INTRADERMAL ADMINISTRATION OF RIVAX, A RICIN VACCINE

APPROVED BY SUPERVISORY COMMITTEE

Ellen S. Vitetta, Ph.D.

Philip E. Thorpe, Ph.D.

Joan E. Smallshaw, Ph.D.

Michelle A. Gill, M.D., Ph.D.

Kim Orth, Ph.D.

DEDICATION

To my father.

INTRADERMAL ADMINISTRATION OF RIVAX, A RICIN VACCINE

by

PRAVEENA S. SELVADURAY

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

April, 2010

ACKNOWLEDGEMENTS

Most importantly, I must thank my mentor, Dr Ellen Vitetta. It is rare to find a mentor who acts as a mentor, and not as a boss. Ellen taught me to do science *and* how to function and survive in science. I am grateful to have a mentor who has, and will continue to, help me navigate my career.

I would also like to thank my thesis committee for their scientific advice during the last twp years, this includes Drs. Philip Thorpe, Kim Orth, Michelle Gill, and Joan Smallshaw.

The Cancer Immunobiology Center (CIC) has been like a family during my time in graduate school. Most important were those people whom I worked with closely for the last two years in order to complete this project; namely Joan Smallshaw, Steve Ruback, Phyllis Barron and . Jue Yang. Joan sees many of the details in experimental design that have allowed me to carry out my experiments. . Her tireless coordination of the BLS 3 facility was essential to my work. Steve taught me 95% of the assays in this project. He is a phenomenal teacher at the bench and a true asset to a student. Phyllis put many hours of her time into my project. More important than giving me much needed help with hundreds of mice, she provided me with excellent friendship and encouragement during my many hours in the lab. I also give many thanks to Jue for her hours of work with me in the BSL 3; it is exhausting work which was made much easier with her help.

The students in the CIC have acted as a support group, sounding board and have been my cheerleaders. I have endless memories of them which will always bring a smile to my face. I look forward to continuing to have them in my professional support system as we all continue in various avenues of science. Thank you very much to Dr. Kimberly Brooks, Dr. Lydia Tsai, Dr. Pavitra Chakravarty, Andrew BitMansour, Angela Collins, Angela Desmond and Megan Wachsmann for making every day fun.

Numerous others in the CIC have also contributed to my time here, providing scientific advice, technical support and a listening ear, these include Dr. Elaine Coleman, Drew Ivey, Kelly Mapes, Ayesha Ahmed, Dr. John Gu, Ryan Willingham, Dr. Radu Marches, Nancy Lane, Dr. Charlie Liu, Dr. Iliodora Pop.

The CIC would not run if it were not for Linda Berry, Katherine Holloway, and Rhonda McCracken. Their work allows us to concentrate on research without having to worry about the logistics of doing science. They also give the CIC a great deal of warmth, by providing sympathetic ears and coordinating many much- needed and appreciated social events.

Last, but definitely not least, I am very thankful for the unconditional help and support of Dr. Laurentiu Pop. When I ask for five minutes of his time, he will often give me fifteen. His help is not only technical and intellectual; his personality

gives the CIC the comicrelief it needs in order to deal with the stress of doing science. Every student in this lab has benefited from his selflessness and I am grateful to have been one of them.

At UT Southwestern I have a number of people to thank for administrative support, including Nancy McKinney, Karen Kazemzadeh and Harmony Hilton, I would like to thank those who have provided me with much needed professional support when I was in dire need of it, specifically Drs. John Schatzle, Melanie Cobb and Michelle Gill.

My greatest assets in life are the remarkable people who love me. First, is my father for encouraging me to think like a scientist since I was a child; I would not have made it to this point in my life if not for him. Secondly, I must thank my mother; though she does not always understand my motives shesupports me unconditionally. Third are my friends whom I love like family; I am greatly indebted to Megan, Diana, Dana, Lori, Alethia, Leila, Alex and Odette for their unconditional encouragement. I must also thank my family in Malaysia and Romania; though we cannot spend much time together, you never forget that we are family and therefore always support me. Lastly, I thank my husband, Andrei. He is truly my partner in life, accepting all of my challenges as though they are his, while giving me full credit for my successes. Earning this degree was made much easier by having him in my life.

INTRADERMAL ADMINISTRATION OF RIVAX, A RICIN VACCINE

PRAVEENA SELVADURAY, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

ELLEN S. VITETTA, Ph.D.

Ricin toxin is a CDC Level B Biothreat due to its extreme toxicity and ease of production. The most effective method for minimizing ricin toxicity in humans is prophylactic vaccination. We have previously described the efficacy and safety of RiVax, a recombinant mutant of ricin A chain (RTA). RiVax has no residual toxicity from either its ribotoxic site or its vascular leak-inducing site. When administered by intramuscular (IM) injection, it was safe and immunogenic in mice, rabbits, and humans. A three dose regimen of IM administered RiVax also protected mice from an LD_{50} dose of ricin delivered by injection, gastric gavage or aerosol. In this study we have attempted to increase the utility and immunogenicity of RiVax. To this end, we have compared intradermal (ID) vs. IM

administration of RiVax by evaluating the following parameters of vaccine efficacy: (1) short-term antibody responses and protection of mice from a 10X LD_{50} of ricin following a three dose vaccine regimen; (2) long-term antibody responses and protection of mice from a 10X LD_{50} of ricin following a three dose vaccine regimen; (3) protective effect of a single high dose of RiVax from a 10X LD_{50} dose of ricin; (4) the minimum dose of ricin at which fully vaccinated animals are no longer protected; (5) the rate of antigen trafficking to draining lymph nodes (DLN) following administration of RiVax. In the short term, when RiVax was delivered with alum, very low doses of vaccine administered ID were superior to the same low doses administered IM, with regard to both antibody production and protection against ricin delivered by injection, gavage, or aerosol. Low doses of ID vaccine were also superior in maintaining lung function in mice exposed to aerosolized ricin. Comparing the same parameters in the long term or after a single dose of RiVax, ID and IM vaccinations were equally effective. Both ID and IM vaccination were also similar in their ability to protect mice from a supra-lethal challenge with injected ricin. One possible explanation for the improved efficacy of low doses of RiVax administered ID was that the vaccine trafficked more effectively to the DLNs. This appeared to be the trend, albeit not a statistically significant one. Given the increased efficacy of low doses of ID vaccine in protecting mice against ricin delivered to the lung and gut, we suggest that it should be considered for testing in humans.

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PRIOR PUBLICATIONS

Marconescu PS, Smallshaw JE, Pop LM, Ruback SL, Vitetta ES. *Intradermal Administration of RiVax Protects Animals from Mucosal and Systemic Ricin Intoxication*. Vaccine (2010), doi:10.1016/j.vaccine.2010.05.045

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LIST OF ABBRE*VIA*TIONS

Ab – antibody

APC – antigen presenting cell

BAL – bronchial alveolar lavage

BCG – Bacillus Calmette Guerin

CFA – complete Freund's adjuvant

CNS – central nervous system

DC – dendritic cells

dgRTA – deglycosylated RTA

DLN – draining lymph node

DMSO – dimethyl sulphoxide

dsRNA – double stranded RNA

EDEM – ER degradation enhancing α mannose I-like protein

ER – endoplasmic reticulum

ERAD – ER associated degradation pathway

FACS – fluorescence-activated cell sorting

FCS – fetal calf serum

GI – gastrointestinal

GMT – geometric mean titer

IC_{90} – inhibitory concentration to 90% of the cells

ID – intradermal

IFA – incomplete Freund's adjuvant

IM – intramuscular

IN – intranasal

iNOS – inducible nitric oxide synthase

IP – intraperitoneal

IT – intratracheal

J-chain – joining chain

LD_{50} – lethal dose to 50% of the group

LN – lymph node

LPS - lipopolysaccharide

MMR – measles/mumps/rubella

MPL – monophosphoryl lipid A

NLR – nod-like receptor

ODN - oligodeoxynucleotides

ON – overnight

PLA – polylactide

PLGA – poly (lactide-co-glycolide)

PBS – phosphate buffered saline

pIgR – polymeric Ig receptor

RES – reticuloendothelial system

RIP – ribosome inactivating protein

rPA – recombinant anthrax protective antigen

RT – room temperature

RTA – ricin toxin A chain

RTB – ricin toxin B chain

SC – secretory component

SC – subcutaneous

sIgA – secretory IgA

TLR – toll-like receptor

USAMRIID – U.S. Army Medical Research Institute of Infectious Diseases

WT – Wild type

CHAPTER ONE
Introduction

PART A. RICIN

History and cultivation

Ricin is a toxin produced by the beans of the castor bean plant, *Ricinus communis*. Today, *Ricinus communis* is most extensively cultivated for its beans in order to make castor oil, but it is also found as a decorative plant in homes. Castor oil is used as an industrial lubricant, but has also been used medicinally as a laxative since ancient times. When the oil is produced from the beans by heat extraction, it is non-toxic and ricin can be easily purified from the remaining by products [1]. It is estimated that approximately 1 - 5% of each bean is ricin [2].

The medicinal benefits of ricin go well beyond the application of its oil as a laxative. In the late 1800s, Paul Ehrlich injected small non-toxic doses of ricin into animals. He found that the treated mice were protected against larger, lethal doses of toxin and that they produced “serum proteins” which precipitated the toxin; today we refer to these “serum proteins” as antibodies. In essence, Ehrlich’s was the first ricin vaccine.

More advanced applications of ricin in medicine took place in the mid to late 1900s when it was investigated as an anti-cancer therapeutic. Originally, the toxin was applied to tumor sites or injected into tumor bearing animals. In Phase I clinical trials, intravenously (IV) administered ricin given to patients with various types of cancers yielded a response in only 1 out of 54 patients. The doses of ricin were generally well tolerated with significant fatigue being the most prominent side effect, but at the same time a Phase I trial with abrin, another plant toxin with the same mechanism of action as ricin, led to two deaths [3]. Since then, ricin toxin alone has not been studied as a cancer therapeutic.

Ricin as an Agent of Bioterroism

Ricin has a long history of use in espionage. Anecdotes from ancient times describe the use of the toxin to poison people. More recently, both the US and UK have investigated how to best weaponize the toxin. In the 1970s a toxin, presumed to be ricin was used by the KGB and other agencies in attempts to kill various people in the Eastern Bloc. Georgi Markov, a Bulgarian journalist, was injected with a ricin filled platinum capsule *via* the tip of an umbrella and subsequently died. This delivery method was used several times although it was only successful in the

case of Markov. Other attempts failed since the capsules did not leak enough ricin into the body [1].

Within the past 20 years, ricin has been in the news on several occasions. It has been found in the possession of several individuals who have intended to use it destructively (FBI Weapons of Mass Destruction cases page, http://www.fbi.gov/hq/nsb/wmd/wmd_cases.htm). A threatening letter intended for Congress containing ricin was discovered in a South Carolina post office in 2003 [4]. Overall, there have been no known incidents of ricin poisoning in recent years, but there have been a number of close calls and threats. The US Department of Defense has been concerned that it will be used to poison soldiers and have been working on a vaccine for many years.

Ribosome Inactivating Proteins (RIPs)

Paul Ehrlich discovered the first RIP proteins, ricin and abrin [5-6]. Since then, numerous other RIPs have been identified and they have been classified into Type I, II and III. These proteins are found in plants, fungi, bacteria, and algae. They can be produced in a variety of anatomical structures including seeds, leaves, bark, roots and fruits. All RIPs act as N-glycosidases to irreversibly inactivate eukaryotic ribosomes by cleaving

adenine 4324 of the 28s rRNA in the 60S subunit of the ribosome. Type I and Type II RIPs are very similar in both structure and function, Type II RIPs simply have an additional chain as compared to Type I RIPs. Type III RIPs are more unique and will be described below.

Type I RIPs - Type I RIPs are classified as RIPs consisting of a single polypeptide chain that is approximately 30kD. They have enzymatic activity, but lack any means of entering cells. Therefore, in cell-free assays they are quite potent in inhibiting protein synthesis, but in cells and animals they are relatively non-toxic [5-6].

Type II RIPs – Type II RIPs consist of two chains, an A chain and a B chain. Both chains are about 30kDa in size. The A chain has enzymatic activity while the B chain is a lectin. The B chain allows the Type II RIPs to gain entry into the cell by binding to cell surface glycolipids and glycoproteins. This allows the enzymatic chain to enter the cytosol and inhibit protein synthesis. The presence of a lectin for cell entry makes Type II RIPs the most toxic of the RIP family in that it can act in cell-free systems, in intact cells and in animals. Ricin falls into this family and is described more extensively below [5-6].

Type III RIPs – The Type III RIP family consists of two proteins. One is a maize protein b-32. B-32 is expressed as a zymogen and activated by the removal of an internal inhibitory 25 amino acid peptide [7]. The other Type III RIP is an enzymatic barley protein (similar to a Type I RIP) which may or may not be associated to another protein of equal size and unknown function [5-6].

Ricin Structure and Function

Ricin Structure- The ricin toxin consists of two disulfide linked subunits, an A chain (RTA) and a B chain (RTB) (**Figure 1.1**) each with a molecular weight of 30-32 kDa. RTA is a 267 amino acid chain consisting of 3 domains. Domain one is found at the amino terminus and includes residues 1 – 117; itconsists of a 5 β stranded sheet. Domain two spans residues 118 – 210, the main architectural feature of this domain is 5 α helices. The third domain of RTA includes residues 211 – 267 which interacts with the first and second domains in addition to RTB. RTB, is the lectin subunit of ricin, and consists of 267 amino acids containing l two domains that fold identically, but only have 32% sequence homology. Domain one spans residues 1 – 135 while domain two spans residues 136 – 267. Each contains a galactose binding site. The disulfide linkage

between RTA and RTB forms between residue 259 of RTA and residue 4 of RTB [8].

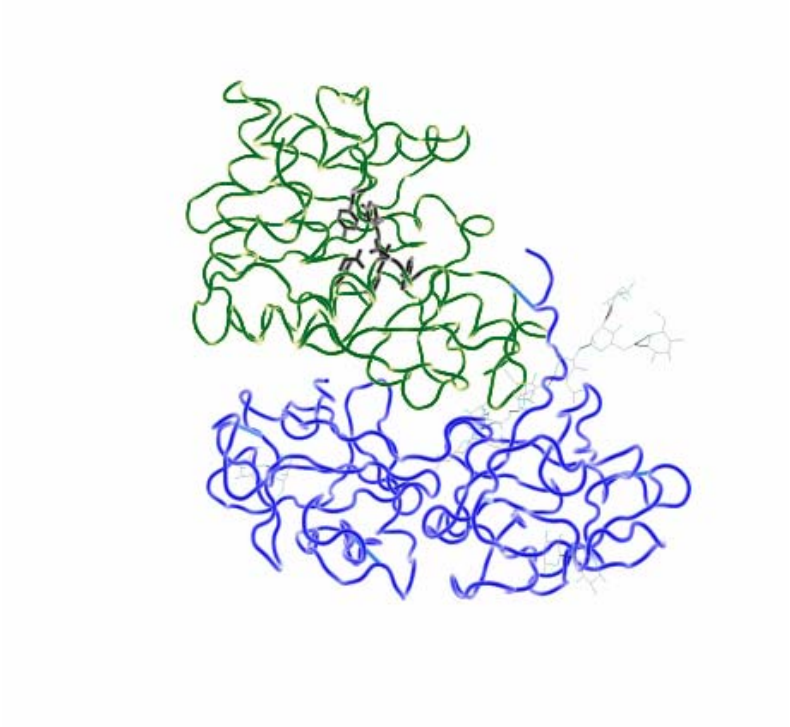


Figure 1.1: Structure of Ricin Ricin holotoxin, green = RTA, blue = RTB, black = active site residues, white = carbohydrate moieties.

Ricin Trafficking and Function – RTB binding of galactose-containing glycolipids and glycoproteins expressed on the cell surface facilitates entry of the toxin into the cell [9] [10]. It is evident that there are many routes by which the toxin can enter the cell, including *via* both clathrin dependent and clathrin independent endocytosis, but these routes are still not clearly elucidated. It is probable that all of these routes converge in the early endosome [11].

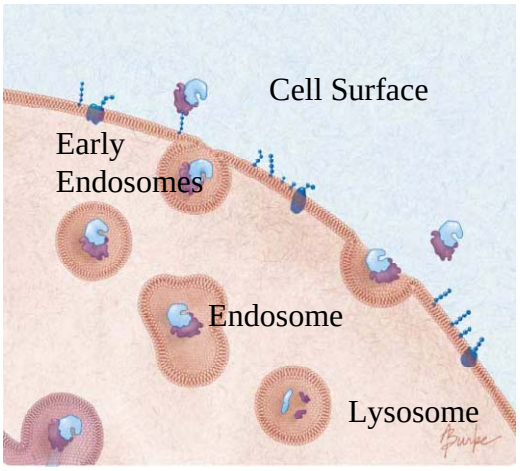
Once endocytosed, ricin undergoes retrograde transport to the endoplasmic reticulum (ER) [12]. *In vitro* experiments show that the majority of endocytosed ricin is found in the endosomes and the second largest proportion of toxin is found in lysosomes. Minor amounts are found in the Golgi stacks and trans Golgi network, in fact only 5% of internalized ricin reaches the trans Golgi network. It can therefore be assumed that the bulk of endocytosed ricin is destroyed and only a small fraction is responsible for its cytotoxicity [13]. Ricin that is not directed to the lysosome is shuttled from the endosome to the trans Golgi network with the assistance of the phosphatidylinositol 3-kinase hVps34 and the sorting nexins SNX2 and SNX4 [14].*In vitro* studies using RNAi knockdown have shown that SNARE complexes are also needed for the effective

retrograde transport of ricin; in fact, knockdown of syntaxin 5 or syntaxin 16 decreases the effect of ricin on protein synthesis [15].

Ricin, like other Type II RIPs, acts on ribosomes in the cytosol. It has been found that ricin enters the cytosol *via* the ER, as proven in studies where the attachment of a KDEL sequence (KDEL is an ER retention sequence, standing for lysine-aspartic acid-glutamic acid-leucine) to ricin increases its toxicity [16]. Once in the ER, protein disulphide isomerase reduces the ricin holotoxin into RTA and RTB [17]. This exposes a hydrophobic portion of RTA which is critical for its interaction with the ER membrane. RTA then unfolds in the lumen of the ER, potentially making it a target of the ER-associated degradation pathway (ERAD); ERAD is an endogenous system which deals with misfolded proteins in the ER [18]. Ricin toxicity has been shown to be dependent on the ER degradation enhancing α-mannose I-like protein (EDEM), a member of the ERAD pathway. Ricin has also been found to interact with Sec61, an ER protein translocator [19]. It is therefore presumed that these two proteins play a role in the retrotranslocation of RTA. Finally, once RTA is unfolded and transported to the cytosol, it must refold into a productive enzyme. This refolding is orchestrated by various proteins. Specifically, BAG-2 and Hip promote the folding of an active RTA, while Hsc70 and Hsp 90 (which are activated by

BAG-1, Hop and CHIP) promote folding of an inactive RTA [20]. Once the properly refolded RTA is in the cytosol, it irreversibly inactivates ribosomes by acting as an N-glycosidase and cleaving an adenine at position 4324 in the 28S rRNA of the 60S ribosomal subunit [21]. A general schematic of ricin trafficking through the cell is shown in **Figure 1.2**.

A



B

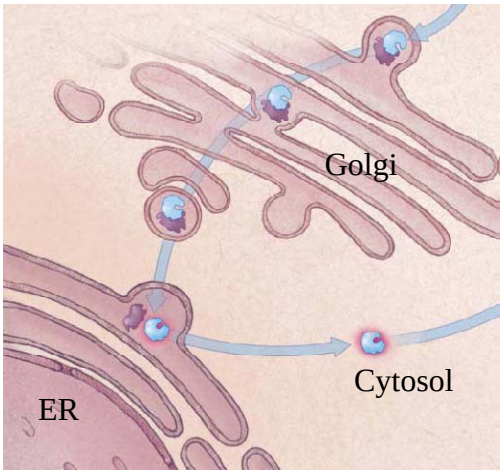


Figure 1.2: Ricin Trafficking RTA is shown in blue and RTB is in purple. (A) RTB allows for entry into the cell by binding to cell surface glycolipids and glycoproteins, which is followed by receptor mediated endocytosis; (B) following entry, the holotoxin undergoes retrograde transport and is reduced in the ER, RTA is then released into the cytosol where it can act on ribosomes. Figure modified from [2].

Ricin Toxicity in Humans and Animals

The specific mechanism of ricin toxicity in animals and humans has not been elucidated and cause of death is generally described as multi-organ failure. Studies of ricin intoxication in humans are rare, therefore much of our understanding of ricin intoxication comes from animal studies. Importantly, the symptoms of ricin intoxication are dependent upon the route of exposure. Since ricin would most likely be encountered by ingesting castor beans, by food/water poisoning or by release of ricin powder into the air, toxicity *via* ingestion and inhalation are the most extensively studied routes of exposure. Topical administrations of ricin, have shown no toxicity so it will not be covered here[22].

