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Renal Excretion of Recombinant Immunotoxins Containing *Pseudomonas* Exotoxin

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Abstract

Recombinant immunotoxins BL22 (CAT-3888) and LMB-2, composed of Fv fragments of anti-CD22 and CD25 MAbs, respectively, have produced major responses in patients with hematologic malignancies, and are also associated with renal toxicity, particularly with BL22. Characterization of the renal excretion of recombinant immunotoxins, which have 2–4 h half-lives in plasma, has not been reported in humans. To study the renal excretion of recombinant immunotoxins, urine from patients treated with BL22 was collected and the recombinant protein visualized after trichloroacetic acid (TCA) precipitation or anion exchange chromatography. BL22 viewed by immunoblot was found in the urine of patients within 8 h after dosing as an intact protein, and progressively degraded to fragments of <20 kDa within 1 day. We studied the stability of BL22 and LMB-2 added to urine at different time points and pH. When exposed to urine *ex vivo*, BL22 time-dependent proteolysis was similar to that observed in treated patients. By N-terminal sequencing, proteolysis was documented at positions 348–249 and 350–351 of BL22, and 339–340 and 341–342 of LMB-2, and other proteolytic sites were observed as well. Our data suggest that BL22 is excreted into the urine in a potentially cytotoxic form, even after its plasma level declines, and may remain intact long enough to cause renal toxicity.

Graphical Abstract

BL22 (CAT-3888) and LMB-2 are recombinant immunotoxins that have been tested in patients with leukemia and lymphoma.^{1–4} BL22 contains an anti-CD22 Fv fragment fused to

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truncated *Pseudomonas* exotoxin A (PE).⁵ LMB-2 is composed of the Fv fragment of the anti-Tac mAb, recognizing the α -subunit of the IL-2 receptor (CD25), fused to the same truncated toxin as BL22.⁶ The truncated toxin in both BL22 and LMB-2 is called PE38 and contains PE amino acids 253–364 and 381–613. Domain III, containing amino acids 395–613, contains the ADP ribosylation activity which catalytically inactivates elongation factor-2 (EF-2). Residues in domain II, amino acids 253–364 and 381–394, contain a proteolytic cleavage site and residues which translocate domain III to the cytosol.⁷ PE38 is missing domain Ia, the binding domain of PE, which binds to normal cells.

LMB-2 induced major responses in a variety of B- and T-cell CD25+ hematologic malignancies, including hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL), Hodgkin's disease (HD), and adult T-cell leukemia (ATL).^{1,2} BL22 had a complete remission rate of 47–61% in purine analogue-resistant HCL and has also shown some activity in CLL.^{3,4,8} The dose-limiting toxicity of BL22 was due to a reversible hemolytic uremic syndrome (HUS) with kidney damage observed in 11% of the phase I patients. LMB-2 did not cause HUS, but did result in mild renal toxicity in some patients.

There is little known about the renal excretion of recombinant immunotoxins. In mice, at 45 and 90 min after an injected dose of LMB-2, 42.1% and 22.3%, respectively, of the injected dose per gram, was found in the kidney, a higher concentration than any other tissue.⁹ However, anti-Tac(Fv)-PE38KDEL, a more toxic version of LMB-2, showed no histologic evidence of renal toxicity even at a dose which caused hepatic toxicity.¹⁰ In humans, the half-lives of LMB-2 and BL22 are 2–4 h in the blood.^{2,4,8} Characterization of urinary excretion of recombinant immunotoxin in humans has not yet been reported. As a first step to better understanding the mechanism of HUS with BL22, we decided to first characterize BL22 and LMB-2 after renal excretion. To determine stability of recombinant immunotoxins in urine, we examined urine from patients treated with BL22 and LMB-2, and also exposed the immunotoxins *ex vivo* to normal urine.

MATERIAL AND METHODS

TCA Precipitation.

Aliquots of 1520 μ L urine were treated with 380 μ L of 60% TCA (final 12% TCA). After a brief vortex, the samples were centrifuged at 17 000 g for 5 min at 4 °C. The supernatant was aspirated and the pellet washed with 1 mL of 6% TCA. The samples were centrifuged, as above, for 2 min and the pellets washed with 1 mL of 93% acetone. The samples were centrifuged again for 2 min and the pellets resuspended in 10 μ L of reducing SDS loading buffer and heated to 100 °C. Before loading the samples on SDS-PAGE, they were centrifuged at 17 000 g for 4 min at room temperature (RT).

