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IMMUNOLOGIC APPROACH TO THE IDENTIFICATION AND DEVELOPMENT OF VACCINES TO VARIOUS TOXINS

ANNUAL/FINAL REPORT

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The extreme toxicity of the protein synthesis inhibitor ricin, and the sodium channel blocker saxitoxin render them unsafe as vaccines in the elicitation of active and protective immunity against their toxicity. For this reason, we have investigated the feasibility of the anti-idiotype antibody-based vaccine approach for inducing protective immunity against saxitoxin and ricin. Because of the proteinaceous nature of ricin, we have also investigated the synthetic peptide subunit vaccine strategy for eliciting protective immunity against this toxin. Protective murine monoclonal and goat polyclonal anti-ricin antibodies have been generated, and are being utilized to produce anti-idiotype reagents with antigenic mimicry, which may be effective as vaccines against ricin toxicity. Two peptides homologous to ricin chain A sequences, and two homologous to ricin chain B sequences have been synthesized. Studies are underway to assess their potential, either used singly or in combinations, in inducing protective anti-ricin immunity. Protective burro polyclonal anti-STX IgG have also been used to generate anti-idiotype antibodies capable of antigen mimicry for the elecitation of active and

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19. Abstract (Continued)

protective immunity against saxitoxin toxicity. Our results are encouraging thus far, and we are confident that our research work will in the near future lead to safe and effective vaccines against the in vivo toxicity of both saxitoxin and ricin.

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FOREWORD

In the conducting of the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources - National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. INTRODUCTION

The sodium channel blocker **saxitoxin** (STX), produced by the dinoflagellates of the genus Gonyaulax, and the protein synthesis inhibitor **ricin**, produced by the castor plant Ricinus communis, are extremely toxic biological compounds. The main objectives of this investigation are to generate specific and protective monoclonal antibodies (mAbs) with high binding affinity constants, to develop sensitive immunoassays for the specific detection of these toxins, and to assess the feasibility of anti-idiotype (anti-Id) based vaccines for the induction of active and protective immunity against the in vivo toxicity of these compounds.

Idiotypes or Id determinants are antigenic determinants or amino acid sequences associated with the variable (V) region of an antibody molecule. Anti-Id antibodies (or Ab2) are specific anti-immunoglobulin (Ig) antibodies that can be used to serologically define the Id (or Ab1). Anti-Id antibodies can be classified into three different categories based on their binding specificities (1). Anti-Id antibodies binding to framework determinants not associated with the antigen-binding site of the Ab1 (anti-antigen), and therefore not inhibited by the antigen are referred to as Ab2a. Anti-Id that are inhibited by antigen, and can display similar tertiary conformation to that of the nominal antigen (antigenic mimicry) are designated Ab2b, or internal image anti-Id. Whereas, those anti-Id that are inhibited by antigen but lack biological mimicry are referred to as Ab2y. It is the Ab2b with its antigen mimicry that have been proposed as potential antibody-based vaccines (2). Indeed, anti-Id vaccines have been shown to induce protective immunity against a variety of experimental bacterial, viral and parasitic infections.

For nonproteinaceous, low molecular weight biological and chemical toxins such STX, the anti-Id vaccine approach may represent the only safe and effective strategy for vaccine development since their extreme toxicity precludes their use as immunogens. This anti-Id-based vaccine approach was successfully demonstrated in our laboratory in inducing protection against the in vivo toxicity of the trichothecene mycotoxin T-2 (3).

Because ricin is a protein toxin composed of two disulfide-linked glycoprotein chains whose primary amino acid sequences have been determined, it is also proposed, in addition to the anti-Id approach, to develop subunit vaccines based on synthetic peptides homologous to antigenic sequences of the ricin A and B chains.

This report describes our research work in the generation of

mAbs against STX using different conjugation methods for preparing STX-conjugates, and different immunization schemes. Monoclonal anti-ricin antibodies have also been isolated by immunizing mice with a variety of antigens (ricin whole molecule, purified chain A, purified chain B, and synthetic peptides homologous to the ricin A and B chains). Passive and active protection experiments against the in vivo toxicity of ricin using anti-ricin mAbs and synthetic peptides, respectively, are also discussed. Finally, we will report on our progress in the monoclonal and polyclonal anti-Id approaches in the STX and ricin systems, with the ultimate aim of inducing active and protective immunity against toxicity.

II. RESULTS

A. MONOCLONAL ANTI-SAXITOXIN ANTIBODIES

We had previously generated two murine mAbs specific for STX with approximate affinity constants (K_a values) of $10^6 M^{-1}$, using formaldehyde as the coupling reagent in the preparation of STX-conjugates (4). These anti-STX mAbs were used in a sensitive enzyme-linked immunosorbent assay (ELISA) for the specific detection of STX. They were able to displace [^{3}H]STX binding to rat brain membranes in vitro, and to partially protect against STX-induced reduction of peripheral nerve action potential in rat tibial nerve in situ. It should be noted that this protection was only partial, which could be accounted for by the relatively low K_a values of the anti-STX mAbs generated.

We have taken two approaches in attempting to enhance the K_a of the generated mAbs. The first approach involves our continued effort to design more immunogenic STX-conjugates by coupling STX to the protein carriers keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) via the linker N-succinimidyl 3-2 (pyridyldithio) propionic acid (SPDP). The propionic acid linker is thought to enhance immunogenicity of the hapten-protein conjugates by rendering STX more solvent-exposed and accessible for immune recognition. The conjugates were prepared by adding 50 mg of KLH or BSA in 15 ml of borate buffered saline (BBS, pH 7.2) to 5.2 mg of SPDP in 0.2 ml dimethyl formamide. The mixture was incubated for 1 hr at room temperature and dialyzed overnight against BBS at 4°C. Dithiothreitol (3.85 mg) in BBS was added to the KLH-SPDP, and dialysis against 2 liters of BBS containing 3 mM sodium borohydride was done for 1 hr at 4°C. The buffer was changed to BBS, and dialysis was continued for another hour at 4°C. The STX derivative (decarbamoyl STX), prepared by drying 5 mg STX under vacuum followed by heating at 110°C for 3 hr in the presence of 7.5 N HCl and drying under vacuum, was added to the dialysate. The STX-SPDP-carrier conjugates were reacted for 3 hr at room temperature and dialyzed for 3 days with daily changes of the dialysis buffer. Routine binding ELISA indicates the presence of STX in the conjugates, however the amount present and the ratio of conjugation are not known. Five and 4 fusions have been performed with spleen cells from BALB/C mice immunized with STX-SPDP-KLH and STX-SPDP-BSA, respectively. A total of over 3,000 resulting hybrid culture supernatants have been screened for binding to STX using the ELISA described previously (4). Among these, about 49 hybrids were found to be positive in the binding ELISA, however none had K_a values, as determined by IC_{50} competitive inhibition enzyme immunoassay (4,5), significantly higher than the two anti-STX mAbs previously generated (4). Five

fusions were also performed with spleen cells from BALB/C mice immunized with STX coupled to KLH or BSA using formaldehyde. Although specific anti-STX mAbs were generated as above, none showed K_a values higher than $10^6 M^{-1}$.

B. MONOCLONAL ANTI-RICIN ANTIBODIES

One of the major emphasis of the present study is to generate anti-ricin mAbs of high binding affinity that would protect against ricin in vivo toxicity. For this purpose, BALB/C mice were immunized with either native ricin antigens or with synthetic peptides homologous to amino acid sequences of the two ricin chains.

1. Monoclonal Antibodies Raised Against Native Ricin: Groups of BALB/C mice were immunized intramuscularly with whole ricin (1.0ug/mouse), or purified ricin A chain (10 ug/mouse), or with purified ricin B chain (10 ug/mouse). The native ricin antigens were purchased from Sigma Chemical Co., St. Louis, MO. The first injection was done in Freund's complete adjuvant (FCA), the second in Freund's incomplete adjuvant (FIA), and subsequent injections were in phosphate buffered saline (PBS, pH 7.2). Immunizations were given at 2-week intervals. The mice were bled before and after each immunization, and their sera tested for ricin reactivity as follows. Microtiter wells were coated with a pretitrated amount of whole ricin (0.05 ml/well of a 10 ug/ml solution), or ricin A chain (10 ug/ml), or ricin B chain (10 ug/ml) overnight at 4°C. The wells were washed with PBS supplemented with 5% normal goat serum (PBS/NGS), and blocked with PBS/NGS for 1 hr at 37°C. The wells were washed and 0.05 ml of serial dilutions of preimmune or immune sera were added. After 1 hr incubation at 37°C and washing, 0.05 ml of a 1:2500 dilution of goat anti-mouse Ig conjugated to horse radish peroxidase (HRP, Fisher Scientific, Orangeburg, NY) were added to the wells. After 1 hr incubation at 37°C, reactivity was detected by addition of the substrate ABTS (2,2-azino-bis-[3-ethyl-benzthiazoline-6-sulfonate]). Positive reactivity is defined as optical density (OD_{410nm}) greater than 3x that of preimmune sera. TFTA-1 and TFTB-1 hybridoma (ATCC, Rockville, MD) culture supernatants specific for ricin chains A and B, respectively, were used as positive controls. Culture supernatant from HD11 hybridoma secreting an IgG_1 mAb specific for T-2 mycotoxin served as the negative control (6,7). Hybrid culture supernatants yielding positive reactivity in binding ELISA were selected and further tested in an inhibition ELISA as follows. Dilutions of positive supernatants giving approximately 50% binding in the ELISA described above were incubated with an equivalent volume of inhibitors (whole ricin, chains A or B at 200 ug/ml) for 1 hr at 37°C. Following incubation, the mixtures were added to the wells which had been precoated with the immunizing antigen, and the ELISA was continued as

