overcome is nonspecific liver toxicity. In the current study, we have analyzed the mechanism of this toxicity using a mouse model. Mice that were injected with a lethal dose of LMB-2 showed severe hepatic necrosis. Immunohistochemistry revealed that LMB-2 accumulated in Kupffer cells in the liver, suggesting that the damage to the hepatocytes was indirect. When we examined the effects of LMB-2 on peritoneal macrophages, cells in the same lineage as Kupffer cells, we found that LMB-2 induced the production of TNF-α by these cells. Following LMB-2 administration to mice, the levels of TNF-α in the liver increased to very high levels, whereas the rise in serum levels was modest. In addition, the LMB-2-induced liver toxicity was blocked by a specific TNF binding protein (TNFαRp55). Liver toxicity was also blocked by indomethacin, which also blocked the rise of TNF-α in the liver. Both TNFαRp55 and indomethacin treatment protected mice against a lethal dose of LMB-2. These data indicate that TNF-α produced in the liver by Kupffer cells has an important causal role in the nonspecific liver toxicity of LMB-2. These findings have important clinical implications for the use of immunotoxins in the therapy of patients with cancer. The Journal of Immunology, 2000, 165: 7150–7156.

Anti-Tac(Fv)-PE38 (LMB-2)² is a recombinant immunotoxin that contains the Fv portion of an Ab recognizing the α-subunit of the IL-2 receptor (CD25) fused to a 38-kDa form of Pseudomonas exotoxin A (PE) (1, 2). In a recently completed clinical trial, LMB-2 produced eight major responses in patients with leukemia or lymphoma, including one complete remission lasting more than a year (3). A prominent side effect in this trial that needs to be controlled is liver toxicity (4). Recently, we reported the use of site-directed mutagenesis and molecular modeling to decrease the isoelectric point (pI) of the Fv portion of LMB-2 from 10.21 to 6.82 and found that a mutant of LMB-2 with a pI of 6.82, M1(scFv)-PE38, was more than 3-fold less toxic to mice, yet had the same specific cytotoxic activity and the same antitumor activity as the parental LMB-2 (5). This approach represents one way to diminish side effects of the immunotoxin. Although lowering the pI of the Fv allowed the use of larger amounts of the immunotoxin, liver damage was still the dose limiting toxicity in mice. The current study addresses the mechanism of LMB-2-induced liver toxicity.

PE is a bacterial toxin that binds to many types of mammalian cells. It inhibits protein synthesis by the ADP ribosylation of elongation factor 2 (EF2) (6). PE38, the fragment of PE used to make LMB-2, is missing the cell blinding domain of PE, and therefore should not be toxic to liver cells. Therefore, the liver toxicity of LMB-2 in mice must be caused by the nonspecific intake of LMB-2 by cells in the liver, because the Fv fragment used to make LMB-2 binds to human but not mouse cells.

Bacterial toxins play a central role in septic shock and sometimes cause hepatic damage due to the systemic inflammatory processes. LPS plays a major role in the processes. LPS is known to stimulate monocytes to produce TNF-α, IL-1β, and other proinflammatory cytokines that mediate multiorgan failure and cause cell death in experimental animal models (7). PE has also been shown to induce apoptosis in the liver, and this apoptosis is accelerated in the presence of TNF-α (8). Furthermore, acute hepatotoxicity of native PE is partially mediated by TNF-α (9).

In this study, we show that LMB-2 injected into mice accumulates in Kupffer cells in the liver and that TNF-α produced in the liver plays an important role in LMB-2-induced nonspecific toxicity. Furthermore, the toxicity of LMB-2 is blocked by a specific TNF-α binding protein or by indomethacin, which blocks the rise in TNF-α produced by LMB-2.

Materials and Methods

Immunotoxins and therapeutic reagents

LMB-2 was prepared as described previously (5). LMB-2E553D, an inactive mutant form of LMB-2, was prepared by a similar protocol to LMB2 using plasmid pRK79 M. LMB-2E553D did not induce liver damage and was not toxic to mice when 100 μg/mouse was given by i.v. injection (data not shown). LPS content of all immunotoxins used was <10 Eu/mg and meet FDA criteria for human use. The amount of LPS in 0.9 mg/kg LMB-2 does not induce TNF-α levels in mice (data not shown). The LPS content of immunotoxins was determined using Limulus amebocyte lysate.
(Associates of Cape Cod, Woods Hole, MA). TNF-α-soluble receptor P55 (TNFαR55) and IL-1 receptor antagonist (IL-1Ra) were obtained from R&D Systems (Minneapolis, MN).