Q-Sepharose Purification.

Urine (100 mL) with or without exogenous BL22 1 μ g/mL was diluted 5 $\times$ with Buffer A (20 mM Tris pH 7.4 + 1 mM EDTA) at 4 °C, centrifuged in a 1 L bottle at 6000 g for 10 min at 4 °C, and then filtered over a cellulose acetate 0.2 μ m filter. The filtrate was then applied to

5 mL of Q-Sepharose Fast-Flow anion exchange resin (GE Healthcare, Piscataway, NJ), washed in buffer A, and the protein eluted with buffer A containing 30% (v/v) buffer B (buffer A with NaCl 1 M) in 4 mL fractions. The fractions with peak protein concentration, as assessed by Pierce BCA assay (Thermo Scientific, Rockford, IL), were then assessed by immunoblot.

Immunotoxin Stability in Urine.

To observe the stability of the immunotoxins in the urine, BL22 or LMB-2 was added to patient or healthy donor urine at a final concentration of 1 $\mu\text{g/mL}$, and incubated at 37 °C for 1, 8, and 24 h. The stability was tested at pH 5, 7, and 8. Urine samples were taken before adding immunotoxin, immediately after adding BL22 or LMB-2, and after 1, 8, and 24 h of incubation at 37 °C. The samples were TCA precipitated following the above protocol, loaded on SDS-PAGE 8–16% gradient gels (Invitrogen, Carlsbad, CA), and then detected by Western blot. For the Western blot, the primary antibody was affinity-purified rabbit anti-PE, the secondary antibody was horseradish peroxidase (HRP)-conjugated affinity-purified goat antirabbit immunoglobulin (JacksonImmuno Research, West Grove, PA), and the kit for detection of HRP was the SuperSignal West Pico Chemiluminescent assay (Thermo Scientific).

ADP-Ribosylation Assay.

Wheat germ extract (10 μL) was incubated in 250 μL aliquots containing 50 mM Tris pH 8.0 and EDTA 1 mM, and $\{^{14}\text{C}\}$ -nicotinamide adenine dinucleotide (NAD), with sample added last, as described.¹⁷ BL22 or LMB-2 was incubated in urine (pH 7.0) at 100 $\mu\text{g/mL}$ for 24 h at 37 °C, and complete cleavage verified by SDS-PAGE. 10 μL (1 μg) samples were added to the 240 μL wheat germ and $\{^{14}\text{C}\}$ -NAD mixtures, and incubated for 15 min at 25 °C. The 250 μL aliquots were treated with 1 mL of 12% cold TCA, centrifuged, and the pellets washed twice with 1 mL 6% TCA. The pellets were each dissolved in 0.1 mL of 0.1 N NaOH, transferred to vials, neutralized with 0.5 mL of 0.4 M acetic acid, and finally mixed with scintillation fluid and counted.

RESULTS

The relatively small size (63 kDa) and short half-life (2–4 h) of recombinant immunotoxins suggest their rapid excretion into the urine. If so, their renal toxicity could in part be due to intoxication of the cells lining the collecting system, which could increase the concentration and exposure time of immunotoxin to glomerular endothelium. To characterize the urinary excretion of recombinant immunotoxins, urine from patients receiving immunotoxin was analyzed for the presence of toxin as well as their breakdown products, and stability of BL22 and LMB-2 was tested after exposure to urine.

Immunoblot Analysis of Urine from Patients Treated with BL22.

To determine whether BL22 is excreted into the urine of patients, urine was analyzed before and at different time points after i.v. injection of 30–40 $\mu\text{g/kg}$ of BL22. Figure 1A shows an immunoblot where TCA-precipitated protein, reduced in 2-mercaptoethanol, was analyzed by SDS-PAGE and exposed to polyclonal rabbit anti-PE. The major band 1–4 h after the end

of the 30 min infusion corresponded to VH-PE38 (51 kDa), the reduced form of BL22 missing VL. As expected, the 13 kDa VL fragment was not visualized with the anti-PE polyclonal antibody. A contaminant present in pretreatment urine was visualized at >100 kDa. At 4–7 h after BL22 infusion, a ~17 kDa fragment first appeared and became the dominant band by 7–12 h after infusion. At 20–28 h after infusion, the VH-PE38 band decreased and bands at 17 and 6–8 kDa increased. However, even after 20 h after infusion, a significant percentage of full-length VH-PE38 remained. At 12–20 h, an unidentified band was also observed slightly higher than 51 kDa. Another representative patient is included in Figure 1B, showing the major band at 51 kDa initially at 1–9 h, and then by 9–33 h a decrease in the 51 kDa band combined with an increase in the 17 kDa and smaller fragments. Interestingly, after 33 h, the 17 kDa and smaller kDa fragments were less visible for this patient, indicating further degradation and/or excretion.

