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The specific aim of this project was to use the technique referred to as SELEX (Systematic Evolution of Ligands by EXponential enrichment) to generate stabilized RNA ligands ("aptamers") against the potential bio-warfare agents Ricin A and Protective Antigen. SELEX experiments using RNA pools containing 2'-F substituted pyrimidine nucleotides were performed, and aptamers to both targets were generated. The Ricin A aptamer binds native, glycosylated, Ricin A with an equilibrium dissociation constant of approximately 60 nM. The aptamer also binds deglycosylated and recombinant Ricin A with similar affinity. Three families of Protective Antigen aptamers were isolated, using non-stringent selection conditions (low salt, low pH). The consensus sequence from one of the families binds in the c-terminal region of protective antigen (pH 7, 100 mM NaCl, 1 mM $MgCl_2$, and 0.1 mM EDTA) with moderate affinity (~ 200 nM).

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ARO summary

Introduction

The specific aim of this project was to use the technique referred to as SELEX (Systematic Evolution of Ligands by EXponential enrichment) to generate stabilized RNA ligands ("aptamers") against the potential bio-warfare agents Ricin A and Protective Antigen.

Ricin, the toxin produced by the castor bean *Ricinus communis*, is a bipartite toxin composed of A and B chains. The B chain binds to the cell surface and mediates the endocytosis of the disulfide-linked A chain, which depurinates a specific conserved adenosine found in eukaryotic 23 – 28 S rRNA, shutting down translation. Ricin is an extremely potent toxin, which is lethal when injected at a level of 3 – 5 μg/kg (Eitzen, 1998).

Protective antigen is one component of the tripartite exotoxin utilized by the causative agent of anthrax, *Bacillus anthracis*. The three proteins of the exotoxin include protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa) (Dixon, 1999). The toxin is representative of the classic AB model in which PA (the 'B' component) acts to deliver the active A components, LF and EF, to the target cell. PA/LF forms the lethal toxin and PA/EF forms the edema toxin. Following initial binding to a cell surface receptor, PA83 is cleaved by proteases to generate a membrane associated 63 kDa fragment (PA63) which self-associates into a heptamer that is capable of binding LF or EF with high affinity (Bradley, 2001). Heptameric PA63 and associated LF and/or EF is internalized by receptor-mediated endocytosis. Acidification causes the PA63 heptamer to insert into the endosomal membrane and allows LF or EF translocation into the cytoplasm. Once in the cytoplasm, the catalytic activities of EF as an adenylate cyclase and LF as a MAPKK1-targeting protease lead to death of the intoxicated cell.

High affinity aptamers against these agents could be used as detectors for the toxins as well as anti-toxin agents. Aptamers can be configured such that binding to their target molecule results in signal generation (Hamaguchi, 2001) or incorporated into allosteric ribozymes that also couple binding and signaling events (Robertson, 1999; Soukup, 1999). An aptamer to recombinant Ricin A is a potent inhibitor of the toxin's depurination activity (Hesselberth, 2000). This aptamer contains all natural (2'-OH) ribonucleotides and therefore it is expected to have poor stability in biological media, limiting its use as either a detector or anti-toxin. RNA can be stabilized to nuclease degradation by incorporation of 2'-F pyrimidine residues (Pieken, 1991). A single point mutant in T7 RNA polymerase mutant (Padilla, 1999) permits efficient incorporation of 2'-F pyrimidine residues under standard conditions for in vitro transcription.

Several lines of evidence indicate that protective antigen is a good target for therapeutic development to treat post-symptomatic inhalational anthrax. In vaccination studies, generation of anti-PA antibodies correlates well with immune protection and PA is the primary immunogenic component of the approved AVA vaccine (Friedlander, 1997). Peptides (Mourez, 2001) and antibodies (Little, 1996) which bind to PA to block binding of the catalytic toxin subunits (EF, LF) or dominant mutant PAs that block translocation (Sellman, 2001) have been shown to promote survival in animal intoxication studies. Recent studies with recombinant antibody fragments show that protection against toxin correlates well with affinity (Maynard, 2002).

Here we describe use of SELEX to produce 2'-F pyrimidine containing aptamers to Ricin A and Protective Antigen.

In a typical aptamer selection, 10^{13}-10^{14} RNA molecules are contacted with the target of choice and the RNA molecules that specifically recognize the target are physically partitioned from those that do not. The bound RNA is next amplified by reverse transcription, PCR (RT-PCR), and *in vitro* transcription to yield an enriched pool of RNA which can serve as the starting point for a second round of selection. A complete SELEX experiment typically requires approximately 10 rounds of iterative selection / amplification. Once more than 10% of the applied pool binds specifically to the target protein, the pool is cloned and individual members are assayed for binding affinity, specificity, and other desired characteristics.