Mice

Six- to 8-wk-old female NIH Swiss mice were obtained from the National Cancer Institute (Frederick, MD). Animals received humane care according to National Institutes of Health guidelines under an approved protocol.

Animal treatment

All reagents were injected in a total volume of 200 μl/20 g mouse. LMB-2 (0.9 mg/kg, lethal dose) or LMB-2E553D (0.9 mg/kg) was injected i.v. in pyrogen-free PBS containing 0.2% human serum albumin (HSA) (10). In the experiments blocking the toxic effects of LMB-2, mice were treated as follows: 1) 10 mg/kg i.p. of TNFαR55 in PBS/0.2% HSA 15 min before injection of LMB-2 and 24 h after injection of LMB-2 (11); 2) 10 mg/kg i.p. of IL-1Ra in PBS/0.2% HSA 15 min before injection of LMB-2 and 24 and 48 h after injection of LMB-2 (12); 3) 12.5 mg/kg or 1.25 mg/kg i.p. of indomethacin (Sigma, St. Louis, MO) in 100 μl of olive oil (Sigma) 24 h before injection of LMB-2 and 24 and 48 h after.

Liver homogenate preparation

Mice were anesthetized with methoxyflurane (Scherling-Plough Animal Health, Madison, NJ) and the inferior vena cava severed to remove blood from the organs. Livers were removed from the mice, washed with PBS, and weighed. Livers were homogenized in cold buffer (10 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM PMSF, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin A; Sigma). The homogenates were centrifuged at 13,000 × g for 20 min. The supernatant was used to detect cytokines in the liver.

Analysis of liver enzymes and cytokines

Liver damage was assessed by measuring plasma enzyme activity of alanine aminotransferase (ALT) measured by Analysts (Gaithersburg, MD). The cytokines TNF-α, IL-1β, and IL-6 were determined by ELISA (R&D Systems).

Histological study

NIH Swiss mice were sacrificed 18 h after injection of immunotoxic. The livers and kidneys were fixed with 70% ethanol. Paraffin-embedded sections from each of these organs were stained with hematoxylin and eosin and examined histologically. For immunohistochemistry, the liver and kidneys were frozen in liquid nitrogen and kept at −70°C. Six-micrometer cryostat sections were prepared, postfixed using 10% formaldehyde, then labeled using a primary immunohistochemical step consisting of the presence or absence of an affinity-purified rabbit Ab (2 μg/ml) reactive with PE. This was followed by affinity-purified secondary anti-rabbit IgG conjugated to HRP (25 μg/ml) and developed with diaminobenzidine without counterstain. The Ab incubations were conducted for 30 min (23°C) in the presence of 1% BSA.

Macrophage collection

NIH Swiss mouse peritoneal exudate cells (5 × 10⁶) obtained 4 days following the i.p. injection of 2 ml of 3% thiglycollate medium were plated in 96-well plates (4 × 10⁴ cells/well). Nonadherent cells and nonviable cells were removed by washing with RPMI 1640 (Quality Biological, Gaithersburg, MD) after incubation at 37°C for 1 h. Adherent cells were found to be ~90% macrophages by light microscopy (13). Also, indomethacin-pretreated exudate cells were obtained 4 days following the i.p. injection of 2 ml of 3% thiglycollate medium and 1 day following the i.p. injection of 100 μl of indomethacin in olive oil (2.5 mg/ml).

Cytotoxicity analysis

The cytotoxicity of LMB-2 was assessed on ATAC-4 cells by a protein synthesis inhibition assay (inhibition of incorporation of tritium-labeled leucine into cellular protein) in 96-well plates as previously described (14). The activity of the immunotoxin is defined by the IC50, compared with cells that were not treated with toxin.

Statistical analysis

The results were analyzed using Student’s t test. Survival estimates were calculated using the Kaplan-Meier life table method (15).

Results

LMB-2-induced liver damage

The i.v. injection of 0.9 mg/kg of LMB-2 into NIH Swiss mice induces severe liver damage. Eighteen hours after injection, there is an increase in the level of plasma transaminase (Fig. 1). There are also severe morphological alterations in the liver as determined by histological analysis of the livers of mice sacrificed 18 and 24 h after immunotoxin injection (Fig. 2). At 18 h, hepatocyte necrosis is clearly evident. At the 24-h time point, severe hemorrhage is also evident.