Injection – Following injection, toxicity is observed both at the injection site and systemically. Locally, there is tissue necrosis. Systemically, ricin causes hepatocellular necrosis, elevated liver enzymes and liver failure. In addition to the liver, there is rhabdomyolisis, acute kidney failure and hemolysis. A more general symptom of ricin intoxication following injection of the toxin is systemic inflammation which will cause fever and hypotension. These symptoms are reflected in serological tests following exposure where there are alterations in liver transaminases, amylase,

creatinine kinase, bilirubin, myoglobin. These serological tests also suggest that there is damage in the gut [2] [23]. In mice the LD_{50} of injected ricin has been reported to be 10 µg/kg [24].

Ingestion - Toxicity following ingestion varies greatly from one individual to another due to differences in gastric uptake which is most likely influenced by the amount of food present in the gastrointestinal tract or the degree to which the seed is chewed and processed. In animals, LD_{50} values have been reported to be 20 mg/kg [22], but when animals are fasted the LD_{50} is 10 µg/kg [24].

Following ingestion of ricin, there are also both local and systemic reactions. In the gut there is necrosis and apoptosis of the epithelial cells which leads to intestinal hemorrhagic lesions and inflammation. These can then cause diarrhea, infections of the gut, nausea, vomiting and abdominal pain. Diarrhea can cause a significant loss of fluid which can lead to electrolyte imbalance, dehydration, and hypotenion which and eventually kidney failure. Systemic effects of the toxin are seen most significantly in the liver in the form of hepatitis with hepatocellular death [25]. In one case of ingestion of castor seeds by a child, vomiting was observed in addition to an increase in liver enzymes, which went down

with time [26]. These alterations to liver function can lead to liver failure and eventual death [2]. The presence of systemic symptoms indicates that ricin escapes from the gut and enters the blood[27].

Inhalation – Aerosol is the most toxic route of ricin exposure. Importantly the size of the aerosol particle correlates inversely with the severity of lung damage. The smaller particles can travel deeper into the lungs thereby causing more damage [2]. Following exposure the aerosolized ricin, the bulk of the damage is seen inside the lungs, suggesting that little ricin escapes from themucosa. The predominant pathologies in the lungs are necrosis and inflammation. These will lead to non-cardiogenic pulmonary edema and infiltration of immune cells. Subjects die of respiratory failure. Despite the localization of ricin to the lungs, systemic inflammation is still observed and leads to arthralgias. Understandably, it has been observed that pulmonary exposure to ricin upregulates genes involved in inflammation and tissue remodeling, as well as various cytokines and chemokines [28] [29]. The LD_{50} of inhaled ricin has been determined to be 4 μg/kg in mice [24].

Attempts to treat and/or protect animals from ricin intoxication have been numerous and varied and fall into three categories: (1) post exposure

passive immunization, (2) post exposure treatment with small molecules, and (3) prophylactic immunization. Post-exposure administration of anti-ricin antibody is highly effective but it must be given within hours of exposure, before there are symptoms of intoxication [30-32]. Unfortunately these symptoms mimic those of many other diseases and therefore would not be easily recognized in the setting of bioterrorism. Inexpensively manufactured small molecule inhibitors of ricin have also been studied and have yielded promising results when tested *in vitro*. However, these inhibitors would also require administration soon after exposure. In addition, the majority of them have not yet been studied in animals, and those that have do not confer 100% protection [33-34]. Most recently, a paper describing a newly discovered small molecule inhibitor of ricin was published, though it requires prophylactic administration of 200 mg/kg in order to protect from an intranasal (IN) administration of an LD_{90} of ricin. This dose is completely impractical for any real-life setting [35]. Prophylactic immunization provides the most reliable method for inducing protection against ricin toxicity.

PART B. VACCINATION

Mechanism of Vaccine Protection

Vaccines work by inducing an immune response against an innocuous antigen that then protects individuals against a pathogenor toxin. To this end, vaccines are designed to deliver protein or carbohydrate epitopes of a pathogen or toxin. Once this antigen is delivered it is taken up by dendritic cells (DCs) that then traffic to the regional lymph node (LN) and present the antigen to resident T cells. Ideally, this will induce a robust cellular and humoral immune response against the antigen, so that when the individual encounters the active pathogen and/or toxin, the blood will contain neutralizing antibodies and antigen specific killer T cells while the LNs and bone marrow will contain memory cells for a rapid secondary response. Long lived plasma cells can also reside in the bone marrow and secrete antibody for years [36]. In general, vaccines can rely on one of two major mechanisms to fight infection, including either $CD8^+$ cytotoxic T cells or Th_2 mediated antibody responses from activated B cells. Historically, vaccines aimed at improving $CD8^+$ T cell responses are relatively new and no FDA approved vaccines work solely by this mechanism. For the

purpose of this body of work, we will focus on antibody mediated protection.

Antibody Mediated Protection

Historically, vaccines are designed to induce high levels of long lasting, high affinity circulating antibody in order to confer protection. These antibodies will then quickly neutralize toxins or pathogens when they enterthe host, preventing any damage or infection that could be caused by the binding of infectious agents or toxins to target cells.

Antibodies have two distinct regions, the Fc and Fab' regions (**Figure 1.3**); there is one Fc region and two identical Fab' regions commonly referred to as $F(ab)_2$. The Fab' regions recognize antigen while the Fc portion mediates the downstream functionality and isotype classification of the antibody. Different antibody isotypes mediate antigen clearance differently. Class switching is the process by which different isotypes are produced and is the result of irreversible DNA splicing of heavy chain DNA in a B cell clone. The four isotypes of secreted Ab are as follows: IgM, IgG, IgA and IgE. There are also subclasses of IgG and IgA.

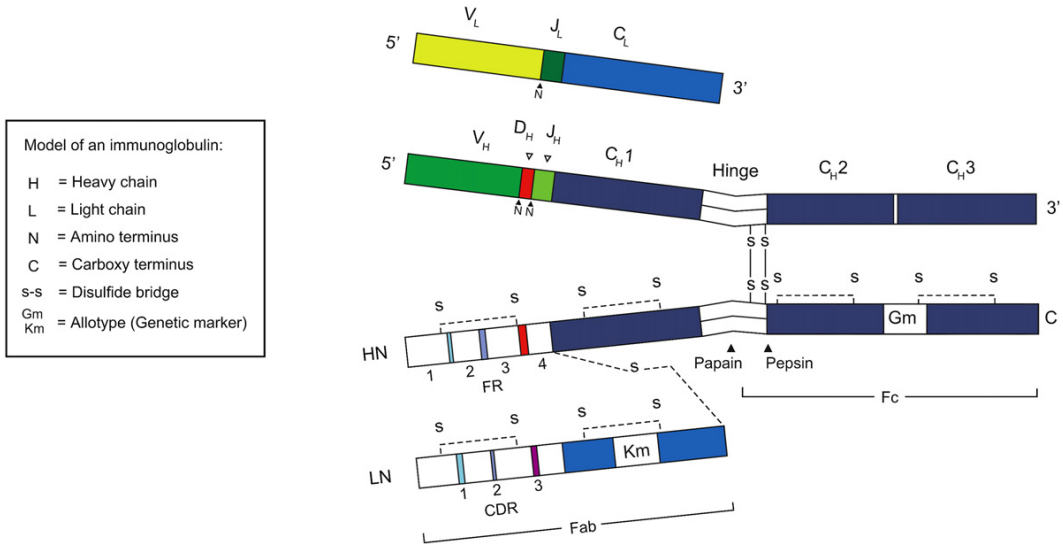


Figure 1.3 The structure of an antibody. The antigen binding portion is referred to as the Fab; shown in white, green, yellow, light blue. The isotype and downstream functionality of the antibody is referred to as the Fc portion, shown in dark blue. Figure from [37].

The portion of DNA encoding the IgM Fc portion is closest to the VDJ regions of the DNA which encode the specificity of the Fab regions of the antibody. As such, the expression of IgM requires no class switching and is therefore the first antibody isotype to be expressed during an immune response. The levels of IgM in the serum are generally highest immediately after antigen exposure and wane with subsequent exposure; this is aided by its relatively short half life of just 10 days. Secreted IgM associates into a pentamer *via* disulfide bonding in the constant regions. The pentamer is also associated with the joining-chain (J-chain). IgM activates the complement pathway which mediates inflammation. Due to its size, IgM is intravascular and rarely seen at the mucosa. [38] [37].

The most prevalent antibody in the serum is IgG. Unlike IgM, it is secreted as a monomerand is composed of 4 subclasses. In humans, these are IgG1, IgG2, IgG3, IgG4 and in mice they are IgG1, IgG2A, IgG2B and IgG3. While their Fc portions are relatively similar to one another, the various IgG subclasses mediate different responses. In mice the IgG subclasses fix complement to varying degrees, with IgG2A being the strongest, then IgG2B, then IgG3 and lastly IgG1. As such, IgG1 is the most prevalent subclass of IgG found at the mucosa.

The most prevalent antibody in the body is IgA. IgA is found largely at the mucosa and in various bodily secretions (tears, breast milk, sweat etc.) in the form of a dimer; this dimer is also joined by a J-chain. In the serum IgA is a monomer. Mucosal IgA is produced largely by plasma cells residing on the basolateral side of the mucosal epithelium. These cells secrete dimeric IgA which then binds to the polymeric Ig receptor (pIgR) which translocates it across the cell, depositing it into the mucosa; a portion of the pIgR called the secretory component remains associated with the IgA dimers; this complex is referred to as secretory IgA (sIgA). IgA does not fix complement and therefore does not induce inflammation in the mucosa, although it can be opsonized. Most importantly, the binding of sIgA can inhibit antigen binding to the mucosa [37-38]

The least prevalent Ab in the serum is IgE. IgE is generally found on the surface of mast cells, basophils, Langerhans cells and eosinophils which engage the IgE with very high affinity *via*their FcεRI. Engagement of FcεRI bound IgE by antigen leads to degranulation and inflammation which is often the cause of allergic and asthmatic reactions. In addition to mediating allergy and asthma, IgE plays an important role in fighting infections with parasites [37].

Protection at the Mucosa

Historically, vaccines have been designed to induce high levels of long lasting, high affinity circulating antibody. An additional concern in vaccine design is that the most likely routes of exposure to a pathogen/toxin best be protected. In the case of ricin, this would be the respiratory and digestive tracts. The mucosal immune system works slightly differently than the systemic immune system.

In the serum, IgG antibodies are the most prevalent, while at mucosal surfaces (lung, gut, nasal and genito-urinary mucosa) sIgA is the prevailing antibody. It is most important that a vaccine meant to protect against a toxin elicit antibodies that can disrupt toxin/pathogen entry into the cell and facilitate clearance by the reticuloendothelial system (RES).

Generally, it is thought that the best method for inducing a mucosal immune response is through immunization at mucosal surfaces. This dogma stems from the fact that the mucosa has its own specialized secondary lymphoid organs, such as the Peyer's patches and mesenteric lymph nodes associated with the gut. Similarly, the lungs and nasal passages have bronchus and nasal associated lymphoid tissues around the lungs and nasal passages. These secondary lymphoid tissues function

in the same manner as regional lymph nodes. Specifically, mucosa sampling DCs carry antigen to the mucosal associated lymphoid tissue where T and then B cells are primed. Plasmablasts can then migrate back to the mucosa where antibodies will be readily transported to the mucosa. It is commonly thought that systemic immunization would only produce a very weak immune response at the mucosa.

Types of Vaccines

There are many types of vaccines that rely on different mechanisms of action in order to confer protection and each has advantages and disadvantages. The following vaccine types will be reviewed here: (1) live attenuated including vectored vaccines, (2) dead vaccines including protein/subunit vaccines, conjugate vaccines and DNA vaccines.

Live Attenuated Vaccines

Live attenuated viruses or bacteria are highly homologous non-virulent counterparts of virulent pathogens. They have been engineered or manipulated to be harmless. The most widely administered and well known live attenuated vaccines are BCG, Influenza (Flu-Mist), measles/mumps/reubella (MMR), oral Polio and varicella. The advantage

of these vaccines is that they induce strong $CD4^+$ and $CD8^+$ T cell immune responses, inducing both humoral and cellular immunity. They also induce herd immunity, such that one person infected with an attenuated virus has the potential to infect, and thereby vaccinate, many other people with the attenuated virus as well. Live attenuated viral vaccines can be relatively expensive to produce because the virus must be grown in tissue culture. Live viruses cannot be administered to immune compromised patients because their immune systems cannot safely fight even an attenuated pathogen. In some cases, the organism can revert to a virulent form and cause disease [39].

Vectored vaccines

Another type of live vaccine is vectored vaccines. Vectored vaccines are viruses which have been modified to be non-pathogenic while carrying and expressing surface proteins from a pathogenic virus of the same family. One such example of this is a dengue fever vaccine which is under development. It uses an attenuated yellow fever virus as a vector, into which dengue antigens have been added [40].

Dead Vaccines

Protein and subunit vaccines

Protein and subunit vaccines consist of toxins or molecules from pathogens that are rendered non-toxic by treating them with heat, chemicals, or by site directed mutagenesis. In theory, protein vaccines more robustly activate the humoral arm of the immune system since they are not found in the cytosol for MHC I processing and subsequent $CD8^+$ T cell priming, although they can be cross-presented by DCs. On the other hand, protein vaccines are easy to produce and in the case of mutagenized proteins, are very safe.

Conjugate vaccines

Conjugate vaccines make use of two or more covalently-linked antigens . They are most important in childhood vaccines due to an inability of children to respond to certain antigens, particularly carbohydrates. Hemophilus, meningococcus and pneumococcus vaccines are all conjugate vaccines. These vaccines consist of bacterial capsular polysaccharides that are easy to produce and good antigen targets for the humoral immune system. These polysaccharides normally elicit a T cell-independent immune response. Unfortunately, in children T-independent responses are weak, while T-dependent responses are much stronger. Therefore, vaccine manufacturers have conjugated these T cell

independent antigens to T cell dependent antigens such as tetanus toxoid so that they behave as “haptens” and induce a T cell dependent response to the capsular polysaccharide.

DNA vaccines

DNA vaccines consist of DNA plasmids encoding antigens of pathogens. It was found that this injected DNA can be transcribed and translated into protein that is taken up by antigen presenting cells. This provides a very inexpensive method of vaccine production. Unfortunately, it was found that antibody production in response to the proteins encoded in these plasmids was not robust in primates. More recently, attempts have been made to improve the delivery method and to engineer adjuvant sequences into the DNA.

Adjuvants

The purpose of an adjuvant is to improve the immune response to a vaccine. To this end, it can do one of several things, including boosting antibody production, reducing the dose of vaccine needed to protect, reducing the number of vaccine boosts needed, increasing the percentage of individuals protected, and extending the longevity of a response.

Scientists have been trying to boost immune responses for at least 100 year by adding adjuvants to antigens. Everything from agar to bread crumbs have been tested [41] The most promising adjuvants will be reviewed.

Toll-Like Receptor (TLR) Ligand Adjuvants

TLRs are transmembrane receptors found on and in many cells of the immune system (e.g. macrophages, DCs, B cells, and neutrophils) and recognize motifs found in bacteria, viruses, fungi and parasites. To date, there are 11 known TLRs which work either as homo or heterodimers. Upon engagement, TLRs cause the production of inflammatory cytokines and up regulate the expression of MHC molecules and co-stimulatory molecules. Their engagement has also been found to increase cross presentation and memory CD4 T cell production in the draining lymph nodes[42]. These characteristics are advantageous in augmenting the immune response to a vaccine. The most promising and extensively tested TLR adjuvants are discussed below.

(i) Lipopolysaccharide (LPS) - LPS is found on the surface of gram negative bacteria, and it binds to TLR 4. The active component of LPS, lipid A, has been extensively studied as an adjuvant. Chemical

modification of the lipid A molecule of *Salmonella minnesota* rendered it non-toxic while maintaining its activity as an immune activator; this modified molecule is monophosphoryl lipid A (MPL). Extensive studies using MPL as an adjuvant have been carried out and it has been found effective and safe in both prophylactic and therapeutic vaccines. It has been approved for use in a Hepatitis B vaccine in Europe [41].

(ii) Bacterial DNA - Bacterial DNA, as compared to human DNA, contains unmethylated CpG motifs at a far greater frequency. TLR 9 is an endosomal receptor that recognizes CpG, and synthetic oligodeyoxynucleotides (ODNs) analogues to CpG have been explored for their use as potential vaccine adjuvants. ODNs do not always elicit an adjuvant effect, but when they, do they are robust. They provide excellent protection at mucosal surfaces when administered intranasally or orally, by inducing significantly robust antigen specific antibody titers, increasing the avidity of those antibodies and protecting animals. Clinical trials in humans have shown varying results; ODNs increase the immunogenicity of vaccines in most cases, but not all. There are several safety concerns associated with ODNs including fears of autoimmunity, tissue damage and increasing susceptibility to toxic shock as a result of over stimulation of the immune system. In animals, when high and/or extensively repeated doses

of ODNs have been given, serious pathology was observed. However, when lower doses, consistent with adjuvant administration, were given such side effects were not noted, either in animals or humans [41].

(iii) Other TLR Adjuvants - TLR 3 and TLR 5 ligands have also been explored as potential vaccine adjuvants. TLR 3 is also found in the endosome and recognizes viral double stranded RNA (dsRNA). Initial studies used a synthetic analogue of dsRNA, poly I:C that was found to be toxic to patients in Phase I and II clinical trials. Modifications of the molecule have since been made and animal studies look promising [43]. Flagellin, a component of bacterial flagella, is a TLR 5 ligand and has been fused to various protein antigens. Studies in animals showed increased antibody titers and cytotoxic T cell activity [43].

Oil-in-Water Emulsions

Oil-in-water emulsions have been used as adjuvants for many years. The first was developed by Freund in the 1930s. Both complete Freund's adjuvant (CFA) and incomplete FA (IFA) consist of 10% mannide monooleate either with or without killed mycobacteria, respectively. Freund's adjuvants were found to be much more potent than alum adjuvants (to be discussed in the next section). However, studies have

determined that both CFA and IFA were not safe for human use due to induction of severe injection site reactions [41].

More recently, oil-in-water emulsions have been revisited and new formulations have been quite successful. MF59 is an oil-in-water emulsion where the squalene oil component is combined with the surfactants Tween 80 and Span 85 for stabilization in addition to citrate buffer [41]. MF59 has been shown to have effects that are similar to most other adjuvants, namely the ability to induce innate immune responses at the site of injection by recruiting and activating DCs [44] [45]. MF59 is safe both in humans and rodents and increases the immunogenicity of a variety of antigens. MF59 has been approved for use in Europe [46].

Aluminum Hydroxide/Aluminum Phosphate Adjuvants

Aluminum hydroxide and aluminum phosphate adjuvants, commonly referred to as alum, are the only adjuvants currently approved in the United States. Alum is a common component of most dead vaccines.

Alum was originally used as a vaccine adjuvant nearly 100 years ago, though its mechanism(s) of action are still not completely understood. The fact that proteins adsorb onto alum *via* electrostatic attraction is one

possibly reason that alum works so well. It is thought that this causes the protein to remain at the injection site and slowly leak into the circulation and hence provides continuous immune stimulation; this is commonly referred to as the “depot effect” and has been supported by results from experiments showing the presence of antigen at the injection site for up to weeks [41]. The effect of alum on DCs has also been studied, and shown that alum recruits and activates DCs at the site of injection [42]. Recently, studies investigating the potentiating effect of alum in inflammation have implicated the NALP3 inflammasome in the process. These reports further support the notion that the innate immune system is triggered when antigen plus alum is given to mice. NALP3 is a member of the nucleotide-binding oligomerization domain-like receptor family known as NOD-like receptors (NLRs) which, similar to TLRs, sense danger [47]. Additionally, it was seen that adaptive immune responses were altered in NALP3 deficient mice, but the present data in this area is inconsistent on whether NALP3 is necessary for an adaptive immune response [44].

In general, administration of vaccines in alum is thought to be a safe, reliable method of boosting vaccine specific immune responses.

Routes of Vaccine Administration

The most extensively used routes of vaccination are oral/nasal, sub-cutaneous (SC), IM and ID; each serves a different immunological purpose.

Oral/Nasal – Oral and nasal administration of vaccine are generally reserved for live attenuated vaccines. The rationale is that most harmful pathogens and toxins are generally encountered at mucosal surfaces and would most likely be captured and eliminated by mucosal sIgA. It is therefore hypothesized that by inducing an immune response at mucosal surfaces that the mucosal sIgA response would be robust. Mucosal doses of vaccines would be very easy to administer. Since vaccination with a live attenuated vaccine leads to viral replication, it is possible for vaccine infected individuals to “infect” others, thereby vaccinating them as well. Due to the potential advantage of boosting mucosal immunity, there have recently been numerous attempts to administer protein vaccines *via* the mucosa. The major issue with mucosal vaccines, particularly in the gastrointestinal (GI) tract, is that they often require specialized adjuvants in order to facilitate uptake and avoid degradation in the harsh environment of the GI tract, making it a difficult option for dead vaccines [48].

Subcutaneous – SC vaccination is also reserved for live attenuated vaccines. It is thought to provide a good physiological space for viral/bacterial replication which will lead to systemic immunity. This route of vaccination has been evaluated for delivery of protein vaccines, but it was seen that administration of alum at this site was not well tolerated and it was not as immunogenic as IM administration [49].

Intramuscular – IM vaccination is the most common route of administration for dead and subunit vaccines both with and without alum. Antigen injected into the muscle is taken up by resident DCs to initiate an adaptive immune response.

