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ACTIVE ANTITOXIC IMMUNIZATION AGAINST RICIN USING SYNTHETIC PEPTIDES

Final Report

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Over the project period, 22 ricin peptides (14 from ricin A-chain and 8 from ricin B-chain sequence) have been synthesized. Peptides were conjugated to BSA or KLH, and free peptides as well as conjugates were used to immunize mice. Sera were tested by ELISA for their binding to corresponding immobilized peptides, corresponding ricin subunits, and intact ricin. Five of the ricin A-chain peptides showed significant binding to antiricin A serum. Additional peptides with overlapping sequences were synthesized and tested as above. Challenge studies were performed on peptides eliciting ricin-binding antibodies, however, none protected against ricin challenge. Longer peptides covering the loop regions, used along with T-cell epitopes, may provide protection.

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CONTENTS

TABLES

INTRODUCTION

Statement of Problem

Many different plants contain cytotoxic proteins, which are among the most poisonous compounds known. The best known of these toxins is ricin, isolated from the seeds of the castor plant Ricinus communis. Because of the high toxicity of ricin, ingestion of only a few seeds can be fatal if the seed coat is broken. Extracts from castor beans have been used since ancient times for criminal purposes and biochemical warfare. No antidote for ricin poisoning is known, and no truly effective treatment exists for patients who have ingested ricin. The objective of this research is to investigate the potential of synthetic peptides derived from ricin A- and B-chain sequences to serve as immunogens for the production of protective "antitoxic" antibodies.

Background and Rationale

Ricin, a plant toxin, consists of two peptide chains, A and B, linked together by a disulfide bond. The B-chain binds the toxin to receptors on the cell surface, and the A-chain enters the cytoplasm and inactivates the 60 S ribosomal subunits, causing disruption of protein synthesis and cell death. Montfort et al. (1987) determined the three-dimensional structure of ricin and observed a reasonably prominent cleft in the A-chain enzyme assumed to be the active site.

Sera produced against a toxoid of ricin effectively protect animals and cells in culture against intoxication with the ricin toxin. Antibodies directed against the A- and B-chains have been found to be equally efficient in preventing the effect of the toxin on animals and on cells in culture. It has also been demonstrated that anti-B-chain antibodies efficiently inhibit the binding of the B-chain to cell surfaces and that anti-A-chain antibodies prevent the enzymatic activity of the A-chain on ribosomes in a cell-free system. In experiments performed by Godab et al. (1983) and Fodstad et al. (1984), the presence of circulating immune complex was clearly demonstrated in mice, but no indications of side effects attributable to immune complexes were observed in mice or humans (cancer patients). Also, no significant level of specific IgE was found in patients' sera, suggesting that the probability of anaphylactic reactions is minimal.

So far, no work on determining the antigenic sites and mapping the epitopes on ricin chains has appeared in print.

EXPERIMENTAL METHODS

Peptide Synthesis

Peptides from ricin A- and B-chain sequences were synthesized. To predict antigenic sites, hydrophilicity and β-turns were considered. Hydrophilicity plots and secondary structural predictions are shown in SRI's original proposal. Peptides that were synthesized, along with their sequences, are listed in Tables 1 and 2. From A-chain, peptides with overlapping sequences were also synthesized (Table 3). The peptides were synthesized on a Beckman 990C Automated Peptide Synthesizer using t-BOC chemistry. Crude peptides were purified using high pressure liquid chromatography (HPLC). Purity of the peptides was checked by analytical HPLC and amino acid analysis. The final purified peptides were obtained in 20 to 40% yield in >95% purity by HPLC. Amino acid analysis of all peptides agreed within ±10% of the theoretical values.

For immunization, peptides were conjugated to BSA or KLH. The extent of conjugation, calculated from the increase in weight of the conjugate, was from 20 to 35 molecules of peptide per molecule of BSA or KLH.

