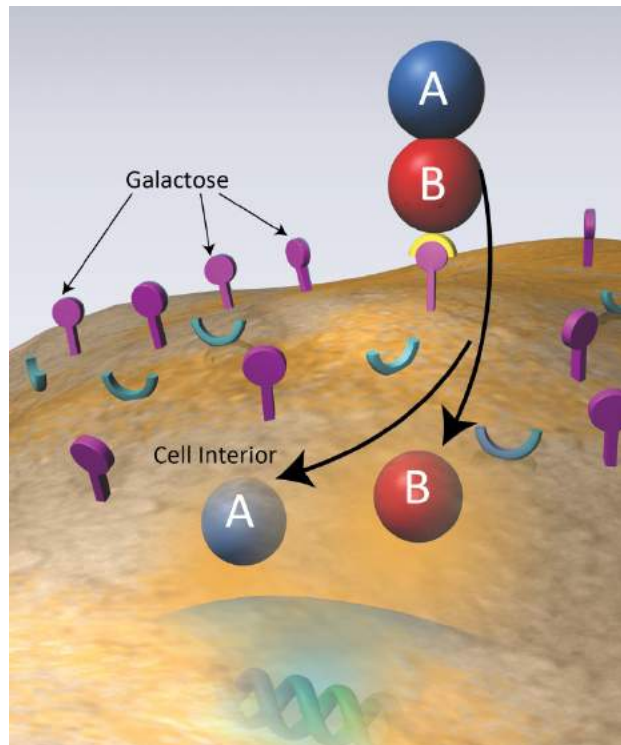# Ricin Toxin

Editor:
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Ricin Toxin

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Editor's Biography

Dr. John W. Cherwonogrodzky is a scientist at the Defence Research and Development Canada, Suffield Research Centre. He has researched countermeasures against biothreats for 30 years (*e.g.* brucellosis, tularemia, anthrax, glanders, melioidosis, plague, ricin, botulinum toxin). His B.Sc. (1974) and Ph.D. (1983) are from the U. Toronto, his M.Sc. from the U. Alberta (1977), Canada. He has 50 publications, 50 applied/approved patents (for USA patents, see www.uspto.gov), and 100 conference presentations. Two special assignments (1994) have been UNSCOM (NY) and the BTWC (Geneva). His greatest satisfaction is using research projects to develop the skills and novel ideas of students during their work terms.

FOREWORD

From pre-historic coated spear tips to a modified umbrella and beyond, ricin toxin from the castor plant has been used as a biological weapon by and against humans for millenia. It is a highly toxic, potentially-fatal threat not only to military but also civilian personnel, both through accidental exposure (*e.g.* ingestion) and terrorist-/state-sponsored intentional release. Ways to detect and treat toxin exposure are essential in protecting a population and form the basis of this book.

Here, Dr. John W. Cherwonogrodzky presents contributions of world experts to provide a text which covers the historic use of ricin, its detection and opportunities for intervention. This volume details recent advances in the development of various approaches to neutralise the toxin as well as highlighting further lines of investigation.

The reader is encouraged to broaden his/her understanding and appreciation for the ability of innovative researchers that have used the toxin to exploit our own cellular machinery to cause damage, and similarly, our ability to exploit the characteristics of ricin, to develop treatment strategies and even modify it to target cancer cells. Accordingly, the book serves as a good resource for toxin researchers, with a wider appeal to those studying cell and cancer biology, medicinal and protein chemistry and vaccine development. The book is informative without being overwhelming, provides a few key contributions to a few key topics, and will likely be worthwhile for reader to learn how far progress has reached against the toxin threat.

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PREFACE

This book focuses on the wide range of subjects needed to beat the biological threat of ricin, beginning with background information (Chapter 1) followed by toxin detection (Chapters 2, 3). *"If you know your enemies, you will not be imperiled"* (Sun Zi, 544-496 BC). This then leads to reducing the threat by creating cultivars that have greatly reduced amounts of toxin (Chapter 4) or generic antibody therapies that neutralize the toxin regardless of its cultivar source (Chapter 5). This in turn leads into not one but several breakthroughs in medical countermeasures against the toxin, be it vaccines (Chapter 6), antibodies (Chapters 7-9) or repurposing abandoned drugs to find an antidote (Chapter 10). The book ends on a positive note, developing antisera against the toxin without using the toxin but instead using a harmless genetically engineered toxoid (Chapter 11). It finishes with ricin's past benefits as an anti-cancer drug (Chapter 12) so as not to lose sight of opportunities to *"beat swords into ploughshares"*.

All of the authors were selected for their expertise and fresh approaches to defeating the threat of ricin. This book is not intended to be a comprehensive textbook on the subject of ricin. Indeed, some topics may have to be addressed in another volume (*e.g.* regulations, emergency responses, forensics, histology and immunity). Regardless, the selected contributions show how innovative "out of the box" scientific-based thinking can shift the bio-threat advantage away from those who wish to intentionally cause harm. Novel methods and discoveries have advanced rapid toxin detection and identification, protection of first responders and military forces by vaccines, rescuing casualties by neutralizing antibodies or repurposed drugs, and possibly frustrating the terrorist should they prepare a useless extract from cultivars that no longer express the toxin. We do not have these measures in place yet, but the book does show how investments in research have paid off towards achieving these goals. With additional support for development and regulatory approvals of these discoveries, the ricin threat can be mitigated.

This book took longer to compile than anticipated. It would not have been possible to complete without the authors, and especially the staff at Bentham

Science Publishers, giving their continuous support. Their patience, enthusiasm and encouragement were a greatly appreciated source of strength during its extended preparation. Perhaps of greater importance, than the book and its many contributions, were the remarkable people that formed a unique collaborative team to make it happen.

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PART 1: BACKGROUND

Ricin - From Pharaohs to Bioterrorists and Beyond

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Abstract: From the time Pharaohs first presided during the dawn of civilization until the present day, the castor bean plant, *Ricinus communis*, and its seeds have played a significant role in the advancement of science, industry, and war. From ancient times, the seeds have been a valuable source of oil used for light and medicinal purposes. Also co-existing in the seed is a toxin (ricin) that has been used in beneficial, and sometimes iniquitous, ways. This chapter provides a review of the history of ricin use (with background information on its source the castor plant and its seeds) that shows the impact that *Ricinus communis* has had upon many facets of civilization.

Keywords: *Ricinus communis* history*,* castor beans, castor oil, beneficial uses, bioterrorism, biocrime.

INTRODUCTION

Ricinus communis is a species of the spurge family (*Euphorbiaceae*) and is the sole member of the genus *Ricinus* (www/ars/usda.gov). Since ancient times, the plant has been treasured for its valuable oil used both commercially and medically. The plant was often called "*Palma Christi*" because the red leaves resembled the palms of Christ and because of the plant's amazing healing powers [1].

In the tropics, the plant can reach the height of a small tree, being over 40 feet high with foliage 15 feet in diameter, and with a woody trunk often a foot in diameter (Fig. **1**). The plant grows rapidly, appearing to spring up spontaneously, growing along river banks and other areas that have suitable moisture. Because of its rapid growth and wide leaves that offer much needed shade from the tropical

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sun, the plant is also known as the "African Wonder Tree" [2]. In ancient Egypt, the plant grew along the banks of the Nile River and both the Egyptians and Israelites were impressed by its ability to grow into a fully-grown tree in such a short period of time. The plant "*kakiyon*" (Hebrew), mentioned in the Bible (Jonah 4: 6-7) as the plant that God made grow into a tree overnight to shade Jonah from the hot sun, was probably the castor bean plant as the growth characteristics of the *kakiyon* and the castor plant appear very similar [3]. In temperate climates, the plant grows more shrub-like, reaching heights of 4-6 feet, and spreads rapidly by the dispersal of its seeds (http://www.ansci.cornell.edu/plants/toxicagents/ricin/ricin.html). The colorful and distinctive leaves, in addition to the ease of its growth, make the plant a popular shrub for ornamental purposes (Fig. **2**). The plant is often planted to deter moles from colonizing a garden or yard as castor oil and ricinine, an alkaloid found in the plant, are known to repel moles. Indeed, because many commercial mole repellents contain castor oil for this reason, the plant is sometimes named as "The Mole Plant."

Figure 1: In tropical areas, castor bean plants grow up to 40 feet tall with the stalks resembling the woody trunks of trees. Image from www.library.illinois.edu, courtesy of M. Williams.

Figure 2: In sub-tropical areas, castor bean plants grow 4-6 ft tall. The color-ful plant is often used in gardens for its ornamental beauty. Photograph credit to Dr. Tom Ombrello at Union County College, Cranford, NJ, USA.

In addition to the plant's ornamental qualities and its valuable oil, the poisonous nature of the plant has also been known for thousands of years. Castor beans (seeds) contain a glycoprotein (ricin) that is one of the most toxic natural substances known. Ricin is produced in the seed's endosperm tissue and is stored within protein bodies [4]. During post-testa maturation (post seed-coat formation), ricin may comprise 3-5% of the seed weight [5, 6]. Ricin is water soluble and easily extracted in concentrated form. The toxin is a ribosomal-inhibiting protein (RIP) that removes an adenine from eukaryotic ribosomal RNA and thereby prevents further addition of amino acids onto the polypeptide chain [7-10]. The end result of this irreversible reaction is inhibition of protein synthesis followed by cell death.

For centuries, both the oil and toxin have been utilized for various purposes, revealing a rich history of the plant's influence upon mankind. Because the

history of the oil and toxin is, in many ways, closely intertwined, this review will present a history of both, providing a glimpse of the journey that both traveled together and their impact, in both beneficial and malevolent ways.

CASTOR BEANS

The castor bean plant originated in Africa and there are records of its use since the time of ancient Egypt [2, 3]. The seeds have been found in Egyptian tombs that date back to 4000 B.C. The common name, castor bean plant, was somewhat accidental in that English traders while in the West Indies and Jamaica, confused the plant with another shrub, *Vitex agnus-castus*, and called the plant the "Castor bean" or "Castor" plant. The scientific name was given by the well known botanist, Carl Linnaeus, who thought the seeds of the plant resembled the Mediterranean sheep tick, Ioxdes ricinus, and named the plant "Ricinus" after the tick. The species name "communis" was chosen because of its world-wide distribution.

The castor bean is actually not a bean but a seed within a spiny seed pod containing three carpels with a seed in each. The seed pod splits open and the seeds are forcibly ejected (http://www.ansci.cornell.edu/plants/toxicagents/ricin/ricin.html). The seeds are shiny, displaying a mosaic of muted black, grey, brown, yellow-brown, maroon and white colors with each seed having its own unique pattern (Fig. **3**).

Figure 3: *Ricinus communis* (castor bean) seeds. Copyright 2002 Floridata.com LLC.

The seeds, varying slightly in size, are approximately a half-inch long and contain a spongy caruncle at one end. Intoxication by ingesting the seeds is not uncommon, with most intoxication causing transient mild-to-severe gastrointestinal problems. Death from seed ingestion is rare with less than an 8% mortality [11-13]. Reports indicate that as few as 2 seeds can be fatal to children while in adults 5-10 seeds may lead to mortality [2, 14]. In all cases, intoxication increases if the seed is chewed or if the shell is broken as the toxin becomes able to leak out of the seed. Intoxication by ingestion is often a problem for livestock such as horses and cattle if they eat the plant growing in fields, since, in addition to the toxin, the plant contains the alkaloid, ricinine, which makes the entire plant toxic [15].

From a historical perspective, castor beans were predominately used to extract the oil and poison, and not for ornamental purposes. The seeds from another plant, *Abrus precatorius*, are more colorful and used in jewellery, containing the closely related toxin, abrin. As the mechanism of action and the threat of use for the two toxins are so intertwined, a brief discussion is presented on abrin for comparative background information.

Abrus precatorius originated in Southeast Asia and, like the castor bean plant, grows in tropical and sub-tropical regions in many areas of the world [3, 16]. *Abrus* seeds contain little usable oil, therefore their cultivation has not been extended commercially. The toxin, abrin, is found in the seeds and is reportedly far more toxic than ricin [16, 17]. A member of the pea family, *Fabaceae*, the plant grows vine-like, twining around trees, and is commonly known as "*Gunja, jequirity,* crab's eye or rosary pea" [3; http://www.ansci.cornell.edu/ plants /alphalist.html].

Unlike the size and color variation seen with castor seeds*, Abrus* seeds are uniform in size and color (red or white) and are known by the names "*gunchi*" in Hindi and "*gunja*" in Sanskrit (Fig. **4**). Because of their uniform size, the seeds were very important in ancient India as a system of weights (*Ratti*) for weighing gold and jewels [18]. The seeds formed the basis for the Ganda system of weights that was based on multiples of four *Abrus* seeds. Several weight systems can be traced back to *Abrus* seeds including the *Varaha* or *ducat* (4^2 seeds), the *shekel* (4^3 seeds), the old ounce (4^4 seeds), and the *ser* (4^5 seeds).

In addition to their role as a basis for weights, their uniform size and distinctive color (bright red with a black spot) have made the seeds popular in the making of rosaries. Even today, the seeds are widely used for making necklaces and other ornamental objects. The poison abrin is more potent than ricin, but the hard shell prevents the seed from being digested and exposing the toxin [3, 19]. Cases of abrin poisoning are primarily associated with chewing the seeds that are in rosaries or other jewelleries, but there have been incidences from a worker pricking a finger when drilling holes in the seeds [20-23].
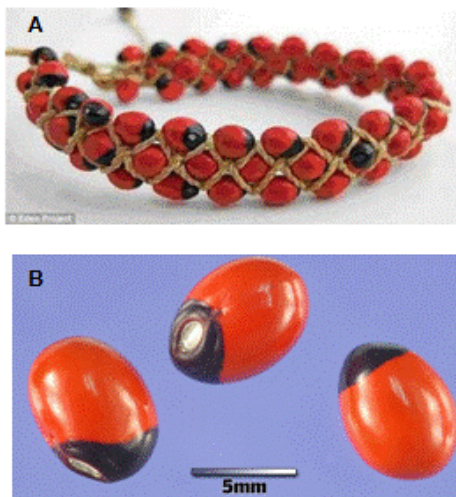
Figure 4. (A) Bracelets made from *Abrus* seeds. Thousands were recalled when the Eden Project in Cornwall, UK found that the seeds were highly toxic. (B) *Abrus* seeds showing their conformity in size, color and shape. Craig Mackenzie, Mail Online. http://www.dailymail.co.uk/news/article-2078362

CASTOR OIL

In the Ebers Papyrus, an ancient Egyptian medical treatise (about 1550 B.C.), castor oil is mentioned as a medicine for various illnesses. Herodotus described a plant, *Kiki* (or *CiCi*), and noted that the plant was grown because of its oil used in wick lamps [2]. Since mature castor seeds contain 40-60% oil that is easy to separate from the bean mash, castor oil became a precious commodity for early civilizations in Northern Africa and the Middle East. Pedanius Dioscorides (70 AD) described in his *De Materia Medica* an oil extraction process and noted that neither the oil nor the mash was fit for food. During ancient times, the oil was also

valued as an emollient (lotion), unguent (skin remedy), as well as for various other medicinal purposes [2, 3]. Cleopatra used castor oil around her eyes and to soothe her skin. The oil was also used to help induce hair growth and prevent head lice infestation, something for which the oil is still used today. The oil was also used as a laxative, purgative, and cathartic. Early Egyptians found that the oil could be used to cleanse their bodies and purged themselves regularly by drinking the oil mixed with beer. The poisonous nature of the seeds led the Greeks and Romans to only use the oil externally.

By the Middle Ages, the plant had spread to Europe where the oil became a popular remedy for many illnesses. Albertus Magnus, Bishop of Ratisbon (13th century) cultivated the plant, harvested the seeds and stalks for medical uses. Gerard described a plant "*kiki*" in his book, "Herball" (1597), noting that the oil, "*Oleum cicinum*", was good for skin diseases, but was a harsh purgative. By the 18th century, European cultivation of the plant became very limited and most Europeans began to import the seeds and oil from Jamaica, although some cultivation continued in Greece and Asia.

While castor oil continued to be used as lamp oil and for various medicinal purposes, the development of automobiles and airplanes provided new demands for this product [24]. In 1899, Charles Wakefield started a new company that sold lubricants for trains and heavy machineries (http://www.wakefieldtrust.org.uk/about-charles-wakefield.php). He became interested in the new motorized vehicles and felt that better lubricants could be developed to better meet the demands of these new engines. Most oils and lubricants at that time were waxy, viscous and would thicken under low temperatures. The engines needed oils that were liquid for cold start-ups but would be thick enough to work at high temperatures. Wakefield and colleagues discovered that adding castor oil allowed lubricants to work despite the wide variation in temperature. With their product, "Castrol", they paved the way for more efficient automobiles and permitted the advancement of a new mode of travel, the airplane.

With the advent of airplanes, World War I opened a new era in warfare with the introduction of flying machines and their new battlefield arenas. Most of the early airplane engines at this time were rotary engines that required sufficient

lubrication with an oil that would not burn easily. Castor oil provided the engines with a high burning, stable lubricant, even though it emitted a unique smell on heating. Unfortunately, a drawback for the airplane engines was that these engines spewed oil. Much of the oil landed in the open cockpits, spraying the pilots and the plane's instruments with burnt castor oil. The long silk scarves that pilots wore, while perhaps giving the aces a debonair look, served an extremely useful purpose. The scarves were used to wipe oil from their faces, goggles, and instruments. In spite of the smell, castor oil lubricated engines worked so well that producing the oil became an important industry, providing a product that is still valued today [25].

During World War II, there was a shortage of good lubricants for war planes. The American federal government encouraged farmers in the Midwest to grow the castor bean plant as an oil seed crop. However, widespread cultivation of castor bean plants in the Midwestern United States stopped because the plant's pollen, a potent allergen, caused a significant increase in hay fever and asthma. In spite of the allergens associated with the plant, cultivation continues. Today, castor oil remains one of the most versatile natural products known with many different products containing the oil (Table **1**).

While being a valuable commodity throughout history, the oil has also been used nefariously. Many children may have thought their mothers being wicked when giving them a dose of castor oil*,* but that small dose was no comparison to what happened to many unfortunate Italians during World War II [26]. During the First World War, the Italian poet D' Annunzio was given credit for first suggesting that castor oil could be used as a means of punishment (http://www.britannica.com/ eb/article-27762/Italy). Using D'Annunzio's suggestion, the Italian dictator Benito Mussolini and his Fascist militia (*Camicie Nere*) used castor oil to coerce Italian citizens to comply with Fascism. Dissidents were often forced-fed up to a liter of the oil that was sometimes mixed with gasoline or other caustic liquids. The resulting diarrhea, dehydration, and caustic burns caused many to die extremely painful deaths.

Table 1: Applications for Castor Oil and its Derivatives*

Agriculture	Food	Textile Chemicals	Paper
Organic Fertilisers	Surfactants Viscosity Reducing Additives Flavourings Food Packaging	Textile Finishing Materials Dyeing Aids Nylon, Synthetic Fibers & Resins Synthetic Detergents Surfactants, Pigment Wetting Agents	Flypapers Defoamer Water Proofing Additive
Plastics & Rubber	**Cosmetics & Perfumeries**	**Electronics & Telecommunications**	**Pharmaceuticals**
Polyamide 11 (Nylon 11) Plastic Films Adhesives Synthetic Resins Plasticizers Coupling Agents Polyols	Perfumery Products Lipsticks Hair Tonics Shampoos Polishes Emulsifiers Deodorants Textile Finishing Materials	Polymers for Electronics & Telecommunications Polyurethanes Insulation Materials	Antihelmintic Antidandruff Cathartic Emollient Emulsifiers Encapsulants Expectorant Laxatives & Purgative Surfactants
Paints, Inks & Additives	**Lubricants**	**Castor Products and Derivatives Used**	
Inks Plasticizer for Coatings Varnishes Lacquers Paint Strippers Adhesive Removers Wetting & Dispersing Additives Finishing Materials	Lubricating Grease Aircraft Lubricants Jet Engine Lubricants Racing Car Lubricants Hydraulic Fluids Heavy Duty Automotive Greases Fuel Additives Corrosion Inhibitors	Castor Oil Castor Oil Esters Undecylenic Acid Castor Wax Zinc Ricinoleate Heptaldehyde Heptanoic Acid Undecylenic Acid Heptyl Alcohol Ethyl Heptoate Heptyl Acetate	

*From the "Comprehensive Castor Oil Report", Castor Oil Clixoo Anugraha, 41, NH Road, Nungambakkam, Chennai-600034, Tamil Nadu, India www.castoroil.in

After World War II, the medicinal use of castor oil decreased. With the advent of milder laxatives and antimicrobials, the use dropped significantly. The new therapeutics, more chemically defined, caused the less refined remedies, such as castor oil, to be relegated to the class of folk remedies. Within the last 50 years, however, several investigations have unraveled castor oil's mode of action. These studies found that, upon ingestion, lipases from the intestine digest the oil, releasing ricinoleic acid into the lumen. The free ricinoleic acid causes changes to intestinal villi which in turn produces the laxative and labor-inducing effects [27, 28].

More recently, while screening various fatty acids for their ability to bind cellular receptors, Sorin Tunaru and colleagues [29] found that ricinoleic acid, the main component of castor oil, binds to certain cellular receptors. Further screening, using a large library of compounds with conformation characteristics known to block cellular receptors, indicated that ricinoleic acid bound to the prostaglandin receptors, EP3 and EP4. The investigators confirmed that ricinoleic acid bound to EP3 receptors *in vitro* and further characterizations showed that the acid was a selective agonist of the EP3 and EP4 receptors.

EP3 and EP4 receptors are found on the intestines and uterus. When the investigators gave ricinoleic acid to mice, they found that normal mice showed a laxative effect to the drug but that mice that lacked EP3 and EP4 receptors ($EP^{-/-}$ mice) were non-responsive [29]. Experiments were also conducted to show the effect of ricinoleic acid as a labor-inducing drug by measuring the contractility of uteri in non-pregnant and pregnant mice. After being given the drug, there was a strong increase in the frequency of contractions in normal mice while there was little effect of the drug on $EP^{-/-}$ mice. These studies indicated that castor oil, or at the least ricinoleic acid, may once again provide therapeutic applications for specific conditions.

The many uses of castor oil and the possible therapeutic uses of ricinoleic acid, indicate that the demand for castor oil will not decrease. The current expanded interest in biofuels may lead to renewed interest in the oil, possibly leading to more cultivation of castor plants and the extraction of this oil from the seeds [1, 30]. This, in turn may lead to the generation of larger quantities of castor bean mash that could conceivably result in the problem of having the potent toxin ricin as a by-product [15].

RICIN - THE TOXIN

While the poisonous nature of castor beans has been known for centuries, recent discoveries indicate that the beans may have been used as a poison far earlier than previously thought [31, 32]. Recent archeological discoveries at the Border Cave located in South Africa indicate that ricin may have been used as a weapon 20,000 to 40,000 years ago. During excavations at the cave site, researchers found a lump

of beeswax mixed with a toxic resin that, when analyzed, was found to be approximately 35,000 years old. It is thought that the beeswax-resin mixture was used to help attach stone points onto wood to make arrows and spears. The team found additional artefacts, including a thin wooden stick with distinctive, man-made scratches. Experimental analysis, revealed traces of ricinoleic acid, the major component of the oil found in castor seeds. The investigators believe that the stick was used to dab poison onto stone tips or spearheads to improve the weapons' lethal capabilities. The poison applicator appears to be approximately 20,000 years old, making this finding the earliest use of poison so far known.

There exists no evidence that prehistoric cultures used the seeds in medicine. However, the presence of ricinoleic acid on ancient tools suggests that, as these cultures had knowledge of the plant and seeds, it is possible that the seeds were used in medicines as well. Certainly, castor beans have been valued for their healing powers since the times of ancient Egypt and Asia [2, 3]. The seeds were mentioned in the Sanskrit treatise on medicine, *Susruta Ayurveda* (6th century BC) and were called "*erranda*" in ancient India, a Sanskrit name that is still used in many parts of Asia. In ancient Egypt and Asia, the seeds were known to contain a poison that in large quantities (5-8 seeds) could kill a person, but in small quantities (no more than 2-3 seeds) was found useful as an emetic and antibiotic for adults. The ancients knew that to be effective, the seeds had to be crushed as the hard shell was not always digested, allowing the seed to pass through the digestive tract without any harm.

Similar to castor beans, *Abrus* seeds have also been used for centuries, although, unlike the castor plant, only the seeds of the plant contain the toxin abrin [16, 20]. In ancient Asia, ground *Abrus* seeds were used to poison fish and to make arrows poisonous as to be used in battlefields. In times of conflict, abrin was used to poison humans and cattle by placing seed paste onto needles (*sui*) and injecting these poisonous needles into the target (*sui*-pricking) [19, 33, 34].

Abrus seeds were also used as a medicine in many primitive cultures from Asia to South America and in many areas these seeds are still used medicinally [20]. In the 1880s, Louis de Wecker suggested that a paste of *Abrus* could be used to treat trachoma and other eye diseases; however, the treatment caused severe

inflammatory reactions [35, 36]. During this time, the toxins in seeds were thought to be bacterial toxins produced by "ferment" in the seeds. Based upon this assumption, de Wecker thought that the inflammation from abrin was instead caused by an infection and decided against using the toxin to treat eye infections.

Extensive investigations into the nature of bacterial toxins, and the findings that some toxins were released from bacteria ("ferment"), led to the idea that the accumulation of toxins found in abrin and castor seeds were due to the presence of bacteria [37, 38]. The "bacterium" thought to be in the *Abrus* seed was called the "*jequirity bacillus*" [39]. There were skeptics of this theory and finally, in their treatise "Non-bacillar Nature of *Abrus* Poison", Warden and Wadell disproved the theory by demonstrating that the poisonous substance in *Abrus* seeds was a "proteid" body that they named "Abrin" [40]. The investigators were also able to precipitate the toxic component from the water-soluble fraction of the seed mash. Further studies performed by Sidney Martin indicated that there were two "proteids" contained within the seed, albuminose and globulin [41].

Around the same time, Dixson used sodium carbonate to neutralize a hydrochloric acid extract of castor seeds and found that the precipitate contained the toxic substance of the seed [42]. After further purification, he showed that the final preparation had increased in both the toxicity and the protein content. The investigations and findings of the protein nature of the poisons in *Abrus* and castor seeds were confirmed by the hallmark studies of Peter Stillmark [43]. Stillmark precipitated a sodium chloride extract of castor seeds with sodium sulfate. After dissolving the precipitate in water, he dialyzed the salts out of the solution and determined that the protein was probably globulin in nature. Further studies showed that the protein agglutinated erythrocytes and was highly toxic. He named the isolated toxin "*Ricinus communis* agglutinin" or "ricin" and with these studies, ushered in an intense interest in plant hemagglutinins [38, 43].

Approximately 10 years after Stillmark's discovery, and based upon studies that ricin was chemical in nature, another German scientist, Paul Ehrlich, used ricin and abrin to demonstrate that animals developed immunity against chemicals introduced into the body [36]. His seminal research established the fundamental tenants of immunology that remain as the cornerstone of modern-day immunology.

Prior to the noted discoveries, for centuries, farmers in parts of India knew that they could protect their cattle from castor plant poisoning if they fed the animals nonlethal doses of seed mash over a period of time [15]. Whether Ehrlich knew about this practice is not clear, but he knew of the important investigations by Edward Jenner and Louis Pasteur who discovered that individuals and animals could become "immune" to certain diseases by giving them previously attenuated microorganisms [44]. Using the scientific knowledge shown by these scientists, Ehrlich furthered the understanding of immunity by introducing his "side chain theory" (German, *Seitenkettentheorie*) in which he proposed that the chemical binding to cell receptors elicited the immune reaction.

Ehrlich believed that animals developed immunity by the chemical binding onto receptors located on specific cells which then led to recognition of the same chemical [36]. To prove his theory, Ehrlich moved away from using micro-organisms and instead designed his experiments to use two substances that he thought to be chemicals, ricin and abrin. Ehrlich fed mice with small, but increasing doses of ricin. After these test animals received several doses over a period of time, Ehrlich believed that these had become "ricin-proof" and showed that the mice were protected from a lethal challenge of ricin [45]. He found also that the "immunity" lasted for several months and that the immune substance was not cellular because mixing the cell-free serum, from an immune animal, with the toxin neutralized the toxin.

Further studies showed that the protection could be transferred to other animals by giving them sera taken from the "ricin-proof" mice. Another aspect of immunity that his side-chain theory predicted was that the immune state was specific; a point he proved by showing that animals "immune" to ricin were not protected from a challenge with abrin and that animals vaccinated against abrin were not protected from abrin intoxication [46].

Taking his investigations further, Ehrlich found that feeding a lactating mouse with increasing sub-lethal doses of the toxin not only protected the mother, but also protected the pups from a ricin challenge. His elegant investigations laid the framework and understanding of modern immunology with many of tenets of his theory supported by future research efforts. In 1908, he was awarded the Nobel

Prize in Physiology and Medicine for his achievements and contributions to the field of medicine and immunology.

After Stillmark's and Ehrlich's discoveries, investigations continued to reveal the secrets contained within this new class of proteins, "toxalbumins". With further investigations, the toxalbumins appeared to differ from other previously characterized proteins. For example, proteins were thought to be susceptible to enzymes, such as those found in the intestines, so the protein and its activity would be destroyed by treatment with proteolytic enzymes. Stillmark [43] found that an 18 hour treatment with trypsin did not reduce ricin's activity. Investigations showed also that incubating abrin or ricin with intestinal juices did not affect their toxic nature [4, 47, 48]. However, Osborne and colleagues found that the agglutination and toxic properties of their more purified preparation of toxin were both diminished by extended (>24 hours) digestion with trypsin [49]. These studies and others indicated that the plant toxalbumins were resistant but still susceptible to proteolytic digestive enzymes, leading Osborne to suggest that the toxalbumins formed a unique class of proteins [50].

The inability to separate the toxin from the agglutinin led to the suggestion that perhaps the two properties were contained in the same substance [49, 51]. This assumption was supported by studies in which castor seeds acquired from localities around the world were collected and compared for their toxin and agglutinin properties. All seeds tested contained both the toxin and the agglutinin. Although the amount of protein varied for seeds collected from different areas, the ratio of toxin to agglutinin always appeared the same [52, 53]. This finding that the agglutinin was always present with the toxin provided an assay, the agglutination of erythrocytes, for detecting the presence of the toxin in cases of suspected poisoning [54, 55].

The physical characterization, indicating that one component contained both agglutinating and toxic properties, was also supported by investigations in which the immune sera from ricin-treated rabbits were found to inhibit both properties [45, 46, 56, 57]. However, more detailed animal experiments using anti-ricin serum indicated the existence of two proteins [58]. Additionally, the agglutination substance appeared to be more sensitive to degradation by pepsin and it was found

that, during germination, the agglutination properties disappeared before that of the toxic properties [59, 60].

As discussed by Hunt and colleagues [61], the controversy as to whether the agglutination and toxin properties were found in the same substance or whether these were two individual components continued throughout the early 1900s [49, 51, 57, 62-67]. Kabat and Heidelberger [68] purified and characterized the toxin using more modern physiochemical techniques. Their studies confirmed that the castor bean contained two proteins, one being toxic and the other being non-toxic. These findings were supported by Ishiguro and coworkers [69] who classified the toxin into two components: a highly toxic, non-agglutinating compound that they named Ricin D and a nontoxic hemagglutinin that he named castor bean hemagglutinin. Later studies showed that the toxin was a protein with a molecular mass of 62 kDa [70-73]. Further purification of the toxin revealed that the toxin was composed of two components in which one, the ricin B chain (RTB), bound to the cell while the other, the ricin A chain (RTA), inhibited protein synthesis [8, 74, 75].

Interest in ricin for medical purposes was revitalized with the investigations by Lin and coworkers [70, 71, 76] in which they found that a single dose of ricin injected intra-peritoneally into mice would completely inhibit the growth of Ehrlich ascites tumor cells. Refsnes and colleagues [17] studied ricin's entry into these cells and found that the ricin B chain bound to the cell and helped the transport of the ricin A chain into the cell. Other studies had previously shown that the A chain inhibited protein synthesis, and thus they concluded that it was the ricin A chain which killed the tumor cells [74, 76-78].

Isolation of toxins such as ricin brought in a new era of "directed" chemotherapy in which the toxin was conjugated to anti-tumor antibodies [79]. Problems arose, however, because both the polyclonal antibodies and the ricin B chain lacked the specificity required for *in vivo* targeting of tumor cells. This problem was solved when investigators coupled the toxic ricin A chain to a monoclonal antibody directed against tumor antigens, thus creating a ribosomal inhibiting conjugate that would be transported only into the tumor cell [80-82].

Immunotoxin development using the ricin A chain slowed, however, when clinical trials and animal studies indicated that intravenous injection of RTA could result in life-threatening vascular leak damage [83-85]. Whether vascular damage was due to the cytotoxicity of the A-chain entering the vascular endothelial cells, an immune complex type of reaction due to the A-chain binding to endothelial cells by specific tri-peptide moieties, or a combination of these, has not yet been determined.

Although ricin's applications to health-related fields have been important contributions to science, ricin has also provided a tool for understanding various aspects of cell biology. Tracing the toxin's endocytosis through the Golgi has helped in the characterization of basic endocytes [86-89]. Characterizing the mechanisms by which ricin inhibits protein synthesis has led to a greater understanding of transcription and translation, as well as a further understanding in the field of enzymatic reactions [70, 90-91]. Intraneural (subepineurial) microinjection of ricin has demonstrated retrograde transport to the perikarya followed by cell death in the local area. These "suicide transport" experiments have resulted in the observation of pathway-specific lesions that have been used to investigate cellular localization of neurotransmitter receptors [92]. Overall, ricin's contributions to science are noteworthy and reveal how the use of a simple toxin has been an attribute to scientific discovery.

SYMPTOMS OF RICIN INTOXICATION

Stillmark's discovery of ricin's agglutination properties led many to believe that its toxic effects were due to its ability to agglutinate red blood cells [43, 49]. This view was soon abandoned upon further investigations [51, 92, 93]. A detailed pathological assessment of mice, rabbits and guinea pigs intoxicated with ricin was undertaken by Flexner who concluded that ricin intoxication affected cells and tissues in a way similar to that caused by bacterial toxins [94]. Flexner performed a series of animal studies intoxicated with abrin and stated that, "*except for Ehrlich's important observations, I should feel inclined with Kobert to regard them as physiological identical*" [94].

Flexner observed that the presentation of illness depended partially upon the dose, but more upon the route of intoxication. In all cases, there was a slight rise in temperature,

diarrhea with bloody stools, albumin in the urine, general weakness, opisthotonos, and severe convulsions. Death was usually preceded by a decrease in temperature. Flexner gave detailed descriptions of the necropsies done on these test animals and evidenced many similarities with the intoxication caused by bacterial toxalbumins. He noted, however, that while bacterial toxalbumins affect the Malpighian bodies more than the splenic pulp, the reverse was the case with ricin and abrin.

Since Flexner's investigations, there have been numerous studies describing symptoms of ricin intoxication [61, 95-98]. Several recent review articles are available that provide excellent descriptions of poisoning by this toxin [99-102]. The main concern, as these studies indicate, is that there are no definitive symptoms that could be used as a definitive diagnosis of exposure to ricin. This capability gap is a major problem in the rapid identification of poisoning or acts of bioterrorism on casualties requiring treatment [103].

RICIN AS A WEAPON OF WAR

During World War I, the United States Bureau of Mines investigated two possibilities for weaponizing ricin [61, 104, 105]. The first was to enhance the lethality of projectiles, either by coating them with ricin to contaminate resulting shrapnel or to place the toxin within bullets. Hunt and coworkers [61] were able to show that firing caused no loss in its toxicity and preparations of ricin could be made to adhere to bullets. In his report, Hunt stated "*It is not unreasonable to suppose that every wound inflicted by a shrapnel bullet coated with ricin would produce a serious casualty, i.e., a casualty much more severe than from the bullet without the ricin. Many wounds which would otherwise be trivial would be fatal*" [61, pg. 112].

These findings faced some disapproval as many felt that using ricin-coated shrapnel would constitute "poisoning" because the bullets pierced the skin. Such a weapon would violate the Hague convention of 1899 (adopted into US law in 1903). It was therefore decided that the "poisoned" ammunition would only be used in retaliation if the Germans used similar weapons.

Aerosol delivery was a second possibility for weaponization. At that time, this was considered an acceptable form of chemical warfare because aerosolization

was not considered a "poison" and so would not violate treaties or laws [105, 106]. Preliminary investigations by Williams [as cited in 61] indicated that delivery of ricin by a dust cloud was not effective nor could the delivery of consistent doses be ensured. Results of his studies did not find ricin as excessively toxic when introduced into the respiratory tract, suggesting that further experimentation was needed to preserve its toxicity under field conditions.

With the onset of WWII, many nations investigated ricin as a possible chemical weapon. The major advantage for its use was that it was more controllable than mustard and chlorine gases that were previously used in WWI. The thought behind ricin use was to develop a lethal weapon that would be more effective in its action and one where the dispersal area could be more easily contained. During this time, Great Britain and Canada investigated the potency of ricin powder placed within 4-lb bursting bomblets [105, 107]. Initially the U.S. and France thought that without an anti-toxin to protect their own troops, it was too dangerous to use. In 1942, the U.S. renewed its interest in ricin and collaborated with England and Canada on the development of a ricin bomb.

A key factor in the development of weaponized ricin was the availability of crude toxin. The yield of ricin was approximately 1 g of ricin in every kg of cold-pressed castor bean cake and the American production of castor oil provided enough bean cake from which to extract the necessary quantities of the toxin. To produce a substance suited for aerosolization, ricin needed to be fluidized or milled into a fine powder [105]. Infusing ricin into a volatile liquid diluted the toxin and this in turn limited the amount of toxin that could be dispersed. Milling ricin into a fine powder proved difficult in that milling by friction heat-inactivated a large portion of the toxin. A process by which the agent was spray-dried and then milled in a chilled-air grinder solved the heat-inactivation problem. This final process and formulation resulted in a highly toxic dry powder known as "Agent W."

At least three field trials were conducted at the Dugway Proving Ground (DPG, Utah, USA) using Agent W. Two of the trials utilized the 4-lb bursting bomblet for dispersal while the third used a spray ejected from the tail of a plane. The tests conducted at DPG indicated that ricin's effectiveness as a poison was limited in scope and that hundreds of pounds of ricin would be required for massive

destruction. During 1943, based upon the assumption that castor oil production would produce sufficient quantities of castor bean cake, a manufacturing plant was planned for the production of 26 pounds of Agent W per day. The plan for ricin production was based upon an improved crystallization method that yielded a higher quality toxin that was more potent. Although there was extensive research and future plans proposed for ricin production, ricin was not used during the war, primarily because it offered no serious advantage over other threat agents and because there was no antidote available against accidental ricin intoxication [108].

In addition to bomb delivery, a ricin dust cloud released from a plane flying over the battlefield was considered a possible weapon. However, tests on the dust cloud delivery system suggested that there was more of a possibility for incapacitation by ricin's blinding effects than lethality from delivery of an inhalational dose of toxin. Initial tests indicated that aerosolized ricin was effective over areas smaller than that originally anticipated and that during these tests any toxicity by the "lung effect" could not be confirmed. The results of these studies suggested that if ricin was ever to be an effective biowarfare agent, the toxin needed to be highly purified. Thus, the military enlisted scientists at universities to help isolate and purify ricin to facilitate weaponization. One of these defense contracts was given to Michael Heidelberger (Columbia University, New York, NY) and his group both improved upon the purification of the toxin and collaborated with two other laboratories for the crystallization of the toxin [68, 109].

The lack of clarity in its use, and more pertinently the lack of methods to protect American and allied troops from accidental exposure, put the further development of ricin as a weapon on hold until an antitoxin could be developed. Although four manufacturing plants were arranged for ricin production, and although the U.S. Army was interested in conducting field trials to develop its use, the too many unknown reasons and the end of the war prevented the use of a ricin weapon in the field.

Several years after the end of WWII, research continued on improving the production and purity of ricin, leading to a patent detailing with the preparation of

ricin toxin that was submitted to the U.S. patent office [110]. The patent was later classified, removed and no longer made available by the U.S. Patent Office.

In 1970, President Nixon banned the use of biological warfare (BW) and by 1972 all BW agents in the United States were destroyed. The original ban did not include toxins but, by 1975, BW toxins were also banned. Since that time, ricin use has been highly regulated under the "Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and Their Destruction" (usually referred to as the Biological and Toxin Weapons Convention, or BTWC, entered into force in 1975) and the "Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and Their Destruction" (usually referred to as the Chemical Weapons Convention, or CWC, entered into force in 1997). Both conventions prohibit the development, production, acquisition, stockpiling, retention, transfer, and use of these weapons, both include ricin as it is considered a biological toxin under the BTWC and also a Schedule 1 chemical under the CWC.

RICIN AND THE COLD WAR

Theoretically, amassing chemical and biological weapons stopped worldwide with the implementation of the BTWC in 1975 and the CWC in 1997. However, during this time, there were reports that the Soviet Union included ricin in its biological programs. Although not used as a weapon in the conventional sense, ricin became a BW agent whose use was documented during the Cold War [111, 112]. Perhaps the most notable case of ricin use as a poison was that on the Bulgarian dissident, Georgi Markov (1929-1978) who was assassinated while working in London, England. The murder resembled an Ian Fleming "James Bond" espionage thriller in which a man is murdered and, except for the suspicious nature of the event, would have gone undetected with the cause of death remaining unknown [113, 114].

Georgi Markov, originally a close friend of the Bulgarian president Todar Zhivkov, became disenchanted with communism and moved to England. Not one to remain silent, he used his BBC broadcasts to criticize communism, the Bulgarian leadership and its government. Afraid that Markov was offending

Bulgarian citizens (more likely, Zhivkov feared that his citizens would awaken to the harsh reality of his regime), Khivkov stated that he wanted Markov "physically removed." Although Yuri Andropov agreed, he distanced himself from the order and gave Sergei Golubev, Intelligence Directorate security head, the task of removing Markov in a manner that could not be traced to the Bulgarian government. The KGB's Operational and Technical Directorate laboratory developed a pellet that contained ricin which would be lethal to its victim and untraceable.

Using a spring-loaded dart concealed in an umbrella or pen, the ricin pellet was initially tested on a horse and then on a prisoner. Even though the outcome of these tests was variable (the horse died while the prisoner did not) the KGB decided to move forward with the ricin dart. On September 7th, 1978, their assassin struck Markov who was waiting for a bus at the Waterloo Bridge. Later Markov stated that he remembered a sting to his thigh and saw a man pick up his umbrella, apologize to Markov, and then leave in a taxi. By nighttime, Markov became extremely ill, experiencing muscle cramps, dehydration, and fever. He was hospitalized and continued his decline with no one being able to identify his illness. He died on September 11th, four days after the umbrella incident. Because there had been previous attempts on his life, and due to the suspicious nature of his death, Scotland Yard requested an autopsy during which a small metal pellet was found in his thigh (Fig. **5**). The pellet measured 1.7 mm in diameter and had two holes drilled through it having an X-shaped cavity in its center.

Figure 5: An iridium-platinum pellet measuring 1.7 mm in diameter with 2 holes drilled to form an X-Shaped cavity. The pellet was filled with ricin and encased in a waxy substance that melted at 37°C.

Upon hearing about Markov's case, another Bulgarian dissident, Vladimir Kostov, recounted an incident that happened to him personally two weeks before the murder [114-116]. On August 26th, 1978, Vladimir felt a sharp jab and stinging sensation in his back as he left the Metro station under the Arc de Triomphe in Paris, France. He developed a fever, but recovered. An X-ray of Kostov's back showed a pellet similar to the one found in Markov's body. The pellet was removed and because the pellet did not penetrate beneath the adipose tissue, the waxy substance covering the two holes had not completely melted, kept the toxin entrapped. There was enough ricin remaining inside the pellet for diagnostic identification. From the second pellet, authorities at Scotland Yard estimated that it took only 425 micrograms of the poison to kill Markov.

The Markov murder became known as the "Umbrella Murder" although there is some doubt as to whether an umbrella was the actual murder weapon (Fig. **6**). Initial tests performed on the feasibility of the weapon, taking into account that Vladimir Kostov was stabbed in the upper back, suggested that this was probably too high for an umbrella weapon to be used effectively. Markov's own injury was located in an area of the body that would have been more easily jabbed with a pen than the tip of an umbrella. The umbrella that Markov saw the man pick up was never located, so whether an umbrella, or another instrument, was used will always remain a mystery.
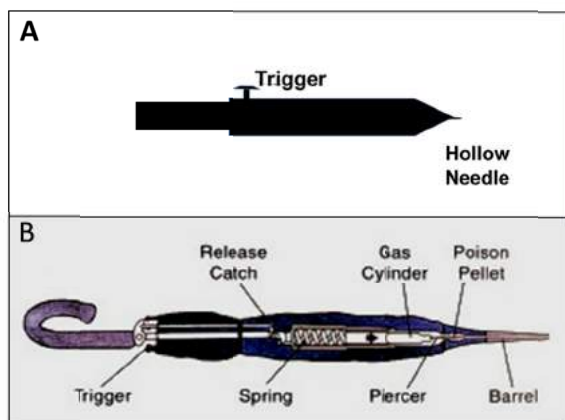

Figure 6: Weapons for delivering a pellet filled with ricin. The weapon consisted of a spring-loaded piston that would drive the firing pin forward to propel the pellet out of the hollow tip. A) Schematic representation of a basic KGB-designed "poison" pen with release. B) The umbrella gun that was supposedly used to kill Markov (from van Keuren, R.T. Chemical and Biologic Warfare, an Investigative Guide, Washington, 1990, Ref 116).

The ricin pellet was reportedly used again in another assassination attempt, this time more likely carried out by the KGB. Boris Sielicki-Korczak was a Polish National and now a U. S. citizen. In 1964, he escaped Communist Poland and fled to Denmark. It was in 1973 when he started working for the U.S. Central Intelligence Agency (CIA) as a field operative. Because Korczak was fluent in Russian, he infiltrated the KGB within that country and became a double agent. By 1979, he had reached the rank of Major within the KGB, but at about this time he was exposed as a double agent. He fled to the U.S. and eventually was able to bring his wife and family to live with him. The CIA did not acknowledge Korczak and denied his working for them. In 1981 while shopping in a Giant Food Store in Vienna, VA, he was shot, probably with an air gun, that lodged a pellet containing ricin into his kidney. Shortly afterward, he felt as though he were passing a kidney stone and recovered a small pellet in his urine. Although he became deathly ill, he survived, probably because his kidney treated the pellet as a stone and expelled it, thereby limiting his exposure to the toxin.

RICIN AS A WEAPON OF TERROR

Bioterrorism, "the deliberate or threatened use of bacteria, viruses or toxins to cause disease, death, disruption, or fear," has been documented since 700 BC when Assyrians poisoned water wells of their enemies with rye argot [116]. Although there appear to be no documented reports of ricin being used as a bioterrorist agent before 1978, the toxin is listed as a Category B biothreat by the U.S. Centers for Disease Control and Prevention (CDC). The reason for identifying it as a threat is because it is readily available world-wide, fairly easy to produce and disseminate, causes moderate morbidity rates, and requires CDC's specialized diagnostics capacity and enhanced disease surveillance for its identification (www.bt.cdc.gov/agent/agentlist-category.asp). Incidences (see Appendix 1) indicate that ricin poses a serious threat as a poison and has been used by criminals and terrorists targeting specific individuals or groups. Public fear about the poison, and the expense of HAZMAT clean-ups, make ricin a recognized bioterrorist threat agent. Possession of the toxin without proper certification forms is a serious offense and making ricin without regulatory approval may be considered a criminal activity [117]. Diligent detective work and surveillance have prevented completion of these plots, but the number of incidents

by those of modest skill and means show ricin as a possible "poor man's weapon of terror".

In addition to the reports listed in Appendix 1 that describe various incidents in which ricin was used as a weapon of terror or crime, the use of ricin on a wider bioterrorism scale has been suggested since the 1980s [118]. UNSCOM's investigations in Iraq (1990s) uncovered 155 mm artillery shells filled with ricin and the UN Food and Agriculture Organization found that Syria had produced 5-18 tons of castor beans annually between 1980 and 1995, presumably for ricin extraction. (http://data. mongabay.com/commodities/category/1-Pr/1-Crops/265-castor+oil+ seeds/51-Production+Quantity/212-Syrian+Arab+Republic). There were allegations that ricin was used during the Iraq and Iran war (1980s), although there was no evidence to support this claim. While anthrax spores and botulinum neurotoxin were uncovered in Iraq during the 1990s, UNSCOM did not find stockpiles of ricin. United States forces found traces of crude ricin in hideouts of the Al Qaeda in Afghanistan, but there was no evidence that ricin was ever used by this group either before or after September 11, 2001 [119, 120].

A high profile incident within the United Kingdom was the January 2003 raid of a flat in Wood Green, north London, in which five North African men were arrested as part of a "ricin ring" thought to exist from Europe to Afghanistan. The men were later acquitted of conspiracy to commit a chemical attack because the jury found that there was insufficient evidence to convict. Others throughout the UK were also arrested within a few weeks and one man was found guilty of "conspiracy to commit a public nuisance by the use of poisons or explosives to cause disruption, fear, or injury" in addition to a murder charge for killing a policeman during his arrest. Although there does not appear to have been a "ricin ring", the incidents listed show ricin as both a serious biothreat and biocrime agent (http://www.historycommons.org/entity.jsp?entity=kamal_bourgass_1).

The use of ricin as a weapon of bioterror is well documented, but its use as a weapon of mass destruction (WMD) is doubtful. The amount of ricin needed to produce mass casualties would exceed the amount that could be easily dispersed. For example, biological weapons experts estimate that 8 tons of ricin would be needed to kill 50% of the people in a 100 sq. km area [121]. It would be virtually

impossible to produce a bomb that could deliver this amount of ricin. Furthermore, to produce mass casualties, ricin would have to be delivered so that lethal doses of ricin would be inhaled. As wind currents and physical barriers would likely interfere with the dispersal of ricin droplets, the possibility of such a scenario appears to be fairly remote [122].

While ricin may not be considered a WMD, Heather Barker (2010) developed a theoretical analysis of ricin as a bioterrorism agent [123]. From her studies, she concluded that while it may not be possible to deliver 8 tons of ricin by using a rocket or bomb, a plane such as a crop duster could feasibly release this amount of ricin over a 100 sq. km. area. Even if a successful delivery does not kill 50% of the people, the chaos and panic caused by a ricin cloud would certainly affect the entire region. Additionally, the expense for decontaminating an area of this size, if even possible, would be catastrophically costly and time-consuming.

A critical aspect for any weapon of terror is for it to be produced in enough amounts without arousing suspicions in the world community. As a by-product of castor oil production, the ability to mass-produce ricin is feasible. Approximately 1 million tons of castor beans are processed annually and the amount of ricin in the by-product mash could be around 40,000 tons, providing enough toxin for weapons development [106]. While aerosolized ricin is considered the route of choice for a bioterrorist attack, a terrorist plot was recently uncovered in which al Qaeda was planning to intoxicate large number of individuals by slipping ricin or cyanide into salad bars and buffets [124]. The availability of large quantities of ricin could make it feasible for a terrorist to slip enough toxin into food or a water supply that, if not lethal, would result in severe illnesses (http://www.cbsnews.com/8301-18563_162-7169266.html). These new threats show the great need for developing rapid sensitive methods of detection in a variety of matrices and for new, less costly methods of decontamination should a large scale incident of its use occur [125].

