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Resolution of Two Overlapping Neutralizing B cell Epitopes within a Solvent Exposed, Immunodominant α -Helix in Ricin Toxin's Enzymatic Subunit

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Abstract

Residues Y₉₁-T₁₁₆ of ricin toxin's enzymatic subunit (RTA) constitute an immunodominant loop-helix-loop motif that is the target of two potent toxin neutralizing monoclonal antibodies (mAbs), PB10 and R70. To define the exact epitope(s) recognized by these mAbs, we affinity enriched from a phage-displayed peptide library 12 mers that bound one or both of these mAbs. We report that PB10 recognizes a distinct but overlapping epitope with R70, in which residues (Q₉₈), E₁₀₂, T₁₀₅, and H₁₀₆ are central to mAb recognition.

Keywords

Toxin; Neutralizing Antibodies; B cell Epitope; Phage Display

Ricin toxin is a member of the RNA N-glycosidase family of ribosome-inactivating proteins (RIPs) that includes abrin and Shiga toxins. Immune-based countermeasures against ricin are focused on the toxin's enzymatic subunit (RTA), a 267 amino acid glycoprotein that consists of three distinct folding domains (O'Hara et al., 2010; O'Hara et al., 2012; Rutenber et al., 1991). Folding domain I (residues 1–117) is dominated by five stranded β sheets, but terminates in a solvent exposed, loop-helix-loop motif that spans residues Y₉₁-T₁₁₆. The loop-helix-loop motif of RTA is conserved among almost all known RIPs, although its function in RNA N-glycosidase activity remains unclear (Lebeda and Olson, 1999; O'Hara et al., 2012). Nonetheless, we now know that this loop-helix-loop motif is the target of several potent ricin-neutralizing mouse monoclonal antibodies (mAb) (O'Hara et al., 2012). The first murine mAb shown to recognize this region of RTA is UNIVAX 70, herein referred to as "R70" (Lebeda and Olson, 1999; Lemley et al., 1994). R70, administered passively by intraperitoneal or intravenous injection, is sufficient to protect mice against systemic and mucosal ricin challenge (Lemley et al., 1994; Neal et al., 2010). While the R70's exact epitope is not known, pepscan analysis indicated that the mAb is capable of

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recognizing a 12-mer peptide (NQEDAEATHLF) corresponding to residues N₉₇-F₁₀₈ of RTA (Aboud-Pirak et al., 1993; Neal et al., 2010; O'Hara et al., 2010). In 2010, we described a second murine mAb, known as PB10, that binds the same peptide (NQEDAEATHLF) and that is as effective as R70 at neutralizing ricin *in vitro* and *in vivo*, even though its affinity for ricin is slightly lower than R70's (O'Hara et al., 2010). A third "R70-like" neutralizing murine mAb, WB2, has recently been isolated and partially characterized in our laboratory (J. O'Hara, D. Vance, and N. Mantis, unpublished results).

Immune profiling studies conducted in our laboratory aimed at defining the nature of the neutralizing antibody responses elicited by a candidate RTA-based subunit vaccine suggest that residues N₉₇-F₁₀₈ constitute one of the most immunodominant regions of RTA ((O'Hara et al., 2010); J. O'Hara, R. Brey and N. Mantis, manuscript in preparation). For this reason we wished to define in high-resolution the epitope(s) recognized by the mAbs R70, PB10, and WB2. To take an unbiased approach to epitope identification, we screened the PhD-12 phage displayed peptide library (New England Biolabs (NEB), Beverly, MA) for phage that bound PB10, R70 and/or WB2 (each at 2 µg/ml) immobilized in NUNC Maxisorb Immuntubes (Krackeler Scientific, Albany, NY). Phage were allowed to bind for 60 min, washed and then mock eluted with 50 µg/ml of an isotype control antibody (MOPC-21; Sigma-Aldrich, Co., St. Louis, MO) specific to phosphocholine. Phages that bound to R70-, PB10- and WB2-coated tubes were eluted with 20 µg/mL soluble R70, PB10 and WB2, respectively. The resulting eluates were amplified in *Escherichia coli* strain ER2738, as recommended by NEB. This process, known as panning, was repeated two additional times, after which unamplified eluted phage were plated on LB agar plates. Individual plaques were picked, amplified and tested for the ability to bind R70, PB10 and/or WB2 by ELISA. Phage DNA was isolated from all clones using the QIAprep Spin M13 Kit (Qiagen, Valencia, CA) and then submitted for sequence analysis.

