Summary of the NIAID
Ricin Expert Panel Workshop
April 1-2, 2004
Bethesda, MD

EXECUTIVE SUMMARY

Ricin is classified as a Category B priority pathogen by the Centers for Disease Control and Prevention (CDC) and is one of the most toxic biologic agents known. Ricin may be delivered by a variety of routes, including injection, ingestion (contaminated food and water), and inhalation (exposure to aerosols)—all routes that pose major threats from a bioterrorist perspective. Derived from the bean of the castor plant, *Ricinus communis*, the toxin is relatively easy to produce in massive quantities at minimal cost in a low-technology environment. Ricin is an example of a multi-chain microbial ribosome-inactivating protein (RIP) toxin. These toxins inhibit protein synthesis, preventing new growth and leading to cell death. Currently, there is no licensed vaccine or therapeutic for ricin. Confirmation of inhalational ricin exposure is best obtained through a nasal swab within 24 hours following exposure. Identification of ricin in blood and body fluids is difficult due to its rapid protein binding and metabolism before excretion.

Recognizing the need to develop therapeutics, vaccines, and diagnostics for ricin to protect civilian populations in the event of the toxin's use as a bioterror weapon, the National Institute of Allergy and Infectious Diseases (NIAID) convened an Expert Panel Workshop in Bethesda, Maryland on April 1 and 2, 2004. The purpose of the meeting was to:

- discuss our current knowledge of the mechanisms of ricin activity, and identify knowledge gaps;
- identify potential targets for clinical intervention;
- provide an overview of the current status of discovery of new therapeutics, vaccines and diagnostics;
- evaluate the technical opportunities, as well as the constraints, to the development of countermeasures; and
- identify research resources that are needed to advance discovery efforts.

This report is a summary of the discussions addressing these objectives.

Ricin is known to cause toxic effects through two mechanisms: inhibition of protein synthesis and induction of vascular leak syndrome. The relative contribution of each of these to overall pathogenesis (morbidity and mortality) is not well characterized. In addition, the damage to specific cell types or organ systems has not been well defined, especially in the context of the route of exposure to the toxin. The pharmacokinetics of the ricin following various routes of exposure is unclear. The development of diagnostics is constrained by the same gaps in our basic knowledge of ricin biology. The development and application of defined animal models is needed to begin to address these fundamental gaps in our knowledge.

The development of vaccines to protect against ricin is underway in several laboratories and preliminary results are promising. These vaccines have the potential to protect laboratory workers and others at high risk of exposure by maintaining sufficiently high levels of antibodies that neutralize toxin prior to it entering cells.

It is currently thought that ricin-specific antibodies provide protection, especially if present pre-exposure. However, the therapeutic benefit of post-exposure treatment with antibodies is unproven and the therapeutic window undefined. An important goal of pharmacokinetic studies would be to define the potential therapeutic windows and the viability of specific therapeutic approaches for post-exposure treatment.

In summary, there are still considerable gaps in our knowledge of the biology, the pharmacokinetics and the mechanisms and sites of action of ricin. Efforts to better understand this toxin through basic biology and pharmacokinetic research will contribute important knowledge toward the development of effective vaccines and therapeutics against ricin.

Background
Ricin is a potent protein toxin found in castor beans, which are the seeds of the plant *Ricinis communis*, and the source of castor oil. The beans are pressed and castor oil is separated from the hulls and waste mash; ricin is a small component of the waste products. Ingestion of castor beans or ricin is followed by a latent period of a few hours. Early signs of intoxication include fever, thirst, sore throat and headaches, followed by nausea, vomiting and abdominal cramps, and subsequently severe diarrhea, gastrointestinal hemorrhage and vascular collapse. Necrosis in the liver, spleen, kidneys and draining lymph nodes have been observed. Remarkably, most people survive the ingestion of castor beans when given supportive care.

The procedures to isolate ricin from the waste products of the industrial process or from whole castor beans are uncomplicated and the required reagents are easily obtained. Significant amounts of crudely purified yet highly toxic ricin could easily be prepared and delivered in a variety of bioterrorism scenarios. While the specific activity of ricin may limit its ability to be used as an aerosol delivered weapon of mass destruction (WMD) on a large scale, this protein has proven potential to be used as a weapon of assassination and terror. Protection from the effects of ricin by the oral, intraperitoneal and inhalation routes by vaccine and passive antibody administration has been demonstrated in animal studies.