CONCLUDING REMARKS

Since the time of Pharaohs and perhaps thousands of years before, ricin has played a significant role in many areas of medicine, science, and war. Part of its

uniqueness lies in its high toxicity and ease of extraction from castor beans that provided a product long before the development of molecular biology and highly purified proteins. Another unique quality relates to its ability to bind to the majority of human cells and, after the A chain enters the cell, to cause rapid death of that cell. The availability and unique qualities of ricin are probably the main reasons for its rich history and likely continued use as a biothreat in the future.

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CONFLICT OF INTEREST

The author discloses that she has had no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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APPENDIX 1, INCIDENCES OF RICIN USE

1983: William Chanslor was convicted of attempting to murder his wife. Previously he had requested information *via* a paramilitary journal to meet an expert in poisons and chemical agents. When the Houston, Texas police learned of the request, they set up a sting operation. From a "source" (undercover policeman), Chanslor purchased two "ricin" tablets, a surgical mask, gloves, and tweezers. The tablets were Vitamin C and Chandler was arrested, sentenced to three years in prison and fined $5,000 (http://cns.miis.edu/reports/ricin_chron.htm.)

1983: The FBI arrested two brothers who had produced an ounce of pure ricin, The material was destroyed at the U.S. Army laboratories at Fort Detrick (http://cns.miis.edu/reports/ricin_chron.htm).

1983: A 21-year-old student ingested 30 ricin seeds in a suicidal attempt. He recovered after symptomatic treatment by infusion of saline and glucose solutions [126].

1985: Orange Country, Florida. Todd Meeks had a classmate purchase ricin from Aardvark Enterprises, Louisville, Kentucky. Instead of giving Meeks the ricin, the classmate gave him a vial of water. When Meeks poured the liquid into a glass of water for his father, he was convicted of attempted murder and solicitation for the murder of his father (http://cns.miis.edu/reports/ricin_chron.htm).

1992: Members of the Minnesota Patriots Council (a tax-resistance anti-government group produced ricin. There was a theory that the ricin was intended to be used against a U.S. deputy marshal and a deputy sheriff. Member of the group were arrested in 1995 without the ricin being used (http://cns.miis.edu/reports/ricin_chron.htm).

1995: Dr. Michael Farrar was hospitalized with an unknown illness on three different occasions. It was thought that Farrar became ill after consuming food that his wife, Deborah Green, has laced with ricin. She was charged with attempted murder by poison and is serving a life sentence for two additional murders (http://cns.miis.edu/reports/ricin_chron.htm).

1995: Dr. Ray W. Mettetal, Jr., a neurologist in Harrisonburg, Virginia, was apprehended in Nashville, TN for the attempted murder of Dr. George S. Allen, a former supervisor when he was a neurology resident during the 1980s. Searching a rented storage shed, police found toxic chemicals, a small jar of ricin, books describing production of chemical and biological agents, maps of Dr. Allen's home and workplace. From information gathered by police, it is thought that Mettetal planned to soak pages of a book with a ricin-solvent mixture and take the book to Dr. Allen (http://cns.miis.edu/reports/ricin_chron.htm).

1995: Thomas Lewis Lavy was arrested at the Canadian border when Canadian customs officials found 130 grams of ricin in his possession. Mr. Lavy was arrested for possession of a lethal poison, but the intended use of the toxin was unknown (http://cns.miis.edu/reports/ricin_chron.htm).

1997: The FBI arrested James Kenneth Gluck (Tampa, Florida) for making threats against two Colorado judges. Inert materials that could be used to make ricin were found at his residence, although no ricin had been made (http://cns.miis.edu/reports/ricin_chron.htm).

1997: Authorities in Janesville, WI. were called to a residence concerning a man who had shot his son in the face. While there authorities discovered 0.67 grams of ricin and nicotine mixed in a solvent that would allow the toxin to penetrate the skin. Thomas Leahy received a 8 yr prison sentence for the shooting and 6 ½ yr for possession of a dangerous substance (http://cns.miis.edu/reports/ricin_chron.htm).

2002: Kenneth Olson produced small amounts of ricin. He was found guilty of possession of a biological toxin for use as a weapon and was sentenced to 13 years in prison (http://cns.miis.edu/reports/ricin_chron.htm).

2002: Reports emerged that Ansar al-Islam, a Sunni militant group, was testing poisons and chemicals including ricin. According to one report the group tested ricin powder as an aerosol on animals such as donkeys and chickens and perhaps even an unwitting human subject. No more specific details have been released (http://cns.miis.edu/reports/ricin_chron.htm).

2003: After an informant's lead, British law-enforcement arrested six men of African descent. A search of a north London apartment yielded traces of ricin and manufacturing equipment (http://cns.miis.edu/reports/ricin_chron.htm).

2003: Ricin was detected in a small metallic container and letter found in a Greenville, South Carolina mail facility. The letter was signed "The Fallen Angel," but no one has been identified and the FBI has offered a $100,000 reward for information leading to the individual's arrest (http://cns.miis.edu/reports/ricin_chron.htm; http://ftp.cdc.gov/pub/epr/planning/prepconf2005/presentations).

2003: The Secret Service discovered an envelope addressed to the White House which contained ricin. A letter with similar demands as those requested in the Greenville incident and signed "the Fallen Angel" was found with the ricin. The ricin was found to be relatively crude and therefore would probably not be lethal (http://www.foxnews.com/story/0,2933,110496,00.html).

2004: Ricin was found in the mailroom of Senate Majority Leader Bill Frist which is located on the fourth floor of the Dirksen Senate Office Building, Washington, D.C. No one was harmed and it is thought that the ricin came from the same source as the Greenville incident. (http://www.cnn.com/2004 /US/02/03/senate.hazardous/; http://www.cbsnews.com/stories /2004/02/06/terror/main598441.shtml).

2004: Ricin was found in jar of Gerber's baby food in Irvine, CA. The FBI investigated the incident but no arrests have been made. Analysis of the baby food found ground up castor beans and trace amounts of ricin. The amount of ricin present in the jars was probably not lethal (http://articles.latimes.com/2004/jul/29/local/me-ricin29).

2005: The FBI arrested a man in Ocala, FL for possession of ricin in his home (http://www.ocala.com/article/20050113/NEWS/50113037).

2006: Mashed castor beans (crude ricin) were found in a home in Richmond, VA. Chetanand Sewraz was preparing the toxin to kill his estranged wife (http://web.archive.org/web/20060311045325/http://wtvr.com/Global/story.asp?S=4457615)

2006: Survivalist Kurt Saxon was arrested in Phoenix AR and sentenced to 7 yrs in prison for attempting to manufacture ricin (http://news.google.com/newspapers?nid=1314&dat=20030306&id=02VWAAAAIBAJ&sjid=o_IDAAAAIBAJ&pg=6978,5810678).

2008: Roger Von Bergendorff, hospitalized with an unknown illness, lapsed into a coma. A relative came to retrieve Bergendorff's luggage from a Las Vegas motel room where Bergendorff had been staying. Finding firearms and anarchist material in the room, the police were called. Upon searching the room, several vials of ricin were found and hazmat teams were called to ensure no ricin had been spread in the room. A search of Bergendorff's residence in West Jordan, UT produced no further ricin. Bergendorff woke from the coma and was charged with possession of a biological toxin and two weapons offenses.

2009: Eleven gay bar establishments in the Capitol Hill area of Seattle received letters claiming he had 67 grams of ricin that would be used to kill 5 individuals from each bar. No arrests have been made and no ricin has been discovered. (http://slog.thestranger.com/slog/archives/2009/01/06/gay_bars_receive_threatening).

2009: A jar containing a small amount of ricin was seized from the home of Ian and Nicky Davidson. Ian was arrested under the 2000 Terror Act and sentenced to 10 yrs in prison for possessing material useful to commit terrorism. His son Nicky was placed on 2 yrs youth detention (http://news.bbc.co.uk/2/hi/8086701.stm).

2009: Ricin was found in a home in Evertt WA and the wife's urine tested positive for ricinine that is found in ricin. The woman told police that she had been sick for the last year and thought that her husband was trying to poison her. The husband was arrested for attempted murder (http://www.komonews.com/news/local/46984857.html).

2009: A 49-year-old man who committed suicide by intravenous and subcutaneous injection of a castor bean extract. He died of multi-organ failure approximately 35 hrs later [127].

2011: Jeffery Boyd Levanteris was indicted by a Federal Grand Jury of making and possessing ricin. Levanteris told FBI agents that he wanted to see if he could make the toxinhttp://www.newsnet5.com/dpp/news/local_news/fbi-says-tests-confirm-ricin-found-in-coventry-township-house).

2011: Asim Kauser, Bolton, Greater Manchester, England, was arrested for possessing a recipe for ricin and documents about how to make bombs; he was sentenced to two years and three months in prison. (http://www.newsnet5.com/dpp/news/local_news/fbi-says-tests-confirm-ricin-found-in-coventry-township-house).

2011: Four elderly men, members of an anti-government militia, were accused of plotting attacks on government buildings. The four men were arrested after federal agents found they were

attempting to extract ricin from castor beans. (http://news.yahoo.com/feds-online-novel-played-role-ga-militia-plot-111437565.html).

PART 2: DETECTION

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CHAPTER 2

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Current Technologies for the Detection of Ricin in Different Matrices

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Abstract: Ricin is a protein made of the castor bean plant, *Ricinus communis*, and found primarily in its seeds. Ricin accounts for about 5% of the proteins in the mature seeds and is one of the most poisonous naturally occurring substances. With the worldwide increase of castor bean production for biofuels and petrochemical replacements, it has been of increasing concern that ricin may become a major instrument of bioterrorism because of its heat stability, accessibility, and ease of production in massive quantities. To assure a safe food supply, it is necessary to have detection methods applicable to important food matrices. This review highlights detection of ricin in different matrices using methods ranging from classic animal bioassays to cutting edge molecular approaches.

Keywords: Ricin, immunoassays, receptor-based assays, ricin activity, ricinine.

INTRODUCTION

Ricin is derived from the seeds of the castor plant, *Ricinus communis*, and comprises approximately 1-5% of the weight of the castor bean mash that remains after oil extraction [1, 2]. In its crude form, ricin is often found in association with a lectin, *Ricinus communis* agglutinin (RCA-I or RCA_{120}), which has hemagglutinating activity but greatly reduced toxicity compared to ricin [3]. RCA-I is a tetramer containing two non-covalently bonded ricin-like dimers. Ricin is a member of the Type II ribosome inactivating proteins, known as dimeric or AB toxins, of which the Shiga and Shiga-like toxins are members [4].

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The toxic heterodimer, a 32 kDa A-chain and a 32 kDa B-chain, are normally linked by a single disulfide bond [5]. Both chains are glycoproteins containing mannose carbohydrate groups. The ricin A chain is a highly active *N*-glycosidase that is specific for the depurination of a single adenosine in the 28S ribosomal RNA at position 4324, resulting in the cleavage of the RNA [6]. When cleaved, the RNA is no longer capable of binding Elongation Factor 2, which is needed for the synthesis of protein [7, 8]. Once in the target cell, a single ricin molecule can inactivate more than 1500 ribosomes per minute. Ricin kills cells through inhibition of protein synthesis. The ricin B chain is a lectin that allows it to bind to galactose-containing glycoproteins or glycolipids on the surface of target cells and help facilitate toxin entry into cells *via* receptor-mediated endocytosis.

Ricin is poisonous if inhaled, injected, or ingested and the levels of toxicity vary depending on the route of exposure. In laboratory mice, the approximate dose that is lethal to 50% of the exposed population (LD_{50}) and time to death are, respectively, 3 to 5 µg/kg and 60 hours by inhalation, 5 µg/kg and 90 hours by intravenous injection, 22 µg/kg and 100 hours by intraperitoneal injection, 24 µg/kg and 100 hours by subcutaneous injection, and 20 mg/kg and 85 hours by intragastric administration. The lethal dose for an adult human is about 0.35-0.7 mg by inhalation, whereas the lethal oral dose has been estimated to be between 1 to 20 mg of ricin/kg body weight [9]. The large discrepancy between oral and systemic toxicity is likely due to the harsh digestive conditions found in the stomach and epithelial, and innate immune barriers present in the intestinal track [10]. Persons exposed to ricin usually exhibit symptoms of nausea, vomiting, diarrhea, collapse of major organ systems then death if exposed to a sufficient dose. Diagnosis of the ricin poisoning is difficult due to the similarities with more commonly encountered illness, such as foodborne, chemical or infectious gastroenteritis. There is currently no effective vaccine, antibody or specific medicine available to treat ricin exposure. Treatment for ricin exposure is generally supportive and designed to bolster the body's natural immune response.

The annual worldwide production of castor beans is approximately 1 million tons [8]. The residual castor meal, that is left after extraction of the oil, represents about one-half of the weight of the castor bean [11] and it has a protein content of 34-36% [12], which could be a good source of protein for animals. However,

castor meal has not found a place as a protein supplement due to the high content of ricin.

The high toxicity and large quantities of raw materials readily available to produce ricin-rich preparations in a simple laboratory have made ricin a documented biothreat (42 CFR 72) [8]. Possession, transfer, and use of ricin are restricted under the Public Health Security and Bioterrorism Preparedness Act of 2002. However, it is legal to possess or transfer castor beans, or castor plants, because agents on the select agent list are exempted if they are in their natural state. Both castor beans and castor plants are openly sold within the United States. Because of the high risk of bioterrorism with ricin, more and more recent research efforts have been directed to the design of sensitive methods for the detection of this toxin in complex matrices, such as castor meal, food and human biological samples. Such methods will also be useful for farmers to determine if the castor meal can be used as fertilizers and livestock feed after detoxification. This chapter focuses on the detection technologies for ricin and challenges encountered for validating existing analytical methods for the detection of ricin in foods and other biological and environmental samples.

ASSAYS

Antibody-Based Methods for the Detection of Ricin

Antibody-based immunological detection technologies have been broadly used for the detection and quantification of substances such as peptides, proteins, antibodies and hormones. A variety of immunoassays have been developed for the detection of ricin. The sensitivity and specificity of these immunoassays chiefly rely on the quality of antibodies used. High-affinity monoclonal antibodies that specifically bind ricin have been generated by different groups [13-15]. These antibodies have been incorporated and used extensively in different assay formats. One limitation of antibody-based assays is that they lack the ability to differentiate active ricin from inactive ricin [16].

Radioimmunoassay

Radioimmunoassay was one of the earliest methods used for the detection of low concentrations of ricin [17]. It successfully detected ricin in blood to

concentrations as low as 50-100 pg/mL [17, 18] and had been used for pharmacological studies of ricin in mice and humans. Critical information regarding the distribution and fate of ricin following systemic intoxication could be obtained using this method [19]. However, the location and intensity of the radioactive label (either from intact or degraded toxin) could not be discerned. The other drawback was that this method requires the use of hazardous radioisotopes, and the handling and disposal of radioisotopes presented environmental challenges. Also, it was not practical for diagnostic use in human biological samples.

The Enzyme-Linked Immunosorbent Assay (ELISA)

Among the different antibody-based assays available, the ELISA has been and continues to be used the most frequently. Though not the most sensitive assay, the ELISA provides important benefits. Notably, it requires only small volumes and hence lesser amounts of reagents and it is easily adapted to 96-well microtiter plates used with many different detection systems. Therefore, it has become a common technique for basic research and high throughput applications. It can immobilize reactants to the microplate surface, which facilitates separation of bound from non-bound material during the assay. The ability to wash away non-specifically bound materials makes the ELISA a powerful tool for measuring specific analytes within complex matrices. Furthermore, all reagents and equipments needed by the ELISA are available to most laboratories. There are two basic ELISA formats: the "direct ELISA", immobilizing the antigen by direct adsorption to the assay plate, and the "sandwich ELISA", immobilizing the antigen *via* a capture antibody that has been attached to the plate. The antigen is then detected either directly (labeled primary antibody) or indirectly (labeled secondary antibody). The sandwich ELISA is more popular because it is more sensitive and robust compared to the direct ELISA. In these types of assays, the antigen of interest is bound to the surface of a microplate through an antibody-antigen interaction; the binding is oriented and highly specific. In contrast, the attachment of antigens to the plate in the direct ELISA is random, and non-specific. A number of sandwich ELISA formats have been developed for the detection of ricin [13, 20-24]. We have compared three different sandwich ELISA formats (Fig. **1**) using the same pair of antibodies [25] and found that the ELISA

using a biotinylated primary detection antibody and streptavidin-linked horseradish peroxidase (HRP) system was most sensitive with limits of detection (LOD) of 25 pg/mL in phosphate buffered saline (PBS), 50 pg/mL in non-fat milk or mouse serum, and 100 pg/mL in whole milk. The second best was the ELISA using a streptavidinylated primary detection antibody and biotin-HRP system. Its LOD for ricin was 100 pg/mL in PBS and milk, and 1 ng/mL in serum. The ELISA using a non-tagged primary detection antibody and HRP-labeled secondary antibody was least sensitive among all and the LOD was 1 ng/mL in PBS, milk and serum. Fig. **2** shows the linear range of detection by three ELISA methods. Compared with the direct ELISAs (without using the capture antibody), the sandwich ELISAs were 50 to 500-fold more sensitive in PBS buffer. Estimation of the accuracy of these immunoassays using the Coefficient of Variability (CV) showed that the most sensitive ELISA format also had the lowest inter- (4.28%) and intra-assay CV (2.15%), although the inter- and intra-assay CV for the other two ELISAs were less than 10% and 6%, respectively, well below the maximum acceptable level. To increase sensitivity and reduce background noise, traditional chromogenic substrates have been replaced by electrochemiluminescence [20, 26] and electrochemical read-out [27].
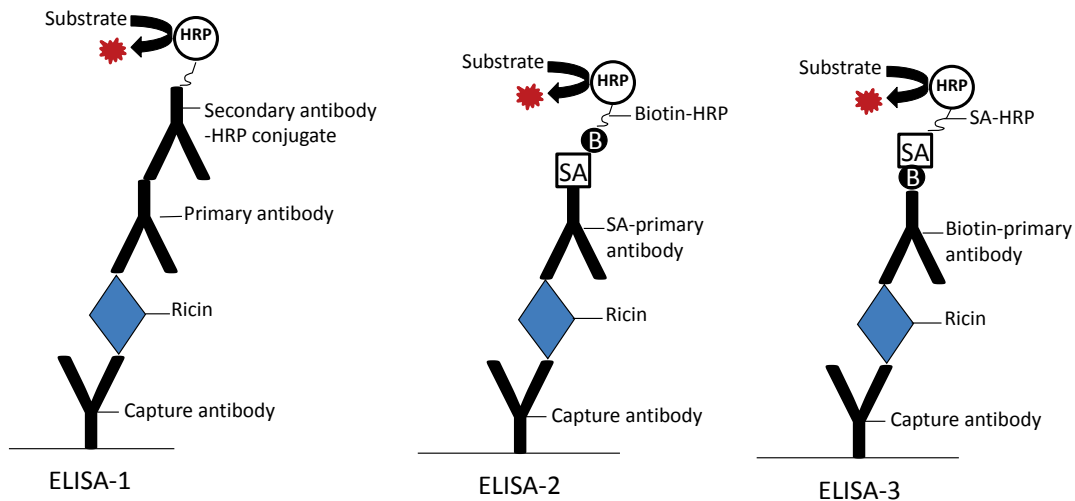
Figure 1: Schematic representation of the three ELISA formats, depicting the analytical complexes on the surface of an assay well. ELISA-1: Indirect sandwich ELISA using an unlabeled primary detection antibody and HRP-conjugated secondary antibody; ELISA-2: Indirect sandwich ELISA using a streptavidin (SA) tagged primary detection antibody and biotin (B)-HRP; ELISA-3: Indirect sandwich ELISA using a biotin tagged primary detection antibody and SA-HRP.
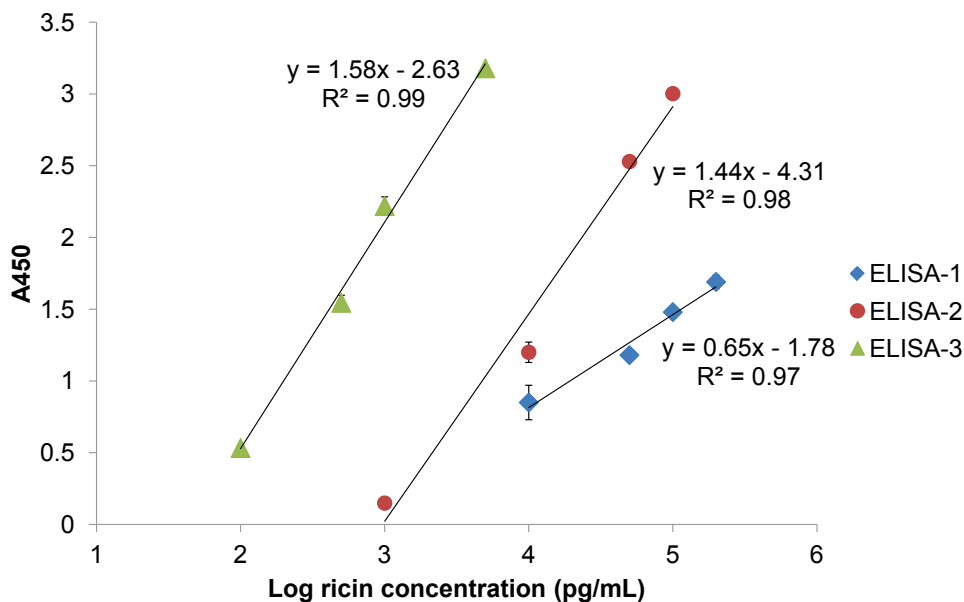
Figure 2: Comparison of the performances of three ELISA formats. Signals are presented as the average absorbance readings at 450nm for three replicate measurements of ricin in PBS. The semi-logarithmic scale was used to show the slopes of straight lines.

The ELISA has been proven to be one of the most versatile techniques, however, there are significant issues of cross-reactivity and "hook effects" in some assays for ricin, even in relatively simple liquid matrices [28]. The discrimination of ricin and RCA is still technically not feasible. Furthermore, most ELISAs are time-consuming; require hours to perform and are also not sensitive enough, both for forensic purposes and monitoring blood levels of ricin in patients, because ricin binds quickly and is often metabolized before excretion.

Lateral Flow Technology

To meet the demand for fast and easy on-site detection, Shyu *et al*. [29] developed a lateral flow assay (LFA). This assay was based on the sandwich format using two monoclonal antibodies with distinct specificities. One antibody, directed against ricin B-chain, was immobilized to a defined detection zone on a porous nitrocellulose membrane, while the other antibody, directed against ricin A-chain, was conjugated to colloidal gold particles which served as a detection reagent. The ricin-containing sample was added to an absorbent area, where ricin reacted

with the gold-labeled anti A-chain antibodies. The complex of ricin and gold-labeled antibody was then passed along the nitrocellulose by capillary action and captured by the immobilized anti B-chain antibody in the detection zone, where a visible red line developed with an intensity proportional to the ricin concentration. In the absence of ricin, no immunogold was bound to the antibody in the detection zone. With this method, 50 ng/mL of ricin was detected in less than 10 minutes and the assay sensitivity was increased by silver enhancement to 100 pg/mL. This assay has been used for the diagnosis of ricin poisoning [30] and screening castor plants for the presence of ricin [31]. Garber *et al*. used commercial diagnostic kits to successfully detect three select agents, ricin, amanitin, and T-2 toxin, at concentrations below what might be a health concern [27]. LFA are usually around 1,000 times less sensitive than standard sandwich ELISA and the limit of detection is around 1-50 ng/mL [29, 31]. Although simple LFA is not as sensitive as other methods, the high speed (it takes only a few minutes to obtain the test results) and simplicity for testing (it requires little or no sample/reagent preparations) has made it more advantageous in many applications. It can be applied to urine, saliva, blood, stool, food, or environmental samples.

Biosensor Devices

Rapid detection of ricin has important implications in the case of criminal use of ricin in a potential bioterrorism scenario. Most antibody-based methods are time-consuming and often expensive. The LFA is rapid and simple but lacks sensitivity. Therefore, demand is increasing for sensitive and accurate assays with rapid detection systems for ricin. A hand held biosensor would give fast, reliable results for identifying and quantitating ricin in different samples. In this technique, bioreceptors, selected due to their high specificity to ricin, are immobilized onto a transducer to form a functional sensor. Antibodies are the most common bioreceptors. The transducer surface is usually tethered by a linker molecule, which is often gold, carbon, silicon or hydrogels, using a suitable method including streptavidin/biotin affinity, silanisation, protein A or direct attachment. The immobilization of the bioreceptor is fundamental for functionality and integrity of the sensor, *i.e.* the antibody epitope binding domain must be orientated to facilitate binding to the target protein. Often steric hindrance and denaturation of the bioreceptor are an issue for reproducibility and sensitivity.

Another problem can be cross-reactivity between bioreceptors and non-target molecules. This can be addressed by selecting highly specific bioreceptors and by blocking the non-specific binding sites of the sensor surface with a protein before use. Surface plasmon resonance-based biosensors have been used for the detection of ricin and the limit of detection ranged from 0.5 ng/mL to 200 ng/mL [32-35].

Receptor-Based Assays for Detection of Ricin

As described above, ricin is composed of A- and B-chains. The B-chain has two carbohydrate-binding sites, each of which binds to the β-D-galactopyranoside (β-Gal) or β-D-N-acetylgalactosamine (β-GalNAc) residue in host glycoconjugates. Based on this property, different biosensors have been developed. Uzawa *et al.* reported that they were able to detect ricin at a concentration as low as 10 pg/mL within 5 min [36] using a sugar-probe biosensor. Stine *et al.* compared biosensors using glycosphingolipids and antibodies as receptor molecules for the detection of ricin and found that the glycosphingolipids offered more sensitive detection limits than antibodies [37]. Recently, Liu *et al.* [38] developed galactose-functionalized silanized magnetic iron oxide nanoparticles (Gal-SiMNPs) for capturing ricin based on the specific recognition between galactose-binding sites of ricin B chain and the Gal-SiMNPs. Ricin in PBS and serum could be enriched 10-25 fold using these particles and the limit of detection by a simple colorimetric assay following this enrichment step reached 2-4 ng/mL. Compared with the ELISA, this method is much faster.

Activity-Based Assays

While the above-mentioned technologies are useful for detecting the presence of ricin, these lack the ability to discriminate inactive (non-hazardous) *versus* active (hazardous) toxins. Besides animal bioassays, a number of functional assays for ricin have been developed since the molecular mechanism of ricin's depurination was deciphered [39, 40].

Mouse Bioassays

The mouse bioassay used to be the "gold standard" for the detection of ricin [18, 41, 42]. The acute toxicity of ricin was measured on the basis of the mean lethal dose, the dose that is lethal to 50% of the exposed population (LD_{50}) and the time

to death at different doses [18, 43]. Mice are usually injected intraperitoneally or intravenously with 100 µL of ricin sample in a dilution series, and then monitored over several days for signs of intoxication and death. In laboratory mice, the approximate LD_{50} and time to death have been found to be 3 to 5 µg/kg and 60 hours by inhalation; 5 µg/kg and 90 hours by intravenous injection; 22 µg/kg and 100 hours by intraperitoneal injection; 24 µg/kg and 100 hours by subcutaneous injection, and 20 mg/kg and 85 hours by intragastric administration [44]. The precision and reproducibility of the LD_{50} value are influenced by a considerable number of factors, such as the species of the experimental animal, the injection route, observation time, age, sex and feeding conditions. Such factors limit the accuracy of the assay. While the mouse bioassay has high sensitivity, can measure active toxin and is amenable to be used in different matrices, it has many drawbacks. The bioassays are not practical for use in most laboratories because these are expensive, time-consuming, require special animal care facilities, and are unrealistic for high-throughput applications.

Cell-Based Assays

Assays based on the cytotoxicity induced by ricin to cells in culture are alternative assays. These do not need to use live animals and the full activity of ricin, including the binding to cells, uptake and expression of toxicity, can be tested. The uptake of ricin by rat liver non-parenchymal cells [45], bone marrow macrophages [46] and mouse peritoneal macrophages [47], *via* both galactose-containing structures on the cell surface and mannose receptor-mediated endocytosis, suggests that many different kinds of cells are sensitive to the toxin [48]. The two most frequently used cell lines for ricin activity assays are *Vero* cells [49, 50] and *Hela* cells [51, 52]. A typical protocol for ricin cytotoxicity assays was described as by Neal *et al*. [53]. The cells were first trypsinized and adjusted to approximately 0.5×10^5 to 1.0×10^5 cells per ml, then seeded (100 µL/well) onto 96-well plates, and allowed to adhere overnight. Cells were then treated with ricin (10 ng/ml), or medium alone (negative control) for 2 h at 37°C. The cells were washed to remove non-internalized toxin and then incubated for 40 h. Cell viability was assessed using CellTiter-Glo reagent (Promega) according to the manufacturer's instructions. Luminescence was measured with a luminometer. A 100% viability was defined as the average value obtained from

wells in which cells were treated with medium only. Jackson *et al*. [54] applied a cell-based assay to determine the effects of heat treatments on the detection and toxicity of ricin added to milk and soy-based infant formulas. The cell-based assays can give faster results than regular mouse bioassays, but these still require lengthy incubation times.

Cell-Free Translation Assays

As described above, ricin is one of the plant ribosome-inactivating proteins, it possesses *N*-glycosidase activity and it can be monitored with *in vitro* translation assays. Principally, in these assays ricin activity is determined by measuring the inactivation of the ribosomes and therefore decreases in the yield of active proteins compared to the control [55, 56]. Hale developed a microtiter-based assay for evaluating the activity of ricin using luciferase as a reporter [57]. Compared with other cell-free assays, Hale's assay started with the translation step and did not involve transcription. It therefore reduced erroneous results associated with proteins from heterogeneous mRNA contamination. To better prepare for the intentional adulteration of food with ricin by bioterrorists, we validated this method for the detection of ricin in different food matrices [58]. It was found that ground beef has very little matrix effect on the assay, whereas low-fat milk and liquid chicken egg showed clear interference on the protein translation. A simple dilution in phosphate-buffered saline (PBS) effectively eliminated the translational inhibition from these foods (Fig. **3**). The concentrations inhibiting 50% of luciferase translation derived from the this study was 0.01 nM for the pure ricin A chain, 0.02 nM for pure ricin, and 0.087 nM for crude ricin in PBS. This method was also used to study the thermal stability of pure and crude ricin in different matrices and has been shown to have a high level of precision within and between runs [59]. In summary, cell-free translational assays are very useful for the detection of ricin activity. These are rapid and sensitive and have the potential for high-throughput studies. However, these also have drawbacks. One of these is that the assays cannot discriminate ricin from other ribosomal inactivating proteins. These also require ricin-specific antibodies to block the cytotoxicity to confirm the presence of ricin. The other drawback is that the translation systems are very sensitive to some matrices.
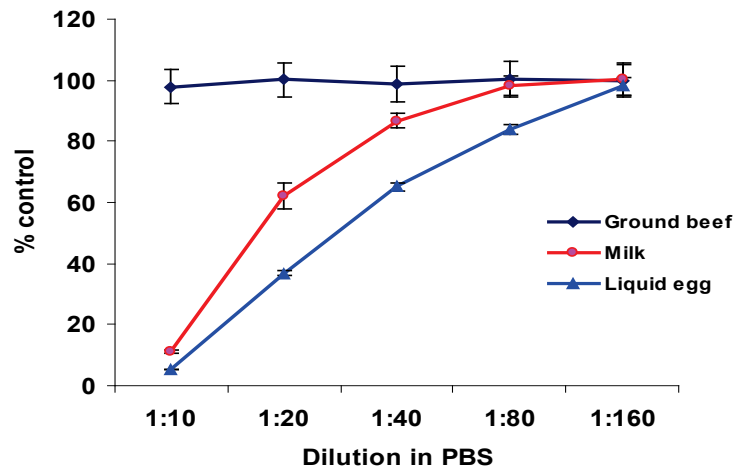
Figure 3: Effects of food matrices on cell-free translation. Beef extract, milk and liquid egg were each diluted in PBS at the ratios indicated. Aliquots of the diluted food samples (3 µL) were mixed with the translation lysate (15 µL) and incubated for 90 min at 30ºC. Luminescence was measured. Values were calculated as % control activity [(cps from food matrix/cps from PBS control) x 100]. Results represent the means of three replicates from one representative experiment ± standard deviation. Three individual experiments were performed.

Assays Based on Enzymatic Activity on Nucleic Acids

Ricin is a ribosomal inactivating protein. The mechanism of the inactivation is the hydrolysis of the *N*-glycosidic bond adjacent to the α-sarcin site in 28S rRNA [39]. Many assays have been developed based on ricin activity on nucleic acids since Endo *et al*. [39, 40] reported that ricin A chain depurinates A4324 on a GAGA stem-loop region of eukaryotic 28S RNA. These assays were performed using ribosomal or small RNA containing GAGA loop sequences as substrates and the activities were quantified by measuring the free adenine released by ricin [60-62]. Methods for adenine quantification vary from fluorescence, colorimetry, and electrochemiluminescence [62-64]. These assays measure directly the adenine released by ricin, so these are highly specific, extremely rapid and quantitative. However, it was found that there was no correlation between enzymatic activity on nucleic acids and cytotoxicity measured by cell assays [61].

Assays Based on Surrogate Markers of Ricin

Ricin exhibits lectin activity that can cause unexpected losses, or conversely high background binding, in some cases. It can be useful to use a marker for the presence of ricin, rather than measure the toxin itself. The use of surrogate markers could

provide a second tier of testing for suspected positive samples. It would also be necessary to develop sample preparation methods for these marker molecules. Castor genomic DNA and ricinine, a small alkaloid, have been used as surrogate markers of ricin.

Polymerase Chain Reaction for Castor Genomic DNA

Based on the incidences that have occurred in real cases, it seems likely that crude, rather than purified, ricin would be used as a bio-weapon. It is currently feasible to employ a polymerase chain reaction (PCR) to identify the presence of castor genomic DNA that remains associated with crude ricin preparations. We developed a real-time PCR for the detection of crude ricin in ground beef, milk and liquid egg [65, 66]. In this study, a pair of castor specific primers, Ricin-F4/R4, was identified. Using this pair of primers, an amplicon, with an estimated size of 69-bp, was amplified from milk and egg samples spiked with castor material. No PCR products were observed in samples without spiking with castor material. The presence of equal amount of DNA in these samples was confirmed by PCR using a pair of primers, 18S-F/R, derived from conserved DNA sequence (Fig. **4**). It was found that
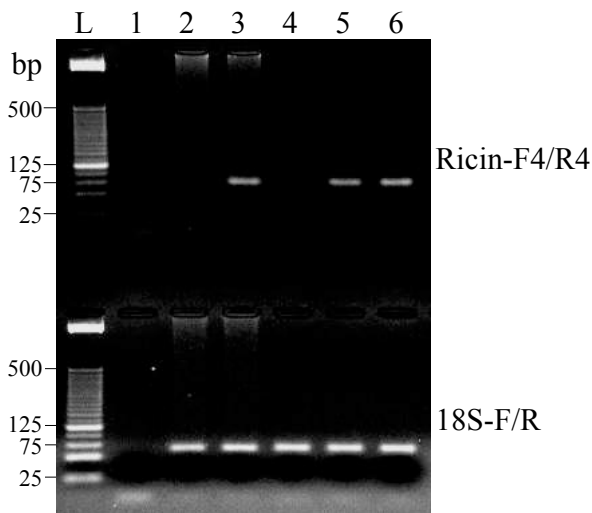
Figure 4: Real-time PCR was performed using Ricin-F4/R4 (top panel) and 18S-F/R (bottom panel) primers and DNA (5 μL) extracted with the CTAB method from castor, milk or liquid egg as template. The ethidium bromide stained 3% agarose gel with 10 μL of real-time PCR end products is shown. L, 25-bp DNA ladder; 1, nontemplate control; 2, DNA from milk; 3, DNA from milk spiked with 0.1% castor bean acetone powder (CAP); 4, DNA from egg; 5, DNA from egg spiked with 0.1% CAP; 6, DNA from castor.

the acetyl trimethylammonium bromide (CTAB) method yielded the highest quality of DNA from ground beef, milk and egg. Using this RT-PCR, the limits of detection for castor acetone powder were less than 10 μg/g (corresponding to 0.5 μg of ricin) in ground beef; 100 ng/mL (corresponding to 5 ng of ricin) in milk and liquid egg, all well below the toxic dose for humans.

Ricinine

Ricin is a large heterogeneous glycosylated protein, made difficult to analyze when it is present in complex matrices. Ricinine is a toxic alkaloid ($C_8H_8N_2O_2$, MW 164.1) that is derived from the leaves and seeds of the castor bean plant [67]. Because ricinine is unique to the castor plant and can be extracted from the same source as the ricin, ricinine may serve as a surrogate marker for the presence of ricin. The advantage of using ricinine as a marker is that ricinine can be easily extracted and detected due to its small size. The standard analytical method for ricinine in crude castor bean extracts uses GC/MS and/or LC/MS [68]. In contrast to the analysis of ricin, ricinine can be measured much more rapidly because it does not require the tryptic digestion step. Ricinine is also more resistant to heat, solvent, and acidic or basic pH conditions. In a fatal case of castor bean ingestion in a female puppy, ricinine was monitored by using LC/MS to confirm ricin exposure [69]. The presence of ricin has been confirmed by the detection of ricinine from food, feed and clinical samples [70-72].

CONCLUSIONS

Ricin is a protein toxin that has been used as a biological weapon in bioterrorism [8]. Intentional contamination of food has also happened in the past. To defend against ricin toxin, reliable, sensitive, and high-throughput methods, that can monitor ricin in contaminated food and human clinical samples in a timely manner, are required. Although, multiple methods have been developed for the detection of ricin, improvement of the current methods is needed because challenges (such as food matrix effect, rapid absorption and internalization of ricin within the tissues, early detection for treatment) are still present when real incidences of ricin intoxication occur.

One of the promising approaches to increase the sensitivity of detection is to combine several assay methods which have different advantages. Recently, we developed a novel immune-polymerase chain reaction (IPCR) assay for ricin [73]. This assay combined the advantages of the flexible and robust immunoassays with the exponential signal amplification power of PCR. Fig. **5** shows the schematic diagrams of two IPCR approaches. The sandwich IPCR allowed for the detection of as little as 10 fg/mL of ricin in PBS buffer, 10 pg/mL in liquid egg and milk, and 100 pg/mL in ground beef extracts. Using this method, the distribution dynamics of ricin
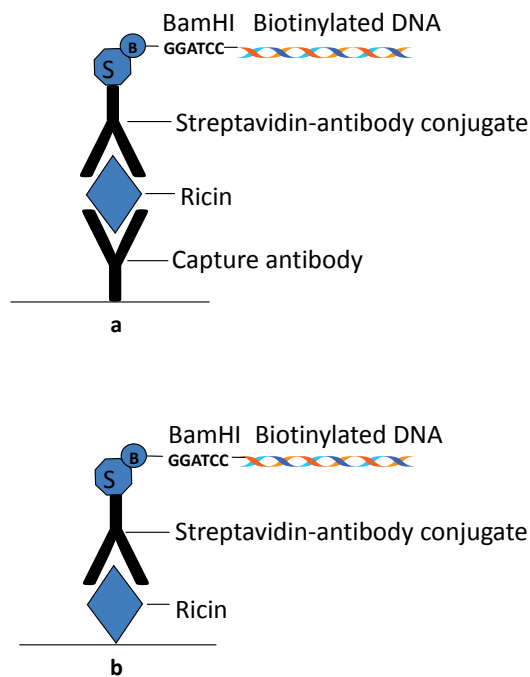
Figure 5: Schematic representation of the two IPCR approaches, depicting the analytical complexes on the surface of an assay well. (a) Sandwich IPCR using a capture antibody, antigen (ricin) and streptavidin-conjugated detection antibody. DNA marker was linked to the immunocomplex through biotin-streptavidin interaction. (b) Direct IPCR, same as the Sandwich IPCR without using a capture antibody. B, biotin; S, streptavidin.

after oral or systemic intoxication was able to be monitored in mice [43]. Limitations of the IPCR method directly resulted from the unique advantages of this method. Because the PCR signal amplification is extremely sensitive, the presence of only very low amounts of non-specifically bound DNA marker molecules can lead to strong background signals. Therefore, avoidance of cross-contamination, well-

optimized assay protocols and adaption of additional sample dilution steps are essential in this assay. Becher *et al*. [74] developed a method combining an immune-affinity step with the enzymatic activity detection by LC/MS that led to a specific assay for the entire functional ricin protein with a limit of detection of 0.1 ng/mL. This method is applicable to milk and tap water samples. Research to improve ricin detection is ongoing, which holds great promise towards the successful treatment of intentional or unintentional intoxications with ricin.

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CONFLICT OF INTEREST

The author discloses that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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CHAPTER 3

Ricin: Sorption by Soils, Minerals, Textiles, and Food; Soil Infiltration and Dust Transport

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Abstract: Ricin is a highly toxic globular protein (lectin) found in castor (*Ricinus communis*) seeds. Inhaled ricin dust or intravenously injected ricin is ~1000 times more toxic than orally ingested ricin. Interactions between ricin, soil minerals, and other materials control the environmental fate of dispersed ricin. Ricin was sorbed by the common soil clay minerals, montmorillonite (~350 g/kg), illite (~50 g/kg), and kaolinite (10 g/kg). Ricin also effectively sorbed to other soil materials: ferrihydite, goethite, calcite, and Ca-humate, but sorption by activated carbon was minimal. Montmorillonite sorbed 5 times more ricin at pH 5 than at pH 10. Much of the ricin sorbed at pH 7 could be desorbed using pH 10 buffer solutions. X-ray diffraction patterns showed that ricin sorption expanded montmorillonite interlayers and shifted the basal spacing from ~ 11.8 Å to ~21.2 Å. Textiles, fruit, and vegetables sorbed ricin, but much less ricin was sorbed than by soil materials. Breakthrough curves for ricin leached through soil columns indicate that dissolved ricin is strongly sorbed by soils and most readily moves in sandy soils. Ricin readily moved in the dust from sandy soils and was concentrated in the fine dust particles. Ricin less readily moved in clayey soil dust and was less effectively concentrated in the fine dust. Experiments using PM10 (< 10 μm) and PM2.5 (< 2.5 μm) dust samplers demonstrated that significant quantities of respirable-size ricin particles were generated in fine soil dust. Dust control chemicals, particularly polyacrylamide, reduced the generation of fine ricin dust particles.

Keywords: Ricin toxicity, ingestion routes, adsorption to solids, pH-dependent sorption, soils, clay mineralogy, textiles, fruits, vegetables, toxin mobility in water, movement in dust, and dust control agents.

INTRODUCTION

Ricin Toxicity and Ingestion Route

Ricin is a highly toxic protein (lectin) that is present in the seed of castor (*Ricinus*

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communis). The castor seed contains 1-5% ricin by weight [1]. The median lethal dose to kill 50% of the population (LD50) for intravenous ricin injection into mice is 3.7 μg/kg and it is 50 to 100 times more toxic when administered as an aerosol than when administered intravenously [2]. Orally ingested ricin is much less toxic. The median lethal dose for orally ingested ricin in mice is 30 mg/kg or about 1000 times greater than for injected or inhaled ricin [3]. In humans, the lethal oral ricin dose was estimated to be 1 to 20 mg/kg of body weight [3]. Ricin is a 2-chain protein cytotoxin, which consists of an A-chain and a B-chain linked by a disulfide bond (Fig. **1**). The B-chain binds ricin to the surface of cells allowing the toxic A-chain to enter cells. A single molecule of ricin A-chain can inactivate 1500 ribosomes per minute, which rapidly inhibits protein synthesis and leads to cell death [3]. A site was identified on ricin A-chain that induces vascular leak syndrome (VLS) in humans [4]. There is no antidote to ricin poisoning, but a recombinant A-chain vaccine was developed in which two amino acids in the ricin A-chain were genetically engineered to inactivate the ribotoxic site and the VLS site [4]. Ricin is a probable weapon of choice by terrorists because a large quantity of castor seed could easily be obtained by cultivation or other means. Separation of the toxin from castor seed can be accomplished using low-tech equipment and materials available in a kitchen or garage. See Chapter 5 of this book, Hilsen *et al.* (2012) for a more detailed description of the toxic properties of ricin from different cultivars.
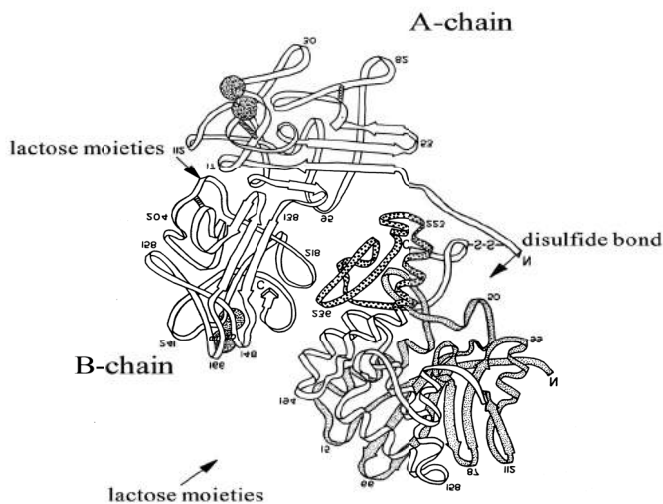
Figure 1: Ribbon depiction of ricin protein structure. Lactose moieties are sites on the B-chain that allow ricin to attach to cell surfaces. (With kind permission for use: © 1987 The American Society of Biochemistry and Molecular Biology. Montfort W, *et al.,* J Biol Chem 262(11):5400, Fig. **2**).

History of Ricin as a Potential Terrorist Threat

Castor is widely cultivated in Iraq and other countries. In 1989, about 10 liters of concentrated ricin solution were manufactured at the Salman Pak biological weapons facility just south of Bagdad [5]. Even before the events of September 11, 2001, the US Centers for Disease Control and Prevention (CDC) had identified ricin as one of the high-priority threat agents for biological and chemical terrorism. Ricin was placed on the CDC's "Category B" list because of its ease of dissemination and moderate morbidity [6]. After the events of 9/11, biosecurity issues have been an increasing concern. These security issues led to the passage of The Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (the Bioterrorism Act). The Bioterrorism Act (2002) takes steps to protect the public from a threatened or actual terrorist attack on the US food supply [7]. There already have been limited biological attacks on fresh fruit and vegetables. The Rajneeshees cult contaminated salad bars in Oregon with *Salmonella typhimurium* to influence the outcome of an election [8]. In 2003, there were arrests in England for the potential contamination of military food supplies with ricin [9]. These two examples demonstrate that intentional contamination of fresh fruit and vegetables can occur. The simplicity of contaminating food supplies is that it is a very "low technology" threat.

Ricin Properties Related to Sorption and Environmental Mobility

Like the synthetic polymers, nylon, polyethylene, and polyacrylamide, proteins are polymers with rather high molecular weights. Neutral synthetic polymers and biopolymers are thought to non-specifically sorb to mineral surfaces *via* weak van der Waals bonds. The terms, sorb and sorption, are used here to indicate removal from solution without specifying a particular mechanism, such as chemisorption or adsorption to a surface. Although the individual polymer bonds are weak, the collective effect of multiple bond attachments to the surface can result in strong sorption, particularly for high-molecular-weight polymers. Polymer sorption is considered essentially irreversible in that most of the adsorbed polymer cannot be desorbed. The presence of cationic groups on a polymer causes even stronger sorption to a negatively-charged mineral surface. Proteins, such as ricin, have amino, carboxylic acid, and other functional group side chains that cause the solubility to vary with pH. At a particular pH, a protein can be anionic, cationic,

or have both anionic and cationic groups (*i.e.* a zwitterion). There are twenty different side chains (*i.e.* from the 20 amino acids present in all living organisms) common in proteins [10]. The isoelectric point of a protein is the pH where the molecule has a net charge of zero (*i.e.* equal number of cationic and anionic groups). The isoelectric point of ricin is 7.1 [11]. At pHs below the isoelectric point, ricin has a net positive charge and a net negative charge at pHs above the isoelectric point. A pH that maximizes the number of cationic groups would be expected to maximize sorption to materials with a high cation exchange capacity (CEC). Similarly, a pH that maximizes the number of anionic groups would maximize sorption to a high anion exchange capacity (AEC) material.

Ricin is classified as a lectin, which is a protein that specifically binds to particular sugars. Lectins are highly specific for particular sugar moieties. Ricin (RCA60), *Ricinus communis* Agglutinin (RCA120), peanut agglutinin (PNA), and hairy vetch lectins are galactose-binding lectins. The larger and much less toxic RCA120 lectin occurs with ricin in castor seeds. Other lectins specifically bind to mannose, fructose, and other sugars. Much of the research conducted on lectins concerns the specific binding to cell surfaces [12, 13]. Ricin has been attached to antibodies in research to kill cancer cells. Ricin use as an anti-cancer medication will be discussed in Chapter 12 of this book by De Virgilio and Degryse. Several articles have reported that the clay component of soils and sediments effectively sorbs proteins. The clay mineral, sepiolite, sorbed collagen protein and the extent of sorption was affected by solution pH and ionic strength [14]. Collagen is positively charged in acid solution, which increases electrostatic interactions between the negatively-charged sepiolite clay surface and the positively-charged protein. Electrostatic interactions dominate in protein adsorption to goethite, illite (or clay mica), and montmorillonite, as well as in sorption to marine sediments [15]. Clay minerals common in soils were effective in binding ricin [16]. Ricin sorption was greatest at pHs ≤ 7 (*i.e.* below ricin 7.1 isoelectric point), but much less ricin was sorbed at pH 10. At alkaline pHs, ricin might sorb to positively-charged minerals, such as hydrotalcites. At pH 7, ricin sorption to montmorillonite was ~79 times greater than ricin sorption to kaolinite [16].

Ricin need not be "weaponized" to contaminate food [17]. The letter containing ricin (~October 23, 2003) had not been weaponized [18]. Terrorists can be clever

by the subtle use of low technology methods. Contamination of fresh fruits and vegetables by ricin would be an example of a sophisticated, low technology terrorist attack [1]. The food supply chain is vulnerable to this type of attack from farm production through transportation and delivery all the way to consumer sales [9]. It is a standard practice to keep vegetables fresh in the supermarket with water rinses. If the rinse water were contaminated with ricin, the consumer could be harmed without immediate knowledge. This type of biological attack on food security could easily occur because supermarket employees might contaminate the food or customers might surreptitiously contaminate the food as was done by the Rajneeshees cult.

Sorption to natural soils and other materials affects the migration and fate of toxins in the environment. Soils contain inorganic minerals, organic matter, and microorganisms. These materials can sorb or degrade aqueous toxins. Man-made or synthetic materials can also sorb and affect the migration of toxins. Soils contain a wide variety of materials with different sorptive properties and a wide range in particle size. The sand (2 to 0.05 mm) - and silt (0.05 to 0.002 mm) -size fractions usually consist largely of quartz and feldspars. The clay (<0.002 mm) fraction usually consists mostly of silicate clay minerals and iron oxides. The clay-size minerals have a large specific surface area, which in part, explains the greater capacity of clays to adsorb a large variety of substances. The sand and silt fractions have low specific surface areas and are generally rather poor sorbents. Soil organic matter consists of a wide variety of materials that range from recalcitrant, highly-altered materials, such as humus, to fresh plant material and animal remains. Humic acid is a component of humus that has been shown to sorb a variety of organic compounds, such as pesticides. Humic acid is the name (or misnomer) given to polydisperse biopolymers that occur in soil organic matter, weathered lignite coal, and other materials. Calcium humate is the form of humic acid that commonly occurs in weakly acid to alkaline soils.