described above. This concentration of inhibitors (200 ug/ml) was selected based on observations that mAbs not inhibited by this concentration of inhibitors would in all likelihood possess relatively low K_a values. Culture supernatants whose binding was inhibited by greater than 30% were further selected for the EL-4 in vitro protection assay. The EL-4 cells, obtained from Dr. J. Hewetson at USAMRIID, were used to determine the in vitro toxicity of ricin. EL-4 in log phase growth were washed and resuspended at a cell density of $2x10^6$ cells/ml in Dubbelco MEM (DMEM) supplemented with 10% fetal bovine serum (FBS). Triplicates of 0.05 ml containing 10^5 cells/well were added to 96-well flat bottom tissue culture plates. Different concentrations of ricin in 0.05 ml of leucine-free DMEM medium were added to the wells which were incubated overnight at 37°C. After incubation, 0.05 ml of DMEM containing 1.0 uCi of [^{3}H]leucine were added to the wells, which were incubated for 4 hr at 37°C. The cells were then harvested and processed for scintillation counting. The concentration of ricin inhibiting approximately 50% of [^{3}H]leucine uptake was determined to be 6.0 ng/ml, which is routinely used in our laboratory for assessing antibody protection. In this in vitro protection assay, the proper concentration of ricin is incubated with an equivalent volume of anti-ricin mAbs for 1 hr at 37°C. The mAb/ricin mixture was added to the EL-4 cells, and the assay preformed as described above.

Using this approach, a number of hybrids specific for ricin have been isolated (Table 1). Among the 7 mAbs raised against whole ricin, 6 were of the IgM isotype, whereas one (BG11-G2) was an IgG_1. The six IgM mAbs reacted strongly in binding ELISA with ricin- and ricin A-coated wells. E4, E7 and D6 mAbs reacted to a lesser extent with ricin B chain-coated wells, whereas A1, 3F2 and WR1C4 did not. The BG11-G2 IgG_1 mAb was specific for whole ricin, and did not react with chains A or B. The binding of A1 and 3F2 were inhibited by A chain, but not by ricin or B chain. The binding of the other 4 IgM hybrids were inhibited most strongly by ricin A chain followed by whole ricin, and to a lesser degree by ricin B chain. BG11-G2 binding was only inhibited by whole ricin, suggesting that the expression of the antigenic determinant involved requires both chain in their native conformation. All 7 mAbs, with the exception of A1, protected EL-4 cells against the in vitro ricin toxicity, with BG11-G2 being the most efficient, as determined by titration experiments (data not shown).

Two mAbs (A2H11 and 3B8) were isolated from fusions of chain A-immune spleen cells. Both mAbs reacted with whole ricin and ricin A-coated wells, and not with ricin B-coated wells. Their binding were inhibited by whole ricin and chain A, and to a lesser extent by chain B. It was surprising that chain B was able to inhibit their binding, however one likely explanation would be the contamination of the purified chain B by whole ricin or chain A. Both

mAbs showed some protection against the in vitro ricin toxicity in EL-4 cell assay.

Three mAbs (7F3, FD10 and HC7) were generated from spleen cells of mice immunized with purified ricin B chain. They possess similar reactivity patterns in that they reacted with both whole ricin and ricin chain B, and not with chain A. Their binding were inhibited by whole ricin and chain B, and not by chain A. They only partially inhibited the in vitro ricin toxicity as assessed by the EL-4 cell assays.

In summary, mAbs raised against whole ricin molecules appear to provide a higher level of protection against ricin toxicity. Among these mAbs, BG11-G2 appears to be the most promising mAb. As will be discussed later, this IgG_1 anti-ricin mAb was capable of protecting against the in vivo toxicity of ricin.

2. Monoclonal Antibodies Raised Against Ricin Synthetic Peptides: A number of synthetic peptides homologous to either ricin chain A or B have been synthesized in our laboratory. The rationale for the selection of these sequences for synthesis will be discussed later. Two of the synthetic peptides, namely A-18 and B-18, were conjugated to KLH and used to immunize mice for the production of mAbs. The A-18 sequence (Figure 1) which is homologous to an amino acid sequence of the ricin A chain starting at position 18, defines a solvent-exposed alpha-helical region. The B-18 sequence (Figure 2) which is homologous to a sequence on the ricin B chain starting at position 18 contains one of the 2 galactose-binding domains of the B chain. Three mAbs were isolated from A-18-KLH-immune spleen cells that bound to whole ricin and chain A, but not to chain B. Their specificity was demonstrated by the inhibition of their binding by ricin and chain A (Table 1). Likewise, the two mAbs produced from B-18-KLH-immune spleen cells reacted with ricin and chain B, but not chain A, and were inhibited by ricin and chain B. None of these mAbs protected against ricin toxicity by more than 50%. Thus, although anti-ricin mAbs specific for either chains A or B could be generated from synthetic peptide antigens, they do not appear to be highly protective. Although two more synthetic peptides are currently available in our laboratory (see below), mAbs against them have not been generated. Our plan is to investigate these peptides as well as others that we have proposed in order to derive mAbs that are protective either singly or in combinations.

C. BALB/C IMMUNE RESPONSES AGAINST RICIN SYNTHETIC PEPTIDES

In addition to the strategy of generating anti-Id antibodies which may be effective as vaccines against ricin toxicity, we also

propose to develop synthetic peptide-based subunit vaccines because of the proteinaceous nature of ricin. Based on the known primary sequences of the A and B chains of ricin, we have selected four sequences thus far for synthesis. These sequences are chosen based on their high hydrophylicity/hydrophobicity indices, alpha-helical structures, beta-turns, galactose-binding domains of the B chain, and high potential exposure to solvent or accessibility for immune recognition. Two amino acid sequences homologous to the A chain were selected for synthesis because they represent regions of solvent-exposed, potentially immunogenic alpha-helical structures. They are designated ricin A-18 and A-95 (Figures 1 and 3, respectively). Two sequences were also selected from the ricin B chain. Designated B-18 and B-230, they encompass the galactose-binding domains of the ricin molecule B chain (Figures 2 and 4, respectively). It is believed that these sequences represent the binding sites of ricin to complex sugars on the cell membrane, allowing the internalization of the ricin molecule into the cell cytoplasm where the A chain exerts its toxicity. Three of these peptides (A-18, A-95 and B-230) were synthesized as free peptides followed by conjugation to KLH by carbodiimide reaction to render them immunogenic. The fourth peptide (B-18) was synthesized on a backbone of branching lysine core as described by Posnett et al. (8), and referred to as multiple antigenic peptides or MAP (Figure 5). Peptides synthesized as MAP have been demonstrated to render the need for carrier proteins obsolete in generating high titered anti-peptide and anti-native antibodies. This approach is attractive because the possibility exists for the synthesis of MAP with multiple sets of peptide arms, or the synthesis of polymers containing two or more different peptide epitopes. The latter property may be significant in the development of effective vaccines in instances where there is a requirement for more than one protective immunogenic epitopes. Groups of BALB/C mice were immunized with the synthetic peptides in various adjuvants as shown below:

A-18 Peptide
(6 mice/group)

1. A-18 in alum
2. A-18-KLH in alum
3. A-18-KLH in FCA

A-95 Peptide
(5 mice/group)

1. A-95 in alum
2. A-95-KLH in alum
3. A-95-KLH in FCA
4. A-95-KLH in FIA

B-230 Peptide
(5 mice/group)

1. B-230 in alum
2. B-230 in FCA
3. B-230-KLH in alum
4. B-230-KLH in FCA

B-18-MAP Peptide
(5 mice/group)

1. B-18-MAP in PBS
2. B-18-MAP in alum
3. B-18-MAP in FCA
4. B-18-MAP in FIA

Each mouse received 10 ug per injection of peptides A-18 or A-95, or 50 ug of peptides B-230 or B-18-MAP intraperitoneally on a two-week interval. The reason for increasing the doses of the latter two peptides was the relatively low antibody responses obtained with 10 ug of A-18 and A-95 peptides (see below). However, it became apparent that higher doses did not in this case result in enhanced antibody responses, and that peptide synthesized as MAP induced a significantly stronger immune response.