Effect of Urine on the Integrity of Recombinant Immunotoxin.

Since degradation of BL22 could occur prior to entering the collecting system, or upon exposure to urine, or both, the composition of BL22 was analyzed after *ex vivo* exposure of BL22 to urine. The calculated isoelectric point (pI) of BL22 is 5.08, based on the full sequence missing the carboxyl terminal lysine residue, which is rapidly removed upon exposure to plasma.¹¹ To determine whether the degradation of BL22 in the presence of urine is related to pH, possibly by inducing conformational changes close to the isoelectric point, the urine tested was adjusted to pH 5, 7, and 8. BL22 was incubated with urine at a final concentration of 1 $\mu\text{g}/\text{mL}$ for 1 h at 37 °C, and the TCA-precipitated protein analyzed by Western blot. As shown in Figure 2A, normal urine at all 3 different pH conditions did not degrade the BL22 to lower molecular weight bands. Urine from the CLL patients produced slight degradation of BL22, producing 2 lower bands of molecular weights which were similar between patients BC01 and CL10. However, as shown by Coomassie-stained gels (Figure 2D–F), even normal urine is able to degrade BL22 after 24 h incubation, particularly at pH 7 and 8. On the Coomassie gel, the reduced VL fragment at 13 kDa is visible as shown in the BL22 control band. Figure 2G–I shows significant degradation of LMB-2 by urine at 24 h, which appeared less at pH 8. Since LMB-2 contains a linker rather than a disulfide bond connecting the VH to VL, no lower band representing a variable domain was expected.

Characterization of Degradation Products of Recombinant Immunotoxins.

To determine which regions of the PE sequence are susceptible to urinary proteases, LMB-2 and BL22 were incubated with urine for 16 h at 37 °C and the products analyzed. As shown in Figure 3, BL22 was cleaved into 3 visible bands on reducing SDS-PAGE. The lowest band (C) co-migrated with the 12 kDa VL band. The upper band (A) was lower in molecular weight than the 35 kDa PE35 protein, indicating that, if the Arg279-Gly280 Furin protease site in BL22 was cleaved outside the cell, producing PE35, at least one additional site within this sequence was also cleaved. Cleavage of the Arg279-Gly280 Furin protease in PE is known to occur with proteases other than furin.¹² As shown in Figure 3, the size of the upper band (A) in cleaved BL22 was similar to upper band (E) in cleaved LMB-2. The size of the lower band (F) in LMB-2 is similar to the size of the 12 kDa VL band C in BL22 plus the slightly larger band B in BL22. Thus, the degradation products in Figure 3 are compatible

with BL22 and LMB-2 each being cleaved once on the C-terminal side of the Arg279-Gly280 Furin cleavage site, with the difference between BL22 and LMB-2 being reduction in BL22 of the engineered disulfide bond linking Cys44 of VH with Cys100 of VL.

Identification of Urinary Protease Sites in Recombinant Immunotoxins.

To determine the exact locations of cleavage in LMB-2 and BL22 by urinary proteases, the bands in BL22 and LMB-2 from Figure 3 were subjected to amino terminal sequencing. The N-terminal sequences are listed in Table 1. Bands B and F contained sequences corresponding to the N-terminus of the VH fragment of reduced BL22 and the N-terminus of LMB-2, respectively. The amino terminal end of the upper band (A) in BL22 indicates that the toxin is cleaved at 2 locations only 2 amino acids apart, Glu348-Arg349 and Phe350-Val351. Surprisingly, in LMB-2 the toxin was also cleaved in 2 locations 2 amino acids apart, but the cleavage sites, Ala339-Leu340 and Thr341-Leu342, were slightly different than those of BL22. Thus, even though the urine sample and toxin sequences were identical, BL22 and LMB-2 were cleaved within the toxin slightly differently.

ADP-Ribosylation Activity of Cleaved Recombinant Immunotoxins.

