Chapter 16

RICIN

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SUMMARY

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INTRODUCTION

Ricin is a potent toxin derived from the ornamental and widely cultivated castor plant, *Ricinus communis L* (*Euphorbiaceae*). Ricin is mostly concentrated in the seed of the plant—popularly known as the castor bean—that, despite its name, is not a true bean. The purified ricin toxin is a white powder that is water soluble; it inhibits protein synthesis leading to cell death. Ricin is stable under normal conditions, but can be inactivated by heat exceeding 80°C. After oil extraction and inactivation of ricin, the defatted mash and seed husks are used as animal feed supplement and fertilizer, respectively.[1]

In 1978, the lethality of ricin was overtly established after the high-profile assassination of Bulgarian dissident Georgi Markov.[2] Numerous incidents involving ricin or castor seeds for nefarious purposes have been reported since 1978.[3-9] More recently, various extremists and terrorist groups have also experimented with ricin; some involved mailing ricin-tainted letters to the offices of US politicians. These events have heightened concerns regarding ricin's potential for urban bioterrorism, and thus prompted its constant inclusion in weapons of mass destruction investigations.[10] The wide availability of the castor plants; the relative ease of toxin production; and the toxin's lethality, stability, and media coverage fortify the appeal of ricin for those in quest of retribution and public attention.

In the United States, the possession or transfer of ricin and genes encoding its functional form is regulated by the Centers for Disease Control and Prevention (CDC) Select Agents and Toxins Program. CDC has classified ricin as a category B threat agent. Category B agents, which are the second highest priority agents, are moderately easy to disseminate, result in moderate morbidity and low mortality rates, and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance.[11,12] Investigators must register with the CDC before using nonexempt quantities of ricin in their research. No federal regulations restrict the possession of castor plants; however, some states or cities (eg, Hayward, CA) prohibit possession of castor plants or seeds.

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, or Biological and Toxin Weapons Convention, and the 1997 Chemical Weapons Convention.[13,14]

HISTORY, BIOLOGICAL WARFARE, AND TERRORISM

History

The castor plant, also known as the Palm of Christ, was initially indigenous to the southeastern Mediterranean region, eastern Africa, and India, but is now widespread throughout temperate and subtropical regions.[15–17] *Ricinus* is a Latin word for "tick" to describe the castor seed's appearance that resembles a tick (*Ixodes ricinus*), and *communis* meaning "common" to describe its worldwide distribution. For centuries, the castor plant has been cultivated for numerous economically important products, primarily castor oil.[17] In ancient Egypt, Europe, India, and China, castor oil was used for lighting and body ointments, and as purgative or cathartic, and other ethnomedical uses. Castor oil was also reportedly used as an instrument of coercion by the Italian Squadristi, the Fascist armed squads of Benito Mussolini.[18] Political dissidents and regime opponents were forced to ingest large amounts of castor oil, triggering severe diarrhea and dehydration that often led to death.[19] Presently, castor oil has abundant commercial applications including medicinal and industrial purposes.[20–24] Castor seeds are being produced in more than 30 countries in the world because of their economic benefits and myriad uses. In 2013, world castor oil seed production totaled 1.86 metric tons, and the leading producers include India, China (mainland), and Mozambique.[25]

In 1888, Peter Hermann Stillmark, a student at the Dorpat University in Estonia (Stillmark 1888, as cited in Franz and Jaax[5]), discovered ricin. During Stillmark's extensive research, he observed that ricin caused agglutination of erythrocytes and precipitation of serum proteins.[17] In 1891, Paul Ehrlich studied ricin and abrin in pioneering research that is now recognized as the foundation of immunology.[17] Ehrlich found that animals vaccinated with small oral doses of castor beans were protected against a lethal dose of the toxin. Additional experiments using abrin and ricin showed that the immunity was specific, was associated with serum proteins, and could be transferred to the offspring through milk. Further research on ricin showed that the toxin described by Stillmark was actually two proteins, one with an agglutinin with a molecular weight of 120 kDa (*R communis agglutinin I*) possessing little toxicity and the other, *R communis agglutinin II*, a smaller molecular weight protein (60 kDa) with little agglutinating capacity

but extremely toxic. Nearly a century after Stillmark's original discovery, Olsnes and Pihl[26] demonstrated that the 60 kDa toxic protein (ricin) inhibited protein synthesis and that the 60S ribosomal subunit is the toxin's molecular target.[27]

Although ricin is considered a possible biological threat (see below), its potential medical applications have been also explored. During the past decade, ricin has been used extensively in the design of therapeutic immunotoxins, often called "magic bullets." Specifically, ricin, ricin A chain (RTA), or a related toxin is chemically or genetically linked to a binding ligand such as an antibody or used in other conjugates to specifically target and destroy cancer cells, and also as alternative therapies for AIDS and other illnesses.[28–30] Ricin-based immunotoxins conjugated to either the anti-CD22 antibody RFB4[31,32] or its Fab fragment[33] have been reported to provide enhanced therapeutic efficacy and improved antitumor activity.[34–36] However, the US Food and Drug Administration has placed a hold on the clinical testing of RTA-based immunotoxins because they caused vascular leak syndrome (VLS) in humans, a life-threatening condition in which fluids leak from blood vessels leading to hypoalbumina, weight gain, and pulmonary edema.[37] Although progress has been made in understanding the mechanisms of immunotoxin-mediated VLS, significant effort is still required to understand VLS and generate RTA-derived immunotoxins that do not cause VLS but also maintain RTA's potent antitumor activity.[38]

Ricin as a Biological Weapon

During World War I, the United States, which was aware of the German biological warfare program, examined ricin for retaliatory intentions.[39] Two methods of ricin dissemination were described in a 1918 technical report: (1) adhering ricin to shrapnel bullets for containment in an artillery shell, and (2) production of a ricin dust cloud (Hunt et al, 1918, as cited in Smart[39]). The thermal instability of ricin constrained its initial use in exploding shells, and ethical and treaty issues limited its use as a poison or blinding agents. World War I ended before the toxin could be weaponized and tested. During World War II, ricin was evidently never used in battle despite its mass production and being armed into ricin-containing bombs (also known as W bombs), because its toxicity was surpassed by the even more potent biological agents of the time.[5] Interest in ricin diminished with the production and weaponization of other chemical agents, for example, sarin. During the Cold War, the Soviet Union studied ricin as a possible biological weapons agent. A former top Russian official who defected to the United States in 1991 asserted that Russia developed ricin as a weapon, and that the toxin used against the Bulgarian dissidents Georgi Markov and Vladimir Kostov was formulated in Russian laboratories.[40] Iraq reportedly manufactured and tested ricin in animals and used it as payload in artillery shells.[41–44] Syria and Iran were believed to have produced unknown quantities of the toxin.[45] Ricin was also found in Afghanistan in 2001 after the collapse of the Taliban government.[46,47]

Although ricin's potential use as a military weapon was investigated, its utility over conventional weaponry remains ambiguous. Despite its toxicity, ricin is less potent than other agents such as botulinum neurotoxin or anthrax. It has been estimated that eight metric tons of ricin would have to be aerosolized over a 100 km^2 area to achieve about 50% casualty, whereas only kilogram quantities of anthrax spores would cause the same effect.[48] Furthermore, wide-scale dispersal of ricin is logistically impractical. Thus, while ricin is relatively easy to produce, it is not as likely to cause as many casualties as other agents.[49]

Ricin as a Terrorist Weapon and Use in Biocrimes

The well-publicized "umbrella murder" of the Bulgarian writer and journalist Georgi Markov in 1978[2] represents the first documented case of a modern assassination using a biological agent,[50] although this remained unproven. Markov defected to the West in 1969 and was a vocal critic of the Bulgarian communist regime. The Bulgarian secret police had previously attempted to kill Markov twice, but failed. However, on September 7, 1978, Markov was assaulted with an umbrella tip while waiting at a bus stop in London. He subsequently developed severe gastroenteritis and a high fever, and died on September 11, 1978. The autopsy revealed a small platinum pellet with an X-shaped cavity. Further examination of the pellet revealed ricin.[2,51] Prosecutors have failed to identify, arrest, or charge anyone for the crime. On September 11, 2013, a news report disclosed that Bulgaria was abandoning its investigations of the notorious case 35 years after the cessation of the absolute statute of limitations.[52]

Days before Markov's assassination, an attempt was made to kill another Bulgarian defector, Vladimir Kostov.[2] However, the pellet lodged in the fatty tissue in Kostov's back prevented the toxin from being released from the sugar-coated pellet; he survived the incident. Several cases involving the possession, experimentation, or planned misuse of ricin by bioterrorists and extremist groups have been investigated or prosecuted by law enforcement agencies worldwide.[5-7] Recent related incidents include the following:

- In April 2013, letters containing ricin were mailed to Republican Senator Roger Wicker (of Mississippi) and the White House.[53,54] A Mississippi martial arts instructor, Everett Dutschke, was charged with sending those ricin-tainted letters.[55]
- In May 2013, five letters, three of which that tested positive for ricin, were mailed from Spokane, Washington, to a local judge, downtown Spokane post office, President Barack Obama, Fairchild Air Force Base, and the Central Intelligence Agency in McLean, Virginia.[56]
- On June 7, 2013, actress Shannon Richardson was arrested for allegedly sending ricin-tainted letters to New York Mayor Michael Bloomberg and President Barack Obama.[57]

These reports further substantiate ricin's image as an attractive lethal poison, and ostensibly, a biological weapon of choice by extremist groups and individuals.

DESCRIPTION OF THE AGENT

Biochemistry

While ricin is a well-known toxin that can be extracted from castor bean mash, most do not realize that it is related in structure and function to the bacterial Shiga toxins and Shiga-like toxin (also known as Verotoxin) of *Shigella dysenteriae* and *Escherichia coli*. Antibiotic-resistant Shiga toxin-producing *E coli* was responsible for 54 deaths in Germany in 2011[58]; the Shiga toxin gene encoding the toxin was carried by this infectious pathogen. Ricin is noninfectious; however, both the structure and enzymatic activities of ricin and Shiga toxins are similar (Figure 16-1). These protein toxins belong to a family of toxins known as ribosome inactivating proteins (RIPs). More than 60 different plant and bacterial species produce RIPs.[59–61] Type I and II RIPs include the plant toxins ricin, abrin, mistletoe lectins, volkensin, modeccin, saporin, trichosanthin, luffin, and the bacterial Shiga toxin and Shiga-like toxin.