Experimental procedures and results

Ricin A

Pool preparation. Selection for anti-Ricin A aptamers was initiated with a nucleic acid pool containing 2'-fluoropyrimidines. A DNA template with the sequence 5'-GCCTGTTGTGAGCCTCCTGTCGAAN$_{40}$TTGAGCGTTTATTCTTGTCTCCCTATAGTGAGTCGTATTA-3' was synthesized using an ABI EXPEDITE™ DNA synthesizer, deprotected by standard methods and purified using a Poly-Pak™ (Glen Research) purification cartridge (N_{40} denotes a random sequence of 40 nucleotides built uniquely into each aptamer). The pooled templates were amplified with the primers YW.42.30.A (5'TAATACGACTCACTATAGGGAGACAAGAATAAACGCTCAA3') and YW.42.30.B (5'GCCTGTTGTGAGCCTCCTGTCGAA3') and then used as a template for in vitro transcription with Y639F T7 RNA polymerase, which efficiently incorporates 2'-F containing nucleotides.

Selection conditions. Selection was performed by incubation of 3 x 10^{14} RNA pooled molecules with commercially available Ricin A purified from castor beans (Sigma) (100 pmoles) for 30 minutes at room temperature in binding buffer (PBS, plus 5 mM $MgCl_2$). Complexed and free RNA molecules were separated using 0.2 μicron nitrocellulose filter disks (Tuerk and Gold, 1990; Conrad et. al., 1996). RNA:Ricin A complexes were expected to be retained on the nitrocellulose membrane, while unbound RNA would pass through. RNA was eluted from the nitrocellulose membrane by submerging the membrane in 7 M urea, 100 mM sodium acetate, 3 mM EDTA that had been pre-heated to 90°C, incubating briefly, then collecting the supernatant. The elution process was repeated twice, followed by extraction of the eluate with phenol and ethanol precipitation of the eluted RNA. After annealing to the 3' primer YW.42.30B (5'GCCTGTTGTGAGCCTCCTGTCGAA3'), the RNA was amplified by reverse transcription at 50°C for 30 minutes (Thermoscript™ RT, Invitrogen) followed by PCR under standard conditions (Taq polymerase, Invitrogen) using the primers YW.42.30B and YW.42.30A, yielding the corresponding DNA templates for the round two of selection. Subsequent rounds of selection were conducted using a similar procedure, except that the pooled RNA was passed through a nitrocellulose filter prior to incubation with Ricin A to remove molecules that bound to nitrocellulose. In addition, the concentration of Ricin A was decreased in later rounds of selection to increase binding stringency. After 10 rounds of selection, the pool was significantly enriched for Ricin A binding (Figure 1).

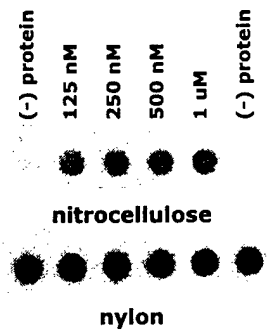

Figure 1. SELEX enriched the pool for Ricin A binders. The pool RNA from selection round 10 was incubated with varying concentrations of Ricin A in binding buffer. The mixtures were passed through a nitrocellulose-nylon filter sandwich using a dot blot apparatus. The protein:RNA complexes are captured on the nitrocellulose membrane and the unbound RNA is captured on the nylon filter.

The template DNA from Round 10 was cloned using the TOPO TA cloning kit (Invitrogen). Template DNA from 48 individual clones was amplified by PCR and used to charge 48 individual run-off transcriptions. Clone RNA's were initially screened for binding to Ricin A by incubation with the 100 nM protein in selection buffer for 30 minutes at room temperature followed by passage through a nitrocellulose-nylon filter sandwich using a dot blot apparatus to separate free from bound RNA. Twelve clones showed greater than 10% binding (6 showed 20%). Five candidate clones were chosen to characterize further. In order to estimate dissociation constants for these clones, RNA binding was measured at various concentrations of Ricin A. Since the binding curves saturate at values lower than 100%, K_d's were estimated as the protein concentration that elicited half-maximal binding (Figure 2). The estimated dissociation constants for each clone were roughly the same (45 – 80 nM), but clone D2 bound to a higher extent, suggesting it may fold into a more stable secondary structure.