To determine if the cleaved LMB-2 and BL22 fragments would retain ADP-ribosylation activity, they were incubated as in Figure 2F and I, in urine at pH 7.0 at 100 $\mu\text{g/mL}$ for 24 h at 37 °C, when SDS-PAGE as in Figure 2F,I verified essentially complete cleavage. No cleavage was observed in urine at time 0. As shown in Figure 4, incubation of LMB-2 or BL22 with urine for either 0 or 24 h did not significantly decrease enzymatic activity. Figure 4 shows that 100 ng of LMB-2 alone had 21% less activity than 1 μg of LMB-2 alone and 19% less than completely cleaved LMB-2 after 1 h incubation with urine. Thus, in this semiquantitative assay, cleaved fragments of LMB-2 and BL22 retain 20–100% of their original ADP-ribosylation activity.

DISCUSSION

To study the renal toxicity of recombinant immunotoxins containing truncated PE, anti-CD25 and anti-CD22 recombinant immunotoxins LMB-2 and BL22 were detected and characterized in urine, either after i.v. injection of BL22 in patients or after incubation of LMB-2 or BL22 with urine. We found evidence of proteolysis of both LMB-2 and BL22, which varied with both time and pH. N-terminal analysis showed that incubation with urine resulted in cleavage of LMB-2 at Ala339-Leu340 and Thr341-Leu342, and cleavage of BL22 at Glu348-Arg349 and Phe350-Val351. Incubation with urine did not destroy enzymatic activity of the immunotoxins.

Cleavage Locations within BL22 and LMB-2 for Urine Proteases.

Based on SDS-PAGE (Figure 3) and N-terminal sequencing (Table 1), a model of proteolysis of recombinant immunotoxins by urine is shown in Figure 5, where BL22 and LMB-2 are cleaved prior to Arg349 and Leu340, respectively. Reduction of BL22 is expected to produce a free VL (Figure 5, fragment C), consistent with the ~13 kDa band C in Figure 3. Fragment A if containing amino acids 349–364 and 381–613 would be consistent with band A at 29 kDa. The remainder of VH-PE38 would include VH followed

by the C3 connector (ASGGPE)⁶ followed by amino acids 253–348 of PE, with estimated molecular weight of ~24 kDa. Since band B in Figure 3 is much smaller than 24 kDa, it is possible that BL22 is also cleaved between 253 and 348 such that the carboxyl terminal fragment (Figure 5, fragment D) would be too small to be visible. If the second proteolysis site were the Furin cleavage site between Arg279 and Gly280, fragment B would be ~16 kDa, most consistent with band B in Figure 3. Because of the covalent (G₄S)₃ linker between VH and VL of LMB-2, reduction alone would not result in fragmentation of LMB-2. Proteolytic cleavage of LMB-2 by urine before Leu340 would result in a carboxyl terminal fragment of ~30 kDa, represented by fragment E in Figure 5 and possibly corresponding to the upper band E in Figure 3 lane 5. If LMB-2 were also cleaved at the Arg279-Gly280 Furin site, the amino-terminal fragment F in Figure 5 would contain VH and VL connected by the linker, followed by the C3 connector, and finally followed by PE amino acids 253–279 (~28.5 kDa). In that case, the 7 kDa fragment G in Figure 5 would be too small to see on the gel. Based on their size, fragments A and E, each with known amino-terminal sequence, should contain the ADP-ribosylating activity present in amino acids 395–602.¹³ However, gel analysis could not exclude additional proteolysis just before amino acid 602, which could destroy ADP ribosylation activity. Therefore, urine-cleaved LMB-2 and BL22 were tested directly for ADP ribosylation activity, which was found to be retained (Figure 4). Thus, for either BL22 or LMB-2, the cleavage products in urine leave open the possibility that a fragment with cytotoxic activity may be nonspecifically internalized into renal endothelial cells or cells lining the collecting system, resulting in renal toxicity.

Renal Toxicity with Recombinant Toxins.

