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CONTRACT NO: DAMD17-91-C-1108

TITLE: PRODUCTION AND CHARACTERIZATION OF AN AVIAN RICIN ANTITOXIN

PRINCIPAL INVESTIGATOR: Douglas C. Stafford, Ph.D.

CONTRACTING ORGANIZATION: Ophidian Pharmaceuticals, Inc.
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13. ABSTRACT (Maximum 200 words)

This study was designed to demonstrate the feasibility of producing a ricin antitoxin consisting of highly purified specific avian antibodies. Antitoxins consisting of avian antibodies have significant advantages when compared to conventional mammalian (e.g., horse) antibodies; a) avian antibodies do not fix human complement, eliminating the associated risk of inflammatory reactions, and, b) avian antibodies can be manufactured more economically. This study also utilized affinity chromatography to increase antibody purity, increasing effectiveness and reducing the risk of serum sickness in the patient. Ricin toxoid produced by formalin treatment was injected into laying hens followed by collection of eggs bearing hyperimmune antibody. Antibody was extracted from the yolks and assayed by enzyme immunoassay. When suitable antibody was measured in the eggs, extracted immunoglobulin was further purified by affinity chromatography using immobilized ricin. Electrophoresis showed that the purified antibodies were >99%

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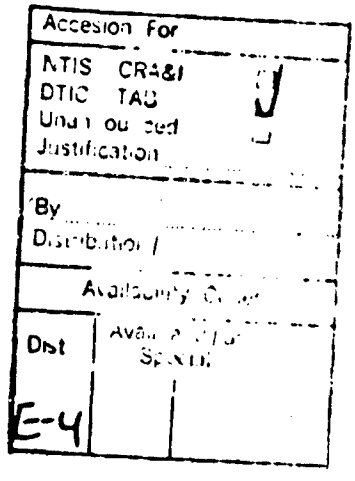
immunoglobulin. The affinity purified antibodies were evaluated in a mouse model for toxin-neutralization, showing that mice given a lethal dose of ricin were rescued by the specific antibody at low doses. Highly purified therapeutic antibodies, as produced in this study, should offer enhanced clinical effectiveness, greater safety, and reduced manufacturing costs as compared to other technologies.



U.S. DEPARTMENT OF DEFENSE

APPENDIX B

SMALL BUSINESS INNOVATION RESEARCH (SBIR) PROGRAM
PROJECT SUMMARY

TOPIC NUMBER: A91-027

PROPOSAL TITLE: Production and Characterization of an Avian Ricin Antitoxin

FIRM NAME: Ophidian Pharmaceuticals, Inc.

PHASE I or II PROPOSAL: I

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

This study was designed to demonstrate the feasibility of producing a ricin antitoxin consisting of highly purified specific avian antibodies. Antitoxins consisting of avian antibodies have significant advantages when compared to conventional mammalian (e.g., horse) antibodies; a) avian antibodies do not fix human complement, eliminating the associated risk of inflammatory reactions, and, b) avian antibodies can be manufactured more economically. This study also utilized affinity chromatography to increase antibody purity, increasing effectiveness and reducing the risk of serum sickness in the patient. Ricin toxoid produced by formalin treatment was injected into laying hens followed by collection of eggs bearing hyperimmune antibody. Antibody was extracted from the yolks and assayed by enzyme immunoassay. When suitable antibody was measured in the eggs, extracted immunoglobulin was further purified by affinity chromatography using immobilized ricin. Electrophoresis showed that the purified antibodies were >99% immunoglobulin. The affinity purified antibodies were evaluated in a mouse model for toxin-neutralization, showing that mice given a lethal dose of ricin were rescued by the specific antibody at low doses. Highly purified therapeutic antibodies, as produced in this study, should offer enhanced clinical effectiveness, greater safety, and reduced manufacturing costs as compared to other technologies.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

Technology utilized in this study has wide application in developing antitoxins and antivenoms against a wide variety of threats. This technology should make a greater number of clinically superior antitoxins available for both military and civilian use, especially for rare poisonings, because of reduced development/production costs.

List a maximum of 8 Key Words that describe the Project.

Ricin	Affinity chromatography
Antitoxin	Avian
Antidote	Immunoglobulin

Problem Area to be Studied

Because of the potential use of inexpensive naturally-occurring plant and/or bacterial toxins as biological threat agents, effective antitoxins are needed for either prophylactic or causal immunotherapy. A polyspecific antitoxin composed of high purity polyclonal antibodies would provide an important defense against a variety of possible agents or against a single agent that might not be readily identified in hostile field environments.

To fulfill requirements for purity, effectiveness, potential for polyspecificity, and manufacturing economy, the feasibility of an avian antibody production system will be studied. As a model for this system, an avian antitoxin against the potent phytotoxin, ricin, will be developed and evaluated.

The production of therapeutic antibodies in avian species (e.g., chickens) coupled with affinity purification methods offers distinct manufacturing and clinical advantages as compared to conventional mammalian antibody production methods, including:

a) Production costs: the laying hen is more efficient than large mammals, resulting in the production of hyperimmune antibody at a significantly lower cost than mammalian systems. Efficiencies include lower immunogen consumption and higher antibody output (based on the yield of antibody per body weight).

b) Development costs: because chickens are less expensive to purchase, maintain, and hyperimmunize than large mammals, prototype development and process optimization are far simpler and more economically feasible.

c) Reduced side effects: therapeutics derived from mammalian antibody present the risk of serious inflammatory side effects due to non-specific complement fixation. This problem is often addressed by enzymatically removing the antibody Fc fragment by proteolytic digestion, which reduces the final product yield and adds to manufacturing cost. Because chicken antibody does not fix human complement this risk is not present and Fc removal unnecessary.

d) Serum sickness: antitoxins that are derived by fractionation of hyperimmune animal plasma often cause serum sickness due to the large amounts of protein impurities. The use of affinity purification methods can yield therapeutics that are >99% specific immunoglobulin and therefore drastically reduce the risk of serum sickness.

e) Final product formulation: various affinity purified monospecific polyclonal antibody preparations can be separately produced which can then be blended to form the desired polyspecific antitoxin product.