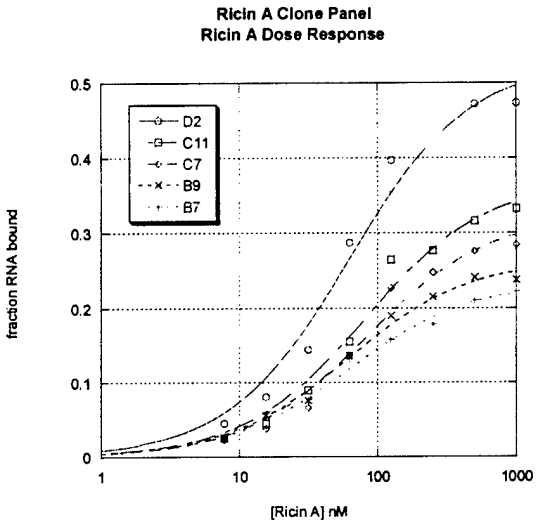

Figure 2. Ricin A binding clones and dissociation constants Dissociation constants were measured using the technique described in Figure 1. Dissociation constants (K_ds) were estimated fitting the data to the equation: fraction RNA bound = amplitude*K_d/(K_d + [Ricin A]).

Clone D2 was compared with 31RA, the all 2'- OH containing Ricin A aptamer described by Ellington et. al. D2 bound to fully glycosylated Ricin A isolated from castor beans approximately 10-fold more tightly than 31RA which was selected against recombinant Ricin A (Figure 3).

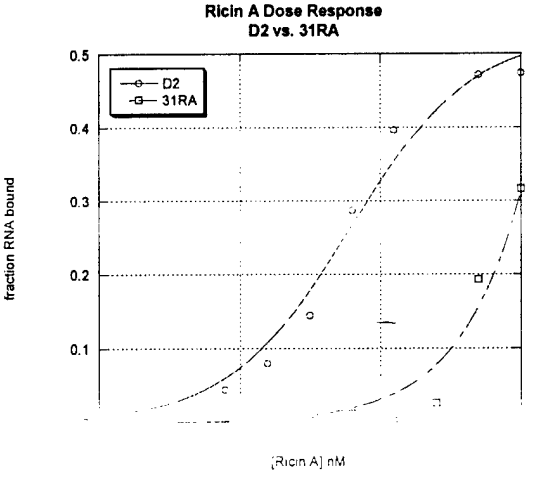

Figure 3. Comparison of clone D2 and 31RA. Clone D2 or 31RA RNA (~ 5 nM) were incubated in binding buffer with various amounts of Ricin A for 30 minutes prior to passage through a nitrocellulose/nylon filter sandwich as described in the Figure 1 legend.

Clone D2 binds to glycosylated and deglycosylated Ricin A from castor beans

and recombinant Ricin A ($K_d^{gly} \approx K_d^{degly} \leq K_d^{rec}$). The complexes are stable in the presence of competitor tRNA up to.1mg/ml. In order to both optimize clone D2 for Ricin A binding affinity and to the key Ricin A binding elements, a second selection was conducted. The template sequence for clone D2 was synthesized with each residue originating from the random region doped at a 30% level, i.e. at each position, the residue has a 70% chance of being the wild type sequence and a 30% chance of being one of the other three nucleotides (Figure 4).

5′ GGGAGACAAGAATAAACGCTCAA*TGTTGTGTCCGGATAAGGGGACCCTTAGATTCACCTCCTC*
TTCGACAGGAGGCTCACAACAGGC 3′

Figure 4. Parent sequence for a doped pool based on clone D2. The nucleotides shown in italics are doped such that at each residue there is a 70% probability of the wild type nucleotide and a 30% chance of a mutation.

The template and RNA pool for the second selection were prepared essentially as described above, except that approximately 3×10^{13} sequences were prepared. The selection was carried out as described above, except that the concentration of Ricin A used was 50 nM for all 7 rounds.

Protective Antigen (PA)

Pool preparation. Selection for anti-PA aptamers was initiated with a nucleic acid pool containing 2'- fluoropyrimidines. A DNA template with the sequence 5'-GCCTGTTGTGAGCCTCCTGTCGAAN$_{40}$TTGAGCGTTTATTCTTGTCTCCCTATAGTGA GTCGTATTA-3' was synthesized using an ABI EXPEDITE™ DNA synthesizer, deprotected by standard methods and purified using a Poly-Pak™ (Glen Research) purification cartridge (N_{40} denotes a random sequence of 40 nucleotides built uniquely into each aptamer). The pooled templates were amplified with the primers YW.42.30.A (5'TAATACGACTCACTATAGGGAGACAAGAATAAACGCTCAA3') and YW.42.30.B (5'GCCTGTTGTGAGCCTCCTGTCGAA3') and then used as a template for in vitro transcription with Y639F T7 RNA polymerase.