1. Immune Responses Against Ricin A-18 Peptide: The binding ELISA results of mice immunized with A-18 peptide, determined as described above, are shown in Table 2. The majority of the animals immunized with unconjugated peptide, or with A-18-KLH in alum or in FCA developed demonstrable anti-ricin titer after the 3rd immunization. The sera reacted not only with the immunizing peptide, but also with whole ricin and chain A. No significant reactivity was observed with chain B (data not shown). The reactivity against A chain (with titers ranging from 1:40 to 1:1280) appeared to be somewhat higher than those against A-18 peptide (1:20 to 1:320) and against whole ricin (1:20 to 1:320). With few exceptions, the serum titers of all three groups of mice did not increase significantly with booster injections and remained rather modest up to the 5th and 6th immunizations. No obvious differences were noticed with various adjuvants employed.

2. Immune Responses Against Ricin A-95 Peptide: The serum reactivity of mice immunized with ricin A-95 peptide is presented in Table 3. Unlike peptide A-18, conjugation of A-95 peptide to KLH followed by immunization as alum precipitate or as FCA emulsion did not result in significant anti-peptide or anti-ricin reactivity, even after the 6th immunization. On the other hand, unconjugated A-95 peptide immunogen given in alum or FCA induced a detectable anti-peptide and anti-ricin reactivity after 2 to 3 injections. The reason for this is unclear. It was somewhat surprising that the free peptide was immunogenic, however it should be noted that these are rather large peptides composed of 20 or more amino acids (Figure 3). As is the case with A-18 peptide, A-95-immune sera reacted with the homologous peptide, chain A and ricin, but not with chain B. The antibody titers of all groups did not increase significantly after the 6th immunization, and different adjuvants did not influence the anti-ricin antibody titer.

3. Immune Responses Against Ricin B-230 Peptide: Groups of BALB/C mice were also immunized with ricin B-230 peptide, which encompasses one of the two galactose-binding domains of the ricin B chain. The binding ELISA results which are presented in Table 4 are somewhat confusing and disappointing. Unconjugated B-230 peptide in alum or FCA induced primarily an anti-peptide antibody response which was apparent only after the 3rd or 4th immunization.

No reactivity was observed with the native ricin B chain, whereas only minimal reactivity was obtained against whole ricin following the 5th immunization. B-230-KLH in alum induced an anti-ricin response after the 5th injection, however no reactivity was observed with the homologous peptide or with ricin B chain. Immunization with B-230-KLH in FCA resulted in a strict anti-B-230 response with some anti-whole ricin reactivity after the 5th injection, and no detectable anti-native B chain antibody response.

4. Immune Responses Against Ricin B-18-MAP: As mentioned earlier, a synthetic peptide homologous to the second galactose-binding domain of the B chain (residues 18-48) was synthesized on a backbone of lysine, designated B-18-MAP. The ELISA binding results of sera of mice immunized with B-18-MAP are shown in Table 5. Unlike the three other peptides which required 2 to 3 injections before detection of a low antibody response, ricin B-18-MAP administered in PBS or in alum induced a detectable although low reactivity after the primary immunization. The anti-peptide and anti-native antibody responses increased dramatically with subsequent booster injections. Two weeks after the 4th immunization, the antibody titers reached levels of 1:160 to greater than 1:10,000, which were not achieved with 5 or 6 injections with the other peptides not synthesized as MAP. The 2 groups of mice immunized with peptide in FCA or in FIA gave low responses following the 2^{nd} and 3^{rd} immunizations. The reason for this is not clear, however, it is noteworthy that B-18-MAP peptide did not emulsified well in these adjuvants because of their low solubility. Nevertheless, after the 4th immunization, these 2 groups of animals developed titers comparable to those of the PBS or alum groups. The immune responses induced were specific in that no significant reactivity was obtained against chain A (data not shown). These results suggest that B-18-MAP peptide induces significantly higher anti-peptide and anti-native antibody responses that could be obtained with the other peptides studied which were not synthesized as MAP. Sera from the B-18-MAP-immune mice were able to protect against the in vitro ricin toxicity using the EL-4 cell assays (data not shown), whereas sera from the other groups did not. Whether this enhanced immune response and associated protection are due to the MAP synthesis or due to the inherent immunogenic property of the B-18 peptide is not known. To attempt to answer this question, we synthesized A-18 and B-230 peptides as MAP, and immunized BALB/C mice as follows:

A-18-MAP (6 mice/group)	**B-230-MAP** (6 mice/group)
1. A-18-MAP in alum	1. B-230-MAP in alum
2. A-18-MAP in FCA	2. B-230-MAP in FCA

The mice received 50 ug/each per injection intraperitoneally on a 2-week schedule. They were bled before and after every immunization, and reactivity was assessed in ELISA as described above. Peptide A-18 synthesized as MAP induced an earlier and significantly better antibody response compared to that obtained with A-18-KLH immunization (Table 6). There did not appear to be any significant differences between the use of alum or FCA as adjuvant. Immunization with B-230-MAP (Table 7), however did not result in a significantly higher antibody response than immunization with B-230-KLH, suggesting that peptide B-230 sequence may not be very immunogenic, at least in BALB/C mice.

5. Immune Responses Against Ricin Peptide Combinations: In order to determine how BLAB/C mice would respond to different combinations of ricin peptide-MAP (e.g., antigenic competition), groups of 6 mice each were immunized with 50 ug of each peptide in alum as follows:

Combinations of Peptide-MAP

1. A-18-MAP and B-230-MAP
2. A-18-MAP and B-18-MAP
3. B-18-MAP and B-230-MAP
4. A-18-MAP, B-18-MAP and B-230-MAP

The results of their sera reactivity are presented in Table 8-11. In summary, no antigenic competition was observed. Ricin peptide-MAP that induced a good antibody response when given singly also induced a good antibody response when given together with other peptide-MAP. The converse was also true. Thus, the sera of the first group of mice immunized with A-18-MAP and B-230-MAP gave high antibody reactivity against chain A and whole ricin, but low reactivity against chain B (table 8). This was not surprising due to the poor immunogenicity of the B-230 peptide even when synthesized as MAP. It is noteworthy that mice immunized with all three ricin peptide-MAP gave significant titers against whole ricin, chains A and B. This may represent a good combination subunit vaccine in an attempt to induce active and protective immunity against ricin in vivo toxicity.

D. SYNTHETIC PEPTIDES AS SUBUNIT VACCINES AGAINST RICIN IN VIVO TOXICITY

Encouraged by the anti-ricin antibody titers obtained above with the MAP system, we investigated the ability of some of these ricin peptides in inducing an active protective immunity against the in vivo toxicity of ricin. A group of 20 female, age-matched

BALB/C mice were immunized as above with 50 ug each of A-18-MAP in alum. Two weeks after the 3rd booster injection, the mice were bled and their sera tested on ELISA as described, to ensure that the immunization process is properly done. The titers of the 20 mouse sera against chain A and whole ricin ranged between 1:64,000 to 1:128,000 and between 1:8,000 and 1:16,000, respectively (data not shown). The mice were boosted twice more, and 10 days following the 5th immunization, the immune mice and a group of age-matched unimmunized controls were challenged subcutaneously with various doses (0.2 ug, 1.0 ug and 5.0ug/mouse) of ricin (Table 12). The rationale for selecting these particular ricin doses is that we had previously determined the LD_{50} dose of ricin in BALB/C mice to be approximately between 0.1 and 0.5 ug/mouse (data not shown). After challenge, the number of dead mice in each group and the elapsed time between challenge and onset of death were recorded. All mice that received 5.0 ug/mouse died. At the 1.0 ug/mouse dose, 4/5 nonimmune mice died, whereas 3/7 immune mice did. All the mice receiving 0.2 ug/mouse survived the challenge. It is important to notice that it took considerably longer for the peptide-immune than the nonimmune mice to succumb to the challenge, whether they received 1.0 or 5.0 ug of ricin. This delay in elapsed time between challenge and death can be best illustrated in the survival graph presented in Figure 6. Thus, 52 hr after administration of 1.0 ug of ricin, only 20% of the control mice survived, whereas there was a 100% survival in the immune group. Thus, immunization with a single ricin peptide (A-18-MAP) in alum was partially protective against the in vivo ricin toxicity, as assessed by the survival ratio as well as by the delay in onset of death. Another group of 25 mice have been immunized with a combination of A-18-MAP, A-95-MAP and B-18-MAP in alum. B-230 peptide was not included because our results thus far suggest that B-230 is not very immunogenic. As of this writing, the mice have received 3 immunizations and are showing good antibody responses to ricin, to ricin chains A and B. They will be boosted twice before a challenge experiment will be performed.