Table 1

AMINO ACID SEQUENCES
OF THE PEPTIDES SYNTHESIZED FROM A-CHAIN

No.	Sequence
1.	1 I F P K Q Y P I I N F T 12
2.	17 T V Q S Y T N F I R A V R G R 31
3.	32 L T T G A D V R H E I P V L P N R V 49
4.	50 G L P I N Q R F I L V E L Q N H A E 67
5.	84 Y R A G N S A Y F F H P D N Q E D 100
6.	102 E A I T H L F T D V Q N R Y T F A 118
7.	119 F G G N Y D R L E Q L A G N L R E N 136
8.	137 I E L G N G P L E E A I S A L Y 152
9.	154 Y S T G G T Q L P T L A R 166
10.	175 I S E A A R F Q Y I E G E M R T R 191
11.	192 I R Y N R R S A P D P S V I T L 207
12.	208 E N S W G R L S T A I Q E S N Q G A F 226
13.	227 A S P I Q L Q R D G S K E S V Y D 243
14.	254 V Y R C A P P P S S Q F 265

Note: Underlined regions predict the probable location of reverse or β-turns.

Table 2

AMINO ACID SEQUENCES
OF THE PEPTIDES SYNTHESIZED FROM B-CHAIN

No.	Sequence
1.	20 C V N V R D G R F N H G N 33 A
2.	40 K S N T D A N Q L T L K R D N T I 57 R
3.	86 A D R E I W N N G T I I N P R 101 S
4.	163 C S E K A E Q Q W A L 174 Y
5.	175 A S G N I N P Q Q R R D 187 N
6.	188 C L T S D S N I R E T V V 201 K
7.	206 G P A S S G E R W M F K N D G T 222 I
8.	245 L Y P L W G H D P N 255 Q

Note: Underlined regions predict the probable location of reverse or β-turns.

Table 3

AMINO ACID SEQUENCES OF THE OVERLAPPING PEPTIDES FROM RICIN A-CHAIN

Peptide	Sequence
Candidate peptide	^{17}T V Q S Y T N F I R A V R G ^{31}R
Overlapping peptide	^{13}T A G A T V Q S Y T N F I R ^{27}A
Overlapping peptide	^{21}Y T N F I R A V R G R L T T ^{35}G
Candidate peptide	^{84}Y R A G N S A Y F F H P D N Q E ^{100}D
Overlapping peptide	^{80}Y V V G Y R A G N S A Y F F H P ^{96}D
Overlapping peptide	^{88}N S A Y F F H P D N Q E D A E A ^{104}I
Candidate peptide	^{102}E A I T H L F T D V Q N R Y T F ^{118}A
Overlapping peptide	^{98}Q E D A E A I T H L F T D V Q N ^{114}R
Overlapping peptide	^{106}H L F T D V Q N R Y T F A F G G ^{122}N
Candidate peptide	^{175}I S E A A R F Q Y I E G E M R T ^{191}R
Overlapping peptide	^{170}I C I Q M I S E A A ^{180}R
Overlapping peptide	^{178}A A R F Q Y I E G E ^{188}M
Overlapping peptide	^{186}G E M R T R I R Y N ^{196}R
Candidate peptide	^{227}A S P I Q L Q R D G S K E S V Y ^{243}D
Overlapping peptide	^{221}S N Q G A F A S P I Q ^{232}L
Overlapping peptide	^{230}I Q L Q R D G S K F ^{240}S
Overlapping peptide	^{238}K F S V Y D V S I L L ^{249}P

Production of Anti-ricin Antisera

First, ricin toxoid was prepared by inactivating ricin with 10% formalin. After extensive dialysis, mice were immunized with this toxoid. These mice produced antibodies that bound to ricin A-chain, ricin B-chain, and ricin. Mice were also immunized with ricin A-chain, and these mice produced antibodies that bound to both ricin A-chain and ricin, as determined by immunodiffusion. Similarly, mice immunized with ricin B-chain produced antibodies that bound to both ricin B-chain and ricin.

Production of Anti-peptide Antisera

Mice were immunized with 10 or 100 µg of each immunogen (peptide, peptide-carrier conjugate, ricin subunits, or ricin) emulsified in complete Freund's adjuvant. Following primary immunization, mice were given one to three booster immunizations, as appropriate, to elicit high-titer antiserum. Control sera and sera from the immunized mice were tested in a solid-phase immunoassay (ELISA) to determine binding activity to the immunizing peptide, the corresponding ricin subunit, and intact ricin.

Determination of LD_{100} of Ricin

We conducted five experiments to determine LD_{100} of ricin. In two experiments, untreated mice were injected with various doses of ricin intraperitoneally (ip) and in three experiments, intravenously (iv). From the number of surviving mice in each group, the LD_{100} was calculated to be 350 ng when injected ip and 200 ng when injected iv.