Selection conditions. Selection was performed by incubation of 6×10^{14} RNA pooled molecules with purified recombinant PA (100 pmoles) for 30 minutes at room temperature in binding buffer (20 mM Hepes, 1 mM $MgCl_2$, 1 mM EDTA, pH 6.0). Complexed and free RNA molecules were separated using 0.2 μicron nitrocellulose filter disks (Tuerk and Gold, 1990; Conrad et. al., 1996). RNA:PA complexes were expected to be retained on the nitrocellulose membrane, while unbound RNA would pass through. RNA was eluted from the nitrocellulose membrane by submerging the membrane in 7 M urea, 100 mM sodium acetate, 3 mM EDTA that had been pre-heated to 90°C, incubating briefly, then collecting the supernatant. The elution process was repeated twice, followed by extraction of the eluate with phenol and ethanol precipitation of the eluted RNA. After annealing to the 3' primer YW.42.30B (5'GCCTGTTGTGAGCCTCCTGTCGAA3'), the RNA was amplified by reverse transcription at 50°C for 30 minutes (Thermoscript™ RT, Invitrogen) followed by PCR under standard conditions (Taq polymerase, Invitrogen) using the primers YW.42.30B and YW.42.30A, yielding the corresponding DNA templates for the round two of selection. Subsequent rounds of selection were conducted using a similar procedure, except that the pooled RNA was passed through a nitrocellulose filter prior to incubation with PA to remove molecules that bound to nitrocellulose. In addition, the concentration

of PA was decreased in later rounds of selection to increase binding stringency. After 10 rounds of selection, the pool was significantly enriched for PA binding (Figure 5).

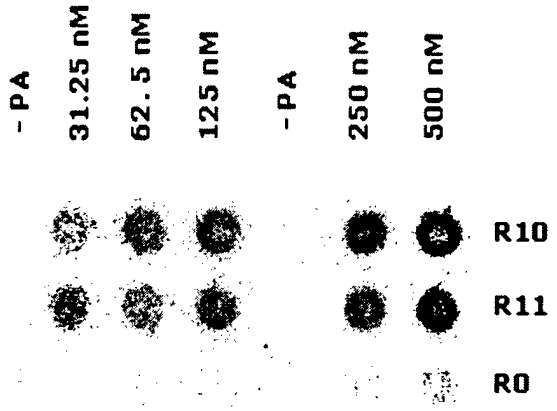

Figure 5. SELEX enriched the pool for PA binders. The pool RNA from round 10, 11 and round 0 of the selection were incubated with varying concentrations of PA. The mixtures were passed through a nitrocellulose-nylon filter sandwich using a dot blot apparatus. The protein:RNA complexes are captured on the nitrocellulose membrane and the unbound RNA is captured on the nylon filter. After 10 rounds of selection, the extent of binding to PA had significantly increased relative to the starting pool.

After the eleventh round of selection, the pooled template DNA was cloned using the TOPO TA cloning system (Invitrogen). 48 individual clones were sequenced (LARK Technologies). Individual clones were screened in duplicate for their ability to bind PA using a 96-well nitrocellulose (Protran®, Schleicher and Schuell) - PVDF (Hybond P, Amersham Pharmacia Biotech) sandwich filter binding assay. As both bound and free RNAs are captured, the fraction bound at a fixed concentration of PA can easily be measured, allowing segregation of clones on the basis of binding affinity. Clones that were retained efficiently on nitrocellulose in the absence of PA were discarded. Equilibrium dissociation constants for clones can be carefully measured using the same assay by titrating the concentration of PA over a broad range.

Aptamer clones fell into three distinct sequence groups (Figure 6) and bound specifically to protective antigen versus unrelated proteins (MEK or thrombin) in the presence of competitor nucleic acid (Figure 7).

A: GGGAGACAAGAAUAAACGCUCAAGAGGUUUUCAACUGCUGUGAUGAGUAACAGGCACGAAUCCUUCGACAGGAGGCUCACAACAGGC
B: GGGAGACAAGAAUAAACGCUCAAUUGGGGUGACCGACAAUUAUGGGAGUCGAAUUGUUGUGAGUUCGACAGGAGGCUCACAACAGGC
C: GGGAGACAAGAATAAACGCUCAACGUCCUGUAGCUUGGGUAAGAUAAAGAGUGAUCCUUCGACAGGAGGCUCACAACAGGC

Figure 6. Consensus sequences for each of the 3 protective antigen binding groups. The underlined regions originate from the random region of the pool RNA.