Intradermal – Smallpox and BCG are the only two vaccines commonly given ID; these are both live attenuated vaccines. While there are no dead protein vaccines approved for ID administration presently, ID and transcutaneous administration of vaccines have been studied as an alternative route for administering protein vaccines [50]. Transcutaneous refers to administration of vaccine onto the skin such that it can migrate through the skin. ID administration of protein vaccines has been used to reduce the dose of some vaccines [51] [52] [53]. Dose sparing and enhancing immunogenicity have been found to be particularly useful in

patient populations that are difficult to immunize, i.e. the elderly and hemodialysis patients [54] [55]. Additionally, ID vaccination can be done using a needle free ID gun which administers vaccine *via* a high pressure stream, thereby eliminating the cost involved with needles and needle disposal. While ID administration with a needle and syringe is a very precise technique requiring a trained healthcare professional, ID administration with an ID gun does not require skilled personnel. Previous designs of ID guns allowed for mass vaccination without the need to change vials or needles between vacinees [56]; unfortunately, this method lead to cross contamination and was implicated in the spread of disease and was therefore taken off the market. Newer designs of ID 'guns' have corrected this issue [57]. These guns can be adapted to inject liquid ID, SC or IM and have been used by diabetic patients to administer insulin and in children to deliver growth hormone (www.bioject.com).

PART C. EFFORTS TO MAKE A RICIN VACCINE

Efforts to protect people against ricin intoxication and morbidity have been ongoing for several decades, predominantly by the Department of Defense. As discussed earlier, due to the limitations of post exposure treatments, prophylactic vaccination provides the most efficient method of preventing ricin toxicity. In considering the necessary requirements of a ricin vaccine, the population that will receive the vaccine as well as the most likely route of ricin dissemination must be considered. First, ricin is generally thought of as a weapon of espionage or bioterrorism. Therefore military personnel and first responders will be the most likely population to benefit from a ricin vaccine. The method of dissemination that would affect the most people would be either contamination of food/water sources or release of aerosolized powder. Therefore, it is important that a ricin vaccine be easily administered, have the potential for dose sparing so that large amounts need not be made and that it protects mucosal surfaces. To that end, several attempts have been made to develop a ricin vaccine and the 4 most prominent are described.

(i) Griffiths et al.

This group's first attempt at a ricin vaccine utilized a ricin toxoid that they prepared by treating ricin holotoxin with 10% formaldehyde at 37ºC for 28 days. They administered 125 µg of toxoid per kg body weight *via* the SC route once every 3 weeks for 6 weeks, then challenged with a 5X LD_{50} 3 weeks after the last vaccination. The group worked with rats in these studies and did a test bleed on the day of each vaccination. Total antibody titers increased by 4 fold following each boost. Serum specific antibody isotypes after the first vaccination showed 55% IgG, 33% IgM and 12% IgA. By the time the third vaccination was administered, these proportions shifted away from IgM and toward IgG, although the percentage of IgA remained the same; isotype proportions were 79, 9, and 12 % of IgG, IgM and IgA, respectively. These results were not surprising given that IgM is the first antibody isotype produced an adaptive immune response, followed by IgG. Additionally, 100% of toxoid vaccinated mice survived aerosol challenge with what the group classified as a 5X LD_{50} dose of ricin (6µg toxin/kg body weight). These surviving mice did suffer respiratory distress immediately after challenge, but this resolved within one week. The group did not do histological analysis on the lungs of these mice [58].

Griffiths et al continued to work with their toxoid preparation, by combining it with either alum or encapsulating it in liposomes at either a high (500 µg

vaccine/kg body weight) or low (100 µg vaccine/kg body weight) dose. The vaccine regimen was reduced to 2 doses given 6 weeks apart. In an attempt to improve mucosal protection the vaccine was administered intratracheally (IT). Seven weeks following the last vaccination animals were challenged with a 3X LD_{50} of ricin *via* the IT route because the group did not have access to the large volumes of ricin needed for an aerosol challenge. Of the three treatment groups, (liposomal toxoid, toxoid + alum and toxoid alone), at both the high and low dose levels, liposomal preparations induced the highest antibody titers which were significantly higher than the alum group. Interestingly, at the high dose level the addition of alum did not induce significantly higher antibody titers as compared to toxoid alone. At the low dose level the addition of alum significantly enhanced the antibody response as compared to toxoid alone. This study also looked at Ab isotype distribution in the serum and bronchial alveolar lavage (BAL) of animals receiving the high vaccine dose of 500 µg vaccine/kg body weight. In the serum, their observations were similar to their previous paper, in that, IgM decreased with subsequent boosts, while the IgG proportions increased with each boost; IgA and IgE remained constant. In general, following two vaccinations, serum Ab isotype proportions were as follows: ~80% IgG, ~10% IgM, ~10% IgA, and ~3% IgE. The proportions of each Ab isotype in the serum did not vary

significantly between the liposomal, alum and non adjuvanted vaccine groups. Evaluation of Ab isotype in the BAL was only done 2 weeks after challenge, but at both the high and low dose levels. In general, BAL antibody titers among all isotypes did not vary significantly between the high and low dose level animals. IgG, IgM and IgE titers among the liposomal, alum and non adjuvanted vaccine groups were not significantly difference. The greatest difference in antibody titer was seen for IgA in BALs where the liposomal formulation induced significantly higher titers than the alum or non-adjuvanted vaccine groups. Regardless of dose level, survival following vaccination with liposomal and alum adjuvanted toxoid was 100%, while it was only 66% with toxoid alone. Theese investigators were concerned that their vaccine did not provide sufficient protection of lungs following aerosol ricin exposure. They therefore assessed lung function by visually determining the degree of labored breathing. Through these assessments they determined that animals receiving the liposomal vaccine did not suffer from labored breathing. Overall, based on Ab titers in the BAL, survival data, and subjective assessment of lung function, the group drew the conclusion that the liposomal delivery of toxoid *via* IT administration provided excellent protection from ricin related morbidity and lung damage [59].

While Giffiths et. Al. had determined that a liposomal administration of toxoid prevented ricin related lung damage and morbidity, the group attempted to develop an improved vaccine. To do this they investigated the usage of an RTA vaccine and compared it to their toxoid vaccine. They studied both immunogens with and without liposomes, and also compared IT to SC vaccine administration. Rats were again vaccinated twice then challenged *via* an IT administration of ricin; unfortunately the paper does not detail the time course of vaccine administration and subsequent ricin challenge. In comparing SC administration of toxoid *vs* RTA without liposome, they found that at the 100 μg/kg dose level, toxoid was superior in eliciting serum specific antibody titers 3 weeks after the last boost. They then did the same comparison, but following an IT vaccine administration at the 500 μg/kg dose level and again they found the toxoid to be superior in eliciting serum specific antibodies 3 weeks after the last boost. When liposome was added to the toxoid or RTA and subsequently administered IT at the 250 μg/kg dose level the two vaccine formulations were found to be equal in their ability to induce serum specific antibody titers 1 week following the last vaccination. They evaluated the BAL- specific Ab titers following challenge, finding again that the toxoid and RTA formulations were equivalent. Lastly, the group evaluated the Ab isotypes in the BAL of rats that were vaccinated with 250

μg/kg of toxoid or RTA with liposome then challenged with ricin. BAL was collected on days 1, 3, 7, and 14.Compared to the RTA preparation, the toxoid preparation induced similar proportions of IgE and IgM (1% and 0% respectively) in the BAL as compared to the RTA preparation. The major difference was seen in IgG and IgA levels, where vaccination with toxoid induced about 70% IgG and 30% IgA, while RTA vaccination induced 85-99% IgG and 1-15% IgA. Overall, the authors showed that toxoid in liposome was superior to RTA with liposome in its ability to induce a mucosal IgA response and that the vaccine was not inducing an allergic response (as indicated by low IgE levels) as ricin is known to do [60].

In 1999, Griffiths et al published their last paper on a ricin toxoid vaccine. They had already demonstrated the efficacy of the vaccine in inducing serum and BAL antibody titers and in protecting animals from aerosol and IT ricin challenge. In their final paper, they were interested in continuing the comparison between a ricin toxoid and RTA vaccine. Again, rats were given vaccine on days 0 and 21 and were challenged 1 week following the last vaccination with what they determined to be a 3X LD_{50} of ricin given IT. Since they were already confident in the vaccine's ability to protect the animals from ricin-related morbidity, they focused solely on lung damage. To do this, 3 parameters were studied in the lung following challenge: total

protein, total white blood cells, and total polymorphonuclear leukocytes. These comparisons were made between 3 vaccine groups: liposomal toxoid given IT, liposomal RTA given IT and liposomal RTA given SC. In general, they found that animals receiving liposomal toxoid, as compared to those receiving RTA by either route, had significantly less protein, white blood cells and polymorphonuclear cells in their lungs; in fact these data resembled those of sham vaccinated mice. There were no differences seen between liposomal RTA given IT *vs* SC. These results suggested that a toxoid vaccine was significantly better than an RTA vaccine in conferring mucosal protection following ricin challenge [61]. While these studies were promising, toxoids can develop some toxicity unless they are stored carefully. Specifically, it was found that if all the formaldehyde was removed from the toxoid, toxicity could return; therefore it is necessary to store the toxoid with a specific amount of formaldehyde which is not toxic to patients, but also prevents the toxoid from regaining toxicity [62].

(ii) US Army Medical Research Institute of Infectious Diseases (USAMRIID)

Since a ricin vaccine will be most needed by the military, extensive research into developing a ricin vaccine has been undertaken by the

USAMRIID. In the last 15 years they have published papers which have taken one of four approaches to a ricin vaccine: (1) administration of an anti-idiotype vaccine, (2) oral and intranasal (IN) administration of microsphere encapsulated ricin toxoid, (3) administration of deglycosylated RTA (dgRTA) or recombinant RTA with novel mucosal adjuvants, (4) vaccination with a truncated recombinant RTA both with and without alum.

In their earliest studies in developing a prophylactic treatment for ricin toxicity, USAMRIID developed an anti-idiotype antibody vaccine. The group already had access to a mouse anti-ricin antibody (BG11-G2), which they then injected into rabbits in order to raise rabbit anti-BG11-G2 antibodies. These antibodies were then administered as a vaccine to naïve animals *via* 5-50 µg intraperitoneal (IP) injections in alum, each given 3 weeks apart. Following the last vaccination, animals were challenged with various doses of ricin given *via* SC injection. While the anti-idiotype vaccine induced significantly greater antibody titers than the control, it only protected animals from a ricin dose of 20 µg of toxin/kg body weight. Doses of 35 and 50 µg of toxin/kg body weight killed all vaccinated animals. Animals that survived challenge did go on to survive

re-challenge. Given the cost and minimal efficacy of this vaccine, it would not prove to be a good method for prophylactic vaccination[63].

The next paper from USAMRIID took a totally different approach, though one that was similar to Griffiths'. They investigated the efficacy of a ricin toxoid vaccine administered with a microsphere delivery system/adjuvant. Specifically, they formalin inactivated ricin then encapsulated it in one of two microspheres: poly (lactide-co-glycolide) (PLGA) or polylactide (PLA). The paper investigated numerous aspects of vaccinology. First, they found that their PLGA adjuvant induced significantly higher serum IgG2A titers than toxoid alone following intranasal (IN) administration of vaccine.Two doses of 50 μg of toxoid in PLGA given three weeks apart was superior to one dose of 50 μg in PLGA in eliciting serum IgG2A. These results correlated well with survival following an aerosol ricin challenge with 60 μg of ricin toxin/kg body weight given 6 weeks after the last vaccination. 100% of animals receiving the two dose vaccine regimen in PLGA survived while only 70% receiving one dose survived. Additionally, fewer than 50% of those receiving either one or two doses of toxoid *without* adjuvant survived. Knowing that PLGA was protective in a 2 dose regimen, the group then compared PLGA to PGA using the two dose regimen and dropped their vaccine dose down to 25 μg of toxoid. Forty

weeks post vaccination, they found that toxoid with either PLGA or PLA was superior to toxoid alone in inducing serum specific IgG2A. No significant differences were noted between PLGA and PLA. In comparing survival between the two adjuvants and the two dosing regimens at the 25 μg dose level, it was found that PLGA protected 70% of animals in a single dose, and 87% of animals following two doses. Single dose administration of PGA was not successful in protecting animals from aerosolized ricin, with only 30% survival, but the two dose regimen protected 70% of animals. They then combined the two adjuvants and administer it in a two dose regimen with 50 μg of toxoid; then then waited 1 year to challenge with 60 μg of toxin/kg of body weight. They found that the combined adjuvant with 50 μg of toxoid was superior to a 25 μg dose level with only one of the adjuvants. Lastly, they investigated the Ab isotypes found in the lung and serum following either one or two doses of toxoid alone or toxoid administered with both PLGA and PLA. The most marked difference between the adjuvant and the control group was the presence of extremely elevated levels of serum IgG. Overall, the paper did an extensive review of the effects of PLGA and PLA as adjuvants, but they did not provide us any information regarding the preservation of lung function in vaccinated mice following aerosol challenge [64].

In USAMRIID's second and last paper to evaluate ricin toxoid as a vaccine candidate. Only PLGA was used as an adjuvant, and the investigation focused only on the efficacy and oral administration of vaccine. Their first comparisons were between a 7 x 15 μg oral vaccinations in PLGA (given on days 1, 2, 3, 28, 29, 30 and 49) and a single 15 μg SC dose in PLGA; they also had non adjuvanted controls for each group. In comparing serum specific total IgG, serum specific IgG2A, and serum specific IgA, the oral administrations with PLGA were equivalent to the SC administrations with PLGA; as expected the presence of PLGA induced significantly higher titers than without. Comparisons of survival following aerosolized ricin challenge 10 weeks postimmunization showed that both SC and oral administrations of toxoid in PLGA conferred 100% protection, while toxoid alone, regardless of vaccination route, did not protect more than 10% of animals. The group then compared two different oral dosing regimens (Schedule A: vaccination on days 1, 2, 3, 14, 15, 16, 30; Schedule B: vaccinations on days 1, 2, 14, 15, 30, 32, 32) with 25 μg of toxoid to a single SC administration with 15 μg of toxoid. Similar levels of serum specific IgG were elicited in all groups while only the SC group was 100% protected from an aerosol ricin challenge 10 days post immunization. Overall, their oral vaccine regimens required many boosts in order to confer minimal protection. Additionally, it required significantly more toxoid

than their SC administration. SC administration of toxoid in PLGA did appear to be efficacious, although they again did not evaluate lung function in vaccinated animals post aerosol challenge with toxin [65].

The next strategy attempted by USAMRIID was to use dgRTA along with a mucosal adjuvant called LTR72. LTR72 is a mutant of *E. coli* heat-labile enterotoxin. They administered two doses of vaccine *via* the IN route, 28 days apart, then challenged 19 days later with 60 μg of toxin/kg body weight *via* aerosol exposure. Initially, they did a dose titration of dgRTA alone, and found that 100% of animals receiving two doses of 40 μg of dgRTA were protected, and only 50% of mice receiving two doses of 20 μg were protected. Serum antibody responses were dose dependent. Based on these results, they chose to administer 8 μg of dgRTA in increasing doses of LTR72, specifically 4, 2 and 1 μg. Holding the vaccine dose constant, the group found that the antibody response was dependent on the adjuvant dose both in the serum and the BAL and that production of neutralizing antibodies was also dependent on the adjuvant dose. Ninety percent of all animals receiving 8 μg of dgRTA with adjuvant survived, while those not receiving adjuvant died. Evaluations of adjuvant related pathology in the nasal passage, lungs, central nervous system (CNS) and lymph nodes showed only mild, transient reactions to the

dgRTA LTR72 vaccine. Following challenge, vaccinated animals showed significantly less pathology than unvaccinated animals, but those receiving adjuvant showed equal pathology to those receiving unadjuvanted dgRTA. This implies that while LTR72 decreases ricin related morbidity, it does not decrease ricin related lung damage[66].

The group continued studies with dgRTA, and compared LTR72 and LTR63. LTR63 is a different mutant of the heat labile E.coli enterotoxin. The studies in this paper were similar to those in the last paper with some exceptions. A three dose vaccine regimen (vaccinations on days 0, 28, 56) given IN was used, followed by a test bleed 20 days after the last vaccination, and aerosol challenge with 60 μg/kg at 22 days after the last vaccination. When 40 μg of vaccine was administered with increasing doses of LTR72 and LTR63, the two adjuvants elicited similar levels of serum IgG. LTR63 induced serum IgA and neutralizing antibody. The two adjuvants also protected animals from ricin toxicity equally well; >80% of mice receiving vaccine plus adjuvant survival. Histological analysis following LTR63 showed that it was safe inducing only minimal to mild lesions in the nasal passages. Following challenge, animals still had lung damage as determined by histological analysis following vaccination with dgRTA in either LTR72 or LTR63. While the group has shown that

application of dgRTA as a vaccine is efficacious, they were not able to protect animals from ricin related lung damage. This vaccine still maintains the vascular leak inducing site found in RTA [67].

In a recent change in strategy, USAMRIID has studied a truncated RTA vaccine (RTA 1-33/44-198) in which a surface loop and the C-terminus of recombinant RTA were removed (**Figure 1.4**). The group preferred this design for a ricin vaccine due to its increased stability which was conferred when then hydrophobic C-terminus was removed. They vaccinated animals with 10 μg of their vaccine either with or without alum *via* the IM route once every 4 weeks for 8 weeks. Four weeks after the last vaccination, mice were challenged with a 10X LD_{50} of either IP or aerosolized ricin. Regardless of the presence of adjuvant in the vaccine or the challenge route, 100% of animals were protected from ricin related death. While this was shown to be efficacious, this group did not evaluate

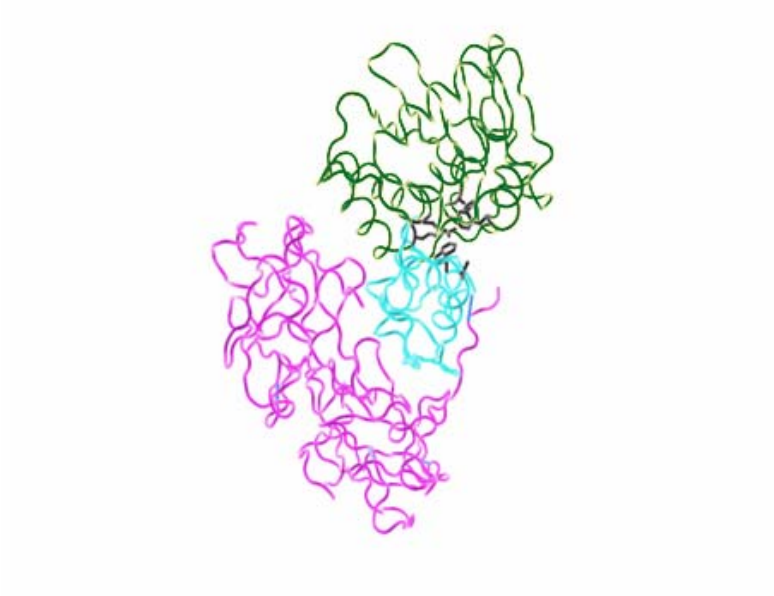


Figure 1.4: USAMRIID Truncated Vaccine. Blue + green = RTA, purple = RTB, green alone = USAMRIID truncated ricin vaccine construct

lung function following aerosol challenge. And they did not look look at injection site toxicity which is important given that RTA's vascular leak inducing site was still intact[68]. To continue this work, the group then investigated the storage buffer that would allow optimal adsorption of their vaccine onto alum while also remaining stabile and potent following various storage conditions. They found that storage in a succinate buffer, as compared to a phosphate buffer allowed for better alum adsorption over time and that the new formulation was more immunogenic in animals [69].

(iii) Marsden et al.

Another attempted ricin vaccine was described by Marsden et al. [70]. This group took a cue from nature where a Type III RIP is produced as a zymogen that has an amino acid insert which interferes with the active site of the RIP. The vaccine candidate was a recombinant RTA containing a 25 amino acid inactivating insertion administered in alum. Rats received 2 vaccinations of 100 μg per kg of body weight at 3 week intervals. Six weeks following the last injection, animals were challenged with a 5X LD_{50} of ricin administered *via* the IT route. 100% of animals receiving this

regimen survived challenge, but residual catalytic activity made it an unlikely candidate for a human vaccine [70].

(iv) RiVax

The Vitetta lab has developed a recombinant mutant RTA vaccine called RiVax that emerged from their work on immunotoxins. Using dgRTA conjugated to antibodies (immunotoxins) as cancer therapeutics, it was found that the dose limiting side effect was vascular leak syndrome (VLS) which caused a variety of symptoms including hypoalbumenimia, edema, decrease in blood pressure and weight gain [71]. Subsequent work to delineate the cause of immunotoxin-induced VLS mapped it to an (*x*)D(*y*) motif at amino acids L74, D75, V76 [72]. RiVax, was designed to eliminate toxicity mediated by both the ribotoxic and VLS-inducing sites. Three combinations of active site and VLS site targeted mutations were considered and all were assessed to be safe and immunogenic. But due to issues of yield and ease of production, the Y80A/V76M RTA mutant was selected for extensive development (**Figure 1.5**) [73]. This mutant lacks both ribotoxic and VLS-inducing activity, but is still immunogenic and retains its native structure as determined by X-ray crystallography [74].