The National Institute of Allergy and Infectious Diseases (NIAID) convened a Blue Ribbon Panel on October 22 and 23, 2002 to discuss and propose a "Biodefense Research Agenda for the Category B and C Priority Pathogens". This meeting led to specific recommendations for immediate, intermediate and long-term research and development activities for the Category B and C priority pathogens and toxins, including ricin. The research agenda is published and is available at: http://www.niaid.nih.gov/biodefense/research/categorybandc.pdf. A Progress Report was published in June 2004 and is available at: http://www.niaid.nih.gov/biodefense/research/category_bc_progress_report.pdf.

Currently, there is no Food and Drug Administration (FDA) approved vaccine or treatment for ricin exposure. Diagnostics are primarily aimed at detecting ricin from nasal swabs in the event of an aerosol exposure. The Blue Ribbon Panel research agendas pointed to the need for vaccines, post-exposure therapeutics, and diagnostics to counter the threat of ricin.

The purpose of this meeting was to discuss in greater detail the technical opportunities as well as the constraints to the development of ricin-specific countermeasures. The meeting included the following sessions:

- Basic Biology
- Therapies to Block Toxin Action
- Therapies to Treat Disease
- Vaccines
- Diagnostics
- Research Resources.

BASIC BIOLOGY
Moderator: Dr. Leonard Smith, United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Maryland

Ricin belongs to a group of toxins that have been designated type II ribosomal inactivation proteins (RIPs). Type II RIPs are composed of an A chain (RTA) that has enzymatic activity and a B-chain (RTB) that has lectin properties and binds carbohydrates. Both the genetic and protein sequences for ricin have been published, as have X-ray crystallographic studies. Once the toxin is internalized, RTB binds to carbohydrate ligands on cell surfaces and the toxin is endocytosed. There can be millions of binding sites for ricin on a cell surface, making ricin a difficult toxin to counteract. The intracellular trafficking of the toxin is not well characterized but at least some is translocated to the cytosol, where RTA is able to reach its substrate, a site on 28S ribosomal RNA. The enzymatic cleavage of the adenine 4324-ribose linkage prevents binding of the ribosomal elongation factor and prevents protein synthesis in the cell, which ultimately results in cell death.

In addition to the toxicity associated with the inhibition of protein syntheses, the RTA has the ability to cause a phenomenon known as vascular leak syndrome. Dr. Vitetta and others describe this toxicity as responsible for damage to the vascular endothelium, which results in the movement of fluids and proteins into interstitial spaces.

Inactivation of the protein synthesis inhibition site does not result in the inactivation of the ricin A chain's ability to cause vascular leakage. This toxicity presents an additional challenge to the development of countermeasures to ricin toxin.

Clinical diagnosis of ricin exposure is difficult since there are few specific signs, symptoms or clinical laboratory parameters unique to ricin intoxication. Hypoglycemia has been observed in laboratory animals exposed to ricin but is not a unique finding to ricin intoxication. The presence of ricene (a unique nontoxic material also found in castor beans) in urine following ingestion of castor beans and crude preparations of ricin may have some utility as a surrogate marker of ricin exposure. In laboratory animals, the oral lethal dose of ricin has been estimated to be approximately 20mg/kg.

In laboratory animals, pulmonary and intravenous toxicities have been experimentally determined to be 3-5 ug/kg. Findings following pulmonary exposure in animals include a dose dependent latent period of 8-24 hours with the development of labored breathing, cough, nausea, hypothermia, cyanosis and acute pulmonary edema. Necrosis in the primary and secondary airways is also observed. Death occurs 36-48 hours after exposure.

Discussions addressed the potential use of various countermeasures in a terrorism scenario. Candidate vaccines and specific antibodies have been useful in preventing the tissue damage caused by ricin exposure in animal models of ricin exposure. There are no FDA approved therapeutic agents for the treatment of ricin exposure but there are limited research efforts attempting to identify candidate therapies.

Knowledge gaps identified in this discussion included the lack of information on the pharmacokinetics of ricin absorption from the intestine or lung and mechanism of distribution to other organs, the temporal relationship from oral or pulmonary exposure to cellular, tissue and organ injury and the lack of well characterized animal models. Additionally, once ricin has been ingested/inhaled the responses of various cell types and the corresponding local and systemic chemokine and cytokine responses are poorly understood. From a clinical diagnostic point of view, the lack of clear case definition criteria makes the early diagnosis of ricin intoxication quite difficult. Proteomic studies in model systems may offer insights into potential diagnostic keys as well as a clearer understanding of local and systemic responses. Panelists discussed the need for a simple yet rapid diagnostic assay, and the potential of well-characterized cellular and animal models of intoxication to contribute to the development of countermeasures to ricin.