Ricin, a type II RIP, consists of two glycoprotein subunits: a catalytic A-chain (RTA) and a lectin B-chain (RTB), which binds cell surface oligosaccharides containing galactose.[59,62–64] The RTA and the RTB, which are of approximately equal molecular mass (~32 kDa), are covalently linked by a single disulfide bond. The protein-coding region of ricin consists of a 24 amino acid N-terminal signal sequence preceding a 267 amino acid RTA. The RTB has 262 amino acids. It consists of two major domains with identical folding topologies,[62] each of which comprises three homologous subdomains (α, β, γ) that probably arose by gene duplication from a primordial carbohydrate recognition domain.[65] RTB binds terminal β 1,4, linked galactose and *N*-acetyl galactosamine (Gal/GalNac)[66] that are on the surface of most mammalian cells. A 12-amino acid linker in the pre-protein joins the two chains. The carboxyl-terminal end of the RTA folds into a domain that interacts with the two domains of the B chain.[62]

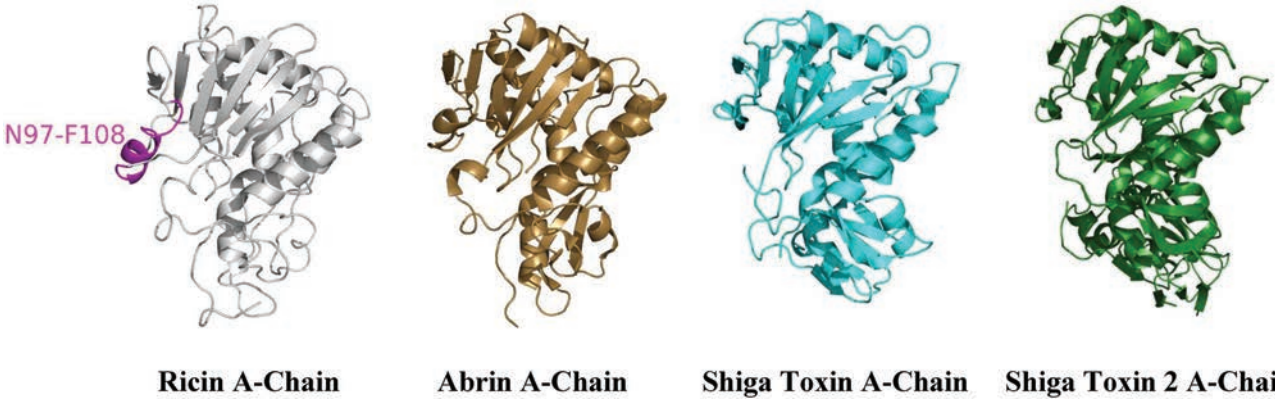

Figure 16-1. Structural and functional similarities among RIPs. The A-chains of plant RIPs such as ricin (PDB 3HIO) and abrin (PDB 1ABR) are structurally and functionally related to the bacterial Shiga toxin A-chains (PDB 1R4Q and 1R4P). The A-chains catalyze the same reaction to inactivate ribosomes and halt protein synthesis.
Data sources: (1) Ho MC, Sturm MB, Almo SC, Schramm VL. Transition state analogues in structures of ricin and saporin ribosome-inactivating proteins. *Proc Natl Acad Sci U S A*. 2009;106:20276–20281. (2) Tahirov TH, Lu TH, Liaw YC, Chen YL, Lin JY. Crystal structure of abrin-a at 2.14 A. *J Mol Biol*. 1995;250:354–367. (3) Fraser ME, Fujinaga M, Cherney MM, et al. Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J Biol Chem*. 2004;279:27511–27517.

A disulfide bond is formed between amino acid 259 of the RTA and amino acid 4 of the RTB.[59,63,64] Thirty percent of the RTA protein is helical. The RTA folds into three somewhat arbitrary domains. The active site cleft of the RTA is located at the interface between all three domains.

Pathogenesis

Pathogenesis resulting from ricin intoxication is a two-step process. The first phase occurs at the cellular level in which the toxin kills cells in a cell-cycle independent fashion; the second phase is primarily caused by systemic reactions that develop in response to cell death and tissue damage.

The cell binding component of the toxin (RTB) binds to cell surface lipids and proteins with exposed terminal β-1,4-linked galactose molecules that are found on most mammalian cells, permitting ricin to bind indiscriminately to most cells in the body.[17,66,67] In addition to binding to surface glycoproteins, ricin contains three mannose oligosaccharide chains, two in RTB and one in RTA, that provide another route for ricin binding to the cell via mannose receptors located primarily on macrophages and dendritic cells.[68]

Once RTB binds to the cell, it is endocytosed.[69] At this point, three possible fates exist for ricin:

1. entry into endosomes and recycling to the cell surface;
2. degradation via the late endosomes; or
3. entry to the trans-Golgi network and entry to the endoplasmic reticulum (ER) via retrograde trafficking.

In the ER, a protein disulfide isomerase reduces the toxin into RTA and RTB components.[70] The low lysine content of RTA probably enables the molecule to evade the ER-associated protein degradation pathway and chaperone proteins, such as calreticulin, and to transport RTA from the Golgi apparatus to the ER; dislocation of RTA from the ER may involve the translocon component Sec61p.[71–73] Postdislocation of RTA in the cytosol probably involves Hsp70, which may also aid the protein in binding to its ribosomal substrate.[74] Additionally, the ribosome itself may act as a suicidal chaperone by facilitating proper refolding of RTA, which is required for the catalytic activity of the enzyme.[73]

Extensive investigations on reactions controlling RTA's binding to ribosomes provide detailed information on RTA's enzymatic functions.[17,75] RTA catalyzes the hydrolysis of a specific adenine in the ricin-sarcin loop of the 28S ribosomal RNA (rRNA) (Figure 16-2). The ricin-sarcin loop interacts with eukaryotic elongation factor EF-2. The binding of EF-2 to the ricin-sarcin loop is required for the translocation of the peptidyl-tRNA from the A-site to the P-site on the ribosome during protein synthesis. The depurinated ricin-sarcin loop fails to bind EF-2 and the ribosome stalls with the peptidyl-tRNA stuck in the A-site.[76,77] The overall effect is a halt in protein translation and cell death.

Analysis of reactions resulting from mixing purified rat ribosomes with RTA shows that the RTA reaction follows classical Michaelis-Menten enzyme kinetics, and the enzymatic action has been calculated to be 0.1 mol/L.[78] Furthermore, these studies predict that one RTA molecule would depurinate 1,500 ribosomes per minute, thus making one ricin molecule sufficient to kill the cell. Site-directed mutagenesis and the development of transition state mimics have yielded mechanistic information. The hydrolysis reaction catalyzed by RTA is thought to proceed via a dissociative mechanism with an oxocarbenium transition state.[79] Glu-177 in the active site stabilizes the developing positive charge on the ribosyl ring while Tyr-80 and Tyr-123 have been proposed to activate the leaving group by pi-stacking with the adenine[80] (Figure 16-3). The enzymatic activity of RTA is the primary source of toxicity and therefore must be attenuated in RTA subunit vaccines by incorporating the Y80A mutation[81] or removing the C-terminal residues (residues 199–267).[82] The mutations interfere with rRNA binding.

Activation of apoptotic processes is one method by which RTA kills cells, but the apoptotic pathways are somewhat cell dependent.[81–83] Evidence indicates that some cells have novel ricin-specific pathways for activating apoptosis. Wu and colleagues[84] found that RTA binds to a novel binding protein (BAT3) that is found in the cytoplasm and nucleus of many cells. BAT3 possesses a canonical caspase-3 cleavage site that appears to be exposed when RTA binds to BAT3; apoptosis is then activated with caspase-3 cleavage. The finding that BAT3 may play a role in ricin-induced apoptosis could identify new targets for preventing ricin toxicity.

Ricin intoxication has been shown to activate numerous signaling pathways including mitogen-activated protein (MAP) kinases and subsequent secondary signaling pathways, such as the stress activated protein kinase family.[85] MAP kinases regulate activation of cytokines such as interleukin (IL)-8, IL-1β, and tumor necrosis factor-α that, in turn, cause inflammatory reactions and tissue damage. Although inflammatory responses caused by ricin have been described previously, pathways and resulting cellular responses were only recently examined.[86] Korcheva et al[87] demonstrated that intravenous administration