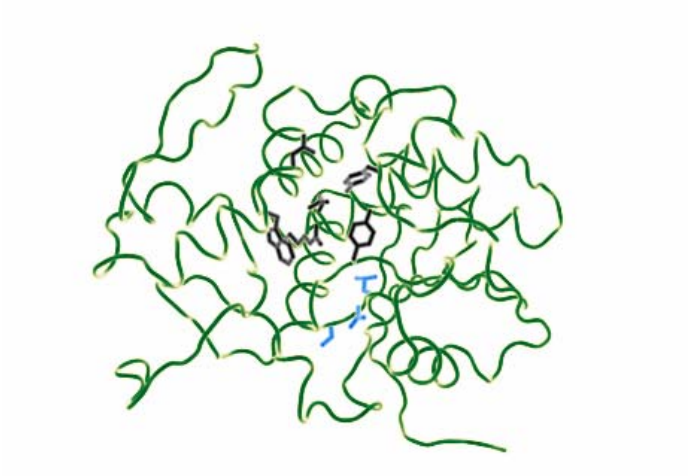


Figure 1.5: RiVax. Mutated RTA; black residues show the ribotoxic site, blue residues show the VLS-inducing site

Aside from toxicity, the lab was primarily concerned with developing a vaccine that would provide a robust antibody response to ricin toxin, therefore it was important to preserve the B cell epitopes when designing a vaccine. The dominant B cell epitope of RTA is at residues 163 to 174 [75] [76]. Since RTB is responsible for cell entry, one would consider that an RTB vaccine would be most advantageous. However, it has been found that RTB is less immunogenic than RTA RTB vaccines elicits significantly less specific antibody than RTA vaccines and do not protect animals from ricin challenge as well as an RTA vaccine [77].Therefore our lab chose to make a recombinant RTA vaccine. The goals in designing this vaccine were to induce not only specific antibodies, but also neutralizing antibodies. Whether neutralizing antibodies prevented endocytosis or celluar processing of ricin was not considered, so long as they provided protection in an in vitro cell culture model of ricin poisoning. In this study, neutralizing antibody titers were not studied due to technical difficulties, but our protection data suggest that neutralizing Abs are elicited. Protection of animals was the main goal, and while passive protection experiments with RiVax specific antibodies have been done, the only mechanism of antibody protection that has been studied is neutralization. Therefore, it is possible that RiVax induced antibody facilitates clearance of ricin by the RES.

In initial studies with RiVax, animals were vaccinated IM once per week for 4 weeks with 0.5 µg of RiVax/g body weight. One week following the last vaccination, animals were challenged *via* IP injection with a 10X LD_{50} of ricin (100ng toxin/g body weight). 100% of vaccinated animals survived [73].

With a potentially efficacious and safe vaccine in hand more extensive safety and immunogenicity testing was carried out. It was found that administration of 10 µg of RiVax IM once per week for 4 weeks protected mice from an IP challenge with 10X LD_{50} dose of ricin given one week after the last vaccination. Examination of the injection site in mice showed no long term pathology following IM vaccination with RiVax.
Vaccination with extremely high doses or repeated doses of RiVax did not result in significant weight loss in mice. The same was true when these studies were carried out in rabbits. While rabbits in these studies only received IM doses of vaccine at 10 or 100 µg, they were sacrificed and their serum neutralizing titers were determined. Neutralizing titers were found to be dose dependent. Rabbit sera combined with ricin, and injected IP into mice demonstrated that the sera contained protective antibodies. While these studies showed the efficacy of RiVax against an IP ricin

challenge, it still needed to be tested in the more realistic setting of a mucosal ricin challenge [78].

With the safety data from the previous paper, the lab began Phase I clinical trials. All volunteers received 3 IM doses of RiVax without alum at monthly intervals; the three doses administered were 10, 33, and 100 μg. No volunteers reported serious side effects in response to the vaccine. 1/5, 4/5 and 5/5 volunteers seroconverted in the 10, 33, and 100 μg dose levels, respectively. All volunteers who seroconverted also had neutralizing antibodies. Unfortunately, titers waned with time, and by 250 days post immunization, serum titers were extremely low. As in the previous paper, passive protection experiments were undertaken. Various dilutions of sera from subjects in the middle and high dose groups were combined with the equivalent of a 5X LD_{50} of ricin, then injected IP into mice. Mice receiving 62.5 and 25 μg of antibody survived, while those receiving 12.5 and 5 μg did not. Here it was clearly shown that RiVax without adjuvant is safe and immunogenic in humans though serum antibody titers wane with time [79].

The next step was to define how well RiVax protected mucosal surfaces while also doing a dose titration of the vaccine. To do this, animals were

vaccinated IM with 10, 3.3, 1.0, 0.3 and 0.1 μg of RiVax once every 4 weeks for 8 weeks. One to two weeks following immunization, animals were challenged with a 10X LD_{50} administered by either IP injection, gastric gavage or aerosol. Evaluation of serum specific antibody titers showed a clear dose response curve. Following IP ricin challenge, 100% of mice from the 10, 3.3 and 1.0 μg dose levels survived, while fewer than 50% survived in the 0.33 and 0.1 μg dose levels. Animals in surviving groups lost no more than 10% of their body weight. 100% of animals challenged *via* gastric gavage in the 10 and 3.3 μg dose level survived, while ~75% of those in the 1.0 and 0.33 μg dose level survived and only 20% in the 0.1 μg dose level survived. Again, surviving animals lost no more than 10% of their body weight. Lastly, following aerosol ricin challenge, 100% of animals in the 10 and 3.3 μg dose level survived, 80% in the 1.0 μg dose level survived and fewer than 30% of those in the 0.33 and 0.1 μg dose level survived. Animals in surviving groups lost no more than 10% of their body weight. Lung function on days 1, 2, 3, 4, 7, 10, and 14 post-challenge was also accessed *via* whole body plethysmography There was a dose dependent maintenance of lung function following aerosol ricin challenge. There was also a positive correlation between lung function and serum specific antibody titers. Overall, this study showed that

doses of 1.0 μg and above protect mice from both systemic and mucosal ricin challenge [41].

Most recently, our group has tested the stability of lyophilized RiVax and shown that it remains active for at least 1 year following storage at either 4^{o}C or 25^{o}C. This paper also showed that a 10-fold lower dose of IM administered RiVax on alum (ie 1.0 and 0.1 μg) protects mice from a 10X LD_{50} of ricin delivered *via* IP injection [80].

PART D. STUDY OBJECTIVES

The goal of this study was to determine whether ID administration of RiVax both with and without alum could be advantageous compared to IM administration, and if so, to try to understand why. The specific objectives of the study were: (1) to compare the immunogenicity of RiVax both with and without alum when administered *via* ID or IM injection; protection following challenge with a 10X LD_{50} dose of ricin *via* IP injection, gastric gavage, and aerosol was also studied, (2) to determine if ID *vs.* IM administration of RiVax on alum induced a longer lasting immune response, (3) to evaluate the ability of a single dose of RiVax on alum administered either ID *vs.* IM to protect mice against a 10X LD_{50} ricin challenge *via* IP injection, (4) to compare the ability of ID *vs.* IM administered RiVax on alum to protect mice against increasing doses of ricin administered *via* IP injection, (5) to determine the kinetics of RiVax trafficking to the injection site draining lymph node following either ID or IM administration of RiVax with and without alum.

CHAPTER TWO
Materials and Methods

Experimental Design

Female Swiss Webster mice (Taconic, Hudson, NY) were injected either ID or IM with RiVax, prepared as previously described [78] [73, 80]. The vaccine formulation consisted of 0.2 mg/mL RiVax in 20% trehalose (Sigma, St. Louis, NJ) and 0.04% Tween 80 (Fischer, Fair Lawn, NJ). This was then lyophilized and sealed under vacuum, [80] and stored at 4°C.

Vaccine that was reconstituted with sterile distilled water and diluted with phosphate buffered saline (PBS) was administered in a volume of 50 μL either with or without 1 mg/mL alum (Alhydrogel 1.3%, Brenntag Biosector, Denmark) at one of three dose levels. RiVax with alum was administered at 1.0, 0.1 and 0.01 μg three times at 4 week intervals; RiVax without alum was administered at 10, 1.0 and 0.1 μg 3 times at 4 week intervals. Control mice were injected with formulation alone or formulation plus alum.

Vaccine, and subsequently ricin, was administered in various regimens, as stated. Mice were challenged with various multiples of a previously determined 10 X LD_{50} dose (**Table 2.1**) of ricin by either IP injection, gastric gavage or aerosol [24]. Weights and survival of all mice were followed for 14 days following challenge.

Table 2.1. LD_{50} of Ricin in Mice

Route of Exposure	LD_{50} (µg toxin/kg body weight)
IP Injection	10
Gastric Gavage	10
Aerosol	4

Adapted from [24].

Radioimmunoassay (RIA) to Determine Titers of RiVax-Specific Antibody

RIAs were carried out using ninety-six well, U- bottom, vinyl plates (Thermo, Millford, MA) coated with 100 µL of RiVax in PBS overnight (ON) at 4°C. Plates were washed three times in distilled water and blocked with 10% fetal calf serum (FCS) (HyClone, Logan, UT), 0.05% sodium azide in PBS for 2 hours at room temperature (RT) and then frozen until use. Plates were thawed, washed three times in distilled water and coated with 100 µL of a known amount of affinity purified mouse anti-RiVax (1-1000 ng/mL) to provide a standard curve or test serum serially diluted in 10% FCS, 0.05% sodium azide in PBS, incubated ON at 4°C, washed three times in distilled water and incubated with ^{125}I- labeled rabbit anti-mouse IgG (10^5 cpm/100 µL per well). Plates were incubated for 2 hours at RT and washed two times with PBS and 3 times with distilled water. The wells of the plates were cut out, individually placed into 12 x 75mm glass tubes and the radioactivity in each tube was measured on a Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Waltham, MA). To determine the concentration of RiVax-specific antibody, we compared the radioactivity of our samples to that of the RiVax standard curve. Using the radioactivity of the points on the standard curve and multiplying by the

appropriate dilution factor, we were able to determine the concentration of RiVax specific antibody in our serum samples.

Fluorescent Labeling of RiVax

RiVax was labeled using Alexa Fluor 488 carboxylic acid 2, 3, 5, 6 tetrafluorophyenyl ester (TFP) (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Briefly, 100 µL of pH 9.0 0.1 M sodium bicarbonate buffer was added to every 1 mL of 0.9 mg/mL RiVax in pH 7.2 PBS. Lyophylized Alexa Fluor 488 TFP was reconstituted in dimethyl sulphoxide (DMSO) to a concentration of 10 mg/mL. While stirring, reconstituted Alexa Fluor 488 was added to the basic RiVax solution in a 1:9, mg of reactive dye to each mg of protein; this ratio was optimized. The mixture was incubated for 1 hr at room temperature, and was then dialyzed in PBS to remove any unreacted dye.

Lymph Node Harvest, Cell Surface Staining and Flow Cytometry

Inguinal lymph nodes that drained the site of either IM or ID injection were harvested, and a single cell suspensions was prepared by mashing the tissue between the frosted end of two microscope slides in Hank's

balanced salt solution (Gibco, Grand Island, NY) with 2% FCS. Cells were strained with a cell strainer to remove debris. Cells were then analyzed on a BD FACS Calibur for the presence of Alexa-488+ cells.

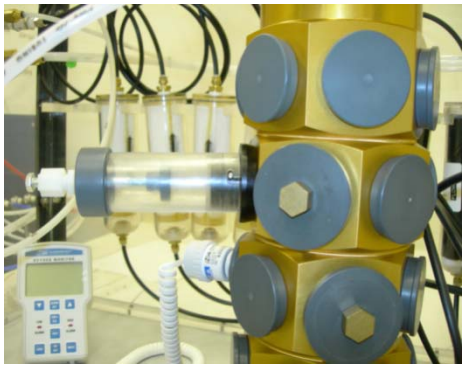
Vaccination and Ricin Challenge

Female Swiss Webster mice, age 6-8 weeks old, were injected ID or IM, with RiVax either with or without alum. IM vaccinations were administered in the left flank. The skin was prepped with an alcohol pad prior to ID vaccination either on the belly or on the left flank in order to see the skin; the formation of a 'blister' after injection confirmed ID delivery of the vaccine. Following challenge by either IP injection, gastric gavage or aerosol, mice were euthanized if moribund or after having lost >25% of their pre-challenge body weight.

Aerosol Challenge

Mice were exposed to aerosolized ricin in a nose-only exposure chamber (InTox, Moriarty, NM) as previously described [24] (**Figure 2.1 A)**. The air flow rate in the system was 10 L/min and was measured using a DryCal airflow meter (Bios, Butler, NJ). The average particle size was 2 μm and

A



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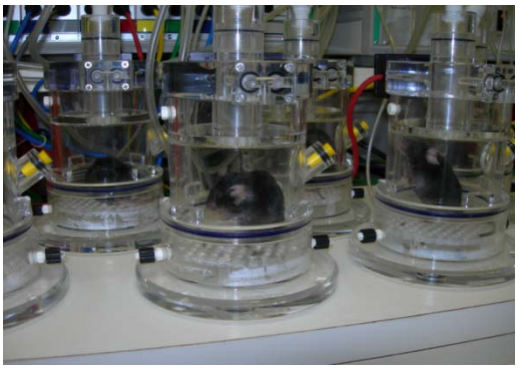

Figure 2.1: Aerosol Challenge Model. (A) Nose only exposure restrainer for exposing mice to aerosolized ricin. (B) Whole body plethysmograph chambers for measuring lung function.

was generated using a Lovelace nebulizer (Intox). The amount of ricin delivered was estimated by measuring the weight of the ricin before and after exposure. The amount of ricin delivered to each mouse was determined using three parameters: (1) the volume of air inhaled per minute by the mouse, which was calculated using the formula $\log_{10}(V)$ (mL min^{-1}) = -0.899 +1.725 ($\log_{10}$body weight) (g), (2) the ricin concentration in each liter of air, (3) the particle retention time, and (4) the length of exposure [24]. Once mice were loaded into the nose-only exposure system, they were not allowed to remain there for more than 45 minutes so as not to overly stress the animals. All equipment was thoroughly cleaned with 10% bleach following each exposure to ensure safety and that there was no residual ricin at the time of subsequent exposures. Lung function was measured using a 12 chamber whole body plethysmograph (Buxco, Wilmington, NC) (**Figure 2.1 B**). Animals were put into the chambers and allowed to acclimate for 5 minutes before lung function was measured. Penh was the parameter that we used to evaluate lung function. Penh is a unit-less measure of the length of the pause between the passive and active phases of expiration. The longer the pause, the higher the Penh and the more labored the breathing.

Gavage Challenge

Mice were challenged by gastric gavage as previously described [24]. Briefly, mice were fasted and moved to a clean cage 20 hours before challenge. They were dosed with 100 μg/kg ricin in a volume equal to 1% of their body weight in PBS using a feeding needle delivered into their stomach while being restrained by hand (**Figure 2.2**). Mice were then fasted for an additional 4 hours. They were monitored for 14 days for weight loss and survival.

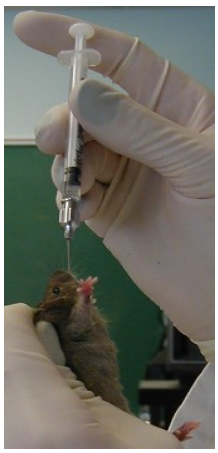

Figure 2.2: Gavage Challenge Model. Photo showing the method used to gavage the mice.

IP Challenge

Mice were injected IP with 100 µL ricin at 100 µg/kg in PBS and weight loss and survival were monitored for 14 days.

Statistical Analysis

Statistical significance was considered to be $P < 0.05$ in all cases. To determine significant difference in survival the Mantel-Cox log-rank test was used. To determine significant difference in titer data and Penh levels, a 2 tailed Student's t test was used. For titer data, the geometric mean of the titers were calculated, for Penh data the average of the values were calculated. All error bars represent one standard deviation from the mean.

CHAPTER THREE
Results

Many factors can play a role in determining the ultimate success of a vaccine. These include: (1) the required dose when considering how much vaccine needs to be produced to protect a given population, (2) the amount of vaccine that must be administered in one injection (3) the longevity of the response and hence the need for boosts, (4) knowledge of the amount of pathogen/toxin that the recipient is protected against *vs.* how much exposure is likely and (5) most effective protection against the most likely route of exposure

PART A. SHORT TERM PROTECTION OF MICE FROM RICIN INTOXICATION FOLLOWING ID *VS.* IM ADMINISTRATION OF RIVAX

To begin our studies in comparing RiVax administered *via* the ID *vs.* IM routes, we studied protection and immunogenicity in the short term. To this end, mice were vaccinated on days 0, 28, 56; blood was collected on day 69, and mice were challenged on day 70 either by IP injection, gastric gavage or aerosol. In the case of aerosol challenged mice, lung function was assessed pre-challenge, and monitored frequently post-challenge. For all mice, 25% weight loss was an indicator of insufficient protection and these mice were sacrificed. This regimen allowed us to assess a number of factors. First, we were able to specifically evaluate vaccine induced serum antibody titers, since adaptive immune responses are boosted by ricin administered at the time of challenge. We were able to determine not only the survival of mice, following aerosol challenge, but also the degree of lung protection without having to sacrifice the animal for histopathology. This experimental setup allowed us to compare various parameters of ID *vs.* IM administration of RiVax both with and without alum

RiVax-Specific Antibodies

The ability to elicit high levels of circulating, antigen specific antibodies is an important indicator of an effective vaccine. When studying vaccine efficacy in human subjects, this is often the critical readout of immunogenicity. In order to compare the ability of ID *vs.* IM vaccinations to induce specific antibodies, RiVax was administered to mice as described in the Materials and Methods. Mice were bled one day prior to challenge with ricin to measure the titers of anti-RiVax antibodies. As shown in **Figure 3.1**, the geometric mean titers (GMT) of RiVax-specific antibodies in mice vaccinated with RiVax in the absence of alum (**Figure 3.1 A**) were dose dependent; the addition of alum to the vaccine significantly increased these titers (**Figure 3.1 B).** In comparing titers in the ID or IM vaccine administrations, we observed significantly higher titers in ID vaccinated mice at the 0.01 μg dose level ($p < 0.01$) (**Figure 3.1 B)**. Comparisons between ID and IM vaccinations at all other dose levels showed that ID vaccination was as effective as IM vaccination in inducing RiVax-specific antibodies. Overall, at low doses ID vaccinations were significantly better than IM vaccinations in inducing RiVax-specific antibodies suggesting that lower doses of vaccine could be used for effective protection when the ID route is used.

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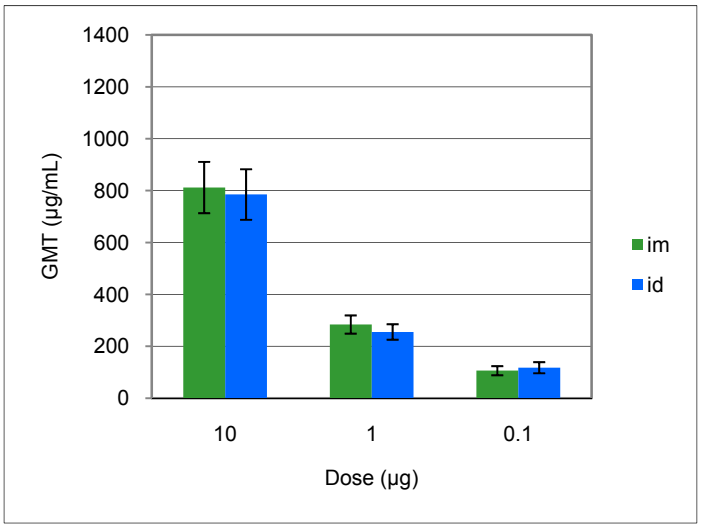

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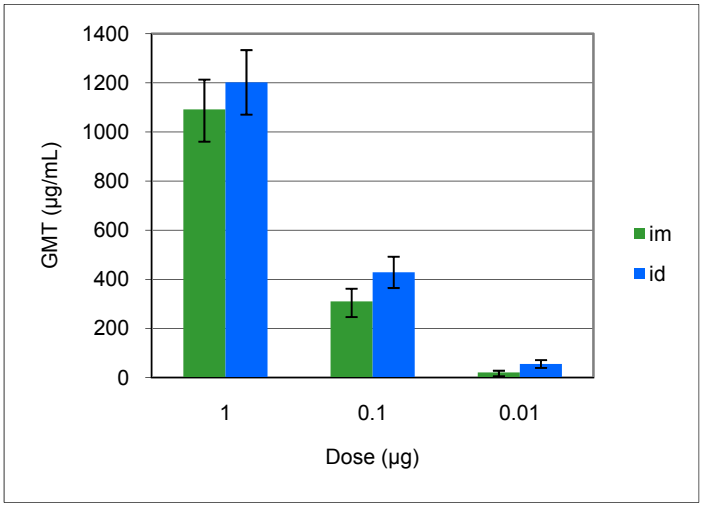


Figure 3.1: RiVax- Specific Serum Antibody Titers Mice were vaccinated once every 4 weeks for 8 weeks with the dose levels indicated on the *x*-axis. Two weeks after the last vaccination mice were bled before ricin challenge and sera were analyzed for RiVax-specific antibody, green bars = IM, blue bars = ID; all data represent 6 experiments of 8 mice each combined into groups of 48 for analysis. (A) without alum, (B) with alum, comparing 0.01 IM and 0.01 ID with alum, $P < 0.01$. Vehicle control vaccinated mice had negligible titers and therefore are not shown

Post Challenge Survival After ID or IM Vaccination with RiVax

While levels of circulating antibodies, and neutralizing antibodies, are good predictors of vaccine efficacy, these antibodies must protect animals. In order to determine the ability of ID *vs.* IM vaccinations to protect mice against ricin intoxication, we carried out a series of experiments where mice were vaccinated with RiVax as described in the Methods. Two weeks after the last vaccination mice were challenged with a 10 X LD_{50} dose of ricin either by IP injection, gastric gavage, or aerosol.