THERAPIES TO BLOCK TOXIN ACTION
Moderator: Dr. John Robertus, University of Texas

Ricin intoxication proceeds from ingestion/inhalation through a series of events that ultimately result in cell death. Countermeasures that have demonstrated capability to disrupt this process include vaccines and antibody therapy. Both rely on the ability of antibody to prevent the binding of ricin to cell receptors. To ensure maximum protection, vaccine must be given prior to exposure and sufficient antibody must be produced. Similarly, administration of preformed antibodies affords maximum protection if antibody is present before exposure. The ability of antibody to protect against the toxic effect of ricin diminishes as the interval from exposure to treatment increases. Additional studies are needed to more clearly define the dose time relationships of intoxication with ricin and the ideal characteristics of antibodies used for therapy. Antibody appears to be effective in preventing the toxic effects of pulmonary and parenteral exposure to ricin. Little information is available on the ability of antibodies to protect the gastrointestinal tract following ricin ingestion.

Few chemotherapeutic agents have been identified that have activity against the toxic action of ricin. There are a number of strategies where chemotherapeutic intervention could be applied. As with antibodies, the initial point of intervention for chemotherapeutics would be the elimination of ricin prior to cellular binding. There was general agreement that any chemotherapeutic should affect the binding region of the ricin molecule since there is a high multiplicity of binding sites on most cell surfaces. The high multiplicity of cellular receptors would also require that inhibitors be highly specific and have high binding constants.

Participants were not aware of any candidate therapeutics with favorable toxicity profiles and the ability to cross the cell membrane. An additional data gap that further complicates the development of potential therapeutic agents is the lack of studies that describe the kinetics of binding, cellular intoxication and, ultimately, cell death.

Cellular interactions with ricin or the RTA where therapeutic intervention might be considered are listed below.

- Blocking translocation of the RTA through the cell membrane
- Prevention of RTA attachment to the golgi or endoplasmic reticulum
- Prevention of RTA escape from the endoplasmic reticulum
- Attack of RTA in cytosol
- Alteration of intracellular pH to preclude the RTA chain from unfolding intracellularly
- Development of small molecular weight compounds that could act intracellularly
- Development of agents to block the action of the RTA chain on the ribosome
- Development of ribosomal protectants or mimics

THERAPIES TO TREAT DISEASE
Moderator: Dr. Tom Obrig, University of Virginia

Discussions in this session addressed animal models of ricin intoxication and similarities of ricin to other RIPs, such as shiga toxin. Interactions of ricin and mucosal surface epithelial cells represent the initial point of contact of this toxin following ingestion or inhalation. Animal models to evaluate injury following inhalation of ricin have been developed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) but similar models for ingestion have not been developed. Therefore, little is known about the sequence of events and the pathophysiology of the injuries that follow the ingestion of ricin. It would be useful to understand if ricin enters the circulation, if it is carried extracellularly or intracellularly to other organs, and whether there are organs, tissues and types of cells that are at higher risk of injury than others. Animal models that have been useful in defining some of these issues for shiga toxin include mice, rats, ferrets, pigs, dogs and nonhuman-primates. Additionally, the cytokine and chemokine responses to intoxication are poorly defined and their contributions to further injury or recovery are not well characterized. The panel agreed that there is a need for well-defined animal models to study the disease process and to evaluate countermeasures.

VACCINES
Moderator: Dr. Ellen Vitetta, University of Texas Southwestern Medical Center

Vaccines have been successful in preventing diseases such as tetanus and diphtheria that are caused by microbial toxins. Similarly, researchers have attempted to develop vaccines to protect against ricin. Initial attempts involved the evaluation of a toxoid developed by treating ricin with formaldehyde. Others attempted to produce a vaccine using deglycosylated RTA. Neither of these efforts produced viable vaccine candidates. More recently, since the active sites for the ribosomal and vascular leak toxicity have been identified, mutant forms of the ricin molecule have been evaluated as candidate vaccines. Dr. Vitetta described her efforts to develop a ricin vaccine using recombinant technologies to introduce mutations in the genes for each toxic site and produce the vaccines using recombinant bacterial cells. Preliminary animal protection studies with these vaccines have been promising. No vascular leak or ribosomal toxicities have been observed in cellular or animal models. Efforts to complete preclinical studies and initiate clinical evaluations are underway.