A total of 44 phage from the PB10 affinity enrichment were subjected to DNA sequencing. The deduced phage-encoded peptides revealed 10 unique sequences that, when aligned, clustered into two "consensus" groups, QExLG and QxHxExLTH (Table 1; Fig. 1). Six different phages (#13-1,-2,-3,-5,-8,-23) displayed the consensus QExLG, while two additional phages displayed QExxG (#13-7) and QxxxG (#13-29). Clones #13-7 and -29 also contained hydrophobic residues immediately upstream of the C-terminal glycine, alanine and methionine respectively, matching the hydrophobic leucine of the QExLG sequence. Furthermore, the center residues in 6/8 peptides contained a ringed structure (e.g., H, W, Y). Although represented by only two phages (#13-4,-36), the second consensus sequence, QxHxExLTH, aligned closely with residues of RTA's loop-helix-loop motif (Table 1).

The phage displayed peptide library was also subjected to three rounds of panning using R70 or WB2 as bait. We sequenced 24 clones from each screen, identifying four different peptide sequences (#14-1,-2,-5,-8) from the R70 screen, and eight different peptide sequences (#18-1,-3,-4,-6,-12,-14,-22,-24) from the WB2 screen (Table 1; Fig. 1). Remarkably, two sequences were common to both screens: VNQQLHAEALTH, represented by phages #14-1 and 18-3, and SEQEMMETKTHH, represented by phages 14-6 and 18-2. Even more remarkable is the fact that the former sequence was also isolated from the library by panning with PB10, as reflected by phage 13-4.

Alignment of the ten unique sequences from the R70 and WB2 screens revealed a consensus peptide consisting of an N-terminal ExxTH motif, arguing that the ExxTH residues are essential for R70/WB2 recognition. Moreover, eight of the ten peptides had a Q situated four residues proximal to the ExxTH motif; the remaining two (#14-8, 18-22) had an E (a

conservative substitution) in this position. Five of the ten peptides had a negatively charged residue situated three residues proximal to the ExxTH motif.

Alignment of the peptide sequences from the panning experiments against PB10 (13-4,-36), R70 (#14-1,-2,-5,-8) and WB2 (#18-1,-3,-4,-6,-12,-14,-22,-24) further argues that the common motif recognized by all three mAbs is ExxTH, with Q four residues proximal, and a negative charge three residues proximal (Table 1). When compared to the sequence of RTA, it is clear that the core epitope common to all three mAbs consists of residues Q₉₈, E₉₉, E₁₀₂, T₁₀₅, and H₁₀₆. These residues constitute the majority of the surface exposed area in the loop-helix-loop motif of RTA that spans residues Y₉₁-T₁₁₆ (Fig. 2). It is striking that the spatial representation of residues QExxExxTH was conserved in all of the peptides identified in all three panning experiments (excepting clones displaying the QExLG motif). Recent work by Dai et al. further validates Q₉₈, E₁₀₂, T₁₀₅, H₁₀₆ as being the core residues recognized by another ricin neutralizing monoclonal antibody against this loop-helix-loop region (Dai et al., 2011).

We next examined the ability of select phages (#13-1,13-4,14-2,14-8) representing consensus motifs identified from the three different panning experiments to be cross-recognized by PB10, R70 and WB2 in an ELISA format (Table 1; Fig. 1). Clone #13-4 bound R70, WB2 and PB10 equally well, which was not surprising because this phage was isolated in all three panning studies. Phage 14-2 (SEQEMMETHTH) also bound all three mAbs, while phage 14-8 (LHLEEWSELNTH) bound R70, WB2, but not PB10, demonstrating that PB10 recognizes slightly a different epitope than does R70 or WB2. We postulate that the failure of PB10 to recognize phage 14-8 is likely due to the Q to E substitution located four residues proximal to the ExxTH motif. While this hypothesis was not tested directly, the fact that PB10 also failed to recognize phage 18-22 (NEERWEAQTHHR), which carries that same relative Q to E substitution as phage 14-8, supports the model that the Q₉₈ on RTA is critical for PB10 recognition. R70 and WB2, on the other hand, tolerate a substitution at position 98, as long as it is conserved.