E. PASSIVE PROTECTION AGAINST RICIN TOXICITY

To determine if one can passively protect mice against ricin toxicity, we selected one of the better anti-ricin mAb generated, namely BG11-G2 (see above), and injected the hybrid cells into BALB/C mice to produce ascites. The BG11-G2 ascitic fluid was harvested and the IgG_1 mAb was purified by caprylic acid precipitation. The purified IgG_1 mAb (10.0 mg/mouse) was injected intraperitoneally into groups of BALB/C mice, which were challenged the following day with various doses of ricin (Table 13). We had previously determined that the circulating BG11-G2 reached optimum levels approximately 12-24 hr after i.p. injection of the mAb (data

not shown). Although all untreated and treated mice died from challenge with either 2.5 ug or 5.0 ug of ricin, a considerable delay in the onset of death was observed with the groups that were pretreated with BG11-G2 anti-ricin mAb. A larger amount of purified BG11-G2 mAb is being produced, and the experiment will be repeated with a higher dose of BG11-G2. However, these results suggest that it is indeed possible to delay the onset of death by pretreatment with a specific anti-ricin mAb.

F. ANTI-IDIOTYPIC ANTIBODIES

The other proposed strategy for the induction of active protective immunity against the in vivo toxicity of ricin and STX is the anti-Id-based approach. The general scheme for this approach is presented in Figure 7. The first requirement for a successful anti-Id vaccine is the generation of a high binding affinity and protective anti-toxin (Ab1) antibody. This Ab1 is then utilized to produce a second generation antibody (Ab2) of the internal image class that has the ability to mimic the tertiary conformation of the nominal antigen (toxins), and thus can be used to induce the production of an anti-toxin immune response.

1. Ricin Monoclonal Anti-Idiotypes: We have thus far selected 3 anti-ricin mAb1 (see Table 1) for the generation of monoclonal anti-Id antibodies:

a. WR1C4, an anti-ricin mAb derived from a fusion of spleen cells of mice immunized with ricin.
b. A2H11, an anti-ricin mAb derived from a fusion of chain A-immune spleen cells.
c. BG11-G2, a recently derived anti-whole ricin mAb, and probably the best mAb generated thus far.

Groups of syngeneic BALB/C mice were immunized i.p. with 10 ug/each of WR1C4 or A2H11 anti-ricin mAbs in alum. Injections were done every other week. Sera from these mice obtained 2 weeks after immunizations were tested for anti-Id reactivity employing a "sandwich assay" as follows. Briefly, purified Ab1 anti-ricin mAb (WR1C4, A2H11 or BG11-G2) were used to coat microtiter wells at a concentration of 10 ug/ml. After blocking unreactive sites, serial dilutions of the anti-Id (Ab2)-containing sera were added to the wells, which were incubated for 1 hr at 37°C. After incubation, the wells were washed followed by the addition of biotinylated Ab1, and incubated at 37°C for 1 hr. The wells were then washed, and avidin-HRP was added, and the wells incubated for another hour at 37°C. Reactivity was detected by addition of the ABTS substrate. No anti-Id reactivity was detected after 5 immunizations of WR1C4. On the other hand, some anti-Id reactivity was observed with sera

obtained two weeks after the 5th immunization with A2H11 (Table 14), although the anti-Id antibody titers were quite low. The mice were boosted, and three independent fusions have been performed. Approximately 750 resulting hybrids were screened for anti-Id reactivity using the sandwich ELISA described above. None of the tested culture supernatants showed any significant anti-Id reactivity. It should be mentioned that the Ab1 were used as unconjugated immunogens in a syngeneic system (BALB/C into BALB/C). This may account for our inability thus far to induce significant anti-Id reactivity. Experiments are underway to immunize BALB/C mice with WR1C4 and A2H11 Ab1 conjugated to KLH. BG11-G2, a recently derived anti-ricin mAb, has also been conjugated to KLH for this purpose.

2. Ricin Polyclonal Anti-Idiotypes: In addition to the monoclonal anti-Id approach, it was thought advantageous to also pursue the polyclonal anti-Id strategy, as suggested by Dr. J. Hewetson (USAMRIID). For this purpose, we have immunized 2 New Zealand White rabbits with ricin (50 ug/injection). After 4 immunizations given at two week-interval, the rabbits were bled and their sera tested for anti-ricin reactivity as described above. The sera titers of the rabbits ranged from 1:5,000 to 1:10,000 (data not shown). The rabbit anti-ricin IgG was purified by adsorption to and elution from a protein A-agarose column, and tested for protection against ricin toxicity in EL-4 cell assays. Results obtained with rabbit No. X-840 are presented in Table 15. As mentioned before, in our hands, a final concentration of 6.0ng/ml of ricin routinely inhibits approximately 40%-60% of the [^{3}H]leucine uptake by EL-4 cells. When rabbit X-840 purified IgG was added to the EL-4 cultures in the presence of ricin, there was a pronounced reduction in the percent inhibition of leucine uptake, suggesting a protective effect of the rabbit anti-ricin IgG. The same concentrations of preimmune rabbit X-840 IgG did not have any effect. Similar results were obtained with rabbit X-839 and are not shown. Although these rabbit anti-ricin IgG are available, we have not begun immunization for anti-Id production. We have instead elected to work with a better characterized polyclonal goat anti-ricin IgG preparation generously provided to us by Dr. J. Hewetson (USAMRIID). This goat anti-ricin IgG preparation has been demonstrated to protect against ricin in vitro toxicity (9,10). BALB/C mice were immunized i.p. every other week with 50 ug/mouse of protein G-purified goat anti-ricin IgG (generously provided by Dr. J. Hewetson) precipitated in alum. Following the 3rd immunization, the sera of the 5 mice were pooled and adsorbed over a normal goat IgG-agarose column in order to remove anti-isotype and anti-allotype reactivity. After adsorption, mouse anti-goat Ig reactivity was detected as follows. Microtiter wells were coated with a predetermined optimum concentration of goat Ig (2.0 ug/ml), and blocked. Serial dilutions of the pooled, adsorbed mouse sera were added. The wells were incubated for 1 hr at 37^{o}C. After washing,

0.05 ml of a 1:2,500 dilution of goat anti-mouse Ig-HRP were added. After incubation and washing, reactivity was developed by the addition of the substrate ABTS (Table 16). Most if not all anti-isotype and anti-allotype reactivity had been adsorbed, as no reaction was observed with wells coated with normal goat IgG. On the other hand, the same sera reacted strongly with wells coated with goat anti-ricin IgG, suggesting a significant anti-Id antibody titer. The Ab2-containing adsorbed mouse sera were precipitated with saturated ammonium sulfate to purify the Ig fraction, which was alum precipitated. This purified Ig fraction was alum precipitated, and used to immunize a group of 6 BALB/C mice for the production of anti-ricin Ab3. However, after 5 immunizations of 50 ug Ab2/mouse given i.p. on a two-week schedule, only minimal anti-ricin reactivity was observed (data not shown). Although these results are disappointing, it should be pointed out that whereas the generation of Ab2 was successful and was done in a xenogeneic system (e.g, goat Ab1 into BALB/C mice for Ab2 generation), the production of Ab3 (anti-ricin) was done in a syngeneic system (e.g., BALB/C Ab2 into BALB/C mice for the production of Ab3). This may well account for the lack of a strong Ab3 antibody responses observed presently. For this reason, we have initiated an experiment in which New Zealand White rabbits were immunized with the purified goat anti-ricin IgG. The generated rabbit anti-Id sera will be adsorbed as above to remove anti-isotype and anti-allotype reactivity. The adsorbed sera will be purified by protein A- or protein G-agarose to obtain rabbit anti-Id IgG Ab2, which will then be alum precipitated and used to vaccinate BALB/C mice for the production of anti-ricin antibodies. It is anticipated that this strategy will yield a significantly higher anti-ricin Ab3 response than obtained above.

3. Saxitoxin Polyclonal Anti-Idiotypes: Since the 2 anti-STX mAbs thus far generated had K_a values of only $10^6 M^{-1}$, and because of the availability of purified burro anti-STX IgG (provided by Dr. J. Hewetson) which has been shown to protect against STX toxicity in vitro (11), we decided to utilize this purified burro anti-STX IgG as the Ab1 preparation for the generation of anti-Id antibodies. The approach taken was similar to that discussed above with the goat anti-ricin IgG, and yielded similar results. BALB/C mice were immunized as above with protein G-purified burro anti-STX IgG. The mouse immune sera were pooled and adsorbed over a normal horse IgG-agarose column. Normal horse IgG was used because normal burro IgG is not readily available, and because the horse is probably the closest species to the burro in evolutionary terms. The adsorbed mouse sera were tested in ELISA employing wells coated with a predetermined optimum concentration (4ug/ml) of normal horse IgG or burro anti-STX IgG (Table 17). As is the case with the ricin system, the adsorbed mouse sera lost its reactivity with normal horse IgG, but reacted strongly with burro anti-STX IgG, even at a 1:10,000 dilution. As discussed with the ricin system above, the

adsorbed sera were purified by ammonium sulfate precipitation, alum precipitated and used to immunize BALB/C mice for the generation of an Ab3 anti-STX antibody response. The results obtained were similar to the ricin situation discussed above, in that no significant anti-STX activity was induced after 5 immunizations of the mouse Ab2. Therefore, we have immunized New Zealand White rabbits with the purified burro anti-STX IgG, and will take the approach described above in which we will attempt to induce a protective anti-STX antibody response in BALB/C mice by immunizing them with purified rabbit Ab2 IgG specific for the burro anti-STX IgG.