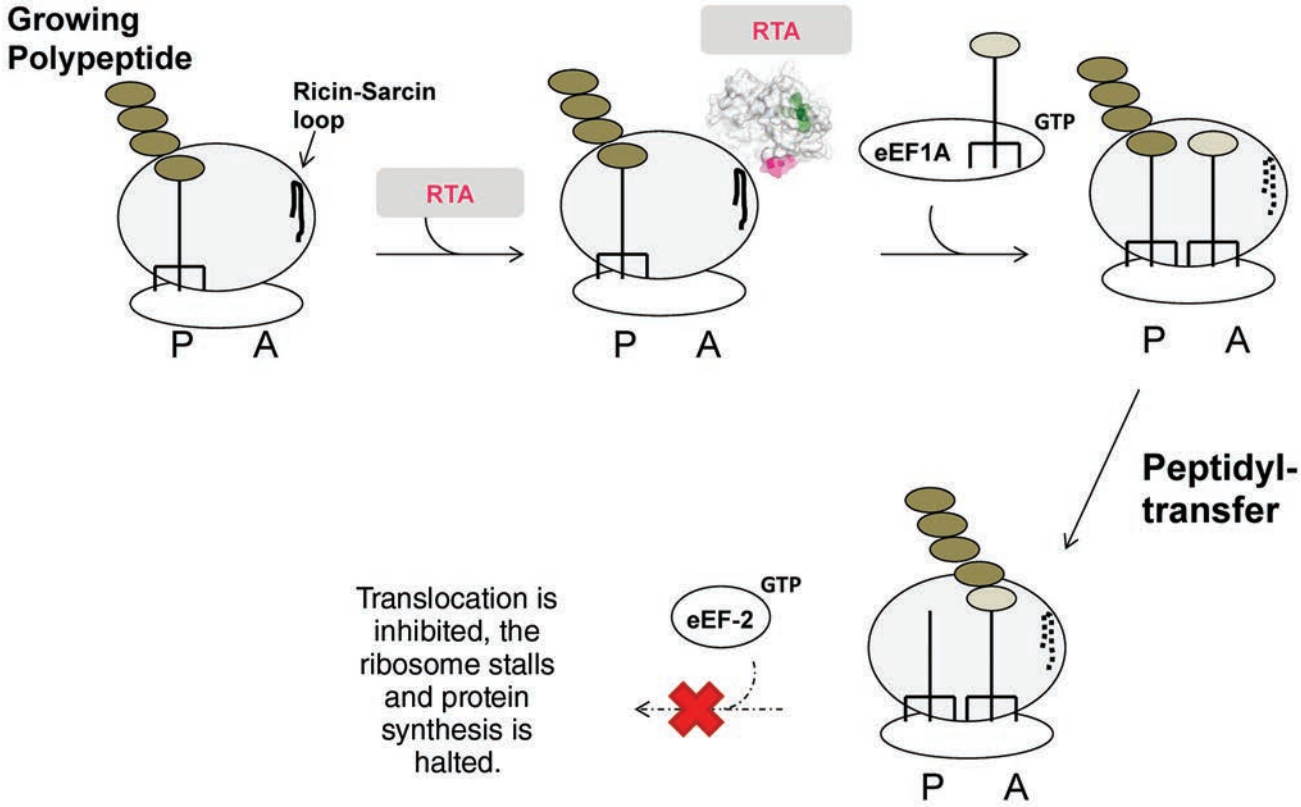

Figure 16-2. The ricin A chain catalyzes the hydrolysis of an adenine in the ricin-sarcin loop. The depurinated rRNA is shown as a dotted line. The aminoacyl-tRNA is delivered to the A-site by eukaryotic elongation factor eEF1A and peptidyl transfer follows. The binding of eukaryotic elongation factor eEF-2 carrying GTP is required for the peptidyl-tRNA to translocation from the A-site to the P-site; this movement requires eEF-2. The depurinated loop fails to bind eEF-2, and the ribosome stalls with the peptidyl-tRNA in the A-site. GTP: guanosine triphosphate
Data source: Figure adapted from Mansouri S, Nourollahzadeh E, Hudak KA. Pokeweed antiviral protein depurinates the sarcin/ricin loop of the rRNA prior to binding of aminoacyl-tRNA to the ribosomal A-site. *RNA*. 2006;12:1683–1692.

of ricin in mice resulted in cellular signaling pathway activation and a significant increase in serum proinflammatory cytokine levels. Additional research in which ricin was instilled by an intratracheal route showed similar signaling pathway activation as well as an increase in proinflammatory cytokine levels, although more inflammatory reactions and tissue damage were observed in the lungs.[88] Although these studies have initiated the systemic pathogenesis characterization of ricin intoxication, further efforts aimed to determine the cellular responses induced by ricin will lead to a greater understanding of its pathogenesis and may also enable the development of new treatment strategies to combat the effects of intoxication.

CLINICAL SYMPTOMS, SIGNS, AND PATHOLOGY

Experimental animal studies reveal that clinical signs and pathological manifestations of ricin toxicity depend on the dose as well as the route of exposure.[5,27,89] The common routes of entry are oral intoxication (ingestion), injection, and inhalation. The differences observed in pathology among various routes likely result from the fact that RTB binds to a wide array of cell surface carbohydrates.[90] Once bound, RTA is internalized and results in the death of intoxicated cells. Although symptoms may vary, in most cases, there is a time-to-death delay of approximately 10 hours, even with a high dose of toxin.[91] Additionally, in animals and humans intoxicated either by injection or oral ingestion, a transient leukocytosis is commonly observed, with leukocyte counts rising two to five times above their normal values. The LD_{50} and time to death for animals by various routes have been reported, and the values for humans were estimated based on animal experiments and accidental human exposures.[5,7,40]

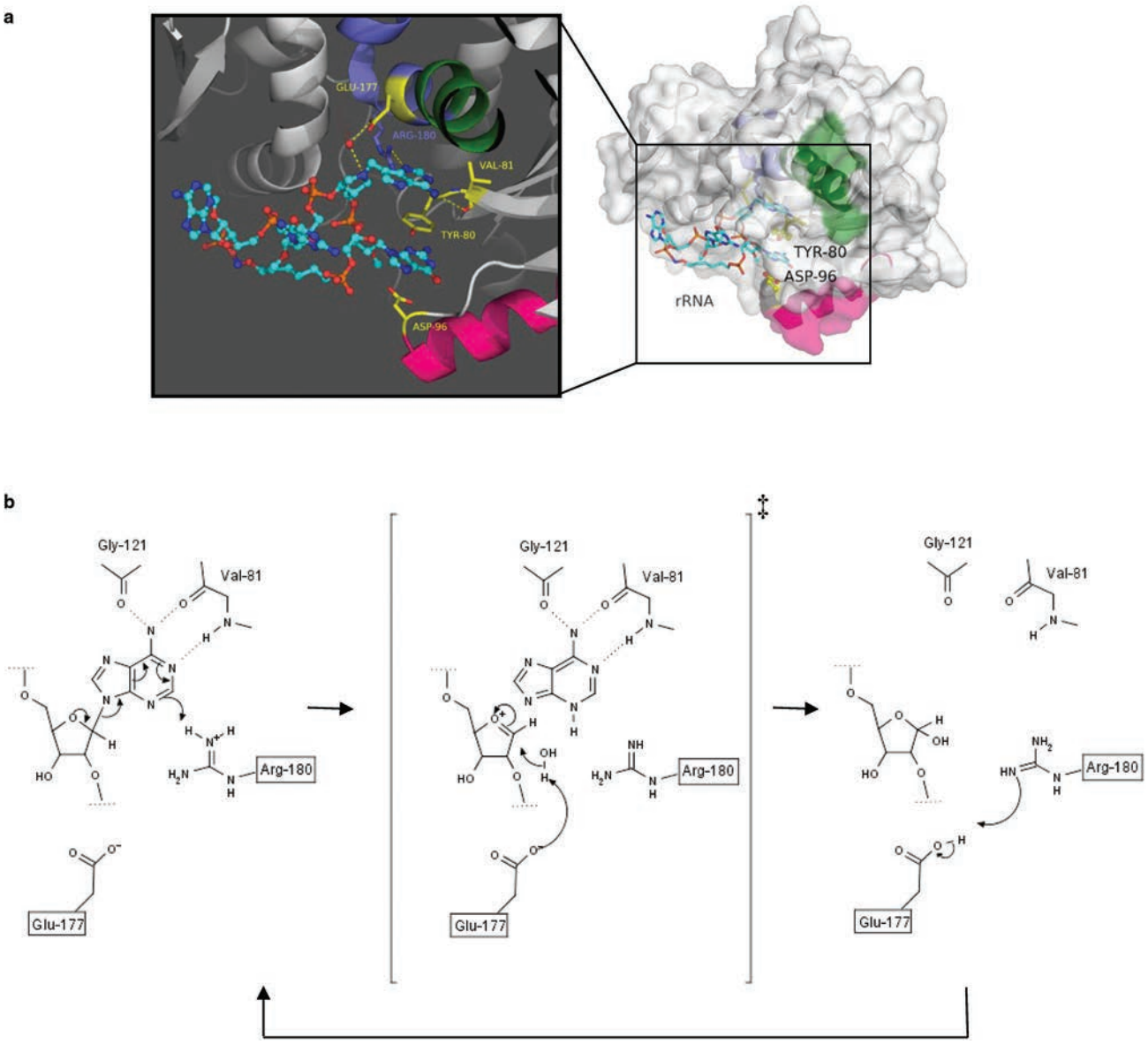

Figure 16-3. RTA catalyzed depurination reaction. (**a**) Structure of a cyclic G(9-DA)GA 2′-OMe transition state mimic determined by Ho et al. (PDB 3HIO). A methylene carbon between the nitrogen of the aza-sugar and the adenine mimics the increased riobsyl-adenine distance in the dissociative transition state. (**b**) Proposed mechanism of the RTA catalyzed depurination reaction. The hydrolysis reaction is thought to proceed via a dissociative mechanism with an oxocarbenium transition state. Arg-180 protonates the leaving group (adenine) and the N-glycosidic bond is broken. Glu-177 deprotonates the hydrolytic water that attacks at carbon to complete the depurination reaction.
Data sources: (1) Ho MC, Sturm MB, Almo SC, Schramm VL. Transition state analogues in structures of ricin and saporin ribosome-inactivating proteins. *Proc Natl Acad Sci U S A*. 2009;106:20276–20281. (2) Roday S, Amukele T, Evans GB, Tyler PC, Furneaux RH, Schramm VL. Inhibition of ricin A-chain with pyrrolidine mimics of the oxacarbenium ion transition state. *Biochemistry*. 2004;43:4923–4933.

Oral Intoxication

Oral or intragastric delivery is the least effective and least toxic route, reportedly 1,000 times less toxic than parenteral routes. The reduction in toxicity may result from poor absorption of the toxin across the epithelium and slight enzymatic degradation of the toxin as it traverses the gastrointestinal tract. Ingestion of castor beans is the most common route of poisoning for humans and domestic animals. Worbs and

colleagues[92] provide an updated list of ricin intoxication in humans worldwide. Since the late 1880s, 875 cases of accidental poisoning and 13 fatalities were reported in the literature (1.5% death rate); there were 11 intentional poisonings, five of which were fatal (45.5% death rate). In recent years, ricin intoxication resulting from intentional poisoning using mashed seeds or crude preparations of ricin has become a major bioterror weapon as well as a method of suicide.[8,9]

Great variability exists in the effects from seed ingestion, which is probably related to the number of seeds, the degree of mastication that releases ricin from the seeds, the age of the individual, and—to a lesser extent—the cultivar of the castor bean plant.[92,93] In addition, an accurate description of ricin intoxication in humans is complicated by other factors including the presence of other somewhat toxic components, such as the ricin agglutinin protein and the alkaloid ricinine, that is found in castor seeds and crude preparations of ricin.[92] These substances can also cause tissue damage and contribute to pathological manifestations.

Despite numerous differences that may play a role in oral toxicity, all fatal or serious cases appear to have a similar clinical history; a recent case study is presented in this reference (Exhibit 16-1).[94] Within a few hours, an onset of nausea, vomiting, and abdominal pain occurs, which is followed by diarrhea,

EXHIBIT 16-1

CASE REPORT: RICIN POISONING CAUSING DEATH AFTER INGESTION OF HERBAL MEDICINE

A 42-year-old male Saudi patient presented to the emergency department with a 12-hour history of epigastric pain, nausea, repeated attacks of vomiting, chest tightness, and mild nonproductive cough.