Protection Studies

In these experiments, mice were immunized with 100 µg of peptide in complete Freund's adjuvant (CFA) on Day 0, then boosted with an equal dose of peptide in incomplete Freund's adjuvant (IFA) on Days 21 and 35. Control animals were consistently boosted with PBS in IFA at the time the other animals in the experiment were receiving booster injections of peptide in IFA. Mice were then challenged intravenously with 200 ng of ricin and the survival of the mice was monitored.

RESULTS AND DISCUSSION

Results of binding assays on ricin A- and B-chain peptides are summarized in Tables 4 and 5. Antibody titers of these peptides are shown in Tables 6 and 7.

As can be seen from the antibody titers, eight A-chain peptides, 17-31, 17-31-BSA, 32-49, 32-49-BSA, 84-100-BSA, 1[illegible]91-BSA, 186-196, and 186-196-BSA, elicited anti-peptide, anti-ricin-A, as well as anti-ricin antibodies whereas peptides 1-12, 50-67, 154-166, 175-199, 227-243-BSA, 254-265 and 254-265-KLH elicited only antipeptide and antiricin antibodies. However, none of these peptides protected mice against a lethal dose of ricin (Table 8). This lack of protection may be due to the relatively low titer of antibody (10-350, compared with 500,000 for toxoid-immunized mice). Immunogenicity of these peptides may be enhanced by developing suitable carriers and adjuvants and by synthesizing longer or cyclic peptides.

Among the B-chain peptides, most of the BSA conjugates exhibited significant serum antibody titer against peptides, but none had any titer against ricin B-chain or intact ricin.

Table 4

SUMMARY OF BIOLOGICAL RESULTS FOR A-CHAIN PEPTIDES

Peptide	Binding of Antipeptide Antisera to			Protection Against Challenge
	Peptide	Ricin A	Ricin	
1-12	+	-	+	-
1-12-BSA	+	-	-	nt
17-31	+	+	+	nt
17-31-BSA	+	+	+	-
32-49	+	+	+	-
32-49-BSA	+	+	+	-
50-67	+	-	-	nt
84-100	+	-	+	nt
84-100-BSA	+	+	+	-
102-118	-	-	nt	nt
102-118-BSA	-	+	+	-
119-136	+	-	-	nt
119-136-BSA	-	-	-	nt
137-152	-	-	-	nt
137-152-BSA	+	+	-	nt
154-166	+	-	+	-
154-166-BSA	+	+	-	nt
170-180	-	-	+	nt
170-180-BSA	-	-	-	nt
175-191	+	-	+	-
175-191-BSA	+	+	+	nt
178-188	-	-	-	nt
178-188-BSA	-	-	-	nt
186-196	+	+	+	-
186-196-BSA	+	+	+	-
192-207	+	-	+	nt
208-226	-	-	-	nt
221-232	+	-	-	nt
221-232-BSA	+	-	-	nt
227-243	+	-	-	nt
227-243-BSA	+	-	+	-
230-240	-	-	-	nt
230-240-BSA	+	-	-	nt
238-249	+	-	-	nt
238-249-BSA	+	-	-	nt
254-265	+	-	+	nt
254-265-KLH	+	-	+	nt

nt = not tested

\+ = anti-peptide serum dilution yielding an optical density equal to that of normal mouse serum plus 2 standard deviation intervals.

Table 5

SUMMARY OF BIOLOGICAL RESULTS FOR B-CHAIN PEPTIDES

	Binding of Antipeptide Antisera to		
Peptide	Peptide	Ricin B	Ricin
20-33	-	-	+
20-33-KLH	+	-	-
40-57	-	-	-
40-57-BSA	+	-	-
86-101	-	-	-
86-101-BSA	+	-	-
163-174	-	-	-
163-174-BSA	-	-	-
175-187	-	-	-
175-187-BSA	+	-	-
188-201	+	-	-
188-201-BSA	-	-	-
206-222	-	-	-
206-222-BSA	+	-	-
245-255	-	-	-
245-255-BSA	+	+	-

+ = anti-peptide serum dilution yielding an optical density equal to that of normal mouse serum plus 2 standard deviation intervals.