The Model System

Ricin has been described as one of the five most toxic materials known (tetanus toxin, botulinum toxin, diphtheria toxin, and gramicidin are the others), with its lethal effects described in the scientific literature as early as the 1880's. Animal studies have shown that the minimum lethal dose of ricin in laboratory animals is extremely low; 0.35-0.5 ug/Kg in rats, 0.4-0.5 ug/Kg in guinea pigs, and 0.3-0.6 ug/Kg in rabbits. In Dogs, the minimum lethal dose is within the range of 1.6-1.75 ug/Kg. In man, a dose of between 0.05 and 0.1 mg is considered very dangerous with lethality occurring with subcutaneous administration of 3 mg of purified ricin. Lethal doses of ricin may also result from the ingestion of as few as eight castor oil seeds.

Ricin is a member of a group of at least 20 plant toxins that have been described which inhibit the translation of proteins in eukaryotic cells. Other toxins in this group include abrin, viscumin and modeccin, and are isolated from different plants yet have common biochemical structure, including similar molecular weight and subunit structure. The A subunits of these four toxins all enzymatically inactivate 60S ribosomal subunits and the B subunits all share galactosyl lectin activity. However, analysis of the neutralizing ability of antisera against these toxins has shown that they are immunochemically distinct. Therefore, even though there are structural and functional similarities among these toxins, neutralizing antibody raised against one toxin may not be useful in neutralizing other related toxins. An antitoxin that neutralizes a wide range of these toxins would require multiple antibody specificities found in a polyclonal serum raised against the target toxins.

Biochemical analysis has shown that ricin is a heterodimer consisting of two glycoprotein chains (A and B) linked by a disulfide bond and strong hydrophobic interactions. The 32,000 dalton B chain is a lectin having binding specificity for galactosides found in complex carbohydrates (such as on the surface of cells) and as simple sugars. It is believed that the B chain binds to the surface of target cells with galactosyl receptors and triggers endocytotic uptake of the toxin followed by deposition into the cytosol. Once deposited into the cytosol, the 32,000 dalton A chain becomes free from the B chain where it acts by interrupting the cells protein synthesis mechanism.

This is accomplished by inactivation of the 60S ribosomal subunit by modification of 28S ribosomal RNA by specific endonuclease activity of the A chain. It has been reported that the disruption of protein synthesis by the internalization of a single molecule of ricin A chain can result in death of the cell.

Purified ricin has been shown to be immunogenic in various research animals. The most widely reported methods for the generation of antiricin serum in animals is by immunization with a formalin toxoid of whole (i.e., both chains) ricin typically mixed with adjuvant. Furthermore, monoclonal toxin-neutralizing antibodies have been produced from spleen cells obtained from mice hyperimmunized with ricin toxoid.

The avian antitoxin system has applicability for the development and large-scale production of a wide number of antitoxins. The development of a ricin antitoxin followed by demonstration of its effectiveness in animals should serve as a model for the further development of antitoxins against a wide range of toxic substances. Because ricin *a)* is a highly characterized protein, *b)* can be made into an immunogenic toxoid, and *c)* has been shown to elicit neutralizing antisera in numerous research animals, it should serve as a highly useful model for demonstrating the effectiveness of the avian system against toxins that act in extremely low doses. Once proved using the ricin model, development in Phase II could then be expanded to include the production of multi-specific toxin-neutralizing antitoxins with increasingly broader specificities and/or the large-scale development of a single antitoxin, such as antiricin.

This research project will include the following activities:

a) preparation of ricin toxoid followed by immunization of laying hens,

b) purification of ricin-specific antibodies using affinity purification methods,

c) immunochemical analysis of the resulting antibodies,

d) analysis of the toxin neutralization ability of the purified antitoxin using standard mouse protection assays.

Work Plan

The project was broken into a sequence of several discrete steps as described in the PERT and Gantt charts shown in ATTACHMENT A. Completion of each of these steps was reported to USAMRAA and

USAMRIID in the preceding five monthly technical reports. All elements of the project were successfully completed on time, as projected in the attached plans.

As required in the SBIR Phase I contract, a sample of affinity purified avian antiricin antibodies was supplied to the Department of the Army (LTC Paul Lemley, Pathophysiology Division, USAMRIID, Fort Detrick) on 17 December 1991.

Materials and Methods

Ricin was obtained from Sigma Chemical Company as purified intact Ricin (RCA_{60}) in aqueous buffer. Sigma lot no. 109F-4025 containing ricin at 3.4 mg/mL was used throughout this study.

Toxoid preparation: Biologically active ricin was brought to 0.6 mg/mL in 50 mM potassium phosphate, pH 7.5, containing 0.9% sodium chloride (PBS). To this solution, 1% (volume/volume) formaldehyde was added and held at 37±1 C for 96 hours. Following formaldehyde treatment, the reaction mixture was dialyzed twice, each dialysis step using 100 volumes PBS for 24 hours at 10 C. The toxoid was concentrated by ultrafiltration using a 10,000 molecular weight cutoff membrane. Approximately 20 mg of toxoid was produced in a single batch.

Toxoid safety tests: In order to comply with the Biological Safety and Animal Use and Care requirements of the University of Wisconsin-Madison, where chickens were housed for this study, a residual toxicity test is required prior to injection of the chickens. This was accomplished by injecting five mice (C57B/6, weighing approximately 20-25 grams, i.p. injection) with toxoid as prepared above representing at least 50 mouse lethal doses of unmodified ricin. These mice showed no obvious signs of intoxication for an observation period of five days. This indicated that at least 98% of the toxicity was removed by the formaldehyde treatment. These results were reported to the University of Wisconsin-Madison Biological Safety Officer where it was determined that chicken immunization with the toxoid could commence.