These symptoms were preceded by a 5-day history of constipation for which the patient ingested a large amount of a mixture of herbal medicine preparation 2 days before his admission. A review of systems was unremarkable. He had no history of any medical illnesses and medication use except for the herbal medicine. Initial examination showed a mild elevation of temperature (38°C), with generalized abdominal tenderness and hyperactive bowel sounds. His respiratory system examination showed equal bilateral air entry and no added sounds. The rest of his systemic examinations were unremarkable.

Laboratory investigations on admission showed mild leukocytosis of 14 x 10^9/L, a normal platelet count of 200 x 10^9/L, and normal hemoglobin level of 15.8 g/dL. Liver enzymes initially showed mild to moderate elevation of alanine transaminase (ALT) 86 U/L (normal range up to 37 U/L), aspartate transaminase (AST) 252 U/L (normal range up to 40 U/L), and serum lactate dehydrogenase 281 U/L (normal range 72–182 U/L), and the renal function was normal. The initial coagulation profile was impaired as documented by a prolonged prothrombin time (19 seconds, control 12 seconds) and a prolonged activated partial thromboplastin time (56 seconds, control 32 seconds). Electrocardiogram showed a right bundle branch block, and a chest radiograph was normal.

After 4 hours of admission, the abdominal pain became worse, and the patient started showing subcutaneous bleeding at the intravenous sites and upper gastrointestinal bleeding, manifested as hematamesis. The patient was managed by intravenous fluid therapy, fresh frozen plasma and platelet transfusion, and gastric decontamination with activated charcoal. A gastrointestinal consultation was requested in which endoscopy was planned after stabilization of the patient, but was not performed because of the patient's rapid deterioration.

In the second day after admission, his liver enzymes increased to a level of 5980 U/L for ALT and 7010 U/L for AST. Serum albumin was 31 g/L (normal range 38–50 g/L), total protein was 59 g/L (normal range 66–87 g/L), and the platelet count dropped to 85 x 10^9/L. His renal function also deteriorated, elevating the creatinine level to 150 Umol/L (normal range up to 123 Umol/L), and urea to 110 mmol/L (normal range 1.7–83 mmol/L). His blood and sputum cultures and sensitivity were negative for bacterial pathogens, and an abdominal computerized tomography scan was normal. The patient was managed conservatively with supportive measures as maintained earlier; however, he remained persistently hypotensive necessitating inotropic support. On the third day, he developed cardiopulmonary arrest and was resuscitated; however, he could not be revived. The sample of the herbal medicine powder was sent to the university lab. The chemical contents were extracted by the liquid chromatography/mass spectrometry technique, revealing the presence mainly of ricin powder that was further identified by the immuno-polymerase chain reaction assay that confirmed the presence mainly of ricin with no other significant contaminants. This finding could be implicated as the cause for the patient's fulminant clinical course.

Data source: Assiri AS. Ricin poisoning causing death after ingestion of herbal medicine. *Ann Saudi Med*. 2012;32:315–317.

hemorrhage from the anus, anuria, cramps, and pupil dilation.[95] Fever develops, followed by thirst, sore throat, and headache, leading to vascular collapse and shock. Death usually occurs by day three or thereafter. Common histopathological findings during autopsy are multifocal ulcerations and hemorrhages in the stomach and small intestinal mucosa. Significant lymphoid necrosis occurred in intestinal associated lymph nodes, lymphoid tissue, and spleen. Necrosis was also observed in cells of the reticuloendothelial system resulting in liver damage and nephritis. Macrophages and macrophage-derived cells appear to be very susceptible, probably because of the large numbers of mannose receptors present in the cell membrane.[68,96]

Injection

Pathological damage caused by injection of ricin depends largely upon the dose. Results of a clinical trial in which 18 to 20 μg/m^2 of ricin were given intravenously to cancer patients indicated that the low dose was fairly well tolerated, with flu-like symptoms, fatigue, and muscular pain as the main side effects.[97] Some patients experienced nausea and vomiting, but after 2 days, they had recovered and experienced no more side effects. At low doses, intramuscular or subcutaneous injections may result in necrosis at the injection site possibly resulting in secondary infections.[98] High doses by either route cause severe local lymphoid necrosis, gastrointestinal hemorrhage, diffuse nephritis, and splenitis. Targosz and colleagues[99] describe a suicide case in which an individual injected himself subcutaneously with a large dose of ricin extracted from castor beans. The 20-year-old man was admitted to the hospital 36 hours after injection. He experienced severe weakness, nausea, dizziness, headache, and chest pain. Clinical exams showed hypotension, anuria, metabolic acidosis, and hematochezia. The patient was observed with hemorrhagic diathesis and liver, kidney, cardiovascular, and respiratory systems failure requiring endotracheal intubation and artificial ventilation. Although given maximal doses of pressor amines and treated for hemorrhagic diathesis, treatments were ineffective and the patient developed symptoms of multiorgan failure followed by asystolic cardiac arrest. Resuscitation was not effective, and the patient died shortly thereafter. A postmortem examination revealed hemorrhagic foci in the brain, myocardium, and pleura.

In the case of Georgi Markov,[2] the lethal injected dose was estimated to be 500 μg. Markov experienced severe local pain after the injection, which was followed by a general weakness 5 hours later. Fifteen to 24 hours later, he had an elevated temperature, felt nauseated, and vomited. He was admitted to the hospital with a high fever and signs of tachycardia. While his blood pressure remained normal, lymph nodes in the affected groin were swollen and sore, and a 6-cm diameter area of induration was observed at the injection site. Just over 2 days after the attack, he suddenly became hypotensive and tachycardic with a pulse rate of 160 beats/minute and a white blood cell count of 26,300/mm.[3] He became anuric developing gastrointestinal hemorrhaging and complete atrioventricular conduction block. Shortly thereafter, Markov died from cardiac failure complicated by pulmonary edema; the time of death was 3 days after he was initially poisoned.[2]

Inhalation

No reports exist in which humans have been subjected to ricin by accidental inhalation or premeditated aerosolized exposure. Most of the human data comes from descriptions of workers being exposed to castor bean dust in or around castor bean processing plants.[100] Allergic manifestations induced by ricin dust were first described in 1914.[101] Symptoms and clinical signs of intoxication were later differentiated from the allergic syndrome and further investigations showed that the allergens and toxin were two different molecules.[102–104]

Because no data exist for human exposure, it is important to determine whether a consistency exists between rodents and nonhuman primates (and other animal models) that can be used to extrapolate an accurate representation of inhalational ricin in humans. Unlike other routes of intoxication, damage caused by an aerosol exposure is greatly dependent on particle size, and to a lesser extent on the dose and cultivar from which ricin was obtained.[86] Ricin extracted from *R communis var. zanzibariensis* was twice as lethal as ricin extracted from the Hale Queen variety.[86] The differences are more than likely related to variations in the isotoxins of ricin found in the seeds from different cultivars.[86]

For ricin to reach the lung, the particles would need to be a size that could move around the nasal turbinates and flow with the airstream to the lung. Roy and colleagues[105] compared the outcome of mice receiving 1 μm versus 5 μm particle size by an aerosol challenge. With the 1 μm particles, the majority of ricin was found in the lung and by 48 hours, lung tissue show significant lesions with alveolar edema, fibrin, and hemorrhage. Seventy-two hours postexposure, all of the mice had died. Conversely, no deaths were observed when mice were exposed to ricin with a 5 μm mass median diameter. Most of the toxin was found in the trachea, and little lung damage was observed in histological sections of lung tissue taken 48 hours postexposure.

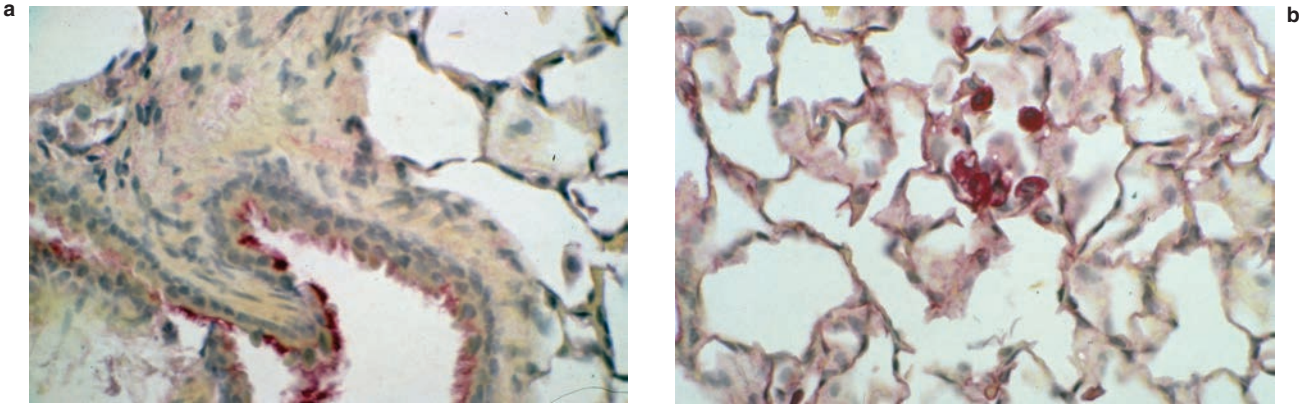

Figure 16-4. Histological sections of lungs from CD1 mice exposed to ricin by aerosol showing (**a**) perivascular edema and pulmonary epithelial necrosis. Hematoxylin and eosin stain at original magnification × 25; (**b**) pulmonary epithelial cell necrosis, hematoxylin and eosin stain at original magnification × 100.
Photographs: Courtesy of Lieutenant Colonel (Retired) Catherine L. Wilhelmsen, Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

When rats were exposed to a sublethal dose (LCt_{30}) of ricin with particle sizes less than 1 μm, damage was limited to the lung and no histological changes were noted before 8 hours postchallenge.[86,106] By 48 hours, pathological changes observed included necrosis and apoptosis in bronchial epithelium and macrophages present in the alveolae septae. Photographs of tissue sections from CD1 mouse lungs 48 hours after exposure show perivascular edema and pulmonary epithelial cell necrosis (Figure 16-4). Three days post-exposure, there was significant diffuse alveolar edema, and severe capillary congestion and macrophage infiltration of the alveolar interstitium. By day four, there was a rapidly resolving pulmonary edema and renewal of the bronchial epithelium, even though severe pas-sive venous congestion existed in all solid peripheral organs. Fourteen days postexposure, all animals survived. Examination of tissue sections from sacrificed animals were similar to control tissues, except for focal areas of intraalveolar macrophage infiltration.[106] Additionally, when rats and mice were given lethal doses of ricin by aerosol, no indication of lung damage was observed during the first 4 to 6 hours.[14,106,107] By 12 hours, there was an increase in total protein and polymorphonuclear cells in the bronchial lavage, indicating damage to the epithelial cell barrier. Thirty hours after challenge, alveolar flooding was apparent, along with arterial hypoxemia and acidosis. Histopathology showed lesions throughout the respiratory tract, spleen, and thymus. A median lethal dose of ricin by inhalation was determined to be 1 μg/kg body weight for both Sprague Dawley rats and BALB/c mice.[107] Further characterization of inhaled ricin exposure was performed by examining lung tissue sections for the presence of ricin.[5] Immunohistochemical studies showed that ricin binds to the ciliated bronchiolar lin-ing, alveolar macrophages, and alveolar lining cells.[14] This finding further substantiates the importance of the lung epithelium and alveolar macrophages in the inhaled ricin intoxication process.