The cation- and anion-exchange capacities of materials can strongly affect the sorption of many compounds. Cationic compounds strongly adsorb to materials with a high CEC. Anionic compounds strongly adsorb to materials with a high AEC. Because like charges repel, small anionic compounds are not effectively sorbed by the negatively-charged surfaces of high CEC materials nor are small

cationic compounds effectively sorbed by high AEC materials. Large organic compounds, such as natural and synthetic polymers, sorb to a variety of materials. Proteins are natural polymers that can exist as cations, anions, or zwitterions depending on solution pH. The sorption of proteins and other charged polymers is affected both by charge and the polymeric character.

The silicate clays are classified into 1:1 and 2:1 types based on crystal structure and chemistry (Table **1**). Kaolinite and halloysite are examples of 1:1 silicate clays, which have low CECs. Montmorillonite, vermiculite, and illite are examples of 2:1 silicate clays. Montmorillonite and vermiculite are expandable clays with high CECs. Montmorillonite is the principal mineral in most bentonites, which are mined worldwide and are extensively used commercial products. Montmorillonite is used as an excipient in pharmaceuticals, in food products, in animal feed, in well drilling, in metal casting, in paints, in pet litter, and other uses. Illite (or clay mica) is a non-expandable clay with an intermediate CEC. Kaolinite and illite are non-expanding minerals and the nitrogen gas (N_2) "external" surface area is about the same as the ethylene glycol monoethyl ether (EGME) "total" surface area (Table **1**). The "total" surface areas of smectites measured using EGME is much greater than the "external" surface areas. Smectite total surface areas can be as high as 800 m^2/g. The EGME can adsorb to both external and interlayer sites. The dry conditions required for N_2 surface area measurements collapse the interlayers of expandable minerals, which prevents N_2 access to the interlayers. Minerals, such as quartz (SiO_2), have a fixed composition. In contrast, vermiculite and montmorillonite chemical compositions and CECs vary within limits for different samples. Sepiolite and palygorskite are fibrous clay minerals that have a crystal structure similar to the 2:1 clays and intermediate CECs. Most clays in temperate region soils have significant CECs and minor AECs. Amorphous or non-crystalline clays, which are more common in tropical climates or in temperate areas blanketed by volcanic deposits, have large surface areas and a pH-dependent charge (AEC at low pH, CEC at high pH). Iron oxide minerals, such as hematite, goethite, lepidocrocite, and ferrihydrite, also have a pH-dependent charge. Iron oxides occur in most soils, but are more abundant in the soils of tropical regions.

Table 1: Properties of minerals and other potential ricin sorbents

Sample	Material	N$_2$ (external) Surface Area m^2/g	EGME[†] (total) Surface Area m^2/g	CEC Cmol/kg
Ca-montmorillonite high charge	2:1 expanding clay	76	761	120
Na-montmorillonite low charge	2:1 expanding clay	27	630	76
Illite	2:1 non-expanding clay	20	-	~15
Kaolinite	1:1 clay	11	12	2
Sepiolite	Fibrous clay	317	452	~15
Palygorskite	Fibrous clay	178	334	~20
Goethite	Fe-oxide	31	-	~0*
Hematite	Fe-oxide	39	-	~0*
Ferrihydrite	Fe-oxide	227	-	~0*
Calcite powder	CaCO$_3$	0.6	-	-
Quartz sand	Quartz	<0.1	-	~0
Ca-humate	Humic acid	0.1	-	~200*
Glass powder	Pyrex glass	<0.1	-	<0.1
Concrete	Portland cement	<0.1	-	<0.1
Decolorizing carbon Norit-A	Activated carbon	658	881	~0

*CEC and AEC values are pH dependent.
†EGME = ethylene glycol monoethyl ether.

The objectives of this chapter of the book are to (1) quantify ricin sorption to soils, soil minerals, synthetic materials, building materials, textiles, and food; (2) to examine the movement of dissolved ricin through soil columns; and (3) measure dry ricin movement in soil dust and measure the effects of dust control agents.

MATERIALS AND METHODS

Nitrogen surface areas were measured by the single point method using a Micromeritics Flowsorb II model 2300 surface area meter with a 30% N$_2$/70% He carrier gas. The ethylene glycol monoethyl ether (EGME) method of specific

surface area measurement was also used (19, method 16-3). Ricin and peanut lectin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) method [16, 20]. Batch ricin adsorption isotherms were prepared by adding aliquots of ricin stock solution to weighed samples in centrifuge tubes. Samples were shaken overnight, centrifuged, filtered, and ricin equilibrium concentrations measured by ELISA. Breakthrough curves for ricin and peanut lectin were prepared using soil columns prepared by packing soil samples into 60-mL syringes. Castor seed and raw peanuts were extracted with acetone to remove castor and peanut oils. The oil-free castor and peanut seed materials were extracted with phosphate buffered saline (PBS) to prepare castor and peanut extracts. Batch ricin and peanut sorption to clays and other materials were prepared by adding ricin or peanut lectin to weighed samples in centrifuge tubes and measuring unsorbed ricin or peanut lectin concentrations in the supernatants. Ricin stock solution (*i.e.* castor extract) and peanut extract were passed through the soil columns and the effluents were collected using a fraction collector [21]. Ricin and peanut lectin concentrations in the fractions were measured by ELISA using standards prepared from purified ricin (RCA60) and purified peanut lectin (PNA) purchased from Sigma-Aldrich Chemical Company. Clay samples with and without ricin were dried on glass slides to prepare oriented films for X-ray diffraction examination. X-ray diffraction patterns were collected by scanning from 2 to 20°2θ using CuKα radiation and a Phillips X-ray diffractometer. A miniature dust generator/collector system was constructed based on the large dust generator/collector system used by U.S. Department of Agriculture, Agricultural Research Service scientists [22, 23].

RESULTS

Ricin Expansion of Montmorillonite and pH-Dependent Sorption

Montmorillonite, illite, and kaolinite are common soil minerals in temperate region soils. At pH 7, ricin sorption to montmorillonite was many times greater than to illite or kaolinite (Fig. **2**). X-ray diffraction of oriented montmorillonite samples treated with pH 7 buffer and pH 7 buffer with ricin were examined. Ricin sorption at pH 7 shifted the montmorillonite basal spacing from 11.8 Å to 21.2 Å (Fig. **3**). This indicates interlayer expansion of montmorillonite by adsorbed ricin. A similar plot of montmorillonite treated with pH 10 buffer with and without ricin

(not shown) produced no shift in basal spacing [16]. This suggests that at pH 10 ricin only adsorbed to the edges of montmorillonite particles. The interlayers account for ~80-90% of montmorillonite particle surface area. Ricin sorption at pH 5, 7, and 10 to montmorillonite indicate a strong pH dependence to sorption (Fig. **4**). The sorption data were fitted to the Langmuir adsorption model. A good fit to the Langmuir model suggests that ricin adsorbs to a surface. Also, ricin sorption to a variety of materials is proportional to surface area, which also suggests ricin adsorbed to a surface. From a fit to the Langmuir model, monolayer ricin adsorption capacities (X_m) were calculated for the three pHs [24]. Ricin adsorption at pH 5 was ~5 times greater than adsorption at pH 10 based on the monolayer capacities. The decolorizing activated carbon powder adsorbed much less ricin than Ca-humate or montmorillonite (Fig. **5**). Ricin sorption to building materials (not shown), such as concrete and glass was minimal. Ricin adsorption by granular activated cocoanut charcoal (not shown) was much less than decolorizing carbon (Fig. **5**). This suggests that activated carbon would not be effective in treating patients that had swallowed ricin. However, the recommended treatment for ricin ingestion is a single dose of activated charcoal [1, 6, 25]. The intended purpose of the activated charcoal is to adsorb ricin and prevent ricin from absorbing from the digestive tract into the blood stream. The mycotoxin, aflatoxin B1, adsorbs to bentonites from water and mixing ~1% bentonite with animal feed can prevent aflatoxin toxicity [26]. Activated carbon also effectively adsorbs aflatoxin B1 from water, but animal feeding studies indicate that activated carbon mixed with animal feed does not prevent aflatoxin toxicity [27]. A sorbent might effectively bind a toxin from pure water, but not effectively bind ingested toxin. Many organic compounds are hydrophobic and readily adsorb to activated charcoal, but ricin and other proteins are hydrophilic and do not readily adsorb to activated charcoal. Activated carbon does not work effectively for organic compounds that are highly water-soluble or polar [28]. Based on the ricin adsorption isotherms, ingesting activated carbon after swallowing ricin would have little or no effect on the toxicity. Increasing solution ionic strength 10-fold from 0.018 to 0.180 decreased ricin adsorption to montmorillonite (at pH 7) from ~350 g/kg to ~250 g/kg (Fig. **6**). Ricin adsorption was rapid and mostly complete within 1 minute.
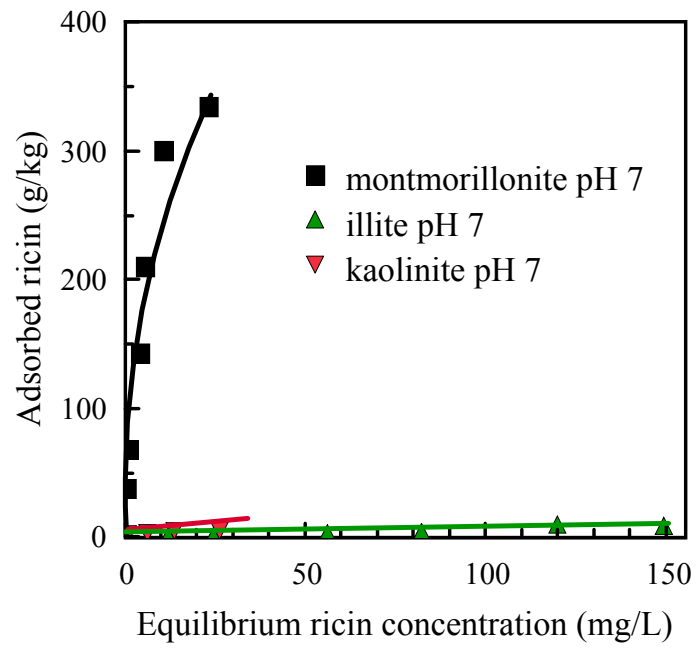
Figure 2: Ricin sorption to common soil clay minerals at pH 7.
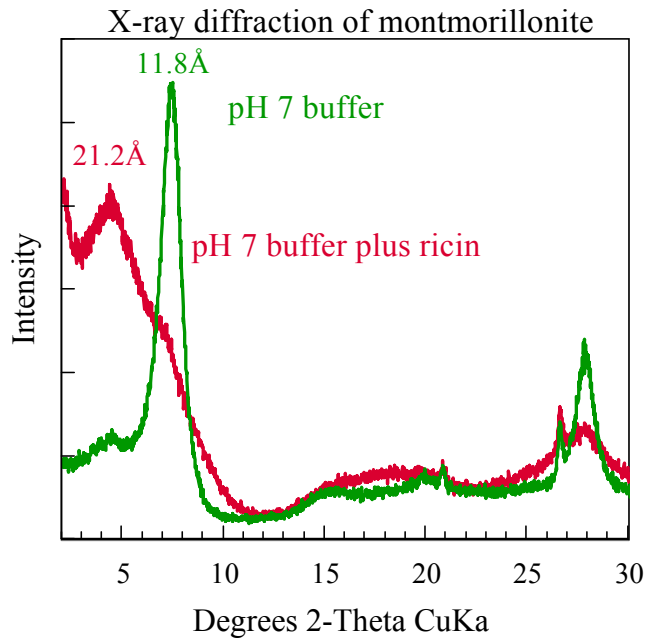
Figure 3: X-ray diffraction patterns of pH 7 montmorillonite before and after ricin sorption.
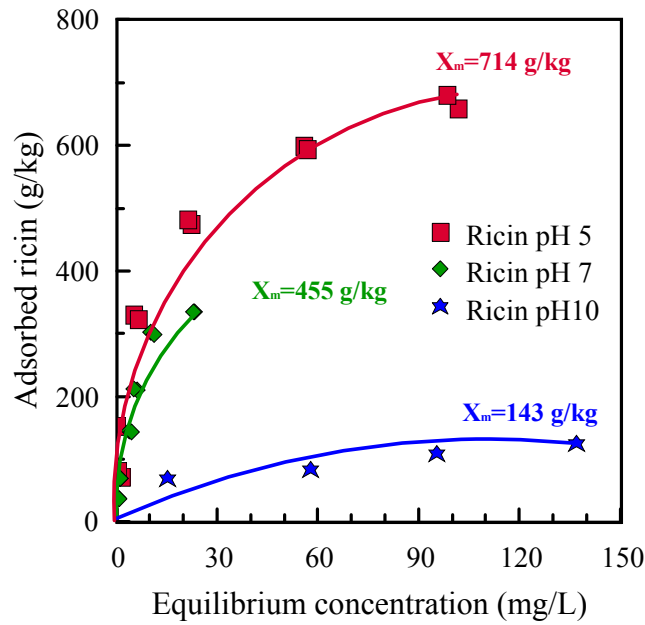
Figure 4: Ricin adsorption to montmorillonite at pHs 5, 7, 10, and monolayer adsorption capacities (X_m) calculated from fit of Langmuir model to data.
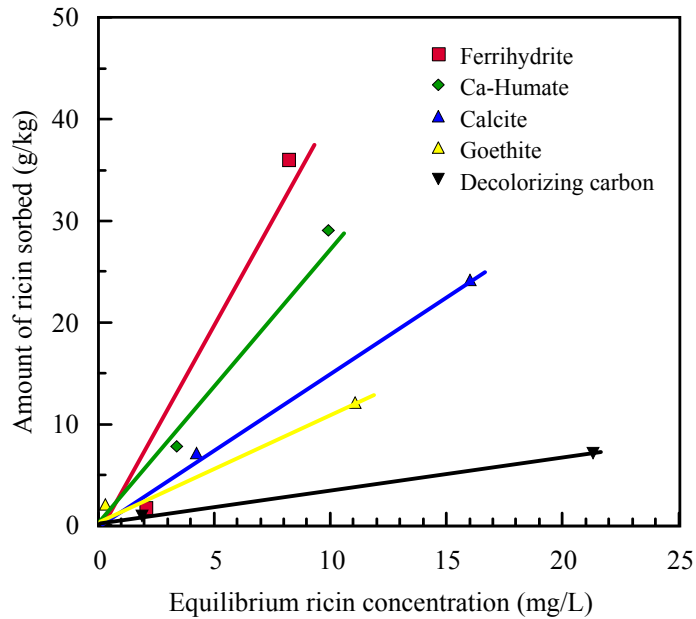
Figure 5: Ricin adsorption at neutral pH to decolorizing activated carbon, Ca-humate, calcite, goethite, and ferrihydrite.
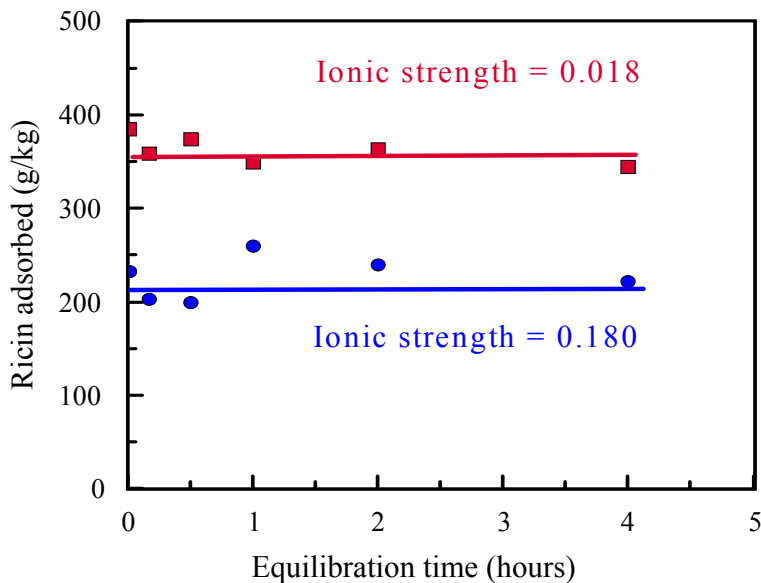
Figure 6: Kinetics of ricin adsorption at pH 7 to montmorillonite and the effect of ionic strength.

Ricin Sorption and Movement Through Soil Columns

At pH 7, ~350 g ricin/kg montmorillonite was adsorbed, but only ~50 g peanut lectin was adsorbed at a comparable equilibrium concentration (Fig. **7**). The peanut lectin molecule has a molecular weight of ~110,000, which is about twice the ~65,000 molecular weight of ricin. Larger molecules are generally more strongly adsorbed than smaller molecules. Ricin and peanut lectin are both galactose-binding lectins, but ricin was much more readily adsorbed to montmorillonite. Of the 529 amino acids in the A and B chains of ricin, 42 contain acidic side-chain amino acids (glutamate, aspartate) and 43 contain basic side-chain (histidine, lysine, arginine) amino acids [29, 30]. Of the 1028 amino acids in peanut lectin (a tetramer made of four identical 257-amino-acid subunits), 204 have acidic side chains and 80 have basic side chains [31]. The greater ratio of acidic to basic amino acids in peanut lectin (204/80 = 2.55) relative to ricin (42/43 = 0.98) probably explains the smaller amounts of peanut lectin sorbed by montmorillonite. At pH 7, the amine side chains of lysine and arginine are positively charged (histidine either neutral or positively charged) and the carboxyl side chains of glutamate and aspartate are negatively charged [10]. Therefore at pH 7, peanut lectin should have a greater proportion of negative charge sites than ricin.
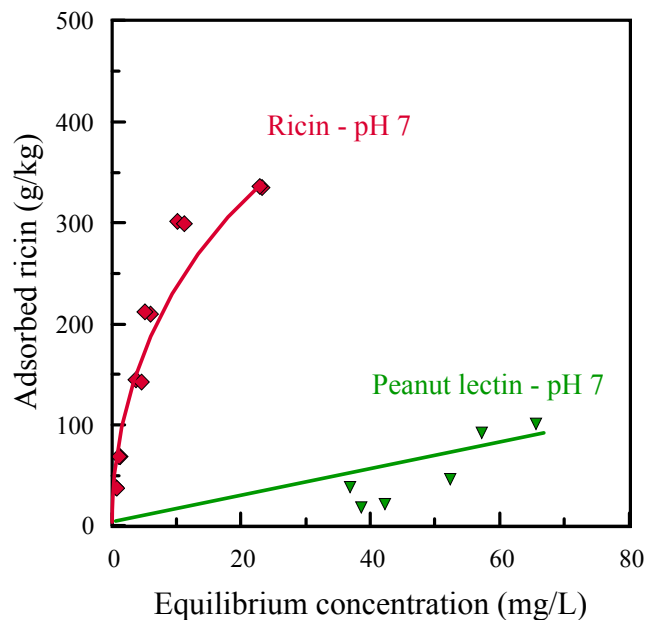
Figure 7: Ricin and peanut lectin (PNA) adsorption to montmorillonite at pH 7.

Differences in the breakthrough curves for ricin and peanut lectin leached through loamy fine sand and sandy clay loam soil columns (Fig. **8**) are consistent with the adsorption isotherms (Fig. **7**). Peanut lectin is not as strongly sorbed to soil as ricin and more rapidly moved through the soil columns (Fig. **8**). In Fig. **8**, Ce = equilibrium ricin or peanut lectin concentration and C0 = initial ricin or peanut lectin concentration. Breakthrough was achieved when the ricin or peanut lectin concentration passing out the bottom of the soil (Ce) column was equal to the initial concentration (C0). Breakthrough curves are used to measure how effectively a soil can prevent movement of a contaminant into surface or groundwaters. Breakthrough (Ce/C0 = 1) for peanut lectin in loamy fine sand was achieved after <10 pore volumes, but breakthrough was not quite attained (Ce/C0 = 0.9) for ricin after 50 pore volumes. For the sandy clay loam soil column, >55 pore volumes were needed for peanut lectin breakthrough, but ricin breakthrough was not even approached (Ce/C0 = 0.1) after 130 pore volumes. The breakthrough curves indicate that ricin dissolved in pH 7-8 water is not very mobile in soils, but is more mobile in loamy fine sand than in sandy clay loam. In acid soils, expected ricin mobility would be even less. At pHs >8, expected ricin mobility would be greater, but few soils (*e.g.*

sodic soils ~pH 9) are more alkaline than pH 8. The soil clay fraction accounts for most of the peanut lectin and ricin sorption. The loamy fine sand soil contains much less clay (13%) than the sandy clay loam (28%) soil. Peanut lectin was used as a non-toxic simulant for ricin. However, peanut lectin clearly behaves much differently than ricin. Ricin toxoid might be a better simulant for ricin. Ricin toxoid can be prepared from ricin by partial denaturation using formaldehyde [32]. Research using non-toxic simulants can aid method development without exposure to toxins, but actual toxins must be used in experiments to verify that results derived from simulants are applicable to the toxin. Research based entirely on simulants might be irrelevant to the actual toxins.
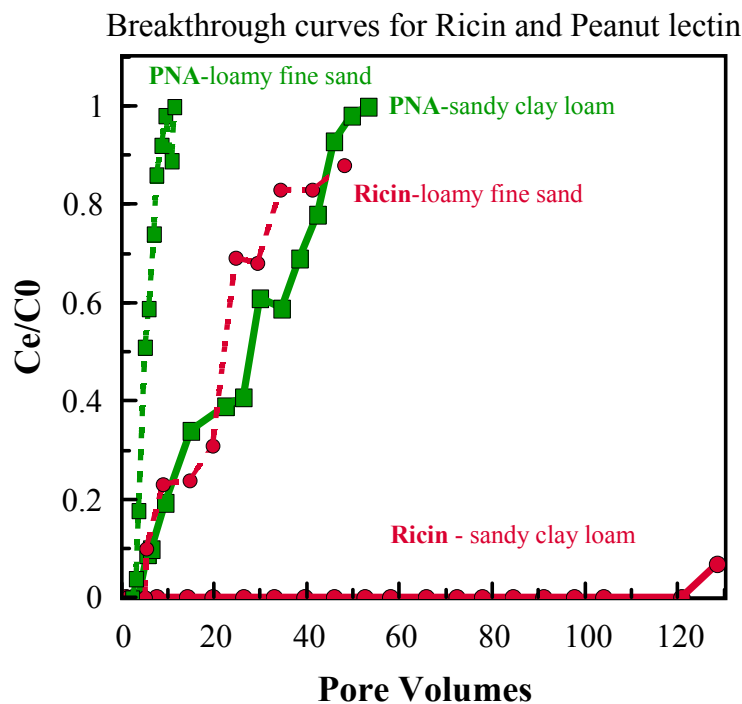
Figure 8: Breakthrough curves for ricin and peanut lectin (PNA) leached through soil columns of Brownfield loamy fine sand and Amarillo sandy clay loam. A neutral to slightly alkaline solution pH of 7-8 was controlled by the soils.

Ricin Sorption and Desorption from Clays and Soils

Sorbed compounds can to some extent be desorbed into solution. Small compounds are usually easier to desorb than high molecular-weight compounds,

such as polymers. Smectites are a group of expanding 2:1 minerals that includes montmorillonite, saponite, nontronite, hectorite, and beidellite. Smectite group minerals have a similar range in layer charge and CEC, but differ chemically. Ricin dissolved in pH 7.4 phosphate-buffered saline (PBS) was equilibrated with smectite (montmorillonite) and illite (Fig. **9**). After equilibration, the samples were centrifuged and ricin concentrations were measured in the supernatants. Based on the differences between initial and equilibrium ricin concentrations and the sample weights, amounts of ricin sorbed to the clays were calculated. To try to desorb the sorbed ricin, a fresh sample of PBS was added to the centrifuge tubes, equilibrated, centrifuged, and ricin concentrations were measured in the supernatants. Two more desorption steps were performed using pH 10 buffer. The amounts of sorbed ricin did not change much from the initial amounts after the desorption with PBS. However, the first desorption with pH 10 buffer desorbed significant amounts of ricin from both smectite and illite. The second desorption with pH 10 buffer desorbed much less ricin. At neutral to acid pHs, ricin has a net positive charge. At pH 10, ricin should have a net negative charge. Smectite retained ~100 mg ricin/g of clay after one PBS and two pH 10 desorption treatments, but most ricin sorbed to illite was desorbed. Smectite has both interlayer and external surface adsorption sites, but illite is not expandable and only has external surface sites. Ricin sorbed to interlayer sites appears to be more resistant to desorption than ricin sorbed to external sites. In Fig. **10**, ricin sorption/desorption data were presented using the ricin concentrations in the extracts rather than calculating the amounts sorbed. Twenty mL of ~200 μg ricin/mL were added to all samples. The concentration of ricin in the "Initial" smectite extract was <10 μg/mL, which indicates that 10 mg of montmorillonite sorbed more ricin than 50 mg of illite, 250 mg of Amarillo fsl soil, and 1000 mg of kaolinite. The "PBS" extract concentrations were comparable or higher than the "Initial" extract concentrations, which indicates some ricin was desorbed. The first pH 10 buffer wash desorbed the greatest amounts of sorbed ricin. This probably occurred because the net charge on ricin molecules at acid pHs is positive, but the net charge is negative at alkaline pHs. The negative charge of the clay surfaces acts to repel negatively-charged molecules. Very little additional ricin was desorbed by the second pH 10 wash.

Sequential sorption and desorption from clays
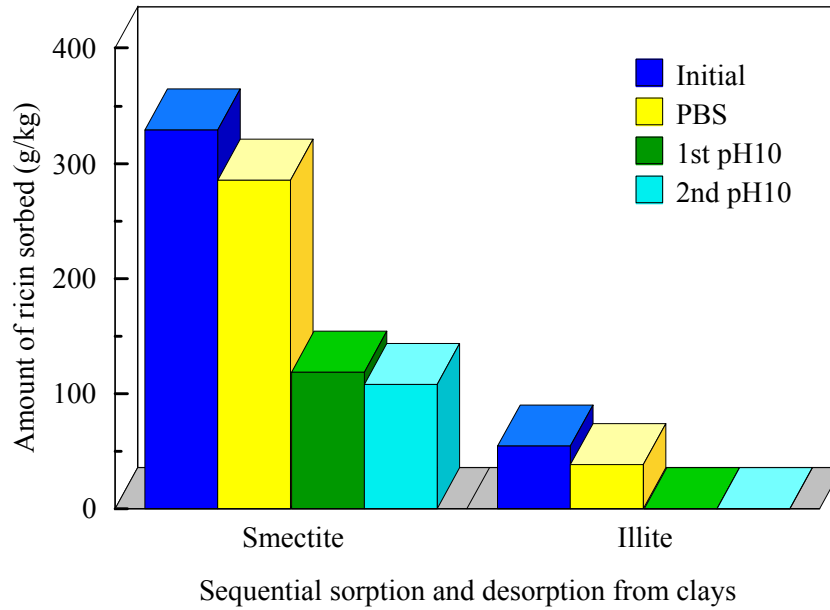
Figure 9: Ricin sorption/desorption to smectite (montmorillonite) and illite.

Ricin concentrations in extracts
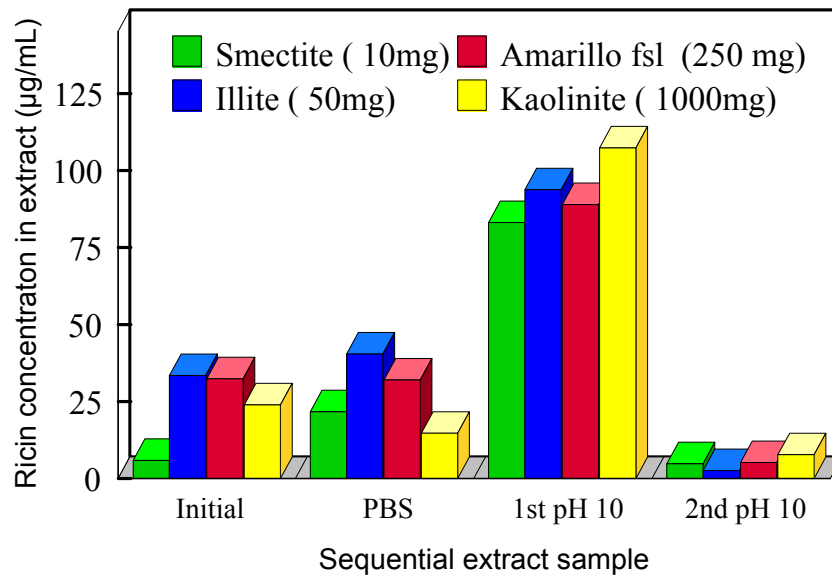
Figure 10: Concentrations of ricin desorbed from clays and a soil in sequential extracts.

Ricin Sorption/Desorption from Textiles

Ricin sorption/desorption by textile samples were examined in Fig. **11**. The wool sample sorbed more ricin than nylon or cotton, but far less (4 g/kg *vs.* 350 g/kg) than the smectite in Fig. **9**. Unlike nylon and cotton, wool is a protein. The amino acids in wool with acidic and basic side chains can impart negative and positive charges to wool depending on pH. Desorption using PBS reduced the amount of sorbed ricin, particularly for nylon and cotton. The pH 10 buffer washes desorbed most of the ricin sorbed to nylon and cotton. Wool retained the most sorbed ricin after PBS and pH 10 buffer washes. Nylon sorbed the least amount of ricin and was more completely decontaminated by the PBS and pH 10 buffer washes than the other textiles. The pH of laundry detergent is ~8 and should act like the pH 10 buffer to desorb the sorbed ricin [33].
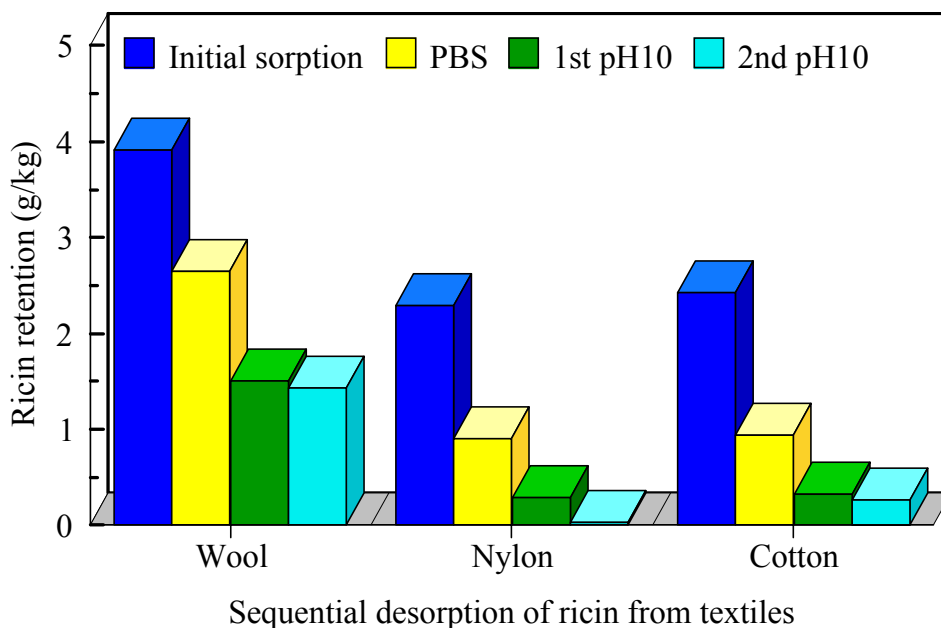
Figure 11: Ricin sorption/desorption to textiles.

Ricin Sorption/Desorption to Fruits and Vegetables

Small fruit and vegetable samples were placed in 50-mL centrifuge tubes to measure ricin sorption/desorption (Fig. **12**).

Figure 12: Centrifuge tubes with fruits and vegetables used to measure ricin sorption/desorption.

In Fig. **13**, broccoli (*Brassica oleracea*), lettuce (*Lactuca spp.*), carrots (*Daucus carota*), and celery (*Apium graveolens*) sorbed 60, 55, 43, and 33 µg ricin/g of sample, respectively (*i.e.,* 0.060, 0.055, 0.043, and 0.033 g/kg).
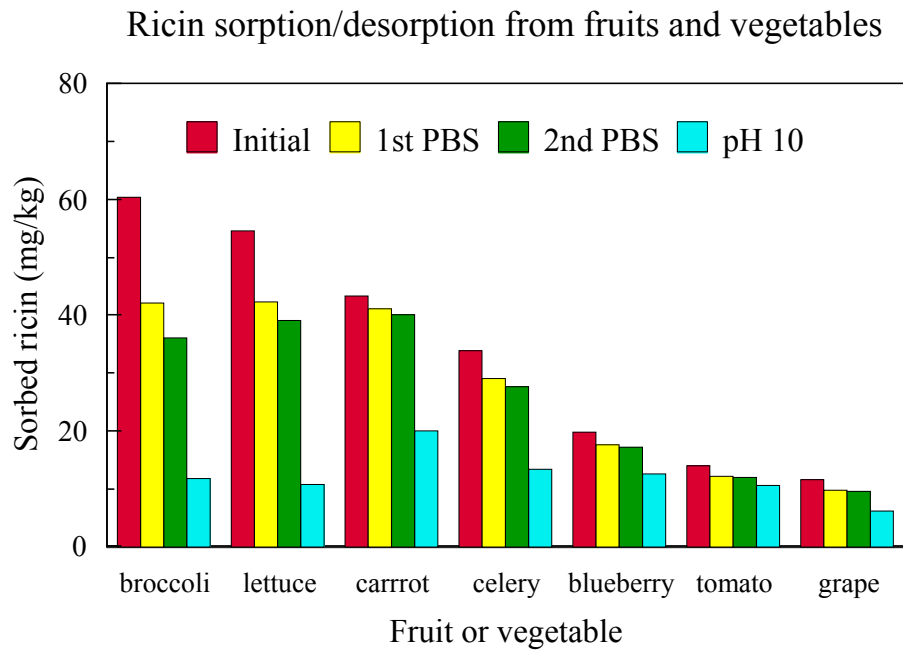
Figure 13: Sorption/desorption of ricin from fruits and vegetables.

The waxy surfaces and spherical shape (*i.e.* lower surface area) of grapes (*Vitus spp.*), tomatoes (*Lycopersicon esculentum*), and blueberries (*Vaccinium spp.*)

probably limited ricin sorption (11, 13, and 20 µg ricin/g) relative to the vegetables. Ricin sorbed to the fruits and vegetables was most effectively desorbed by the pH 10 buffer wash. This experiment was designed to model the intentional contamination of fruits and vegetables by ricin added to the spray wash solution. A spray wash is typically used in supermarkets to keep displayed produce moist and fresh. Although all of the fruits and vegetables sorbed ricin, the amounts were ~100 times less than the textiles (Fig. **10**) or ~5000 times less than smectite (Fig. **9**). Based on a minimum lethal dose of 1 mg ricin/kg of body weight [3] for orally ingested ricin, a 150-lb person would require 68 mg of ricin or 4 to 8 castor seeds. Consumption of ~2.5 lbs (1.13 kg) of broccoli or lettuce with ~60 mg ricin/kg would be needed to get a lethal dose. This assumes that all of the sorbed ricin is bioavailable. Consumption of contaminated fruits or a smaller quantity of contaminated vegetables might cause severe illness. Hence, a large vegetarian meal contaminated with ricin from a supermarket might contain just enough ricin to cause severe illness or death. However, washing the fruits and vegetables at home with water or an alkaline wash solution prior to consumption would significantly reduce the amount of ingested ricin. Cooking the vegetables should completely eliminate the toxicity. Greater amounts of a toxin are needed to do harm than the *Salmonella typhimurium* bacteria used to contaminate salad bars by the Rajneeshees cult. Ricin delivered intravenously or as an aerosol to the lungs is ~1000 times [3] as toxic as orally ingested ricin and multiple lethal doses might easily be ingested. Ingestion of a fatal ricin dose from eating contaminated fruits and vegetables is possible, but ricin dust or injected ricin could more easily deliver a lethal dose.

Ricin Transport in Dust

Inhaled or injected ricin is ~1000 times more toxic than orally ingested ricin [3]. Hence, ricin in dust is potentially far more dangerous than orally ingested ricin. A dust generator/collector system was built to simulate ricin movement in dust (Fig. **14**).

The 4 rpm rotation of the dust generator serves to temporarily suspend soil samples in air. The ball valves of the dust generator limit air entry to the cylinder at the top of the rotation. A vacuum pump draws air in and through the collector system of six glass bottles from the sixth bottle. The first glass tube in each

Figure 14: Dust generator rotating glass cylinders with ball valves and six dust trap bottles.

collection bottle forces entering air downward to deposit dust and a second glass tube moves the air to the next bottle. The glass tubes lengthen from bottle one to bottle six. Coarser particles remain as residual in the dust generator glass cylinders. The particle size of collected dust decreases from bottle one to bottle six. The PM10 and PM2.5 dust samplers were inserted into the fourth bottle. The fifth and sixth bottles contain 5-10 mL of water to trap fine dust particles that would otherwise exit the system. To prevent any ricin dust from entering the laboratory air, a 500-mL Erlenmeyer filter flask containing 100 mL of water is placed after the sixth bottle to trap any dust particles that have passed through the system. A 4000-mL Erlenmeyer flask containing 1500 mL of water traps any remaining dust before air reaches the vacuum pump. Glass tubes in the Erlenmeyers and bottles five and six force air to hit the water surface before exiting. As a final precaution, the dust generator/collector system was operated inside a hood to ensure no ricin dust entered the laboratory.

Dust from Dry Ricin Powder Mixed with Soil

Powdered castor extract (5%) mixed with Brownfield loamy fine sand soil was placed into the dust generator/collector system and dust fractions collected

(Fig. **15**). Air flow rate is an important factor in dust generation, but was not measured with this small dust generator/collector system. The fixed flow rate was controlled by the vacuum pump. Higher flow rates in the dust generator/collector and higher winds in the field would generate more dust. However, a much higher flow rate would likely shatter the glass components of the small dust generator/collector. The dust collector used in Figs. **15** and **16**, with only four dust traps, was an earlier version than the one depicted in Fig. **14**. The later version of
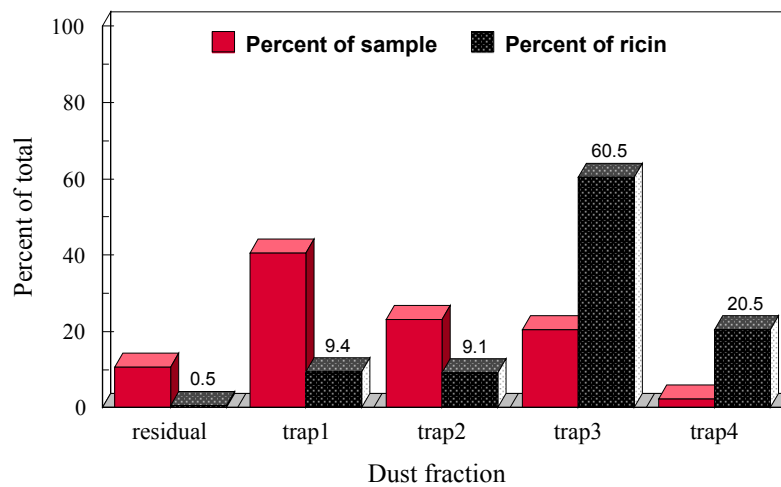
Figure 15: Dust from Brownfield loamy fine sand (lfs) with 5% castor [35].
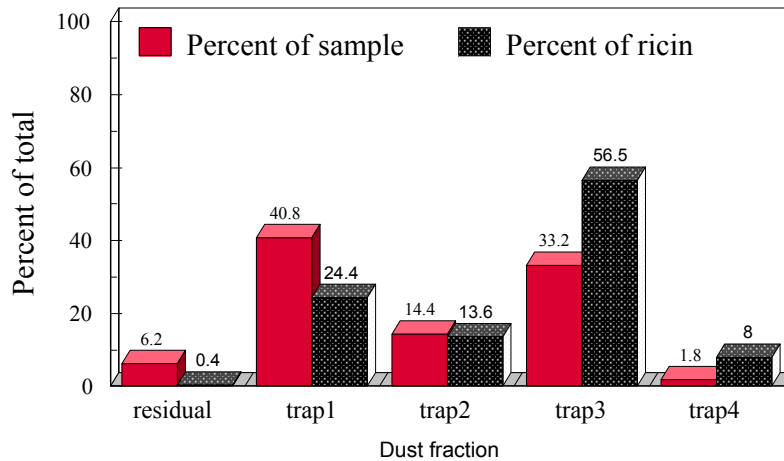
Figure 16: Dust fractions from Pullman clay with 5% castor.

the dust collector (Fig. **14**) added trap 5, trap 6, and dust traps between vacuum pump and dust generator/collector. Because the volume of the 4-trap dust collector was smaller, the flow rates were somewhat higher than in the later 6-trap version. Ricin was depleted in residual, trap 1, and trap 2 fractions and concentrated in trap 3 and 4 (Fig. **15**). The powdered castor extract clearly moved independently of the soil particles. Both particle size and density affect particle movement in dust. The average density of soil particles is ~2.5 g/cm^3, but the density of castor extract powder is only ~1.2 g/cm^3. Hence, like a feather in the wind, castor extract particles should more readily move than soil particles of equal diameter. Most (~80%) of the ricin was collected in traps 3 and 4. Trap 4 contained 20.5% of the ricin, but only 2.4% of the total sample weight. Hence, ricin was 8.5 (*i.e.* 20.5/2.4) times more concentrated in the fine dust in trap 4 than in the original sample. If no concentration of ricin in the dust occurred, the % sample and % ricin values should be equal. Most (~64%) of the ricin also moved to traps 3 and 4 in the Pullman clay soil dust (Fig. **16**). However, more Pullman soil material moved along with the ricin. Ricin in the Pullman clay trap 4 dust was only 4.4 (*i.e.* 8.0/1.8) times more concentrated than in the original sample. Based on the ricin adsorption data presented above, ricin in inhaled soil dust should adsorb to the soil particles. Moisture in the respiratory tract would dissolve ricin and facilitate adsorption to soil particles. Ricin adsorbed to soil particles should be non-toxic or much less toxic than free ricin. Dust with the greatest ricin concentrations and least soil material should be the most hazardous. Hence, the more concentrated ricin in dust from Brownfield loamy fine sand should be more hazardous than dust from Pullman clay. After each dust storm in a natural soil, soil and ricin particles would settle on the ground and can subsequently be re-dispersed into air with each dust storm or passing vehicle.

PM10 and PM2.5 Respirable Dust

The MSP Corporation Model 200 personal environmental monitor air pump and PM10 and PM2.5 dust samplers were used to collect dust samples at the bottle 4 position of the dust collection system (Fig. **17**). The PM10 and PM2.5 dust samplers only collect dust particles in the <10 μm and <2.5 μm size range. Dust samplers were added to determine the actual size of the dust particles. Dust particles, whether

Figure 17: Personal Environmental Monitor air pump and PM10 and PM2.5 dust particle samplers. The modified dust collector bottles allow PM10 and PM2.5 dust collection from the fourth bottle in the dust collector.

toxic or non-toxic, in the 10 μm (PM10) and 2.5 μm (PM2.5) size range are small enough to be inhaled into the lungs and are considered health hazards. The PM2.5 dust is more hazardous because particles of 2.5 μm or less can be inhaled deeply into the lungs. The PM10 and PM2.5 dust samplers collect a fraction of the dust at bottle 4 in the dust collector. The flow rate through the PM10 and PM2.5 dust samplers was only ~1/5 of the total flow in the dust collector. Hence, the estimated total amounts of PM10 and PM2.5 dust should be about 5 times the measured values. Castor extract powder (2%) was mixed with a white quartz sand (100% particles of 50-2000 μm diameter) and placed in the dust generator. The PM2.5 dust sampler was placed inside dust collector trap 4 and dust samples were collected (Fig. **18**). Some of the ricin was separated from the dust in the residual fraction and was concentrated in the finer dust fractions. Most of the ricin remained in trap 1 and 2. The dust in trap 4, PM2.5, trap 5, and trap 6 mostly consisted of castor powder with very little soil material. About 20% of the ricin was collected in traps 5 and 6, which contained 5-10 mL of water to trap dust that would otherwise exit the collection system. Similar results (not shown) were obtained using the PM10 dust sampler. Based on the dust samplers, significant amounts of respirable (PM10, PM2.5) dust particles of ricin were generated. Dust samples were also collected for a clay sample

(100% primary particles <2μm). The pure clay sample was prepared by removing the sand- and silt-size particles from a soil. The clay sample consisted of clay aggregates of <100 μm diameter (Fig. **19**). Most ricin in the clay dust fractions was in traps 1 and 2 and was not effectively separated from the clay. Small amounts of ricin were collected in traps 3, 4, PM2.5, 5, and 6. Ricin was effectively separated from sand in the dust, but much less effectively from clay. Clearly, dust generated from a sandy soil forms respirable-size (PM10 and PM2.5) dust particles. Respirable-size ricin particles could enter the lung, be ingested, and produce toxic effects. Coarser dust particles would more likely be ingested through the mouth and nose and enter the digestive tract where ricin toxicity is much lower.
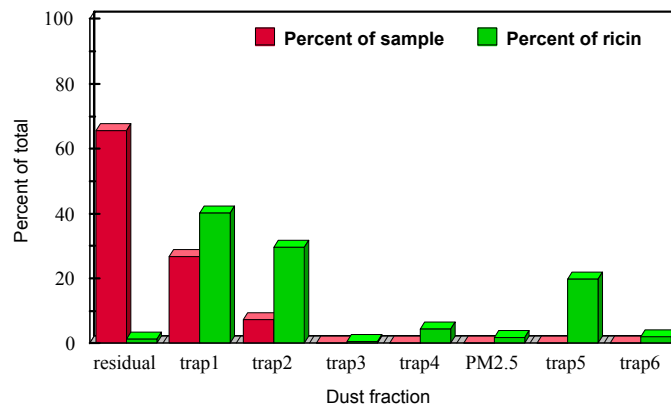
Figure 18: Dust fractions from sand with 2% castor, PM2.5 sampler.
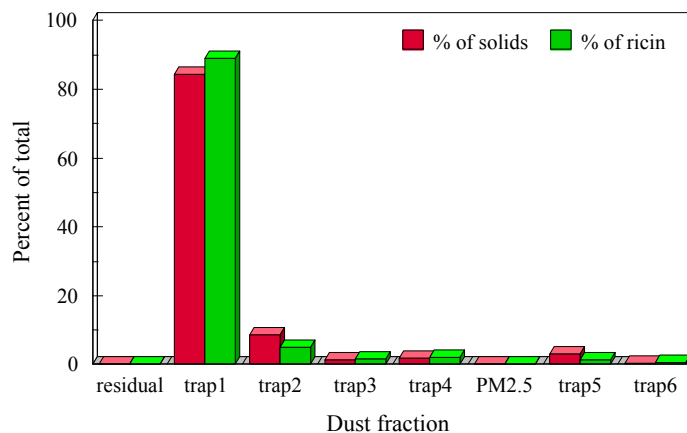
Figure 19: Dust fractions from clay with 2% castor, PM2.5 sampler.

Dust from Dry Ricin Powder *Versus* Aqueous Ricin Mixed with Soil

Dry ricin powder mixed with soil was concentrated in fine dust (Figs. **15**, **16**, **18** and **19**). However, rainfall would dissolve ricin, which might subsequently adsorb to soil particles and limit ricin dispersal in dust. Aqueous castor extract (2%) was added to Brownfield loamy fine sand soil and air-dried. The dried soil/castor extract mixture was then placed in the dust generator/collector system and dust fractions were collected (Fig. **20**).
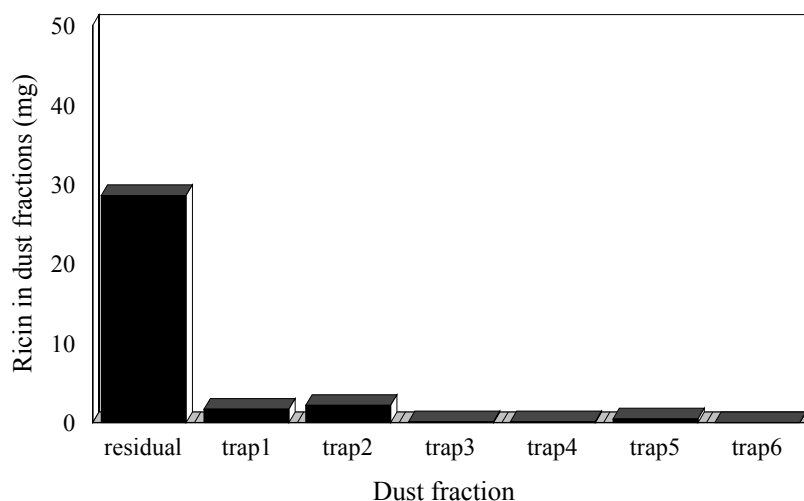
Figure 20: Ricin content in dust fractions of Brownfield loamy fine sand plus 2% aqueous castor extract.

Much less of the fine ricin dust (*i.e.* sum of traps 3, 4, 5, 6) was generated from the soil treated with aqueous ricin than with dry ricin powder. Some of the ricin was sorbed to soil particles and no longer moved in the dust independently of the soil particles. Some of the ricin attached to soil particles might later detach (*i.e.* ricin attached to sand and silt grains), but ricin adsorbed to clays would likely remain attached to the clays. A control treatment without castor extract (not shown) generated more dust than the soil treated with aqueous castor extract. Hence, the ricin in aqueous castor extract acted to bind soil particles together (*i.e.* act as a dust control agent) and reduce dust formation. The commercial dust control agents, polyacrylamide (PAM), Haul Road Dust Control (HRDC), Chemloc 101 (Chemloc), and Soiltac, were used in dust experiments to measure

the effects in reducing dust generation [34]. The commercial dust control agents (0.015%) were dissolved in water and added along with (2%) aqueous castor extract to Brownfield loamy fine sand soil, mixed thoroughly, and air-dried. The dried samples were loaded into the dust generator/collector and dust fractions were collected. The Brownfield sample treated only with aqueous castor extract yielded the most fine ricin dust, which indicates that all of the dust control agents reduced ricin dust formation to some extent (Fig. **21**). However, PAM was much more effective than the other dust control agents. Sufficient amounts of rainfall (or irrigation water) should effectively mitigate the hazards of ricin-contaminated soils. Ricin dissolved in rain would move deeper into soils where vehicular traffic or wind cannot generate dust. The dissolved ricin would also encounter and adsorb to soil clay particles as it moves deeper into the soil. In arid climates or during dry months, however, ricin-contaminated soils might remain hazardous for a long time.
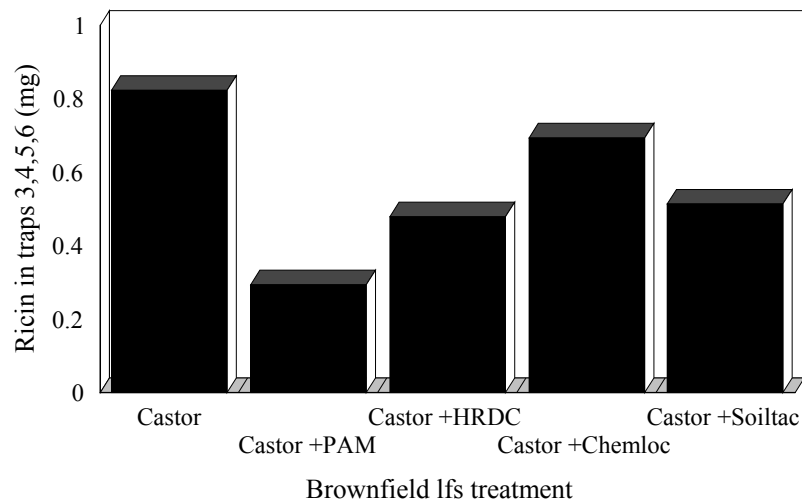
Figure 21: Ricin in respirable dust fractions of Brownfield lfs treated with aqueous 2% castor extract and 0.015% aqueous dust control agents.