Table 6

BINDING OF SERUM FROM MICE IMMUNIZED WITH
RICIN A-CHAIN PEPTIDES TO THE PEPTIDE, RICIN A, AND RICIN

	Serum Antibody Titer Against:		
Immunogen	Peptide	Ricin A	Ricin
1-12	100	0	70
1-12-BSA	100	0	0
17-31	300	25,000	50
17-31-BSA	300	20,000	250
32-49	10,000	1,000	200
32-49-BSA	10,000	1,000	200
50-67	90	0	100
50-67-BSA	nd	nd	nd
84-100	10	0	100
84-100-BSA	1,000	300	100
102-118	0	0	100
102-118-BSA	0	100	40
119-136	30	0	0
119-136-BSA	0	0	0
137-152	0	0	0
137-152-BSA	500	100	0
154-166	500	0	100
154-166-BSA	500	100	0
170-178	0	0	100
170-178-BSA	0	0	200
175-191	500	0	40
175-191-BSA	10	1,000	10
178-188	0	0	50
178-188-BSA	0	0	200
186-196	100	50	100
186-196-BSA	700	500	350
192-207	10	0	200
192-207-BSA	nd	nd	nd
208-226	0	0	0
208-226-BSA	nd	nd	nd
221-233	50	0	0
221-233-BSA	100	0	0
227-243	20	0	0
227-243-BSA	600	0	100
230-240	0	0	0
230-240-BSA	50	0	0
238-249	60	0	0
238-249-BSA	80	0	10
254-265	70	0	70
254-265-KLH	1,000	0	30

Table 6 (concluded)

Immunogen	Serum Antibody Titer Against: Peptide	Ricin A	Ricin
Ricin A	N/A	500,000	500,000
Ricin B	N/A	500	100,000
Ricin toxoid	N/A	500,000	500,000

Note: Serum from mice immunized with peptides or peptide-protein conjugates was assayed for antibody titers by ELISA. Results are presented as titer, which is expressed as the reciprocal anti-peptide serum dilution that yields an optical density equal to that of normal mouse serum plus 2 standard deviation intervals.

nd = not determined. N/A = not applicable.

Table 7

BINDING OF SERUM FROM MICE IMMUNIZED WITH
RICIN B-CHAIN PEPTIDES TO THE PEPTIDE, RICIN B, AND RICIN

	Serum Antibody Titer Against:		
Immunogen	Peptide	Ricin B	Ricin
20-33	0	0	500
20-33-KLH	90	0	0
40-57	0	0	0
40-57-BSA	500	0	0
86-101	0	0	0
86-101-BSA	80	0	0
163-174	0	0	0
163-174-BSA	0	0	0
175-187	0	0	0
175-187-BSA	2000	0	0
188-201	100	0	0
188-201-BSA	0	0	0
206-222	0	0	0
206-222-BSA	100	0	0
245-255	0	0	0
245-255-BSA	50	50	0
ricin A	N/A	1,000	500,000
ricin B	N/A	100,000	100,000
ricin toxoid	N/A	250,000	500,000

Note: Serum from mice immunized with peptides or peptide-protein conjugates were assayed for antibody titers by ELISA. Results are presented as titer, which is expressed as the reciprocal anti-peptide serum dilution that yields an optical density equal to that of normal mouse serum plus 2 standard deviation intervals.

N/A = not applicable.

Table 8

RESISTANCE OF PEPTIDE-IMMUNIZED MICE
TO A LETHAL CHALLENGE WITH RICIN

Immunogen	No. Survivors/No. Total Mice per Group
PBS	0/10
BSA	0/10
Ricin toxoid	10/10
A(1-12)	0/10
A(17-31)-BSA	0/10
A(32-49)	0/9
A(32-49)-BSA	0/9
A(50-67)	0/8
A(84-100)-BSA	0/10
A(102-118)-BSA	0/8
A(154-166)	0/10
A(186-196)	0/10
A(186-196)-BSA	0/10
A(227-243)-BSA	0/10

Note: Groups of mice were immunized with the various immunogens emulsified in CFA on Day 0. Mice received booster immunizations on Days 21 and 35 and were challenged intravenously with 200 ng ricin on Day 49. Survival was monitored for 30 days.

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