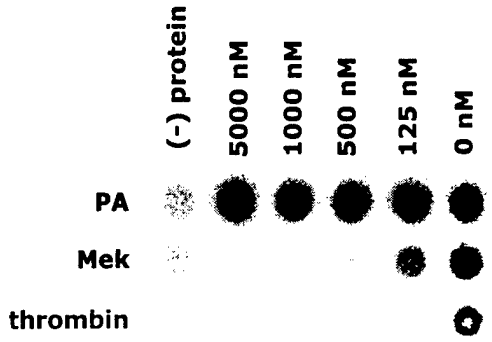

Figure 7. Specificity of the aptamer 1:PA complex. 5 nM the pool RNA from Round 11 was incubated with 100 nM PA, MEK, or thrombin in the presence of varying concentrations of naïve pool RNA that contains 5' and 3' fixed sequences flanking a randomized 40 nt region 20 mM Hepes, 1 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl, pH 6.0). The protein:RNA complexes were captured on a nitrocellulose filter. The PA:aptamer 1 complex is stable in the presence of a 1000 – fold excess of competitor RNA while the MEK and thrombin complexes are disrupted at a significantly lower competitor concentration.

We evaluated binding of the consensus sequences from each of the three families to PA_{83}, PA_{63}, and PA_{32}, which contains the two c-terminal domains of protective antigen. The aims were to identify aptamers that are functional at pH 7.0 and physiological salt,

as well as to identify the binding site(s) of the aptamer. Table 1 shows the results. The Group B sequence binds protective antigen at pH 7, and it appears to bind to the c-terminal region of the protein.

construct	PA83		PA63		PA32	
	pH 6	pH 7	pH 6	pH 7	pH 6	pH 7
Group A	(+)	(-)	(+)	(-)	(-)	(-)
Group B	(+)	(+)	(+)	(-)	(+)	(+)
Group C	(+)	(-)	(+)	(-)	(-)	(-)

Table 1. Results of primary screen for PA domains binding at 10 mM Hepes pH 6 or pH 7, 100 mM NaCl, 0.1 mM EDTA, 1 mM $MgCl_2$. In each case, the protein concentration was 500 nM.

The dissociation constant of the Group B consensus sequence for PA83 was measured in the presence and absence of 0.1 mg/mL competitor tRNA. Presence of competitor had little effect on the K_d, which is approximately 400 nM under both conditions (Figure 8).

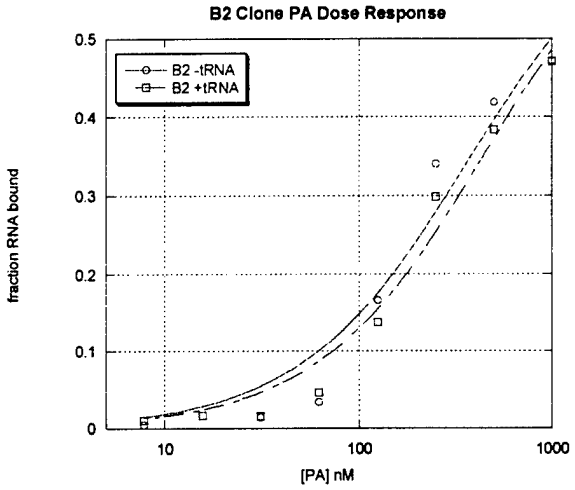

Figure 8. Binding curve for the Group B consensus sequence binding PA83. Trace internally ^{32}P-labeled Group B sequence was incubated with varying concentrations of PA83 for 30 minutes at room temperature in the presence or absence of 0.1 mg/mL tRNA (20 mM Hepes pH 7, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA). The protein:RNA complexes were then captured on a nitrocellulose filter and unbound RNA was captured on a nylon filter. Kd's were determined by best fit to the equation fraction RNA bound = amplitude*K_d/(K_d + PA_{83}).

Summary

SELEX was used to generate RNA aptamers containing stabilizing 2'-F pyrimidines against the potential bio-warfare agents Ricin A and Protective Antigen. The Ricin A aptamer binds native, glycosylated, Ricin A with a Kd of approximately 60 nM. The aptamer also binds deglycosylated and recombinant Ricin A with similar affinity. Three families of Protective Antigen were isolated, using non-stringent selection conditions (low salt, low pH). The consensus sequence from one of the families binds in the c-terminal region of protective antigen (pH 7, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA) with moderate affinity (~ 200 nM).

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