CONCLUSIONS

Ricin sorbs to a variety of materials, but smectite minerals, such as montmorillonite, sorb the most ricin. Ricin sorption is pH dependent with the greatest sorption to soil clays in acidic solutions. Ricin adsorption isotherms to

montmorillonite have a Langmuir form, which suggests that ricin adsorbs to surfaces and more is adsorbed to high-surface area materials. Ricin adsorption occurs rapidly to montmorillonite and high ionic strength suppresses adsorption. Ricin adsorption expands the interlayers and shifts the basal spacing of montmorillonite. Very little ricin sorbs to activated carbon, which suggests that montmorillonite rather than activated carbon should be recommended as an antidote to oral ricin ingestion. Ricin is also sorbed to textiles, such as nylon, cotton, and wool, but washing with a laundry detergent should effectively remove sorbed ricin from most clothing. Small amounts of ricin are sorbed by fruits and vegetables, but the consumption of a large quantity of contaminated fruits and vegetables would be required for a toxic oral dose. Ricin and peanut lectin are both lectins that specifically bind to galactose. Peanut lectin has been used as a non-toxic simulant for ricin. However, adsorption isotherms and breakthrough curves indicate that ricin and peanut lectin act much differently in soils. Ricin sorption to clays greatly limits ricin movement in soils and the potential for groundwater or surface water contamination is low except for very sandy soils. Inhaled ricin is the most toxic form and wind generated dust in soils can produce respirable-size (<10 μm, <2.5 μm) ricin particles. Ricin in the dust generated from dry ricin powder mixed with soils can be greatly concentrated in respirable-size dust particles, particularly for sandy soils. Dust control agents applied to ricin-contaminated soils can greatly reduce the formation of respirable ricin particles.

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CONFLICT OF INTEREST

The authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript chapter.

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PART 3: RICIN AND CASTOR PLANT CULTIVARS

CHAPTER 4

Risk of Ricin from Commercial Castor Production in North America

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Abstract: Commercial production of castor, as a source of highly valuable hydroxyl fatty acids, has been limited by both the real and perceived risks of commercial castor production in North America. Crop commodity groups, regulatory governmental agencies, and much of the general public may have reservations about the large scale production of an oilseed crop which produces a seed meal with high concentrations of the toxin ricin as an accidental contaminant in feed grains or human food commodities, and as a potential source of chemical weapons by terrorist organizations. A successful castor industry in North America has to provide assurance that castor production will not impact the quality of existing crop commodities or create public safety concerns. Both the genetic detoxification of castor seed and the development of vertically integrated production systems, to functionally isolate castor seed and its commercial products, are being developed by researchers in Texas and California.

Keywords: Ricin, castor oil, commercial, production, cultivar, processing detoxification, genetic detoxification.

INTRODUCTION

Castor (*Ricinus communis* L.) is one of the oldest cultivated crops [1], but currently represents only 0.15% of the vegetable oil produced in the world [2].

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The oil produced by castor is essential to the global specialty chemical industry because it is the only commercial source of hydroxylated fatty acids. Over the past decade, India, China and Brazil have produced the majority of the world's castor oil [3]. Although the U.S. had significant castor production on the Texas High Plains (~30,000 ha), the industry collapsed in the early 1970's due to low global oil prices for castor oil [4]. Castor has tremendous potential as an oilseed crop in the U.S. because of its high seed oil content; capacity to produce very high oil yields; broad adaptation across North America; and its unique ability to grow on marginal land [5-7]. The increasing demand, for the use of castor oil in the production of specialty chemicals, biodiesel, and RFS2 renewable fuel, has generated interest by several companies in developing commercial castor production in this country [6-9].

Commercial production of castor in this country was initially limited due to the lack of mechanized technologies to produce and harvest the crop [5]. The incorporation of several simply inherited traits has allowed the development of commercial castor cultivars that can be grown without hand labor. The variety "Hale" carries a single gene for semi-dwarf internodes derived from a breeding line, RA 11-5-4, which reduces plant height to 1.0 - 1.2 m and has been used as a common parent in varieties of castor developed for mechanized harvest [4]. Capsule drop resistance and seed dehiscence were shown to be controlled by only one or possibly two genes [10]. At least one of these genes appears to be closely linked to the short pedicel trait. Researchers in Russia have also shown the reduction of dehiscence of capsules and pedicel length are simply inherited and can be rapidly transferred in castor [11]. Research spanning a period of over 50 years developed the cultivars and the production techniques to allow fully mechanized production and the direct harvest of castor [6]. More recent research on plant populations, weed management, volunteer castor control, growth regulators, soil fertility and planting dates have shown commercial castor production which could be profitable in many production environments across North America [12].

Crop commodity groups, regulatory governmental agencies, and much of the general public are or will be concerned by both the real and perceived risks of commercial castor production in North America. Currently, USDA grain

standards allow a limited number of castor seeds in feed and food grains (two seeds per 2 kg sample of corn and one seed per kg sample of wheat, soybean or sorghum). The utilization of both feed grains and human food commodities grown in the same general area with castor could potentially suffer from consumer concern about both detectable and perceived castor contamination. A successful domestic castor industry in North America will need to provide assurances that castor production will not impact markets or the quality of existing crop commodities. The two strategies being considered to answer these concerns are the genetic detoxification of castor and the development of vertically integrated production systems that functionally isolate castor seed and it commercial products.

REVIEW

Genetic Detoxification

Ricin is a protein toxin found only in the endosperm of the castor seed that can represent up to 5% of the meal weight remaining after oil extraction. This toxin has historically limited the use of castor meal as an animal feed and has recently been a concern as a potential bioterrorism agent [6, 7]. Development of commercial castor cultivars with low levels of ricin may generate a meal that could be used as a high protein supplement in animal feeds and also significantly improve both the economics and the perception of commercial castor oil production in North America. Ricin has both an A and a B chain linked together by a disulfide bond. The ricin gene was reported to be produced by a single gene (preproricin) that is 1695 base pairs long [13]. The B chain facilitates the movement of the A chain into the cell [14]. The A chain is capable of enzymatically destroying approximately 1,500 eukaryotic ribosomes per minute. Initially, ricin was hypothesized to be controlled by a gene family of six [15] or eight ricin-like genes [16]. However, the recently published DNA sequence has identified 28 ricin-related genes or pseudo-genes in castor [17].

Castor is a diploid (1n = 10, 2n = 20) [1] with a very small genome (~320 Mb) that was sequenced in 2010 [13]. One of the objectives of the sequencing process was to provide tools for a "gene silencing strategy" to reduce ricin content in castor seed. Castor has been successfully transformed [18, 19] but the registration

cost for a transgenic castor variety would likely be cost prohibitive given the small initially anticipated acreage of this crop in North America. Currently, the research and extension team at Texas AgriLife and Texas Tech University, with direct cooperation of the USDA-ARS Laboratory at Albany, CA, are using a conventional genetics and mutational approach for developing commercial castor cultivars with low levels of ricin.

Conventional genetics were used to reduce the levels of ricin in a semi-dwarf internode castor variety using crosses with two accessions from the Soviet Union, PI 258 368 and PI 257 654, which had been previously selected for reduced levels of ricin [20]. In subsequent segregating generations, individual plants were selected for semi-dwarf internode growth habit and reduced levels of ricin using a Radial ImmunoDiffusion (RID) assay [21-23]. In 2003, twelve F_8 lines with low levels of ricin where intercrossed to develop a broad-based, synthetic population adapted to mechanical harvest. From 2004 to 2009, this experimental population was screened for semi-dwarf internode growth habit and shattering resistance. This process developed a new experimental castor variety, 'Brigham' which has a seven to ten fold reduction in the level of ricin (Table **1**). The reduced levels of ricin in Brigham seed represent a significant reduction from the levels of ricin produced in the seed of historic cultivars like Hale. Such a reduction would make purification of ricin to be used as a bioterrorism weapon from seed of Brigham which is much more difficult and expensive.

Table 1: Quantification of ricin concentration using SDS-Page on Coomassie-stained NuPAGE Novex Bis-Tris 4-12% gel. (Dr. Xiaohua He, USDA-ARS Laboratory at Albany, CA).

Sample or Variety	Band Density	Ricin Quantity (µg)	Proportion of Ricin Soluble Proteins (%)
Ricin Std 1	2763528	8.00	
Ricin Std 2	1659458	4.00	
Ricin Std 3	1242174	2.00	
Ricin Std 4	717365	1.00	
Brigham	678582	0.46	3.07
Kaiima 93	930741	1.36	9.10
Kaiima 75	936686	1.39	9.24
BRS Energia	1116491	2.03	13.53
Hale	1117959	2.04	13.57

Genomic and protein analyses indicate that ricin genes are highly susceptible to inactivation by mutagenesis [24]. The mutagen, Ethyl Methanesulfonate (EMS), reacts with guanine causing misreading during replication, resulting in a random substitution of arginine and proline in enzymes that effectively silence the gene. The transcript responsible for the synthesis of the mature ricin molecule has arginine in the active site and is relatively rich in both arginine and proline across the protein [25]. Mutational substitution of specific arginine residues in ricin has reduced production up to 100 fold [26]. Proline plays a critical role in the formation of the secondary, tertiary, and quaternary protein structure. Proline has been shown to be necessary for the bonding of the A and B chains of ricin as well as the formation of the protein fold that is the active site of ribosome degradation. Consequently, EMS mutagenesis provides an excellent non-transgenic approach for even further reduction in ricin content in castor seed.

The USDA-ARS Laboratory has developed enzyme-linked immunosorbent assays (ELISA) using antibodies to identify individual seeds that have extremely low levels of ricin in the seed [27]. They have also shown that Western Blotting (WB) in concert with ImmunoPCR (IPCR) would be an excellent tool to screen segregating lines of castor for very low levels of ricin [28]. Our current objective is to identify 150-200 genetically diverse lines that produce less than 1 mg of ricin/seed through selection of lines derived from either sexual hybridization or mutagenesis. This dual approach, of using both existing genetic variation in combination with single base substitutions that eliminate ricin production, will hopefully help to ensure the future success of this on-going research.

Vertically Integrated Production

The concept of Vertically Integrated Production is predicated on the absolute commitment of the producers, contractors, crushers, and handlers of castor to ensure that castor is not introduced into facilities that transport, store, or process food, feed or other crop products. Even the perception of castor contamination would be devastating to other crop commodity markets. Castor seeds contain three separate products that could negatively impact human and livestock health. Ricin is a seed borne toxin that is dangerous when inhaled, injected, or ingested. In addition, castor seeds contain *Ricinus communis agglutinin* (RCA_{120}) which

can also be toxic at high concentrations. The vegetative tissues also produce a potent allergen which can cause a reaction ranging from a minor skin irritation to a more serious anaphylactic shock reaction that occurs in only a small percentage of individuals.

The State of Oklahoma recently approved legislation banning the production and processing of castor and any castor products out of a perceived threat of contamination of the State's other crops. Much of the information considered by the Oklahoma Legislature in this process was not based on scientific evidence but on public perception. This concern about castor may spread to other states. Therefore it is essential to have scientifically reviewed publications to be considered in the political discussions on the potential threats and attributes of castor.

At this time, no special licensing or permission is required in much of the U.S. for growing castor seed for oil production or as an ornamental plant. Texas AgriLife Extension has drafted recommendations for safe production, processing, and transportation of castor and castor by-products due to many potential or perceived issues relating to castor and ricin toxicity. Castor production should be conducted in a prudent and sound fashion, but unfounded paranoia should be avoided. Thousands of acres of castor were safely cultivated in the High Plains of Texas for over 30 years with no significant safety problems. These guidelines may serve as the basis for encouraging safe handling and heightened awareness for processing this valuable industrial crop. Texas AgriLife has agreed to prepare a two-hour course in health, safety and handling for growers, contractors, processors, and transporters of castor seed. General worker protection and safety standards will be incorporated into this training course. All educational, production, handling and safety guidelines will be web based at http://castor.tamu.edu, available for viewing, printing, and download on demand.

Since the seed storage protein ricin surrounds the embryo underneath the seed coat, whole seed that has not been cracked or broken do not have ricin present on the surface of the seed. Consequently, handling whole seed manually is not dangerous. If the seed is broken or cracked during processing there may be traces of ricin on the seed and seed handling surfaces. A common misunderstanding

persists for ricin and ricinoleic acid. Although both of these products are obtained from castor seed these are two distinct compounds. Ricin is a protein while ricinoleic acid is a fatty acid component in the oil. Castor seed should be stored in cool, dry conditions to maintain seed germination. If castor is treated with commercial seed treating equipment that is also used on other crops, then this equipment must be thoroughly cleaned after use.

Once a planter has been used for planting castor, all planter boxes should be removed from the planter and disassembled to clean out any remaining seed that may be trapped inside the planter box or in the air-vacuum disk housing. Traces of castor residues and possibly oil could build up on the planting equipment. The residues do not pose a threat to subsequent planting seed of other crops. As a precaution, power washing planter boxes and disc/plate housings may be used to eliminate any castor residues from the planting equipment prior to subsequent handling or maintenance. Other production equipment that has been used in castor production up to its harvest do not require special handling or cleaning. Workers in castor fields should be alert for possible allergic reactions. For a period of up to two years after the production of a castor crop, care must be taken to remove any volunteer plants that could contaminate subsequent crops. The use of crops with selective herbicide resistance could facilitate the removal of volunteer castor plants.

Combines should be used exclusively for castor harvest to reduce the risk for contamination of other food and feed grains. Since combines are very difficult to clean out, it is essential that once a combine has been used for castor harvest then it should be exclusively dedicated to castor and not allowed to harvest other crops. Equipment in contact with castor seed may in time become 'gummy' due to the accumulation of small amounts of castor oil and dust.

Castor seeds can be hauled in farm trucks and semi-truck trailers to designated castor receiving points or to a similarly dedicated crushing facility. Grain trailers and trucks should be cleaned out after transportation and it is important to ensure that rear gates or bottom hopper gates are closed tightly so that seed does not leak through. Tarps or covers should be used to prevent seeds from being blown off the truck during transit. After unloading, the inside of the hopper or truck bed should

be swept out to physically remove any residual castor seed. At the end of a castor harvest, the trucks and trailers should be power-washed with hot water and detergent to remove any castor residues. Castor should never be hauled in rail cars since these cannot be readily inspected or cleaned.

Castor should not be introduced into elevators that store food and feed grains. There are too many areas where small amounts of castor seed will be retained in these facilities to ensure clean out of essentially all of the castor seed. Crushing facilities used for castor should be used exclusively for castor or other industrial oilseed crops that have no food uses. Castor oil is essentially free of the potentially toxic products found in castor seed but the meal residue remaining after oil extraction must be treated with caution. Historically, the meal has been used as a high value, organic fertilizer which reduced soil pathogens such as nematodes [29]. The use of genetic, chemical, and physical methods to detoxify the meal may also allow it to be used as a high protein supplement in livestock rations.

However, complete elimination of ricin during oil extraction would obviate the problem of ricin and could be implemented by an oilseed processing plant in a reasonably short time. Numerous studies have indicated that the ricin protein is degraded by heat [30]. Industrial processes, used to extract oil from castor by applying significant heat and/or pressure to the seed, degrade the ricin remaining in the meal. Private industry has been successful in direct extraction and conversion of lipids to biodiesel utilizing supercritical fluid technology. A supercritical fluid is any substance at a temperature and pressure above its critical point. It can effuse through solids like gas and dissolve materials like liquid.

Inventure's technology (U.S. Patent No. 7943792) involves taking the target biomass, such as castor seed, reducing its size and then generating a slurry with alcohol and water. This slurry is then fed to a continuous plug flow reactor where the temperature is increased above the critical point for the target alcohol. Temperature, residence time (less than 30 minutes), reaction zone and pressure are controlled. The resulting solids are esterified and hydrolyzed effectively and a complete liquid is discharged from the reactor. The condition of super critical water hydrolysis is achieved at the critical temperature and pressure of alcohol

(temperature and pressure roughly half that of super critical water). Control samples of surrogate protein biomass containing toxins, such as phorbol esters, have been processed using the Inventure technology and the toxins have been completely eliminated in the post-reaction product. Preliminary evaluations have demonstrated that at the bench scale this processing technique can eliminate ricin from castor meal samples. In addition, Inventure has demonstrated that the amino acids, peptides and derivatives are substantially intact and potentially usable as an animal feed. This technology is flexible, fast, projected to be low cost and has demonstrated the ability to destroy protein based biological toxins but still render the amino acids usable as animal feed.

FUTURE RESEARCH

Rapid Ricin Detection

While the ELISA and WB are useful to detect the presence of ricin, these lack the ability to measure the activity of the toxin; *i.e.,* the ability to discriminate inactive (non-hazardous) *versus* active (hazardous) material. Functional assays for ricin have traditionally been done using mouse bioassays that have prolonged assay times; required specialized facilities with trained staff; and used a large number of animals with death as an endpoint. To measure the effect of different processing techniques on detoxification of ricin, we will develop *in vitro* enzymatic activity and cytotoxicity assays based on the existing protocols [31, 32]. A small subset of results will be confirmed by mouse bioassay [33].

Ricin Detoxification During Processing

Samples of the meal residue remaining after processing need to be evaluated to quantify any residual ricin toxin remaining after potential oil extraction processes. Once a technology has been identified as promising, it needs to be tested in replicated livestock animal feeding trial. TX AgriLife has shown interest to conduct these trials once funding is approved. These trials will require several tons of meal, several livestock animals, and will be very expensive.

Genetic Detoxification

Genetic work continues on the development of future varieties of low ricin castor. Recently our program at TX AgriLife and Texas Tech University has identified

several castor breeding lines that appear to have more than a 95% reduction in ricin content. One potential concern is that lower ricin lines appear to be more susceptible to seed feeding insects than conventional high ricin lines. Genetic enhancement research is very expensive and dependent upon finding suitable industry or governmental agencies willing to support this research thrust.

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CONFLICT OF INTEREST

The authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript chapter.

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Generic Antibody Therapy, Polyclonal and Monoclonal, on Ricin Toxin Extracted from Several Cultivars of the Castor Plant (*Ricinus Communis*)

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Abstract: Castor beans, from 10 different cultivars of *Ricinus communis*, were acquired from different commercial sources from three different countries. Ricin was extracted from 25 grams of beans from each cultivar. Resulting toxin yields varied from negligible to relatively high. Two methods of extraction were used, with one method yielding on average 7-fold more toxin than the other. Reducing SDS-PAGE showed that most of the ricin extractions had not only the A and B chains, but also additional bands of comparable molecular weights similar to those noted in the historical literature. Results from intra-peritoneal injection (*i.p.*) of mice, given the same amount of ricin extracted from different cultivars, varied in toxicity up to 10-fold. Regardless of the different sources of ricin, both polyclonal goat anti-ricin antisera and monoclonal mouse antibody (mD9) inhibited all of these, rescuing mice from 5 LD_{50} of toxin when given *via i.p.* an hour later.

Keywords: Castor plant, cultivars, ricin, extraction methods, polyclonal, monoclonal, antibodies, rescue, generic therapy.

INTRODUCTION

In August 2011, President Barack Obama was warned by security officials that the al-Qaeda in Yemen was planning to use ricin toxin in bomb attacks in the USA [1]. Many in the public are likely to know about ricin, either through the famous assassination of the Bulgarian defector, Georgi Markov, in London by a tiny pellet injected from a weaponized umbrella [2], or from incidences in the international news. For example, in the United States, "poison letters" were

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mailed to the White House and others in 2003, 2004 and 2013 [3-5], in 1993 Canadian Customs officials confiscated a ricin preparation at the Yukon border [6], in France two small flasks with traces of ricin toxin were found in a locker at a railway station in 2003 [7], and in the United Kingdom 22 castor beans, equipment and recipes for ricin and other poisons were found at the apartment of a suspected al-Qaeda operative in north London in 2003 [8]. Twenty-two beans may not appear to be much, but given that 5% of a bean could be ricin [9], these beans could have had enough toxin to kill several people if given by the proper route of exposure.

As a preventative measure, several countries are developing medical countermeasures against ricin as a possible terrorist biothreat. Once these therapeutics are been developed, there arises the need to know whether these have limited applications, such as against ricin from one castor plant cultivar, or broad generic uses such as against ricin from any source. For the former, because of the wide geographic distribution, different cultural conditions and different agricultural purposes, there is a wide variation in cultivars [10]. Also, seeds from different castor plant cultivars express different ricins, some having bands showing chains A1 and A2 [11], B [12], CI and CII [13], D [14], E [15] chains as well as the haemagglutinin [16]. Given these variances, one might assume that having a generic countermeasure against the toxin from different cultivars is unrealistic if not impossible. However, even with the large phenotypic differences observed between castor plants, surprisingly these are genetically similar, all castor plants being monotypic and easily interbred regardless of their source [10]. Also, if one looks in depth at the ricin molecule, it matters little if toxins extracted from different cultivars have structural or antigenic differences. As long as the toxin's active site [9] is conserved between cultivars and the therapeutics act on this conserved region, medical countermeasures are likely to have broad applications.

With the availability of castor beans through common commercial sources, it was possible to complete an antibody therapy study on the ricin extractions from each of the different cultivars acquired. For this study, two methods of extraction were compared for yields of toxin and the ricin preparations were compared for appearance on reducing sodium dodecyl sulphate polyacrylamide gel

electrophoresis (SDS-PAGE) and toxicity by LD_{50} (*i.p.*) in mice. For some of the ricin extractions, groups of mice were each given 5 LD_{50} of ricin and treated with either goat anti-ricin polyclonal antiserum or a mouse anti-ricin monoclonal antibody to show mouse survival from ricin toxin by antibody therapy. The following gives insights, both on the ricin extractions prepared from each cultivar and on the effective treatment of these toxins in a mouse model.

CHARACTERIZATIONS OF RICIN FROM BEANS OF DIFFERENT CASTOR PLANT CULTIVARS

Extraction Methods and Yields

Castor beans were acquired from seed catalogues, internet websites or seed exchange services. Fig. **1** shows a few of the *Ricinus communis* plants, seed pods and beans of the different cultivars used in this study. Note the diversity, especially for the colour and morphology of the seed pods and beans of the different cultivars.

For the extraction of ricin, two very different methods were used. One method followed that in the open literature, using laboratory equipment and reagents. Another method followed that readily available on the internet and used equipment, materials and reagents freely available to the public. Both methods yielded partially purified ricin toxin. For security reasons, it is not disclosed if the open literature or the internet information is Method 1 or Method 2.

The yields of ricin from both methods were analyzed for ricin concentration. The amount of total protein was determined using a Bio-Rad (Hercules, CA) bicinchoninic acid (BCA) protein assay, using bovine serum albumin as a standard and a spectrophotometer reading at A_{590}nm. Each sample was also run against two ricin standards on reducing SDS-PAGE and the amount of ricin protein in each band on the gel was determined from its density as a measure of intensity. A purified ricin standard was obtained from Dr. Sylvia Worbs, Centre for Biological Security, Microbial Toxins (ZBS3), Robert Koch-Institut, Berlin, Germany. A DRDC Suffield ricin standard was obtained using Method 1 with one kilogram of castor beans originating from Asia.

Figure 1: Plants, seed pods and beans of different cultivars of *Ricinus communis* © Copyright, photographs courtesy of the Government of Canada, by DRDC Suffield Research Centre.

The total grams of ricin extracted from the beans of each cultivar was calculated from the estimated ricin concentration from SDS-PAGE results and the final volume extracted from 25 grams of beans (final volumes differed for each cultivar). Percent ricin was calculated by dividing the total ricin concentration (SDS-PAGE data) by the total protein (BCA assay data). Our findings are that noted in Table **1**.

Table 1: Yields of ricin from beans of different cultivars of the castor plant (*Ricinus communis*)

CULTIVAR	METHOD 1			METHOD 2		
	Total mg amount of extracted PROTEIN (from 25 grams of beans)	Percent of extracted RICIN	Total mg amount of extracted RICIN (from 25 grams of beans)	Total mg amount of extracted PROTEIN (from 25 grams of beans)	Percent of extracted RICIN	Total mg amount of extracted RICIN (from 25 grams of beans)
1	390	4	15	47	34	16
2	270	30	82	84	6	5
3	287	21	62	18	1	0.2
4	147	19	29	3	35	1
5	70	9	6	ND*	ND	ND
6	341	38	130	72	24	18
7	270	27	74	71	7	5
8	322	32	104	152	13	20
9	17	6	1	ND	ND	ND

*ND = not determined.

LD$_{50}$ (*i.p.*)

The published LD$_{50}$ value for intra-peritoneal injection of ricin in mice is approximately 2 to 20 ug/kg [11,17,18]. The LD$_{50}$ for each of the ricin toxins in Table **1** extracted using Method 1 was determined in mice (BALB/c female, 19-21 grams, from Charles River, QUE). Only the ricin extracted by Method 1 was used as there was more available for assessment. Briefly, ricin extracts were diluted in sterile phosphate buffered saline (PBS) to give a range of concentrations from 1 to 10 µg ricin/ml followed by one in ten dilutions. A tenth mL of each dilution of each ricin extract was injected *i.p.* into mice in a group of 5 mice and mice monitored for 3 weeks. Concentrations of toxin were subsequently adjusted to narrow the range and confirm the LD$_{50}$ value in groups of 10 mice. The work was done under an approved Study Approval Form, an approved Animal Care Committee protocol, in a secure area using certified equipment. Toxicity of the ricin extracts from each cultivar is noted in Table **2** along with the toxicity of the DRDC Suffield ricin standard. One can see from the table that for the same amount of ricin, there were differences in the level of toxicity.

Table 2: The toxicity ricin, from beans of different castor plant cultivars, in BALB/c mice[a]

CULTIVAR	Ricin LD$_{50}$ (µg/kg)	Ricin LD$_{50}$/µg
1	1	50
2	1	50
3	10	5
4	7	7
5	10	5
6	1	50
7	5	10
8	5	10
9	1	50
DRDC Suffield standard	10	5

[a]Mice averaged about 20 grams.

Appearance of the Different Ricin Extracts in Reducing SDS-PAGE

Upon running the different ricin preparations on reducing SDS-PAGE (Fig. **2**), differences were evident. Many of the ricin cultivars had not only two subunits, the A and B chains with molecular weights around 32 kDa and 34 kDa respectively, but two additional bands. This is understandable given that historical literature cites ricin of different composition, some having bands showing chains A1 and A2, B, CI and CII, D, E chains as well as the haemagglutinin [11-16]. It is interesting to note that a DRDC Suffield ricin standard extracted previously during another study, and a purified ricin standard from the Robert Koch-Institut in Germany, had predominantly only 2 bands in the A and B chain region. The Robert Koch-Institut and DRDC Suffield ricin extracts were both prepared from a cultivar extensively grown throughout the world.

ANTIBODY RESCUE OF MICE FROM 5 LD$_{50}$ RICIN FROM DIFFERENT CULTIVARS

From the above results and calculations, different groups of mice (female, BALB/c, 19-21 grams) received *i.p.* injections of approximately 5 LD$_{50}$ of different ricin extracts in 0.1ml sterile saline. An hour later, one group received saline, another group received goat polyclonal anti-toxoid antiserum, and another group received purified mouse monoclonal anti-ricin antibody.

Molecular

Weight
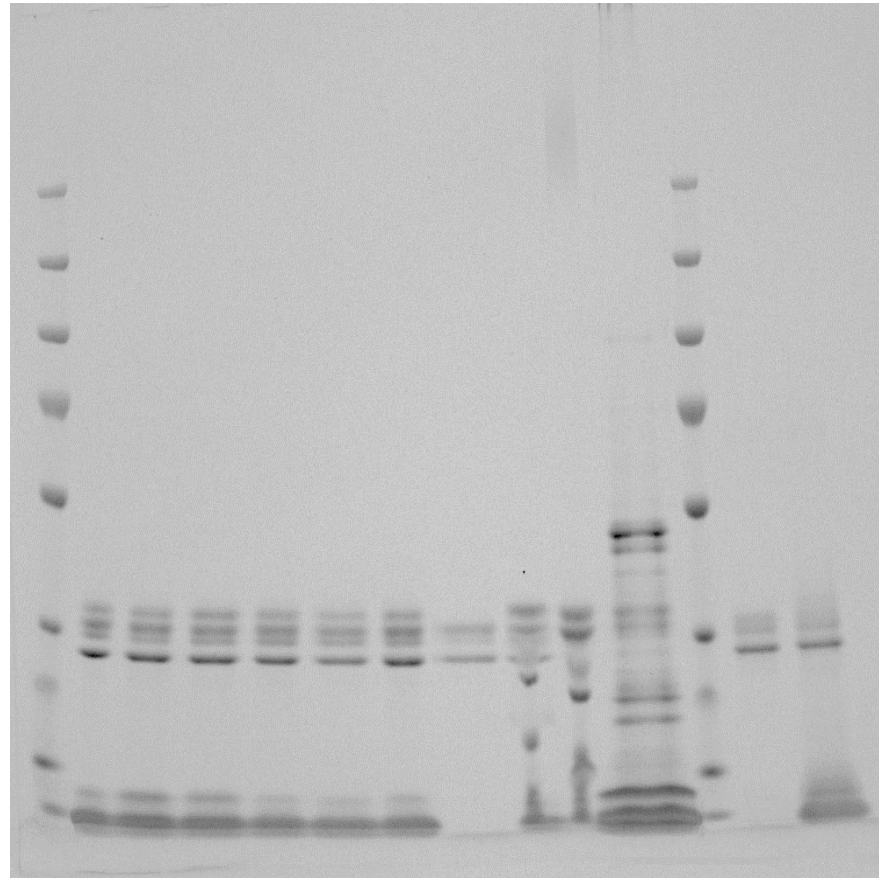
Markers

Figure 2: SDS-PAGE of ricin enriched extracts from beans of different castor plant cultivars. [a]The lane numbers in Fig. **2** are out of sequence to correlate with data in Tables **1** and **2**. Lane A: molecular weight markers, Lanes 2-9: enriched ricin extracted from beans of different castor plant cultivars, Lane G: purified ricin standard from Germany, Lane D: ricin stock from DRDC, Suffield Research Centre. © Copyright, Government of Canada, DRDC Suffield Research Centre.

The polyclonal antiserum was acquired from Cangene Corporation (under Centre for Security Sciences/Defence Research and Defence Canada project CRTI 02-0007TA, 2003-2005) that involved goats boosted multiple times with large doses

of harmless single-chain toxoid (Twinstrand Therapeutics Inc., collaborator in CRTI 02-0007TA). The toxoid has an altered A and B chain linker that maintains the protein in a single-chain inactive form, has a genetic sequence 96% similar to ricin, and is produced in recombinant yeast then purified. This goat anti-serum was found to have 6 mg/ml IgG (unpublished results). During an immune response, about 2-5% of the IgG may be directed against the antigen [19, 20], and so it is likely that mice received about 60 µg goat anti-ricin IgG/0.1ml by *i.p.* injection. For the mouse anti-ricin monoclonal antibody, mice were vaccinated against increasing amounts of DRDC, Suffield Research Centre's ricin standard (0.2, 1, 5, 25 LD_{50} *i.p.*), followed by fusing the mouse spleen cells with a myeloma cell line to create 2000 hybridoma, and from these, the best mouse monoclonal antibody (mD9) was selected by *in vitro* and *in vivo* methods [21].

Results were similar for all ricin extracts prepared from Method 1 for each of the different cultivars. Results showed that mice survived when given either polyclonal antiserum or monoclonal antibody against ricin. A representative graph of one of the 9 ricin toxins extracted from one of the castor plant cultivars is given in Fig. **3**.
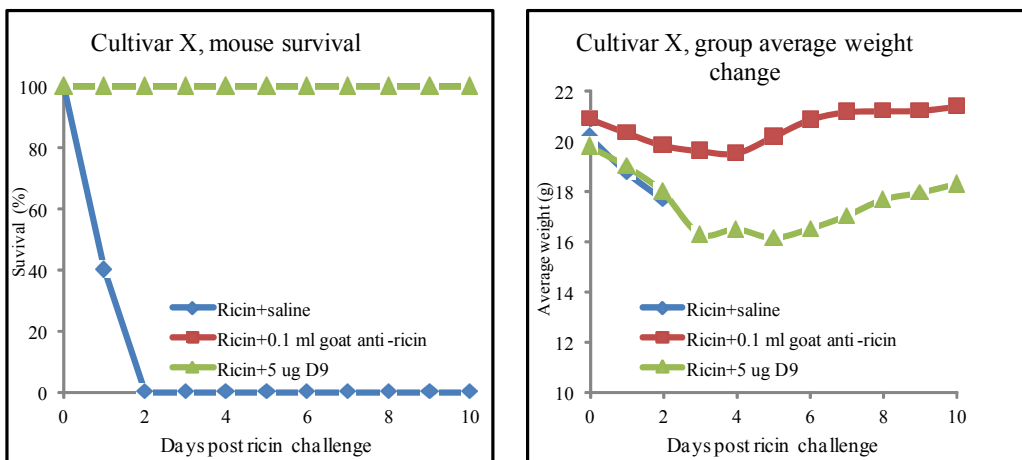
Figure 3: Rescue of mice given ricin (*i.p.*) and then saline (*i.e.* control), anti-ricin goat polyclonal antiserum or anti-ricin mouse monoclonal antibody.

In the first two days, there did not appear to be a difference in weight loss between the controls given saline and the mice given anti-ricin monoclonal antibody. The main difference was that the monoclonal antibody prevented death

and allowed recovery. Goat polyclonal antiserum prevented death for the ricin poisoned mice, but also reduced weight loss as compared to the controls and those rescued with mouse anti-ricin monoclonal antibody. Polyclonal antiserum will have several antibodies with affinity to different regions of the toxin, and it is known that multiple antibodies act synergistically against ricin [22].

DISCUSSION

There have been several incidences of terrorists either having used ricin or attempting to do so. Ricin is indeed a potential threat to civilian or military targets, but how much of a threat? In the previously noted incident in north London, UK, suspects of an al-Qaeda cell were arrested in possession of 22 castor beans. How serious is a threat involving 22 castor beans?

We do not intend to minimize a potentially very serious bioterrorist threat, but in this study we have found that, even when the method of the extraction is done in a well-equipped laboratory, ricin toxicity can differ 7-fold. This implies that if done wrong, achieving a high yield of toxic ricin may be extremely difficult. Ricin is a protein and proteins are denatured from the effects of heat, solvents, harsh pH, and contaminants secreting proteinases. Some or all of these negative effects are more likely to happen when a less scientific extraction process is used.

The yield of ricin from a castor bean has been reported to be 5% of the bean, but as shown in our study, this could drop to an insignificant amount depending on the cultivar and the method of extraction used. Also, as found for the Aum Shinrikyo sarin attack in the Tokyo subway system, the impact of the assault was lessened by the poor preparation and dispersal of the chemical agent. Should ricin ever be used in a similar way, even a minor amount of aggregation, caused by either the extraction or dissemination methods, might render it harmless [23].

With several different cultivars of castor plants available, some expressing ricin with different toxicities, one could be concerned that a medical countermeasure to one ricin variant may be inadequate for another. Fig. **1** shows variation of size and colour of castor beans even from the same cultivar. We have not had the opportunity to investigate these to determine if the ricin or its amount varies

accordingly. As previously mentioned, the literature cites different ricin extractions with different composition, some having A1 and A2, B, CI and CII, D, E chains, as well as the haemagglutinin. As noted in Fig. **2**, we have also noted variations in the number, amount and characteristics of subunit chains separated on SDS-PAGE. Could exposure to some of these ricin variants be unaffected by antibody therapies? Although our study was limited to assessing ricin extracts from only 10 different castor bean cultivars, results were consistent. It appeared that both the polyclonal goat anti-ricin antiserum and the monoclonal mouse anti-ricin antibody had efficacy against all ricins. Perhaps this is an indication that regardless of the toxin variation, if the active site on the A chain is conserved to maintain ribosomal RNA N-glycosidase (adenine depurination) activity [9], then so will its vulnerability to antibody neutralization. These preliminary results suggest that no matter which castor bean cultivar the ricin is extracted from, antibody therapeutics, either polyclonal or monoclonal, may be able to rescue the casualty.

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CONFLICT OF INTEREST

All authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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PART 4: MEDICAL COUNTERMEASURES

Ricin Toxin, **2014,** 110-129

CHAPTER 6

Progress in the Development of Vaccines Against Ricin Intoxication

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Abstract: Ricin is a potent phytotoxin that presents a significant public health concern due to its potential use as a bioterrorism agent. Exposure to ricin results in local tissue necrosis and general organ failure leading to death within several days. Currently, there is no U.S. FDA-approved drug or vaccine against ricin poisoning. Because vaccination offers a practical prophylactic strategy to protect selected populations at risk of ricin exposure, there has been a great deal of interest in developing a safe and effective vaccine to protect humans, in particular soldiers and first responders. Generation of non-toxic derivatives of ricin or ricin A chain (RTA) for use as vaccines has been initially attempted by several groups using formalin treatment, chemical deglycosylation, or mutagenesis by substitution or insertion. Most of these efforts resulted in unstable protein products that aggregated in solution, had residual toxicity, or expressed poorly in recombinant form. At present, two leading recombinant RTA vaccine candidates, RiVax (University of Texas) and RV*Ec* (USAMRIID), are in advanced development in clinical trials. This chapter reviews the efforts, challenges, and progress toward the development of ricin vaccines.

Keywords: Ricin, recombinant ricin vaccine, RV*Ec*, RiVax, ricin toxoid, RTA, dgRTA.

INTRODUCTION

Ricin is a potent toxin derived from the castor plant *Ricinus communis* L., which is grown throughout the world for commercial purposes. The toxin is synthesized

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as a single polypeptide in maturing castor seeds, and accumulates in the storage granules. The ricin holotoxin (Fig. **1**) is composed of two dissimilar polypeptide chains, designated the A chain (RTA) and the B chain (RTB), linked by a disulfide bond that joins cysteinyl residues near the carboxy terminus of RTA and the amino terminus of RTB [1-3]. The RTA (Fig. **2**) is composed of 267-amino acids that contain eight alpha helices and eight beta sheets [4]. The RTB has 262-amino acids and is a lectin that binds to galactose-containing glycoproteins and glycolipids expressed on the cell surface [5]. The RTA chain can gain access to the cell and inhibits protein synthesis by irreversibly inactivating a specific nucleoside (A4324) of the 28S ribosomal RNA. Such depurination results in the inhibition of elongation factor-2 (EF-2) dependent GTPase activity of the ribosome which halts translation and leads to cell death [2,6]. Ricin is extremely toxic; once internalized, a resident molecule can inactivate 1777 ribosome per minute, sufficient to kill the cell [7]. When the RTA is separated from the RTB and administered parenterally to animals, it has little or no toxicity [8-10]. RTA is ~1000-fold less toxic than natural ricin when administered parentally in mice [10].
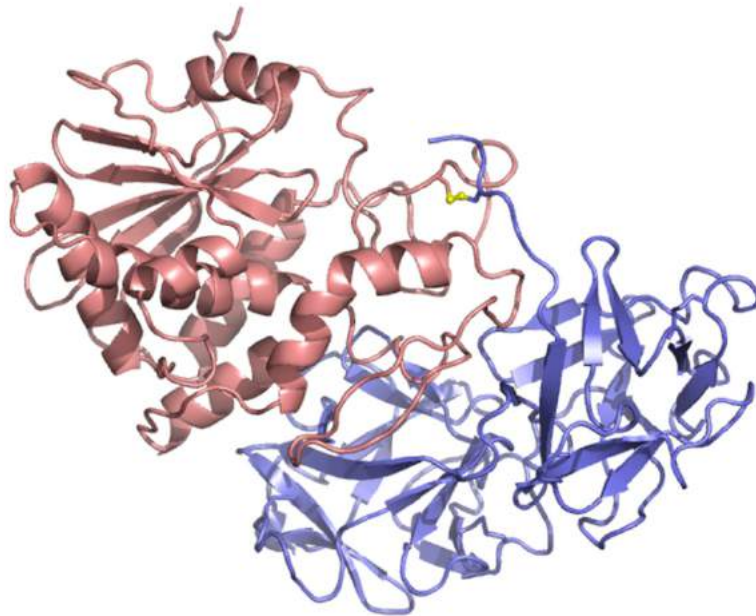
Figure 1: Three-dimensional representation of ricin. The A chain (RTA) is depicted in red, the B chain (RTB) in blue, and the disulfide bond in yellow. Image courtesy of Dr. Mark A. Olson, Integrated Toxicology Division, USAMRIID, Fort Detrick, MD.

Figure 2: Ribbon representation of RTA. The RTA has three structural domains and exhibits a substantial amount of secondary structure. Color schemes: cyan = helix; magenta = strands; red = coil regions. Image courtesy of Dr. Mark A. Olson, Integrated Toxicology Division, USAMRIID, Fort Detrick, MD.

The Centers for Disease Control and Prevention (CDC) has classified ricin toxin as a Category B threat agent. Category B agents are the second highest priority agents and include those that are moderately easy to disseminate, can cause moderate morbidity and low mortality rates, and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance [11, 12].

Ricin is listed as a Schedule 1 toxic chemical under both the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction (usually referred to as the Biological Weapons Convention (BWC), or Biological and Toxin Weapons Convention (BTWC), and the 1997 Chemical Weapons Convention, (CWC) [13, 14]. In the U.S., the possession or transfer of ricin toxin or the genes encoding its functional form is also regulated by the CDC Select Agents and Toxins Program.

Although ricin is on the list of dangerous biological warfare (BW) agents, it has never been used in combat nor in a mass attack on civilians. However, the toxin's

significance as a potential threat relates to its toxicity, the wide availability of its sources, and relative ease of production. Ricin has received significant attention since 1978 after the assassination of the Bulgarian dissident Georgi Markov publically demonstrated the extreme lethality of this toxin [15]. Numerous cases of human poisoning with ricin or castor seeds, including suicide attempts and biocrimes, have been reported [1, 16-20]. Ricin has also been cited as one of the most prevalent agents involved in weapons of mass destructions (WMD) investigations [21]. Recent attempted uses of ricin by various extremists and radical groups have heightened concerns regarding ricin's potential for urban terrorism.

RICIN TOXICITY AND SYMPTOMS OF POISONING

Ricin's toxicity is dependent on a number of factors including route of exposure [inhalation, parenteral (injection), ingestion, dermal contact, or ocular contact], amount of toxin administered, and animal species. In mice, the approximate dose that is lethal to 50% of the exposed population (LD_{50}) and time to death are, respectively, 3-5 μg/kg [17] and 60 h by inhalation [22], 20 mg/kg and 85 h by ingestion [17], 5 μg/kg and 90 h by intravenous injection [17], and 24 μg/kg and 100 h by subcutaneous injection [17]. Limited information is available regarding human toxicity. Based on animal experiments and accidental human exposures, the approximate LD_{50} and time to death for humans exposed to ricin from either inhalation, ingestion, intravenous, or subcutaneous administration of toxin have been reported [17, 23, 24]. Low oral toxicity is possibly due to poor toxin absorption and partial degradation in the gut.

The clinical signs, symptoms, and pathological manifestations of ricin toxicity vary with the dose and route of exposure. For symptomatic patients, the clinical course presents with the rapid onset of nausea, vomiting, and abdominal pain. Gastrointestinal bleeding, anuria, diarrhea, cramps, and vascular collapse can also occur [25]. Most symptoms develop less than 6 h after ingestion, although the lag time from ingestion of castor seeds to onset of symptoms has ranged from 15 min to almost 10 h. Progression to death occurs within 36 to 72 h of exposure, depending on the route of exposure and the dose received [26].

RTA AND VASCULAR LEAK SYNDROME

The RTA contains an (x)D(y) consensus motif where (x) could be L, I, G, or V and (y) could be V, L, or S [27] that is thought to be responsible for inducing vascular leak syndrome (VLS) [28-36], and has been referred to as the vascular leak-inducing peptide (VLP) [37]. While VLS is not observed in all laboratory animal models, it is a common problem (often dose-limiting) encountered in patients treated with a number of immunotoxins that contain other toxic moieties so far tested in humans, including ricin immunotoxins [34,38], and also cytokines such as interleukin (IL)-2 [38, 39]. VLS is characterized by hypoalbuminemia, weight gain, and edema, resulting from the extravasation of fluids and proteins from the vascular system into the periphery [27, 34, 35, 40-42]. Signs and symptoms of VLS can be reversed by removal of the IgG or by corticosteroid treatment [34]. Because of this potential toxicity risk, the vascular leak issue is consequently of concern when developing a ricin vaccine for human use.

HISTORY OF VACCINE DEVELOPMENT

There has been no concerted effort to produce specific ricin therapies or prophylactic measures until the early 1990s, when it was perceived to be a significant biological warfare threat [43]. Generation of non-toxic derivatives of ricin or RTA for use as vaccines has been attempted by several groups using formalin treatment, chemical deglycosylation, or mutagenesis by substitution or insertion (see below).

Ricin Toxoid

The seminal work of Paul Ehrlich during the 1890s provided the very foundation of the discipline of immunology [Ehrlich, 1891, as cited in 17]. Ehrlich demonstrated that by feeding animals small amounts of castor seeds, specific serum proteins, capable of precipitating and neutralizing the ricin toxin antigens were induced. During WWII, the U.S. Army developed a ricin toxoid vaccine for human use. This vaccine was created by incubating the ricin holotoxin with formaldehyde for 3 days, after which stationary phase, surface to air evaporation was carried out to precipitate the vaccine. This vaccine candidate did not progress past pre-clinical testing.

A second-generation toxoid vaccine composed of denatured ricin adsorbed to aluminum-based adjuvant was developed after the 1991 Persian Gulf War. Animal studies involving this toxoid vaccine [44, 45] and various delivery methods, *e.g.*, subcutaneous [46, 47], intranasal [48], intratracheal [49, 50]; and oral administration [51] were performed. In each case, protection against ricin intoxication was demonstrated. Intranasal or intratracheal instillation of ricin toxoid was also found to initiate good systemic immunity and protect against lethality from aerosolized ricin, but was not effective in protecting against bronchiolar and interstitial pulmonary inflammation [47, 52, 53]. Liposomal formulations were studied to improve localized respiratory immunity [48-51, 55]. Microencapsulation of a single dose of ricin toxoid, administered intranasally, provided both systemic and local immunity that was maintained for at least 1 year [48, 55]. Encapsulated ricin toxoid has been demonstrated to be a much more effective mucosal antigen than aqueous vaccine when delivered by oral administration [51].

Though the ricin toxoid is capable of stimulating long-lasting immunity to ricin that protects animals against lethal ricin exposures, its major shortcomings include potential for reversion to its toxic form, and the difficulty in completely inactivating this vaccine [47, 48]. The residual activity of the ricin toxoid (albeit approximately 1,000-fold lower than native ricin [14]) poses safety concerns for human use [56]. On the other hand, the completely inactivated toxoid was poorly immunogenic, and required co-administration with adjuvant or liposomal delivery [50, 54] or biodegradable poly(lactide co-glycolide) microspheres [48, 55] that have not been approved for human use.

Deglycosylated Ricin A Chain

Wannemacher and colleagues [43] performed modifications on the carbohydrate moieties of the ricin A chain resulting in deglycosylated A-chain, or dgRTA. dgRTA is approximately three logs less toxic than ricin [10]. The ability of dgRTA to elicit protective immunity in mice and rats was tested [57]. Results indicated that dgRTA elicited toxin-neutralizing antibodies in vaccinated animals, and these neutralizing antibodies were detected after two doses of dgRTA vaccine as opposed to three doses of RTA. Improved protection of lungs was observed in dgRTA-vaccinated rats as compared to RTA-vaccinated animals [43].

dgRTA was cleared from the circulation at a slower rate than the native chain [58,59] resulting in a significantly lower rate of removal by liver as compared to native ricin. In contrast to native ricin, the dgRTA did not cause histological lesions in the liver and spleen, but its toxicity in rats and mice was elevated by up to fourfold depending on the extent of carbohydrate modification [60]. By contrast, its toxicity to cultured cells diminished by up to tenfold. Thus, while modification of the carbohydrate portion of ricin greatly reduced its clearance by non-parenchymal cells, it also led to significant elevation of toxicity to experimental animals. In addition to toxicity and residual N-glycosidase activity, the dgRTA vaccine is beset with numerous shortcomings including protein instability (strong tendency of the chemically deglycosylated material to self-aggregate in solution), lack of a reproducible and robust manufacturing process that results in heterogeneity of the final product, and its potential to cause local or systemic VLS. Studies have shown that dgRTA-ITs induced VLS by damaging vascular endothelial cells (VECs) [34,37,61,62] *via* a mechanism distinct from that involved in the inhibition of protein synthesis [37].

Development of Recombinant Vaccines

While the above studies demonstrated that formalin-inactivated toxoid and dgRTA elicited a good immune response and conferred protection against aerosolized ricin, nonetheless, major safety issues precluded them from further evaluation as vaccine candidates. Thus, other approaches to vaccine development have been investigated to develop a safe and efficacious candidate. Several groups have engaged in the conventional protein engineering approach of active-site substitutions as a means of inactivating RTA and rendering it safe for use in a ricin vaccine [36, 63, 64]. Whereas this strategy was effective in reducing enzymatic activity, it failed to settle the problem of instability, *i.e.*, precipitation during production or storage. Additionally, single amino acid substitutions can be problematic for ricin because of the resilient plasticity of the RTA active site in obtaining the catalytic transition state [65]. Therefore, while these mutations often dramatically reduced RIP activity, they still remained cytotoxic [66, 67]. Mutational and modeling studies of RTA and its binding to RNA suggested probable reasons as to why isolated active-site substitutions often fail to produce structurally robust immunogens [68-70]. Recent research has focused on

developing recombinant RTA subunit vaccines devoid of cytotoxicity and other potential deleterious activities [36, 65, 69, 71, 72].