As shown in **Figure 3.2 A,** following IP challenge, 100% of mice vaccinated either by the ID or IM routes, using 3 doses of 10 and 1.0 μg per dose, survived. Of the mice receiving three doses of 0.1 μg, 88% (14/16 mice) of IM vaccinated and 56% (10/16 mice) of ID vaccinated mice survived *(P* > 0.05) demonstrating that ID vaccination was similar to IM vaccination in protecting mice against a systemic ricin challenge.

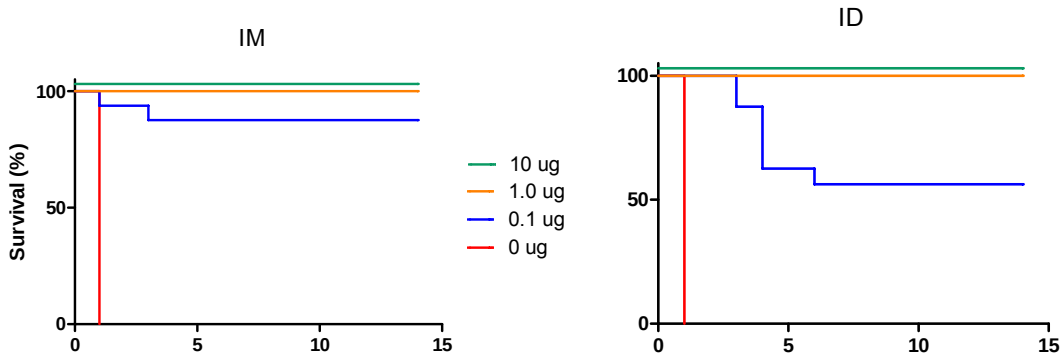
All the mice that received 3 vaccine doses of 10, 1.0 or 0.1 μg survived a gavage challenge with ricin regardless of the vaccination route. In order to find a dose level that might demonstrate a dose-related difference in potency between the two vaccination routes, we repeated the experiment

using 1.0, 0.1, and 0.01 μg per dose. All of the mice at the 1.0 μg and 88% (7/8) at the 0.1 μg dose levels survived. In contrast, ricin was lethal to all the mice vaccinated with 0.01 μg. Overall, the percent survival following either vaccination route was identical. The combined results of the two experiments demonstrated that all the mice vaccinated with three doses of 10 and 1.0 μg each, survived oral ricin challenge. In addition 94% (15/16 mice) receiving 3 doses of 0.1 μg survived. In contrast, none of the mice receiving 3 doses of 0.01 μg survived (**Figure 3.2 B**).

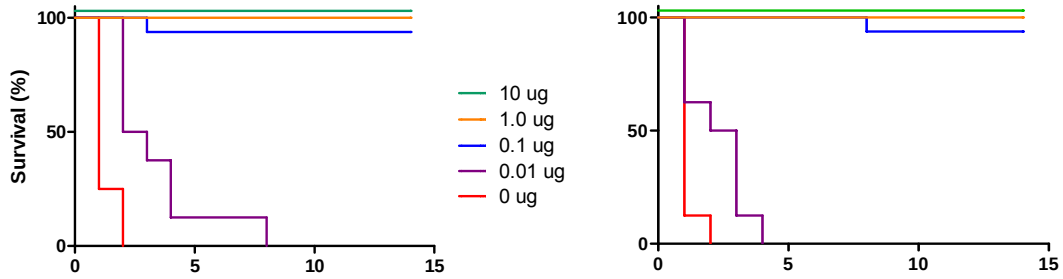
We also investigated the survival of mice vaccinated ID *vs.* IM followed by challenge with aerosolized ricin. At the 10 and 1.0 μg dose levels 100% of the mice in both groups survived. At the 0.1 μg dose level, 69% (9/16 mice) vaccinated *via* the IM route and 66% (10/15 mice) vaccinated *via* the ID route survived (**Figure 3.2 C**). Hence, vaccination *via* the ID and IM route with RiVax alone protected animals from death when ricin was administered systemically, by gavage or by aerosol.

Survival following Ricin Challenge in Mice Vaccinated with RiVax - Alum

A – IP Challenge



B – Gastric Gavage Challenge



C – Aerosol Challenge

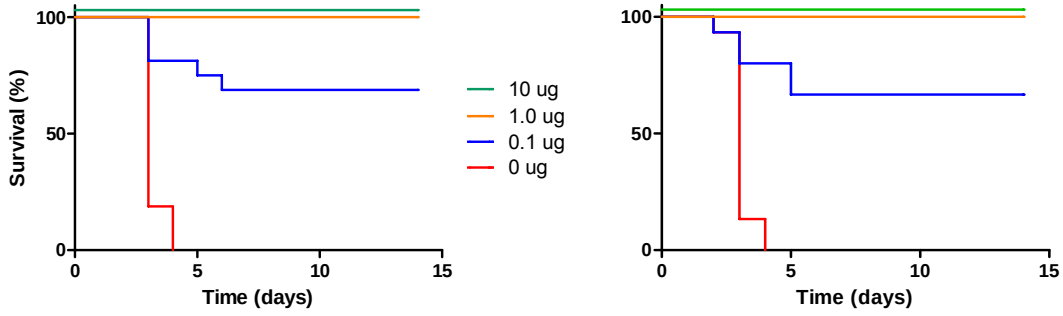


Figure 3.2: Ricin Challenge After Vaccination With RiVax Without Alum. Mice were vaccinated three times at 4 week intervals. Two weeks after the last vaccination mice were challenged with ricin. *P* values compare survival of ID *vs.* IM vaccinated mice within a dose level; the following doses were given at each vaccination; green = 10 µg, orange = 1 µg, blue = 0.1 µg, purple = 0.01 µg, red = 0 µg. Challenge *via* (A) IP injection, at the 0.1 µg dose level, $P = 0.078$, (B) gastric gavage, at the 0.01 µg dose level, $P = 0.218$, (C) aerosol, at the 0.1 µg dose level $P = 0.848$. (A) and (B) represent 2 experiments of 8 mice each combined into groups of 16 for analysis. (C) represents 4 experiments of 4 animals each, combined into groups of 16 for analysis.

Post Challenge Survival After ID or IM Vaccination with RiVax Plus Alum

Despite the fact that survival following vaccination with RiVax was equivalent following vaccination by either the IM or ID routes, several findings led us to postulate that ID vaccination would be advantageous when RiVax was administered with alum: (1) recent work by others suggested that alum works by enhancing activation of DCs [44]; (2) DCs (Langerhan's cells) are highly prevalent in the skin; (3) our data presented in **Figure 3.1 B** which demonstrate that ID *vs.* IM administration with alum is superior in eliciting specific antibodies. To test this hypothesis, mice were vaccinated and boosted either *via* the ID or IM routes with various doses of RiVax in 1 mg/mL alum. Two weeks after the last vaccination, mice were challenged with ricin administered by IP injection, gastric gavage or aerosol.

As shown in **Figure 3.3 A**, when challenged by IP injection 100% of animals vaccinated receiving 3 doses of 1.0 and 0.1 μg each survived, regardless of the vaccination route. 13% of mice receiving 3 doses of 0.01 μg RiVax by the IM route, and 50% of mice vaccinated by the ID route survived. This data indicate that when low doses of vaccine are used ID

administration of RiVax on alum is superior ($P < 0.05$) to IM administration in protecting animals from systemic ricin intoxication.

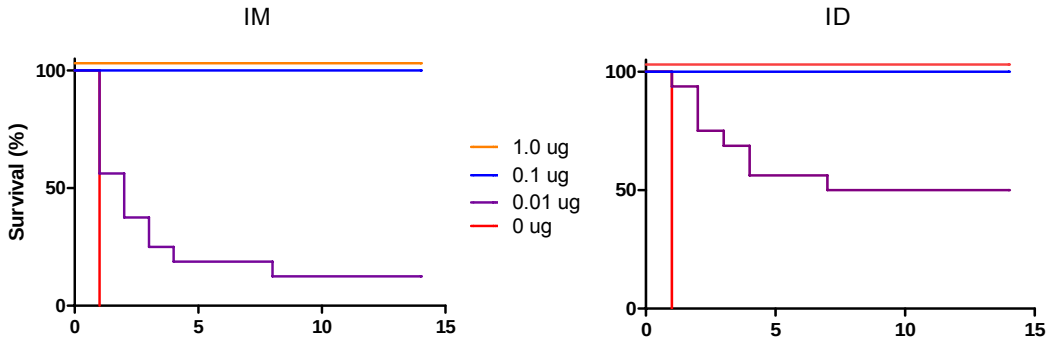
As shown in **Figure 3.3 B**, when challenged with ricin by gastric gavage, > 90% of the mice receiving 3 doses of 1.0 and 0.1 μg each, survived regardless of the route of administration. At the low dose level ID administration of RiVax proved superior with 19% and 56% survival using the IM and ID vaccination routes, respectively. This difference supports the conclusion that ID vaccination using low doses of RiVax with alum is superior ($P < 0.05$) to IM vaccination with alum in protecting animals from gavage challenge with ricin.

Finally, vaccinated animals were challenged with aerosolized ricin. At the high dose level of 3 doses of 1.0 μg each, 100% of mice vaccinated *via* either the ID or IM routes survived. At the middle dose level, 93% of the mice vaccinated *via* the ID route survived, while only 50% of the mice vaccinated *via* the IM route survived. At the low dose level, ID vaccination was again significantly better than IM vaccination at protecting animals with 25% *vs.* 13% survival, respectively (**Figure 3.3 C**). Hence, when low doses of RiVax were administered in alum *via* the ID route protection was

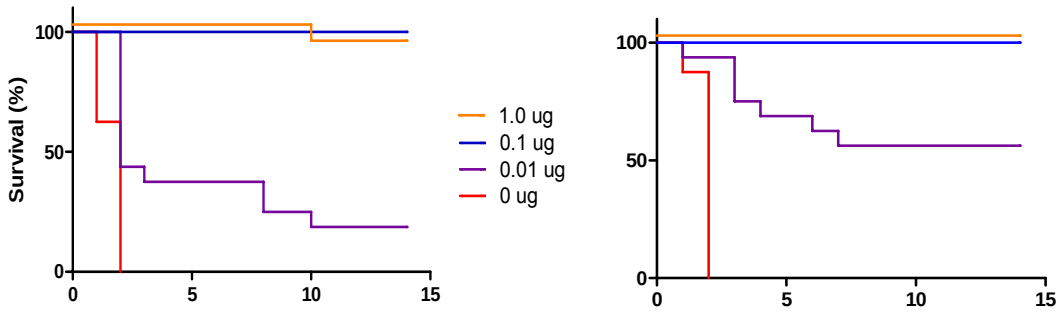
superior ($P < 0.05$) as compared to the IM route in protecting mice against the systemic, gut-mucosal and respiratory toxicity of ricin.

Survival following Ricin Challenge in Mice Vaccinated with RiVax + Alum

A – IP Challenge



B – Gastric Gavage Challenge



C – Aerosol Challenge

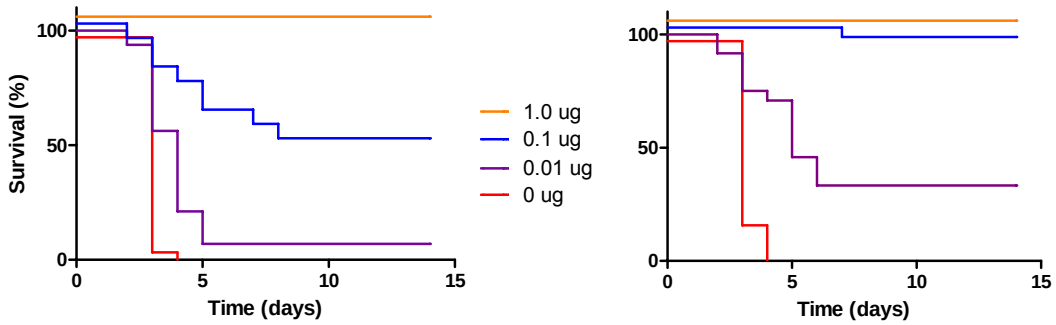


Figure 3.3: Ricin Challenge After Vaccination with RiVax + Alum. Mice were vaccinated three times at 4 week intervals with RiVax in 1 mg/mL alum. Two weeks after the last vaccination mice were challenged with ricin. *P* values compare survival of ID *vs.* IM vaccinated mice within a dose level; the following doses were given at each vaccination; orange = 1.0 µg, blue = 0.1 µg, purple = 0.01 µg, red = 0 µg. Challenge *via* (A) IP injection, at the 0.01 µg dose level, $P < 0.05$, (B) gastric gavage, at the 0.01 µg dose level, $P < 0.05$, (C) aerosol, at the 0.1 µg dose level, $P < 0.05$, at the 0.01 µg dose level $P < 0.05$. (A) and (B) represent 2 experiments of 8 mice each combined into groups of 16 mice for analysis; (C) represents 4 experiments of 4 animals each combined into groups of 16 mice for analysis.

The Relationship Between Survival and Specific Antibody Titers

Effective vaccination induces significant levels of protective antigen-specific antibody. To validate this in our model, we compared the GMT of RiVax-specific antibody in surviving *vs.* non-surviving animals from all dose groups and all challenge groups. We also compared titers according to vaccination route to determine if surviving mice vaccinated ID had significantly higher antibody titers than surviving mice vaccinated IM. As expected, when RiVax was administered without adjuvant, the survivors showed significantly higher antibody titers than the non-survivors following both ID and IM vaccination. In comparing surviving mice vaccinated either ID or IM, there were no significant differences in their antibody levels nor were there differences when comparing ID and IM vaccinated non-survivors (**Figure 3.4, top panel**). The results of this comparison were similar when RiVax was administered with alum (**Figure 3.4, bottom panel**). While more mice vaccinated with RiVax plus alum *via* the ID *vs.* the IM route survived, the survivors in both groups had similar antibody titers. These data support the conclusion that higher levels of RiVax-specific antibodies in the blood correlate with better protection.

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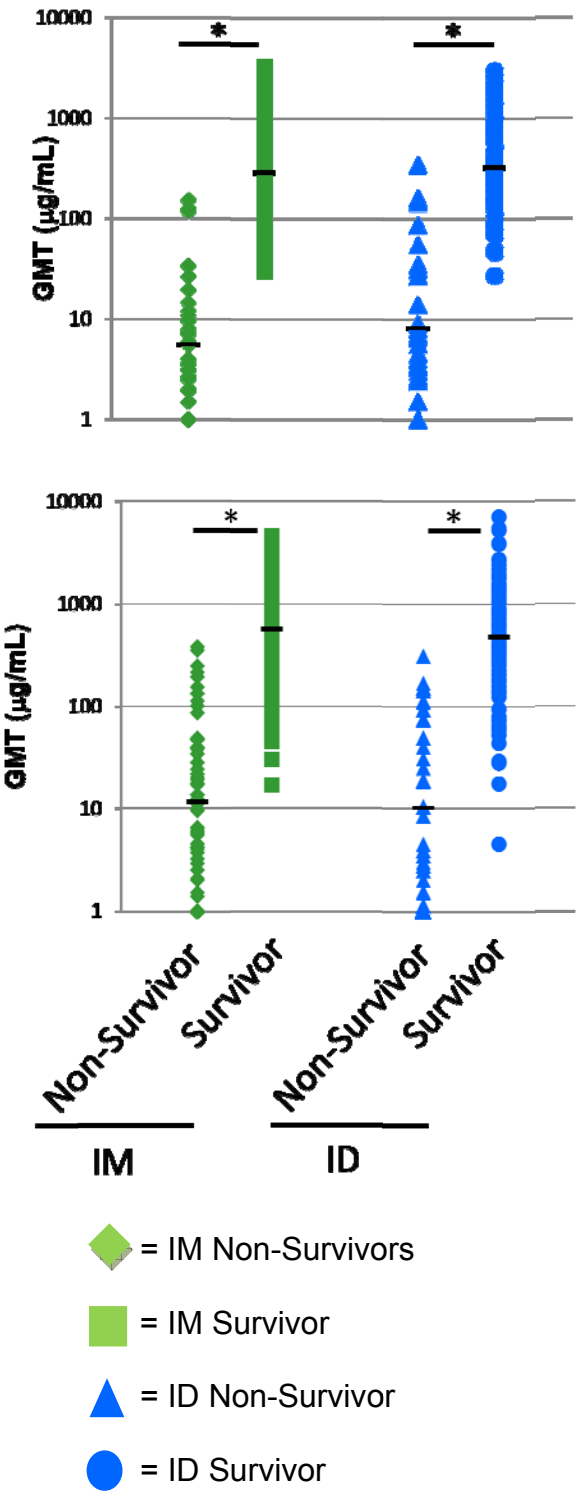
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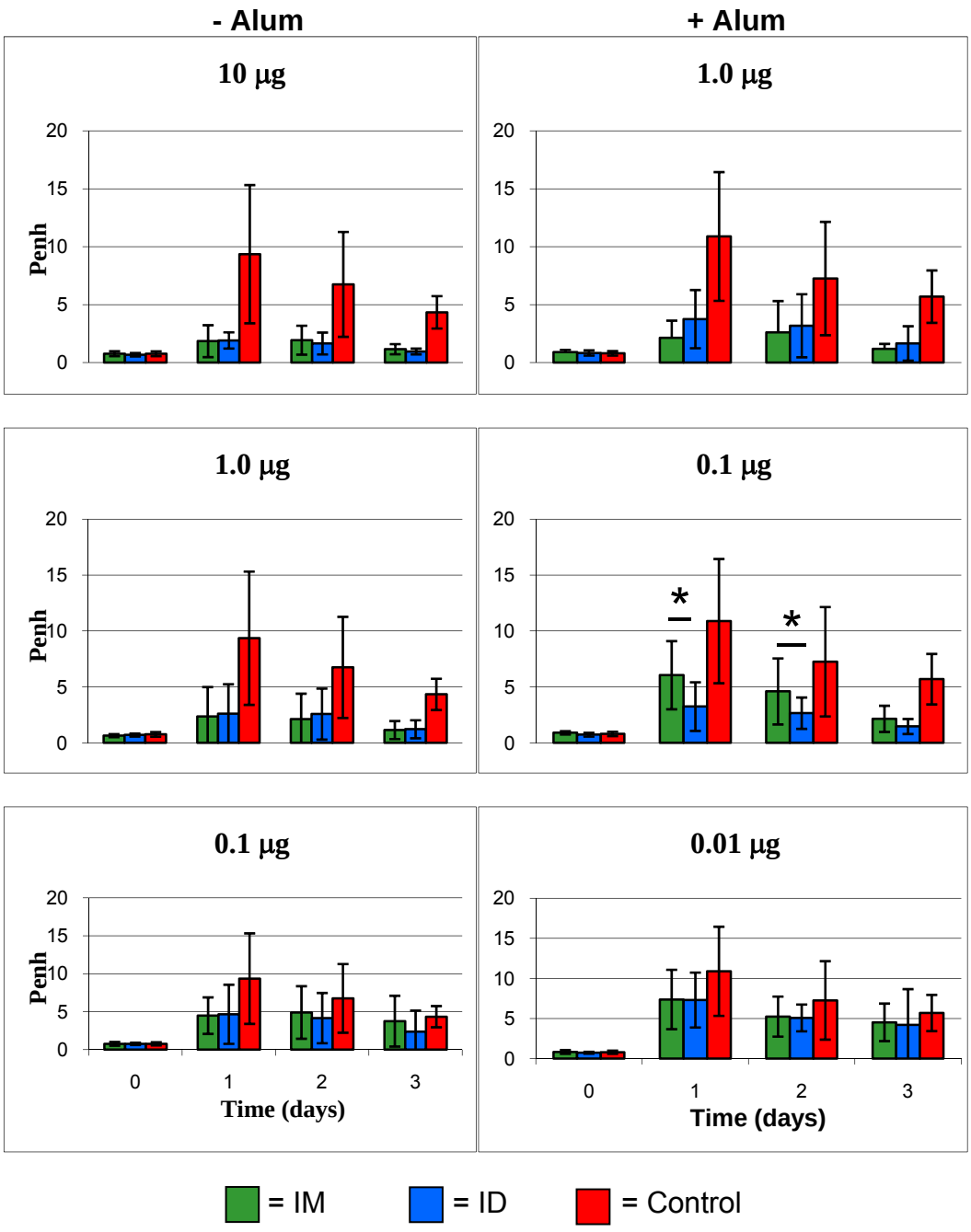
Figure 3.4: Levels of RiVax- Specific Antibodies *vs.* Survival After Ricin Challenge. Titers from animals at all dose levels were compiled, then grouped to compare non-survivors and survivors, each dot represents one animals, the GMT is represented by a horizontal line (Top panel) without alum, IM non-survivors, n = 38; IM survivors, n = 118; ID non-survivors, n = 37; ID survivors, n = 110, (Bottom panel) with alum, IM non-survivors, n = 68; IM survivors, n = 94; ID non-survivors, n = 42; ID survivors, n = 109. In all cases, * indicates $P < 0.05$.