Chicken immunization: Laying Leghorn hens were obtained and allowed to acclimate to housing conditions (at least 7 days prior to initiation of immunization). Three groups of hens were given immunizing doses of ricin toxoid emulsified with Freund's adjuvant, as shown in TABLE 1. Toxoid in PBS was mixed with an equal volume of adjuvant and emulsified prior to subcutaneous and intramuscular injection. The choice of the immunizing doses reflects our experience in obtaining high titers of chicken yolk antibody using other protein

antigens, in addition to doses reportedly used for the production of ricin antibodies in rabbits (refer to studies by Godal, Olsnes, and Phil, *Radioimmunoassays of Abrin and Ricin in Blood*, J. Toxicol. and Environ. Health, 8:409, 1981, and, Pappenheimer, Olsnes, and Harper, *Lectins from Abrus precatorius and Ricinus communis*, J. Immunol. 113:835, 1974).

Egg yolk antibody extraction: All antibody preparations, including the pre-immune sample, were isolated from egg yolk using our standard immunoglobulin extraction procedure, as follows. After cracking the eggs (leaving the yolk intact), a gentle stream of deionized water from a squirt bottle was used to separate the yolks from the whites, and then the yolks were broken by dropping them through a funnel into a graduated cylinder. The yolks were blended with 4 volumes of egg extraction buffer (10 mM sodium phosphate, 100 mM sodium chloride, pH 7.5), and polyethylene glycol (PEG) 8000 (Amresco) was added to a concentration of 3.5%. When all the PEG dissolved, the solution was centrifuged at 9000 x g for 10 minutes to pellet the the protein precipitate. The supernatant was filtered through cheesecloth to remove the lipid layer, and PEG was added to a final concentration of 12% (assuming the supernatant was 3.5%). After centrifugation the supernatant was discarded and the pellet centrifuged twice more to extrude the PEG. The crude IgG pellet was dissolved in the original yolk volume in egg extraction buffer (plus 0.005% thimerosal as preservative) and stored at 2-8 C. This material was analyzed by electrophoresis and enzyme immunoassay (EIA) and subjected to further purification.

Affinity purification: An affinity column was constructed by pouring 6 mL of 4% beaded agarose coupled to native ricin (Sigma) into a glass chromatography support (Bio-Rad, Econocolumn). The column had a bed volume of approximately 6 mL and contained 1.5-3.0 mg ricin/mL of packed resin. The column was washed with PBS and stored in PBS containing 0.005% thimerosal and 0.1 M lactose at 2-8 C, until needed.

PEG extracted immunoglobulin was loaded over the column at a flow rate of 1-2 ml/min. A 1 ml aliquot of the flow-through fraction was collected near the end of the run and saved for analysis by EIA. The column was washed with PBS/thimerosal/lactose to remove any unbound antibody followed by a PBS wash. Non-specifically bound proteins were eluted from the column using BBS-Tween (0.1M boric acid, 0.025M sodium borate, 1M sodium chloride, and 0.1% Tween-20, pH 8.3). The BBS-Tween was washed out of the column with PBS, and specific antiricin antibodies were eluted with 4M guanidine-HCl, pH 8.0. The eluted antibodies were promptly dialyzed against 200 volumes of PBS/thimerosal at 2-8 C over the next 48 hours.

Enzyme immunoassay (EIA): Each well of a 96 well immunoassay plate (Falcon, PRO-BIND) was coated with 180 uL of ricin toxoid diluted in PBS, at a concentration of approximately 2.5 ug/ml. After incubation for at least 2 hours at room temperature, the toxoid coating solution was removed and the wells were filled with blocking solution (PBS containing 0.1% bovine serum albumin and 0.1 M lactose) and allowed to incubate overnight at 2-8 C. Primary antibodies were diluted in a wash/diluent buffer (PBS containing 0.05% Tween 20, 0.05% bovine serum albumin, and 0.025M lactose) and added at 135 uL per assay well. Following incubation at room temperature for 2 hours, the wells were washed 3 times with wash/diluent buffer. Next, 100 uL of a 1:500 dilution (in wash/diluent buffer) of alkaline phosphatase conjugated goat anti-chicken IgG (Sigma) was added to the assay wells and allowed to incubate at room temperature for 2 hours. Following washing of the assay wells with wash/diluent buffer, 175 uL of alkaline phosphatase substrate (Sigma) at 1 mg/mL was added to each well. The plates were incubated at room temperature for 15-30 minutes and color development was measured at 410 nanometers using an EIA plate reader (Dynatech).

Polyacrylamide gel electrophoresis (PAGE): Acrylamide gels were prepared according to methods described by Laemmli (Nature, 227, 1970) and electrophoresis was performed using the Bio-Rad Mini-Protean II system. Antibody samples were dissolved in sample buffer containing sodium dodecyl sulfate (SDS) and 2-mercapto-ethanol, and were heated at 95 C for 5 minutes prior to loading onto 0.5mm thick, 12.5% acrylamide gels. Following electrophoretic separation, the polypetide bands were visualized by staining with coomassie brilliant blue and destaining with methanol/acetic acid. Antibody purity was determined by optical scanning of the stained gels using an ISCO gel scanner.

Neutralization of ricin toxicity in mice: Antibody neutralization of ricin toxicity was demonstrated using a modification of methods previously described (from standard antivenom neutralization methods described in, Progress in the Characterization of Venoms and Standardization of Antivenoms, World Health Organization, 1981, and, Thalley and Carroll, BioTechnology, 10:394, 1990). Briefly, a lethal dose of ricin was mixed with antitoxin over a range of concentrations and allowed to incubate for 10 minutes at room temperature. The experiment was performed as follows:

Mouse Group	Affinity Purif. Antiricin Antibody (ug)	Non-immune Egg Ig (ug)	Ricin (ug)	Injection Volume (uL)
1	0.00	12.00	0.6	250
2	0.12	11.88	0.6	250
3	0.24	11.76	0.6	250
4	0.60	11.40	0.6	250
5	1.2	10.80	0.6	250
6	2.4	9.60	0.6	250
7	6.0	6.00	0.6	250
8	12.0	0.00	0.6	250
9	0.00	12.00	0.0	250

To control for possible neutralization of ricin by avian antibody protein and to control for the total amount of protein in the reaction mixtures, non-immune (normal) purified egg immunoglobulin was incorporated into the reaction mixtures, in the amounts indicated. The ricin/antitoxin mixtures where then injected intraperitoneally into groups of mice (BALB/c either sex) weighing 25-35 grams, followed by observation for signs of animal distress and death. It was determined from previously published results that mice ranging from 25-35 grams would be lethally intoxicated with a ricin dose of at least 0.5 ug. Therefore, experiments to confirm lethal doses (LD_{50}) were not replicated for this study, in conformance with Department of the Army contract requirements stating that "this protocol is not an unnecessary duplication of previous [animal] experiments." Animals were given food and water *ad libitum* during the observation period.