Ricin-MPP

Marsden *et al*. [69] created a vaccine candidate by introducing an inhibitor peptide (25-amino acid maize propeptide) into a surface-exposed loop connecting helices E and D which runs through the core of the RTA, and which forms part of the active site [5,73]. This modified RTA (RTA-MPP; Fig. **3**) has substantially lower catalytic activity *in vitro* (~300-fold less than that of native RTA); however, directed cleavage of the peptide insert restored the catalytic activity of the resulting two peptide A chain to normal levels [69]. RTA-MPP was reassociated with native RTB to create ricin-MPP, and this vaccine was found to be stable, highly protease-resistant, and was not toxic *in vivo* although the authors did not present data concerning lung function or tissue damage. Rats vaccinated with ricin-MPP (*i.m.*) were completely protected against a lethal dose of native ricin [69]. Nevertheless, since the RTA-MPP component of the ricin-MPP still retains residual catalytic activity, its use as a vaccine is limited by safety concern. Currently, it is not known whether this vaccine is undergoing further development [74].
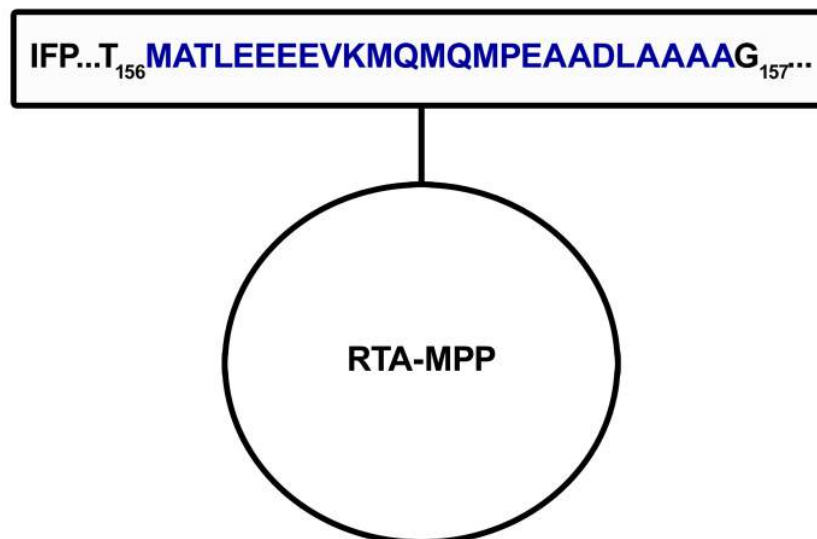
Figure 3: Mutant RTA (RTA-MPP) containing the 25-residue peptide insert from the maize proRIP. This internal pro-peptide (in blue) was introduced between Thr156 and Gly157 in RTA. RTA-MPP was expressed in *E. coli* JM101; for details, see [69].

RiVax

Researchers at the University of Texas have generated a panel of recombinant RTA (rRTA) mutants that altered the VLS-inducing site, and one was chosen for further study [36]. This candidate, RiVax, includes RTA residues 1–267 with two intentional amino-acid substitutions: Y80A mutation to inactivate catalysis, and V76M mutation to ensure the removal of any trace VLS activity from the immunogen. RiVax is at least 10,000-fold less active than wild-type RTA. The x-ray crystal structure of RiVax was recently solved to 2.1 Å resolution (Fig. **4**). It was shown to be superposable with that of the RTA with a root-mean-square deviation of 0.6 Å over 258 C^{α} atoms, demonstrating that the Y80A and V76M mutations do not significantly perturb the overall protein fold or alter the conformation of residues corresponding to known neutralizing epitopes [75].
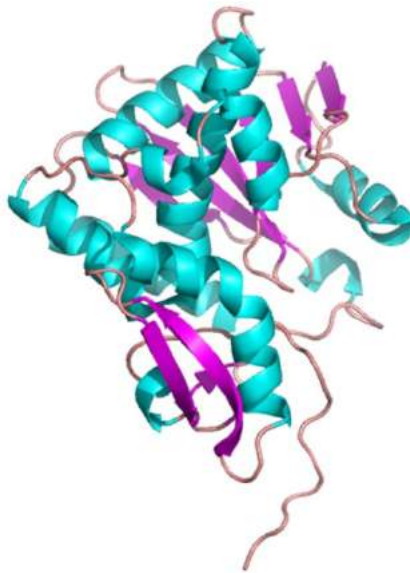
Figure 4: Ribbon schematic of RiVax. The crystal structure of RiVax was solved to 2.1 Å resolution, deposited as PDB entry 3srp [75]. Image courtesy of Dr. Mark A. Olson, Integrated Toxicology Division, USAMRIID, Fort Detrick, MD.

RiVax elicited protective immunity in mice, and had sufficient pre-clinical safety data [76]. When administered *i.m.* in mice, RiVax showed protection against ricin doses given by any of the three challenge routes [77]. Protection was also observed against ricin administered by gavage in mice lacking secretory IgA that

were vaccinated with RiVax/alum administered *s.c.* [78]. There was a marginal improvement in the ability of RiVax to induce protective antibody titers if given intradermally (*i.d.*) compared to *i.m.* route [79]. Results from the initial Phase I human trial of the vaccine without adjuvant in humans showed that RiVax appeared to be immunogenic and well tolerated in humans [80]. However, while such results were encouraging, vaccine formulation and stability remain problematic. Hence, a lyophilized formulation that retained immunogenicity when stored at 4ºC was developed [79,81]. This vaccine has been out-licensed to Soligenix for more advanced clinical trials [74].

RVEc Recombinant Vaccine

To overcome both safety and stability issues simultaneously, researchers at USAMRIID undertook a structure-based solution by reversing evolutionary selection on the RIP-fold of RTA. Olson and colleagues [65] examined the RTA-RTB interfacial region with the equivalent region of monomeric pokeweed antiviral protein (PAP) to determine the relative hydrophobicity of the C-terminal regions. This comparative analysis led to the partitioning of function of the two domains of RTA wherein the N-terminal serves as an anchoring fold, and the C-terminal contributes to RIP activity. In two-chain RIPs region, the C-terminal also provides the hydrophobic interfacial region with the lectin subunit. Exploitation of this structural hierarchy led to the RTA1–198 truncation which eliminated the undesirable hydrophobic surface of RTA that is normally in contact with the RTB, while preserving a previously identified neutralizing epitope [82, 83]. Further modeling studies revealed that a 10 residue loop region (sequence RTA 34–43), unfavorably increased the overall solvent accessibility of the protein, hence it was also removed to create the recombinant rRTA1–33/44–198 as a lead vaccine candidate [65].

The engineered RTA 1-33/44-198 (RV*Ec*) was demonstrated to have increased protein stability under thermal denaturing conditions over the parent RTA subunit. Because this truncated molecule inherently lacks several active site residues, including Glu208, Asn209, Trp211, and Arg213, it showed reduced enzymatic (N-glycosidase) activity (at least three orders of magnitude compared with RTA) [65]. More importantly, RV*Ec* conferred animal protection against supra-lethal

aerosol challenges. Complete mice protection was seen when 10 μg of the vaccine was administered either with or without aluminum hydroxide by *i.m.* injection once every 4 weeks for 8 weeks and the animals were challenged 4 weeks later with a 10X LD$_{50}$ of either *i.p.* or aerosolized ricin [65,84].

The optimal formulation conditions by which RV*Ec* remained stable and potent under various storage conditions were determined. RV*Ec* reformulation from phosphate to succinate buffer increased adherence of the protein to aluminum hydroxide adjuvant from 15% to 91%, with a nearly threefold increase in effective antigenicity in a mouse model [85]. After 6 months, all mice survived an aerosolized ricin challenge of 10 LD$_{50}$ [85]. These results suggest that optimization of adherence of a protein antigen to aluminum adjuvant should be pursued as a means to increase both antigenicity and product stability.

Further studies indicated that RV*Ec* failed to cause a considerable reduction in electrical resistance crossing the endothelial cell layer, suggesting that the vaccine should not induce VLS [86] described in RTA-based immunotoxins in humans. Additionally, the observed lack of cytotoxicity of RV*Ec* adds credence to the safety characteristic of this vaccine.

A cGLP pre-clinical toxicity study of RV*Ec* in New Zealand white rabbits demonstrated that no treatment-related or toxicologically significant effects were observed with RV*Ec* during this study [87]. A phase I clinical study is ongoing at USAMRIID to evaluate the safety and immunogenicity of RV*Ec* in humans [88, 89].

A recent study was conducted to further improve the stability and solubility of the RTA1-33/44-198 by incorporating pairs of novel cysteine residues based on the crystal structure of the truncated protein (Fig. **5**; [90]). Introduction of disulfide bond at either of two positions (R48C/T77C or V49C/E99C) increased the protein melting temperature by ~5°C compared with RTA1-33/44-198 and by ~13°C compared with RTA. Prolonged stability studies of the R48C/T77C variant revealed a >40% reduction in self-aggregation compared with RTA1-33/44-198 lacking the SS-bond. The x-ray structures of the two variants were solved to 2.3 Å (R48C/T77C) and 2.1 Å (V49C/E99C; Fig. **5**) resolutions [90].
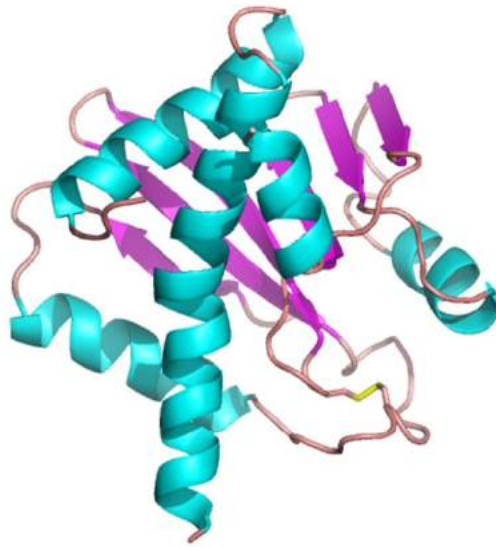
Figure 5: Structure of RTA1-33/44-198 that contains an S-S bond at residues 39 and 68. RTA1-33/44-198 lacking the S-S bond failed to crystallize [90]. The X-ray structure of RTA1-33/44-198 R48C/T77C disulfide-bonded variant was solved to 2.3 Å (PDB entry 3lc9; [90]). Image courtesy of Dr. Mark A. Olson, Integrated Toxicology Division, USAMRIID, Fort Detrick, MD.

RTA AND RTB ANTITOXINS

The potential of either RTA or RTB as an immunotoxin has been investigated. Foxwell *et al.* [91] reported that antibodies (Abs) raised to either RTA or RTB were equally protective in mice against ricin challenge. However, an examination of the ability of anti-ricin antibodies to neutralize ricin cytotoxicity *in vitro* and protect against toxicosis *in vivo* has shown that only a subset of neutralizing monoclonal antibodies (MAbs) produced against RTA were protective *in vivo*; none of the anti-RTB MAbs tested could protect animals against ricin poisoning [92]. RTB could be used as a mucosal vaccine since it has the potential to stimulate antibodies that would prevent ricin attachment to the epithelial surfaces of the respiratory and intestinal tracts [93].

PASSIVE IMMUNIZATIONS WITH ANTI-RICIN ANTIBODIES

Passive exposure with anti-ricin antibodies has been reported to protect animals against ricin intoxication [46, 94]. However, this strategy was found only effective if

the ricin dose was relatively low and the antibody was administered within a few hours after exposure [46, 53, 91]. Recent efforts have involved the development of monoclonal anti-ricin antibodies, and animal studies demonstrated protection against aerosol exposure to ricin [14, 95]. Passive prophylactic administration (i.p.) of a murine IgG1 monoclonal anti RTA antibody, GD12, protected mice against five LD_{50} ricin challenge i.p. [96]. Similar protection was observed when two other MAbs, R70 (anti RTA) and 24B11 (anti-RTB), were passively administered using the backpack tumor model [78]. Additionally, a combination of three MAbs (one anti RTA and two anti RTB) was also found effective in protecting mice against 5 LD_{50} intranasal challenge of ricin [97].

SUMMARY

Ricin is a potent toxin derived from the castor plant, *R. communis* L. which has been widely cultivated for its oil since ancient times. Although ricin has never been used in combat nor in mass casualty attacks, it is regarded as a potential biothreat agent because of its high potency, stability, and wide availability of its source plants. Ricin is also one of the most prevalent agents involved in WMD investigations. Currently, there is no FDA-approved antidote or drug against ricin intoxication; treatment is mainly symptomatic and supportive. Early studies on ricin vaccine development and pretreatment against ricin exposure involved ricin toxoid and dgRTA. However, because of the major shortcomings of both of these candidates, other approaches to vaccine development such as protein engineering and recombinant DNA technology, have been investigated. At present, two ricin vaccines, RiVax (University of Texas) and RV*Ec* (USAMRIID), are in advanced development in clinical trials. The successful development of an effective and safe vaccine may restraint the potential use of ricin as a biological weapon, and could also be used in rapid deployment scenarios in the event of a biological attack. Antibody therapy has also demonstrated protection against ricin intoxication. Ricin MAbs for postexposure treatment are presently undergoing preclinical studies.

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CONFLICT OF INTEREST

The authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript chapter.

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Improving Anti-Ricin Antibodies: Chimerization and Selection of Ricin-Resistant Hybridoma Cell Lines

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Abstract: Antibodies provide the best defense against the effects of biological toxins. We have previously made a panel of 45 different murine monoclonal antibodies (mAbs) to ricin A chain, B chain, and determinants found on both chains. Comparative *in vitro* and *in vivo* studies have identified one anti-A chain mAb, designated RAC18, as the most effective at both *in vitro* neutralization and *in vivo* protection from ricin toxin. Here we describe experiments to improve the utility of this mAb. We have made a chimeric mouse/human Ab by grafting the murine V regions onto human IgG1/K constant regions, and show that it retained full *in vitro* and *in vivo* activity. We also used a novel approach to generate higher affinity Abs, based on our observation that hybridoma cell lines were resistant to ricin in proportion to the affinity of the Ab they produce. We induced AID-dependent V region mutagenesis while selecting cells in increasing concentrations of ricin. We were able to isolate cells that were 100X more resistant to ricin than the parental hybridomas, but the quality of the Ab was no different. Rather, cells had down-regulated the expression of cell surface structures that are bound by ricin. These results demonstrate a unique mechanism whereby cells become resistant to ricin's lethal effects.

Keywords: Ricin, anti-ricin, antibodies, chimeric, ricin-resistant, hybridoma, protection.

INTRODUCTION

Ricin toxin is an important biodefense threat [1-7]. The castor bean, its source, is freely available, and the toxin may be prepared to >90% purity in two simple chemical steps. The toxin is chemically stable. Although the toxin may be placed in food or water, its toxicity is minimal *via* the oral route [6, 7]. The greatest concern for exposure to ricin is by inhalation, where the lethal dose is ~10,000-

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fold less than oral exposure. The concern is mitigated somewhat by the requirement for small particle size [8]. Nevertheless, the development of countermeasures to protect both civilians and military personal is important. Although there has been some effort to develop small molecule inhibitors of ricin's toxic activity [9-11], antibodies (Ab) still represent the most effective protective agents. Both active and passive immunization have been proposed [7, 12-22].

In understanding the mechanism of action of human vaccines, there are very few "truths" that are universally agreed upon. One is that protective immunity to toxins is mediated by antibody. Some of our oldest and most effective vaccines, preventing tetanus and diphtheria, target toxins rather than directing immunity to eliminate the causative organism. The same observation is true in regard to ricin. Numerous studies have demonstrated the efficacy of passive antibody treatment [7, 12-14, 16-20, 22-28]. Almost all protective Abs are directed against the toxin's A chain, although at least one protective anti-B chain antibody has been reported [13]. Similarly, active immunization with the A chain provides greater protection than immunization with the B chain [22], and most active immunization approaches also focus on the A chain [7, 21, 29-36].This presents an interesting paradox, because it is generally believed, and taught to our students, that the mechanism of Ab's protection of cells from toxins is by blocking the toxin's binding to its cellular receptor [37-39]. Yet for ricin, and other toxins, antibodies to the A chain are generally more effective than antibodies to the cell-binding B chain. We and others have resolved this paradox by showing that neutralization of ricin and shiga toxin, which share the same toxic ribosomal N-glycosidase activity, occurs intracellularly [40, 41] (S.H. Pincus, *et al.*, under review).

Ricin vaccines may have utility for the military when they face an enemy known to have ricin in its arsenal. But vaccines will not be acceptable for civilian populations, where the likelihood of any one individual being exposed to ricin is vanishingly small, even though ricin incidents continue to occur with regularity [4, 5]. Passive Ab therapy is most appropriate for this purpose, although its application will require rapid identification of ricin exposure and dissemination of stored Abs from depot sites in order to treat patients in the therapeutic window.

We have shown that passive Ab may be administered as late as 12-24 hrs post exposure and yet remains effective [19, 20].

We have produced a panel of 40 monoclonal Abs (mAbs) to ricin A chain, ricin B chain, or compound determinants on both chains [22]. One of these Abs, designated RAC18, was found to be most effective in both *in vitro* neutralization assays, and *in vivo* assays of protection from ricin's lethal effect. In this chapter, we describe studies performed in an effort to improve the function of RAC18. To make it more suitable for human use, a chimeric version has been created with murine V-regions and human C-regions. To improve its efficacy, we used a novel approach to generate high affinity variants. But instead, we obtained cell lines that were resistant to ricin's effects through mechanisms having nothing to do with the Ab they secreted.

PRODUCTION OF CHIMERIC ANTI-RICIN Ab

RAC18, although highly protective, is a murine mAb. There are several reasons that this would be unacceptable for human use [42-49]. The first is immunogenicity. Although it is unlikely that anyone would require more than a single course of treatment for ricin intoxication, the development of an anti-murine Ig immune response would foreclose any future use of a therapeutic murine Ab. In the presence of a pre-existing anti-murine immune response, anaphylactic or other acute hypersensitivity responses could occur. Additionally, the role of Ab Fc regions in the prevention of toxin-mediated effects has recently been emphasized [50]. Fc-mediated effector functions are optimal in the presence of species-specific Ig. As a first step in developing RAC18 for potential use, we have made a chimeric version (chRAC18) utilizing human gamma-1, kappa constant region genes. Although the murine V-region may retain some degree of immunogenicity, this occurs even when V-regions of the same species are utilized. Such anti-idiotypic responses are part of the regulation of the immune system [51, 52].

The V-region genes from RAC18 were cloned by PCR amplification using an FR1 degenerate 5' primer [53-55] and a 3' murine C-region primer. The heavy chain was encoded by a V_H gene from the J558 family, $D_H Q52$ gene, and J_H3. The

recombined kappa gene utilizes V_k19-17and J_k5. The V-gene sequences have been deposited in Genbank (accession numbers DQ164183.1 and GQ165714.1). The constant region genes were cloned from the human anti-HIV gp41 hynridoma 7B2 [56]. The V and C regions were joined by overlap PCR, and cloned into the pcDNA3.1 expression vector, expressed transiently in 293F cells, and purified by protein A chromatography. Hybridoma murine RAC18 (mRAC18) was grown in culture supernatants with low IgG fetal calf serum, and purified on protein A. All Ab preparations were analyzed for composition by microcapillary electrophoresis under reducing and non-reducing conditions. All preparations were >98% IgG. Assays below were all performed with purified IgG. Fig. **1** shows that both the chimeric and murine Abs bound to the appropriate ricin preparations, A chain and holotoxin, but not B chain, and these were detected with the appropriate secondary Ab. There was elevated background binding to the holotoxin detected with the inappropriate secondary Abs, most likely resulting from the lectin portion of ricin binding to carbohydrate on either the primary or secondary Ab.
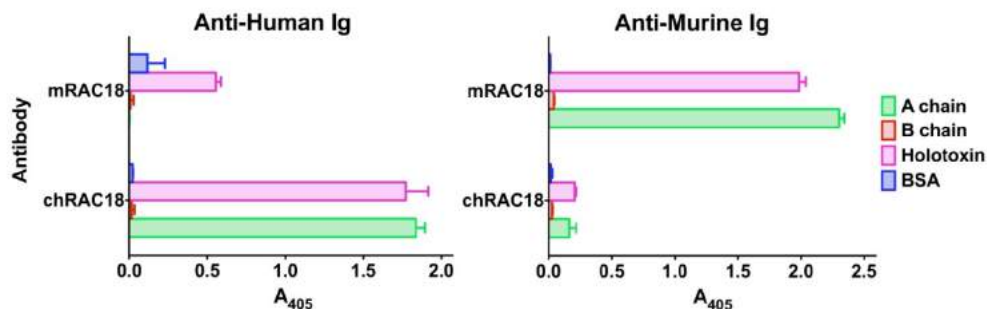
Figure 1: Comparison of binding of murine and chimeric RAC18 to ricin chains and detection by species specific antibodies. ELISA plates were coated with ricin A chain, B chain, holotoxin, or bovine serum albumin. After blocking, the plates were incubated with mRAC18 or chRAC18 overnight, washed and incubated with alkaline phosphatase conjugated goat anti-mouse or anti-human Ig. Following another wash step, the alkaline phosphatase substrate p-nitrophenyl phosphate was added, and the plates read at A_{405}. The results are mean and SEM of triplicate determinations.

This can be abolished if the ELSA is performed in 0.1M lactose (not shown). In Fig. **2** we compare titration curves of the two Abs binding to holoricin. Their virtual overlapping indicates that both Abs bind to ricin with the same relative avidity.
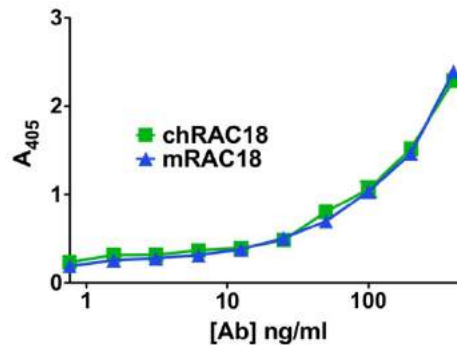
Figure 2: Comparative titration of mRAC18 and chRAC18. Using ELISA plates coated with holoricin, the two Abs were serially diluted and binding detected with the appropriate secondary Ab.

The functional activity of the two Abs was tested both *in vitro* and *in vivo*. The ability of the two Abs to neutralize ricin toxicity was determined on three different cell lines, both murine and human, and on human primary PHA blast cultures. Cell viability was assessed by MTS dye reduction at 72 hrs. The data indicate that both Abs have equivalent neutralizing activity for all cells (Fig. **3**).
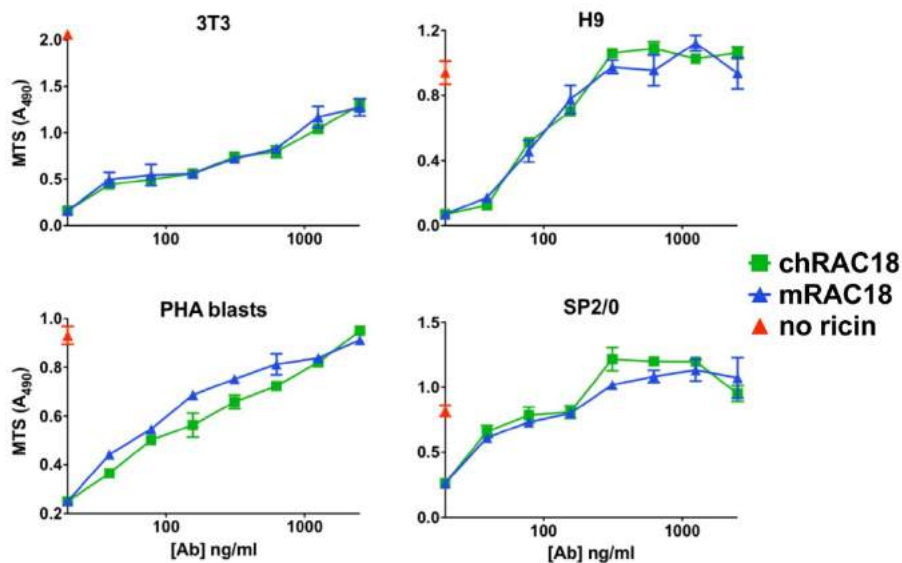
Figure 3: *In vitro* neutralization of ricin cytotoxicity by chRAC18 and mRAC18. Four different cell types were tested: murine 3T3 fibroblast and SP2/0 non-secreting hybridoma, and human H9 T-cell lymphoma and primary PHA blasts. Cells were incubated with the indicated concentration of Ab and then ricin toxin (1-4 ng/ml depending upon the cell line) was added.

Three days later MTS dye was added, and dye reduction was measured as A_{490}. Results are the mean and SEM of triplicate values, with the no cell background subtracted out. The values with no Ab are shown overlying the left axis, no ricin in red, and with ricin in blue and green.

In Fig. **4**, we show that the two antibodies provide *in vivo* protection in mice. As we have reported previously, we have used the development of hypoglycemia as a
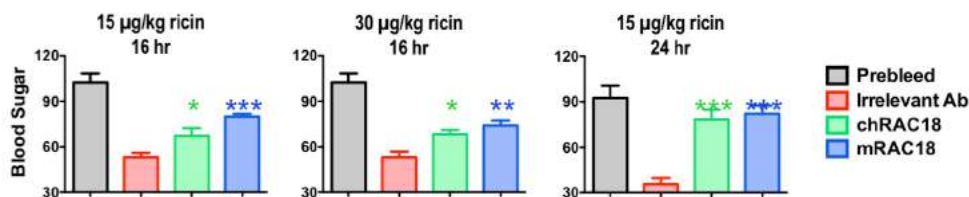
Figure 4: *In vivo* **protection of mice by Ab.** Groups of six mice were injected with the indicated dose of ricin and 0.8 mg/kg of chRAC18, mRAC18, or an isotype matched mouse Ig. At 16 or 24 hrs, the blood sugar was read using a hand-held glucose meter. The Fig. represents three independent experiments and shows mean and SEM. Statistical significance was determined comparing the RAC18 treated groups to the isotype control group using a t test: *$p<0.05$, **$p<0.01$. ***$p<0.001$. Although the blood sugar is slightly less in the chRAC18 treated group than in the mRAC18 treated animals in each experiment, none of the differences are statistically significant.

surrogate endpoint [22, 57], rather than lethality. All assays were approved by the Children's Hospital IACUC, performed in an AAALAC accredited facility, and animals were euthanized if any signs of pain or suffering developed. In three separate experiments, both Abs provided significant protection, when compared to an irrelevant, isotype-matched, Ab control. The murine Ab appears slightly more effective (but not significantly so), as would be expected when tested in mice.

These results showed that the chimeric and murine Abs have equivalent function in terms of binding to ricin, neutralizing ricin *in vitro*, and providing *in vivo* protection. The production of this chimeric Ab is the first step in producing a fully human Ab. Next we will humanize the framework regions of the variable domains. Others have prepared such chimeric Abs [23] and a macaque Fab has been chimerized [17]. Polyclonal, "despeciated" Fab preparations have also been prepared and shown to have protective activity as well [7]. Which of these Abs will be prepared in sufficient quantity for use as a stockpiled ricin antidote remains to be seen. Issues such as cost, stability in lyophilized form, and comparative efficacy will be important in this determination.

SELECTION OF RICIN-RESISTANT HYBRIDOMA CELL LINES

It has been demonstrated for a number of toxins that antibody affinity correlates with both *in vitro* neutralization and *in vivo* protection [22, 58-62]. To enhance the utility of RAC18, we therefore sought to increase its neutralizing ability. To do this we made use of the observation that hybridomas secreting anti-ricin Ab are relatively resistant to ricin's toxic effects [22, 63, 64], and that hybridomas secreting higher affinity Ab show greater levels of resistance [22]. Fig. **5** demonstrates the relative resistance of RAC18 compared to the parental non-secreting hybridoma partner SP2/0.
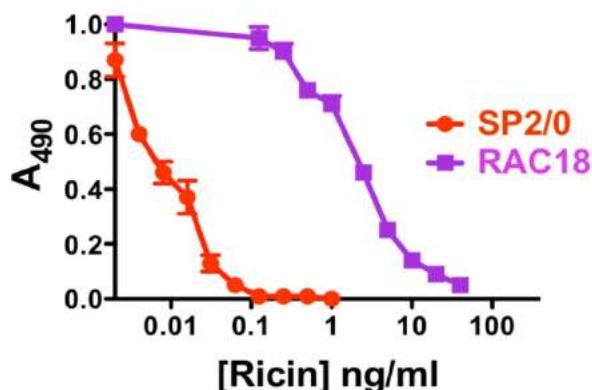
Figure 5: Sensitivity of RAC18 and non-secreting SP2/0 hybridoma cells to ricin. Cells were incubated in the presence of the indicated concentration of ricin for 72 hr prior to the addition of MTS. The RAC18 cells are ~300X more resistant to ricin than SP2/0.

To induce variable region mutations in the hybridoma cells we transfected the cells with the gene encoding murine activation-induced cytidine deaminase (AID) under the control of a tetracycline-inducible promotor. This enzyme is responsible for somatic mutation and class switching in B cells, and its expression in hybridoma cell lines was shown to be sufficient to induce somatic mutation in a similar pattern [65]. To avoid the induction of class switching, we deleted the terminal 10 amino acids from the protein [66, 67]. Our plan was to induce V region mutations in the hybridoma cells by treatment with doxycycline, and then select the cells in increasing concentrations of ricin. Expression of AID and

C_T	mGAP			msAID		
RAC18 Passage 2	23.19	23.08	23.44	32.78	33.78	32.29
AID Transfected	23.34	23.67	23.42	24.13	24.57	24.55
AID Transfected + Dox	23.68	23.67	23.74	20.55	21.12	20.91

Average C_T	mGAP		msAID	
	Mean	StdDev	Mean	StdDev
RAC18 Passage 2	23.24	0.18	32.54	0.35
AID Transfected	23.48	0.17	24.42	0.25
AID Transfected + Dox	23.70	0.04	20.86	0.29

ΔC_T	AID Expression Normalized to GAP	
	Mean	StdDev
RAC18 Passage 2	9.30	0.39
AID Transfected	0.94	0.30
AID Transfected + Dox	-2.84	0.29

$\Delta\Delta C_T$	AID Transfected compared to RAC	
	Mean	StdDev
RAC18 Passage 2		
AID Transfected	-8.36	0.50
AID Transfected + Dox	-12.14	0.49

Fold Change	AID Transfected compared to RAC	
	Mean	StdDev
RAC18 Passage 2		
AID Transfected	328	1.41
AID Transfected + Dox	4498	1.40

Figure 6: Expression of activation-induced cytidine deaminase and somatic mutation in RAC18 cells. The expression of AID was studied by real-time reverse-transcriptase PCR on mRNA samples taken from low passage RAC18 cells, the same cells transfected to express AID but not treated with doxycycline, or transfected cells treated for 7 days (left panel). The threshold cycle (C_T) was determined for AID and the house-keeping gene GAP in triplicate. There was little difference in GAP expression in the different cells. The expression of AID was first normalized to GAP for each cell line, and then the two transfected cell lines were compared to the parental RAC18 cells. The right panel shows the results of 454 pyrophosphate sequencing of PCR amplicons from the mRNA of the V_H gene. Approximately 1000 amplicons were sequenced. The frequency of mutations at each positions are graphed, color coded for the mutant nucleotide, and the overall mutational frequency is shown.

somatic mutation are shown in Fig. **6**. We assessed expression of AID by real-time reverse transcriptase PCR. There was a 4500X increase in expression of AID when transfected cells were treated with doxycycline compared to the parental

hybridoma, but expression was somewhat leaky with a 300X increase in transfected but untreated cells. We then PCR amplified the V genes from the mRNA of the parental hybridoma and transfected cells using primers in the leader sequence and 5' region of CH1, and sequenced the product using 454 sequencing. We have plotted the sites of the mutations and overall mutation frequency in ~1000 sequences from each cell. In low passage untransfected hybridomas, there is a surprisingly high degree of variability (0.19% mutations). When the cells were treated with doxycycline for two weeks, the variation increased to 0.25%, suggesting that somatic mutation may have been occurring. However, the variation did not appear to cluster in the CDRs. Cells were then subjected to repetitive cycles of doxycycline treatment and exposure to increasing concentrations of ricin. As controls, we selected untransfected hybridoma cells, and the parental non-secreting hybridoma cell line SP2/0. Eventually cells arose that were resistant to ricin, as demonstrated in Fig. **7**. However, expression of AID
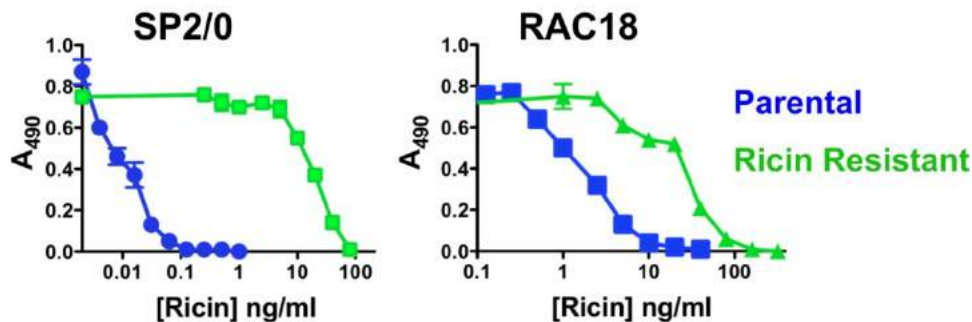
Figure 7: Ricin resistance in SP2/0 and RAC18 cells. Parental cells and cells selected for 4-6 weeks in increasing concentrations of ricin were incubated in serial dilutions of ricin, and MTS dye reduction measured 72 hr later.

had no influence on the rate of variant evolution or on the ultimate level of ricin that could be tolerated (not shown). In fact, the secretion of Ab was not necessary to develop resistance, as shown by the SP2/0 ricin-resistant cells. The lack of relationship between resistance and Ab production was confirmed by showing that neither the rate of Ab secretion, the affinity of Ab as measured by Biacore, nor the isotype of the Ab was different in the parental cells or the ricin resistant cells (not shown).

To understand the basis of ricin-resistance, we measured the binding of ricin to parental and variant cells (Fig. **8**). The results showed a 3-4X decrease in the amount of ricin bound to the surface of the resistant variants, compared to the parental cells. Immunoblots with biotin-ricin, or as a control biotin-ConA, did not show any specific proteins absent (data not shown). We tested the cells for sensitivity to a panel of cytotoxic materials, including cyclophosphamide, sodium azide, and blastocydin, and found that there was no evidence of cross-resistance with ricin (not shown). We therefore conclude that the mechanism of ricin-resistance in these cells is the downregulation of surface molecules that serve as the primary targets of ricin binding.

	SP2/0	RAC18
Parental	7440	7291
Ricin Resistant	1626	2392
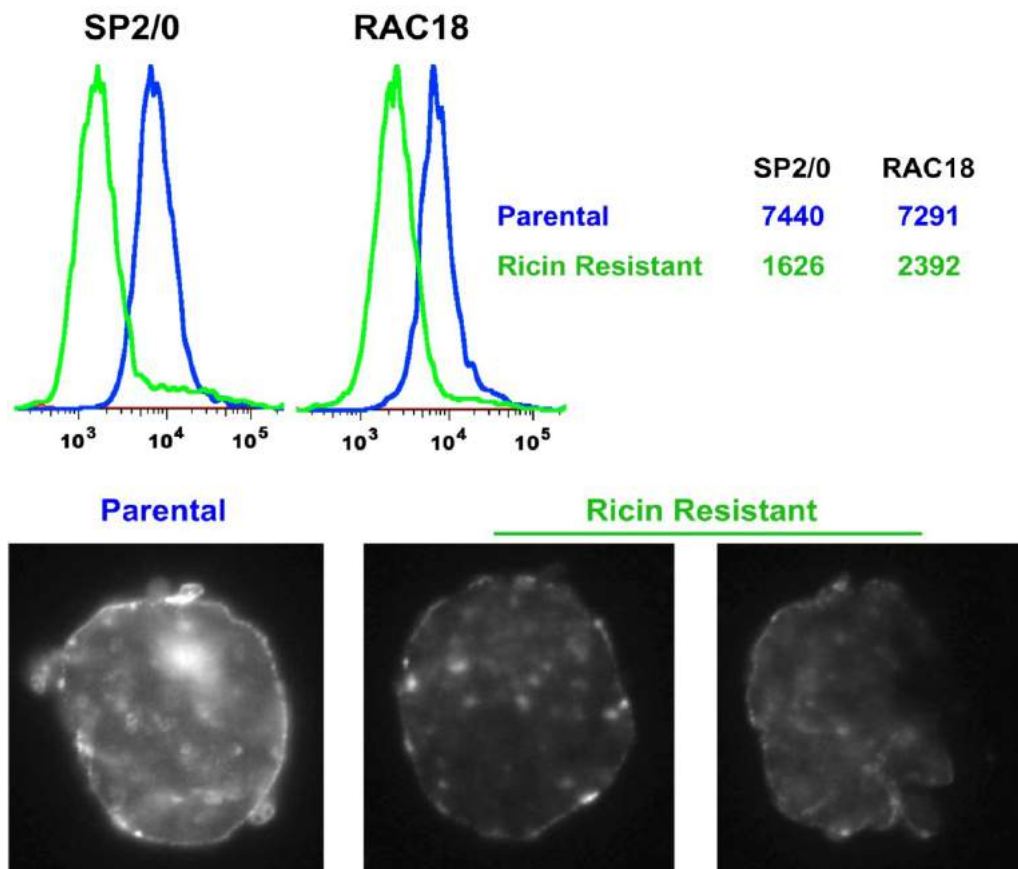
Figure 8: Binding of ricin to parental and ricin-resistant cells. Cells were incubated with Alexa-488 labeled ricin (3 µg/ml) and studied by flow cytometry (top) or confocal microscopy (bottom). The median fluorescence of 10000 cells measured by flow cytometry is shown.

SUMMARY

Our efforts to increase the therapeutic utility of RAC18 have met with mixed success. We were able to produce a chimeric Ab that retained all of the antigen binding characteristics and protective efficacy as the parental hybridoma. However, our approach to selecting for higher affinity Abs yielded results that were not what we were seeking, but in retrospect might have been anticipated. These results do show a previously undescribed mechanism of cellular resistance to ricin toxin, a global decrease in cell surface glycoproteins and glycolipids that are bound by the ricin B chain lectin. To increase Ab binding to ricin toxin, we are currently producing double variable domain antibodies that simultaneously bind to ricin A chain and ricin B chain.

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CONFLICT OF INTEREST

The authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licencsing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript chapter.

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CHAPTER 8

Anti-Ricin Protective Monoclonal Antibodies

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Abstract: Development of anti-ricin protective monoclonal antibodies (mAbs) started in the early 1980s. Much progress has been made since then. Antibodies possess great potential for development as antidotes against toxins. These can be used either prophylactically to prevent, or therapeutically to treat, toxin-mediated intoxications in an emergency situation. Unlike many other therapeutic products, antibodies offer unique and high target specificity, and long half-life in serum. There are several mAbs which are currently in the discovery stage for medical countermeasures against ricin intoxication. This review summarizes these mAbs, including their anti-ricin mechanism, generation, and efficacies *in vivo*.

Keywords: Ricin, protective, monoclonal antibodies, chimerization, humanization.

INTRODUCTION

Antibodies can provide immediate neutralization against toxins. The history of using antibodies as effective antidotes against toxins can be dated back to 1890 [1], when German physiologist, Emil Adolf von Behring, discovered a therapeutic effect against diphtheria toxin using serum. He was then awarded the first-ever Nobel Prize in Physiology and Medicine in 1901 for his contributions [2]. At that time, human hyper-immune sera, containing a small portion of specific antibodies obtained from the convalescent donors, was used as antidotes to combat toxins produced by microorganisms, such as diphtheria and tetanus, with a remarkable record of safety, efficacy, and versatility. At the present time, antibody-based

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products continue to be used as antidotes against toxins [3-5], including tetanus, diphtheria, botulism, and venomous bites. Antibodies can be used either prophylactically to prevent, or therapeutically to treat, toxin-mediated intoxications in an emergency situation. The major advantages of antibody-based products as antidotes against toxins are their exquisite specificity to the target and prolonged half-life in serum.

The development of monoclonal antibodies (mAbs) by mouse hybridoma technology in the late 1970s opened a new era in antibody therapy. MAbs are monospecific, homogeneous, and reproducible [6]. The major benefit afforded by this technology is that it is possible, in principle, to develop a mAb against any target of choice and to produce it in unlimited amounts. MAbs have been developed as therapeutic agents for various clinical applications, initially from murine origin, later chimeric between murine and human, and now humanized or fully human antibodies [7-9]. The purpose of this review is to summarize the development of protective mAbs against ricin intoxication, including their action mechanism, generation, and efficacy *in vivo*. This manuscript reviews the literatures on mAbs that provide protection to mice against ricin intoxication. MAbs that did not have *in vivo* protection data were not included in the review.

MECHANISM OF ANTIBODY NEUTRALIZING RICIN

Antibodies, which are glycosylated proteins naturally produced in the body, have a high specificity and affinity to foreign substances, playing an important role in the immune defence. Practically for any pathogen or toxin, there could be an antibody which functions against it. Antibody biological functions include two principal actions. The first is direct effects [10], which appear to be a function of the antibody antigen-binding alone, such as toxin neutralization, viral neutralization, and interference with microbial attachment or replication. The second is indirect effects [11], which are called effector functions, resulting from the consequence of crosslinking crystallisable fragment (Fc) receptors on complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP). Most antibodies work by indirect effects *via* binding to antigens and sending signals to other parts of the immune system to attack and eliminate the pathogenic

antigens by CDC, ADCC, or/and ADCP. Very few antibodies possess the direct effects, the neutralizing functions. Therefore, antibodies can be divided into two groups based on their functions, neutralizing antibodies and non-neutralizing antibodies. The former not only neutralize the biological effects of antigens, but also flag antigens to destroy these through their indirect effects, while the latter only have the indirect effects to destroy pathogenic antigens.

For development of antibody-based antidotes against toxins like ricin, neutralizing antibodies are desirable. Often the non-neutralizing antibodies are not helpful, but some of these are even harmful due to their assistance to ricin to enter cells, which was confirmed by Colombatti, *et al.,* in 1986 [12]. Therefore, not every single anti-ricin mAb can be developed as an antidote against ricin intoxication.

Ricin is a 60-65 kDa glycoprotein derived from beans of the castor plant [13]. It consists of a ricin toxin A (RTA) protein and a ricin toxin B (RTB) protein linked by a disulfide bond. RTB binds to galactose residues on the mammalian cell surface, not only triggering cellular uptake of ricin [14] but also facilitating transport of the RTA from the endoplasmic reticulum (ER) to the cytosol [15, 16], where RTA then enzymatically cleaves ribosomal RNA to stop protein synthesis [17]. Mounting evidence has shown that antibodies against either subunit can neutralize ricin [18-22]. Regarding neutralizing efficacy, some reports demonstrated anti-RTA neutralizing antibodies were more efficacious than RTB neutralizing antibodies [19], but others showed the opposite, that is, RTB neutralizing antibodies being more effective in protection than RTA neutralizing antibodies [21]. Theoretically, RTB is the logical target for neutralizing antibodies, as these would block the entry of ricin into cells and the transportation of RTA to the cytosol. However, it seems to be more difficult to develop anti-RTB neutralizing mAbs than anti-RTA neutralizing mAbs. One of the reasons is that the immunodominant epitopes on RTB might not provide any neutralizing protection. In other words, RTB is poor in eliciting anti-ricin neutralizing antibodies although it is highly immunogenic in eliciting non-neutralizing anti-ricin antibodies [22]. To date, only a few anti-RTB neutralizing antibodies have been reported [21, 23-26].

IMMUNOGENS

In order to make anti-ricin mAbs, mice need to be immunized with ricin to elicit an anti-ricin immune response. However, ricin is too toxic to be used directly as a primary immunogen to immunize mice. Indeed, primary immunization with either holotoxin or a mixture of purified RTA and RTB is lethal in an immunogenic dose. Instead, purified or recombinant RTA or RTB can be used as an immunogen to immunize mice [18, 19, 27] or a primary immunogen and then boosted with holotoxin [19]. Ricin can also be inactivated with formalin and then the resulting ricin toxoid, which is not lethal, can be used as an immunogen to immunize mice [24]. Furthermore, there is a developing vaccine, RiVax, which is a recombinant RTA subunit with two residue mutations, resulting in attenuated toxicity with retaining immunogenicity. Rivax has been used to immunize mice [28]. In our laboratory, mice were found to survive a large dose of ricin poisoning if the mice were poisoned by a stepwise increase dosage of ricin. In this way, mice were immunized by an intraperitoneal (*i.p.*) injection of ricin from 0.2×LD50 to 25×LD50 and a high anti-ricin antibody titer was obtained [29].

ANIMAL MODELS

Mice are good models for evaluation of mAb efficacy against ricin intoxication. The severity of ricin intoxication in mice depends on species, genders, and ages. In general, inbred, female, and younger mice are more sensitive to ricin challenge than outbred, male and elder mice. As well, ricin challenge routes also contribute to the severity of ricin intoxication. The most lethal challenge route is inhalation, followed by injection and then ingestion. For example, in mouse models, the LD50 for inhalation, injection, and ingestion is around 3-5 μg/kg, 5-10 μg/kg, and 30 g/kg respectively [30, 31].

EVALUATION OF EFFICACY

There are a couple of ways to evaluate antibody efficacy against ricin intoxication. In the earlier publications [24, 27], the antibody titration against a fixed amount of ricin was determined by using a series of different antibody concentrations mixed with a fixed amount of ricin, or *vice versa*, and then the

antibody ricin mixtures were injected into mice to observe mouse survival rates. The lowest antibody dose administered or highest ricin challenge dose, which could provide full protection to the mice, was the antibody efficacy titer.

The therapeutic efficacy of anti-ricin antibody-based treatment is largely dependent on the timing of the administration of rescuing antibodies relative to the ricin exposure. A relatively wide therapeutic window will provide the necessary time for exposed victims to obtain anti-ricin antibody treatment in the event of a ricin attack. Therefore, later on, the therapeutic window of antibodies was used to describe mAb efficacy. The longest time of administration of anti-ricin antibodies after ricin exposure, which can rescue 100% of the ricin-intoxicated mice or keep blood glucose level unchanged, is the antibody efficacy indicator. The blood glucose concentration within 36 hr after ricin challenge might be used, as a surrogate for lethal challenge, as a measure of ricin toxicosis [32]. It is quite understandable that, the wider the therapeutic window is, the better the antibody efficacy.

PROTECTIVE mAbs EVALUATED AGAINST RICIN INTOXICATION *IN VIVO*

The first protective anti-ricin mAb was reported by Colombatti, *et al.,* in 1987 [27]. His group developed a mAb, 75/3B12 (IgG2a), from the mice immunized with purified RTB. This mAb appeared to bind to a galactose-binding domain of RTB. The 75/3B12 antibody antigen-binding fragment (Fab) was evaluated for its efficacy against ricin challenge in AKR mice by a co-incubation approach (mixing ricin with the antibody before administration of the mixture) through titrating the ricin challenge dose against a constant dose of 330 μg or 750 μg per mouse of the antibody. As shown in Table **1** when the mixture of ricin and antibody was delivered by an intravenous (*i.v.*) route, with the antibody dose constant at 330 μg/mouse, the highest ricin challenge dose, with complete protection by the antibody, could be up to 270 μg/mouse. Moreover, in an *i.p.* delivery approach, when the antibody dose was constant at 750 μg/mouse, the highest ricin challenge dose could be up to 2,400 μg/mouse with 100% survival of the mice (Table **2**).

Table 1: Titration of ricin challenge dose against a constant dose of 330 µg/mouse of 75/3B12 Fab in AKR mice by an *i.v.* administration of the mixture of ricin and antibody

	Ricin (µg/mouse)	Survival
Non treatment control	135	none
	45	100%
75/3B12 Fab	270	100%
	135	100%

Table 2: Titration of ricin challenge dose against a constant dose of 750 µg/mouse of 75/3B12 Fab in AKR mice by an *i.p.* administration of the mixture of ricin and antibody

	Ricin (µg/mouse)	Survival
Non treatment control	800	none
	266	65%
	90	100%
75/3B12 Fab	2,400	none
	800	100%

Similarly, Lemley, *et al.,* [24] titrated a mAb, UNIVAX 70/138 (also named R70) developed from the mice immunized with ricin toxiod, against ricin challenge in CD-1 mice (body weight, 25-32 g) *via* an *i.v.* approach. This antibody was IgG1 and specific for RTA. When the ricin challenge dose was fixed to 18 µg/kg (>6×LD50), the minimum antibody dose, which could provide 100% protection to the mice against ricin challenge, was 75 µg/mouse (2.7 mg/kg). It was found that when the antibody dose was held constant at 100 µg/mouse (3.6 mg/kg), the lowest ricin challenge dose with complete protection by the antibody was 25 µg/kg, >8×LD50. Later on, R70 was further demonstrated to be able to provide 100% of protection to the female Balb/c mice when it was administrated (*i.p.*) at 20 µg/mouse (1 mg/kg) 24 hr before *i.p.* injection of ricin (5×LD50, 50 µg/kg) [20, 22] as shown in Table **3**.

Guo *et al.,* [12] developed a mAb that bound to a conformation epitope of RTB, 3E1. As shown in Table **3**, when it was given to adult BALB/c mice (body weight ~20 g) (3 mice per group), 100 µg per mouse (5 mg/kg) (*i.p.* injection) 10 or 20 min post ricin challenge (*i.p.* injection) at 6×LD50 (60 µg/kg), all the mice survived. Non-treatment control mice died within 16 hr. When the ricin challenge dose was increased to 10×LD50 (100 µg/kg) and the time for injection of the antibody was delayed up to 20 min post ricin challenge, all the mice still survived.

However, if the time of antibody administration was further delayed up to 30 min post ricin challenge, the mice did not survive, but death was delayed to 95 hr compared to 14.3 hr for the non-treatment control group. One year later, Guo *et al.,* [33] developed another anti-ricin mAb, 4C13, which recognized a linear epitope of ricin. 4C13 could rescue all the BALB/c mice (4 mice) when it was administered (*i.p.* injection) at the dose of 100 μg per mouse (5 mg/kg) 30 min post ricin challenge (10×LD50) (100 μg/kg) (Table **3**).