Lung Function in Mice Exposed to Aerosolized Ricin

It was important to determine whether ID vaccination would not only increase survival, but would protect mice from lung damage following exposure to aerosolized ricin. There was no reason to expect this unless the higher levels of serum antibody induced by ID administration exudated into the lungs. To this end we monitored lung function in mice vaccinated with RiVax, by both routes with or without alum, followed by an aerosol challenge with ricin. Lung function was assessed by whole-body plethysmography before challenge and on days 1, 2, 3, 5, 7, 10, and 14 post-challenge. Since many mice receiving the lowest doses of vaccine died by day 3, the most relevant comparisons were made prior to that time point. With RiVax on alum, 60% of the low dose group was surviving on day 3; without alum 87% of the low dose group was surviving on day 3. In addition, data from mice surviving 5-14 days post challenge showed no significant difference related to the route of vaccination and therefore are not shown. When comparing ID *vs.* IM vaccinated mice within a dose level and adjuvant group, both were equally protected against ricin-related lung damage. The only exception was at the 0.1 μg dose level, where vaccination by the ID route with alum was significantly better *(P* < 0.05) at protecting mice against lung damage (**Figure 3.5**). Our data demonstrate

that protection of the lungs following IM *vs.* ID vaccination is equivalent when higher doses of vaccine are given. In contrast, when low doses of vaccine on alum are given, ID vaccination is more effective than IM vaccination.


- Alum
+ Alum
10 µg
1.0 µg
1.0 µg
0.1 µg
0.1 µg
0.01 µg
Penh
20
15
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5
0
*
*
0
1
2
3
Time (days)
= IM
= ID
= Control

Figure 3.5: Lung function following aerosol challenge with ricin. Mice were vaccinated three times at 4 week intervals with the doses of RiVax indicated in the panels. Two weeks after the last vaccination, mice were challenged with aerosolized ricin and lung function was measured as the Penh on days 0, 1, 2, 3, 5, 7, 9, 10, 14. Penh values for days 0, 1, 2, 3 are shown. Green bars = IM, blue bars = ID, red bars = control vaccinated mice. * $P < 0.05$. All data represent 4 experiments of 4 mice each, combined into groups of 16 for analysis.

Correlation Between RiVax-Specific Antibody Titers and Penh Values

We postulated that RiVax-specific antibody titers produced as a result of vaccination would protect mice from both death and lung damage following aerosol challenge since some antibodies in the circulation should enter the lung. We therefore compared RiVax-specific antibody titers to day 1 Penh values; day 1 Penh was chosen because mice started to die as early as day 2. In animals vaccinated with RiVax in the absence of adjuvant and then challenged with aerosolized ricin, there was a general correlation between levels of specific antibody and good lung function regardless of the vaccination route (**Figure 3.6 A**). The same was true for mice that were vaccinated with RiVax on alum (**Figure 3.6 B**). Hence, regardless of the route of vaccination or presence of alum, the levels of anti-RiVax antibody in the serum correlated with better lung protection. Therefore, RiVax protected animals from lung damage and also death following exposure to aerosolized ricin. This is significant, since lung damage could lead to subsequent edema, infections, respiratory failure and morbidity.

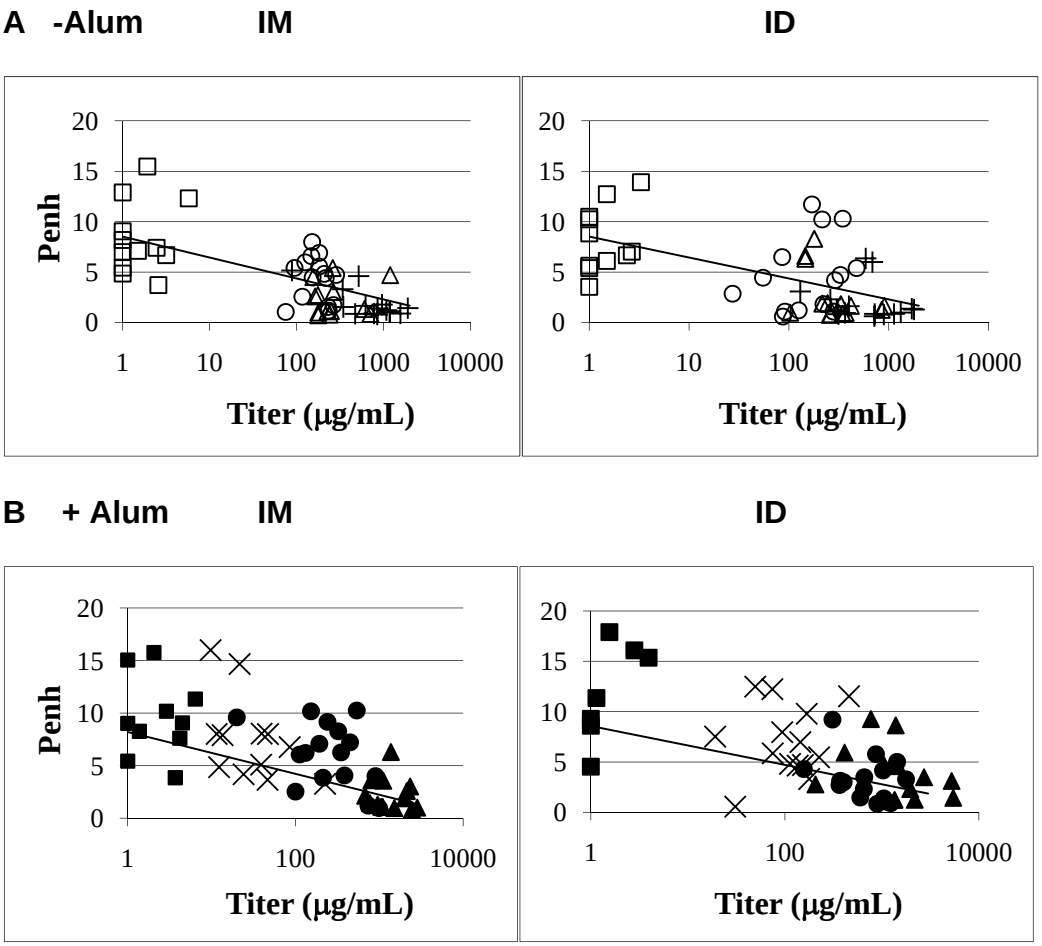

Figure 3.6: Lung Function vs. RiVax-Specific Antibody Titers and Survival. Mice were vaccinated three times at 4 week intervals. Two weeks after the last vaccination, mice were challenged with aerosolized ricin and lung function was measured as the Penh on days 0, 1, 2, 3, 5, 7, 10, 14. These graphs show the Penh values on day 1: (A) Correlation between Penh values and RiVax-specific antibody in mice vaccinated without adjuvant, left panel shows data from IM vaccinated mice, R^2 = 0.40; right panel shows data from ID vaccinated mice, R^2 = 0.40; + = 10 μg, Δ = 1.0 μg, ○ = 0.1 μg, □ = 0 μg, n = 16 per dose level, (B) Correlation between Penh values and RiVax-specific antibody in mice vaccinated with adjuvant, left panel shows data from IM vaccinated mice, R^2 = 0.42; right panel shows data from ID vaccinated mice, R^2 = 0.39; ▲ = 1.0 μg, ● = 0.1 μg, X = 0.01 μg, ■ = 0 μg, n = 16 per dose level.

Survival and Lung Function

Although the specific mechanisms responsible for death by ricin intoxication are not clearly understood and are probably multifactorial, in the case of exposure to aerosolized ricin, it is presumed that lung damage plays a significant role [2]. In order to determine whether this was the case in our experiments, we compared the day 1 post-challenge Penh levels in mice that would go on to survive *vs*. those that would go on to die. In making this comparison we found that regardless of the vaccination route or the presence of alum, mice that ultimately survived had significantly better lung function at day 1 as compared to mice that subsequently died, (**Figure 3.7**). In general, all mice that survived to day 14 post challenge had low Penh values that reflected normal lung function indicating that much of the lung damage had already resolved as we observed histologically in past experiments [24]. While significantly more ID vaccinated mice survived, the Penh levels in surviving mice vaccinated by either route were not significantly different; the same was true in comparing non-surviving mice vaccinated by the ID *vs.* IM route. These results suggest that if ricin-induced lung damage can be minimized, the chances of survival should also improve.

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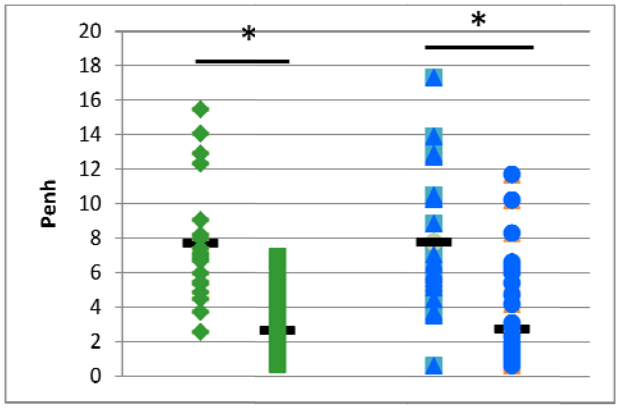
Penh
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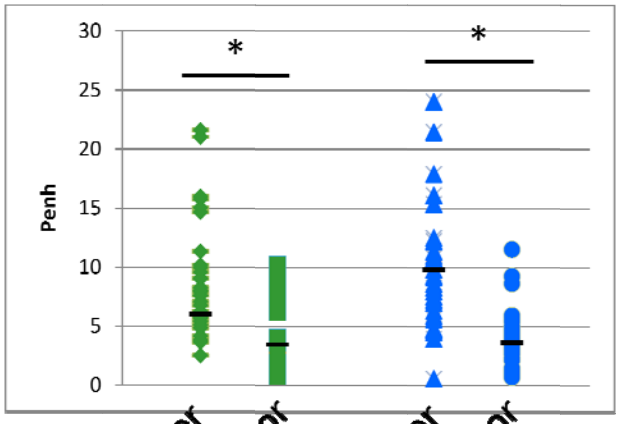
*

Alum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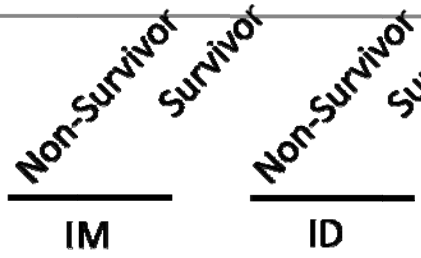

Non-Survivor
Survivor
Non-Survivor
Survivor
IM
ID

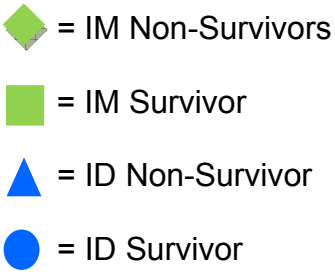

= IM Non-Survivors
= IM Survivor
= ID Non-Survivor
= ID Survivor

Figure 3.7: Lung Function and Survival. Penh values from animals at all dose levels were compiled, then grouped to compare non-survivors and survivors; (Top panel) without alum; IM non-survivors, n = 21; IM survivors, n = 41; ID non-survivors, n = 21; ID survivors, n = 41; (Bottom panel) with alum; IM non-survivors, n = 38; IM survivors, n = 25; ID non-survivors, n = 29; ID survivors, n = 35. * indicates $P < 0.05$.

PART B. LONG TERM PROTECTION OF MICE FROM RICIN INTOXICATION FOLLOWING ID *VS.* IM ADMINISTRATION OF RIVAX ON ALUM

To assess the longevity of the immune response to RiVax following vaccination by either the ID or IM routes, we vaccinated mice on days 0, 28, and 56 and waited for 8.5 months before bleeding them and challenging them with ricin *via* IP injection, gastric gavage or aerosol. Knowing that immune responses tend to wane with time, we chose to adsorb RiVax on alum and only at our two high doses levels, 1.0 μg and 0.1 μg per vaccination. As described previously, we bled the mice one day prior to challenge in order to determine their RiVax-specific antibody titers. **Figure 3.8** shows that 8.5 months after vaccination, a dose response is still evident. At these doses IM and ID vaccination elicited equivalent titers. In comparing **Figure 3.8** to **Figure 3.1 B** it is clear that the RiVax-specific antibody titer has decreased by 80% over time.

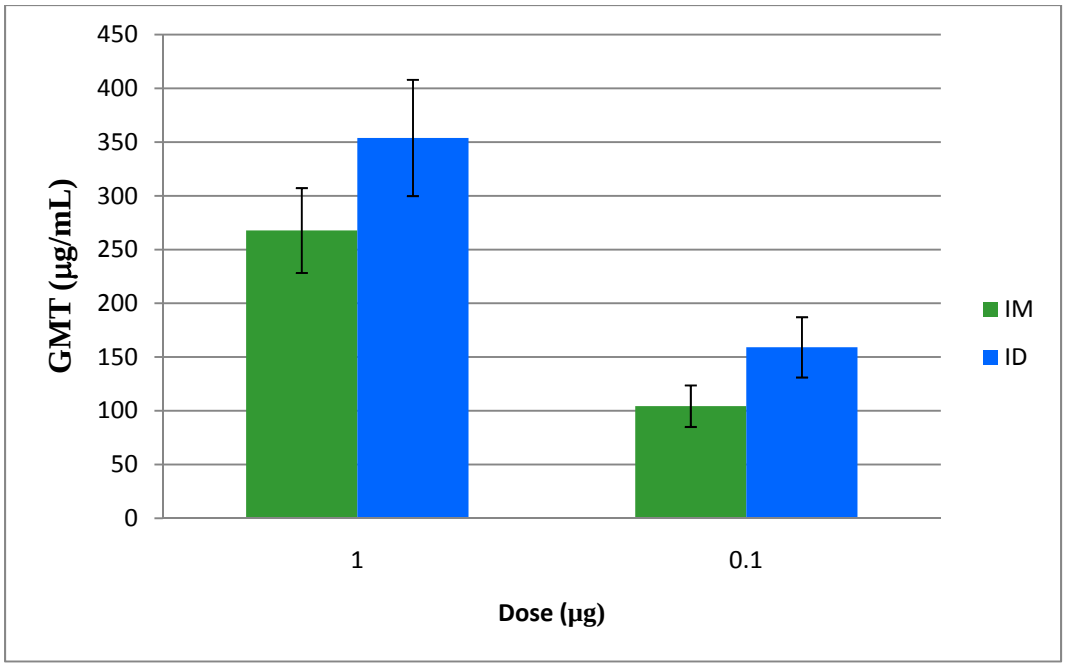


Figure 3.8: RiVax Specific Antibody Titers 8.5 Months Following Vaccination. Mice were vaccinated with RiVax + alum three times at 4 week intervals with the dose levels indicated on the x-axis (µg/mL). Two weeks after the last vaccination mice were bled before ricin challenge and sera was analyzed for RiVax-specific antibody, green bars = IM, blue bars = ID; all data represent 6 experiments of 8 mice each combined into groups of 48 for analysis. Vehicle control vaccinated mice had negligible titers and therefore are not shown

Post Challenge Survival After ID or IM Vaccination with RiVax

The next step in determining the long term efficacy of ID *vs.* IM vaccination was to determine how well it protected animals against a ricin challenge.

In response to ricin challenge *via* IP injection (**Figure 3.9 A**), animals receiving both the high and medium vaccine doses 8.5 months earlier survived regardless of the route of vaccination. With the exception of the 1.0 μg dose level administered *via* the IM route (13/15 mice survived), 100% of all animals in all groups survived.

We then investigated mucosal protection 8.5 months following either ID or IM vaccination. We found that the ID and IM routes of vaccination were equivalent in protecting mice against ricin challenge *via* gastric gavage (**Figure 3.9 B**). 100% of all animals in all groups survived, with the exception of mice receiving 3 doses of 0.1 μg *via* the IM route, where 71% (10/14) mice survived.

In our aerosol model, we also found that ID and IM vaccinations were equivalent in their ability to protect mice from aerosolized ricin 8.5 months

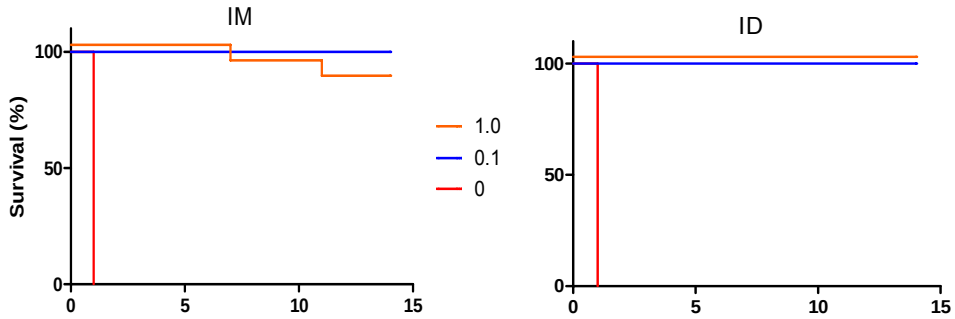
following their last vaccine boost. At the high dose level, 100% and 86 % (12/14) mice survived following ID and IM vaccination, respectively. At the 0.1 µg dose level, 92% (12/13) and 86% (12/14) of the mice from the ID and IM vaccination groups survived, respectively (**Figure 3.9 C**).

Overall, we observe that RiVax on alum administered *via* the ID and IM vaccination routes provided equal protection from both systemic and mucosal ricin challenge 8.5 months later.

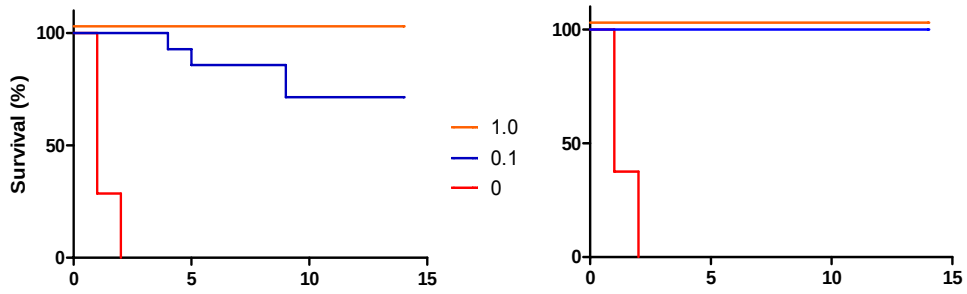
Survival following Ricin Challenge in Mice Vaccinated with RiVax + Alum

8.5 Months Earlier

A – IP Challenge



B – Gastric Gavage Challenge



C – Aerosol Challenge

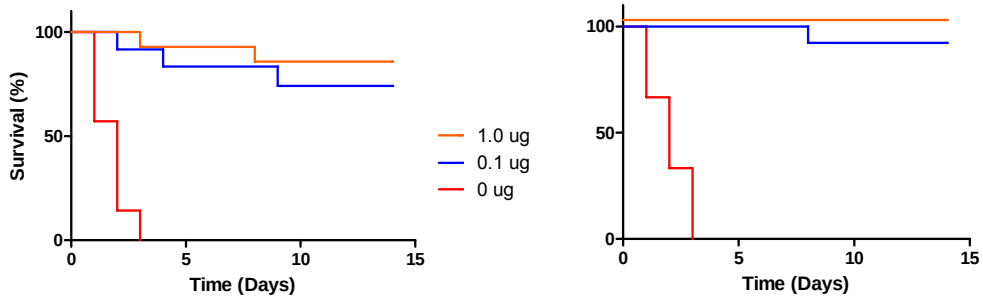


Figure 3.9: Ricin Challenge 8.5 Months After Vaccination with RiVax on Alum. Mice were vaccinated three times at 4 week intervals with RiVax in 1 mg/mL alum. Two weeks after the last vaccination mice were challenged with ricin. Orange = 1.0 µg, blue = 0.1 µg, red = 0 µg. Challenge *via* (A) IP injection, (B) gastric gavage (C) aerosol. All data represent 2 experiments of 8 mice each combined into groups of 16 mice for analysis.

Lung Function in Mice Exposed to Aerosolized Ricin 8.5 Months Following RiVax Vaccination

It is important that vaccinees would not only survive an aerosolized ricin challenge, but would also have minimal injury to their lungs. Therefore, it was important to evaluate the lung function of mice challenged with aerosolized ricin. As described above, Penh values for the first 3 days following challenge were shown. The Penh values of mice that survived returned to baseline (data is not shown). In comparing Penh values at the high dose level of 1.0 μg given three times (**Figure 3.10, top panel**), both ID and IM vaccination with alum were equally effective at protecting lungs from ricin induced damage. The same was true at the low dose level of 0.1 μg given three times (**Figure 3.10, bottom panel**).