In-vivo ricin neutralization: Groups of BALB/c mice weighing from 25-35 grams were injected intravenously with various amounts of antitoxin followed by an intraperitoneal injection of a lethal dose of ricin. The experiment was performed as follows:

Mouse Group	Affinity Purif. Antiricin Antibody (ug)	Intravenous Antibody Volume (uL)	Ricin Dose (ug)	I.P. Ricin Volume (uL)
1	250	250	0.0	100*
2	250	250	0.5	100
3	100	250	0.5	100
4	40	250	0.5	100
5	16	250	0.5	100
6	6.4	250	0.5	100
7	0.0	250	0.5	100

*100 uL of phosphate buffered saline without ricin given i.p.

Antibody was prepared from a 1.0 mg/mL stock solution and diluted into 1.0 mg/mL non-immune purified avian immunoglobulin in phosphate buffered saline to obtain the desired doses. The intraperitoneal ricin challenge was given within one minute of the intravenous antiricin dose. Mice were observed for signs of distress and/or death and were given food and water *ad libitum* during the observation period.

Protein assay: The concentration of protein in aqueous solutions was determined by the Lowry copper sulfate method (J. Biol Chem., 193:265, 1951), or, for purified chicken IgG by absorbance at 280 nanometers (1 mg/mL solution = 1.30 absorbance units at 280 nanometers, 1 cm path).

Results

Immunization:

Three groups of hens were immunized with ricin toxoid as shown in TABLE 1. Initial doses of 0.1, 0.25, and 0.5 ug ricin toxoid emulsified with complete Freund's adjuvant were used, and the animals observed for any dose-dependent adverse reactions. After 14 days no adverse reactions were observed in any group, and booster immunizations were given to all animals consisting of 0.5 mg ricin toxoid emulsified with incomplete Freund's adjuvant. Subsequent booster immunizations were administered as shown, with maximum toxoid doses reaching 1.0 mg. During the course of immunizations, one animal exhibited necrosis near an injection site which subsequently healed completely, suggesting that the wound was unrelated to ricin toxicity.

Development of antibodies to ricin toxoid was measured by EIA beginning with eggs collected at day 21 of the immunization scheme. Eggs collected from each immunization group (day 21, and eggs collected prior to immunization; pre-immune) were extracted using the PEG procedure. The results in FIGURE 1 show that eggs from all three hen immunization groups demonstrate similar specific antibody reactivities and that immunoglobulin from pre-immune eggs are non-reactive in the EIA.

Results of a similar experiment performed using eggs collected on days 52/54 of the immunization scheme are shown in FIGURE 2. Here again, no measurable differences were observed in EIA reactivity among the groups tested. In addition, there was similar reactivity as

compared to antibodies from day 21 eggs, assayed in the same experiment. Therefore, there was no observable increase in EIA antiricin reactivity between the day 21 and day 49 immunizations.

EIA analysis of eggs collected after immunization with 1.0 mg of ricin toxoid showed moderate improvement in antibody reactivity compared to day 21 eggs, indicating that more aggressive immunization will result is greater antibody yields (data not shown).

Antiricin purification:

Based on EIA results, it was determined that specific egg antibody was at suitable concentration to proceed to affinity purification. One egg lain between days 50 and 60 from each of the nine hens was collected, the yolks separated and pooled, followed by PEG extraction. This crude yolk immunoglobulin was loaded over a ricin affinity column followed by elution of specific antibodies, as described in Materials and Methods. FIGURE 3 shows an SDS-PAGE of the starting yolk pool, the PEG extract, and affinity purified antiricin antibodies. The purified antiricin, under reducing conditions, exhibits polypeptide bands with a MWr of 66,000 and a doublet at approximately 24,000, consistent with the heavy and light chains of avian IgG. (The MWr of protein recovered by affinity purification is also consistent with affinity purified egg antibodies to numerous snake venoms, see, Carroll and Thalley, BioTechnology, 10:394, 1990). Densitometric scanning showed that polypeptides corresponding to chicken antibody heavy and light chains represent at least 99% the stained protein in the affinity purified antibody preparation (FIGURE 3).

The EIA shown in FIGURE 4 was performed to evaluate the antiricin reactivity of the egg antibodies at various stages of purification. The results show that affinity purified antibodies possess the greatest specific antiricin reactivity per mg protein (and, therefore, the greatest specific activity) with lesser reactivity in the PEG extract. The column flow-through (e.g., protein that did not bind to the affinity resin) retains low level antiricin reactivity.

Toxicity neutralization effectiveness:

To demonstrate the effectiveness of affinity purified avian antiricin antibodies in neutralizing ricin toxicity, a toxin neutralization study was performed. In this study, ricin is premixed with various amounts of antiricin antibodies followed by injection into mice. The survival of mice injected with the ricin/antibody mixtures during a 20 day observation period is shown in TABLE 2. These results show that the affinity purified avian antibodies are capable of neutralizing a lethal

ricin dose. Survival of mice was accomplished at specific antibody to ricin stoichiometry of less than 2:1.