LMB-2 accumulates in the Kupffer cells in liver

To determine which cell types in the liver accumulated LMB-2, mice were sacrificed 1.5 h after injection of a toxic dose of LMB-2 (2 mg/kg). This high dose of LMB-2 was necessary to obtain a detectable signal by immunohistochemistry. Liver and kidney were removed at various times after injection, rapidly frozen, and used for immunohistochemical studies with an anti-PE Ab. In the liver, LMB-2 was detected in cells that had the location and morphology of Kupffer cells (Fig. 3, E, arrows). This pattern was absent in livers from the PBS-injected control mice (Fig. 3F, arrows) and in LMB-2-injected mouse samples in which the anti-PE Ab was omitted (Fig. 3D, arrow). In the kidney, LMB-2 was detected in numerous small resorptive droplets in the apex of proximal tubular epithelial cells (Fig. 3B, arrows). This pattern was absent in both the PBS-injected control mouse kidney stained with anti-PE (Fig. 3C) and in the LMB-2-injected mouse kidney samples in which the anti-PE Ab was omitted (Fig. 3A).

Cytokine levels in the serum after LMB-2 administration

Because LMB-2 was found to accumulate in Kupffer cells, it seemed possible that LMB-2 could have stimulated the production of proinflammatory cytokines that are known to be made by such cells (16). Therefore, we collected blood samples at various time points after LMB-2 injection and measured the levels of TNF-α and IL-1β in the serum. As shown in Table I, there was a small elevation of TNF-α and IL-1β when compared with mice treated with diluent or a nontoxic mutant of LMB-2 (LMB-2E553D). This mutant has undetectable ADP ribosylation activity and does not produce animal toxicity (data not shown). Table I shows the levels of TNF-α or IL-1β. Both LMB-2 and LMB-2E553D produced a rise in IL-1β. However, there was no significant difference in the elevations produced by LMB-2 or its nontoxic form LMB-2E553D.

LMB-2-induced cytokine production in liver

To determine whether the levels of TNF-α or IL-1β were elevated locally within the liver, liver tissue was removed at various times.
after LMB-2 administration and homogenized. After centrifugation to remove particulate matter, the supernatant was used for cytokine analysis (Fig. 4). TNF-α was found to be elevated 8–10 h after administration of LMB-2. The peak level was 609 ± 56 pg/g at 10 h. PBS/0.2% HSA or LMB-2E553D did not cause an elevation of TNF-α levels in the liver (120 ± 55 pg/g at 10 h). IL-1β levels behaved differently from TNF-α levels. IL-1β was elevated 4 h after LMB-2 injection (Fig. 4B, 3942.0 ± 2.0 pg/g) and remained elevated up to the 10-h time point. Although PBS/0.2% HSA did not increase IL-1β, LMB-2E553D did cause a rise in IL-1β at both the 4- and 6-h time points (2088.6 ± 332.2 pg/g). Overall, the effect of LMB-2 on IL-1β was more prolonged and of greater magnitude than that of the nontoxic mutant.

Cytokine production of Kupffer-like cells by stimulation of LMB-2
To assess the ability of cytokine production of Kupffer cells by stimulation of LMB-2, we used peritoneal macrophages from NIH Swiss mice as a substitute for Kupffer cells, because these cells are the same lineage as Kupffer cells. Peritoneal exudate cells were harvested 4 days following the i.p. injection of 3% thioglycollate medium and were plated in 96-well plates. After incubation at 37°C for 1 h, nonadherent cells and nonviable cells were removed by washing with RPMI 1640 medium (see Materials and Methods). Adherent cells were examined by microscopy for morphology and viability. More than 90% of the adherent cells were macrophages, and their viability was >99%. The cells were incubated

FIGURE 2. Histological sections of a liver taken from a mouse 18 or 24 h after injection of LMB-2 (0.9 mg/kg) with or without indomethacin treatment (1.25 or 12.5 mg/kg, i.p.) The control mouse was treated with PBS/0.2% HSA. All sections were stained with hematoxylin and eosin. Magnification, ×200.