Table 3: Prophylactic and therapeutic windows of protective mAbs against ricin intoxication

mAb	Isotype	Specificity	Mice	Ricin Poisoning	mAb Administration	Survival	Reference
3E1		RTB	BALG/c	100 μg/kg, *i.p.*	5 mg/kg, *i.p.* +20 min(1)	100%	Guo, 2005
4C13		RTA&RTB	BALB/c	100 μg/kg, *i.p.*	5 mg/kg, *i.p.* +30 min	100%	Guo, 2006
RAC17	IgG1	RTA	C57BL/6	40 μg/kg, *i.p.*	0.9 mg/kg, *i.v.* + 6 hr	100%	Roche, 2008
RAC18	IgG1	RTA	C57BL/6	40 μg/kg, *i.p.*	0.9 mg/kg, *i.v.* + 6 hr	100%	Roche, 2008
			BALB/c	16 μg/kg, i.n.	2.5 mg/kg, i.n. + 4 hr	100%	Timothy, 2007
					2.5 mg/kg, i.n. +18 hr	60%	
					2.5 mg/kg, i.n. +24 hr	50%	
Cocktail1(2)	IgG1	RTA	C57BL/6	40 μg/kg, *i.p.*	2.7 mg/kg, *i.v.* +10 hr	90%	Roche, 2008
GD12	IgG1	RTA	BALB/c	50 μg/kg, *i.p.*	0.25 mg/kg, *i.p.* -24 hr(3)	100%	Neal, 2009
					0.5 mg/kg, *i.p.* + 6 hr	100%	O'Hara, 2012
cGD12	IgG1	RTA	BALB/c	100 μg/kg, *i.p.*	5 mg/kg, *i.p.* + 4 hr	100%	O'Hara, 2012
					5 mg/kg, *i.p.* + 6 hr	40%	
R70	IgG1	RTA	BALB/c	50 μg/kg, *i.p.*	1 mg/kg, *i.p.* -24 hr	100%	Neal, 2009
24B11	IgG1	RTB	BALB/c	50 μg/kg, *i.p.*	1 mg/kg, *i.p.* -24 hr	100%	Yermakova, 2011
SylH3	IgG1	RTB	BALB/c	50 μg/kg, *i.p.*	1 mg/kg, *i.p.* -24 hr	100%	Yermakova, 2011
SyH7	IgG1	RTA	BALB/c	50 μg/kg, *i.p.*	2 mg/kg, *i.p.* -24 hr	100%	O'Hara, 2010
PB10	IgG2b	RTA	BALB/c	50 μg/kg, *i.p.*	2 mg/kg, *i.p.* -24 hr	100%	O'Hara, 2010
Cocktail2(4)		RTA&RTB	CD-1	7.5 μg/kg, i.n.	5 mg/kg, *i.v.* +7.5 hr	90%	Prigent, 2011
6C2	IgG1	RTA	BALB/c	50 μg/kg, *i.p.*	0.25 mg/kg, *i.p.* +2 hr	100%	Dai, 2011
6C3	IgG1	RTA	BALB/c	50 μg/kg, *i.p.*	0.25 mg/kg, *i.p.* +2 hr	100%	Dai, 2011
D9	IgG1	RTB	BALB/c	50 μg/kg, *i.p.*	0.25 mg/kg, *i.p.* +6 hr	100%	Hu, 2013
					0.25 mg/kg, *i.p.* -6 w	100%	
hD9	IgG1	RTB	BALB/c	50 μg/kg, *i.p.*	0.25 mg/kg, *i.p.* +6 hr	100%	Hu, 2012
					0.25 mg/kg, *i.p.* +6 hr	50%	

Note: (1) +: Administration of mAb after ricin poisoning;
      (2) A cocktail of RAC 17, 18, and 23;
      (3) -: Administration of mAb before ricin poisoning;
      (4) A cocktail of RB 34, 36, and 37.

Maddaloni, *et al.,* [19] developed 44 mAbs against ricin by immunization of BALB/c mice with purified RTA or RTB. These bound to either RTA [13], or RTB [6], or both of these [33]. The mAbs were evaluated in outbred CD-1 mice by measuring antibody ability against ricin-induced hypoglycemia, rather than lethality [1]. Fifteen µg/kg ricin and 0.8 mg/kg of mAb were premixed and then the mixture was injected to BALB/c mice. Blood sugar was measured at 18 and 34 hr post administration, and mortality was assessed at 34 hr. Only anti-RTA mAbs were protective. Among these, mAb RAC18 was the best in terms of preventing the induced hypoglycemia. Roche, *et al.,* further evaluated these anti-RTA mAbs in male C57BL/6 mice (body weight 22-24 g) [34]. When mice were given 20 µg per mouse (0.9 mg/kg) of a single mAb, RAC 17 or RAC 18 by an *i.v.* injection, 6 hr after ricin challenge (*i.p.* injection) (40 µg/kg, equivalent to the LD100), all the mice survived and the non-treatment controls died within 4-5 days (Table **3**). In addition, 90% of the mice could be protected when administered with a cocktail of anti-RTA mAbs, including 20 µg/mouse (0.9 mg/kg) of each mAb, RAC 17, RAC 18, and RAC23, 10 hr after the ricin challenge. RAC 18 was also evaluated in a ricin challenge lung model using an oropharyngeal aspiration delivery of both ricin and mAb [35]. Male BALB/c mice (body weight 20-25 g) were challenged with 3~5×LD50 (16 µg/kg), the non-treatment control died with 5 days. By contrast, the administration of 50 µg per mouse (2.5 mg/kg) of RAC 18 mAb at 4, 18, and 24 hr after ricin challenge resulted in 100%, 60% and 50% protection respectively, while an anti-RTA polyclonal mouse antibody (50 µg per mouse, 2.5 mg/kg) still showed 100% protection to the mice when the delay of antibody administration for up to 18 hr post ricin challenge.

Mantis's group developed a couple of protective mAbs from female BALB/c mice immunized with ricin toxiod or Rivax. In a pre-exposure prophylaxis setting, BALB/c mice (body weight ~20 g) were administration (*i.p.* injection) of 5 to 40 µg/mouse (0.25 to 2 mg/kg) of a mAb, such as GD12 (IgG1, RTA specific) [20], R70 (IgG1, RTA specific) [20, 22], 24B11 (IgG1, RTA specific) [22], SylH3 (IgG1, RTB specific) [22], SyH7 (IgG1, RTA specific) [28], or PB10 (IgG2b, RTA specific) [28]. Twenty-four hr later, the mice were challenged (*i.p.* injection)

with ricin 5×LD50 (50 μg/kg). All the mice survived over a 3-day period without significant difference of blood glucose level between the treatment group and non-toxin control group within 76 hr post ricin challenge (Table **3**). All the non-treatment control mice died within 2 days.

Prigent, *et al.,* demonstrated that a combination of three anti-ricin mAbs, 2 anti-RTB (RB34 and RB37) and 1 anti-RTA (RA36) protected 90% of the female CD-1 mice (22-25 g) when the three mAbs (5 mg/kg, 110 to 125 μg/mouse) were administered by an *i.v.* injection within 7.5 hr after intranasal challenge of ricin at 5×LD50 (7.5 μg/kg) [21] as shown in Table **3**. Thus, it appears that Prigent *et al*. demonstrated a proof of concept for effective post-exposure prophylaxis to lethal-dose intranasal challenge to ricin.

Dai, *et al.,* [18] developed a group of anti-RTA mAbs by immunization of BALB/c mice with recombinant RTA and tested them both *in vitro* and *in vivo*. 6C2 (IgG1) and 6G3 (IgG1), binding to an alpha-helix comprising the residues 99-106 in RTA, showed the best efficacy against ricin challenge *in vivo*. The i.p administration of 5 μg mAb (0.25 mg/kg) of 6C2 or 6G3 per mouse could protect 100% of the adult BALB/c mice at 2 hr post challenge with ricin (50 μg/kg, *i.p.* injection).

Recently, four mAbs, that bound to conformational epitopes of ricin toxin B (RTB) with high affinity, were developed in our laboratory [29]. The four mAbs were found to have potent ricin-neutralizing capacities and synergistic effects among these as determined by *in vitro* neutralization assay. *In vivo* post-exposure protection assay demonstrated that all the four mAbs had strong efficacy against ricin challenges *in vivo*. As shown in Table **3**, D9 was found to be exceptionally effective. Administration of D9 (*i.p.* injection) at a dose of 5 μg per mouse (0.25 mg/kg), 6 hr after an *i.p.* challenge with 5×LD50 (50 μg/kg) of ricin was found to rescue 100% of the female Balb/c mice (6 week old, body weight 20-25 g). D9 was further evaluated for pre-exposure prophylaxis against ricin challenge *in vivo*, and 5 μg per mouse (0.25 mg/kg) delivered by the *i.p.* route 6 weeks before 5×LD50 (50 μg/kg) ricin challenge (*i.p.* injection) protected 100% of the mice.

CHIMERIZATION AND HUMANIZATION

Murine antibodies cannot directly be used in humans. Although murine antibodies are structurally similar to the human's, the antibody sequence difference between these is sufficient to invoke an immune response in humans when murine antibodies are directly injected into humans. The immune response would result in a rapid removal of murine antibodies from the human blood, systemic inflammatory effects, and possible anaphylaxis, which can sometimes be fatal [36]. To overcome this hurdle, two murine anti-ricin mAbs (C4C13 and GD12) were chimerized by genetically fusing murine antibody variable regions to human antibody constant regions to generate antibody molecules with ~70 % human content [37, 38]. The chimeric GD12 (cGD12) was further evaluated in both pre-exposure prophylaxis and post-exposure rescue settings. As shown in Table **3**, the cGD12 could provide 100% protection to the female mice (8-12 week old) against 5×LD50 (50 µg/kg) of ricin challenge (*i.p.* injection) when *i.p.* administration of 10 µg per mouse (0.5 mg/kg) of the cGD12 24 hr pre-ricin challenge. Administration of 100 µg per mouse (5 mg/kg) of cGD12 at 4 hr post ricin challenge could completely protect the mice against 10×LD50 (100 µg/kg) of ricin challenge (*i.p.* injection). Administration of 100 µg per mouse (5 mg/kg) of cGD12 at 6 hr post ricin challenge conferred partial protection (2/5 survived) and extended the mean time to death to 96 hr.

Chimeric antibodies successfully retained the mouse parental antibody antigen-binding specificity and had diminished immunogenicity. However, chimeric antibodies could still elicit an undesirable anti-antibody variable region response [39]. As molecular biology technology developed, it became possible to further reduce the immunogenicity of the chimeric antibodies by replacing murine variable region frameworks with those of the selected human antibodies using an approach called "complementarity-determined region grafting" [40]. The resulting "humanized" antibodies contain 85-95% human sequences. Numerous clinical studies have confirmed that humanized antibodies are less immunogenic and more therapeutic than murine or chimeric antibodies in humans [41, 42]. A potent anti-

ricin neutralizing antibody, D9 was successfully humanized in our laboratory [43]. The humanized D9 (hD9) exhibited high efficacy *in vivo*. In a female BALB/c mouse model, a dose of 5 µg hD9 per mouse (0.25 mg/kg) could rescue 100% of the mice (6 week old, body weight 20-25 g) up to 4 hr, and 50% of the mice up to 6 hr, after 5×LD50 (50 µg/kg) ricin challenge (Table **3**).

CONCLUSIONS

Continuous development of biotechnology (such as antibody cloning, screening, engineering, expression technologies, and humanization) and understanding of ricin toxicosis will play a pivotal role for the development of anti-ricin antibody-based antidotes. There are some challenges for passive antibody therapy in medical countermeasure against ricin intoxication. One logistical challenge is the large dose of antibodies required. Currently, most antibodies have to be administered by intravenous administrations *via* hour-long infusions and repeated over a long period of time in a specific hospital environment due to a large dose required. Consequently, this approach would be impractical when large populations are exposed to a ricin biothreat. A second challenge is the cost. Therapeutic antibodies are undoubtedly among the most expensive drugs used in clinical practice.

These challenges might be overcome by developing potent anti-ricin antibodies where only a small dosage is required for full protection before, and full rescue after, exposure to toxin. Within our laboratory, to treat mice for both situations the lower limit of either mD9 or hD9 was far lower than that published. This extrapolates to possibly only a few milligrams, not grams, of antibodies needed for humans. This small dose can potentially be delivered with an auto-injector on the field by the victim themselves. Greatly reduced amounts of anti-ricin antibodies required mean greatly reduced costs of therapy per patient. Perhaps of more importance is that where previously a production run may produce enough antibody to save 10 lives of those exposed at a terrorist site, the same amount of hD9 antibody might save 1000-10,000 lives. The benefits to national security may more than justify the investment in the development of such antibodies.

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CONFLICT OF INTEREST

All authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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CHAPTER 9

Antibody Humanization by a Single Cycle of CDR-Grafting

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Abstract: Murine monoclonal antibodies (mAbs) have great potentials as therapeutics developed for clinical applications. The major problem with these mAbs, however, is their immunogenicity due to their foreignness to humans. Humanization, a process to decease the content of murine residues in mAbs, can make it possible to reduce their immunogenicity for clinical uses. One of the humanization strategies, complementarity determining region (CDR)-grafting is well-established and popular, but it generally needs multiple design cycles, which are time-consuming. A CDR-grafting approach now described is based on a comprehensive analysis of the antibody sequence, and its three-dimensional structure from molecular modeling, to identify critical residues in the murine antibody of interest, which guides the completion of CDR-grafting to a human antibody template in a single cycle. The single-cycle structure-based method was used to create the first humanized (>94% human) anti-ricin monoclonal IgG antibody (results published and patented). The steps used in the successful creation of this antibody, hD9, are described to assist the researchers in their quest to develop humanized antibodies against threat agents.

Keywords: Antibody, humanization, CDR-grafting, molecular modeling.

INTRODUCTION

In 1975, the development of monoclonal antibodies (mAbs) by murine hybridoma technology opened a new era in antibody therapy. It was widely believed that mAbs would be the magic bullets for therapy. However, the early excitement was rapidly replaced by disappointment when it was found that mAbs, like animal plasma-derived antibodies, have a serious side effect in humans, namely, "serum sickness" due to their foreignness to humans [1]. All therapeutic settings using

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antibody-based drugs require high and multiple doses. Hence, the immunogenicity of animal antibody given to humans is a critical concern [2,3]. Repeat administration of these mAbs had resulted in rapid clearance of the animal antibodies and anaphylaxis, which was sometimes fatal.

Using the hybridoma method or the Epstein-Barr virus (EBV)-mediated transduction approach to immortalize human B-cells mitigated the problem, but the absence of a suitable fusion partner for human B cells or the instability of EBV-transduced clones made the methods that relied on human B-cell immortalization problematic. Modern alternative strategies now allow the development of fully human antibodies directly from phage-display libraries of human antibody fragments [4,5]. Unfortunately, unlike the antibodies developed *in vivo*, these latter antibodies possess limited antigen-binding affinity due to the absence of the somatic maturation of antibody affinity.

Another approach is to use mice that are transgenic for the human immunoglobulin (IG) locus [6,7]. Immunization of such a transgenic mouse leads to the development of human antibodies, from which hybridomas that produce human antibodies can be generated. However, humanized mice cannot be used effectively when the immunogen is toxic or when the targeted antigen shares a high degree of homology with murine tissues.

To understand the methods of humanization, one must first look at the structure of an antibody, usually IgG. The antibody is a "Y" shaped, having two branches attached to a single stem as showed in Fig. **1**.

As molecular biology technology developed, two approaches were developed in the mid-1980s to reduce the immunogenicity of well-characterized murine mAbs. Some parts of the murine antibody were replaced with human antibody counterparts by chimerization [8-10], and humanization by CDR-grafting [11-13], as shown in Fig. **2**.
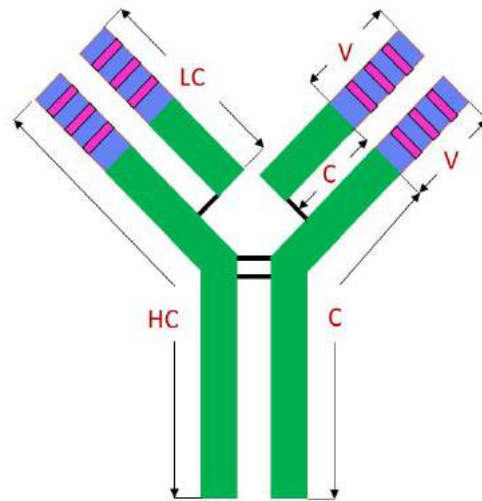
Figure 1: Antibody structure. One antibody molecule consists of four polypeptide chains; two identical heavy chains (HCs) and two identical light chains (LCs) connected by disulfide bonds in black. Each chain is composed of two regions: variable region (V) and constant region (C). The V is responsible for antigen-binding, while the C determines the mechanism used to destroy the antigen. The V can be further subdivided into complementarity determining regions (CDRs) in red and framework regions (FRs) in blue. There are three CDRs and four FRs in each chain. The CDRs are the most important part for binding to antigens with a high variety while the FR regions, which have more stable amino acids sequences, separate the CDRs and serve as a scaffold to hold the CDRs in position to contact the antigen. © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.
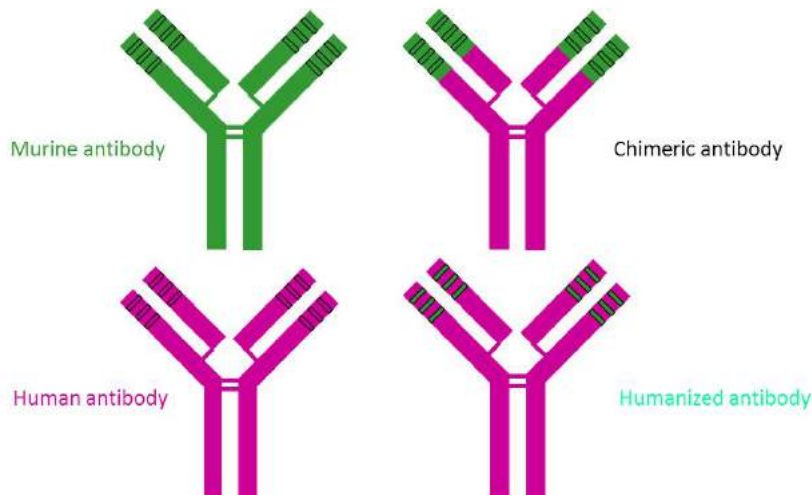
Figure 2: Conceptual representation of murine, chimeric, humanized, and human antibodies. Murine sequences are depicted in green and human sequences in red. © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.

For murine/human chimeric antibodies, the murine C region (about 70% of the total antibody sequence) is replaced by the human C region. Chimeric antibodies successfully retain the original murine antibody antigen-binding specificity and diminish their immunogenicity in humans. Five chimeric antibodies have been approved by Food and Drug Administration (FDA) for clinical applications, such as Remicade for treatment of Crohn's disease, Rituxan for treatment of non-Hodgkin's lymphoma, and Erbitus for treatment of metastatic colorectal, head and neck cancer [14]. However, chimeric antibodies may still elicit an undesirable anti-V region response [15]. In order to further reduce chimeric antibody immunogenicity, humanization was developed by Dr. Greg Winter and his colleagues in 1988, to replace murine FRs with those of the selected human antibodies, using an approach called CDR-grafting [11]. The resulting "humanized" antibodies contain 85-95% human sequences. Numerous clinical studies have confirmed that humanized antibodies are less immunogenic and more therapeutic than murine or chimeric antibodies in humans [3,16]. However, the process of humanization of murine antibodies is much more challenging than construction of murine-human chimeric antibodies. Humanization can result in a loss of antibody antigen-binding activity. Nevertheless, humanization has played a fundamental role in the remarkable progress of antibodies as therapeutics. For example, as of March 2012, there had been 28 antibodies (3 murine, 5 chimeric, 11 humanized, and 9 fully human) in the European Union or the USA approved for therapeutic application [14]. Among these, humanized antibodies are the most common. Therefore, humanization remains an attractive and proven strategy for switching well-characterized and highly specific murine antibodies into clinical therapeutics.

To date, besides CDR-grafting [11-13], there have been other strategies for humanization of xenogeneic (commonly rodent) antibodies, including resurfacing [17,18], de-immunization [19,20], and specificity-determining residues (SDR) grafting [21,22]. However, it is difficult to determine which one is the best, due to sparse data of the immunogenicity of humanized antibodies (only 11 humanized antibodies on the market). Nevertheless, CDR-grafting seems likely most popular.

On the surface, CDR-grafting appears straightforward, grafting the murine CDRs (that are responsible for the desired antigen-binding properties) into human donor

antibody FRs (that are responsible for scaffolding). However, simply grafting does not always reconstitute the binding affinity and specificity of the original murine antibody. Instead, this often results in partial or complete loss of antigen-binding affinity due to incompatibilities between murine CDRs and human donor FRs. Initially, this had been a problem for our laboratory when grafting the anti-ricin binding site of mouse (m) monoclonal mD9 into a human IgG1 to create humanized (h) monoclonal hD9. Upon doing so, the initial version was inactive against ricin.

In order to retain a comparable antigen-binding affinity and specificity after CDR-grafting, the murine CDR conformations have to be preserved in the humanized antibody. How to preserve the murine CDR conformation in the humanized antibody becomes the critical process in reproducing the function of the original murine antibody, which usually includes two steps: the selection of appropriate human antibody FRs as donor, and the substitution of some key residues from human donor FRs into the murine original FRs (back-mutation) to restore antigen-binding affinity. The number of back mutations, the position of residues, and the type of residues to restore antigen-binding affinity vary from antibody to antibody. This requires a trial-and-error iterative procedure to determine back-mutation sites in order to correct the structurally distorting residues and to reconstitute the original antigen-binding affinity of the original murine antibody. In some regards, one has to overcome "the butterfly effect" whereby the grafting of the anti-ricin binding site of mD9 into a human IgG causes a distortion and loss of activity in the created hD9.

A recent approach to antibody humanization, now described, is based on a comprehensive analysis of the available data of the sequence and the three-dimensional (3D) structure of the given murine antibody to identify the key residues both in CDRs and FRs. The analysis guides the choice of the human antibody template and back-mutation positions in FRs to finish CDR-grafting in a single step. The single-step structure-based method is a considerable improvement over the standard CDR-grafting humanization methods, which requires several trial-and-error cycles for error correction and refinement.

SEQUENCE AND STRUCTURAL ANALYSIS OF MURINE Fv

As the initial step, a given murine antibody Fv sequence can be determined by RT-PCR approach from hybridoma cells [23,24]. Sequence analysis can then be performed using online free programs. The highly homologous nature of antibody Fv makes it possible to establish the molecular model of the antibody based on its sequence [25,26].

CDR Determination

In order to humanize a given murine antibody by CDR-grafting, the murine antibody CDRs should be determined first. CDRs (Kabat numbering) can be determined using the online free program "Abnum: Antibody numbering" (http://www.bioinf.org.uk/abs/abnum/) based on murine antibody Fv protein sequence.

Canonical Structure Determination for CDRs

The key for humanized antibodies to retain the original murine antibody antigen-binding affinity, after CDR-grafting, relies on the preservation of the murine CDR conformation by the humanized antibody FRs. The CDR conformation is mainly dependent on CDR canonical structures. In order to keep murine CDR conformation unchanged in the human donor antibody FRs, the human donor antibody FRs should have the same CDR canonical structures as the given murine antibody. The canonical structures can be identified for CDRs 1, 2, and 3 of VL (CDR-L1, CDR-L2 and CDR-L3), and CDRs 1 and 2 of VH (CDR-H1 and CDR-H2) using the online free program "AbCheck - Antibody Sequence Test" (http://www.bioinf.org.uk/abs/seqtest.html) by a few canonical conserved residues located in CDRs and FRs [27,28].

Molecular Modeling

The molecular model for a given murine Fv can be established as shown in Fig. **3** through one of the following modeling softwares [29]: Discovery Studio 3.5 (Accelrys, CA), Molecular Operating Environment (Chemical Computer Group, Montreal, QC), WAM (Web Antibody Modeling, http://antibody.bath.ac.uk), PIGS (Prediction of ImmunoGlobulin Structure, http://www.biocomputing.it/pigs), or

Rosetta   antibody   modeling   server   (rosetta/Antibody, http://antibody.graylab.jhu.edu).
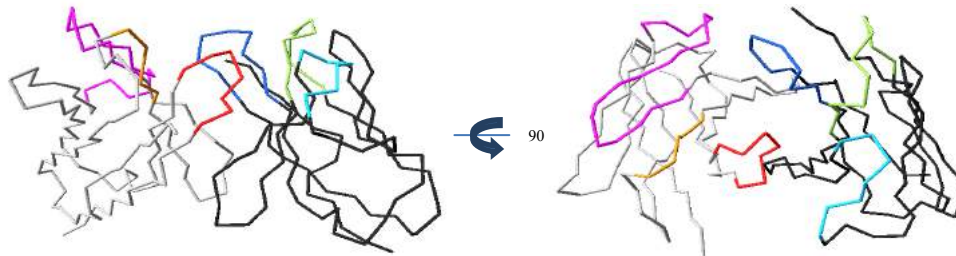
Figure 3: A structural representation of the Fv structure, from the anti-ricin mAb D9, predicted from molecular modeling. CDRs are colored (CDR-L1, green; CDR-L2, blue; CDR-L3, deep blue; CDR-H1, orange; CDR-H2, pink; CDR3-H3, red). FWs are in black for VL and in grey for VH. © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.

Key FR Residue Determination

Most of the residues in FRs do not participate in antigen-binding, but some of these do so either directly or indirectly. These FR residues can be called key FR residues. That is why simple CDR-grafting humanization without consideration of the key FR residues often results in some loss of antigen-binding affinity. The most common approach to restoring high affinity binding is to keep the key murine FR residues in the humanized antibodies. These key FR residues are different from antibody to antibody. These should be determined on a case-by-case basis before humanization.

Unusual FR Residue Determination

Rare FR residues could result from somatic hyper-mutation which may or may not contribute to changes of antigen-binding affinity. Careful consideration should be given when murine CDRs are grafted onto human donor antibody FRs. If the rare FR residues are near the CDRs, as determined from the molecular modeling, these may contact the antigen and therefore the murine residues should be kept in the humanized antibody. If these are not close to the CDR, then these should be replaced by human residues to avoid immunogenic epitopes in the humanized antibody. The unusual FR residues can be determined by comparison with the Kabat subgroup using the online free program "AbCheck - Antibody Sequence Test" (http://www.bioinf.org.uk/abs/seqtest.html).

Prediction of Potential N-Glycosylation Sites

Glycosylation sites may occur as part of the germline or arise through somatic hyper-mutation in the CDRs or FRs. Those carbohydrates may affect positioning of the antigen in the binding pocket. Therefore, their positions should be checked by molecular modeling to ensure these do not interfere with the CDRs. N-glycosylation sites can be predicted using the online free program "NetNGlyc" (http://www.cbs.dtu.dk/services/NetNGlyc).

Determination of Vernier Zone (VZ) Residues

VZ is a platform of FR residues directly located under the CDRs to support CDR conformation. The VZ can be predicted by molecular modeling as shown in Fig. **4**. Since these residues fine-tune the antibody antigen-binding affinity, the platform is called VZ [30]. The VZ residues can be identified by Fv molecular modeling by defining FR residues within 5 Å of CDR residues.
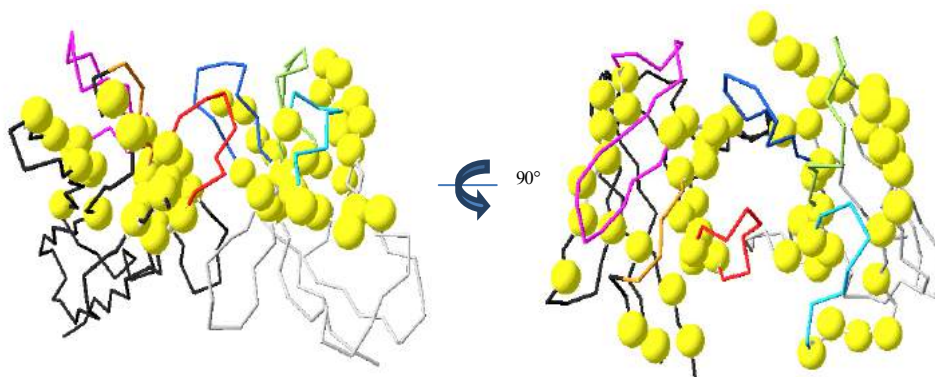
Figure 4: Residues in VZ in yellow identified from a molecular modeling of the Fv, from the anti-ricin mAb D9. CDRs are colored (CDR-L1, green; CDR-L2, blue; CDR-L3, deep blue; CDR-H1, orange; CDR-H2, pink; CDR3-H3, red). FWs are in black for VL and in grey for VH. © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.

Determination of Interface Residues

Residues buried at VL/VH packing interface may affect the relative disposition of CDRs and then affect the antigen-binding function [31,32]. These interface residues can be identified by defining residues in one chain within 5 Å of the other chain using molecular modeling as shown in Fig. **5**.
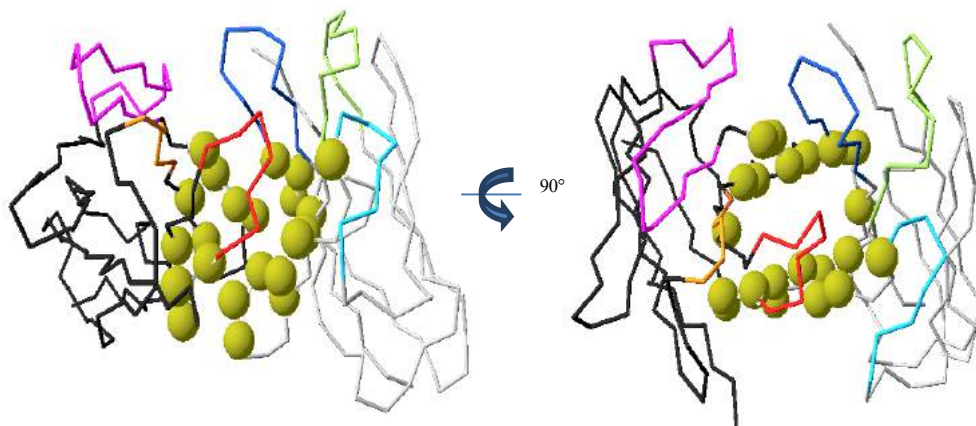
Figure 5: Residues in interface in yellowish green identified from a molecular modeling of the Fv, from the anti-ricin mAb D9. CDRs are colored (CDR-L1, green; CDR-L2, blue; CDR-L3, deep blue; CDR-H1, orange; CDR-H2, pink; CDR3-H3, red). FWs are in black for VL and in grey for VH. © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.

SELECTION OF HUMAN DONOR ANTIBODY FRS

There are two sources of human antibody sequences: mature and germline. Mature sequences result from the recombination of germline genes V, D, and J for VH or V and J for VL [33]. The germline sequence has two advantages over the mature sequences as FR donors for murine CDR grafting. One is that these are less immunogenic, unlike the mature sequences that carry somatic mutations for affinity maturation generated by random processes and which result in potential immunogenicity [34]. The other is its increased flexibility [35,36], resulting in more compatibility between murine CDRs and human donor FRs. Therefore, human germline sequences have increasingly been utilized as a source of FR donors. The human germline genes of IGHV (Table **1**), IGHD, IGHJ (Table **2**), IGKV (Table **3**) and IGKJ (Table **4**) were elucidated during the 1990's [37-40]. Since the murine antibodies are made of 95% κ-type light chains, IGLV and IGLJ are seldom used in humanization protocols. In addition, the D gene only encodes part of CDR3 for VH without any involvement of FR coding, so it is not taken into account in the selection of germline genes as FR donors for humanization of the murine VH.

Table 1: Human germline HC V genes

Locus	FR1	CDR1	FR2	CDR2	FR3	Canonical structure
1-02	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	G--YYMH	WVRQAPGQGLEWMG	WINP--NSGGTNYAQKFQG	RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR	1-3
1-03	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	S--YAMH	WVRQAPGQRLEWMG	WINA--GNGNTKYSQKFQG	RVTITRDTSASTAYMELSSLRSEDTAVYYCAR	1-3
1-08	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	S--YDIN	WVRQATGQGLEWMG	WMNP--NSGNTGYAQKFQG	RVTMTRNTSISTAYMELSSLRSEDTAVYYCAR	1-3
1-18	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	S--YGIS	WVRQAPGQGLEWMG	WISA--YNGNTNYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR	1-2
1-24	QVQLVQSGAEVKKPGASVKVSCKVSGYTLT	E--LSMH	WVRQAPGKGLEWMG	GFDP--EDGETIYAQKFQG	RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT	1-U
1-45	QMQLVQSGAEVKKTGSSVKVSCKASGYTFT	Y--RYLH	WVRQAPGQALEWMG	WITP--FNGNTNYAQKFQD	RVTITRDRSMSTAYMELSSLRSEDTAMYYCAR	1-3
1-46	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	S--YYMH	WVRQAPGQGLEWMG	IINP--SGGSTSYAQKFQG	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR	1-3
1-58	QMQLVQSGPEVKKPGTSVKVSCKASGFTFT	S--SAVQ	WVRQARGQRLEWIG	WIVV--GSGNTNYAQKFQE	RVTITRDMSTSTAYMELSSLRSEDTAVYYCAA	1-3
1-69	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	S--YAIS	WVRQAPGQGLEWMG	GIIP--IFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR	1-2
1-e	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	S--YAIS	WVRQAPGQGLEWMG	GIIP--IFGTANYAQKFQG	RVTITADKSTSTAYMELSSLRSEDTAVYYCAR	1-2
1-f	EVQLVQSGAEVKKPGATVKISCKVSGYTFT	D--YYMH	WVQQAPGKGLEWMG	LVDP--EDGETIYAEKFQG	RVTITADTSTDTAYMELSSLRSEDTAVYYCAT	1-2
2-05	QITLKESGPTLVKPTQTLTLTCTFSGFSLS	TSGVGVG	WIRQPPGKALEWLA	LIY---WNDDKRYSPSLKS	RLTITKDTSKNQVVLTMTNMDPVDTATYYCAHR	3-1/2-1
2-26	QVTLKESGPVLVKPTETLTLTCTVSGFSLS	NARMGVS	WIRQPPGKALEWLA	HIF---SNDEKSYSTSLKS	RLTISKDTSKSQVVLTMTNMDPVDTATYYCARI	3-1
2-70	QVTLKESGPALVKPTQTLTLTCTFSGFSLS	TSGMRVS	WIRQPPGKALEWLA	RID---WDDDKFYSTSLKT	RLTISKDTSKNQVVLTMTNMDPVDTATYYCARI	3-1
3-07	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	S--YWMS	WVRQAPGKGLEWVA	NIKQ--DGSEKYYVDSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	1-3
3-09	EVQLVESGGGLVQPGRSLRLSCAASGFTFD	D--YAMH	WVRQAPGKGLEWVS	GISW--NSGSIGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD	1-3
3-11	QVQLVESGGGLVKPGGSLRLSCAASGFTFS	D--YYMS	WIRQAPGKGLEWVS	YISS--SGSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	1-3
3-13	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	S--YDMH	WVRQATGKGLEWVS	AIG---TAGDTYYPGSVKG	RFTISRENAKNSLYLQMNSLRAGDTAVYYCAR	1-1
3-15	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	N--AWMS	WVRQAPGKGLEWVG	RIKSKTDGGTTDYAAPVKG	RFTISRDDSKNTLYLQMNSLKTEDTAVYYCTT	1-U
3-20	EVQLVESGGGVVRPGGSLRLSCAASGFTFD	D--YGMS	WVRQAPGKGLEWVS	GINW--NGGSTGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYHCAR	1-3
3-21	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	S--YSMN	WVRQAPGKGLEWVS	SISS--SSSYIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	1-3
3-23	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	S--YAMS	WVRQAPGKGLEWVS	AISG--SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	1-3
3-30	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	S--YGMH	WVRQAPGKGLEWVA	VISY--DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	1-3
3-30.3	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	S--YAMH	WVRQAPGKGLEWVA	VISY--DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	1-3
3-30.5	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	S--YGMH	WVRQAPGKGLEWVA	VISY--DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	1-3
3-33	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	S--YGMH	WVRQAPGKGLEWVA	VIWY--DGSNKYYADSVKG	FTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	1-3
3-43	EVQLVESGGVVVQPGGSLRLSCAASGFTFD	D--YTMH	WVRQAPGKGLEWVS	LISW--DGGSTYYADSVKG	RFTISRDNSKNSLYLQMNSLRTEDTALYYCAKD	1-3
3-48	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	S--YSMN	WVRQAPGKGLEWVS	YISS--SSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR	1-3
3-49	EVQLVESGGGLVQPGRSLRLSCTASGFTFG	D--YAMS	WFRQAPGKGLEWVG	FIRSKAYGGTTEYTASVKG	RFTISRDGSKSIAYLQMNSLKTEDTAVYYCTR	1-U

Table 1: contd….

3-53	EVQLVETGGGLIQPGGSLRLSCAASGFTVS	S--NYMS	WVRQAPGKGLEWVS	VIY---SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	1-1
3-64	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	S--YAMH	WVRQAPGKGLEYVS	AISS--NGGSTYYANSVKG	RFTISRDNSKNTLYLQMGSLRAEDMAVYYCAR	1-3
3-66	EVQLVESGGGLVQPGGSLRLSCAASGFTVS	S--NYMS	WVRQAPGKGLEWVS	VIY---SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	1-1
3-72	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	D--HYMD	WVRQAPGKGLEWVG	RTRNKANSYTTEYAASVKG	RFTISRDDSKNSLYLQMNSLKTEDTAVYYCAR	1-4
3-73	EVQLVESGGGLVQPGGSLKLSCAASGFTFS	G--SAMH	WVRQASGKGLEWVG	RIRSKANSYATAYAASVKG	RFTISRDDSKNTAYLQMNSLKTEDTAVYYCTR	1-4
3-74	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	S--YWMH	WVRQAPGKGLVWVS	RINS--DGSSTSYADSVKG	RFTISRDNAKNTLYLQMNSLRAEDTAVYYCAR	1-3
3-d	EVQLVESRGVLVQPGGSLRLSCAASGFTVS	S--NEMS	WVRQAPGKGLEWVS	SI----SGGSTYYADSRKG	RFTISRDNSKNTLHLQMNSLRAEDTAVYYCKK	1-6
4-04	QVQLQESGPGLVKPSGTLSLTCAVSGGSIS	SS-NWWS	WVRQPPGKGLEWIG	EIY---HSGSTNYNPSLKS	RVTISVDKSKNQFSLKLSSVTAADTAVYYCAR	2-1/1-1
4-28	QVQLQESGPGLVKPSDTLSLTCAVSGYSIS	SS-NWWG	WIRQPPGKGLEWIG	YIY---YSGSTYYNPSLKS	RVTMSVDTSKNQFSLKLSSVTAVDTAVYYCAR	2-1
4-30.1	QVQLQESGPGLVKPSQTLSLTCTVSGGSIS	SGGYYWS	WIRQHPGKGLEWIG	YIY---YSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	3-1
4-30.2	QLQLQESGSGLVKPSQTLSLTCAVSGGSIS	SGGYSWS	WIRQPPGKGLEWIG	YIY---HSGSTYYNPSLKS	RVTISVDRSKNQFSLKLSSVTAADTAVYYCAR	3-1
4-30.4	QVQLQESGPGLVKPSQTLSLTCTVSGGSIS	SGDYYWS	WIRQPPGKGLEWIG	YIY---YSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	3-1
4-31	QVQLQESGPGLVKPSQTLSLTCTVSGGSIS	SGGYYWS	WIRQHPGKGLEWIG	YIY---YSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	3-1
4-34	QVQLQQWGAGLLKPSETLSLTCAVYGGSFS	G--YYWS	WIRQPPGKGLEWIG	EIN---HSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	1-1
4-39	QLQLQESGPGLVKPSETLSLTCTVSGGSIS	SSSYYWG	WIRQPPGKGLEWIG	SIY---YSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	3-1
4-59	QVQLQESGPGLVKPSETLSLTCTVSGGSIS	S--YYWS	WIRQPPGKGLEWIG	YIY---YSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	1-1
4-61	QVQLQESGPGLVKPSETLSLTCTVSGGSVS	SGSYYWS	WIRQPPGKGLEWIG	YIY---YSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	3-1
5-51	EVQLVQSGAEVKKPGESLKISCKGSGYSFT	S--YWIG	WVRQMPGKGLEWMG	IIYP--GDSDTRYSPSFQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR	1-2
5-a	EVQLVQSGAEVKKPGESLRISCKGSGYSFT	S--YWIS	WVRQMPGKGLEWMG	RIDP--SDSYTNYSPSFQG	HVTISADKSISTAYLQWSSLKASDTAMYYCAR	1-2
6-01	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	SNSAAWN	WIRQSPSRGLEWLG	RTYYR-SKWYNDYAVSVKS	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR	3-5
7-4.1	QVQLVQSGSELKKPGASVKVSCKASGYTFT	S--YAMN	WVRQAPGQGLEWMG	WINT--NTGNPTYAQGFTG	RFVFSLDTSVSTAYLQICSLKAEDTAVYYCAR	1-2

Table 2: Human germline JH genes

J gene	CDR3	FR4
JH1	---AEYFQH	WGQGTLVTVSS
JH2	---YWYFDL	WGRGTLVTVSS
JH3	-----AFDI	WGQGTMVTVSS
JH4	-----YFDY	WGQGTLVTVSS
JH5	----NWFDP	WGQGTLVTVSS
JH6	YYYYYGMDV	WGQGTTVTVSS

Table 3: Human germline kappa chain V genes

Locus	FR1	CDR1	FR2	CDR2	FR3	CDR3	Canonical structure
O12	DIQMTQSPSSLSASVGDRVTITC	RASQSISS------YLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	2-1-(1)
O2	DIQMTQSPSSLSASVGDRVTITC	RASQSISS------YLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP	2-1-(1)
O18	DIQMTQSPSSLSASVGDRVTITC	QASQDISN------YLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP	2-1-(1)
O8	DIQMTQSPSSLSASVGDRVTITC	QASQDISN------YLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP	2-1-(1)
A20	DIQMTQSPSSLSASVGDRVTITC	RASQGISN------YLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	QKYNSAP	2-1-(U)
A30	DIQMTQSPSSLSASVGDRVTITC	RASQGIRN------DLG	WYQQKPGKAPKRLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP	2-1-(1)
L14	NIQMTQSPSAMSASVGDRVTITC	RARQGISN------YLA	WFQQKPGKVPKHLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP	2-1-(1)
L1	DIQMTQSPSSLSASVGDRVTITC	RASQGISN------YLA	WFQQKPGKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP	2-1-(1)
L15	DIQMTQSPSSLSASVGDRVTITC	RASQGISS------WLA	WYQQKPEKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP	2-1-(1)
L4	AIQLTQSPSSLSASVGDRVTITC	RASQGISS------ALA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQFNSYP	2-1-(1)
L18	AIQLTQSPSSLSASVGDRVTITC	RASQGISS------ALA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQFNSYP	2-1-(1)
L5	DIQMTQSPSSVSASVGDRVTITC	RASQGISS------WLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP	2-1-(1)
L19	DIQMTQSPSSVSASVGDRVTITC	RASQGISS------WLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP	2-1-(1)
L8	DIQLTQSPSFLSASVGDRVTITC	RASQGISS------YLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP	2-1-(1)
L23	AIRMTQSPFSLSASVGDRVTITC	WASQGISS------YLA	WYQQKPAKAPKLFIY	YASSLQS	GVPSRFSGSGSGTDYTLTISSLQPEDFATYYC	QQYYSTP	2-1-(1)
L9	AIRMTQSPSSFSASTGDRVTITC	RASQGISS------YLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSYP	2-1-(1)
L24	VIWMTQSPSLLSASTGDRVTISC	RMSQGISS------YLA	WYQQKPGKAPELLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSFP	U-1-(1)
L11	AIQMTQSPSSLSASVGDRVTITC	RASQGIRN------DLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQDYNYP	2-1-(1)
L12	DIQMTQSPSTLSASVGDRVTITC	RASQSISS------WLA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS	2-1-(U)
O11	DIVMTQTPLSLPVTPGEPASISC	RSSQSLLDSDDGNTYLD	WYLQKPGQSPQLLIY	TLSYRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQRIEFP	3-1-(1)
O1	DIVMTQTPLSLPVTPGEPASISC	RSSQSLLDSDDGNTYLD	WYLQKPGQSPQLLIY	TLSYRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQRIEFP	3-1-(1)
A17	DVVMTQSPLSLPVTLGQPASISC	RSSQSLVYS-DGNTYLN	WFQQRPGQSPRRLIY	KVSNRDS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQGTHWP	4-1-(1)
A1	DVVMTQSPLSLPVTLGQPASISC	RSSQSLVYS-DGNTYLN	WFQQRPGQSPRRLIY	KVSNWDS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQGTHWP	4-1-(1)
A18	DIVMTQTPLSLSVTPGQPASISC	KSSQSLLHS-DGKTYLY	WYLQKPGQSPQLLIY	EVSSRFS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQGIHLP	4-1-(1)
A2	DIVMTQTPLSLSVTPGQPASISC	KSSQSLLHS-DGKTYLY	WYLQKPGQPPQLLIY	EVSNRFS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQSIQLP	4-1-(1)
A19	DIVMTQSPLSLPVTPGEPASISC	RSSQSLLHS-NGYNYLD	WYLQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQALQTP	4-1-(1)
A3	DIVMTQSPLSLPVTPGEPASISC	RSSQSLLHS-NGYNYLD	WYLQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQALQTP	4-1-(1)
A23	DIVMTQTPLSSPVTLGQPASISC	RSSQSLVHS-DGNTYLS	WLQQRPGQPPRLLIY	KISNRFS	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC	MQATQFP	4-1-(1)
A27	EIVLTQSPGTLSLSPGERATLSC	RASQSVSSS----YLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSSP	6-1-(1)
A11	EIVLTQSPATLSLSPGERATLSC	GASQSVSSS----YLA	WYQQKPGLAPRLLIY	DASSRAT	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSSP	6-1-(1)

Table 3: contd….

L2	EIVMTQSPATLSVSPGERATLSC	RASQSVSS------NLA	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYNNWP	2-1-(1)
L16	EIVMTQSPATLSVSPGERATLSC	RASQSVSS------NLA	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYNNWP	2-1-(1)
L6	EIVLTQSPATLSLSPGERATLSC	RASQSVSS------YLA	WYQQKPGQAPRLLIY	DASNRAT	GIPARFSGSGSGTDFTLTISSLEPEDFAVYYC	QQRSNWP	2-1-(1)
L20	EIVLTQSPATLSLSPGERATLSC	RASQGVSS------YLA	WYQQKPGQAPRLLIY	DASNRAT	GIPARFSGSGPGTDFTLTISSLEPEDFAVYYC	QQRSNWH	2-1-(U)
L25	EIVMTQSPATLSLSPGERATLSC	RASQSVSSS-----YLS	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGSGSGTDFTLTISSLQPEDFAVYYC	QQDYNLP	6-1-(1)
B3	DIVMTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSTP	3-1-(1)
B2	ETTLTQSPAFMSATPGDKVNISC	KASQDIDD------DMN	WYQQKPGEAAIFIIQ	EATTLVP	GIPPRFSGSGYGTDFTLTINNIESEDAAYYFC	LQHDNFP	2-1-(1)
A26	EIVLTQSPDFQSVTPKEKVTITC	RASQSIGS------SLH	WYQQKPDQSPKLLIK	YASQSFS	GVPSRFSGSGSGTDFTLTINSLEAEDAATYYC	HQSSSLP	2-1-(1)
A10	EIVLTQSPDFQSVTPKEKVTITC	RASQSIGS------SLH	WYQQKPDQSPKLLIK	YASQSFS	GVPSRFSGSGSGTDFTLTINSLEAEDAATYYC	HQSSSLP	2-1-(1)
A14	DVVMTQSPAFLSVTPGEKVTITC	QASEGIGN------YLY	WYQQKPDQAPKLLIK	YASQSIS	GVPSRFSGSGSGTDFTFTISSLEAEDAATYYC	QQGNKHP	2-1-(1)

Table 4: Human germline JK genes

J gene	CDR3	FR4
JK1	WT	FGQGTKVEIK
JK2	YT	FGQGTKLEIK
JK3	FT	FGPGTKVDIK
JK4	LT	FGGGTKVEIK
JK5	IT	FGQGTRLEIK

Selection of Germline Human Donor V Gene

In order to select human germline V gene candidates as FR donors for murine antibody humanization based on the aforementioned murine CDR canonical structures, a shortlist of germline human antibody V gene candidates, for VH or VL regions sharing the same canonical structures, can be retrieved from Tables **1** or **3**. Such sequences ensure that the human germline donor antibody V gene FRs will support the murine CDR canonical structures. Next, within the shortlist of human germline V genes, those having the highest similarity, in CDRs (CDRs 1, 2 for HC or CDRs 1 to 3 for LC) and key FRs 1 to 3 to the original murine antibody, will be selected as FRs 1-3 donors.

Selection of Germline Human Donor J Gene

For the selection of human donors to provide FR4 for murine antibody humanization, 6 human germline J genes can be retrieved from Table **2** for HC and Table **4** for LC. The similarity of each of 5-6 human candidate sequences to the original murine antibody, regarding CDR3 and key FR 4 residues, should be analyzed and scored. The J gene with highest similarity to the original murine antibody should be selected as the donor of FR4.

Selection of Back-Mutation Sites

Finally, those non-identical or non-conserved residues, at the key FR positions in the selected human donor V and J genes, should be carefully analyzed one by one by using molecular modeling. The analysis should be focused on the size, charge, hydro, accessibility, and neighboring circumstances. If the substitution is favorable, or neutral, based on Table **5**, the back-mutation is not necessary. However, if the substitution is not favorable, the back-mutation should be considered. In other words, this key FR residue should be kept in the humanized antibody being formulated.

Table 5: Residue substitution preferences

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	f	d	d	d	n	d	d	n	d	d	d	d	d	d	d	f	n	d	d	n
R	d	f	n	d	d	f	n	d	n	d	d	f	d	d	d	d	d	d	d	d
N	d	n	f	f	d	n	n	n	f	d	d	n	d	d	d	f	n	d	d	d

Table 5: contd….

D	d	d	f	f	d	n	f	d	d	d	d	d	d	d	d	n	d	d	d	d
C	n	d	d	d	f	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
Q	d	f	n	n	d	f	f	d	n	d	d	f	n	d	d	n	d	d	d	d
E	d	n	n	f	d	f	f	d	n	d	d	d	d	d	d	n	d	d	d	d
G	n	d	n	d	d	d	d	f	d	d	d	d	d	d	d	n	d	d	d	d
H	d	n	f	d	d	n	n	d	f	d	d	d	d	d	d	d	d	d	f	d
I	d	d	d	d	d	d	d	d	d	f	f	d	f	n	d	d	d	d	d	f
L	d	d	d	d	d	d	d	d	d	d	f	f	d	f	n	d	d	d	d	f
K	d	f	n	d	d	f	f	d	d	d	d	f	d	d	d	n	d	d	d	d
M	d	d	d	d	d	n	d	d	d	f	f	d	f	n	d	d	d	d	d	f
F	d	d	d	d	d	d	d	d	d	n	n	d	n	f	d	d	d	f	f	d
P	d	d	d	d	d	d	d	d	d	d	d	d	d	d	f	d	d	d	d	d
S	f	d	f	n	d	n	n	n	d	d	d	n	d	d	d	f	f	d	d	d
T	n	d	n	d	d	d	d	d	d	d	d	d	d	d	d	f	f	d	d	n
W	d	d	d	d	d	d	d	d	d	d	d	d	d	f	d	d	d	f	f	d
Y	d	d	d	d	d	d	d	d	f	d	d	d	d	f	d	d	d	f	f	d
V	n	d	d	d	d	d	d	d	d	f	f	d	f	d	d	d	n	d	d	f

Notes: f: favored; n: neutral; d:disfavored.

CONSTRUCTION, EXPRESSION, PURIFICATION OF FULL-LENGTH HUMANIZED ANTIBODIES

Construction of Full-Length Humanized Antibody Gene

In order to express the full-length humanized antibody, the humanized VH and VL need to be respectively grafted onto human antibody constant regions, CH and CL. Human CL is generally the human κ chain. In contrast, there are not one but five main different CHs: gamma (γ), delta (δ), alpha (α), mu (μ), and epsilon (ε). The γ and α classes are subdivided into six isotypes: γ1, γ2, γ3, γ4, α1, and α2. The CH is not only part of the antibody structure, but also plays an important role in four main antibody effector functions: antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCG), complement-dependent cytotoxicity (CDC), and half-life/clearance rate. However, when the human CH is to be chosen for the engineered therapeutic antibodies, it is γ1, γ2, and γ4 that have mostly been considered [41]. Each of these differs in their ability

to interact with antibody fragment crystallizable region (Fc) receptors and complement to exert effector functions. While choosing among these, careful consideration should be given to the intended outcome of the therapeutic antibodies. If a simple neutralization is desired and antibodies that block a ligand-receptor interaction can function without effector mechanisms, it does not matter which one is selected among the three, γ1, γ2, and γ4 [42]. However, if antibody effector functions were required, such as recruitment of the immune system to destroy the target cells or regulation of cell functions, proper choice among γ1, γ2, and γ4 would be a valuable enhancement to the therapeutic antibodies. Depending on the mechanism of action of a therapeutic antibody, one or more effector functions may play a positive role.