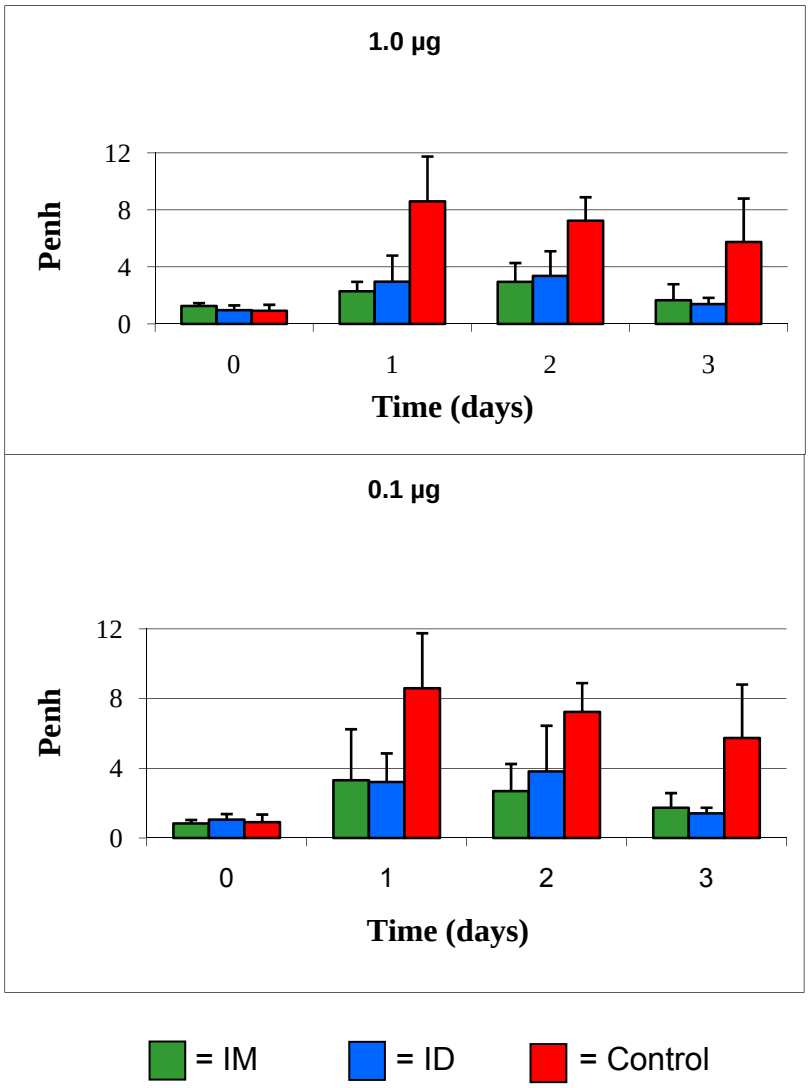


Figure 3.10: Lung Function Following Ricin Challenge 8.5 Months After Vaccination with RiVax on Alum. Mice were vaccinated with RiVax in 1mg/mL alum three times at 4 week intervals. 8.5 months following the last vaccination, mice were challenged with aerosolized ricin. Lung function was measured as the Penh on days 0, 1, 2, 3, 5, 7, 9, 10, 14 post challenge. Penh values for days 0, 1, 2, 3 are shown. Top panel represents the 1.0 µg dose level, the bottom panel represents the 0.1 µg dose level. Green bars = IM, blue bars = ID, red bars = control vaccinated mice. All data represent 2 experiments of 8 mice each combined into groups of 16 mice for analysis.

PART C. PROTECTION OF MICE FROM RICIN INTOXICATION FOLLOWING A SINGLE ID OR IM ADMINISTRATION OF RIVAX ON ALUM

Due to issues regarding patient compliance, especially in military settings, a single protective dose of Rivax would have great value. To this end, we determined the efficacy of RiVax administered in a single dose of 200, 50 or 10 µg in 1 mg/mL alum. Due to the large doses that were administered, animals received two injections of 50 µL each, on day 0. On day 14 mice were bled and levels of anti-RiVax antibody were determined. On day 21 animals were challenged with a 10X LD_{50} of ricin *via* IP injection. Animals were then monitored for 2 weeks for weight loss and survival. With regard to antibody titers, we observed that the antibody responses were dose related and that ID and IM vaccination were equally effective (**Figure 3.11**).

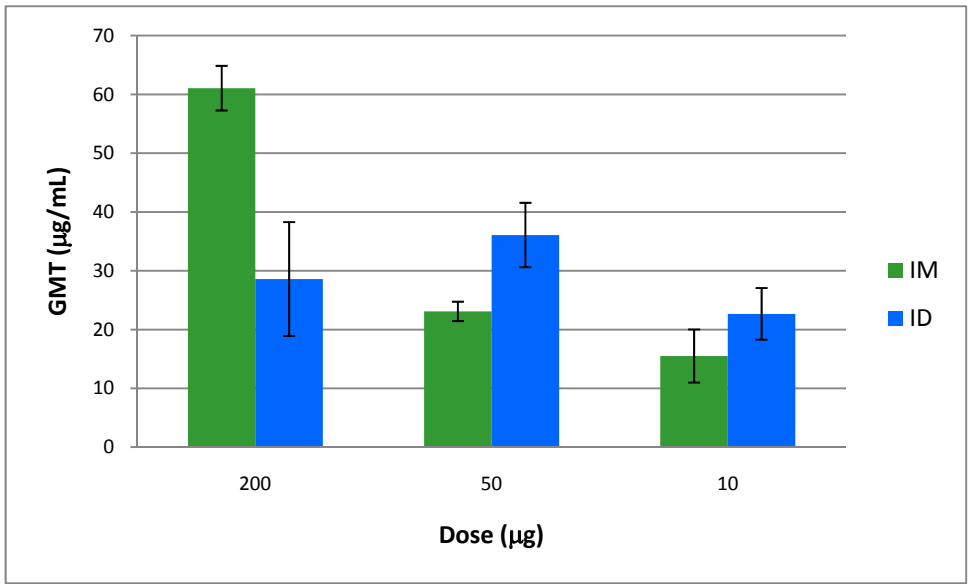


Figure 3.11: RiVax-Specific Antibody Titers Following Administration of a Single dose of RiVax on Alum. Mice were vaccinated once with RiVax in 1mg/mL alum at the given doses, sera were collected two weeks later (one week before ricin challenge) and analyzed for RiVax-specific antibody titers. Green bars = IM, blue bars = ID. All data represent 2 experiments of 4 mice each compiled into groups of 8 for analysis. Vehicle control vaccinated mice had negligible titers and therefore are not shown.

Both ID and IM vaccinations were also similar in their ability to protect mice from ricin challenge. (**Figure 3.12**). At the 200 μg dose level, none of ID and 25% of IM vaccinated mice survived challenge. At the 50 μg dose levels 50% of both the ID and IM vaccinated mice survived. Lastly, at the 10 μg dose level none of the ID vaccinated and 25% of the IM vaccinated mice survived. Regardless of vaccination route or dose, no more than 50% of animals survived systemic ricin challenge and none of the ID survival rates were statistically different from the IM survival rates.

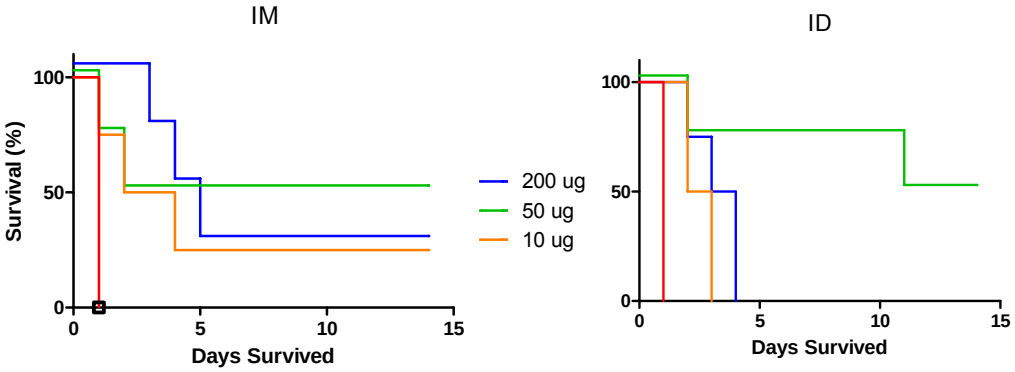


Figure 3.12: Ricin Challenge Following Administration of a Single Dose of RiVax on Alum. Mice were vaccinated once with RiVax in 1 mg/mL alum at various doses. Three weeks later mice were challenged with a 10X LD_{50} of ricin *via* IP injection. Blue = 200 µg, green = 50 µg, yellow = 10 µg, red = 0 µg. All data represent 1 experiment of 4 mice each.

PART D. PROTECTION OF MICE FROM INCREASING DOSES OF RICIN

It was critical to know how much ricin a RiVax vaccine regimen can protect an animal against since the dose likely to be encountered during a bioterrorist attack is difficult to predict. Therefore, we administered 1 μg of RiVax on alum three times at 4 week intervals by either ID or IM vaccination (a dose and regimen we have established to be 100% protective against a 10X LD_{50} of ricin) and then challenged animals with increasing doses of ricin *via* IP injection. Due to technical limitations these experiments were not carried out using the aerosol or gavage ricin challenge models. As shown in **Figure 3.13** there was no significant difference in the antibody titers among all the mice.

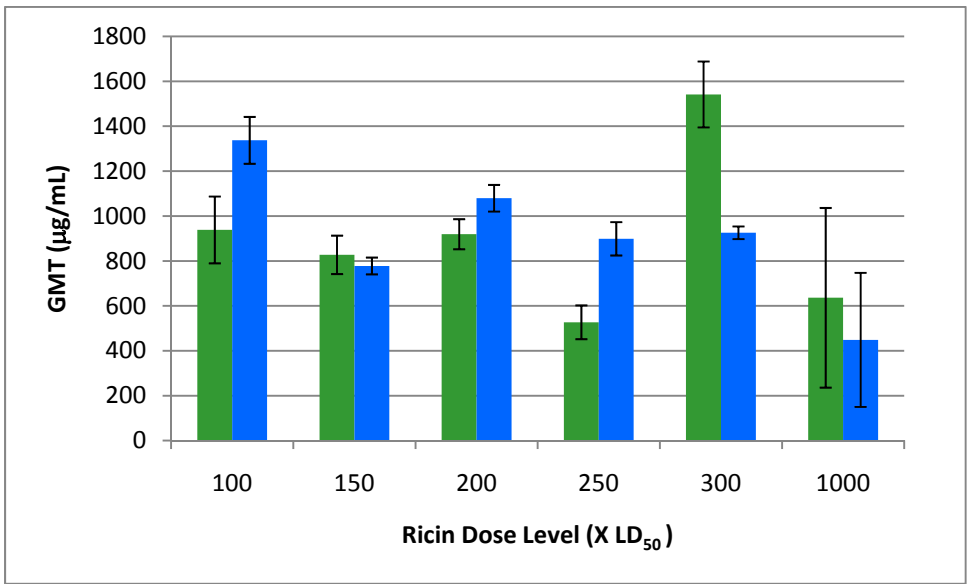
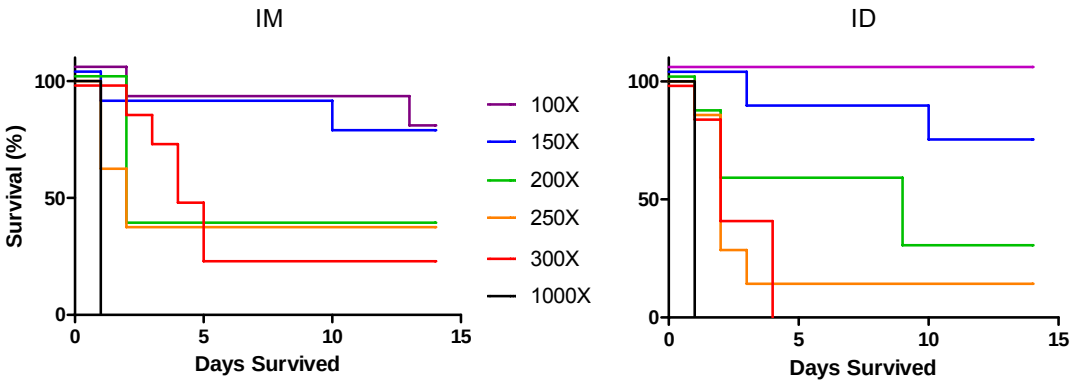


Figure 3.13: RiVax-Specific Antibodies of Mice Challenged *via* IP Injection with Ricin. All mice were vaccinated 3 times at 4 week intervals with 1 µg of RiVax in 1 mg/mL of alum. Two weeks after the last injection, mice were bled before ricin challenge. Sera were analyzed for titers of RiVax-specific antibodies. Green bars = IM, blue bars = ID. Data represents 2 experiments of 4 mice each compiled into groups of 8 for analysis, with the exception of the 1000X LD_{50} ricin dose group which represents 1 experiment of 4 animals. Vehicle control vaccinated mice had negligible titers and therefore are not shown.

Following vaccination with 3 doses of 1 µg RiVax on alum, mice were challenged with increasing doses of the ricin *via* IP injection. Initial experiments were carried out using a 100, 300 and 1000X LD_{50} doses (0.1, 1.0 and 10 mg ricin per kg body weight, respectively) in an attempt to define the limits of protection. While animals in all dose groups received one IP injection of 100 µL, animals in the 1000X dose group received 2 IP injections of 100 µL. We found that a 1000X LD_{50} caused death in vaccinated mice as rapidly as a 10X LD_{50} administered to naïve mice, and therefore did not repeat this experiment. Mice in the 100X dose survived, and mice in the 300X dose level showed minimal survival. No differences in survival were found when comparing ID *vs.* IM vaccinated animals. We then attempted to find a ricin dose level where differences between the two vaccination routes might be evident. To this end, we administered the following ricin doses: 150X LD_{50} (1.5 mg ricin per kg body weight), 200X LD_{50} (2 mg ricin per kg body weight), 250X LD_{50} (2.5 mg ricin per kg body weight) and we repeated the 100 and 300X LD_{50} doses. As shown in **Figure 3.14 A** no significant differences in survival were found in mice vaccinated by either administration route. The survival against increasing doses of ricin is dose dependent (**Figure 3.14 B**).

A



B

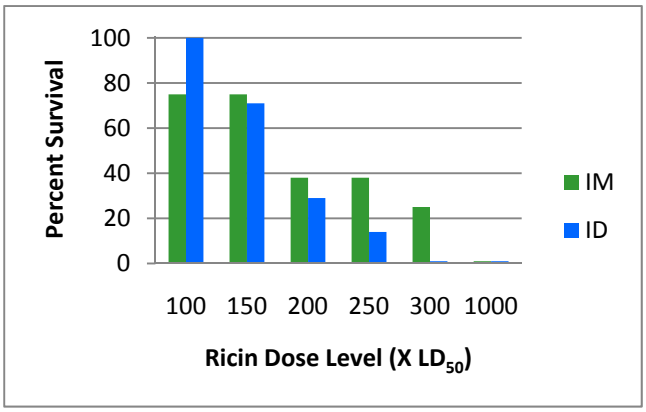


Figure 3.14: Ricin Challenge by IP Injection with Increasing Ricin Doses. All mice were vaccinated three times at 4 week intervals with 1 µg of ricin in 1 mg/mL of alum. Three weeks after the last injection, mice were challenged *via* IP injection with the stated ricin doses. (A) Purple = 100X LD_{50}; blue = 150X LD_{50}; green = 200X LD_{50}; orange = 250X LD_{50}; red = 300X LD_{50}, black = 1000X LD_{50}. (B) Green = IM, blue = ID. All data represent 2 experiments of 4 animals, compiled into groups of 8 for analysis, with the exception of the 1000X LD_{50} ricin dose group which represents 1 experiment of 4 animals.

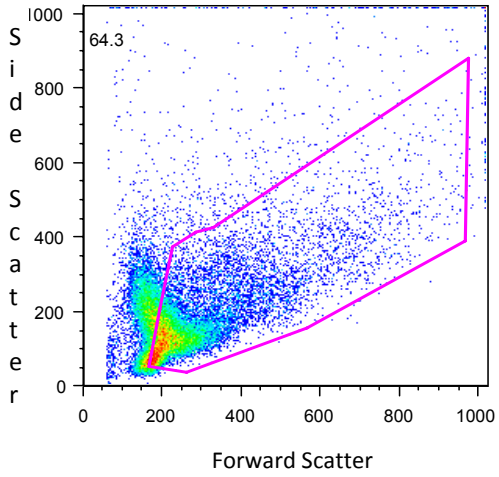
PART E. ANTIGEN TRAFFICKING TO THE DRAINING LN (DLN) FOLLOWING ID OR IM ADMINISTRATION OF RIVAX +/- ALUM

From our experiments in Part A of Chapter 3, we observed that when alum was present, there was a quantitative difference between ID and IM vaccinations in their ability to elicit serum specific antibodies and to protect animals against systemic and mucosal ricin intoxication. This led us to consider the explanation for this observation. While the mechanism of adaptive immune activation involves many steps, as outlined in the Chapter 1, we were manipulating the first step, antigen delivery. Therefore, we hypothesized that differential antigen trafficking to the DLN following ID *vs.* IM vaccination was responsible for the enhanced immunogenicity of ID vaccination. To investigate this question, we labeled RiVax with Alexa-488 and administered it either ID or IM in the left flank. The DLNs were removed at various time points in order to evaluate how many cells in the DLN contained vaccine antigen. This was conducted using flow cytometry. Initially, we had hoped to be able to evaluate which cell types were carrying antigen using cell surface staining and FACS analysis, but due to the very low percentage of RiVax positive cells we eventually had to abandon this goal.

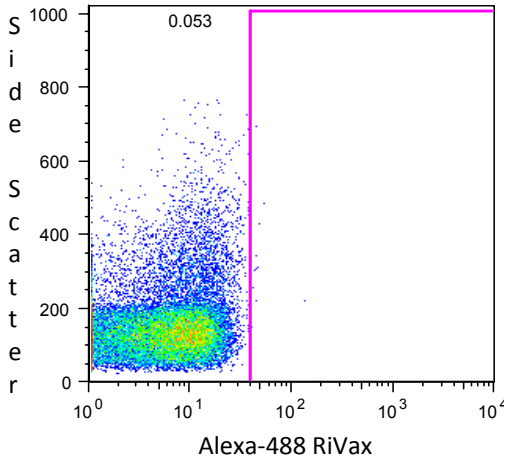
RiVax+ Cells in the DLN Following ID or IM Vaccination

Pilot experiments were first carried out 2, 6, 12, 24, 48 and 72 hours after injection to detect RiVax^{+} cells in both the draining and non-draining LNs. Very low signal at 24+ hour time points in both LNs and low signal in the non-draining LN at all time points were observed (data not shown). This led us to focus on the DLN at time points before 24 hours. We then studied mice at 3, 6, 12, and 18 hours following 1 vaccination with RiVax both with and without 1 mg/mL alum. In all cases, we administered very high vaccine doses as compared to our previous doses, i.e. 25 µg. Previous work by others suggested that high doses of fluorophore would be needed to order to detect antigen in the DLN. Given i our very low signal we chose not to try lower doses [81]. When analyzing data, I first gated on live cells in the forward versus side scatter plot. Of the live cells, the RiVax^{+} cells were then gated in order to determine the percentage of RiVax^{+} cells in the draining lymph node. These gates were drawn on a negative population (lymphocytes from animals that were injected with control vaccine) then applied to sample populations. An example of these gates as they were drawn on a negative population can be seen in **Figure 3.15**.