III. SUMMARY

With respect to the STX project, although mAbs with higher binding affinity than the 2 mAbs we had previously generated, have not been isolated, we have identified a source of polyclonal (burro) anti-STX IgG Ab1, which is protective in vitro. This purified burro anti-STX IgG is currently being utilized to induce rabbit Ab2 which should be able to induce in vivo an active and protective anti-STX antibody response. The mouse Ab2 generated by immunization with burro anti-STX IgG during the course of this investigation appears to be specific for the burro IgG since no reactivity was observed with normal horse IgG following extensive adsorption. The observation that this BALB/C murine Ab2 did not elicit a significant anti-STX in BALB/C mice is most likely due to the syngeneic system employed. The strategy of producing rabbit Ab2 specific for burro anti-STX IgG, which will then be used to immunize xenogeneic BALB/C mice is expected to yield an anti-STX antibody response capable of protecting against the toxicity of STX.

Important achievements have been made in our studies of ricin. A number of anti-ricin mAbs have been produced that have been incorporated into rapid and sensitive immunoasssays for the specific detection of ricin. One of these anti-ricin mAbs, namely BG11-G2, has the potential of passively protecting against the in vivo toxicity of ricin. BG11-G2 mAb, which has been conjugated to KLH, is being used to immunize BALB/C mice for the production of murine anti-Id mAb. Based on our past successful demonstration of the usefulness of the anti-Id-based vaccine approach in the mycotoxin T-2 system (3), we feel that we will isolate an anti-Id mAb with antigenic mimicry which may serve as an effective vaccine against ricin toxicity.

As with the STX system, purified murine Ab2 generated from immunization with goat anti-ricin IgG did not induce significant anti-ricin Ab3 response in BALB/C mice. However, rabbits have been immunized with goat anti-ricin IgG to produce rabbit Ab2. Preliminary results suggest that rabbit Ab2 reactive with the goat Ab1 have been produced. It is anticipated that the polyclonal rabbit Ab2 IgG would be effective in inducing in BALB/C mice an active and protective anti-ricin immune response.

We have also synthesized peptides homologous to amino acid sequences of the ricin A and B chains, and have investigated their ability to induce anti-ricin immune responses in BALB/C mice. Moreover, a single synthetic peptide (A-18) synthesized on a lysine backbone was shown to be capable of inducing an anti-ricin antibody response that at least partially protected against the in vivo tox-

icity of ricin in mice. We are optimistic that a combination subunit vaccine composed of A-18, A-95 and B-18 synthesized as MAP will serve as an effctive subunit vaccine in the induction of an active and protective anti-ricin antibody response.

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Table 1

Characterization of Monoclonal Anti-Ricin Antibodies

MAbs	Immuno-gens	Isotype	Reactivity with			%I[a] by			EL-4[b]
			W[c]	A[d]	B[e]	W	A	B	
A1	W	IgM	+[f]	+	-	2	82	2	28.8
E4	W	"	+	+	±	36	70	16	100.0
E7	W	"	+	+	±	25	90	9	98.2
D6	W	"	+	+	±	41	55	11	96.1
3F7	W	"	+	+	-	10	48	0	84.7
WR1C4	W	"	+	+	-	55	58	5	91.5
BG11-G2	W	IgG_1	+	-	-	95	5	2	100.0
A2H11	A	IgG_1	+	+	-	43	78	24	36.3
3B8	A	IgM	+	+	-	37	47	13	27.9
7F3	B	IgM	+	-	+	54	4	38	45.1
FD10	B	"	+	-	+	43	0	42	36.2
HC7	B	IgG_1	+	-	+	32	6	34	52.3
1C2	A18-KLH	IgG_{2a}	+	+	-	43	78	3	45.2
1D6	"	IgG_{2b}	+	+	-	34	93	0	29.5
4F	"	IgG_1	+	+	-	46	58	1	37.8
BD10	B18-KLH	IgM	+	-	+	36	6	67	34.2
KG7	"	IgG_1	+	-	+	64	9	73	41.8

[a]Mean of triplicate % inhibition of binding defined as [(mean OD without inhibitor) - (mean OD with 200 ug/ml inhibitor) / (mean OD without inhibitor)] x 100.
[b]Mean of triplicate % protection defined as [(mean % inhibition of [^{3}H]leucine uptake without mAb) - (mean % uptake with 50 ug/ml mAb) /(mean % uptake without mAb)] x 100.
[c]Whole ricin.
[d]A chain.
[e]B chain.
[f]Positive reactivity defined as OD 3x > control OD.

Table 2

REACTIVITY OF MICE IMMUNIZED WITH RICIN A18 PEPTIDE

Mouse No.	A18-coated wells				Whole-coated wells				A Chain-coated wells			
	3°	4°	5°	6°	3°	4°	5°	6°	3°	4°	5°	6°
100	40[a]	80	40	80	10	80	20	40	160	40	160	160
122	60	80	40	20	80	80	20	20	160	80	80	80
101 Alum	80	80	80	160	40	160	20	20	80	160	160	640
102	40	40	20	40	320	80	20	20	160	80	80	80
110	80	80	20	20	80	160	10	20	80	80	80	160
120	320	320	40	40	80	320	40	40	160	160	160	80
200	80	40	20	20	160	80	40	40	80	160	640	1280
201	40	160	20	20	40	80	40	40	160	80	320	320
210 KLH	80	80	10	10	40	80	20	20	160	80	40	40
202 Alum	80	80	10	10	40	40	20	20	80	80	40	40
220	80	160	40	40	80	80	20	20	160	80	80	80
222	-	160	20	20	80	80	40	40	80	80	80	80
310	20	40	20	20	40	40	10	10	40	80	40	40
302 KLH	20	40	10	10	40	40	10	10	80	80	20	40
301 FCA	60	160	80	80	40	80	20	20	160	160	160	160
320	40	40	10	10	40	40	10	20	80	80	40	80
300	80	80	40	80	80	160	20	20	160	160	160	160
322	80	160	320	320	40	160	40	40	80	160	1280	320

[a]Reciprocal dilution of serum giving positive reaction (defined as at least three times above the preimmune serum)

Table 3

REACTIVITY OF MICE IMMUNIZED WITH RICIN A95 PEPTIDE

Mouse No.		A95-coated wells				Ricin coated-wells				Ricin A-coated wells			
		2°	3°	4°	5°	2°	3°	4°	5°	2°	3°	4°	5°
900		40[a]	40	80	160	80	40	40	40	80	40	160	160
901		40	40	80	40	40	20	20	40	40	20	160	80
910	Alum	10	20	80	80	40	40	40	40	20	10	80	80
920		40	20	80	80	80	40	40	20	80	80	160	40
902		Mouse Died											
800		0	0	0	0	10	0	0	0	20	0	0	0
801	KLH	0	0	0	0	0	0	0	0	0	20	20	0
802	Alum	0	0	0	20	10	0	0	0	0	20	0	0
810		0	0	0	20	10	0	0	10	20	10	10	0
820		0	0	0	0	20	0	0	0	20	20	20	0
10-00		0	0	0	0	10	0	0	0	20	20	20	0
10-01	KLH	0	0	0	0	20	0	0	0	10	0	0	0
10-10	FCA	0	0	0	0	10	0	0	0	10	0	0	0
10-20		0	0	20	0	10	0	0	0	20	0	0	0
10-02		0	20	10	0	20	0	0	0	10	0	0	0
11-00		10	10	40	40	40	20	40	40	80	40	80	40
11-01		20	20	80	40	80	20	40	40	80	80	160	80
11-10	FCA	20	10	40	80	40	20	40	40	40	20	80	80
11-20		0	0	20	40	20	40	40	40	20	20	80	40
11-02		20	10	80	40	80	40	40	40	160	80	160	80

See legend of Table 2.