Full-Length Humanized Antibody Gene Expression in Mammalian Cells

Antibodies are large molecules composed of two chains that need to be assembled into a four subunit structure to fully exert their functions. To produce recombinant humanized antibodies in mammalian cells, consideration must be given to the possible expression vectors. A number of strategies may be employed to express antibody genes. The expression in cells of two chains by two vectors could result in an imbalance in the production of HC and LC. Unpaired chains usually pile up in the rough endoplasmic reticulum, resulting in the inhibition of protein production, which includes antibody production. Extra chain production might be toxic to the antibody-expressing cells and thus affect the efficiency of antibody expression. This might explain what was previously observed in our laboratory of low antibody yields expressed in mammalian cells using the two-vector approach.

A second approach is to insert an internal ribosomal entry site (IRES) between the HL and LC genes in a bicistronic vector, under a single promoter [43]. Transcription from this bicistronic vector produces a single mRNA molecule encoding both HC and LC. The IRES enables the ribosome to bind to the initiation site of the second gene. In this way, the HC and LC are translated separately from the same mRNA molecule and expression levels of both chains are assumed to be equal. However, the two gene products are not always

expressed equally [44] possibly as a result of variability in ribosome recruitment of the two genes.

A third approach uses a bidirectional cassette, consisting of two promoters orientated in opposite directions driving the expression of both products, but this approach has not been widely tested [45].

Finally the HC and LC genes may be expressed in a monocistronic vector, with a cleavable linker, such as that reported for a foot-and-mouth-disease virus (FMDV)-derived 2A self-cleavage peptide between these. HC and LC are translated as a single polyprotein that is subsequently cleaved within the linker, so that the HC and LC are separated as shown in Fig. **6**. This has proven to be an effective route to produce a balance of HC and LC [46]. The 2A oligopeptide sequence, APVKQTLNFDLLKLAGDVESNPGP is expected to undergo self-cleavage to generate separate HC and LC after translation. The exact mechanism of 2A self-cleavage is still unknown. It has been hypothesized that the 2A sequence impairs peptide bond formation between 2A glycine and 2B proline through a ribosomal skip mechanism [47]. Our previous studies repeatedly demonstrated that the expressed humanized or human antibody HC and LC using 2A expression system were completely cleaved without detectable unpaired chains [23,48,49]. The cleavage is designed to occur at the C-terminus of the 2A sequence, leaving 23 residues of 2A sequence fused to the end of LC and adding one residue to the N-terminus of the leader sequence of HC. Since the leader sequence is immediately cleaved from the HC, once it has been translocated into the endoplasmic reticulum, the one extra residue would be removed with the leader sequence, leaving the HC without any extra residues. A potential drawback of this 2A expression system is that the small, 2A tag (23 residues) left at the end of the C-terminus of LC might affect antibody function or contribute to the antigenicity of antibodies. However, these problems have not been observed [50]. An adenoviral expression system can be used to express the full-length humanized antibody gene. The humanized antibody full-length DNA sequence (2 kb) including an LC leader sequence, the humanized LC (VL+CL), 2A self-cleavage

linker, a human antibody gamma 1 HC leader sequence, and humanized HC (VH+CH1 to 3) can be synthesized and cloned into a pUC57. A recombinant adenovirus vector expressing humanized antibody gene can be constructed and the recombinant humanized antibody can be expressed in HEK 293 mammalian cells using the AdEasy system [23,48,49].
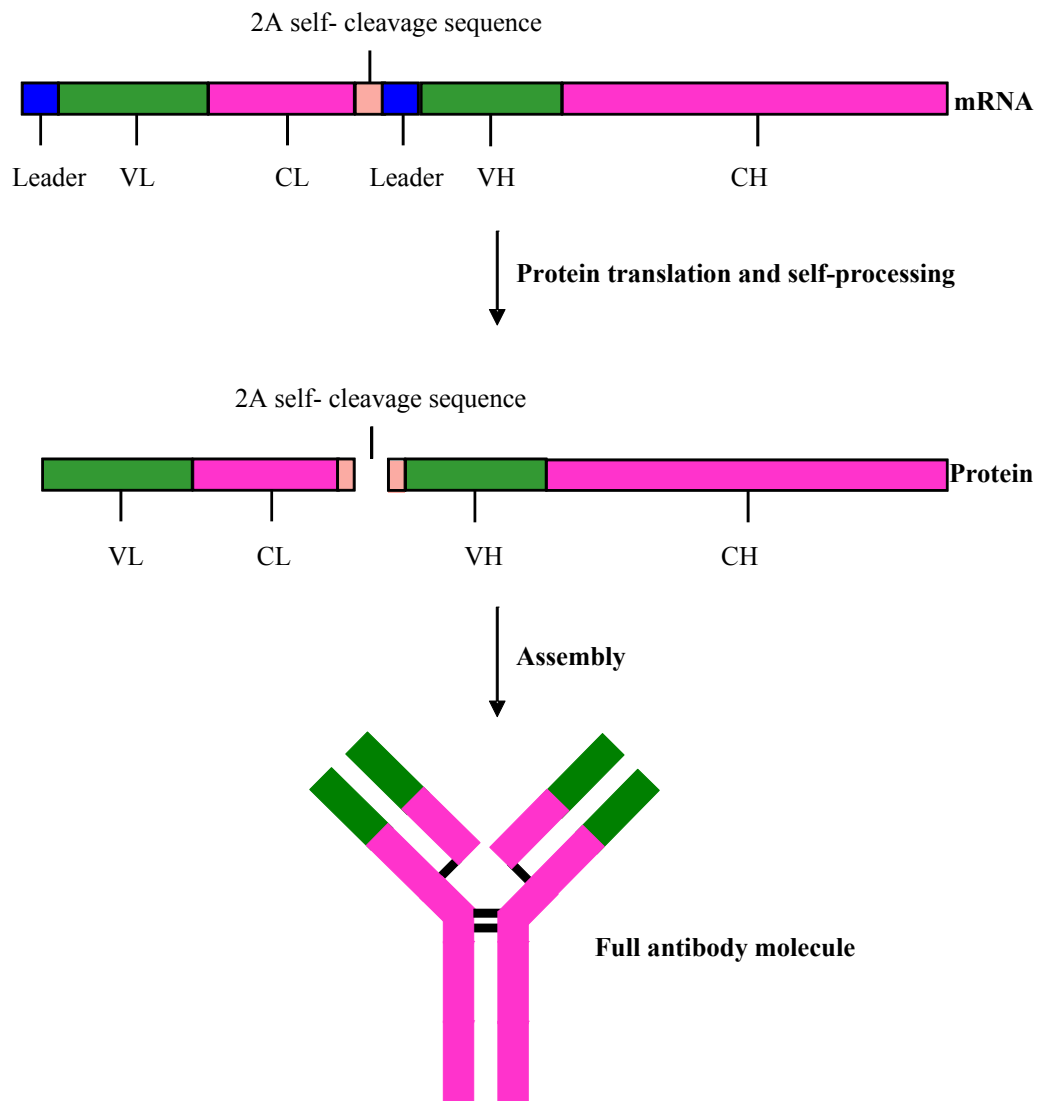
Figure 6: Schematic illustration of full-length antibody expression strategy using a monocistronic vector with 2A self-cleavage sequence as a linker between HC and LC. © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.

Purification

Protein G and A columns are widely used for a quick purification of antibodies because of protein G and A binding to the Fc portion of antibodies. However, protein G and A cannot only bind to human antibodies, but also bind to bovine antibodies. Therefore, these cannot be used for the purification of humanized antibodies since HEK 293 cells are cultured in the medium with 5% fetal bovine serum which contains a high percentage of bovine antibodies. Unlike protein G and A, protein L binds antibodies through interactions with the light chains. Protein L only binds to antibodies containing light chains of type kappa 1, 3, and 4 in humans and kappa 1 in mice. Most importantly, protein L does not bind to bovine antibodies. That is why the protein L column should be chosen to purify the humanized antibodies to eliminate co-purification of any bovine antibodies [23,24,48,49].

CONCLUSIONS

A recent approach to antibody humanization, now described here, is based on a comprehensive analysis of the available data of the sequence and the 3D structure of a given murine antibody. This identifies the key residues both in CDRs and FRs, which guides the choice of human antibody template and back-mutation positions in FRs to finish CDR-grafting into a single step. We have successfully humanized an anti-ricin neutralization antibody, D9, by this single cycle of CDR-grafting approach [49]. The single-step structure-based method is a considerable improvement over the standard CDR-grafting humanization methods, which require several trial-and-error cycles of error correction and refinement.

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CONFLICT OF INTEREST

All authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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Discovery of an Effective Ricin Antidote: An Old Drug for a New Use

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Abstract: A cell-based high throughput screening assay (HTS) was established to screen the Prestwick Chemical Library for candidates that acted against ricin. Of 1120 compounds screened, only 7 were identified as ricin inhibitors. Secondary screening with cell cultures identified only ethotoin as a dose-dependent inhibitor against ricin induced toxicity. Ethotoin was further evaluated in two *in vivo* studies. Study 1: When mice were given intra-peritoneal injections of 5×LD50 ricin (1 µg) pre-incubated with ethotoin (1 mg), all mice survived (monitored for 14 days). In contrast, control mice without ethotoin died within 2 days. Study 2: When mice were given 2×LD50 ricin (0.2 µg) by the intranasal route and then given ethotoin by multiple oral/gavage deliveries (at 1, 2, 4, 6, 8 and 10 hr after intoxication) 40% of the mice were alive at day 14. In contrast, all control mice (those that received sterile saline instead) died between days 3-9. Hence ethotoin, given by one route (oral) could rescue some mice from ricin given by another route (intra-nasal). Our findings suggest that ethotoin, although now an abandoned anti-convulsant drug, deserves further investigation and development as a potential antidote against the ricin biothreat.

Keywords: Ricin, antidote, repurposing, drug, ethotoin, anticonvulsant, rescue.

INTRODUCTION

Ricin is a well-known potent toxin. Aside from numerous publications in the scientific literature that have resolved its mechanism of toxicity *in vitro* [1] and *in vivo* [2], there have also been several incidences where it has been used as a terrorist threat [3-5]. As yet there is no medical countermeasure available either for the protection of first responders or the treatment of casualties from the effects

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of this toxin. Although it is encouraging that there are now drugs and antibody candidates addressing these capability gaps [6,7], the development of any drug for human use is time-consuming, laborious, and costly. It has been estimated that the development of a new drug costs about $1.4 billion and takes 10 years before it can be approved for use [8]. Also, the reality of the high failure rate at each step of product development has made the development of any new drug a high risk venture [9]. The imbalance, between a low reward and a high cost for drug development, has led to a lack of drugs available for the treatment of illnesses and threats [10].

Since the 1980s, many new technologies, such as molecular modeling, computational chemistry and genetic sequencing, have been used to design and select drugs for the treatment of infectious microorganisms or cellular abnormalities [11-13]. The applications of these technologies in drug discovery have in turn resulted in a large number of new drug candidates. However, most of these drug candidates have failed to acquire approval for human use. In 2009, of the 1000s of drugs put forward for development, only 25 new drugs were approved by the United States Food and Drug Administration (FDA) for clinical use [10]. With such a low success rate, an alternative strategy for drug discovery, "old drugs for new uses", has gained interest [14]. This concept takes existing drugs, with known molecular mechanisms on the disease or cell, as well as documented safety data from animal/human use, and utilizes these in novel ways. This approach is called "drug repositioning" or "drug repurposing" [15].

Almost all drugs exert pharmacological side-effects due to their multi-reactive sites on the pathogen or cell [16]. Under certain medical conditions, however, these side-effects may be important for the treatment of a disorder different from that originally targeted by the drug [17-19]. Since an approved drug has already passed a significant number of efficacy, toxicity and safety tests, many of the steps towards its approval for a different use have already been passed. Table **1** summarizes this saving of time and money, combining the stages of product development, using a greatly simplified version of NATO's Technology Readiness Levels for drugs [20], with milestones, either fully or partially, transferrable from a product's past product development to its new purpose [21].

Table 1: Technology Readiness Levels (TRLs) for repurposing a drug

NATO Biomedical Technology Readiness Levels (Drug)	Previous findings of use for repurposing
1. Literature reviews	✔
2. Hypothesis, research plan	
3. Basic research, test hypothesis	
4. Non-GLP research, animal testing	✔
5. GLP safety and toxicity studies in animals, pharmacokinetics	✔
6. Clinical trials (safety) in humans	✔
7. Clinical trials (efficacy, dose)	
8. Expanded clinical trials for risk-benefit, process validation	✔
9. Post-marketing studies	✔

This repurposing approach is designed to increase the return on investment. Since the bioactivities and toxicities of the candidate therapeutic have already been studied and documented, a repurposed drug can bypass the early investment to bring it to market for a second use. As but one example, thalidomide was a drug introduced in 1957 to alleviate the symptoms of morning sickness of pregnant women. It was withdrawn 5 years later when it was found to be a teratogen (causing many different forms of birth defects of newborns). Forty years later, it is now used to treat multiple myeloma (cancer of plasma/white blood cells), erythema nodosum leprosum (inflammation of fat cells under the skin) and symptoms of HIV [22]. In a similar fashion, pharmaceutical companies, such as Biovista™, Numedicus™ and Melior Discovery™ are focusing on drug repositioning, using *in silico, in vitro* and *in vivo* approaches to reassess existing old drugs for new targets [19].

As a source of candidates for repurposing, a drug library is often used that consists of a large number of molecules that are structurally and therapeutically very diverse yet have known safety and bioactivity in humans. The Prestwick Chemical Library contains 1120 small molecules, all previously FDA approved for human use, of high chemical and pharmacological diversity, with known bioavailability and safety data for humans. In our studies now presented, *in vitro*

and *in vivo* studies were used to screen possible "hits" (positive results) from the noted chemical library. The described results will show how 1120 drug candidates were reduced to 1, namely ethotoin, as a possible antidote against ricin toxin.

MATERIALS AND METHODS

Biosafety

All staff using ricin had a high level of security clearance and had been reviewed/approved by the National Authority (Department of Foreign Affairs and International Trade, DFAIT, Ottawa). Ricin was prepared from castor bean seeds at Defence Research and Development Canada (DRDC) − Suffield. The source of the castor beans or method of ricin extraction and purification cannot be revealed for security and Controlled Goods restrictions. The ricin stock was kept in a high security Single Small Scale Facility accessible only to a few security-cleared personnel and then only with card and fingerprint controlled access. Minimized aliquots of toxin working stock (35 µg/0.05 mL) were kept in a locked freezer with documented sign-out. All experiments were conducted in a secure Biosafety Level 2 area (BSL-2) with restricted access. An inspection of facilities and inventory records for compliance is conducted annually by the Biological and Chemical Defence Review Committee (BCDRC). Additionally, our use of ricin and the facilities had also been inspected and approved by CDC-Atlanta on behalf of NIH-NIAID.

Animals

Female BALB/c mice (5-6 weeks old, 18-22 g) were obtained from the pathogen-free mouse breeding colony at DRDC - Suffield research Centre, with the original breeding pairs purchased from Charles River Canada (St. Constant, QC). All animal experiments were performed in strict accordance with the guidelines set out by the Canadian Council on Animal Care (Ottawa)). The animal care protocol was reviewed and approved by the Animal Care Committee - Suffield Research Centre. Where possible, alternative end-points (3+ on a 0 to 5+ symptom scale) were used to minimize stress of the animals.

Preparation of Chemical Inhibitors

The Prestwick Chemical Library was purchased from Prestwick Chemical (Illkirch, France). The library contained 1120 small molecules that were all

previously FDA approved drugs for human use. The stock compounds had been dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, packed in 96-well plates and upon arrival were stored at -20°C. For the *in vitro* primary HTS assay, 10 µL of each compound stock solution was transferred into 90 µL of cell culture medium and kept at 4°C. After the initial screening, the 7 compounds that showed inhibitory action on ricin were purchased as follows: 5-iodo-2'-deoxyuridine, kaempferol, 2-phenyl-1,3-indandione, ticlopidine, trichlorfen (Sigma-Aldrich, Oakville, ON), tracazolate (Ticris Bioscience, Bristol, UK), and ethotoin (Yick-Vic Chemical & Pharmaceuticals, Hong Kong).

Vero Cell Preparation

All cell culture components were purchased from Invitrogen (Burlington, ON, Canada). Vero cells (American Type Culture Collection #CCL-81, Manassas, VA), were maintained in 75 cm^2 Falcon culture flasks under standard culture conditions (5% CO_2, 37°C, media renewed every 2–3 days). The cultures were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). When at 60-80% confluence (estimated visually with a stereomicroscope), the cells were subcultured or used for the HTS assay.

Primary HTS Assay

A suspension of 10^4 Vero cells in 100 µL culture medium was added to each well of 96-well plates. Fifty microlitres of Prestwick Chemical Library compounds were added into each well. After 2 hr incubation in a 37°C incubator, 50 µL of ricin, diluted in DMEM to a concentration of 45 ng/mL, was added into the wells. The final volume for each well was 200 µL, with 250 µM for each compound and 11.25 ng/mL ricin (about 10 LD50 for the Vero cell culture). The plates were kept in a cell culture incubator for 2 days. After this time, 20 µL of Alamar Blue (TREK Diagnostic System, Cleveland, OH) was added into each well and the plate was incubated at 37°C for 6 hours. After incubation, the plate was then read in a microplate reader at absorbance wavelengths of 570 nm and 600 nm. The ratio of A_{570nm} to A_{600nm} was determined. This ratio directly correlated to cell viability. In addition, 8 wells of cell-growth controls (cells only, no ricin, no Prestwick compound) and 8 wells of cell-death controls (cells with ricin, no Prestwick compound) were included in each assay.

In vitro Dose Response of Ethotoin Against Ricin

An *in vitro* dose-response effect of ethotoin against ricin was conducted. Ethotoin at concentrations of 0, 100 μM and 200 μM, was added into cell cultures containing a serially diluted concentration of ricin. The experimental procedure was that described for the primary HTS assay. Cell viabilities were determined using the Alamar blue assay.

In vivo Challenges with Ricin

It was previously determined that for our preparation of ricin given to mice, one LD50 by the intra-peritoneal route (*i.p.*) was 0.2 μg and by the intra-nasal route (*i.n.*) it was 0.1 μg. To be consistent with publications, mice were given 5×LD50 ricin (1 μg per mouse) in 0.1 mL sterile saline *i.p.* and 2×LD50 ricin (0.2 μg per mouse) in 0.05 mL sterile saline *i.n.* When mice were challenged by the *i.n.* route, these were first anesthetized with Metaphane (methoxyfluorane, Medical Developments International Ltd., Springvale, Australia) in a closed chamber (*i.e.* a few mL of this volatile liquid was added to cheesecloth within an open polypropylene bottle). These were then removed unconscious and either saline or ricin was delivered by micro-pipette into the left nostril. Total recovery from the anaesthetic was within a few minutes.

In vivo Assessment of Ethotoin Against Ricin

For the ethotoin/ricin pre-incubation experiment, 1 mg ethotoin in saline was incubated with 5×LD50 ricin at 37°C with constant rotation of 150 rpm for 1 hr. The mixture was then injected *i.p.* into mice. Weights, symptoms and survival of mice were recorded daily for up to 7 days following challenge, survival was observed for an additional week.

For the post ricin exposure rescue study, groups of 5 mice were anesthetized with Metaphane, then given 2×LD50 of ricin *i.n.* Afterwards, ethotoin (either 500 or 50 μg) in saline (0.1 mL) was given by gavage (animal feeding needles, Popper & Sons, New Hyde Park, NY) at 1, 2, 4, 6, 8 and 10 hrs after ricin challenge (*i.e.* mice receiving ethotoin did so at the noted times for a total of 6 doses). Control mice after ricin challenge received only saline by gavage at the same time points. Weights, symptoms and survival of mice were recorded daily for 14 days.

Data Analysis and Hit Selection

The strictly standardized mean difference (SSMD), a statistical parameter described by Zhang [23], was calculated for each HTS assay for quality control and was used for the selection of hits in our assay.

RESULTS AND DISCUSSION

The Alamar Blue Viability Assay (A_{570nm} to A_{600nm} Ratio)

For the initial *in vitro* screen, each Vero cell containing well in a 96 well plate was given a different compound from the Prestwick Chemical Library, followed 2 hours later with 10 LD50 of ricin. Two days later these were given Alamar Blue followed by a 7 hour incubation.

Alamar Blue appears blue in its natural oxidized form (*i.e.* where it absorbs lower energy, red light, at a peak of A_{600nm}) and pink when reduced by the living cell (*i.e.* where it absorbs higher energy, lower wavelength light, at a peak of A_{570nm}) [24]. If the cells had been killed by ricin, Alamar Blue would have accumulated within these cells to give the monolayer a blue colouration. If the compound had neutralized ricin and the cells continued to be viable, then these would have reduced the dye to give a pink appearance. Although the differences in appearance were easily discernible by eye, to obtain an impartial non-subjective numerical value, two absorbance readings were done on the same cell culture given Alamar dye, one at 570 nm and one at 600 nm, and the A_{570nm}/A_{600nm} ratio calculated. The higher the value, the more viable the culture. The assay could likely be improved with better viability dyes, methods, fluorescence *vs* absorbance or the use of colour-filters. For now our assay was adequate for differentiating the effects of a large library of compounds on ricin as evidenced by cell culture viability.

Fig. **1** gives an example of a plate used to screen the first 80 of the 1120 compounds in the Prestwick Chemical Library. Three groups of compounds were observed:

<u>Group 1</u>: The drug was toxic, cells died at the start of the study, and the low number of cells gave an Absorbance Ratio close to 1.0 (*e.g.* Compound #17, Levadopa). These drugs were rejected as unsafe. (In contrast, Compound #55,

acetohexamide, appeared nutritional to Vero cells, giving the cells a very high ratio when ricin was absent).

Group 2: Cells with these drugs, but without ricin, gave a high Absorbance Ratio. However with ricin, cells with these same drugs lost viability and the ratio decreased. These drugs were rejected as ineffective against ricin.

Group 3: The drug protected the cells (*e.g.* Compound #47, Ticlopidine; Compound #51, Trichlorfon). The Absorbance Ratio was unchanged in the presence of ricin. These drugs were selected for further study as anti-ricin candidates.
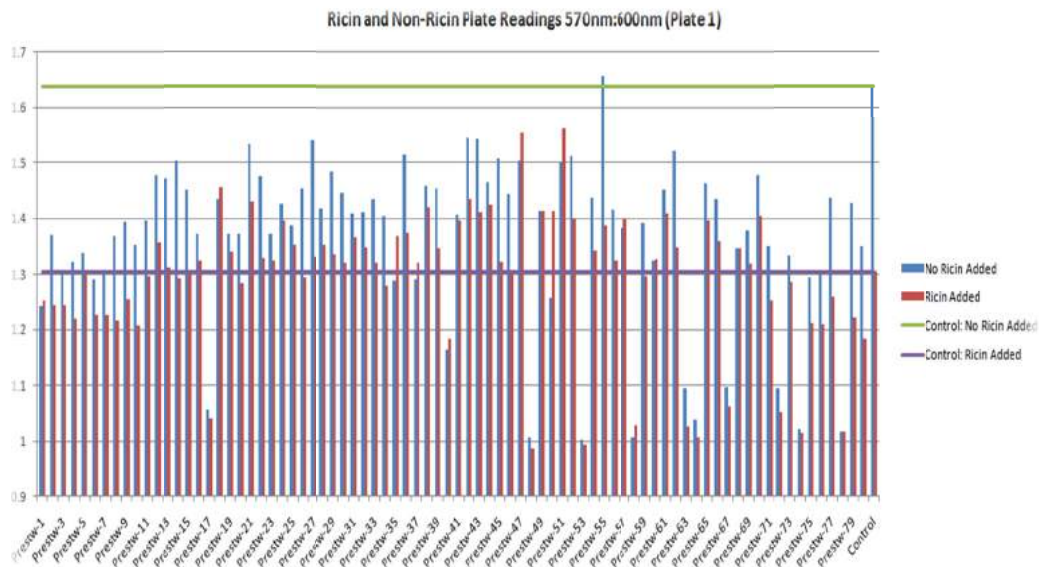
Figure 1: A_{570nm}/A_{600nm} ratios for Vero cells given Prestwick Chemical Library compound (blue bar) or the compound and ricin (red bar) © Copyright, diagram provided by the Government of Canada, DRDC Suffield Research Centre.

Prestwick Chemical Library Primary Screening for Hit Selection

Another method to assess the data is the use of the "strictly standardized mean difference" (SSMD). This was first applied to the HTS assay in 2007 by Zhang *et al.,* for quality control and "hit" selection [23, 25, 26]. In our study, the SSMD scores of all primary HTS assays were between 3.9 and 11.6 with average of 6.4,

indicating consistent results. To select a relatively small number of compound, hits with strong ricin inhibition from the primary screening, compounds with a threshold value of $\beta \geq 7.2$, were chosen. Seven compounds met this criteria.

In vitro Dose-Response Effect of Ethotoin Against Ricin

The selected 7 hits (anti-ricin compounds) were further examined for their effect against a range in concentrations of the toxin. Only 3 of the 7 compounds gave an *in vitro* dose-response against ricin, and of these 3 the best was ethotoin. As shown in Fig. **2**, cells given ethotoin at a concentration of 0, 100 μM or 200 μM, and then exposed to a series of ricin concentrations had the noted viabilities. Cell viabilities were determined with the Alamar Blue assay. (Results of greater than 100% viability were due to some cell growth when the amount of ricin used was very low.) As shown in the figure below, ethotoin inhibited ricin toxicity *in vitro* in a dose dependent manner.
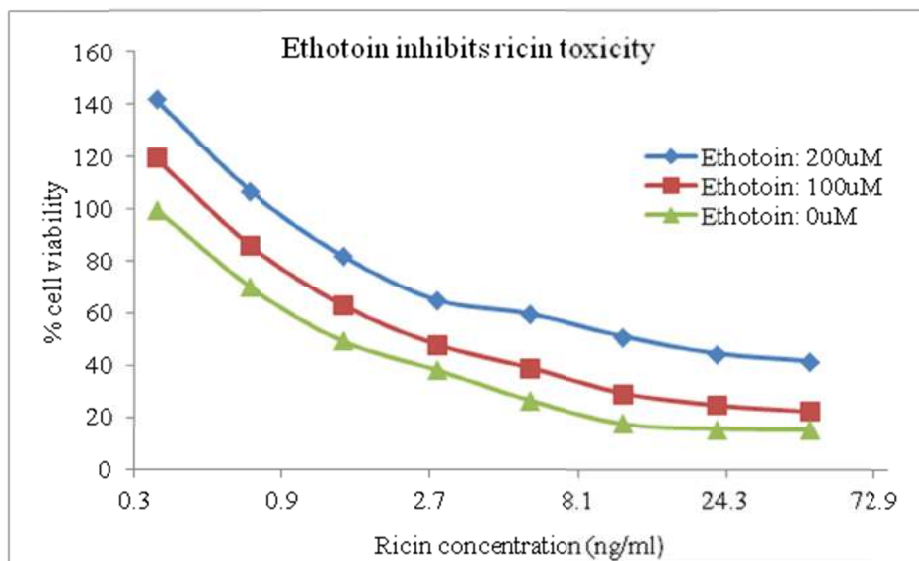
Figure 2: Cell viability when given different amounts of ricin and ethotoin.

Ethotoin Inhibition of Ricin *in vivo*

Ethotoin inhibition of ricin *in vivo* was examined in mice. Ethotoin (1 mg) was pre-incubated with 5×LD50 of ricin, for 1 hr at 37°C, then injected into mice *i.p.* Mice were monitored for survival, weight and symptoms for 7 days, survival for

an additional week. All mice given ethotoin and ricin survived, all control mice without the drug died within 2 days. As shown in Fig. **3**, results indicated that ethotoin strongly inhibited ricin toxin in mice. Differences were considered to be statistically significant at *P<0.05*. Not shown on this figure was the failure of the other 6 compounds, (5-iodo-2'-deoxyuridine, kaempferol, 2-phenyl-1,3-indandione, ticlopidine, trichlorfen, tracazolate) noted in the previous section, that were observed not to protect mice.
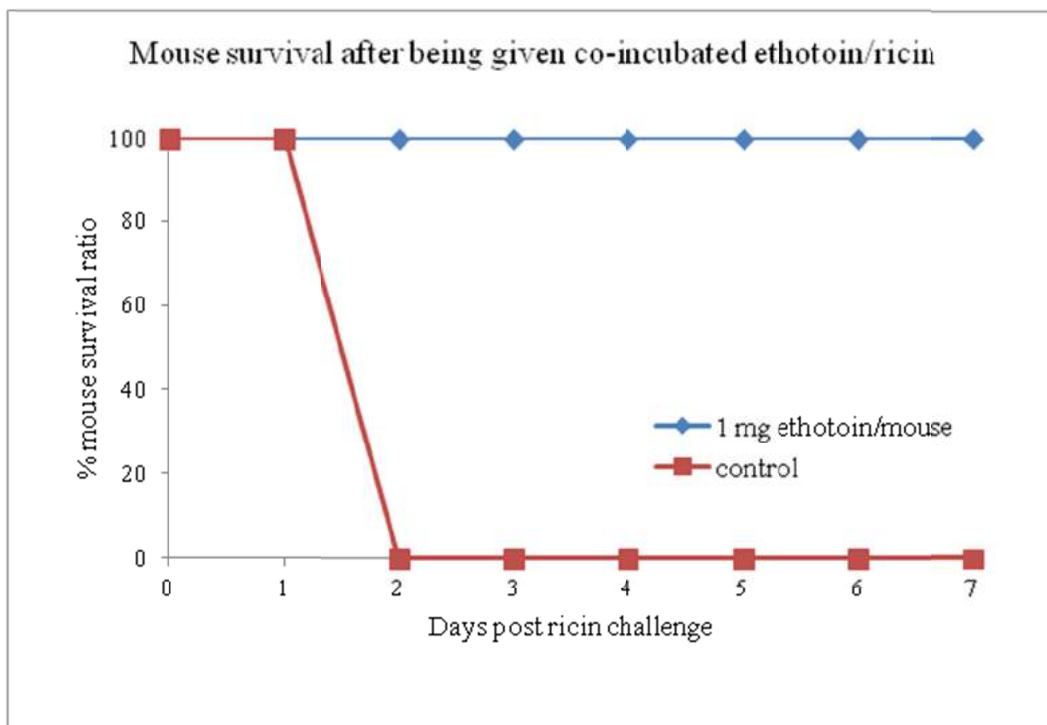
Figure 3: Ethotoin's inhibition of ricin when the two were co-incubated then injected into mice *i.p.*

Ethotoin's Rescue of Mice from Ricin

Ethotoin was further examined to determine if it could rescue mice if given after ricin poisoning. To mimic a scenario where casualties might be exposed to ricin by the respiratory route but treated by a drug given orally, a preliminary study was done whereby groups of 5 mice were exposed to 2×LD50 of ricin given *i.n.*, then treated with either 50 µg or 500 µg ethotoin in 0.1 ml saline by gavage at 1, 2, 4,

6, 8 and 10 hr post-challenge (*i.e.* treated mice received 6 doses of ethotoin). Control mice received the same volumes of saline by the same method at the same time points. Survival, weights and symptoms of all mice were observed daily for 2 weeks. As shown in Fig. **4**, 40% of mice survived at least to day 14 when these received 500 μg of ethotoin, while only 20% of the mice survived if these received 50 ug of ethotoin. All control mice died by day 9. (The extended life for some of the control mice, past the usual 4 days for *i.n.* ricin exposure, may have been due to the positive effects of forced hydration with saline.) The results indicated that ethotoin inhibited ricin toxicity in mice. Differences were considered to be statistically significant at *P*<0.05.
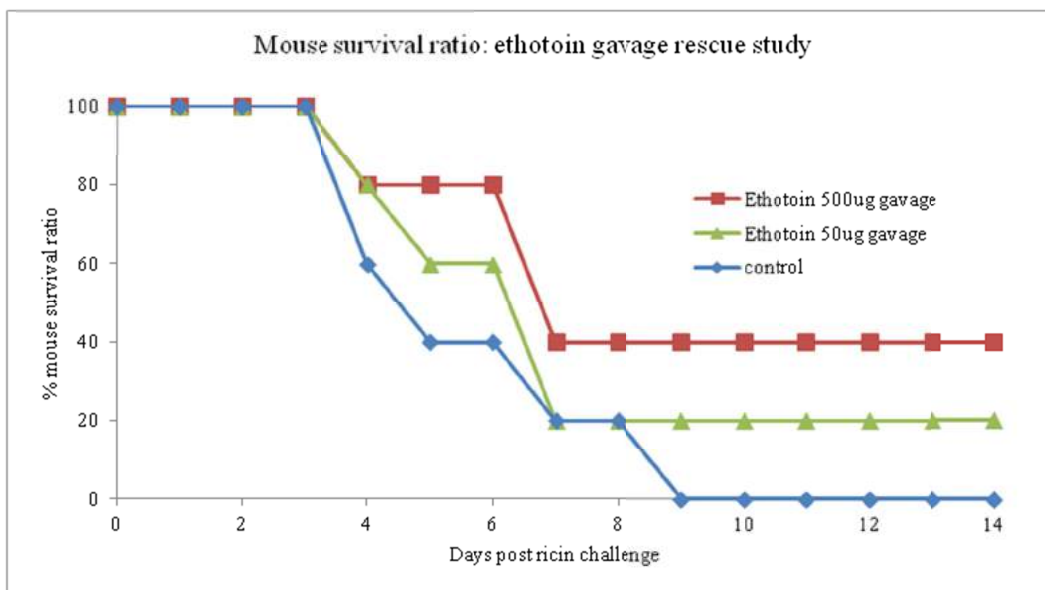
Figure 4: Ethotoin's rescue (gavage/oral) of mice given ricin (*i.n.*).

CONCLUSIONS, RECOMMENDATIONS FOR FUTURE STUDIES

This present study screened the Prestwick Chemical library, consisting of drugs all previously FDA approved, for an anti-ricin antidote. A primary HTS assay reduced this number from 1120 compounds to 7 ricin-inhibitory candidates. An *in vitro* cell viability assay using Amalar Blue, a Strictly Standardized Mean Difference analysis, and *in vivo* survival – all confirmed ethotoin's inhibitory effect against ricin. The results were very encouraging, especially since in one of

the *in vivo* studies, some of the mice could be rescued if the toxin was given by the intra-nasal route (to mimic a respiratory attack) and ethotoin was given by gavage (to mimic oral ingestion of a therapeutic drug in pill form). Ethotoin is an abandoned drug that previously had been used as an anticonvulsant drug to treat epilepsy. Its pharmacokinetics and safety in humans have already been well described and documented. Our discovery of "an old drug for a new use", that ethotoin has anti-ricin effects, should be investigated further, especially since there are presently no countermeasures available against this possible terrorist threat.

Although some mice could be rescued from ricin poisoning if given ethotoin, not all mice survived. Future studies should focus on procedures and supplements to allow all mice to survive after ricin challenge. The pilot studies were successful, but most therapeutics are not given for only a 10 hour period. It should be investigated if there is a better outcome if ethotoin is given over additional days and by better means (*e.g.* doses by gavage every 4 hours, continuousl delivery by Alzet micro-osmotic pump). If there is no improvement with these, then better formulations should be considered (*e.g.* linked to carriers, liposome encapsulation).

With regards to supplements, some of the treated mice may have perished because of refusing to take water or food. In another study involving forced hydration, water by gavage or *i.p.* injections of physiological saline, the injections appeared less stressful. With regards to supplements, it was found that sugar (honey) rather than fat or protein, vitamin E (alpha-tocopherol) and an analgesic (acetaminophen with codeine) had positive effects on mice given ricin. Also, ricin induces apoptosis, inflammation and the expression of toxic cytokines, antagonists of which have a positive effect on the poisoned cell [27-29]. Although these measures may only provide minor relief from the toxin, perhaps with the drug ethotoin a synergistic positive effect can increase the rate of survival of those challenged.

In the presented findings, much has been learned on repurposing, of developing methods to screen libraries of pre-approved drugs to find an antidote against toxins, in this case ricin. However, a gap that needs to be addressed is the

mechanism of action – how does the antidote, ethotoin, act on ricin? Further support is needed to resolve this, especially if ethotoin is going to be approved for use on casualties at a terrorist strike. We do not presently have the answer of ethotoin's mechanism against the toxin, but there are clues. Previously it was reported that the antibiotic, rifampin, inhibited the effects of the toxin, microcystin [30]. Several years ago, several antibiotics were tested for inhibition against ricin and only tetracycline, which has a carbonyl next to a cyclic group, had some inhibitory effect against ricin in mice (Cherwonogrodzky, unpublished data). Ethotoin has a carbonyl next to a cyclic group, as do most of the other six discovered inhibitory compounds cited in the text (*e.g.* iodo-deoxyuridine, kaempferol, phenyl-indandione, tracazolate hydrochloride). It has been reported that the compound retro-2, which has a carbonyl next to cyclic groups [31] and polyphenols in black tea [32] also inhibit ricin. The mechanism behind retro-2 is unclear but believed to be its inhibition of the toxin's transport between the endosome and the Golgi complex, before it can be transported to the endoplasmic reticulum to act on ribosomal RNA. Either ethotoin has the correct composition and structure to insert into ricin's active site to inhibit the toxin, or its effects are more complicated, blocking ricin's transport within the cell similar to retro-2. It is recommended that further studies be done to determine ethotoin's mechanism of action on ricin.

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CONFLICT OF INTEREST

All authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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PART 5: REPLACEMENT, DIFFERENT USES, FOR RICIN

CHAPTER 11

A Ricin-Like Toxoid Used to Raise Goat Anti-Ricin Antibodies

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Abstract: A major difficulty in developing medical countermeasures against ricin (*e.g.* passive antibody therapy) is the need to work with authentic ricin, a Schedule 1 Chemical under the Chemical Weapons Convention. Twinstrand Therapeutics Inc. created a benign but antigenically intact variant of ricin by changing the genetic sequence of the A and B chain linker peptide (representing less than 4% of the gene) to block post-translational processing and activation. The toxoid protein, a single polypeptide chain rather than dimer, was produced in a *Pichia pastoris* expression system in accordance with Good Laboratory Practices (GLP). The toxoid was determined to be 1000-fold less toxic than authentic ricin by both *in vitro* and *in vivo* assays, allowing high immunogenic doses to be administered to test animals. Cangene Corporation oversaw a contract in which goats were inoculated with varying schedules and doses of the toxoid in accordance with GLP. DRDC Suffield Research Centre assessed the activity of the resultant goat antibodies in challenges involving the wild type toxin. The goat anti-toxoid antiserum was found to have high anti-ricin

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Keywords: Ricin, recombinant, toxoid, vaccine, immunoglobulin, anti-ricin, antibody, rescue.

BACKGROUND

Throughout the various chapters of this book, ricin's toxicity, incidences that reveal ricin as a terrorist biothreat, and the development of encouraging medical countermeasures, have been described. Vaccines and anti-ricin monoclonal antibodies are thought to be products most likely to mitigate or negate the ricin threat to humans. A vaccine providing immunity to ricin with demonstrated efficacy in animal models, and safety in human clinical trials would be an ideal protective measure. The effective use of a vaccine, however, would require pre-existing knowledge of 1) "at risk" personnel and 2) timing of a potential exposure - in order to administer an appropriate immunization schedule or booster to ensure that subjects have adequate levels of neutralizing immunoglobulins prior to exposure. Vaccinating a significant percentage of the population against ricin would be costly and would face regulatory obstacles. Monoclonal antibody therapy immediately following exposure to ricin would be more practical and appropriate in circumstances where a small number of people have been exposed to the toxin at an incident site. However, creating panels of monoclonals, selecting the best candidates or combinations of candidates, characterizing their efficacy against different ricin variants, and producing these at scale-up facilities, will involve substantial time, effort and support.

Consequently, an interim emergency countermeasure is needed to bridge gaps until the eventual deployment of anti-ricin vaccines and monoclonal antibodies can be available. Such a countermeasure would emulate the success of anti-snake-venom antiserum which is available for individuals thrust into a life-or-death situation by snake bite. The interim anti-ricin therapeutic described below was developed during the period 2003-2005 in DRDC's CRTI program. The strategy involved the use of a benign toxoid, substituting for ricin toxin, that could be used

repeatedly and in high antigenic amounts to immunize goats without causing illness or death to the animals. Project CRTI 02-0007TA (2003-2005) had 3 stages, each using the exceptional expertise of one partner for a specific objective, then handing their accomplishment to the next partner. The following gives a brief summary of the 3 stage project.

DEVELOPMENT OF AN ANTIBODY THERAPEUTIC AGAINST RICIN POISONING

Twinstrand Therapeutics Inc. Provision of Toxoid (TST 10088)

Ricin is poisonous to eukaryotic cells by inactivating the protein synthesizing ribosomes. Indeed, it is even toxic to the cells of the castor bean plant, *Ricinus communis*, where the toxin is made. The plant protects itself from intoxication by first synthesizing an inactive pro-ricin (a 68 kDa single chain polypeptide consisting of the A chain, a short linker peptide, and the B chain). After the pro-ricin is synthesized, it is translocated into the lumen of the endoplasmic reticulum where it is properly glycosylated and then transported from the Golgi complex to storage vacuoles in the endosperm of the bean. Within the storage vacuoles, the linker is cleaved to convert the molecule into toxic ricin which is stable but of low activity in the low pH environment of the storage vacuoles [1].

Investigations have been reported where the linker peptide sequence was altered and led to insights in ricin transport within the cell [2], stability [3] and activity [4]. Twinstrand Therapeutics Inc. (Burnaby, BC, Canada) took advantage of this knowledge and created variants with the linker peptide resistant to proteolysis [5]. Using recombinant technology, the company constructed and expressed a variant of pro-ricin as TST10088 in *Pichia pastoris* yeast (see Fig. **1**). Advantages for the use of this organism were that it secreted high quantities of expressed pro-ricin protein, into culture medium, with disulfide bonds and glycosylations similar to native ricin [6, 7]. Previously, other investigators have used the A-chain [8, 9], fragments [10] or chemically denatured forms of ricin [11, 12] as antigens but these lacked the native structure of the full-sized toxin. However, as toxoid TST10088 retained the native epitopes of ricin's A and B chain (i.e the toxoid has about 96% the primary structure of ricin [5]), it was found by the company that the toxoid could induce highly effective anti-toxin antibodies in animals despite

the modification that greatly reduced (1000 times less) toxicity [13]. Toxoid TST10088 was produced in gram amounts at a European GMP facility (Eurogentec SA, Belgium) and provided to Cangene Corporation (Winnipeg, MB) for the immunization of goats, also in accordance with GMP.

RICIN	TST10088
A bridging 23 amino acid peptide on pro-ricin is removed by a castor bean protease (note gap at top). Ricin toxin consists of an A chain (glycosidase enzyme) and B chain (lectin to galactose) linked by a disulfide bond.	Pro-ricin, the precursor to natural ricin, is a single continuous chain of the A chain, a 23 amino acid peptide, and the B chain. By contrast TST10088 has a protease-resistant bridging peptide of 8 amino acids that limits the release of the toxic A chain inside target cells.

Figure 1: A comparison of ricin and toxoid TST10088.

Cangene Corporation's Immunization of Goats and Antisera Collection

Cangene Corporation contracted Capralogics Inc. (Gilbertville, MA, USA) to immunize 3 groups of 2 USDA certified scrapie-free goats with different amounts of Twinstrand Therapeutics Inc.'s TST10088 toxoid. The immunization schedule and test bleeds were as noted in Table **1**. Each group consisted of 2 animals that received the amount shown. RIBI adjuvant (Sigma-Aldrich, St. Louis, MO, USA) was used for the initial immunizations *via* the intra-muscular route.

At various time-points (about 30 time-points per goat over the course of 13 months) serum was collected from each goat by plasmapheresis (621 ± 64 mL per goat per collection) to acquire anti-toxoid antisera. From these, aliquots (12.9 ± 4.6 mL) were used to determine anti-ricin titres by ELISA against TST10088

antigen (Capralogics Inc. Customer Report 040651, 9/26/2005). A monoclonal anti-TST10088 antibody was used within the ELISA to formulate a standard curve. Fig. **2** shows the goat antisera titres of anti-TST10088 at various times throughout the vaccination schedule. Although the interpretation of the results is very limited due to the small number of only 2 animals per group, it does suggest that there is considerable variability in the responses (*i.e.* anti-TST10088 antibody titres) of the animals to the toxoid. It also appears that there was no advantage, and perhaps a disadvantage, for multiple vaccinations over extended times, the best titres being after 3 vaccinations over 7 weeks, then declining afterwards. Possible explanations for this decrease in anti-toxoid antibody titres could be the occurrence of immune-tolerance to the antigen [14], or a shift from antibody to other immune responses such as the production of mannose binding lectins acting against the toxoid's mannose glycosylation [15, 16] or expression of cell-mediated cytokines [17]. Regardless of this decrease in anti-toxoid antibody, it should be noted that the titres were still excellent, in high amounts and capable of rescuing mice after several lethal doses of ricin, as will be seen in the next section.

Table 1: The immunization schedule for the production of polyclonal anti-TST10088 antibodies in goats.

Group 1		Group 2		Group 3	
Time of Immunization	Toxoid Dose (μg) Injected	Time of Immunization	Toxoid Dose (μg) Injected	Time of Immunization	Toxoid Dose (μg) Injected
Day 0	500	Day 0	500	Day 0	500
3 weeks	500	4 weeks	500	3 weeks	500
6 weeks	500	6 weeks	500	6 weeks	250
9 weeks	500	8 weeks	500	9 weeks	250
13 weeks	500	12 weeks	500	13 weeks	250
17 weeks	500	16 weeks	500	17 weeks	250
21 weeks	500	20 weeks	500	21 weeks	250

DRDC Suffield Research Centre's Assessment of the Goat Anti-Toxoid Antisera Against Ricin *In vivo*

After the small volume sera aliquots had been assessed, these were pooled and then given to DRDC Suffield for assessment, notably to determine if this antisera raised against toxoid, not toxin, had anti-ricin neutralizing activity.
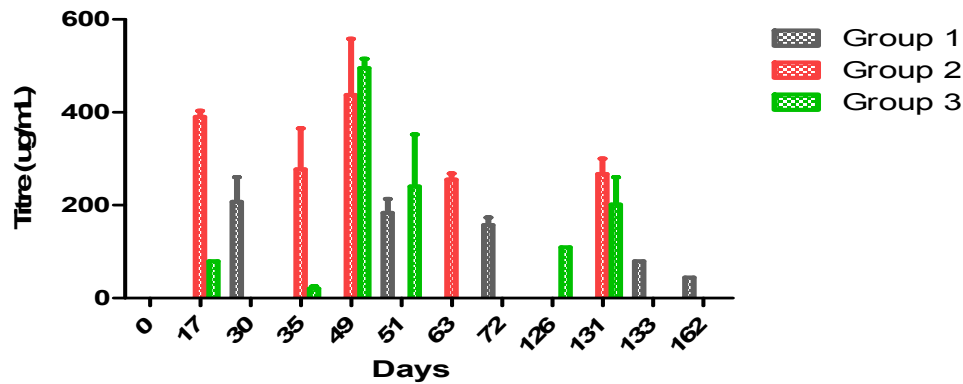
Figure 2: Anti-TST 10088 IgG response (µg/mL) for the goats (2 per group) immunized with the toxoid.

Mice (19-21 gram BALB/c female mice) given 0.1 ml goat antiserum by the intra-peritoneal route (*i.p.*) had <u>protection</u> against toxin as evidenced by their survival and a lack of symptoms when given 5 LD50 of ricin *i.p.* 1 week later. Control mice given saline, instead of goat antiserum, died or had to be terminated 2-4 days after ricin challenge.

For <u>treatment/rescue</u>, mice were first given 5 LD50 of DRDC Suffield's ricin preparation *i.p.* At different time-points after challenge, these were given either 0.1 mL sterile saline or goat anti-toxoid *i.p.* All mice that received 0.1 mL antiserum could be rescued 4 hrs after poisoning and 20-40% could also be rescued 8-16 hours after poisoning (see Fig. **3**).

Curiously, it was found that when ricin-poisoned mice were rescued with the high-titre anti-toxoid goat antiserum, then re-poisoned with 5LD50 of toxin *i.p.* 3 weeks later, the mice survived with only temporary mild illness. The studies were redone with bleeding of mice at different days after ricin challenge then antisera rescue an hour later. Fig. **4** shows that after 9 days, the amount of goat antiserum anti-ricin IgG in the mouse dropped to about 10% of its initial level. For the ELISA, microtitre plates were coated with 0.1 mL per well with 1µg ricin/ml (not toxoid), mouse sera was diluted 1:100 and used at 0.1 mL per well, mouse monoclonal anti-goat IgG-HRP conjugate (Jackson Immunoresearch Lab., Cedarlane Laboratories, Burlington, ON) was diluted 1:3000 and used at 0.1 mL per well.
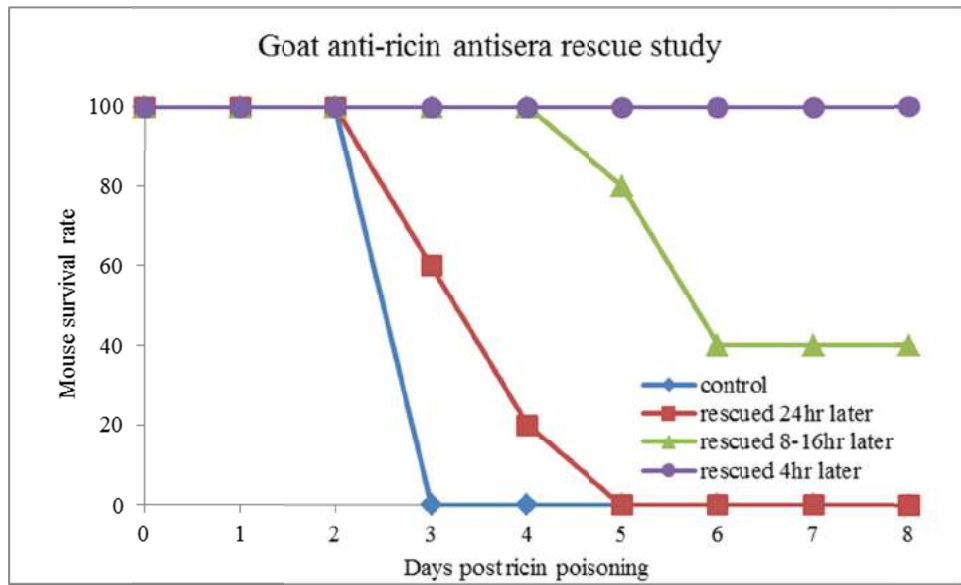
Figure 3: Survival of mice given 5 LD50 of ricin (*i.p.*) then rescued at different times with 0.1 ml goat anti-ricin antiserum.
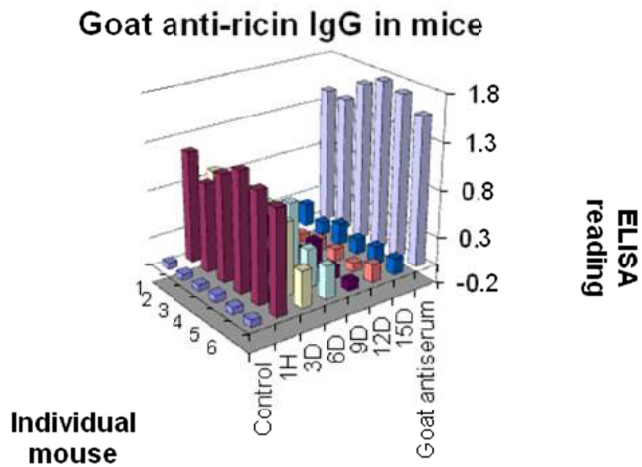
Figure 4: Mice given ricin (*i.p.*), then 1 hr later goat anti-toxoid antiserum (*i.p.*). Individual mice (one mouse represented by one column) were bled at specified times (1 hr to 15 days = 1H to 15D). ELISA (horseradish peroxidase, A_{630nm}) readings are shown for goat anti-ricin IgG in the mouse.