To further demonstrate the ability of the affinity purified avian antiricin antibodies to inhibit ricin toxicity, a mouse rescue study was performed. In this study, mice were given an intraperitoneal injection of a lethal dose of ricin and an intravenous injection of avian antiricin and were observed for 13 days for signs of intoxication. As shown in TABLE 3, the the lowest dose of specific antiricin antibody tested (6.5 ug) was capable of rescuing mice given a lethal dose (0.5 ug) of ricin.

In vivo toxin neutralization demonstrates that the immunizing toxoid and purification resin contain the critical antigenic determinant(s) necessary to produce effective neutralizing antibodies. Because of the low doses of toxin and antitoxin administered, it is unlikely that neutralization occurred by the formation of large immune complexes, and that low dose circulating heterologous antibody can effectively block toxicity.

Discussion and Analysis of Technical Feasibility

Analysis of results:

Results of this study clearly show the feasibility of producing a highly-effective antitoxin by combining an avian polyclonal antibody source and enhanced purification methods. The resulting antitoxin contains the diversity of reactivity found in hyperimmune polyclonal antibodies, superior active antibody purity, and elimination of inflammatory reactions associated with the Fc region of mammalian antibodies.

In this study the laying hen was shown to quickly (within 21 days) develop specific antibodies in response to moderate doses of ricin toxoid. This study confirms the effectiveness of formalin treatment in reducing ricin toxicity while maintaining key antigenic determinants. (Note studies by Judd, Contract No. DAMD17-87-C-7090 where synthetic peptides from the A and B chains were effective in the production of mouse monoclonal antibodies but none were protective in a ricin challenge assay). Quantitative affinity purification data indicated that the yield of yolk antibody reaches up to 1 mg/mL of yolk, with each yolk averaging 15-16 mL. This yield indicates that hens can produce more antibody than large animals in view of their low body weight and high rate of egg production (chickens produce in their egg yolks 200 mL of yolk per Kg per month compared to the approximately 10 mL of sera per Kg per month obtained from bleeding horses and other large mammals). Therefore, if mammals and hens produce equivalent amounts of antibody per volume of serum and yolk,

respectively, the hen is 20 times more efficient as large mammals. In addition, the cost of obtaining and housing hens is less than large mammals, making hens the cost effective production source.

Affinity chromatography is clearly the method of choice to remove both non-immunoglobulin proteins and non-specific immunoglobulins. Results of this study show that antiricin antibodies are readily purified to >99% purity by affinity chromatography and that the resulting antibodies retain their toxin-neutralizing effectiveness. In contrast, conventional salt fractionation procedures yield products (e.g., horse antivenoms) containing <5% reactive antibody (S. Carroll, personal communication). It is generally known that serum sickness is directly related to the amount of foreign protein injected into the patient and occurs in up to 75% of persons receiving horse antivenoms. Therefore, the superior purity of affinity purified antitoxins will likely reduce the incidence and severity of this serious side effect.

Future direction:

Various elements of the antiricin production methods herein described might be optimized, in further studies, to improve antibody yield and/or effectiveness. For example, based on the apparent safety of the ricin toxoid, larger initial immunizing doses could be administered, and a variety of adjuvants are also available that could further enhance antibody yields. In addition, synthetic polymer-based affinity resins might also maximize antibody yields or specific activity. Optimized methods would then be validated in increased scale with the intent of producing multi-kilogram quantities of antibody for potential field use.

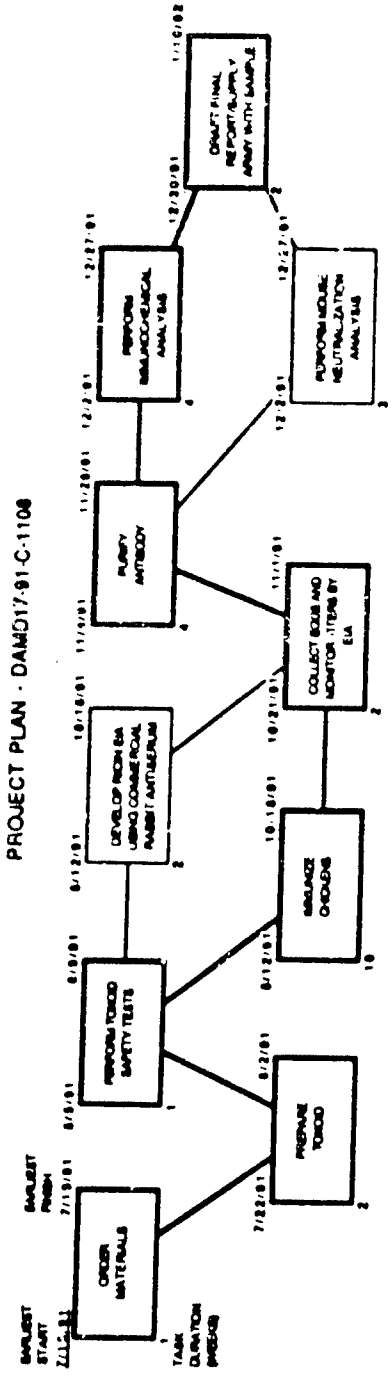
Future analyses of the antibodies developed in this study will be performed at the Department of the Army, Pathophysiology Division, USAMRIID using both *in vivo* and *in vitro* test methodologies. In particular, performance and purity comparisons will be made of purified avian, goat polyclonal, and murine monoclonal antiricin antibodies.

Ophidian Pharmaceuticals has developed various antivenoms/antitoxins based on the scientific principles herein described. This work has shown that the combination of avian antibodies and affinity purification methods is widely adaptable to the production of a broad range of high-quality toxin-neutralizing antibodies. Using these methods, polyspecific antidotes have been developed by blending discrete affinity purified monospecific antibody preparations. Resulting polyspecific antidotes meet performance and purity specifications unobtainable using conventional technologies.

Therefore, it appears feasible that methods developed for the production of an avian ricin antitoxin could be expanded to include the production of polyspecific antidotes capable of neutralizing a panel of relevant toxins (e.g., algal, dinoflagellate, marine invertebrate, or vertebrate toxins).