Another vaccine candidate is under development by the USAMRIID. This vaccine is a deletion mutant of the RTA that was engineered to produce a smaller, more soluble and stably folded version of RTA that retains the neutralizing epitope but does not inactivate ribosomes. Vaccination studies demonstrated the protective efficacy of this vaccine in both intraperitoneal and aerosol challenges of animal models. Reduction of injury has been correlated with increasing antibody titers. This vaccine is continuing its preclinical evaluation.

Dr. Michael Lord discussed his recent work with a novel ricin vaccine candidate that is made by inserting a specific 25-residue peptide from maize seed into the RTA chain. This construct had significantly reduced toxicity when tested in cultured mammalian cells, and was non-toxic when injected into rats. The protein elicited antibodies that protected rats from subsequent challenge with lethal amounts of ricin toxin. This vaccine candidate is still in laboratory evaluation and will need additional studies to evaluate its safety and efficacy profile prior to the initiation of clinical studies.

DIAGNOSTICS
Moderator: Dr. Mark Poli, United States Army Medical Research Institute of Infectious Diseases

Current ricin detection methods are able to attain sensitivity of 100pg/ml. Assay technologies include signal enhanced enzyme linked immunosorbant assay and a variety of chromatographic procedures followed by mass spectrometry. While the assay systems have been developed to detect ricin in clinical materials, questions remain regarding the potential level of ricin that reaches the blood stream following the inhalation or ingestion of ricin. Basic pharmacokinetic studies need to be performed to answer fundamental questions concerning which clinical materials should be used for sampling, what the concentration of ricin would be in these samples and what the timeframe is for the appearance of ricin, or its metabolites, in clinical samples. The utility of nasal swabs needs to be evaluated as a potential method of sampling for detection of aerosol exposure. The panel agreed that most of these studies could be accomplished easily with existing technologies but there is very limited funding available to accomplish these types of studies. The panel agreed that it is critical to have a simple, rapid, yet sensitive assay to conduct patient triage following a bioterrorist incident with ricin.

RESEARCH RESOURCES
Moderator: Dr. Seth Pincus, Research Institute for Children, New Orleans, LA

Three primary research resource areas were identified: standardized reagents, standardized assay systems and a reagent repository to allow the production, characterization and distribution of standardized reagents to the research community. Access to purified and well characterized holotoxin, RTA chain and RTB chain, and antibodies to these proteins, were identified as the most critical reagents. Once standardized reagents are available standardized assay systems can be developed and used in multiple laboratories.

Panelists identified the need for cell free and cell based assays, as well as animal model development and characterization to support basic research and the development of countermeasures. The panel also stated the importance of high throughput assays to identify lead therapeutic compounds.

Finally participants emphasized the need for a reagent repository system. Panelists were made aware that the Biodefense and Emerging Infections (BEI) Research Resources Repository http://www.beiresources.org/ was established by the NIAID to provide scientists with the tools necessary to carry out basic and translational research. The mission of BEI Resources is to acquire, authenticate, store, and distribute state-of-the-art research and reference reagents to the scientific community and to provide current information to facilitate research and product development for biodefense and emerging infectious diseases.

RICIN EXPERT PANEL WORKSHOP
Bethesda, Maryland

A G E N D A

THURSDAY, APRIL 1, 2004

10:00 am to 10:15 am	**Welcome and Introductions** Kathy Taylor and Martin Crumrine
10:15 am to 10:30 am	**Opening Remarks** Ernie Takafuji
10:30 am to 11:30 am	**Basic Biology** Leonard Smith
11:30 am to 11:45 am	**Break**
11:45 am to 12:30 pm	**Basic Biology (continued)** Leonard Smith
12:30 pm to 1:30 pm	**Lunch**
1:30 pm to 3:30 pm	**Therapies to Block Toxin** Jon Robertus
3:30 pm to 4:00 pm	**BREAK**
4:00 pm to 5:00 pm	**Therapies to Treat Disease** Tom Obrig

FRIDAY, APRIL 2, 2004

7:30 am to 8:00 am	**Continental Breakfast**
8:00 am to 9:30 am	**Vaccines** Ellen Vitetta
9:30 am to 10:00 am	**Break/Check-out**
10:00 am to 11:00 am	**Diagnostics** Mark Poli
11:00 am to 11:30 am	**Research Resources** Seth Pincus
11:30 am to 12:00 pm	**Wrap-up** Kathy Taylor and Martin Crumrine

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