FIGURE 3. Immunohistochemical detection of LMB-2 in mouse liver and kidney. Mice were injected i.v. with 2 mg/kg of LMB-2 (A, B, D, and E) or control PBS (C and F) 1 h before sacrifice. Liver (D--F) and kidney (A--C) samples were rapidly frozen, and 6-micron cryostat sections were prepared. These sections were postfixed using formaldehyde, then labeled using a primary immunohistochemical step consisting of the presence (B, E, C, and F) or absence (A and D) (blank control) of an affinity-purified rabbit Ab (2 μg/ml) reactive with PE; this was followed by affinity-purified secondary anti-rabbit IgG conjugated to HRP (25 μg/ml), and dianinobenzidine reaction without counterstain. The Ab incubations were conducted for 30 min (23°C) in the presence of 1% BSA. In kidney, LMB-2 was detected in these brightfield images in numerous small resorptive droplets in the apex of proximal tubular epithelial cells (B, arrows), a pattern that was absent in both the PBS-injected control mouse kidney with anti-PE (C) and in the blank control in the LMB-2-injected mouse kidney (A). In liver, cells consistent in their distribution and shape with Kupffer cells contained LMB-2 (E, arrows), a pattern absent from the PBS-injected control mouse (F, arrows) and in the LMB-2-injected mouse blank control (D, arrow). Magnification, ×590; bar, 17 μm.
with LMB-2, and culture medium was collected at various times for the measurement of TNF-α and IL-1β. Each measurement was done in triplicate. TNF-α was elevated 3 h after ex vivo stimulation by LMB-2 (Fig. 5A). The levels continued to rise and reached a value of 1048 ± 630 pg/ml at the 24-h time point. IL-1β also was increased by LMB-2 incubation but lagged slightly behind TNF-α reaching a level of 206 ± 22.5 pg/ml by 24 h (Fig. 5B).

**TNF-α as a mediator of LMB-2 hepatotoxicity**

Because TNF-α and IL-1β have been identified as mediators of hepatocellular apoptosis and liver damage in experimental mouse models (17, 18), we investigated the possibility that these proinflammatory cytokines could be mediators of LMB-2-induced liver damage. Therefore, mice were pretreated with a specific TNF-α binding protein (TNFsRp55), to inactivate the TNF-α produced following a LMB-2 challenge, or with an IL-1Ra, to antagonize IL-1 action, or with both. As shown in Fig. 6A, TNFsRp55, but not IL-1Ra, significantly inhibited the rise of ALT in the blood. TNFsRp55 combined with IL-1Ra also significantly inhibited ALT release, but the combination was not more effective than TNFsRp55 alone. These data indicate that TNFsRp55 by itself can prevent the rise in ALT in the blood that is known to occur when the hepatocytes are damaged.

The ability of TNFsRp55 to block ALT release was associated with a significant increase in survival of mice treated with 0.9

### Table I. Cytokine levels in the blood in LMB2-treated NIH Swiss mice (n = 3–5)

<table>
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<th>0 h</th>
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<tr>
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<td>4.9 ± 2</td>
<td>7.9 ± 5</td>
<td>67 ± 5</td>
<td>10 ± 6</td>
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<td>LMB2E553D</td>
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<tr>
<td>PBS/HSA</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
<td>Plasma IL-1β (pg/ml)</td>
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<td></td>
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<td></td>
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<tr>
<td>LMB2</td>
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<td>78 ± 30</td>
<td>51 ± 20</td>
<td>53 ± 40</td>
<td>80 ± 80</td>
<td></td>
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<tr>
<td>LMB2E553D</td>
<td>16 ± 6</td>
<td>30 ± 20</td>
<td>110 ± 41</td>
<td>110 ± 40</td>
<td>140 ± 20</td>
<td>100 ± 10</td>
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<td>60 ± 40</td>
<td>31 ± 10</td>
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### FIGURE 4. LMB-2-induced proinflammatory cytokine in liver. NIH Swiss mice received 0.9 mg/kg LMB-2 i.v. or LMB2E553D i.v. Control mice received PBS/0.2% HSA i.v. At the time points indicated, liver was taken and homogenized with protein inhibitors (see Materials and Methods). TNF-α (A) and IL-1β (B) were determined by ELISA. Data are expressed as the mean ± SEM (n = 3–6). *, p < 0.01 vs control mice.