Determining the mechanism of renal toxicity from recombinant immunotoxins can be difficult, since vascular leak syndrome can result in hypotension which can cause renal toxicity secondarily by pre-renal azotemia. However, denileukin diftotox was reported to result in proteinuria and microscopic hematuria in 6% of patients and this appeared dose-related,¹⁴ suggesting that for at least some of the 10% of patients reported with an increase in creatinine, renal toxicity was related to direct intoxication of glomerular endothelium or renal tubules by the toxin. In the phase I trial of LMB-2, increased creatinine and proteinuria were observed in 9% of patients, and no cases of HUS were observed.² In the phase II trial of BL22, one cycle resulted in proteinuria in 33% of patients, microscopic hematuria in 19%, and creatinine elevation in 6%.⁸ Including retreatment cycles, 3 (9%) of 36 phase II and 5 (11%) of 46 phase I patients receiving BL22 had HUS.^{4,8} While the mechanism is unknown, functional plasma ADAMTS-13 was detected in all cases, and HUS during phase II resolved completely without plasmapheresis, suggesting that the syndrome may be due to endothelial damage localized to the kidney without a circulating inhibitor requiring removal. Thus, renal toxicity due to nonspecific internalization of ADP-ribosylation activity into the glomerular endothelium or tubular epithelium could possibly predispose or precipitate HUS. It should be noted that internalization of Shiga-like toxin into renal endothelium can cause HUS, and the toxin itself kills cells by ricin-like inhibition of protein synthesis, much like PE.¹⁵ Based on our data, it may be advisable to maintain adequate hydration of patients for at least 24–48 h after a dose of BL22, and alkalization of urine would not be recommended. We believe that additional knowledge of the behavior of recombinant immunotoxins during renal excretion may be important to ensure optimal safety of these potentially useful agents.

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ABBREVIATIONS

ADAMTS-13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13'
ADP	adenosine triphosphate
ATL	adult T-cell leukemia
CLL	chronic lymphocytic leukemia
CTCL	cutaneous T-cell lymphoma
Fv	variable fragment
HD	Hodgkin's disease
HRP	horseradish peroxidase
HUS	hemolytic uremic syndrome
kDa	kilodaltons
mAb	monoclonal antibody
PE	<i>Pseudomonas</i> exotoxin A
RT	room temperature
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
VH	variable heavy
VL	variable light

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Figure 1. BL22 in urine of patients BH31 (A) and BH36 (B). 1.6 mL aliquots of urine from the indicated hours after end of 30 min infusion of BL22 (30 $\mu\text{g}/\text{kg}$) were subjected to TCA precipitation, and 15 μg of total protein/lane was added (except 3.75 μg at 1–4 h in A).

Figure 2.

Degradation of BL22 and LMB-2 by normal urine. Immuno-blots show BL22 incubated at a final concentration of 1 $\mu\text{g}/\text{mL}$ for 1 h with urine at the indicated pH from a normal donor (A), and CLL patients BC01 (B) and CL10 (C). Controls include 2 ng of BL22 and PE35. Coomassie-stained gels in D-F include urine alone at pH 7 (first lane) followed by 2 μg BL22 added to normal urine incubated at 37 $^{\circ}\text{C}$ for the indicated time periods at the indicated pH. LMB-2 was incubated similarly in G-I and the indicated control lanes included LMB-2, BL22, and PE35.

Figure 3.

Characterization of proteolytic products of BL22 and LMB-2 in urine. BL22 or LMB-2 at 200 $\mu\text{g}/\text{mL}$ was incubated with normal urine for 16 h at 37 $^{\circ}\text{C}$ at pH 7, and 2 μg was added to lane 4 or 5, respectively. Control molecules included 1 μg of LMB-2 (63 kDa, lanes 1 and 6), BL22 (51 kDa + 12 kDa, lanes 2 and 7), and PE35 (35 kDa, lanes 3 and 8).

Figure 4. ADP-Ribosylation activity of urine-cleaved BL22 and LMB-2. LMB-2 (white) or BL22 (black) was tested for ADP-ribosylation activity using the indicated amounts in μg . As indicated, LMB-2 and BL22 were each mixed with urine for either 0 or 24 h at 37 °C, and the final 2 lanes (gray) show PBS and urine alone, respectively.

Figure 5. Model for urine protease fragments of BL22 and LMB-2. Fv and toxin fragments are shown in red and blue, respectively. Fragments labeled A–G represent bands on SDS-PAGE (Figure 3) except for bands D and G, which are too small.

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Table 1.

Amino Terminal Sequencing of Degradation Products of BL22 and LMB-2 in Urine

protein	fragment	sequence	location of N-terminus of fragment
BL22	Band A	R F V R Q G T G N D or V R Q G T G N D E A	Amino acid 349 of PE Amino acid 351 of PE
BL22	Band B	M E V Q L V E S G G G	Amino terminus of BL22 VH
LMB-2	Band E	L T L A A A E S E R or L A A A E S E R F V	Amino acid 340 of PE Amino acid 342 of PE
LMB-2	Band F	M Q V H L Q Q S G A E	Amino terminus of LMB-2 VH