Based on these data, we propose that PB10 anchors itself through hydrogen bonding with Q₉₈, while R70/WB2 likely associate with Q₉₈ and E₉₉ on a basis their hydrophilic nature. What remains perplexing, however, is the significance of PB10's ability to recognize QExLG, which was by far the predominant motif isolated from the phage displayed library as evidenced by the fact that it was displayed by eight different phage and represented by 33/44 isolated clones (Table 1; Figure 1). This motif does not align with the linear sequence of RTA, nor was it recognized by R70 or WB2. It is possible, therefore, that QExLG mimics the tertiary arrangement of residues within and adjacent to the loop-helix-loop motif of RTA that spans Y₉₁-T₁₁₆. Specifically, residues V₄₉, G₅₀, and L₅₁ are spatially in close proximity to Q₉₈ and E₉₉ (Fig. 2). Thus, the motif QExLG could correspond to Q₉₈, E₉₉, V₄₉ or L₅₁, and G₅₀. It is very likely that the N-terminal Q residue is critical for PB10 recognition.

Based on these data, we speculate that PB10 may have a larger footprint on RTA than does R70 or WB2. For example, PB10 and R70/WB2 may share a common heavy chain sequence (or at least key residues within the CDR3) but differ in their light chain specificities, only one of which is able to recognize the extended surface area of V₄₉-L₅₁. The estimated surface area of the N₉₇-F₁₀₈ helix, which is recognized by all three mAbs, is ~180 Å². Amino acids V₄₉-L₅₁ represent another ~25 Å² of surface area, bringing the potential PB10 recognition surface area to ~205 Å². On average, an antibody's binding interface on a protein antigen encompasses 850 Å² (Lo Conte et al., 1999); thus this extra surface area is easily within PB10's "reach". Interestingly, R70 has a ~10-fold higher affinity for RTA than PB10, with K_D values of 3.2×10⁻⁹ and 4.0×10⁻⁸, respectively (O'Hara et al., 2010). Thus, it remains to

be determined whether PB10 can contact this entire surface area when bound to RTA, or whether there are two distinct epitopes with separate PB10 binding orientations.

In summary, we have successfully resolved using a phage-displayed peptide library approach the key residues within RTA's loop-helix-loop motif (Y₉₁-T₁₁₆) that are recognized by three potent toxin-neutralizing mAbs, WB2, R70 and PB10. This information has important implications for the design of immunotherapeutics and vaccines for ricin as well as other ribosome inactivating proteins. For example, the need to preserve residues Q₉₈, E₉₉, E₁₀₂, T₁₀₅, H₁₀₆, as well as the overall tertiary structure of the regions surrounding these residues on the surface of RTA, must be taken into consideration when engineering novel RTA-based vaccine antigens (Compton et al., 2011; O'Hara et al., 2012). Moreover, as mentioned above, Lebeda and colleagues have reported that the loop-helix-loop motif on RTA recognized by R70 is in fact conserved within the larger family of RIPs, including abrin and even Shiga toxin (Lebeda and Olson, 1999). It will be interesting to determine whether antibodies directed against the loop-helix-loop motif regions of other RIPs like abrin and Shiga toxins are in fact neutralizing and/or protective. It should be noted that at the present time, it is not clear why the association of an antibody within RTA's loop-helix-loop motif renders ricin non-toxic. Perhaps the information from this study will provide molecular insight as to the mechanism(s) by which PB10, R70 and related antibodies neutralize ricin.

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Figure 1. Differential phage-displayed peptide binding to PB10 and R70

The ability of specific phages to bind to plate immobilized R70 (black bars), PB10 (dark gray bars), MOPC21 (isotype control, light gray bars), or BSA (white bars) was tested by ELISA. NUNC 96 well plates were coated with indicated antigens (10 mg/ml), blocked with BSA, and then probed with the indicated phage. Following a 1h incubation, plates were washed and then probed with HRP-labeled anti-M13 antibodies (NEB) and developed using SureBlue TMB peroxidase substrate (KPL, Gaithersburg, MD). Phages 13-4 and 14-2 bound both PB10 and R70. Phage 13-1 bound PB10 but not R70, whereas 14-8 bound R70 but not PB10. Phage 14-7 served as a negative control.