### FIGURE 5. Proinflammatory cytokine production of macrophage by stimulation of LMB-2. NIH Swiss peritoneal exudate cells obtained 4 days following the i.p. injection of 3% thioglycollate medium were plated in 96-well plates and nonadherent cells, and nonviable cells were removed by washing after incubation of 1 h at 37°C. Adherent cells were found to be >90% macrophages. After stimulation with LMB-2 (○), TNF-α (A) and IL-1β (B) in the cultured medium were measured by ELISA. For the control, cells were incubated with PBS/0.2% HSA (●). Data are expressed as the mean ± SD (n = 3).
mg/kg of LMB-2 (Figs. 6 and 7). All the mice that were administered LMB-2 (0.9 mg/kg) died within 4 days (n = 20). In contrast, the survival rate of TNFsRp55-treated mice that received LMB-2 at the same dose was 60% when measured 14 days after administration of LMB-2 (n = 10). LMB-2-treated mice receiving IL-1Ra alone did not have an improved survival rate (0% survival, n = 9), and the survival of mice receiving TNFsRp55 and IL-1Ra (55% survival, n = 9; data not shown) was not greater than when TNFsRp55 was used by itself. These data indicate that one cause of the liver damage produced by LMB-2 is TNF-α and most likely the TNF-α produced locally in the liver.

Lastly, we examined the effect of the anti-inflammatory drug indomethacin on liver damage and mouse toxicity produced by LMB-2, because it was reported that anti-inflammatory drug reduced toxicity of TNF-α (19). To do this, mice were treated with indomethacin (12.5 mg/kg) i.p. 1 day before, on the same day, and 1 day after LMB-2 injection. Indomethacin treatment inhibited the ALT elevation produced by LMB-2 administration in NIH Swiss mice (Fig. 6). The livers from the mice treated with indomethacin 12.5 mg/kg and LMB-2 appeared relatively normal. We also investigated the effect of a lower dose of indomethacin (1.25 mg/kg) and observed partial protection against LMB-2-induced liver damage (Fig. 2). This result indicates that indomethacin protects against LMB-2-induced liver damage in a dose-dependent manner. Indomethacin by itself did not affect the histology of the liver and was not toxic to mice in these doses (data not shown).

We then examined the effect of indomethacin on the rise in TNF-α in the liver produced by LMB-2 and found that indomethacin inhibited the elevation of TNF-α in the liver produced by LMB-2 (Fig. 6). This suggests that by suppressing TNF-α production, indomethacin prevented the liver damage (Fig. 6, A and B) and increased the survival of mice treated with a toxic dose of LMB-2 (Fig. 7). These data about the indomethacin treatment support the conclusion that TNF-α is a mediator of LMB-2 hepatotoxicity.

Indomethacin did not prevent the specific cytotoxicity of immunotoxin on cancer cells

To study whether indomethacin affected the specific cytotoxicity of LMB-2 on cancer cell line, we measured the cytotoxic activity of LMB-2 on ATAC4 cells with and without indomethacin (0.0625 mg/ml). Cytotoxic activity was determined by inhibition of protein synthesis using [3H]leucine. This assay reflects the ability of immunotoxins to kill cancer cells (20). There was no difference in the cytotoxic effect of LMB-2 between indomethacin-treated cells and nontreated cells (IC50 = 0.07 ng/ml) (Fig. 8). Thus, indomethacin, by blocking TNF-α production, might improve the therapeutic index of LMB-2 by decreasing its toxicity without blocking its antitumor effect.

FIGURE 6. TNF-α as a mediator of LMB-2 hepatotoxicity. NIH Swiss mice received 0.9 mg/kg LMB-2 i.v. 15 min after pretreated with IL-1Ra and/or TNFsRp55 i.p. Control animals received PBS/0.2% HSA i.p. instead of TNFsRp55 or IL-1Ra. The indomethacin-treated animals received indomethacin 24 h and 30 min before LMB-2 (0.9 mg/kg i.v.). Eighteen hours after LMB-2 administration, blood was drawn for ALT determination. Data are expressed as the mean ± SEM (n = 5–6) (A). At each time point, livers were taken from the indomethacin-treated mice, and homogenized with protease inhibitors. TNF-α was measured by ELISA (n = 3–6) (B).

FIGURE 7. Administration of TNFsRp55 prevents the deaths of LMB-2 (0.9 mg/kg) treated NIH Swiss mice. A. Mice received IL-1Ra (▲) or TNFsRp55 (■) 24 h and 15 min before 0.9 mg/kg LMB-2. B. The indomethacin treated animals received indomethacin (12.5 mg/kg, ■ 1.25 mg/kg ▲), 24 h and 30 min before LMB-2 and 24 h after LMB-2. For the control, mice received LMB-2 only (●). Survival was monitored daily for 14 days. The results represent a compilation of two or three separate experiments.