Table 4

REACTIVITY OF MICE IMMUNIZED WITH RICIN B230 PEPTIDE

House No.		B230-coated wells					Whole Ricin-coated wells					B-chain-coated wells				
		1°	2°	3°	4°	5°	1°	2°	3°	4°	5°	1°	2°	3°	4°	5°
401		0[a]	0	0	0	0	-	-	0	0	80	-	-	10	0	0
402	KLH	0	0	0	0	0	-	-	40	0	160	-	-	0	0	0
410	Alum	0	0	0	0	0	-	-	0	0	80	-	0	0	0	0
420		0	0	0	0	0	-	40	0	0	160	-	0	0	0	0
422		0	20	0	10	0	-		80	0	160	-	0	0	0	0
500(D)		0	80	80	160	80	-	40	0	-	320	-	0	0	0	0
502		0	0	20	80	320	-	-	40	0	40	-	-	40	0	0
510	Alum	0	0	0	80	160	-	-	40	0	40	-	-	40	0	0
520		0	0	80	40	80	-	-	20	0	10	-	20	0	0	0
522		0	0	20		320	-	-	20	0	40	-	20	0	0	0
600		0	0	0	160	160	-	-	20	0	0	-	20	0	0	0
601	KLH	0	0	0	80	640	-	-	20	0	0	-	20	0	20	0
620	FCA	0	0	20	40	160	-	-	20	0	10	-	20	0	0	0
622		0	0	0	80	160	-	-	20	0	0	-	0	0	20	0
700		0	0	0	20	80	-	-	0	0	40	-	0	0	0	0
701	FCA	0	0	0	20	320	-	-	10	0	80	-	0	0	0	0
702		0	0	0	40	160	-	-	0	0	80	-	0	0	0	0
710		0	0	0	20	320	-	-	80	0	160	-	80	0	0	0

[a]Reciprocal dilution of immune sera giving OD_{410nm} at least three times above that of the preimmune sera.

Table 5

Reactivity of Mice Immunized with Ricin B-18-MAP

Mouse No.	Reactivity with							
	Whole Ricin				B Chain			
	1°*	2°	3°	4°	1°	2°	3°	4°
in PBS:								
A-00	40[a]	80	80	160	80	320	160	640
A-10	0	160	160	320	0	40	160	1280
A-02	20	40	320	1280	40	320	1280	10000
A-11	20	80	640	1280	20	640	1280	10000
A-20	20	ND	640	640	80	ND	640	1280
in Alum:								
B-00	10	160	640	10000	20	320	2560	>10000
B-10	20	320	640	>10000	20	320	640	>10000
B-11	40	640	640	5120	40	320	640	>10000
B-02	10	320	1280	5120	20	640	1280	>10000
B-20	20	160	320	2560	40	640	640	>10000
in FCA:								
C-00	0	0	20	640	0	20	320	1280
C-10	0	40	160	1280	0	20	640	>10000
C-11	0	40	320	1280	20	80	640	>10000
C-02	0	20	160	1280	0	20	320	10000
C-20	0	20	20	640	0	20	40	>10000
in FIA:								
D-00	0	0	80	160	0	0	640	10000
D-10	20	20	80	>10000	40	40	1280	>10000
D-11	0	20	160	640	0	40	640	>10000
D-20	0	ND	160	1280	0	ND	640	>10000

*Two-week bleed post number of injections indicated.
[a]Reciprocal dilutions giving positive reactivity defined as OD>3x preimmune OD.

Table 6

Reactivity of Mouse Sera Immunized with A-18-MAP

Mouse No.	Ricin A Chain				Whole Ricin			
	1°	2°	3°	4°	1°	2°	3°	4°
	Group 1: A-18-MAP in Alum							
1-00	500[a]	500	5000	20000	500	500	5000	20000
1-01	50	500	5000	10000	50	50	500	5000
1-10	50	5000	5000	10000	50	500	500	5000
1-11	50	500	5000	20000	50	500	500	5000
1-02	50	5000	5000	5000	50	500	500	5000
1-20	50	5000	5000	20000	50	500	500	20000
	Group 2: A-18-MAP in FCA							
2-00	50	500	5000	5000	500	500	500	10000
2-01	50	500	500	5000	50	500	500	5000
2-10	50	500	1000	10000	500	500	500	5000
2-11	50	500	5000	20000	500	500	5000	5000
2-02	50	500	500	5000	500	500	500	5000
2-20	50	500	5000	20000	500	500	5000	10000

[a]Mean reciprocal dilution of duplicate determinations.
Positive reactivity on B chain-coated wells ranged around 1:20 dilution for all sera.

Table 7

Reactivity of Mouse Sera Immunized with B-230-MAP

Mouse No.	Ricin B Chain				Whole Ricin			
	1°	2°	3°	4°	1°	2°	3°	4°
			Group 3: B-230-MAP in Alum					
3-00	0[a]	50	500	500	0	50	500	200
3-01	0	500	5000	2000	0	50	500	500
3-10	0	50	500	500	0	50	500	200
3-11	0	0	50	200	0	0	50	200
3-02	0	50	Mouse died		0	50		
3-20	0	50	500	500	0	50	500	500
			Group 4: B-230-MAP in FCA					
4-00	0	0	0	0	0	0	0	100
4-01	0	0	20	10	0	0	0	100
4-10	0	0	0	0	0	0	50	100
4-11	0	0	50	100	0	0	50	100
4-02	0	0	0	100	0	0	0	100
4-20	0	0	0	0	0	0	50	100

[a]Mean reciprocal dilution of duplicate determinations.
No reactivity on A chain-coated wells was obtained for all sera.

Table 8

Reactivity of Mouse Sera Immunized with A-18-MAP and B-230-MAP (Group 5)

Mouse No.	Ricin A Chain			Ricin B Chain			Whole Ricin		
	1°	2°	3°	1°	2°	3°	1°	2°	3°
5-00	50[a]	5000	5000	0	0	50	0	500	5000
5-01	50	5000	5000	0	0	50	50	500	5000
5-10	50	5000	5000	0	0	50	0	500	500
5-11	50	2000	5000	0	50	500	0	500	5000
5-02	50	5000	5000	0	0	50	0	500	2000
5-20	50	500	50000	0	0	50	0	50	1000

Cont'	Ricin A Chain	Ricin B Chain	Whole Ricin
	4°	4°	4°
5-00	20000	200	20000
5-01	20000	100	10000
5-10	5000	50	5000
5-11	2000	50	5000
5-02	20000	20	10000
5-20	20000	200	5000

[a]Mean reciprocal dilution of duplicate determinations.

Table 9

Reactivity of Mouse Sera Immunized with A-18-MAP and B-18-MAP (Group 6)

Mouse No.	Ricin A Chain			Ricin B Chain			Whole Ricin		
	1°	2°	3°	1°	2°	3°	1°	2°	3°
6-00	50[a]	500	5000	50	500	5000	50	500	5000
6-01	500	500	5000	500	2000	5000	500	500	5000
6-10	50	5000	10000	50	5000	10000	50	500	10000
6-11	50	500	5000	50	5000	10000	20	500	5000
6-02	50	500	5000	50	500	5000	50	1000	5000
6-20	20	500	5000	50	5000	50000	50	500	5000

Cont'

	4°	4°	4°
6-00	5000	10000	5000
6-01	20000	20000	10000
6-10	20000	20000	10000
6-11	20000	50000	20000
6-02	5000	20000	10000
6-20	10000	20000	20000

[a]Mean reciprocal dilution of duplicate determinations.

Table 10

Reactivity of Mouse Sera Immunized with B-18-MAP and B-230-MAP (Group 7)

Mouse No.	Ricin B Chain				Whole Ricin			
	1°	2°	3°	4°	1°	2°	3°	4°
7-00	10[a]	1000	5000	20000	50	500	5000	10000
7-01	50	500	5000	10000	20	200	2000	5000
7-10	500	1000	10000	20000	50	500	5000	5000
7-11	50	500	5000	20000	20	500	5000	10000
7-02	500	500	10000	50000	500	500	5000	10000
7-20	50	1000	5000	20000	50	200	5000	10000

[a]Mean reciprocal dilution of duplicate determinations.
No significant reactivity was observed with ricin A chain-coated wells (data not shown).

Table 11

Reactivity of Mouse Sera Immunized with A-18-, B-18- and B-230-MAP (Group 8)

Mouse No.	Ricin A Chain			Ricin B Chain			Whole Ricin		
	1°	2°	3°	1°	2°	3°	1°	2°	3°
8-00	50[a]	200	10000	500	5000	5000	50	500	5000
8-01	20	500	5000	200	5000	5000	500	500	5000
8-10	50	500	20000	50	500	10000	50	500	5000
8-11	20	1000	5000	50	5000	5000	50	200	2000
8-02	50	500	5000	50	1000	5000	50	1000	5000
8-20	50	500	10000	50	2000	10000	50	500	2000

Cont'	4°	4°	4°
8-00	10000	20000	5000
8-01	50000	20000	10000
8-10	50000	20000	5000
8-11	20000	10000	10000
8-02	20000	20000	20000
8-20	20000	50000	2000

[a]Mean reciprocal dilution of duplicate determinations.