As with other laboratory animal models, investigations in which nonhuman primates were challenged with an aerosolized dose of ricin indicate that disease progression is proportional to particle size.[108] Inhalational challenge with a particle size of 8 μm was not lethal and did not cause lung damage, suggesting that the upper airways can effectively remove the toxin before it reaches the lung. Inhalational challenge with a particle size of 1 μm presented an entirely different picture with histopathologic changes beginning as early as 4 to 6 hours postexposure.[108,109] By 8 hours, pulmonary changes included alveolar edema, perivascular interstitial edema, lymphangiectasis, alveolar septal necrosis, and hemorrhage. At 16 hours, progression of pulmonary tissue damage continued, and by 24 hours, there was edema, pulmonary congestion, necrotic alveolar septa, and necrotic bronchiolar epithelium (Figure 16-5). Thirty-two hours later, there was marked perivascular and peribronchiolar interstitial edema and alveoli contained fluid (edema) mixed with fibrin and viable or degenerate neutrophils and macrophages. The bronchiolar epithelium was necrotic and often sloughed into the lumen, whereas lymphatics surrounding the airways were moderately dilated and the endothelium of many small vessels had atrophied. In the tracheal mucosa, there was epithelial degeneration with scattered areas of necrosis and subacute inflammation. The cortex of adrenal glands showed mild degeneration and necrosis, and there

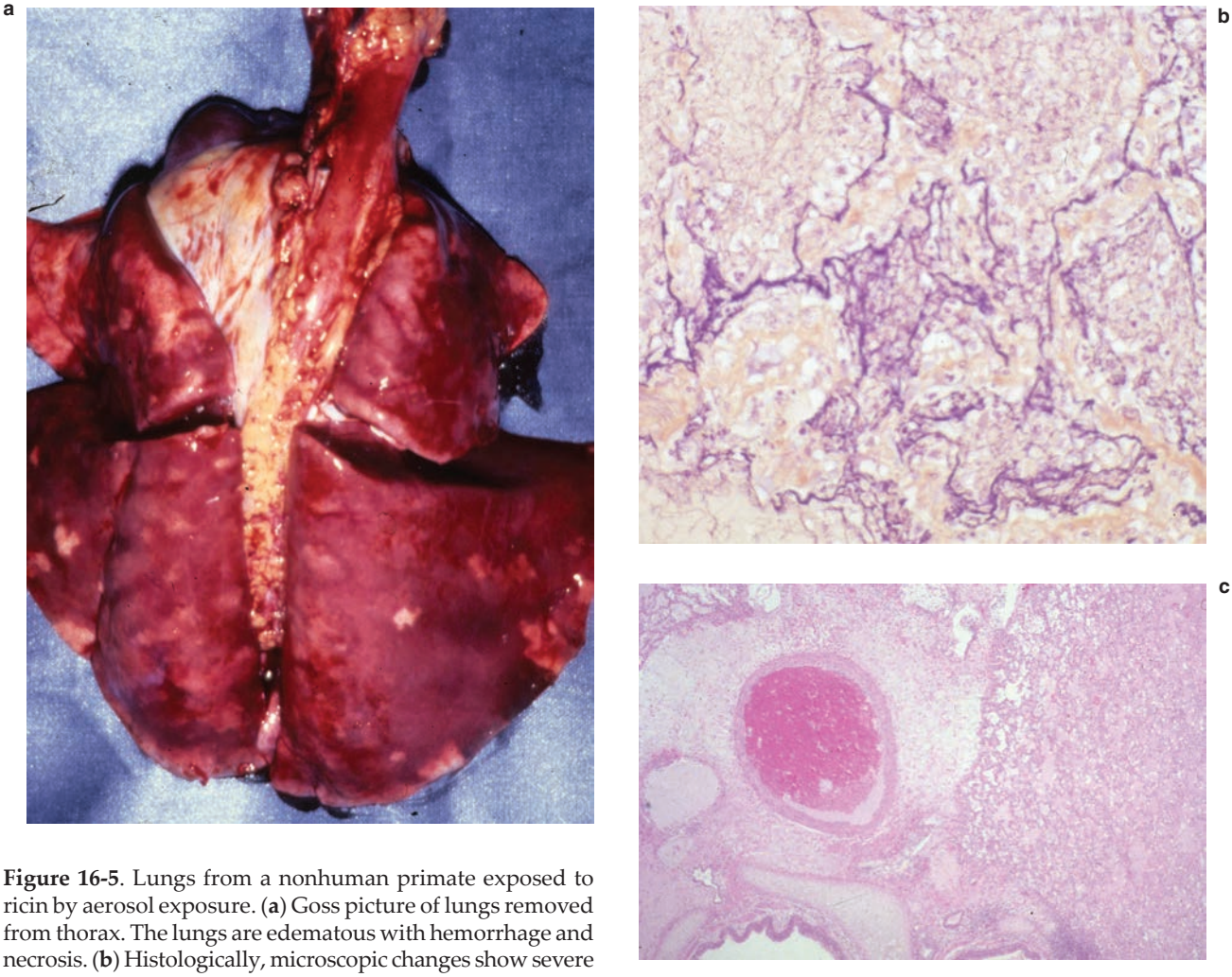

Figure 16-5. Lungs from a nonhuman primate exposed to ricin by aerosol exposure. (**a**) Goss picture of lungs removed from thorax. The lungs are edematous with hemorrhage and necrosis. (**b**) Histologically, microscopic changes show severe perivascular edema; hematoxylin and eosin stain at original magnification × 10. (**c**) Alveolar fibrinopurulent exudate is observed hematoxylin and eosin stain at original magnification × 100. Photographs courtesy of Lieutenant Colonel (Retired) Catherine L Wilhelmsen, DVM, PhD, Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

was lymphoid depletion and lymphocytolysis in the mediastinal lymph nodes. A similar course of disease was observed in an earlier study in which nonhuman primates were challenged with ricin (~1 μm particle size), but the preclinical period varied between 8 and 24 hours in relation to the size of the original challenge dose.[109] This stage was followed by anorexia and decrease in physical activity. The time of death was also dose dependent and occurred between 36 and 48 hours.

Cause of Death

Although the exact cause of death from ricin toxicity is not known, clinical symptoms of individuals exposed to lethal doses of the toxin suggest that death results from a severe inflammatory response and multiorgan failure.[94,95,99] A lethal dose of ricin given to mice by intraveneous injection or intratracheal instillation results in a systemic inflammatory response, thrombocytopenia, hemolytic anemia, renal failure, and microvascular thrombosis, pathologies that are similar to those observed in humans.[87,88] Initially, the fact that macrophages are extremely sensitive to ricin led investigators to believe that macrophages might play a significant role in ricin intoxication.[110] Recent findings demonstrated that pulmonary inflammation caused by ricin required the presence of both macrophages and interleukin-1 signaling pathways.[111] Also, studies using bone marrow derived macrophages showed that ricin inhibition of protein translation led to activation of IL-1β-dependent inflammation by activating innate immune signaling through the

nod-like receptor (NLR) family member, NLRP3.[112] NLRP3 is an innate immune pattern recognition receptor found in the cytosol that is activated by molecular patterns found on many pathogens or other danger-associated proteins. Activation of NLRP3 stimulates IL-1β processing via a multiprotein complex, the inflammasome. More investigations are necessary to understand how ricin activates severe inflammatory responses that lead to multiorgan failure, shock, and death.

DETECTION AND DIAGNOSIS

Early clinical symptoms of ricin intoxication may resemble symptoms caused by other biothreat agents, and therefore it is essential to identify the etiological agent to provide the best treatment for exposed patients. The cellular uptake of ricin is rapid and thus limits the diagnosis of ricin in blood and other fluid samples. Additionally, the ricin concentration may be below the current levels of detection, making diagnosis more difficult.[113] Because of the inability to detect ricin in patients, identifying the toxin in environmental or forensic samples associated with the exposure remains the most reliable method for determining the presence of ricin and the possibility of intoxication. Ricin does not replicate, so detection relies on the ability to identify physical attributes of the toxin within the sample. The most common method for toxin identification uses antiricin antibodies to which ricin would bind. In recent years, several variations of antigen (toxin)-antibody assays have been developed.[114] Physical characterization using liquid chromatography and mass spectroscopy complements the antibody-based methods and permits development of signatures of the toxin preparation.[115]

Enzyme-linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) provides an economical and straightforward method for detecting the presence of ricin in environmental and forensic samples. A capture antibody ELISA is a common method of detection. Ricin is initially "captured" onto the matrix via an antiricin monoclonal antibody (Mab) recognizing RTB. A second anti-ricin Mab, usually recognizing RTA, binds to the immobilized ricin, and the second Mab is then detected by an anti-mouse immunoglobulin 3 conjugated to an enzyme such as horse radish peroxidase that forms a colometric reaction upon the addition of its substrate solution.[86] The limit of detection (LOD) for these assays has been greatly improved by using methods that amplify the detection signal or use a more sensitive signal such as those generated by electrochemiluminescence (ECL). Using slight modifications of these assays, Poli and colleagues[116] reported LODs of 100 pg/100 μL in human serum and urine that had been spiked with various concentrations of ricin. Other studies, such as those by Roy et al,[105] detected ricin in lungs, stomach, trachea, and nares using an ELISA based on time-resolved fluorescence. Although these colorimetric and ECL methods permit detection of high pg or low ng concentrations, sensitivity issues still exist, particularly when assessing foods or biological tissues. Recently, an immuno-polymerase chain reaction assay that uses a polymerase chain reaction to amplify a DNA-labeled reporter system bound to the anti-mouse immunoglobulin G (IgG) permits accurate detection of ricin in these biologic samples ranging from 1 pg/mL to 100 pg/mL.[117] The immuno-polymerase chain reaction may not only offer a method that greatly enhances the ability to detect ricin in environmental samples, but also and more importantly it provides a technique that will accurately determine ricin in tissues from individuals exposed to ricin.