FIGURE 8. Specific cytotoxicity of LMB-2 toward ATAC4 cells. Comparison of cytotoxicity of LMB-2 with and without indomethacin.
Discussion

Recently, a Phase I trial with LMB-2 in patients with leukemias or lymphomas was completed. One of the prominent dose limiting toxicities was liver damage. Despite this toxicity, one complete response and seven partial responses were observed (4). The goal of the current study was to determine the basis of this liver toxicity so that strategies to prevent it could be developed. If larger doses of the immunotoxin can be given to patients, more striking clinical responses should occur.

Our previous hypothesis was that hepatocyte toxicity occurred because LMB-2 nonspecifically bound to hepatocytes and that this nonspecific binding was due to the high pl of the Fv portion of LMB-2. However, when we injected LMB-2 into mice and determined its location in the liver by immunohistochemistry, we were surprised to find LMB-2 concentrated in Kupffer cells rather than in hepatocytes. It is possible that some LMB-2 is also taken up by hepatocytes, but the method used for localization was not sensitive enough to detect it. Having demonstrated the presence of LMB-2 in Kupffer cells, we conducted a series of studies focused on the role of Kupffer cells in LMB-2-based hepatotoxicity. Kupffer cells are macrophages and are known to produce cytokines in response to a variety of stimuli, including LPS. Therefore, we measured cytokine levels in the serum of LMB-2-treated mice and found a modest rise in TNF-α, whereas substances like LPS produce large elevations of this cytokine. It was only when we examined the liver directly that substantially elevated levels of TNF-α and IL-1β could be demonstrated implicating one or both of these cytokines in liver damage.

A role for these cytokines in liver damage was supported by experiments with indomethacin, which prevented the rise in cytokines in the liver, the rise in blood ALT, the damage to the liver, and animal deaths produced by LMB-2. To determine which of the cytokines was involved, we used two specific inhibitors of cytokine function: TNFsRp55 and IL-1Ra. When given by itself, TNFsRp55 prevented LMB-2-induced liver damage and animal death, whereas IL-1Ra did not. We conclude that the liver damage and subsequent death produced by LMB-2 is a consequence of TNF-α production by Kupffer cells in the liver. We wished to directly study the ability of LMB-2 to stimulate TNF-α production by Kupffer cells, but were unable to obtain sufficient number of pure cells for this study. Therefore, we used peritoneal macrophages as a substitute for Kupffer cells and found that the macrophages produced TNF-α after stimulation by LMB-2. Because tissue macrophages and peritoneal macrophages are not functionally equivalent, we plan to carry out in situ hybridization experiments in the future to confirm this data. Our observation that TNF-α levels peaked at 8 h, whereas ALT levels rose from 18 to 30 h, suggests that TNF-α produces progressive damage to liver cells that release ALT. It is of interest that LMB-2 also caused a rise in IL-1β, but IL-1β did not appear to have a major role in liver toxicity, because IL-1Ra did not protect against liver toxicity and because IL-1β levels in the liver were also elevated by LMB-2E553D, a nontoxic mutant derivative of LMB-2.

We also tested whether indomethacin prevents the LMB-2-induced hepatic toxicity. Indomethacin treatment inhibited the LMB-2-induced ALT elevation and TNF-α elevation in liver. Indomethacin protects the liver damage produced by LMB-2 by inhibiting TNF-α-mediated pathway. Because indomethacin did not affect the level of TNF-α stimulated by LPS (21), the mechanism of TNF-α production by LMB-2 should be different from the TNF-α production by LPS. We also tested the effect of indomethacin for TNF-α production in peritoneal macrophage cells in vitro. When macrophages were coincubated with LMB-2 (9 μg/ml) and indomethacin (0.125 mg/ml), the rise in cytokine production (TNF-α and IL-1β) was markedly decreased (data not shown). Because indomethacin enhances the LPS-induced TNF-α production (22), the mechanism of production of TNF-α by LMB-2 should be different from LPS.

Previously, indomethacin and other NSAIDs were shown to prevent the toxic effect of immunotoxin BR96(Fv)-PE40 in rats (23, 24). This immunotoxin, which contains a slightly higher m.w. form of PE than LMB-2, is directed against Le2, an Ag present on epithelial tumors. The major toxicity observed in these experiments was accumulation of fluid in the thoracic cavity and vacuolization of endothelial cells in the lungs. No damage to liver was reported in those studies. It is of interest that several nonsteroidal anti-inflammatory drugs may also protect against toxic side effects of immunotoxin in humans. Clinically, we are now evaluating whether an Ab to TNF-α and a cycoxygenase inhibitor will protect patients against the toxic side effects of some immunotoxins.

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