Figure 2. Refined model of the epitopes on RTA recognized by R70 and PB10

Residues Q₉₈, E₉₉, E₁₀₂, T₁₀₅, and H₁₀₆ dominate the solvent exposed surface area of this region of RTA, and likely constitute the core residues recognized by PB10 and R70 (as well as WB2). Residues V₄₉, G₅₀ and L₅₁ may also be important in PB10 recognition. Figure was generated using PDB sequence 2AAI (Rutenber et al., 1991) and PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC.). Annotations were done using Adobe Photoshop CS5 (Adobe, San Jose, CA).

Table 1

Peptides that bind PB10, R70 and/or WB2 identified from a phage-displayed library

Phage ID	Peptide Sequences												Frequency	Reactivity ^a					
														PB10	R70	WB2			
-PB10 ^b																			
13-1			A	T	M	Q	E	R	L	G	M	A	L	H	9/44	+	-	-	
13-2		K	P	D	I	Q	E	H	L	G	M	G	P		5/44	+	-	-	
13-3			V	S	L	Q	E	V	L	G	N	P	H	A	1/44	+	-	-	
13-5				K	L	Q	E	W	L	G	T	R	Y	P	E	1/44	+	-	-
13-8		H	W	N	V	Q	E	H	L	G	K	P	S		13/44	+	-	-	
13-23	S	P	L	L	F	F	Q	E	Y	L	G	S			1/44	+	-	-	
13-7			W	S	Q	Q	E	H	A	G	M	P	T	P	2/44	+	-	-	
13-29		E	W	S	L	Q		Y	H	M	G	L	Y	H	1/44	+	-	-	
13-4		V	N	Q	Q	L	H	A	E	A	L	T	H		5/44	+	+	+	
13-36			I	T	Q		H	S	E	R	L	T	H	R	3/44	+	+	+	
-R70 ^b																			
14-1		V	N	Q	Q	L	H	A	E	A	L	T	H		10/24	+	+	+	
14-5			T	T	Q	D	T	V	E	A	L	T	H	I	3/24	+	+	+	
14-2			S	E	Q	E	M	M	E	T	K	T	H	H	9/24	+	+	+	
14-8		L	H	L	E	E	W	S	E	L	N	T	H		1/24	-	+	+	
-WB2 ^b																			
18-6		S	E	Q	E	M	M	E	T	K	T	H	H		4/24	+	+	+	
18-4	Q	M	S	Q	E	L	F	E	A	E	T	H			3/24	+	+	+	
18-12			D	Q	L	W	F	E	R	L	T	H	Q	Q	4/24	+	+	+	
18-14		D	T	Q	L	G	R	E	L	L	T	H	R		1/24	+	+	+	
18-3	V	N	Q	Q	L	H	A	E	A	L	T	H			5/24	+	+	+	
18-1		S	D	Q	H	V	L	E	L	L	T	H	L		5/24	+	+	+	
18-24		N	Y	Q	F	L	L	E	L	M	T	H	E		1/24	+	+	+	
18-22			N	E	E	R	W	E	A	Q	T	H	H	R	1/24	-	+	+	
-Consensus																			
14-5		T	T	Q	D	T	V	E	A	L	T	H	I			+	+	+	
13-36		I	T	Q	D	H	S	E	R	L	T	H	R			+	+	+	
14-2/18-6		S	E	Q	E	M	M	E	T	K	T	H	H			+	+	+	
18-4	Q	M	S	Q	E	L	F	E	A	E	T	H				+	+	+	
13-4/14-1/18-3	V	N	Q	Q	L	H	A	E	A	L	T	H				+	+	+	
18-12			D	Q	L	W	F	E	R	L	T	H	Q	Q		+	+	+	
18-14		D	T	Q	L	G	R	E	L	L	T	H	R			+	+	+	
18-1		S	D	Q	H	V	L	E	L	L	T	H	L			+	+	+	
18-24		N	Y	Q	F	L	L	E	L	M	T	H	E			+	+	+	
14-8	L	H	L	E	E	W	S	E	L	N	T	H				-	+	+	
18-22			N	E	E	R	W	E	A	Q	T	H	H	R		-	+	+	
RTA ^c																			
Residue		N	Q	E	D	A	E	A	I	T	H	L	F						
		97	98	99	100	101	102	103	104	105	106	107	108						

^aReactivity of phage with mAb PB10, R70 or WB2 by ELISA; ^bmAbs used as "bait" for affinity panning with the phage displayed peptide library; ^camino acid sequence of RTA with corresponding residue numbers in line below.