Handheld Assay Detection Devices

Although routine capture ELISAs provide accurate diagnostic tools, these assays require a laboratory setting and instruments to measure the signal. Antibody-based handheld assay (HHA) devices were developed to enable first responders to assess the situation in the field.[118] HHAs were initially developed to detect the anthrax in the letters sent through the mail to Senator Tom Daschle's office in 2001. The success of anthrax spore identification initiated development of HHAs for ricin and other biothreat agents. In 2004, HHAs identified ricin in letters sent to the Dirksen Senate Office building.[118] For ricin, HHAs have an antiricin Mab bound as a single line on the matrix bed. The sample is added to one end of the bed, and capillary action causes the sample to flow across the matrix. The toxin binds to the antibody and then another detection antibody is added. If ricin is present, then the detecting antibody causes color development at the line. If samples are positive using a HHA, samples are sent to a laboratory for confirmation and further analysis.

Sample Verification Platforms

Laboratories, such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID) and the National Biodefense Analysis and Countermeasure Center, need capabilities that will accurately

identify ricin. Multiple instrumental platforms with ELISA-based formats have been developed, including the following:

- ECL-based ricin immunoassay (LOD, 0.05 ng/mL);
- Luminex MAGPIX multiplex (LOD, 0.001 ng/mL); and
- MesoScale Discovery (MSD) PR2 Model 1900 ECL (LOD, 0.2 ng/mL).

Both the M1M ECL-based ricin immunoassay and the Luminex MAGPIX use magnetic beads that are labeled with antiricin antibodies.[119] Once ricin binds to the magnetic beads via the antibody, the sample is sent through the instrument where the magnetic beads are captured by an internal magnet. The magnet is set on an electrode that delivers the proper amount of electrical potential resulting in the emission of light identifying that the sample contains ricin. The MSD PR2, which is a highly sensitive ELISA, has the advantage of using less sample amount (25 μL). The detection antibody is conjugated to a chemiluminescent label that allows for ricin detection by ECL.

On May 30, 2013, a multiplexed rapid ricin detection assay was launched by Radix BioSolutions Ltd (Austin, TX) through the CDC's Laboratory Response Network.[120] This assay was developed using Luminex xMAP technology that permits concurrent detection of several agents in an adaptable, multiplexed assay architecture. Following this report, Tetracore Inc (Rockville, MD) publicized the successful study completion and validation of its BioThreat Alert Lateral Flow Assay and BioThreat Alert Lateral Flow Assay Reader by the Department of Homeland Security for ricin detection.[121]

Liquid Chromatography/Mass Spectrometry

Another method for ricin detection includes identification by liquid chromatography/mass spectrometry. The combination of liquid chromatography and mass spectrometry allows for the separation of mixtures in a sample while being able to identify specific substances based on their molecular mass via their mass to charge ratio (m/z).[122] The ionization of the molecules in the sample can either be protonated or deprotonated depending on the characteristics of the analyte and the mode of detection.[123,124] The advantage of using this technique allows for ricin detection when very little sample is available. Picogram amounts of ricin can be detected within a 5-hour timeframe allowing for fast, reliable detection.[125] Liquid chromatography/mass spectrometry can also be used to characterize other components within the sample because they may provide "signatures" that suggest the origin of the agent. For example, a highly pure form of the toxin might indicate that an organized terrorist group, such as Al Qaeda, produced the ricin while a less pure form may indicate fewer organized groups or individuals acting alone.[86]

Ricin Activity Assay

When ricin is detected using an ELISA or other physical types of assays, the ability to determine whether the toxin is active becomes important for forensic evidence. The assay itself needs to accurately detect ricin's biological activity in samples of limited size (about 50 mL) and low toxin concentration (about 10 ng/mL), and preferably, with an assay time less than 6 hours. To meet these criteria, a cell-free translation (CFT) assay was developed at USAMRIID.[126] The CFT assay measures luminescence generated by the enzyme luciferase produced from the translation of luciferase m-RNA in a rabbit reticulocyte lysate system. The amount of luminescence, produced when the luciferin substrate is added to luciferase, is proportional to the amount of luciferase produced in the in vitro translation system. When ricin is added to the mixture, translation of luciferase mRNA is reduced, which decreases the amount of luciferase produced. Since the amount of luminescence developed is proportional to the amount of luciferase present in the CFT mixture, a reduction in luminescence, as compared to a ricin standard control, provides a quantitative assessment of active ricin in the sample.[126] Table 16-1 summarizes the most commonly used techniques for ricin detection and their sensitivity limits.

Diagnosis

Diagnosis of ricin intoxication is challenging because the cellular uptake of ricin is extremely rapid and limits the availability of ricin for diagnosis in blood and other fluid samples to 24 hours postintoxication.[113] Experimental data suggest that the plasma half-life of ricin is biphasic with the early α phase half-life lasting approximately 4 minutes; the longer β phase half-life was determined to last approximately 83 minutes. The biphasic half-life suggests rapid distribution and uptake of the toxin followed by the slow clearance of excess toxin.[113] Additional liquid chromatography/mass spectrometry assessment of urine samples for metabolites, particularly alkaloids such as ricinine that are commonly found in ricin preparations, indicates ricin intoxication if the individual has symptoms

TABLE 16-1

BIOCHEMICAL METHODS FOR RICIN DETECTION AND THEIR LIMITS OF SENSITIVITY

Method*	LOD† (ng/mL)	Time‡	Detection§	Reference
ELISA-based	0.01–10	5–7 h	Ricin	1–4
Handheld	10–50	90 min	Ricin	5
ECL-based ELISA	0.001–10	4–7 h	Ricin	6–8
Immuno-PCR	0.01–0.1	3–5	Ricin	3, 9
LC/MS	0.1–8	5 h	Ricin/ricinine	10–13
CFT	10–50	4–5 h	Biological activity	14

*Each method may include several different assays using similar principles and formats
†The limit of detection is the lowest amount of ricin detected
‡The time required to perform the assay
§The assays detect either the physical form of ricin or determine the biological activity
CFT: cell-free translation
ELISA: enzyme-linked immunosorbent assay
LC/MS: liquid chromatography/mass spectrometry
LOD: limit of detection
PCR: polymerase chain reaction
Data sources: (1) Griffiths GD. Understanding ricin from a defensive viewpoint. *Toxins (Basel)*. 2011;3:1373–1392. (2) Roy CJ, Hale M, Hartings JM, Pitt L, Duniho S. Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. *Inhal Toxicol*. 2003;15:619–638. (3) Bozza WP, Tolleson WH, Rosado LA, Zhang B. Ricin detection: tracking active toxin. *Biotechnol Adv*. 2015;33:117–123. (4) Poli MA, Rivera VR, Hewetson JF, Merrill GA. Detection of ricin by colorimetric and chemiluminescence ELISA. *Toxicon*. 1994;32:1371–1377. (5) Wade MM, Biggs TD, Insalaco JM, et al. Evaluation of handheld assays for the detection of ricin and staphylococcal enterotoxin B in disinfected waters. *Int J Microbiol*. 2011;2011:132627. (6) DHS fund ricin detection. *Homeland Security News Wire*. October 20, 2011. http://www.homelandsecuritynewswire.com/dhs-funds-ricin-detection. Accessed May 29, 2015. (7) Radix BioSolutions News, 2013. Accessed May 29, 2015. (8) GlobalBiodefense.com. Tetracore Completes DHS Validation of Ricin Detector. http://globalbiodefense.com/2013/06/12/tetracore-completes-validation-of-ricin-detector-for-dhs/. Accessed March 15, 2016. (9) He X, McMahon S, Henderson TD II, Griffey SM, Cheng LW. Ricin toxicokinetics and its sensitive detection in mouse sera or feces using immuno-PCR. *PLoS One*. 2010;55:e12858. (10) Thompson M. High-performance liquid chromatography/mass spectrometry (LC/MS). *AMC Technical Brief*. Analytical Methods Committee AMC TB 34. London, England: Royal Society of Chemistry; 2008. (11) Fredriksson SA, Hulst AG, Artursson E, de Jong AL, Nilsson C, van Baar BL. Forensic identification of neat ricin and of ricin from crude castor bean extracts by mass spectrometry. *Anal Chem*. 2005;15;77:1545–1555. (12) Becher F, Duriez E, Volland H, Tabet JC, Ezan E. Detection of functional ricin by immunoaffinity and liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2007;79:659–665. (13) Kanamori-Kataoka M, Kato H, Uzawa H, et al. Determination of ricin by nano liquid chromatography/mass spectrometry after extraction using lactose-immobilized monolithic silica spin column. *J Mass Spectrom*. 2011;46:821–829. (14) Hale ML. Microtiter-based assay for evaluating the biological activity of ribosome-inactivating proteins. *Pharmacol Toxicol*. 2001;88:255–260.

associated with it.[86] Individuals who survive ricin intoxication develop circulating antibodies in their blood that can be used to confirm intoxication. However, these antibodies are not present until approximately 2 weeks postintoxication and, therefore, could not be used in the initial diagnosis.

MEDICAL MANAGEMENT

Despite the history of ricin's use as a weapon, and unlike other toxin-mediated illnesses such as botulism, no Food and Drug Administration-approved therapeutic for ricin exposure exists. Given that ricin does not have cell specific selectivity, treatment of ricin intoxication is dependent on the site or route of entry, is largely symptomatic, and basically supportive to minimize the poisoning effects of the toxin. Medical countermeasures that have demonstrated capability to disrupt the ricin intoxication process include vaccines and antibody therapy. Both rely on the ability of antibody to prevent the binding of ricin to cell receptors. To ensure maximum protection, the vaccine must be given before exposure, and sufficient antibody must be produced.