ATTACHMENT A

Project Work Plan



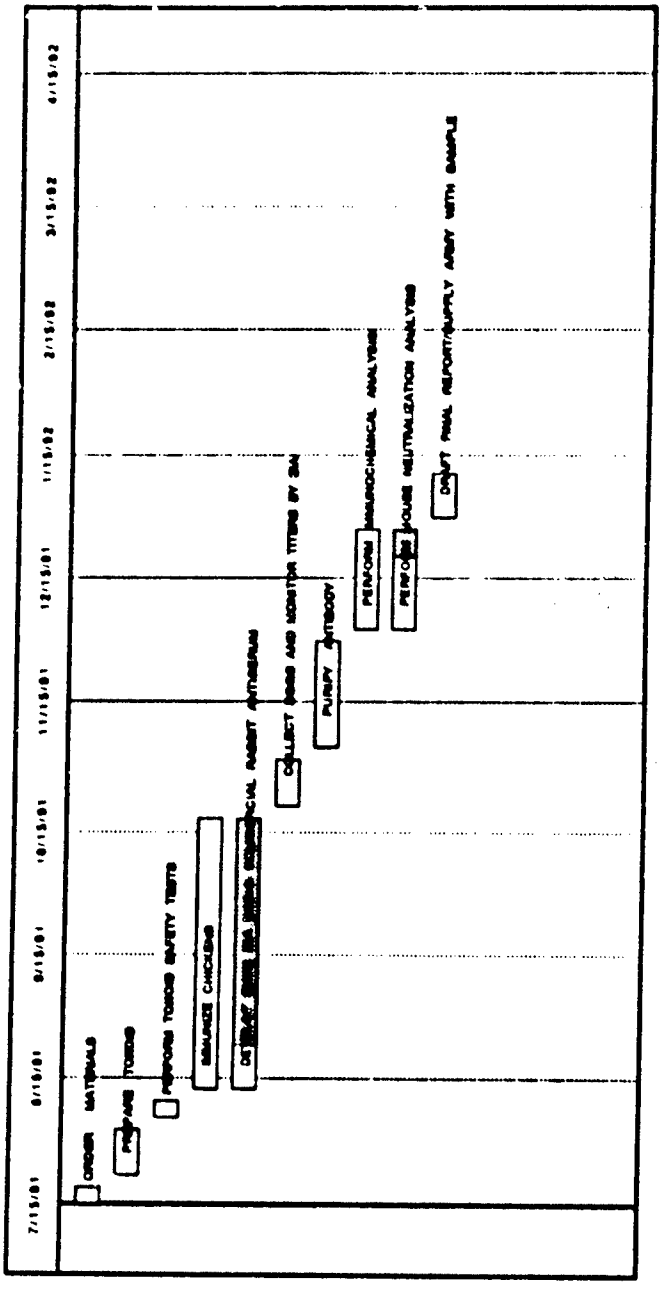


FIGURE 1

Development of Egg Antiricin Reactivity
at 21 Days of Immunization Scheme

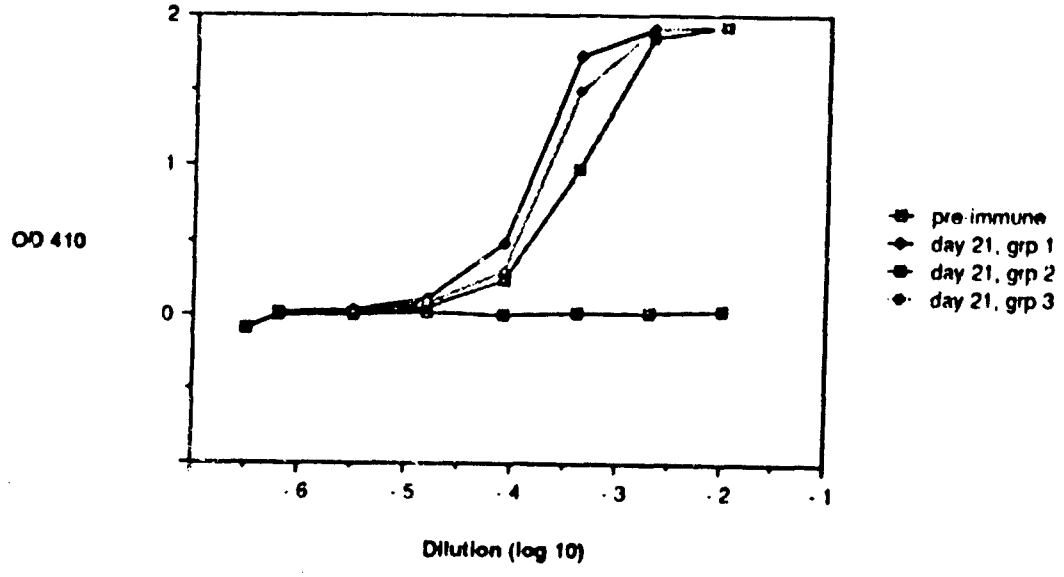
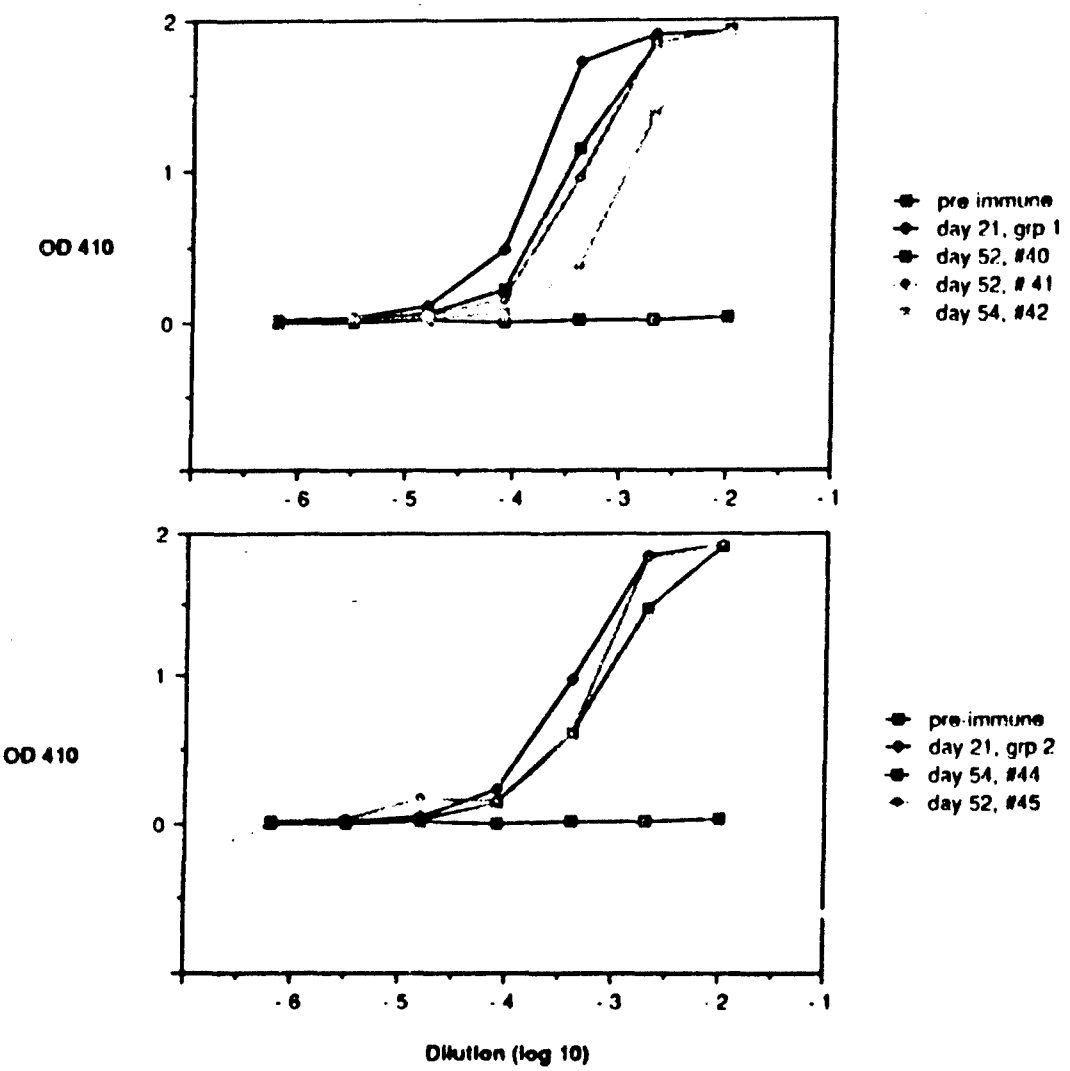