As the concentration of goat anti-ricin IgG decreased in mice over time (Fig. **4**), the titre of mouse anti-ricin IgG increased (see Fig. **5**, conjugate was goat anti-mouse-IgG-HRP at 1:5000), and to a lesser extent mouse anti-ricin IgM (see Fig. **6**, conjugate was goat anti-mouse-IgM-HRP at 1:3000). The rapid vaccination response to ricin neutralized by antibody has been previously reported [18, 19], but this was observed for monoclonal, not polyclonal, antibody. In contrast, our goat anti-TST10088 antibody given *i.p.*, and an hour later 5 LD50 of ricin also given *i.p.*, induced a rapid IgG vaccine response that protected mice from a second challenge on day 10, though with temporary illness and minor weight loss over 4 days afterwards (data not shown). It was not resolved why there was a difference between the published results and those now presented. Regardless, the results suggest that animals (and by extrapolation first responders or casualties) rescued after ricin exposure with antibody may have additional benefits of an acquired vaccination immunity to the toxin.
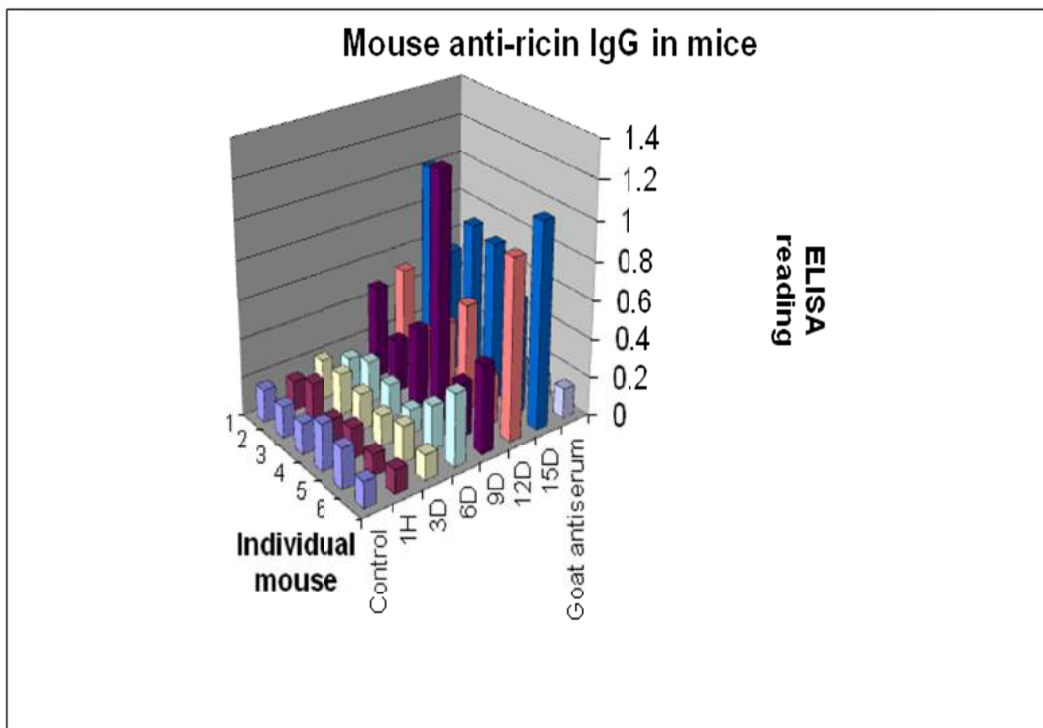
Figure 5: Graph similar to Fig. **4** but of mouse anti-ricin IgG.
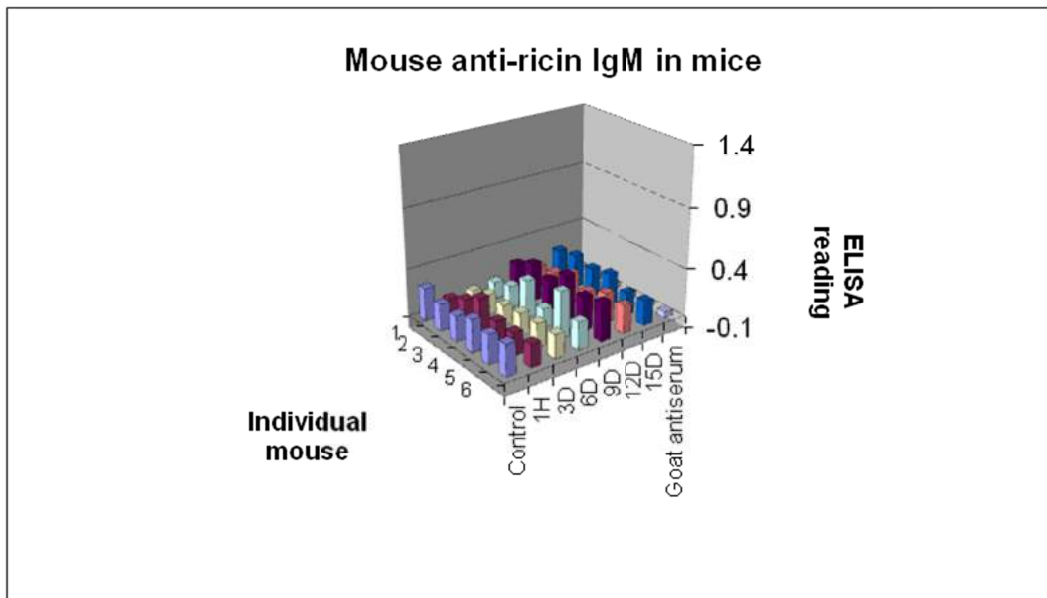
Figure 6: Graph similar to Fig. **4** but of mouse anti-ricin IgM.

From the above results, the approach to use a toxoid, rather than ricin to raise high anti-ricin antibody titres in goats, was successful, circumventing the use of the toxin, a highly restricted Schedule 1 chemical under the Chemical Weapons Convention. The antibodies raised could both protect and rescue mice from multiple lethal doses of the toxin.

0.1 mL of goat anti-toxoid could rescue a 20 gram mouse from 5 LD50 of ricin also given *i.p.* The route of toxin exposure, antisera delivery, and mouse *vs* human treatment, make the studies now reported of little relevance to casualties exposed to ricin in a real situation. However, as a starting point, extrapolating to a 70 kg human, the 100 litres of goat high-titre anti-toxoid produced in this CRTI project might be of use to about 300 casualties (determined by mouse:human weight) and up to 4000 casualties (using Body Surface Area to determine a Human Equivalent Dose, multiply mouse dose by 230) [20].

Further development for the purification of the antibodies in the antisera, and/or removal of the antibody Fc portion, would be recommended to reduce the risk of serum sickness or the remote possibility of viral transmission. Future directions to take advantage of this possible temporary measure against ricin poisoning, before

a superior highly effective and purified humanized anti-ricin monoclonal antibody can be made available, would be to increase its Technology Readiness Level (TRL) by preclinical trials, safety and stability studies towards it being an approved product.

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CONFLICT OF INTEREST

All authors disclose that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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Harnessing the Destructive Power of Ricin to Fight Human Cancer

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Abstract: Ribosome Inactivating Proteins (RIPs) are toxins capable of specifically and irreversibly inhibiting protein synthesis in eukaryotic cells. The plant RIP prototype, ricin, is a vigorous toxin that can be easily extracted and purified from the castor plant *Ricinus communis*. This heterodimeric toxin consists of a catalytic subunit A linked to a galactose-binding lectin subunit B, which allows cell surface binding and toxin entry in most mammalian cells. Ricin exhibits potency in the picomolar range, thereby it is highly toxic at very low lethal doses. Due to its high systemic cytotoxicity, ease of production, and prevalence, ricin is considered as a possible weapon for warfare and terrorism attacks, and therefore included among the potential biological weapons by the United State Centre of Disease Control and Prevention. Since heating denatures the toxin, extracted castor oil has been widely used in the industry; for example, as two-stroke engine oil or lubricant for aviation engine. However, castor oil has also detrimental effects causing violent diarrhea. Thus, even castor oil has been used as a convenient terror weapon under the Italian Fascist regime to convert "red" workers. Nowadays, more peaceful and beneficial medicinal uses have been created. Certainly, the most promising way is to exploit ricin as weapon to combat cancers. Novel molecules in which the toxin moiety of ricin was linked to selective tumour targeting domains have been generated for cancer therapy. The major recent advancements in this field will be discussed in this chapter.

Keywords: Ricin, RIPs, cancer, therapy, immunotoxins.

INTRODUCTION

Ribosome Inactivating Proteins (RIPs) are ribosomal RNA (rRNA) N-glycosidases [1], which catalytically remove a particular adenine residue from the

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28S rRNA (residue A4324 in rat liver 28S rRNA) of the large subunit of eukaryotic ribosomes [2]. The RIP-catalysed depurination leads to the removal of one adenine residue located in a region of rRNA containing one of the most conserved rRNA sequences, the GAGA sequence, which is essential for the ribosomal elongation cycle. The removal of the specific adenine alters the binding site for elongation factors 1 and 2 [3]. Thereby, the modified ribosomes are unable to bind these translation factors, impeding protein synthesis, which will ultimately cause cell death and accounts for toxicity of RIPs [4].

The prototype of plant RIP is ricin (from castor bean plant, *Ricinus communis*). Ricin is a heterodimer composed of a catalytically-active A chain (ricin toxin A or RTA) linked by a single disulphide bond to a B chain (RTB), which is a galactose- and N-acetylgalactosamine-specific lectin [5]. This plant toxin has been extensively characterized concerning the biochemical features, catalytic activity, biosynthetic pathway and intracellular transport. In this chapter, we are summarizing our knowledge on the structure and functions of ricin. We also discuss the therapeutic potential of this RIP with a particular emphasis on the use of ricin to generate chimeric toxins to combat cancers.

MECHANISMS OF RICIN TOXICITY

Structure, Biochemical Characteristics and Functions of Ricin

Plant RIPs are presently classified into three main types: Type I are composed of a single polypeptide chain of approximately 30 kDa; Type II are heterodimers consisting of an A chain, functionally equivalent to Type I polypeptide [6], linked to a B subunit endowed with lectin binding properties [7]; while Type III are toxins synthesized as inactive precursors (Pro RIPs) requiring proteolytic cleavages to form active RIPs [7]. Type III RIPs are not currently in use for therapeutic purposes (Fig. **1**).

Sequence identity between ricin A subunit (RTA) and type I RIPs is rather low. Amino acid sequence among RTA and type I RIPs may vary widely. For example, RTA has only two lysine residues, whereas lysine residues can account for up to 10 % total amino acids in Type I RIPs. Still, several residues are highly conserved among RIPs, including Tyr80, Tyr123, and the key active site residues Glu177, Arg180, and Trp211 of RTA. Moreover, all crystallized RIPs have been shown to share a common three-dimensional (3D) "RIP folding" [4] (Fig. **2**).
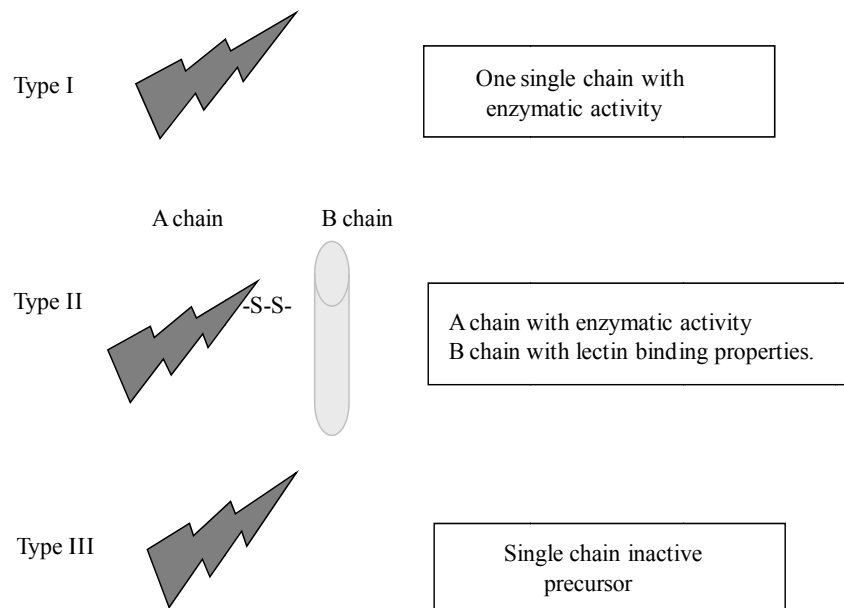
Figure 1: Schematic representation of type I, II, and III plant Ribosome-Inactivating Proteins. Type I: single chain protein with enzymatic activity; Type II: the active domain (A-chain) is cross-linked to the lectin binding domain (B-chain) by a disulfide bond; Type III: synthesized as inactive precursors (ProRIPs) that require proteolytic processing cleavages to form an active RIP.
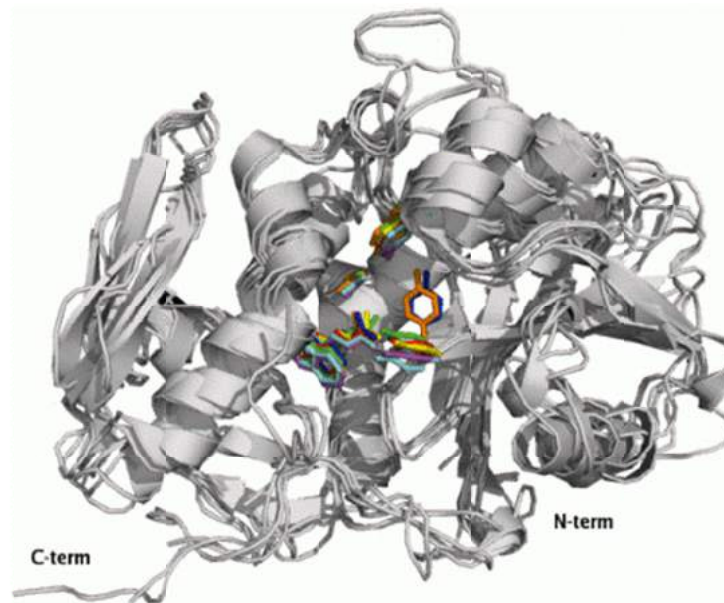
Figure 2: Three-dimensional structure of ricin A chain and several type I RIPs. Superimposition of secondary structure elements of ricin A chain (PDB code 1J1M), thricosantin (PDB code 1J4G),

saporin (PDB code 1QI7), dianthin (PDB code 1RL0), PAP (PDB code 1PAF), bouganin (PDB code 3CTK), and gelonin (PDB code 3KTZ). The five conserved residues in the active site corresponding to saporin residues Tyr72, Tyr120, Glu176, Arg179, Trp208 are highlighted in magenta. The other colours correspond to the other RIPs as follows: ricin in blue, thricosantin in red, PAP in green, bouganin in yellow, gelonin in orange. Crystal structures have been superimposed by the SSM algorithm and this figure was produced by PyMol. This figure was originally published in: de Virgilio *et al*., Toxins (Basel), 2010 [4] (© 2010 by the authors; licensee MDPI, Basel, Switzerland).

The depurinating N-glycosidase mechanism of RTA is well understood. The target adenine of the 28S rRNA substrate is inserted inside the catalytic cleft of RTA. The aromatic ring of adenine becomes sandwiched between Tyr80 and Tyr123 with Arg180, partially or fully protonating N3 of the ribose ring, thus inducing a positive charge stabilization of the intermediate ribose by Glu177. Then, a molecule of water is activated, probably by Glu177 [8], inducing the nucleophilic attack on the N9-C1 glycosidic bond linking the adenine to the ribose ring, finally releasing free A4324. For most of the 3D-structure, the Tyr80 aromatic ring is almost parallel to that of Tyr123/120, as required to form a stack with the adenine of the substrate, while for ricin and gelonin Tyr80 is oriented in such a way that the hydroxyl group forms a hydrogen bond with the Gly121 carbonyl group (note blue and orange residues, respectively, in Fig. **2**). For all the considered 3D structures, crystallographic analysis revealed a higher thermal parameter for the first catalytic residue with respect to other conserved residues, indicating that it could work as a moving door for the adenine entering in the catalytic site [9]. Due to their catalytic activity, RIPs were officially denominated as "rRNA N-glycosidase" (EC 3.2.2.22) [10] as these removed a specific adenine residue located in the universally conserved alpha sarcin/ricin loop, present in 23/26/28S rRNA [11].

The debate is still open as to whether or not ricin possesses enzymatic activities other than N-glycosidase. Ricin A, however, has been reported to have phospholipase activity, to release a single adenine from 80S ribosome [10-14], and to remove adenine from the poly(A) tail of poly (ADP-ribosylated poly(ADP-ribose) polymerase [14]. In plants these enzymatic activities may play a central role in the defence response mechanisms. Therefore it was tempting to speculate that the plant may differentially switch these activities, exerted by the same protein, upon specific environmental/biological requirements.

Ricin is synthesized in the seeds of *Ricinus communis* at the developmental stage along with seed storage proteins, and accumulates in the storage vacuoles of the mature seeds [15, 16]. Storage proteins, including ricin, are hydrolysed during the first few days after germination providing a source of amino acids for the neosynthesis of proteins required at this stage of seedlings development, and disappear as the developing plant is increasingly capable of synthesizing the indispensable amino acids.

The ricin family includes two major members: ricin (collectively known as *Ricinus communis* agglutinin II) and RCA (collectively known as *Ricinus communis* agglutinin I). These are both galactose-specific lectins, each possessing at least two sugar binding sites. RCA is a tetramer of two ricin-like heterodimers, each composed of an A and B chain. RCA is a strong haemagglutinin but a weak cytotoxin, whereas ricin is a weak haemagglutinin but a potent cytotoxin [17, 18]. These properties are a direct consequence of the structures of both ricin and RCA. Ricin is endowed with a single B chain to bind and enter target cells, while RCA has two B chains allowing the simultaneous binding of two target cells causing agglutination of blood cells.

The ricin gene family has been reported to contain 6-8 members [19, 20]. The genome sequence of *Ricinus communis*, however, revealed 28 putative genes in the ricin family, including potential pseudogenes or gene fragments, but only one copy of the gene cluster responsible for castor oil biosynthesis, indicating that there is a selective pressure for the seeds to produce a highly toxic protein [21]. Therefore, ricin is a storage protein with the extra advantage that its potent toxicity deters herbivores from eating the seeds. Moreover, its higher expression in senescent, wounded, or stressed tissues suggests that it could play additional physiological roles and may also be involved in the mechanisms of programmed cell death during plant development.

Ricin Trafficking in Mammalian Cells

The synthesis of ricin by *Ricinus communis* cells is a complicated process chiefly because the plant must avoid autointoxication by the endogenously synthesized ricin. To reach this goal, plant cells synthesize ricin as an endoplasmic reticulum

(ER)-target inactive precursor (proricin) polypeptide, which then within the endomembrane system of the plant cells traffics through the Golgi apparatus to reach storage vacuoles. This complex strategy certainly underlines the crucial physiological functions of ricin [22-24].

On the other hand, to exert its toxic functions in mammalian cells, ricin has ultimately to be delivered into the cytosol, where free RTA will reach its ribosomal target. Obviously, this step is highly dependent on the presence of RTB allowing the efficient binding and internalization of ricin. Indeed, it is the lectin B domain of ricin heterodimer that permits the binding to exposed beta-1,4-linked galactose residues, which are typically abundant on mammalian cells [25]. Then, ricin efficiently enters target cells through receptor-mediated endocytosis.

Ricin is initially internalized into the early endosomes (EEs), where its fate will be sealed. The pool of endocytosed ricin will follow distinct scenarios: a fraction of heterodimers will leave EEs, be directed into recycling endosomes, to then return to the cell surface in a vain entry-exit cycle; another part of dimers will be proteolitically degraded through progression first *via* late endosomes and then into the lysosomes; finally, only a small portion of dimers originally presents in EEs progresses to the *trans*-Golgi network (TGN), to eventually exert the cytotoxic effects of ricin [26, 27]. Then, these ricin dimers undergo retrograde transport from the Golgi complex to the ER [28]. Once the holotoxins have reached the ER lumen, the two subunits of ricin are separated. This step is required in order to permit the released RTA to retrotranslocate in a potentially catalytically-active conformation. The reductive separation of RTA from RTB is catalysed by protein disulfide isomerase [29, 30] even in the oxidizing environment of the ER lumen [31]. When RTA dissociates from RTB, free RTA undergoes a change of conformation mimicking ER-associated degradation (ERAD) substrates in order to become capable of entering the cytosol. Free RTA assumes a partially unfolded conformation exposing a hydrophobic stretch of amino acids close to its C-terminus [32], which in the ricin holotoxin corresponds to a hydrophobic region covered by RTB. When exposed, this latter region allows free RTA interaction with ER membranes, which in its turn promotes RTA unfolding [33]. Prior to dislocation, ERAD substrates must be kept in a soluble form, which explains the requirement of co-chaperones BiP [34, 35] and the ERAD-specific mannosidase

EDEM (ER degradation-enhancing mannosidase-like protein) to modulate ricin toxicity [36]. After being released in the cytosol, RTA most likely escapes degradation because of its atypical low lysine content preventing ubiquitination [37]. Indeed, proteasomal inhibition sensitizes cells to ricin [38]. Since RTA is stabilized in the cytosol, the time period available to inactivate cytosolic ribosomes is prolonged leading in the end to apoptotic cell death.

As illustrated above, the cell trafficking of ricin is very complex. Interestingly, ricin moves in different and sometimes opposite manners in the plant compared to mammalian cells. During its synthesis in *Ricinus communis* cells, ricin traffics in an anterograde manner from the ER, *via* the Golgi complex, to the vacuole [22]. In contrast, during the intoxication of a mammalian cell, ricin undergoes retrograde transport from the cell surface to the ER, where this protein dissociates, and RTA translocates into the cytosol. Thus, ricin holotoxin crosses the ER membrane twice: in the plant cell, during its synthesis from the cytosol to the ER lumen; then in targeted eukaryotic cells, from the extracellular compartment into the ER. In addition, RTA will move once more out of the ER into the cytosol.

Induction of Apoptosis by Ricin

Once internalized, ricin promotes several cytotoxic effects, and in the end cell death. The depurination of RNA by ricin induces the "ribotoxic stress response" characterized by the activation of several protein kinases [39], which trigger the release of Tumour Necrosis Factor (TNF) and other proinflammatory cytokines as observed in cells and animals poisoned with ricin or other toxic type II RIPs [40].

Ricin causes apoptosis and subsequently, or at higher doses, severe necrosis both in cultured cells and in organs of poisoned animals. There is now abundant evidence that ricin induces apoptosis in epithelial, endothelial, lymphoid and myeloid cells *in vitro*, and in multiple organs in animals. However, the liver is the most often affected organ and the site of severe inflammation [41]. Indeed, necrosis is accompanied by the release of CLF chemokines (also called DAMPs, Damage-Associated Molecular Patterns) such as HMGB1, which are potent pro-inflammatory factors [42, 43]. Strong inflammation seems to play an important role in ricin toxicity as illustrated by the fact that the inhibition of inflammation attenuates the lesions and reduces mortality of ricin-poisoned animals [44].

Apoptosis is an energy-dependent process of programmed cell death resulting in physiologic cell death *i.e.* cellular destruction without the induction of an inflammatory response [45]. Apoptosis is the physiological process permitting safe removal of unnecessary cells, which for example, play a critical role during the development of the organism. Alterations of apoptosis are believed to be involved in various pathologies such as tumorigenesis or neurodegenerative diseases. Apoptosis can be initiated through three main different mechanisms: (1), extracellular signals such as TNF mediated by death receptors mediated (extrinsic pathway); (2), intracellular mitochondrial-initiated events (intrinsic pathway); and (3), cell-cell interactions that result in delivery of perforins and granzymes into target cells (perforin/granzyme pathways) [45].

The role of RIPs in the induction of apoptosis has been investigated since 1987 when apoptotic changes in lymphoid tissues and intestine were reported in ricin intoxicated rats [46], and to date several mechanisms have been uncovered. RIP-treated cells undergo apoptosis *via* diverse mechanisms including the loss of mitochondrial membrane, activation of caspases, and regulation of apoptotic proteins [47]. However, the inhibition of protein synthesis also triggers apoptosis through mechanism(s) that remains still unclear. Many studies suggested that both protein synthesis inhibition and apoptosis induction mediated by ricin occur through independent mechanisms. In some cells, ricin B-chain also appears to induce apoptosis. Ricin can activate components of both the extrinsic or death receptor-mediated and intrinsic or mitochondrial-mediated pathways of apoptosis through mechanisms awaiting to be fully characterized [4].

Recently, in human cancer cells, type II RIPs including ricin have been shown to induce apoptosis through the unfolded protein response (UPR) [48]. UPR is a cellular response to the stress caused by excess of misfolded or unfolded proteins, which, if unsuccessful, drives the cell towards apoptosis. Moreover, in mammalian cells, ricin A-chain inhibits UPR by enhancing in that manner its own cytotoxicity [49]. Apoptosis occurs at a low concentration which is capable of activating UPR genes but that is not sufficient for the depuration activity of RTA. Structural motifs of ricin and in particular one LDV sequence within RTA have been demonstrated to induce apoptosis, while other such as the active site are responsible of the blockade of protein synthesis [50].

CHIMERIC RICIN-BASED TOXINS

Ricin-Based Recombinant Chimeric Toxins for Cancer Therapy

Recombinant DNA technology has led to the cloning of several RIPs, and it soon became clear that the potent cytotoxic effects mediated by RIPs could be more specific and powerful if these proteins were linked to targeting moieties.

Chimeric toxins are proteins derived from the fusion between a plant or bacterial toxin in charge of inducing cell death, and a targeting domain which is a natural ligand of a tumour antigen, generally a membrane receptor, directing the chimeric toxin towards its cellular target. Most of chimeric toxins were constructed using RIP domains expressed in bacterial hosts associated to native molecular addresses, or *vice versa* by conjugating recombinantly expressed targeting domains to the native protein toxin. Compared to the classical inhibitors that diffuse into the whole organism and potentially affect most if not all types of cells, chimeric toxins have the major advantage of providing a targeted treatment of the disease. This unique property is shared with another class of compounds represented by immunotoxins (ITs) in which the toxin is fused to an antibody.

Both chimeric toxins and ITs can be built by either chemical conjugation or *via* gene fusion. In a general way, the genetic fusion between targeting and toxic protein domains has a major advantage compared to chemically-conjugated chimeric toxin. The chemical conjugation of the molecular address and the toxin leads to the production of heterogeneous mixture of hybrid molecules that may preclude their successful development into therapeutic molecules.

Despite progress in proteomics and bioinformatics approaches to characterize cell-surface antigens and receptors on tumour cells, it is still difficult to identify novel tumour markers. The antigen targeted on the tumoural cell surface is a major critical factor that determine the successful delivery of ligand based toxins (LTBs) and ITs insuring specificity and thus cytotoxicity at the right place. Ideally, the molecular target should be exclusively expressed by the tumour cell to ensure the specific binding of the chimeric toxin or IT. In addition, the perfect marker must be capable of mediating the internalization of the chimeric toxins and ITs. This latter condition is absolutely required in order to permit the

intracellular access to the toxin moiety. However, genuine tumour-specific antigens are rare. Hence, a convenient alternative is to target antigens or receptors that are significantly over-expressed on the surface tumour cells limiting in that way the peripheral toxicity that will be induced by the toxins.

Ricin has been employed to generate chimeric toxins and ITs. However, one of the difficulties that had to be circumvented was the undesirable affinity of RTA for hepatic cells [51-53]. Since the glycosylated residues were responsible for the unnecessary binding to hepatic cells, the use of deglycosylated RTA was investigated, and in fact deglycosylated A chain (dgA) was chosen for this reason [51, 52, 54]. Deglycosylated native ricin A was the first RIP to be chemically conjugated to RFB4, an anti-CD22 monoclonal antibody. The resulting IT was tested in two phase I trials. One was performed in 15 patients with refractory B-cell lymphoma where partial responses were achieved in 38% of the evaluable patients. However, three patients developed antibodies against A chain of ricin and 25% of the patients had antibodies against both A chains and the mouse immunoglobulin [55]. In a separate clinical phase I trial this particular IT was administered to 26 patients exhibiting B-cell lymphoma relapse after conventional therapies [54]. The outcome was low with five partial and one complete responses for up to 30-78 days. Moreover, due to the vascular leak syndrome (VLS) the efficacy of the IT was limited, and nine patients made antibodies against either dgA or mouse immunoglobulin [56].

The IT 454A12-rRA consists of recombinant RTA associated with a monoclonal antibody against the transferrin (Tfn) receptor [57]. 454A12-rRA anti-tumoural efficacy was explored in eight patients with leptomeningeal spread of systemic neoplasia. The IT was administered intraventricularly to patients, and within 5-7 days four patients displayed a decrease of more than 50% of tumour cell numbers in the lumbar ventricular cerebrospinal fluid (CSF). Unfortunately, after treatment tumour progression was still evidenced in seven out of eight patients [57].

As stated above, the choice of the targeting domain is crucial for the activity of both chimeric toxins and ITs. However, the internalization process that eventually delivers the toxin into the cytosol is equally important. If the chimeric toxins or ITs are not internalized (or even very slowly internalized) the cytotoxic activity of

the toxins will be compromised. This idea is fully supported by data showing that decreasing the cytotoxic activity of a chimeric toxin but increasing its internalization actually resulted in an overall improvement of the efficacy of the chimeric toxin. When the cytotoxic effect of RTA was reduced by adding the N-terminus of vesicular stomatitis virus protein G, this chimera (cRTA), which was expressed in the cytoplasm of *Eschericia coli*, was less effective than unmodified recombinant RTA or even native RTA. Surprisingly, once cRTA was conjugated to human transferrin, the chimeric toxin Tfn-cRTA was shown to be actually more potent than Tfn-rRTA or Tfn-nRTA. This rather unexpected enhanced efficiency of Tfn-cRTA was due to the addition of N-terminal portion of vesicular stomatitis virus protein G, which provided a faster interaction with phospholipid vesicles thereby speeding up the internalization of Tfn-cRTA compared to the other Tfn-rRTA and Tfn-nRTA chimeric toxins [58].

The combination of distinct chimeric toxins or ITs may represent an interesting strategy in order to improve the efficiency of the treatment of cancers. Combotox is a mixture of two ITs prepared by coupling dgA to monoclonal antibodies directed against CD22 (RFB4-dgA) or CD19 (HD37-dgA). Combotox was tested for the treatment of acute lymphoblastic leukaemia (ALL). Pre-clinical data demonstrated that Combotox was effective in killing both pre-B-ALL cell lines and cells from patients with pre-B ALL. In a clinical study on pediatric patients, 3 out of 17 patients experienced complete remission. During administration of Combotox to adults with refractory or relapsed B-lineage-ALL, VLS was the dose-limiting toxicity of the mix of ITs. Still, two out of 17 patients developed reversible grade 3 elevations in liver function test; one achieved partial remission and proceeded to allogenic stem cell transplantation, and all patients with peripheral blasts experienced decreased blast counts indicating that Combotox can be safely administered to adults with refractory leukaemia [59]. Furthermore, in a murine xenograft model of advanced ALL the combination of Combotox with low or high doses of the cytotoxic agent cytarabine (Ara-C) resulted in longer median survival. These findings motivated an ongoing phase I clinical trial exploring this combination of ITs in adults with relapsed or refractory B-lineage ALL (ClinicalTrials.gov identifier NCT01408160) [60].

In addition, another combination of ITs has shown promising activity. Antibody against either CD3 or CD7 was fused to RTA. This IT combination, which was

specifically designed for the treatment of Acute Graft *Versus* Host Disease, acts synergistically *in vitro* eliminating T-cells. Encouraging results have been obtained when this combination was applied as third line therapy. Moreover, extensive biological and clinical responses could be noted in the absence of severe acute toxicities (ClinicalTrials.gov identifier NCT00640497). For a more comprehensive view, the most recent clinical trials using RTA-based toxins are summarized in Table **1** [for recent reviews on ITs generated using RIPs see 100, 101].

Table 1: Clinical evaluations of ricin based immunotoxins

Immunotoxin	Targeted Antigen	Toxin	Disease	Clinical Trial Phase Completed	References
FB4-Fab'-dgA	CD22	dgA	B-cell non-Hodgkin's lymphoma	I	[61]
IGg-RFb4-SMPT-Dga)	CD22	dgA	B-cell non-Hodgkin's lymphoma	I	[62]
HD37-dgA	CD19	dgA	Non-Hodgkin's lymphoma	I	[63, 64]
RFB4-dgA+HD37-dgA	CD22	dgA	Non-Hodgkin's lymphoma; Acute lymphoblastic leukemia	I, on-going	[65, 66]
RFT5-dgA	CD25	dgA	Graft-*versus*-host disease; Hodgkin's lymphoma	I, I/II on-going	[67-70]
Ki-4.dgA	CD30	dgA	Hodgkin's lymphoma; Non-Hodgkin's lymphoma	I	[71]
Anti-B4-bR	CD19	Blocked ricin	B-cell non-Hodgkin's lymphoma	II, III	[72-77]
Anti-CD7-dgA	CD7	dgA	T-cell non-Hodgkin's lymphoma	I	[78]
H65-RTA	CD5	RTA	Cutaneous T cell lymphomas; Graft-*versus*-host disease	I, I/II	[79-81]

Table 1: contd…

RFT5-dgA (IMTOX-25)	CD25	dgA	Melanoma	I	[82]
Anti-CEA-bR	CEA	Blocked ricin	Colorectal cancer	I/II	[83]
N901-bR	CD56	Blocked ricin	Small cell lung cancer	I/II	[84-87]
XomaZyme-Mel (XMMME-001-RTA)	Melanoma antigen	RTA	Melanoma	I	[88-93]
XomaZyme-791 (79IT/36-RTA)	TAA	RTA	Colorectal cancer	I	[94-96]
454A12-rRA	TfR	RTA	Leptomeningeal neoplasia	I	[97]
260F9-rRTA	Breast cancer antigen	RTA	Breast cancer	I	[98, 99]

Molecular Design of Chimeric Ricin-Based Toxins

Designing and constructing a chimeric toxin or an IT using ricin as toxin moiety is not particularly difficult. However, there a few pitfalls that must be avoided for future clinical use. The use of plant RIPs as such or included into chimeric proteins or ITs, could be limited during preclinical and clinical testing by the presence of heterogeneous preparations with several immunologically distinct toxins isoforms, which may also vary significantly in their catalytic activities. Therefore, recombinant DNA biotechnology for the expression of single isoforms is strongly advisable [102]. Recombinant proteins can be expressed in bacterial systems such as *Escherichia coli* and recovered as soluble material or accumulated in inclusion bodies which need to be isolated, purified and solubilized [55, 102]. Using such systems of proteins expression is relatively easy and generally ensures a high yield. However, it is worth mentioning that endotoxin contaminations represent a common problem of bacterial expression systems.

Yet, the potential toxicity of RIPs towards the host cells may prevent or decrease recombinant protein production. Since RTA is not toxic towards prokaryotic ribosomes, it has been successfully produced in *Escherichia coli* [103]. In contrast, type I RIPs, such as saporin or dianthin 32, were found to be active on *E.*

coli ribosomes and 500-times more active on yeast ribosomes [104]. For these reasons, for the expression of type I RIPs, tight regulated host/vector inducible systems are advisable [102-107]. In the last few years, the methylotrophic yeast *Pichia pastoris* has been shown to be a suitable host for high-level expression of various heterologous proteins, especially endowed with clinical and potential high therapeutic value, expressed either intracellularly or in secreted forms [108].

While designing fusion toxins using RIPs, a particular attention should be paid to the mechanism of release of the catalytic domain into the cytosol. An attempt to construct a protein fusion composed of RTA associated to interleukin-2 (IL-2) was unsuccessful because of the use of a cleavable linker. This particular linker contained a proteolytic cleavage site for diphtheria toxin and for clotting factor Xa. Thus, the recombinant chimeric toxin could be extracellularly cleaved, failing to selectively target the cells because the ligand and the toxin moieties were no longer connected [109].

This pitfall may be avoided through the insertion of a flexible peptide linker between the targeting domain and the toxic moiety in order to allow a proper and separated folding of both domains of the chimeric toxin. In this context, the 218 linker is particularly suited. The 218 linker, GSTSGSGKPGSGEGSTKG, employed the VL and VH domains in the antibody moiety of an antimelanoma IT. This linker enhanced the stability of the chimeric proteins towards intracellular proteases, and even further reduced the aggregation of scFv (antibody single chain variable fragment) when expressed in bacterial systems [110, 111]. Similarly, a G_4S peptide linker was designed to connect a bispecific targeted toxin capable of simultaneously and efficiently recognizing human CD22 and CD19 receptors in a mouse model of B-cell metastases [112].

Immunogenicity and Side Effects of Ricin-Based Chimeric Toxins

When administered to the human body, proteins of any origin, including human proteins, may elicit an immune response. This property is called immunogenicity, and may represent an important drawback of therapies using proteins such as chimeric toxins and ITs. Indeed, immunogenicity can exert a number of negative effects on the therapeutic outcome of chimeric toxins or ITs since it may, for

example, reduce the efficacy of the protein by lowering its half-life in the circulatory system because of rapid clearance by immune cells. The protein-induced immune response may also preclude repeated dosing because re-administration would cause a strong immune reaction. The situation may be even worse if the antibodies developed against the delivered chimeric toxins or ITs cross-react with an autologous protein. This problem is so serious that immunogenicity observed with some therapeutic molecules is considered as an obstacle to further development into clinical use. However, a number of guidelines have been established in the attempt to prevent immunogenicity of future drugs. In the case of chimeric toxins or ITs, by using cell culture systems antibodies, neutralizing the cytotoxic effects of the toxins can be detected by determining whether serum from patients block their cytotoxicity.

Furthermore, a number of techniques have been established to address the issue of immunogenicity of therapeutic proteins. PEGylation is the covalent attachment of polyethylene glycol (PEG) to lysine molecules on a protein surface. It was created to prolong the molecule's half-life in the circulatory system and to reduce immunogenicity. PEG is a non-toxic, highly soluble component commonly used in many manufacturing procedures, such as in cosmetic production and the food industry. PEGs are suitable for pharmaceutical applications (no toxicity or immunogenicity) and approved for human use as shown by the commercialization of several PEGylated drugs. PEGs range from 5000 up to 40000, and prolongs half-life by protecting PEG-peptide from proteolysis and also by decreasing renal excretion by increasing molecular mass. PEGylation has also been evaluated for ricin. PEGylated ricin exhibited a lower binding to anti-ricin antibodies without alteration of the enzymatic activity of the toxin [113]. In addition, in the case of free RTA PEGylation was suggested to increase the therapeutic potential [114].

The identification of the epitopes responsible of the immunogenicity of the fused toxins provides another strategy to reduce or prevent this problem. T-cell and B-cell epitopes have been identified on RTA and a peptide scan approach combined with the use of sera of patients treated with an RTA-based IT allowed the identification of a continuous motif forming the B-cell epitope recognized by all patients, which was located close to a T-cell epitope within the RTA sequence [115]. Moreover, all antibodies against this B-cell epitope were capable of

recognizing folded RTA, thereby affecting its biological activity by inhibiting RTA cytotoxicity *in vitro*.

ITs are known to be particularly immunogenic. The antibody moiety is responsible of this enhanced immunogenicity due to the large size of monoclonal IgG moieties, their mouse origin, and their long circulation time period in the body. All these flaws may possibly boost the antigenic features of the toxic domain as well. A number of molecular engineering approaches can be envisaged to prevent these inconveniences. Antigenicity of the antibody moiety can be reduced by humanizing the antibody and modifying the amino acid sequence. Another solution is to minimize the antibody to structures containing the variable domains necessary to bind the target cells. To this purpose, it is possible to generate structures keeping only heavy VH and light VL immunoglobulin chains linked together through a flexible peptide linker. Developing single chain antibody adds further flexibility in terms of the design of the engineered ITs: VH-VL or VL-VH orientation, type and length of the linker peptides.

Due to the IT size reduction, improved tumour infiltration may be also expected. In fact, major concerns have been raised because of poor access of IT to solid tumours, which may require prolonged treatment regimens [116]. IT approaches seem to be better suited for fighting hematologic malignancies, especially the different physiological/biological barriers for local toxin concentration that solid tumours present against IT diffusion, and because patients with such malignancies are often immunocompromised. Indeed, a bottleneck in the treatment of solid tumours may be the high interstitial fluid pressure gradient generated by the architecture of the rapidly formed vascular network that limits the diffusion of large macromolecules such as full-length antibodies. This problem can be bypassed by using smaller antibody formats such as scFvs, for which a number of pharmacokinetics and biodistribution studies have been performed [117-119].

Beside immunogenicity, there are a number of side effects that may occur during ITs administration to patients, which could influence the dose and hence the efficacy of treatments. The allergenicity of *Ricinus communis L.* (castor bean *Euphorbiaceae*) has been described not only to affect laboratory workers but also personnel working in oil processing mills, fertilizer retail, upholstery industry and

other industrial fields. This allergenicity is associated with components of the pollen and the seeds with ricin being the major allergen [120]. In addition, formation of IgE has been observed in rats after administration of ricin [121]. Thus, the generation of ITs using single chain antibodies may offer the possibility to reduce this problem because of their smaller size, the absence of an FC portion, and their more rapid clearance kinetics from the blood circulation.

A further complication observed during IT administration has been the non-specific binding of the toxin domain to vascular endothelial cells leading to VLS [122-126]. VSL is characterized by interstitial oedema, hypoalbuminemia, weight gain, and in most severe cases, pulmonary oedema and hypotension. RTA and some type I RIPs contain a consensus amino acid sequence X-Asp-Y, such as found in Interleukin-2, where X could be Leu, Ile, Gly or Val and Y could be Val, Leu or Ser, which seems to induce vascular damage to human endothelial cells *in vitro* by binding to integrin receptors [123-125]. A similar motif is shared by viral disintegrins, which destroy the functions of integrins [126]. In the case of RTA, molecular modelling suggested that this motif was partially exposed on the surface of the molecule [123]. In the perspective of eliminating VLS during the therapeutical use of ricin-based ITs, RTA mutants causing reduced levels of VLS in mice allowed the identification of Asn97 as a critical residue. Interestingly, Asn97 is located in a region flanking the VLS-responsible motif in the 3D structure of RTA [127]. RTA mutant N97A promoting significantly less VLS in mice was conjugated to RFB4, an anti-CD22 antibody. The dose-limiting toxicity of this mutated IT was five-fold lower than the parental IT counterpart [126]. Unfortunately, a phase I study with Combotox, a mixture of anti-CD19 and anti CD22 RTA-based ITs, revealed unexpected toxicities. Previously undetected protein aggregation caused two drug-related deaths following treatment indicating that more precautions must be taken to ensure the stability of pharmaceutical proteins [127].

Enhancement of the Cytotoxicity Induced by RTA

Studies have been carried out to identify molecules or drugs that could be coadministered with RTA-based ITs in order on the one hand to enhance their cytotoxicity against target cells, and on the other hand to decrease the doses of IT to be dispensed with the aim of reducing side effects.

One manner to reach this double goal is to prevent the proteolytic degradation of ITs and chimeric toxins. The lysomotropic amine, ammonium chloride (NH_4Cl), inhibits the activity of proteolytic enzymes by raising the pH inside acidic organelles such as lysosomes and endosomes, where ricin is routed. The co-administration of NH_4Cl with a serine RTA-based ITs revealed that the amine increased the cytotoxicity of ITs on human cells when the pH was above 7. However, NH_4Cl acted on internalized polypeptides for a very short time suggesting that this enhancer affects an early intracellular step after internalization [128, 129]. The use of chloroquine as therapeutical adjuvant may provide a better alternative to ammonium chloride. Chloroquine is a clinical drug used for the therapy of malaria that was shown to enhance up to 2,500-fold the cytotoxicity of RTA fusion ITs [128]. Similarly, the weak basic amine 1-admantanamine hydrochloride (amantadine) acted as a potent enhancer of the cytotoxic activity of a RTA-conjugated anti-CD5 IT targeting peripheral blood T cells [130]. Since, amantadine is a licensed drug for prophylaxis of influenza, this compound may be more advantageous adjuvant than NH_4Cl.

Carboxylic ionophores are a class of components that exchange monovalent cations across cellular membranes. Exchange of K^+ for H^+ increases the pH of acidic vesicles such as lysosomes [131]. Therefore, the presence of an ionophore is more likely to provide a more suitable compartment for ITs. In line with this idea, the ionophores monensin, grisorixin, lasalocid and nigericin were capable of enhancing the effects of RIPs-based ITs (in particular, those made with RTA). However, other ionofores such as nonactin, valinomycin, and calcimycin had no potentiatory effects on IT-induced cytotoxicity suggesting that the whole mechanism of action of ionophores is not completely understood. In fact, monensin is an immunopotentiator, which at nanomolar concentrations accelerates the kinetics of target cells intoxication induced by RTA-based ITs [132, 133]. Interestingly, at these low concentrations, monensin does not affect lysosomal pH suggesting that distinct mechanisms other than IT stabilization through pH increase may be operating [134]. This idea is further reinforced by the fact that monensin cross-linked to human serum albumin (Mo-HSA) *via* a disulfide bridge was 2-13-fold less toxic than free monensin *in vitro*, and was effective in potentiating both monoclonal antibody-RTA IT and transferrin-conjugated RTA reactive against the transferrin receptor [134].

The possibility to use calcium channel blockers and their derivatives as adjuvant to ITs has been explored. The experimental data were rather positive. Up to a 100-fold increase in RTA-based ITs cytotoxicity has been observed. However, their adjuvant effects are not apparently correlated with the blockage of the functions of calcium channels, but are most probably related to the prevention of lysosomal degradation of the ITs. For example, the calcium-channel blocker, verapamil enhanced up to 40-fold the cytotoxicity of an anti-EGFR (epidermal growth factor receptor)-RTA IT [135]. No influence of verapamil was observed on an anti-CD22-RTA IT suggesting that the effect of the ion channel blocker is in relation with the target antigen or with the isotype of the target cell [135]. Furthermore, verapamil was found to enhance the accumulation in the lysosomes, which may be the consequence of an alteration of cellular membrane permeability induced by the calcium channel blocker that independently affects the translocation of ITs and lysosomal function [135, 136]. Yet, one cannot exclude that difference in the speed of internalization of the two anti-EGFR-RTA and anti-CD22-RTA ITs might actually account for the opposite effects of verapamil [137]. This latter idea is strengthened by the fact that due to the absence of RTB insuring the internalization of ricin-conjugated ITs, the cytotoxicity of RTA-based ITs is irregular compared to their ricin-conjugated counterparts [138].

The Role of The B Chain in Specificity and Cytotoxicity of ITs Based on Ricin

As just stated above, the response of cells to RTA-based ITs is generally more variable than the cellular response promoted by their corresponding counterparts, which include the whole ricin molecule [138]. Most likely the lack of the B-chain, which contributes to ricin internalization and cell surface binding, is responsible for these discrepancies. These experimental data suggest that the more constant internalization of ricin-based ITs is preferable, and certainly represent a plus. For this reason, cytotoxic chimeric proteins were assembled by linking intact ricin to antibodies in a manner that would block the exposure of ricin B-chain galactose-binding site, thereby reducing the non-specific binding of the conjugate to undesirable cells such as endothelial cells [139, 140]. The "blocked" ricin provides a double advantage: high potency, which is often lacking in antibody-conjugated RTA ITs, and high specificity, which is lacking in conjugates using

intact "unblocked" ricin. In line with this idea, the addition of free B-chain *in vitro* [141, 142], or of B-chain coupled to either anti-target antigen antibody or to anti-mouse antibody recognizing the cell surface enhanced the activity of ITs [143, 144].

CONCLUSIONS

Nowadays, producing a chimeric toxin or an IT is relatively simple. However, designing the new molecule requires hard thinking as the success or failure of the novel compound will heavily depend on the critical choices of the targeting domain and toxic moiety.

On the one hand, the targeting domain should fulfil not only the function of directing the chimeric toxin or IT towards the right target but also of ensuring a fast internalization into the cells. Thus, the chosen targeting domain is most of the time a ligand of receptor specifically expressed by the targeted cells.

On the other hand, the toxin must efficiently kill the cell. Among a wide range of plant and bacterial toxins, ricin is a potent poisonous component extracted from plant seeds inducing apoptosis and even necrosis, thereby meeting the conditions for its use as a toxin moiety. The reasons of the presence of ricin in the castor bean seeds most likely rely on the need of preventing the herbivores from eating the seeds ensuring the long-term survival of the castor bean plant. Due to its use as terror weapon including assassination of opponents by secret service agencies, ricin has gained a very bad reputation. However, if employed as a toxic moiety directed against devastating pathologies such as cancer, ricin would have a more beneficial impact for human health.

As a toxin moiety, ricin provides major advantages such as the fact that this toxin triggers multiple signalling pathways leading to cell death. This suggest that ricin-based chimeric toxins or ITs may potentially prevent the apparition of cellular resistance to the compound. Unfortunately, a few pitfalls have to be addressed which are non-specific binding to endothelial cells, immunogenicity and allergenicity. These pitfalls have emerged during laboratory or clinical evaluation of ricin-based ITs or chimeric toxins, which probably will not proceed further than phase I trials. It

is worth mentioning that these trials were prevalently performed on compromised terminally ill patients failing all other available therapies.

However, these experimentations have paved the way for future chimeric toxins or ITs that it is hoped will not have the flaws of their predecessors, and will constitute more efficient drugs against cancers. Indeed, all the evidenced pitfalls are not insurmountable, and can be easily circumvented by modern techniques of molecular biology. In a more general way, chimeric toxins and ITs have great potential to combat cancer, in particular hematologic cancers. Thus, ricin may have a great and peaceful use as a toxin moiety. Presently, scientific knowledge is not the limiting factor for the generation of drugs such as chimeric toxins and ITs. Still, other parameters such as economical or commercial strategy of pharmaceutical companies may further delay their production.

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CONFLICT OF INTEREST

The author discloses that they have no commercial affiliations, consultancies, stock or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript.

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