Ricin Vaccines

Development of a ricin vaccine has previously focused on either a deglycosylated ricin A chain (dgRTA) or formalin-inactivated toxoid.[127] Although both preparations conferred protection against aerosolized ricin, the proteins aggregated and precipitated over time. Additionally, ricin is not completely inactivated by formalin and may retain some of its enzymatic activity (albeit approximately 1,000-fold lower than native ricin). Thus, other approaches to vaccine development have been investigated to develop a safe and efficacious candidate.

Recent research has focused on developing recombinant RTA subunit vaccines to eliminate cytotoxicity and improve the stability of the vaccine[13] (Figure 16-6). Researchers at the University of Texas developed RiVax that contains the Y80A mutation to inactivate catalysis, and the V76M mutation to ensure the removal of any trace of VLS activity from the immunogen.[128,129] RiVax is at least 10,000-fold less active than wild type RTA but has also been shown to protect rodents against aerosol challenge.[129] In 2006, RiVax was tested in phase I clinical trials. Results of these studies showed that RiVax appeared to be immunogenic and well tolerated in humans.[130,131] However, while such findings were encouraging, vaccine formulation and stability remain problematic. Hence, a lyophilized formulation that retained immunogenicity when stored at 4°C was developed.[132,133] RiVax has been out-licensed to Soligenix (Princeton, NJ) for more advanced clinical trials.[134,135]

To overcome both safety and stability issues simultaneously, researchers at USAMRIID structurally modified the RIP-protein fold of RTA to create a nonfunctional scaffold for presentation of a specific protective epitope.[82] The engineered RTA 1–33/44–198 (RVEc) was produced in *E coli* and lacks the C-terminal residues 199–276 as well as a loop between residues 34–43 (Figure 16-6). RVEc contains a number of well-characterized protective B-cell epitopes, but is more stable and less prone to aggregation. Based on preclinical studies, this product was determined to have a reasonable safety profile for use in human studies; it demonstrated no detectable RIP activity or evidence of VLS.[136–139] In April 2011, USAMRIID launched a phase I escalating, multiple-dose study to evaluate the safety and immunogenicity of RVEc in healthy adults, and it was completed in November 2012.[138] The vaccine was well tolerated and immunogenic.[138,139] In June 2013, a phase 1a (Version 2.0) protocol was implemented as a single-dose, single-center clinical study to allow for the administration and evaluation of a fourth boost vaccination.[139] The ELISA and TNA anti-ricin IgG endpoint titers for the four boosted subjects indicated a robust response very soon after a boost vaccine. In conjunction with this study, another protocol was also started in June 2013 for the collection of plasma from previously RVEc vaccinated subjects for passive transfer studies in animal models to demonstrate IgG as a surrogate marker for clinical efficacy. No adverse events have been reported on this study.[139]

The RVEc final drug product passed stability testing through the 48 months.[139] In addition, the potency assay results confirmed the vaccine elicited protective immunity in mice against 5 times the lethal ricin toxin dose, and it was capable of inducing anti-ricin neutralizing antibodies. An end of clinical use stability testing to include the 54-month time point was initiated in October 2013 for both the final drug product and the diluent.[139]

A comparative immunogenicity and efficacy study between RVEc and RiVax has been conducted in mice.[140] Both candidate RTA vaccines were found equally effective in eliciting protective immunity; however, quantitative differences were observed at the serologic level. RVEc was slightly more effective than RiVax in eliciting ricin-neutralizing antibodies. Furthermore, the antisera elicited by RVEc were toward an immunodominant neutralizing linear epitope on RTA (Y91 to F108), whereas those of RiVax were confined to residues 1–198.[140]

Antibody Treatment

Passive protection with aerosolized antiricin IgG has been evaluated as prophylaxis before aerosol challenge. In mice, pretreatment of nebulized antiricin IgG protected against aerosol exposure to ricin.[141] Preclinical studies also have shown the protection afforded by neutralizing monoclonal antibodies against a lethal dose challenge of ricin.[142–144] Researchers at Defence Science and Technology Laboratory in Porton Down, United Kingdom, have developed polyclonal antiricin antibodies that were raised in sheep immunized with ricin toxoid plus incomplete Freund's adjuvant.[145] The protective efficacy of both IgG and $F(ab')_2$ were demonstrated in mice against ricin intoxication when administered 2 hours following either systemic or inhalational ricin challenge, while the smaller Fab' fragment did not prevent death from ricin intoxication.[145,146] This demonstrates the feasibility of producing an effective ovine antiricin antibody for use following ricin intoxication. In a recent study, four chimeric toxin-neutralizing monoclonal antibodies were produced and evaluated for their ability to passively protect mice from a lethal-dose ricin challenge.[147] The most effective antibody, c-PB10, had the lowest IC_{50} (half-maximal inhibitory concentration) in a cell-based toxin-neutralizing assay and was sufficient to passively protect mice against systemic and aerosol toxin challenge.[147]

The use of antitoxins as therapies for toxin exposure has limitations including the following:

- anaphylactoid or anaphylactic reactions;
- requirement of timely detection of exposure; and
- the therapeutic window is dependent on the toxin and the dose received.[145]

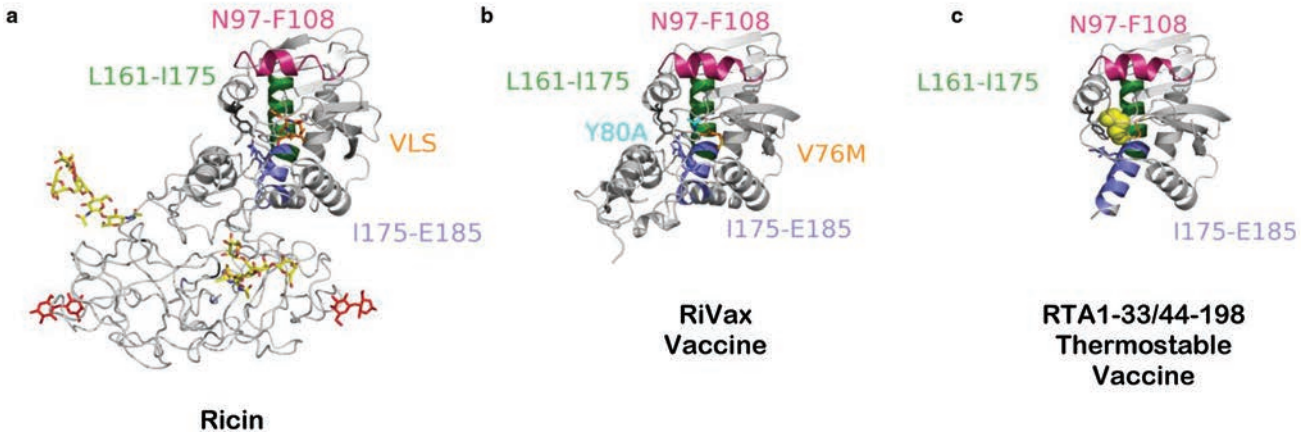

Figure 16-6. Ricin vaccines have been derived from the A-chain of the toxin. (**a**) Ricin consists of an A-chain and a B-chain. The A-chain is shown in *ribbon*, and B-chain in *worm*. (**b**) The ricin A-chain can be produced recombinantly in *Escherichia coli* apart from the B-chain. The structure of RiVax (PDB 3SRP) is similar to the structure of the A-chain of the toxin. (**c**) Truncation of the hydrophobic C-terminal residues of the A-chain and the loop increased the thermal stability of the protein and reduced its propensity to aggregate. The incorporation of disulfide bonds further enhanced the thermal stability of the immunogens (PDB 3MK9, 3LC9, and 4IMV). The protective epitopes are colored on each protein. The UNIVAX R70 epitope is shown in *magenta*; the B-cell epitope recognized by human neutralizing antibodies between Leu161-Ile175 was identified by Castelletti et al (2004) and is shown in *green*. The T-cell epitope between Ile175-Tyr183 is shown in *blue*.
Data sources: (1) Legler PM, Brey RN, Smallshaw JE, Vitetta ES, Millard CB. Structure of RiVax: a recombinant ricin vaccine. *Acta Crystallogr D Biol Crystallogr*. 2011;67(Pt 9):826–830. (2) Compton JR, Legler PM, Clingan BV, Olson MA, Millard CB. Introduction of a disulfide bond leads to stabilization and crystallization of a ricin immunogen. *Proteins*. 2011;7:1048–1060. (3) Janosi L, Compton JR, Legler PM, et al. Disruption of the putative vascular leak peptide sequence in the stabilized ricin vaccine candidate RTA1-33/44-198. *Toxins (Basel)*. 2013;5:224–248. (4) Lemley PV, Amanatides P, Wright DC. Identification and characterization of a monoclonal antibody that neutralizes ricin toxicity *in vitro* and *in vivo*. *Hybridoma*. 1994;13:417–421. (5) Castelletti D, Fracaso G, Righetti S, et al. A dominant linear B-cell epitope of ricin A-chain is the target of a neutralizing antibody response in Hodgkin's lymphoma patients treated with an anti-CD25 immunotoxin. *Clin Exp Immunol*. 2004;136:365–372. (6) Tommasi M, Castelletti D, Pasti M, et al. Identification of ricin A-chain HLA class II-restricted epitopes by human T-cell clones. *Clin Exp Immunol*. 2001;125:391–400.

Supportive and Specific Therapy

The route of exposure for any agent is an important consideration in determining prophylaxis and therapy. For oral intoxication, supportive therapy includes intravenous fluid and electrolyte replacement and monitoring of liver and renal functions. Standard intoxication principles should be followed. Because of the necrotizing action of ricin, gastric lavage or induced emesis should be used cautiously. An aerosol-exposed patient may require the use of positive-pressure ventilator therapy, fluid and electrolyte replacement, anti-inflammatory agents, and analgesics.[148] Percutaneous exposures necessitate judicious use of intravenous fluids and monitoring for symptoms associated with VLS.

Development of Ricin Small Molecule Inhibitors

Reaching intracellular space with a ricin inhibitor provides an ideal pre- and postexposure therapeutic. At a minimum, small molecule inhibitors must possess sufficient safety and efficacy to enable a pathway to licensure. A strong safety profile is critical since no diagnostic capability exists to identify personnel who have received a clinically significant dose of ricin. Ideally, the inhibitor is also self-administered, which would greatly reduce the burden on the healthcare system and allow the provider to focus on patients who require more intensive care and medical resources.

A variety of approaches have been used to identify suitable small molecule ricin therapeutics. Potential compounds fall into three broad mechanisms of action:

1. those that target RTA;
2. those that target the retrograde transport pathway used by ricin to gain access to the cytosol; and
3. a group that alters the cellular stress response following ricin intoxication.