FIGURE 2

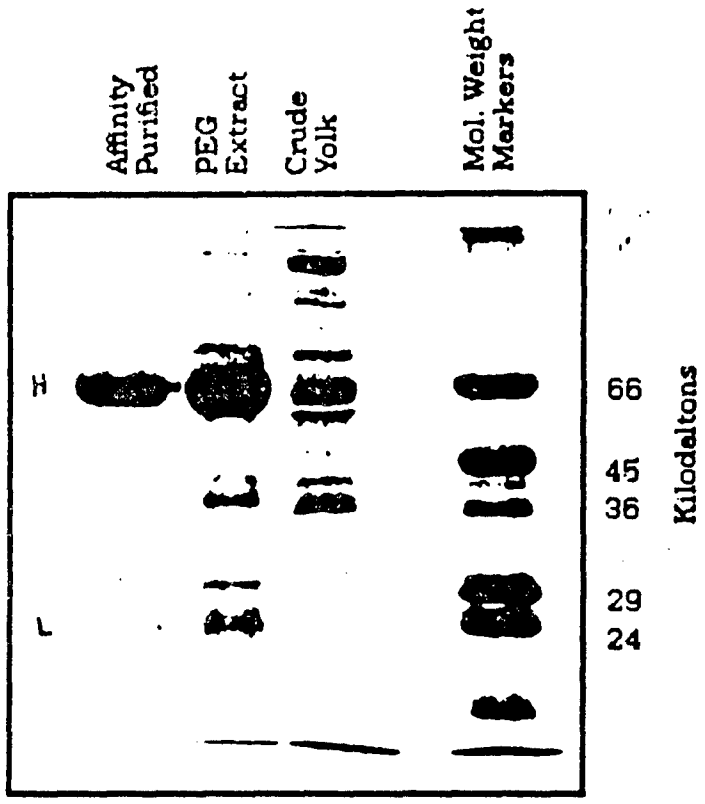
Development of Egg Antiricin Reactivity
at 52/54 Days of Immunization Scheme



Eggs were collected on the days indicated and antibody extracted using PEG prior to EIA analysis. Animals no. 40, 41, and 42 were in immunization group 1 and animals no. 44 and 45 in immunization group 2.