Table 12

In Vivo Challenge of MAP-A18 Immune Mice

Whole ricin (ug/mouse)	# Dead mice/total #		Elapsed time[a]	
	Nonimmune	Immune	Nonimmune	Immune
5.0	5/5	6/6	1/26hrs[b] 2/29hrs 1/31hrs 1/46hrs	1/20hrs 5/46hrs
1.0	4/5	3/7	1/26hrs 2/46hrs 1/52hrs	1/54hrs 1/72hrs 1/118hrs
0.2	0/5	0/7		

[a]Time between ricin administration and death
[b]Number of dead mice/elapsed time

Table 13

Passive Protection Against Ricin In Vivo Toxicity

Ricin (ug/mouse)	No. Dead mice/Total No.		Elapsed time[a]	
	Untreated	Treated[b]	Untreated	Treated
5.0	5/5	5/5	3/20[c] 2/23	2/95 3/115
2.5	5/5	5/5	2/23 1/25 1/26 1/48	1/122 1/137 1/139 1/144 1/161

[a]Time between ricin administration and death
[b]i.p. injection of 10 mg of purified BG11-G2 in PBS as described in text
[c]Number of dead mice/elapsed time in hours

Table 14

Reactivity of Mice Immunized with Monoclonal A2H11 Anti-Ricin

Serum dilution	Mouse Nos.							
	0,0		2,2		0,2		2,0	
	PI[a]	5°	PI	5°	PI	5°	PI	5°
1:10	.05[b]	.29	.04	.19	.06	.17	0	.14
1:20	.03	.08	0	.05	0	.06	0	.02

[a]Preimmune sera; 5° indicates 2 weeks post the 5th immunization
[b]Mean of duplicate determinations
The sandwich ELISA assay was done as described in the report

Table 15

Rabbit Anti-Ricin IgG Protect Against Ricin *In Vitro* Tocxicity

Rabbit X-840 IgG (ug/ml)	EL4 Cells Cultured (cpm)		%I[a]
	Without Ricin	With Ricin[b]	
None	101,512 ± 4,872[c]	48,521 ± 3,267	52.2
Anti-Ricin IgG			
(20.0)	98,045 ± 5,001	102,156 ± 4,913	0
(0.2)	103,527 ± 7,109	88,937 ± 3,296	14.1
(0.02)	95,842 ± 4,901	65,912 ± 3,902	31.2
(0.005)	99,390 ± 3,956	54,937 ± 2,917	44.7
Preimmune IgG			
(20.0)	95,684 ± 4,178	50,034 ± 3,290	47.7
(10.0)	101,256 ± 5,298	47,998 ± 4,712	52.6

[a]Percent inhibition of [^{3}H]leucine uptake was determined using the following formula: %I = [(mean cpm without ricin - mean cpm with ricin)/(mean cpm without ricin)] x 100.
[b]The final concentration of ricin used was 6.0ng/ml.
[c]Mean cpm of triplicate determinations.

Table 16

Anti-Id Reactivity of Mice Immunized with Goat Anti-Ricin IgG

Dilution of adsorbed sera	Wells coated with	
	Normal goat IgG	Anti-ricin IgG
1:10	0.03[a]	0.64
1:20	0	0.68
1:40	0	0.66
1:80	0	0.61
1:160	0	0.44
1:320	0	0.47
1:640	0	0.28
1:1280	0	0.29
1:2560	0	0.11
1:5120	0	0.05

[a]Mean of duplicate determinations

Table 17

Anti-Id Reactivity of Mice Immunized with Burro Anti-Saxitoxin IgG

Dilution of adsorbed sera	Wells coated with IgG from	
	Normal horse	Burro anti-saxitoxin
1:10	0.05[a]	1.11
1:20	0.03	1.13
1:40	0	1.03
1:80	0	0.99
1:160	0	0.86
1:320	0	0.79
1:640	0	0.78
1:1280	0	0.64
1:2560	0	0.51
1:5120	0	0.36
1:10240	0	0.23
1:20480	0	0.10

[a]Mean of duplicate determinations

FIGURE LEGENDS

Fig. 1. Amino acid sequence and physical/chemical characteristics of ricin A-18 synthetic peptide.
Fig. 2. Amino acid sequence and physical/chemical characteristics of ricin B-18 synthetic peptide.
Fig. 3. Amino acid sequence and physical/chemical characteristics of ricin A-95 synthetic peptide.
Fig. 4. Amino acid sequence and physical/chemical characteristics of ricin B-230 synthetic peptide.
Fig. 5. General schematic structure of multiple antigenic peptides (MAP). B-18-MAP is shown.
Fig. 6. Survival curves of BALB/C mice challenged with 1.0 ug of ricin/mouse.
Fig. 7. General scheme of the anti-idiotype-based vaccine approach for toxins.

RICIN A-18 PEPTIDE

SEQUENCE:

N-Terminus-C-V-Q-S-Y-T-N-F-I-R-A-V-R-L-T-T-G-A-D-V-R-COOH-Terminus

AMINO ACID ANALYSIS:

Asp	1	Leu	1
Asn	1	Nle	0
Thr	3	Tyr	1
Ser	1	Phe	1
Glu	0	His	0
Gly	2	Orn	0
Ala	2	Lys	0
Cys	1	Arg	4
Val	3	Pro	0
Met	0	Trp	0
Ile	1		

CHARACTERISTICS:

Molecular Weight:	2584.3
Extinction Coefficient:	0.6
Isoelectric Point:	9.2

Figure 1

RICIN B-18 PEPTIDE

SEQUENCE:

N-Terminus-G-L-C-V-D-V-R-D-G-R-F-H-N-G-N-A-I-Q-L-W-P-C-K-S-N-T-D-A-N-

Q-L-G-G-COOH-Terminus

AMINO ACID ANALYSIS:

Asp	3	Leu	3
Asn	4	Nle	0
Thr	1	Tyr	0
Ser	1	Phe	1
Glu	0	His	1
Gln	2	Pap	0
Gly	5	Orn	0
Ala	2	Lys	1
Cys	2	Arg	2
Val	2	Pro	1
Met	0	Trp	1
Ile	1		

CHARACTERISTICS:

Molecular Weight:	3557.4
Extinction Coefficient:	1.6
Isoelectric Point:	7.3

Figure 2

RICIN A-95 PEPTIDE

SEQUENCE:

N-Terminus-C-P-D-N-Q-E-D-A-E-A-I-T-H-L-F-T-D-V-Q-N-R-Y-T-F-A-F-G-

COOH-Terminus

AMINO ACID ANALYSIS:

Asp	3	Leu	1
Asn	2	Nle	0
Thr	3	Tyr	1
Ser	0	Phe	3
Glu	2	His	1
Gln	2	Pap	0
Gly	1	Orn	0
Ala	3	Lys	0
Cys	1	Arg	1
Val	1	Pro	1
Met	0	Trp	0
Ile	1		

CHARACTERISTICS:

Molecular Weight:	3103.7
Extinction Coefficient:	0.6
Isoelectric Point:	5.4

Figure 3

RICIN B-230 PEPTIDE

SEQUENCE:

N-terminus-C-G-L-V-L-D-V-R-R-S-D-P-S-L-K-Q-I-I-L-Y-P-L-Y-P-L-H-G-D-P-

N-Q-G-COOH-Terminus

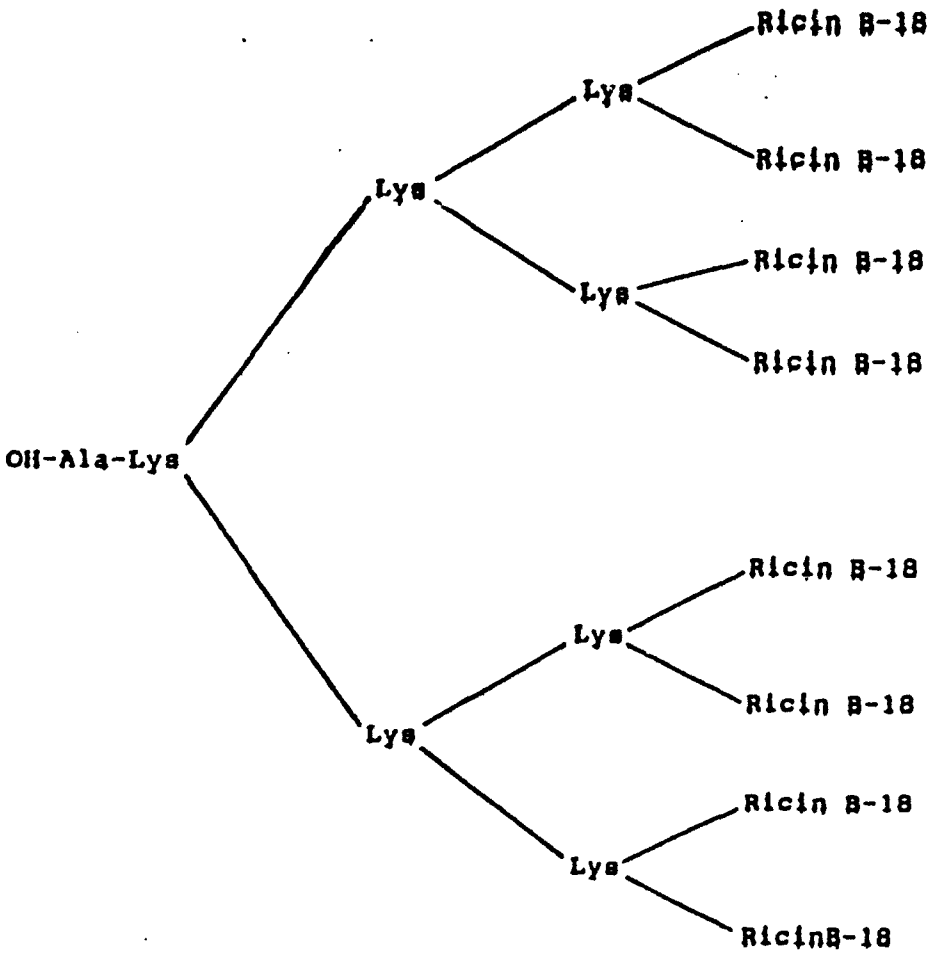
AMINO ACID ANALYSIS:

Asp	3	Leu	5
Asn	1	Nle	0
Thr	0	Tyr	1
Ser	2	Phe	0
Glu	0	His	0
Gln	2	Phe	0
Gly	3	Orn	0
Ala	0	Lys	1
Cys	1	Arg	2
Val	2	Pro	3
Met	0	Trp	0
Ile	2		

CHARACTERISTICS:

Molecular Weigth:	3205.1
Extinction Coefficient:	0.44
Isoelectric Point:	7.1

Figure 4



General Structure of HAP.B-18

Figure 5

Survival Curves of Mice Challenged with 1.0 ug Ricin

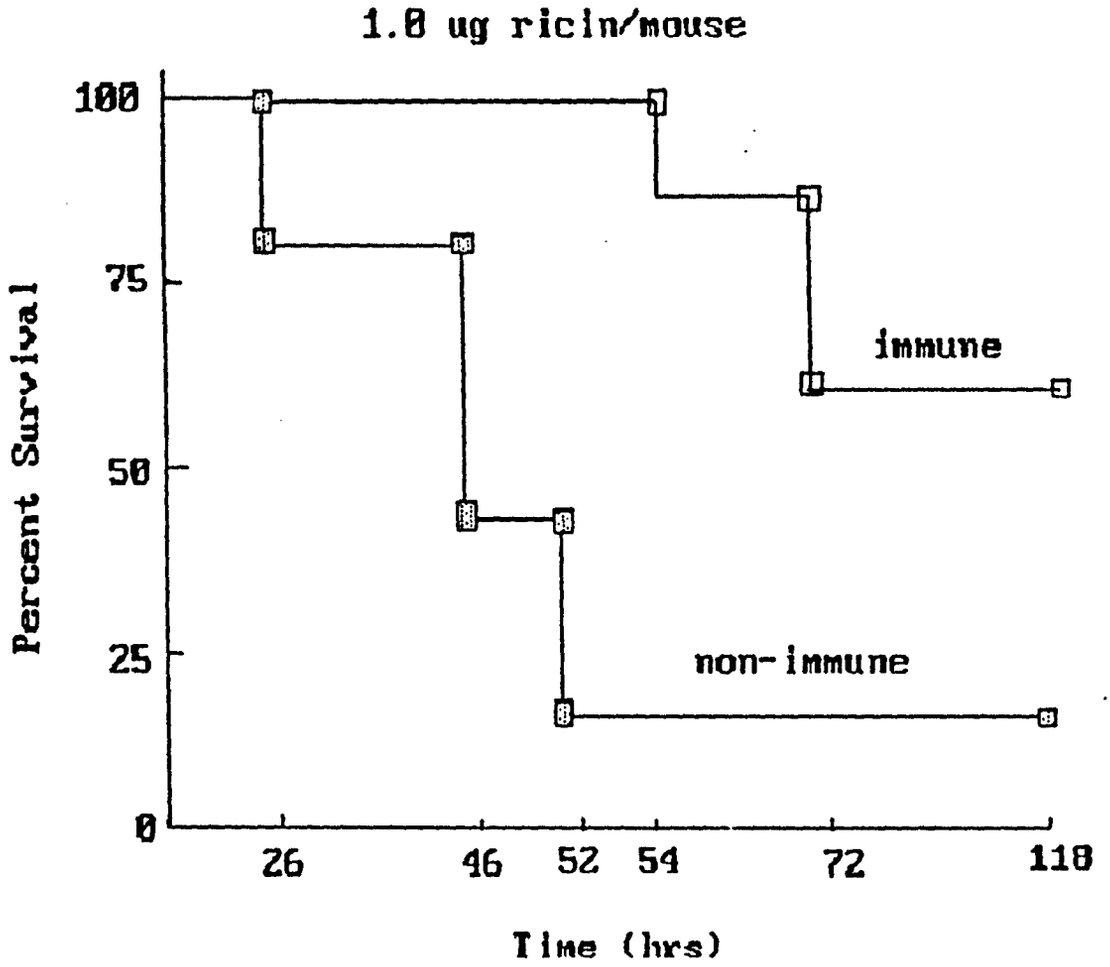
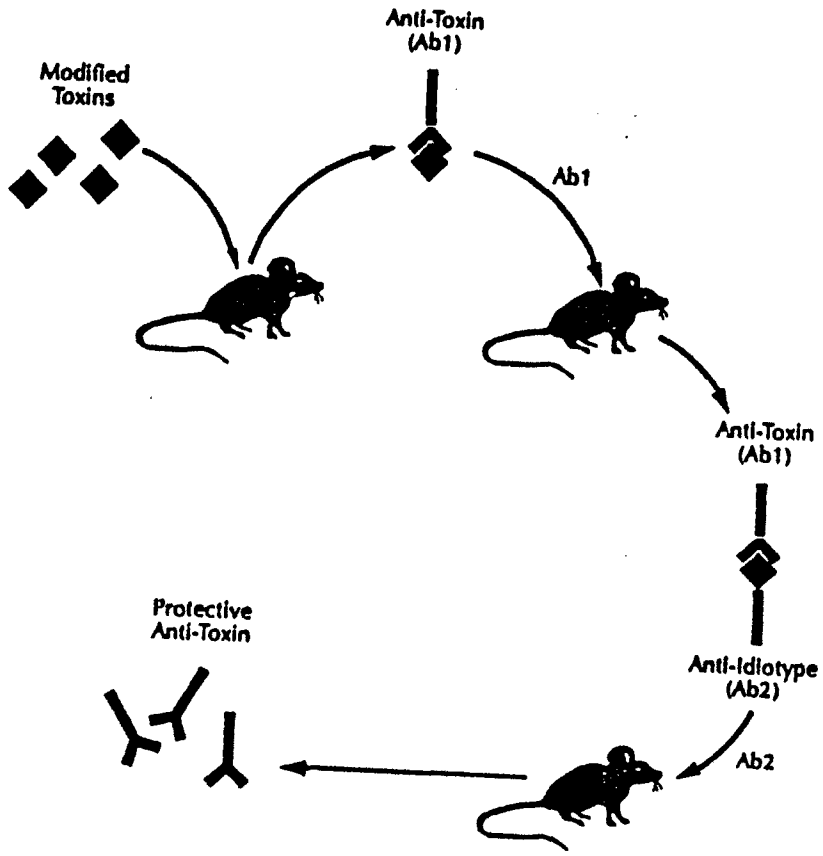


Figure 6



General scheme for producing anti-idiotype vaccines against toxins

Figure 7

PUBLICATIONS

Abstracts:

1. Chanh, T.C., Amrstrong, D.L., and Hewetson, J.F. Anti-idiotype vaccine against biological toxins. 5th Intern. Conf. Immunopharmacol. Tampa, FL. May 26-30, 1991.

Manuscripts:

1. Chanh, T.C., Rappacciolo, G., and Hewetson, J.F. Monoclonal anti-idiotype induces protection against the cytotoxicity of the trichothecene mycotoxin T-2. J. Immunol. 144:4721-4728, 1990.

2. Chanh, T.C., Siwak, E.B., and Hewetson, J.F. Anti-idiotype-based vaccine against biological toxins. Toxicol. Appl. Pharmacol. 108:183-193, 1991.

3. Chanh, T.C., and Hewetson, J.F. Structure/function studies of T-2 mycotoxin with a monoclonal antibody. Immunopharmacol. 21:83-90, 1991.

4. Chanh, T.C., Kennedy, R.C., and Hewetson, J.F. Anti-idiotype vaccine in toxicology. Int. J. Clin. Lab. Res., In press.

ADDITIONAL INFORMATION

List of personnel receiving pay from this contract:

T. Chanh, Ph.D.	35%	
P. Kanda, Ph.D.	5%	
A. Delgado, B.S.	100%	
M. Romanowski, B.S.	100%	
S. Trevino, B.S.	100%	
D. Armstrong, Ph.D.	5%	(UTSA)

No graduate degrees were awarded to personnel working on this contract.

Copies of the first three reprints published listed on page 48 of the final report are enclosed for your information.