A notable absence among published work includes molecules directed against the RTB that might prevent ricin from entering cells. However, the molecular

structure of RTB makes it an extremely difficult drug target. X-ray studies show that RTB is composed of two domains with each domain possessing three subdomains that bind to sugars.[62,149] Selected mutations of RTB suggest that three of the six sites must be inactivated to prevent cellular intoxication.[62] Since these three sites are widely separated on RTB, it would present a formidable challenge in the design of inhibitors that still possess drug-like characteristics. Furthermore, because the RTB carbohydrate binding regions are small and shallow, these features present yet another hurdle for the design of drug-like molecules.[150]

In contrast, RTA presents a more tractable drug target. Although the large, open, and polar nature of the active site makes it a difficult drug target,[151,152] high-resolution X-ray structures of the active site can help in the design of inhibitors.[153] Furthermore, the mechanism of action for ricin is well described and provides additional criteria for the design of drugs that target the active site. Drug discovery approaches for ricin therapeutics have typically relied on virtual screening (VS), or high throughput cell-based assays. Virtual screening uses computational methods to evaluate large numbers of compounds for possible activity against ricin but requires careful consideration of molecular parameters to ensure optimal results, access to libraries of appropriate chemicals,[154,155] and structural data, such as high resolution crystal structures of the target molecule.[156] No single VS software is ideal as each produces different results.[160] Several excellent recent in-depth reviews provide additional background on VS.[157–159] Although it allows for evaluation of a large number of chemicals, VS has not always identified appropriate candidates for drug development.[152,156,160] Regions of proteins, such as the active site of ricin, that have large and polar pockets are difficult drug targets,[151] but successes have been seen in discovering RTA inhibitors.[161,162,163]

Another screening technique, cell-based high throughput screening (HTS), requires an appropriate cellular model of intoxication and a method to identify compounds that have activity against ricin. For cell-based assays the tested compounds should be soluble in cell culture media or with an excipient compatible with cellular growth. The solubility requirement significantly reduces the number of compounds that can be tested in cell-based assays. Furthermore, poor solubility may mask an otherwise useful molecule because it cannot be delivered to the cells in a high enough concentration to have an observable effect. More comprehensive discussion on cell-based HTS can be found in several recent reviews.[164–167] Despite these limitations, the complementary methods of VS and cell-based HTS assays have identified a variety of active compounds. Similar approaches have been used to identify small molecule inhibitors of shigatoxin, a prokaryotic enzyme with related enzymatic activity but limited structural homology to ricin, suggesting pharmacophore discovery is broadly applicable.[151–161,168–170] Some of these VS and HTS successes for RTA inhibitor design will be highlighted in the next sections.

Ricin A Chain Inhibitors

Several research groups used RTA as a target to identify potential lead compounds from chemical libraries. One of the most potent active site inhibitors was developed by a rational drug design process[171] and built on an earlier observation that pteroic acid (PTA) bound to the ricin active site with a modest IC_{50} of 600 μm.[163] Although PTA is not a suitable candidate because of its limited solubility, it served as a platform for designing derivatives. Several derivatives of PTA were found to have increased solubility, and when a linker was included that enabled additional contacts between RTA and the inhibitor, RTA inhibition was enhanced.[172] Saito et al[171] built on this observation by adding di- and tri-peptide linkers to PTA that allowed binding both the specificity pocket and a distant secondary pocket within the ricin active site. The addition of these linkers and the resulting interaction with the secondary pocket provided a 100-fold improvement in the IC_{50}.[171]

Additional successes in identifying RTA inhibitors through virtual screening have been reported by Pang et al[161] and Bai et al.[162] Although both groups' compounds target RTA, Pang's[161] molecules targeted a site distant from the active cleft yielding inhibition of ricin enzymatic activity, whereas Bai's[162] are active site inhibitors. Pang's[161] deliberate choice was based on the recognition that the large size and polar features as well as the multiple electrostatic interactions between rRNA and the active site made it an unattractive and difficult drug target. Pang et al[161] also took advantage of a structural change that occurs in the ricin active site upon binding of the toxin to the α-sarcin-ricin loop in the 28S ribosome, which causes Tyr80 in the active cleft to move to a new position where it participates in the depurination of the ribosome by packing with the bases of rRNA.[153,173,174] Thus, if the movement of Tyr80 is blocked ricin is rendered inactive. Preventing the movement of the Tyr80 is a novel approach to developing RTA inhibitors by avoiding the complication of designing drugs for the ricin active site. This approach to inhibitor design was designated as the "door-stop" approach because it prevents Tyr80 from undergoing the necessary conformational change for enzymatic activity. Pang et al[161] screened more than 200,000 molecules with molecular weights lower than 300 Da and 226 were predicted to block the movement of Tyr80. When evaluated in a CFT assay using firefly

luciferase, several of these compounds inhibited ricin. Unexpectedly, several compounds enhanced the firefly luciferase assay, but were the result of the compounds directly interacting with the firefly luciferase and not RTA. This interaction precluded the determination of the IC_{50} of the compounds and serves to underscore that appropriate controls need to be present when screening chemicals for activity in this reporter assay.[175–178] However, functional studies revealed that the Pang compounds[151] protected cells exposed to ricin, suggesting that ricin inhibition using the "door-stop" approach is a validated model. Furthermore, these results demonstrated that direct competition with the ricin active site, a difficult target, was not essential to achieve inhibition of the ricin catalytic activity.

The VS approach conducted by Bai et al[162] identified several new classes of inhibitors. Bai[162] used two different VS programs, one to identify molecules that could bind to RTA in which the Tyr80 has been displaced and a separate program that identified candidate compounds that bound to the RTA form in which Tyr80 was not displaced. Compounds ranked highly by both programs were selected for further study, and they revealed a variety of new chemical entities for further development.[162] In vitro kinetic studies showed that these compounds possess a potency similar to PTA. Although many of the compounds were cytotoxic, two were identified that protected vero cells exposed to ricin. The best performing compound showed little cytotoxicity and protected about 90% of cells exposed to ricin.[162] Nevertheless, the cytotoxic compounds can still serve as starting points to improve their binding to RTA while reducing their toxicity.

Transport Inhibitors

The second category of inhibitors, transport inhibitors, blocks the retrograde movement of ricin through the cell and may have its greatest utility as preexposure treatments. Compounds that inhibit the retrograde transport of ricin have substantial efficacy in animal models when used in a preexposure setting.[179] However, because of the retrograde pathway taken by ricin to arrive at its cellular target, inhibitors of this normal cellular process also have a potential to exhibit significant toxicity. For example, ilimaquinone (IQ), a marine sponge metabolite, inhibited ricin in a dose-dependent manner in a vero cell assay.[180] However, IQ also caused the Golgi apparatus to fragment into smaller vesicles; yet this effect was reversible when IQ was removed.[181] Additional molecules have been identified that alter retrograde transport and protect cells from ricin challenge; however, the utility of these molecules for continued development is questionable because they also disrupt the Golgi architecture.[182] In spite of the potential toxicity of retrograde transport inhibitors, several groups identified inhibitors of ricin transport, some of which show limited toxicity in cellular and animal based assays of efficacy. Stechmann and colleagues[179] used a protein synthesis cell-based HTS assay to identify compounds that restored normal levels of protein synthesis after ricin exposure. Of more than 16,000 compounds, they identified two that were inhibitors of retrograde transport. Despite functionally blocking retrograde movement, these compounds exhibited no effect on the architecture of the Golgi complex or on cellular transport pathways such as endocytosis, vesicle recycling, degradation, or secretion.[179] These two compounds were further examined in an animal model of intranasal ricin challenge. The compounds completely protected challenged animals when treatment was given 1 hour before ricin exposure; no acute toxicity was observed in animals that received only the test compounds.[179] However, these compounds may not be ideal candidates for further development because of instability.[183]

Cellular Stress Response Inhibitors

Rather than targeting the ricin molecule or the retrograde transport pathway described previously, another target is the cellular response to ricin. When ricin depurinates ribosomes in target cells, these cells enter a condition known as ribotoxic stress response.[184,185] The ribotoxic stress response leads to activation of stress associated protein kinases and other cellular changes.[184] Activation of stress associated protein kinases including p38 mitogen activated protein kinase ($p38^{MAPK}$) can lead to the release of proinflammatory cytokines and the induction of apoptosis in cells.[186–188] A screen of molecules that protected cells from ricin challenge but did not act on ricin or the retrograde pathway identified two molecules for further analysis.[189] One compound reduced the activation of the SAPK $p38^{MAPK}$ by acting upstream of $p38^{MAPK}$ activation. The other compound acted as an inhibitor of caspase 3 and 7 activation, thus blocking a critical step in the induction of apoptosis.[189]

SUMMARY

Ricin is a potent toxin derived from the castor plant, *R communis L*, which has been cultivated worldwide for its oil since ancient times. Because of its potency, stability, wide availability of its source plants, and popularity on the Internet, ricin is considered a significant biological warfare or terrorism threat. Ricin was developed as an aerosol biological weapon during World War II, but was not used in combat nor in

mass casualty attacks. As a biological weapon, ricin has not been considered as useful in comparison with other biological agents such as anthrax or botulinum neurotoxin. Nevertheless, its popularity and its track record in actually being exploited by extremist groups and individuals accentuate the need to be vigilant of its surreptitious misuse.

Despite ricin's notoriety as a potential biological agent, its medical applications have been also explored. Ricin has contributed to early immunology; the understanding of both immunological and cell biological processes; and the treatment of cancer, AIDS, and other illnesses. Clinical manifestations of ricin poisoning vary depending on the routes of exposure. Aerosol exposure represents the greatest threat posed by ricin and can lead to death via hypoxia. Diagnosis of ricin exposure is based on both epidemiological and clinical parameters. No Food and Drug Administration-approved drug or vaccine against ricin intoxication exists; treatment is mainly symptomatic and supportive. Since vaccination offers a practical prophylactic strategy against ricin exposure, considerable efforts have been devoted to develop a safe and effective ricin vaccine to protect humans, in particular soldiers and first responders. Recombinant candidate ricin vaccines are currently in advanced development in clinical trials. Efforts are also underway to develop small molecule inhibitors for the treatment of ricin intoxication. Recent findings suggest that refinement of the newly identified ricin inhibitors will yield improved compounds suitable for continued evaluation in clinical trials.

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