FIGURE 3

SDS-PAGE of Egg Antibody Preparations

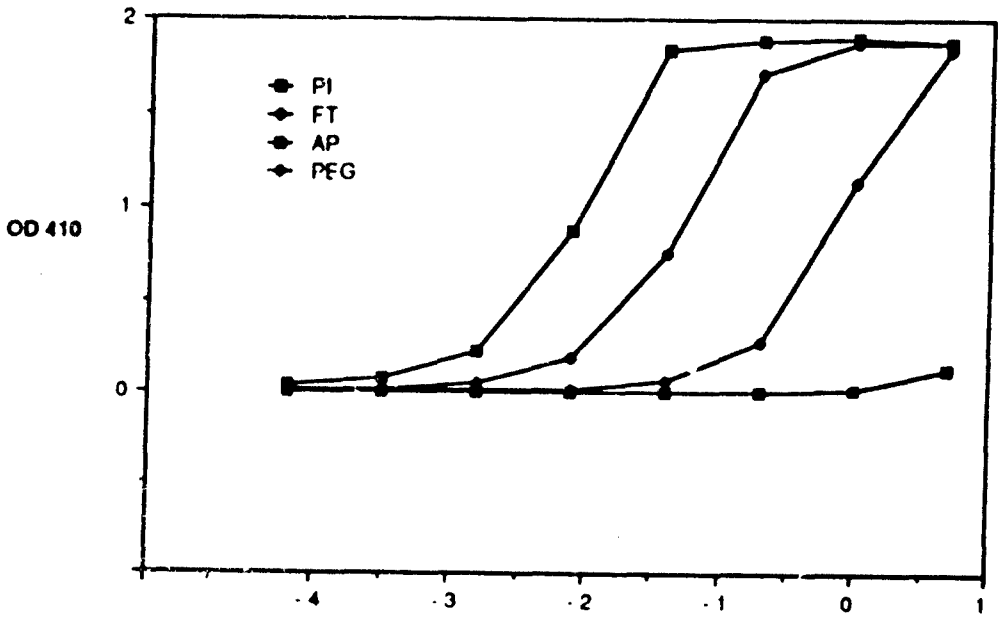


Samples were added to buffer containing SDS and 2-mercaptoethanol and heated at 95 C for 5 minutes prior to loading onto gel. Each lane contains approximately 15 ug of protein.

Chicken IgG heavy chain, H, and, light chain(s), L.

FIGURE 4

Antiricin Reactivity of Egg Antibody Preparations



Log10 Protein Concentration (micrograms/mL)

Protein preparations were adjusted to the concentrations indicated prior to addition to the EIA plate. Samples include, PEG extracted pre-immune egg yolk immunoglobulin (PI), affinity column flowthrough (FT), PEG extracted hyperimmune IgG (PEG), and, affinity purified antiricin (AP).

TABLE 1

Ricin Toxoid Immunization Scheme

Hen Group	Day 1	Day 14	Day 21	Day 49	Day 76	Day 85
1	0.1 mg* CFA** 0.5 mL***	0.5 mg IFA 1.0 mL	0.5 mg IFA 1.0 mL	0.5 mg IFA 1.0 mL	1.0 mg IFA 1.0 mL	1.0 mg IFA 1.0 mL
2	0.25 mg CFA 0.5 mL	0.5 mg IFA 1.0 mL	0.5 mg IFA 1.0 mL	0.5 mg IFA 1.0 mL	1.0 mg IFA 1.0 mL	1.0 mg IFA 1.0 mL
3	0.5 mg CFA 1.0 mL	0.5 mg IFA 1.0 mL	0.5 mg IFA 1.0 mL	0.5 mg IFA 1.0 mL	1.0 mg IFA 1.0 mL	1.0 mg IFA 1.0 mL

Animals were injected with the indicated volume of immunogen distributed over several intramuscular and subcutaneous sites. The immunogen was an emulsion consisting of 50% adjuvant and 50% aqueous ricin toxoid solution.

* Dose of ricin toxoid
** Adjuvant used; CFA, Complete Freund's Adjuvant, or, IFA, Incomplete Freund's Adjuvant
*** Total volume of immunogen injected

TABLE 2

Neutralization of Ricin Toxicity by Affinity Purified Avian Antiricin Antibody

Group	Ricin (ug)	Antiricin Antibody (ug)	Survival (mice alive/total mice per group)* 1 day	2 days	3 days	4 days	5 days
1	0.6	0.00	3/3	2/3	1/3	0/3	0/3
2	0.6	0.12	3/3	3/3	1/3	0/3	0/3
3	0.6	0.24	3/3	2/3	0/3	0/3	0/3
4	0.6	0.60	3/3	3/3	2/3	0/3	0/3
5	0.6	1.2	3/3	3/3	3/3	2/3	2/3
6	0.6	2.4	3/3	3/3	3/3	3/3	3/3
7	0.6	6.0	3/3	3/3	3/3	3/3	3/3
8	0.6	12.0	3/3	3/3	3/3	3/3	3/3
9	0.0	12.0**	3/3	3/3	3/3	3/3	3/3

BALB/c mice weighing 25-35 grams were injected with a mixture of ricin and chicken antibody, as indicated. The mixture was pre-incubated for 10 minutes at room temperature prior to intraperitoneal injection. The total injection volume was 250 uL.

The data are shown as mice surviving at the indicated days post-injection. Animals were given food and water, *ad libitum*.

- All animals surviving at 5 days were alive and appeared normal after an addition 15 days of observation.
- ** Non-immune chicken immunoglobulin was used as control.

TABLE 3

Rescue of Mice Lethally Intoxicated with Ricin by Affinity Purified Avian Antiricin Antibody

roup	Ricin (ug)	Antiricin Antibody (ug)	Survival (mice alive/total mice per group)* 1 day	2 days	3 days	4 days	5 days
1	0.0	250	2/2	2/2	2/2	2/2	2/2
2	0.5	250	3/3	3/3	3/3	3/3	3/3
3	0.5	100	1/1	1/1	1/1	1/1	1/1
4	0.5	40	1/1	1/1	1/1	1/1	1/1
5	0.5	16	2/2	2/2	2/2	2/2	2/2
6	0.5	6.4	3/3	3/3	3/3	3/3	3/3
7	0.5	0.0	4/4	4/4	4/4	2/4	0/4

BALB/c mice weighing 23-35 grams were given an intraperitoneal injection of 0.5 ug ricin in 100 uL of phosphate buffered saline or phosphate buffered saline only (Group 1). Mice were also injected intravenously with indicated amounts of affinity purified specific antiricin antibody in a total volume of 250 uL.

The data are shown as mice surviving at the indicated days post-injection. Animals were given food and water, *ad libitum*.

- All animals surviving at 5 days were alive and appeared normal after an additional 8 days of observation. This experiment was initially designed to include 4 mice in each group, however, mice not receiving an accurate tail vein injection were excluded from the study.