A



B



C

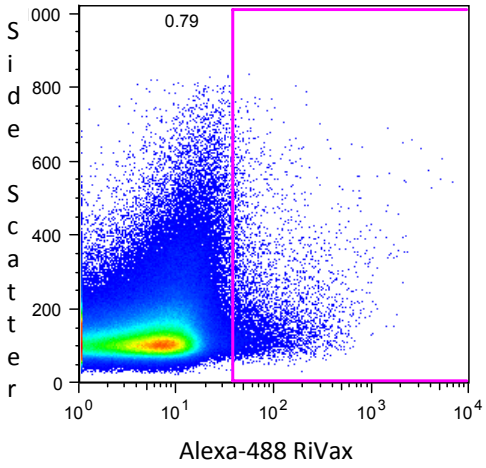
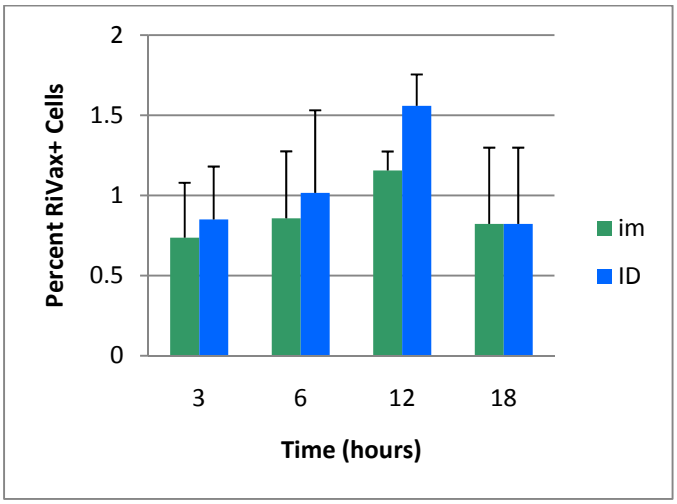


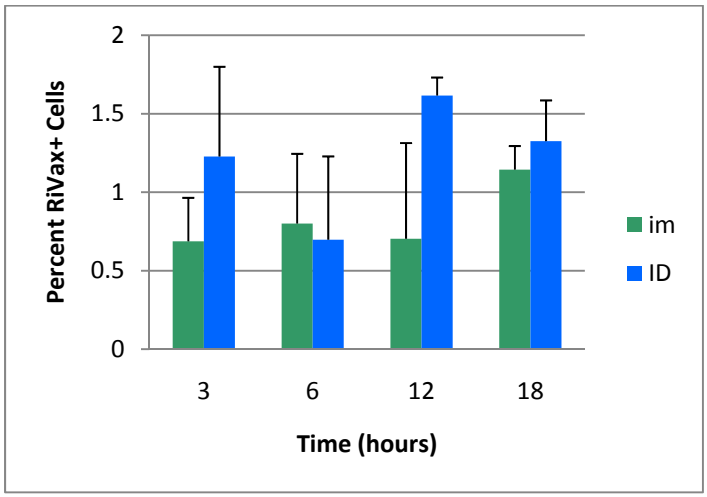
Figure 3.15. RiVax+ Gates. FACS plots of lymphocytes (A) Lymphocytes from a sham vaccinated mouse, the pink shows the gate for live cells. (B) Lymphocytes from a sham vaccinated mouse with the gate from A already applied, the pink gate shows the RiVax^{+} cells. (C) Lymphocytes from an Alexa-RiVax vaccinated mouse with the gate from A already applied, the pink gate shows the RiVax^{+} cells.

In comparing antigen in the DLN following both ID and IM vaccination without alum (**Figure 3.16 A**) we observe a greater percentage of RiVax^{+} cells over time. ID vaccination resulted in more antigen^{+} cells in the DLN as compared to IM vaccination, though not significantly more. This time course was not as prominent following ID *vs.* IM administration of RiVax with alum (**Figure 3.16 B**), but a trend was still seen with antigen traffic peaking at 12 hours. What is most striking is the fact that more antigen arrived in the DLN more quickly following ID vaccination with alum as compared to: IM vaccination with alum, IM vaccination without alum, ID vaccination without alum. This could be one possible explanation for enhanced immunogenicity of RiVax following ID vaccination with alum observed in **Figure 3.3.** It is important to note that given the large standard de*via*tions, this is only a trend. These experiments must be repeated before solid conclusions can be drawn.

A - Alum


Percent RiVax+ Cells
2
1.5
1
0.5
0
3
6
12
18
Time (hours)
im
ID

B + Alum


Percent RiVax+ Cells
2
1.5
1
0.5
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im
ID

Figure 3.15: RiVax^{+} Cells in Draining LN Following ID or IM Vaccination. Animals were injected with (A) 24 µg Alexa 488-RiVax without alum, or (B) 22 µg Alexa 488-RiVax with 1 mg/mL alum. Green = IM, blue = ID. Injections were done in the left flank, either ID or IM and the left inguinal node was harvested for FACS analysis. Negligible amounts of Alexa 488-RiVax were found in the non-draining inguinal node or at 24, 48 and 72 hour time points, therefore neither are shown here. All data represent 2 experiments of 3 mice each.

CHAPTER FOUR
Discussion

PART A. STUDY OBJECTIVES AND MAJOR FINDINGS

We have previously described a recombinant ricin vaccine, RiVax, composed of RTA containing mutations that eliminate all known toxicities [73]. We have demonstrated that when RiVax is administered to mice by IM injection without alum, it was entirely safe and protected mice from ricin-induced death following delivery of the toxin by IP injection, gastric gavage, or aerosol [24]. RiVax was safe and immunogenic in mice, rabbits and humans [78] [79]. Adoptive transfer of human anti-RiVax antibody into mice (at levels found in human serum) protected them from ricin intoxication [79]. Thus far, all of our studies have been carried out using IM vaccination of RiVax with or without alum.

The goal of this study was to compare ID *vs.* IM administration of RiVax by evaluating the following parameters of vaccine efficacy: (1) short-term antibody responses and protection of animals from a 10X LD_{50} of ricin; (2) long-term antibody responses and protection of animals from a 10X LD_{50} of ricin; (3) protective effect of a single high dose of RiVax from a 10X LD_{50} of ricin; (4) the minimum ricin dose at which vaccinated animals are

no longer protected; (5) the rate of antigen trafficking to DLN following administration of RiVax.

The major findings of this study were as follows:

1. In the short term, following a 3 dose vaccine regimen and subsequent challenge by IP injection, gastric gavage or aerosolized ricin:
    a. Both IM and ID immunization with RiVax induced equal titers of RiVax-specific serum antibodies, this occurred both with and without alum. At low doses with alum, the ID route was superior to the IM route in inducing RiVax-specific antibodies.
    b. When RiVax was administered without alum, both IM and ID immunizations were equally effective in protecting animals from a lethal ricin doses given by aerosol, gavage or IP injection.
    c. When RiVax was administered with alum, ID administration was superior to IM administration in protecting animals from a lethal ricin dose given by aerosol, gavage or IP injection.

d. When RiVax was administered without alum, IM and ID immunizations were equally effective in preventing lung damage in mice exposed to aerosolized ricin. At intermediate doses, ID administration of RiVax on alum was superior to IM administration.

e. Serum antibody titers correlated with both survival and lung function in all animals regardless of vaccine dose level or route of vaccination.

2. In the long term (following a 3 dose vaccine regimen and challenge by IP injection, gastric gavage or aerosolized ricin 8.5 months later) we found ID administration of RiVax to be equivalent to IM administration in its ability to induce RiVax-specific serum antibodies, to protect mice from systemic and mucosal ricin challenge, and to prevent lung damage following exposure to aerosolized ricin.

3. RiVax administered on alum in a single high dose, without any subsequent boosts, was found to be partially protective. ID and IM vaccinations in this situation were found to be equivalent.

4. Both ID and IM administration of RiVax were equally effective in protecting animals from challenge with a supra-

lethal ricin dose. However, when ricin was administered at doses higher than 100X LD_{50}, survival was poor, often falling below 50%.

5. Analysis of the kinetics of RiVax delivery to the draining LN following either ID or IM vaccine administration revealed that the two routes were statistically similar to one another, although there may be minor differences between the two that will require further study.

PART B. SHORT TERM PROTECTION OF MICE FROM RICIN INTOXICATION FOLLOWING ID *VS.* IM ADMINISTRATION OF RIVAX

Induction of Serum Antibody

ID or IM administration of RiVax both with and without alum revealed a dose-dependent RiVax-specific serum antibody response. In comparing RiVax administration with and without alum, we observed that alum significantly boosted the antibody responses. These observations were expected and agree with previous data testing IM immmunization of RiVax with and without alum [24, 80].

When RiVax was administered with alum at low dose levels, ID vaccinations were superior to IM vaccinations in terms of both serum titers and survival following challenge. Our previous work with the IM immunizations demonstrated that the low dose level was the only one that conferred less than 100% survival [24, 80]. We were therefore not surprised to observe that it was the only dose level at which we could see differences in antibody titer due to the route of administration.

Reports from other groups comparing GMT following either ID or IM vaccination usually compared a low ID dose to a normal IM dose, both with adjuvant. Such comparisons often showed that low dose ID vaccination was equivalent to the higher IM dose, leading to the conclusion that the ID vaccination was superior [82-86]. However, seroconversion rates and GMT following a low IM dose has rarely been presented. Historically, extensive dose titrations have not been done during vaccine development in order to find the lowest protective dose. Therefore, it is unclear whether lower doses given IM could also induce significant antibody titers. In fact, in one study that compared three dose levels of vaccine given either ID or IM, 3-fold lower vaccine doses given IM (or ID) were equivalent to their full dose counterpart in eliciting serum specific-antibodies [87]. These differences in experimental design make it difficult for us to draw extensive comparisons between our work and that of others.

Systemic and Mucosal Protection from Ricin Intoxication following RiVax Administration without Alum

Extensive studies in our laboratory have shown RiVax to be highly protective when administered *via* IM injection without alum [78] [24]. In this

study we observed that RiVax alone delivered by the ID or IM routes was equally effective at protecting mice from aerosolized, gavage, or IP-injected ricin. Due to the well established advantages to administering vaccines in alum and its extensive history of safety, most new vaccines are rarely studied without alum [41]. Therefore it is difficult to find data comparing ID and IM vaccination with protein vaccines in the absence of alum.

Systemic and Mucosal Protection from Ricin Intoxication following Administration of RiVax with Alum

Administration of vaccines on alum as a method of augmenting immunogenicity has been standard practice for decades. For nearly 70 years, alum has been reported as effective in increasing specific antibody titers, seroconversion rates, and the longevity of these responses [88]. Here we compared RiVax administration alone and with alum (within each administration route) and found that our results are comparable to our previous findings following alum vaccination and IP ricin challenge [80]. While RiVax alone was very effective in inducing seroconversion, we found that the addition of alum significantly increased specific antibody levels while also improving protection from ricin induced death. The

addition of alum to RiVax decreased the dose needed for this protection by at least 10-fold.

We observed that ID administration, as compared to IM, of RiVax on alum significantly improved protection against ricin delivered by gavage or aerosol. This finding is significant for two main reasons: (1) the most likely route of ricin intoxication would be *via* aerosolized liquid or powder, or contamination of food or water sources; (2) most pathogens and toxins enter the body through mucosal surfaces making it important to find novel ways to improve mucosal protection. Hence, research has focused on novel adjuvants and administration routes that enhance mucosal immunity. In our studies we have observed that a protein vaccine, such as RiVax, administered with alum *via* the ID route protects *both* gut and respiratory mucosa as well as, or better than, an equivalent dose administered by the IM route. While IM administration of RiVax on alum was superior to RiVax alone in protecting animals, the observed improvement was greater when the adjuvanted vaccine was administered *via* the ID route. This is not unexpected for several reasons: (1) ID administration of RiVax on alum induced significantly higher antibody titers as compared to IM administration at low doses; (2) in humans, past studies comparing ID *vs.* IM administration of vaccines on alum have

shown that the ID route is 5-fold more effective at inducing specific antibodies [85-86]; (3) recent findings have demonstrated that alum enhances both activation and antigen presentation by DCs [44], and DCs are far more prevalent in the skin than in muscle. Since DCs initiate adaptive immune responses to vaccines, the large numbers of such cells in the skin would suggest that ID administration of a vaccine would be advantageous.

Lung Damage following Exposure to Aerosolized Ricin

As compared to the IM route, administration of RiVax with alum *via* the ID route was also superior in maintaining lung function in animals exposed to aerosolized ricin. While immunization with RiVax protected mice from ricin-mediated death, it was also important to determine whether it led to maintenance of lung function. This was important since poor lung function could result in significant downstream morbidity and mortality. Mucosal protection is mediated primarily by IgG and sIgA. sIgA is produced locally while most IgG exudates into the mucosa by diffusion [89]. Local IgA works primarily by preventing a toxin or pathogen from breaching the epithelium. IgG can neutralize and opsonize toxins in mucosal sites as well [38].

Previous *in vitro* studies have demonstrated that subsets of dermal DCs induce humoral immune responses including the production of IgG and IgA [90]. It has also been shown that transcutaneous vaccination stimulates antibody production at mucosal surfaces [91]. Taken together with the current findings that ID vaccination is more effective at maintaining normal lung function following challenge with aerosolized ricin, it can be concluded that ID vaccination may be superior to IM vaccination at providing mucosal protection against toxin exposure and perhaps pathogen exposure as well.

In addition to the immune cross talk between the skin and mucosal surfaces, we also know that historically, systemic administration of vaccine has provided protection from pathogens that are encountered at mucosal surfaces. Hepatitis A, pneumococcus and chicken pox are good examples of killed, systemically administered vaccines that protect mucosal surfaces from pathogen [92-94].

Correlation of RiVax-Specific Serum Antibodies to Survival and Lung Function

We found that levels of RiVax-specific antibodies in the serum correlated with survival and lung function. Serum antibody levels have long been accepted as an indicator of vaccine efficacy especially in human studies where challenges with toxins or pathogens are not possible. In our animal models of ricin toxicity, we were able to show a correlation between specific antibody levels in the serum and both survival and lung function, regardless of the route of vaccination.

These correlations speak to the issue of the specific mechanism of RiVax protection. Due to a lack of data regarding mucosal antibody levels and Ab isotypes in our model, we cannot be certain of the exact source of protective anti-ricin antibodies, but we can speculate on the mechanism. There are two likely sources of protective anti-ricin antibodies, the serum and the mucosa, and determining which ultimately provides protection will be difficult, but important for future vaccine design. First, we should consider what types of humoral immune responses are present at the mucosa. The most prevalent mucosal antibody is sIgA, which is produced locally, joined into a homodimer by the J chain and transcytosed to the mucosal surfaces *via* the pIgR. Part of the pIgR remains associated with the antibody dimer after transcytosis, and is called the secretory

component. IgG is prevalent in the mucosa, although to a lesser degree. IgM, which also depends on pIgR to form into a secreted multimer, is rarer than IgA or IgG. IgE is extremely rare in the mucosa, under normal conditions. In general, the upper respiratory tract has a higher sIgA:IgG ratio, while the opposite is true in the lower respiratory tract [95]. Due to the inability of sIgA to mediate inflammation *via* complement activation, it is often thought to be the most important antibody involved in mucosal protection[38].

Based on the normal antibody distribution in the lung [95], it would be reasonable to suggest that mucosal sIgA would not be the primary protective antibody in our model; and that systemic administration of RiVax (either ID or IM) protect animals *via* exudation of IgG from the blood. In support of this theory, studies looking at $IgA^{-/-}$ mice revealed that animals can still fight infection without sIgA, and that these animals have increased serum IgG and to some extent serum IgM [96]. This compensation also occurs in humans deficient in IgA [95]. Reports of antibody levels and degree of protection in $pIgR^{-/-}$ mice are inconsistent and no firm conclusions can be drawn from them [97-100]. It is therefore likely, that serum IgG and IgM are capable of conferring protection at mucosal surfaces and perhaps RiVax is only inducing systemic antibodies

which are able to “leak” into the mucosa. In fact, this is consistent with the finding that IgG can be actively transported to mucosal surfaces *via* FcRns [101-102] and passively transported *via* bulk plasma exudation [103] especially following allergic or asthmatic responses [104-105]. Direct mucosal protection by IgG has been extensively tested in passive protection experiments, where antigen specific IgG has been administered systemically to naïve individuals/animals prior to mucosal exposure to a pathogen. Many such studies have found that serum derived IgG, is sufficient to protect humans from mucosal infections [106-107] [108-109]. It is also known that 90% of the immunoglobulins passed from mother to child *via* the placenta are IgGs which provide the primary protection of the developing fetus. Importantly, with regard to our studies, there are reports of IV injected anti-ricin antibodies that protect animals from aerosolized ricin challenge [110]. Another piece of evidence suggesting that systemic vaccination protects the mucosa is the recent approval of the human papiloma virus vaccines which are protein vaccines administered IM with alum. While we know that the vaccine is effective in animals and induces seroconversion in humans, longetivity studies will have to be done to see if the frequency of cervical cancer in the population decreases. But the animal data does point to the fact that systemic vaccination can protect mucosal surfaces [111].

Taken together, these data suggest that sIgA prevents pathogens and toxins from breaching the mucosa, but that it is not essential for protection, particularly when the immune insult is small. In our studies, it is unlikely that we induce a local mucosal immune response although this was not studied. We do know, however, that we induced a systemic antibody response that was capable of protecting animals from a *mucosal* ricin challenge. The next critical step will be to determine how much RiVax-specific IgG and IgA are present at mucosal surfaces following vaccination.

PART C. LONG TERM PROTECTION OF MICE FROM RICIN INTOXICATION FOLLOWING ID *VS.* IM ADMINISTRATION OF RIVAX

In studying ID and IM vaccinated mice 8.5 months following vaccination, we found that animals survived both systemic and mucosal ricin challenges and that lung damage was prevented in animals exposed to aerosolized ricin. We also observed that there was RiVax specific-antibody in the serum, though significantly less than what was found in the serum of animals in our short term studies. In fact, in comparing GMTs of mice from our long term studies to those of mice in our short term studies, we observed that the animals in the former had only 20% of the antibody found in the later. This decrease in antibody titers is in agreement with the literature [112]. Animals in both the 1.0 and 0.1 μg long term dose groups had antibody titers that were similar to those from mice in the 0.1 and 0.01 μg short term dose groups, respectively. Regardless, nearly 100% of the animals in the 0.1 μg long term dose group survived a ricin challenge, while survival of the 0.01 μg short term dose group never exceeded 50%. In fact, survival of the mice in the 0.1 μg long term dose group was similar to that of the 0.1 μg short term dose group, even though their GMTs were significantly different. There are a number of possible explanations for these findings.

First, assuming the antibodies produced the long and short term experiments are qualitatively similar, it is possible that there is a threshold level of antibody needed to confer protection. Hence animals in the 1.0 and 0.1 μg dose groups had titers that reached (and likely, significantly exceeded) this threshold while the animals in the 0.01 μg dose group did not. Therefore, when there was an 80% reduction in the GMTs after 8.5 months, the animals still survived. If this is true, it might be predicted that this survival threshold for serum antibodies is approximately 15 to 70 μg/mL level, correlating to the range of titers in the short term 0.01 μg dose group.

As discussed in Chapter 3, not all antibodies that are specific for an antigen are capable of neutralizing it. An increase in the number of B cell clones producing neutralizing antibodies in the mice receiving the 1.0 and 0.1 μg dose levels as compared to animals in the 0.01 μg dose group could explain the different rates of survival following ricn challenge. The rate at which these neutralizing antibodies are produced may also play a role. Somatic hypermutation is an important aspect of B cell development that occurs from repeated exposure to an antigen [38]. It is possible that animals in the high and middle dose level are more efficiently boosted and

that this, in combination with the depot effect of the alum, results in better protection.

A related explanation for the waning titers, but persistent protection, is that administration of the 1.0 and 0.1 μg dose levels in alum induced a robust population of memory cells and long lived plasma cells. We could predict that the 0.01 μg dose level would not induce such a uniform memory response given that at least 50% of animals in this group did not survive following short term challenge. Long lived plasma cells have been documented to persist in the bone marrow of animals for over one year (nearly half the lifetime of a mouse), without the need for antigen in order to maintain them [113-114]. This leads us to suggest that if protective immunity in RiVax vaccinated animals is maintained after the peak of the secondary immune response, it will most likely continue to persist at later time points than those studied here.

Another mechanism that maintains high levels of circulating levels of antibodies is boosting from exposure to other pathogens or proteins. It has been shown that polyclonal immune activation will cause a certain degree of non-specific activation of memory B cell clones. This is because T cell help in the form of cytokines is non-specific and can therefore activate

many B cell clones in the bone marrow. Hence, those responses that lasted longer might be due to cross-reactive and/or polyclonal boosting. This is a possible explanation for why some individuals have better long term immunity than others, making it difficult to determine how often people should receive vaccine boosts [115-116].

When we take this data together with the results of the short term studies, we conclude that in the short term, ID vaccination is superior to IM vaccination, but that in the long term the two are equal. Therefore, perhaps ID vaccination, as compared to IM, accelerates the immune response rather than increases it. This information is of particular importance when deciding how to administer a vaccine and to whom. In an emergency situation where immediate ricin exposure is of concern, ID vaccination may be the only feasible route of administration. In situations where potential exposure is not anticipated for several months, the two vaccination routes can be considered equivalent. In a military setting these issues are very important. Soldiers being vaccinated immediately before deployment should receive ID vaccinations, while those who will not be deployed for some time, can be vaccinated by either route.

Lastly, in comparing data from other protein vaccines, we observed that long lived immunity is not rare in adult populations. Studies with tetanus vaccine have suggested that some individuals still have circulating levels of anti-tetanus antibodies after 20 years while a small subset of individuals have little or no anti-tetanus antibodies after 7 - 10 years [117]. Boosters every 10 years assures that all individuals should have high levels of circulating antibodies. Another study involving nearly 10,000 subjects concluded that following the typical 3 dose regimen of the hepatitis B vaccine, protection lasted for up to 20 years [118]. It is important to note that life-long follow-ups of antibody titer are rarely done so it is unclear why some individuals have life-long protection while others do not. The lack of such data also makes it difficult to speculate whether vaccine induced immunity does indeed wane after 20 years. In addition, age related waning of the immune response must be considered [119].

PART D. PROTECTION OF MICE FROM RICIN INTOXICATION FOLLOWING A SINGLE ID OR IM ADMINISTRATION OF RIVAX ON ALUM

A significant contributing factor to vaccine efficacy is patient compliance. Vaccines regimens that require numerous boosts in order to confer protection are less likely to be followed by patients. In a military setting this is especially important since soldiers may be deployed quickly, without time to receive all necessary vaccinations; this was a well documented issue during the first Gulf War [120]. We were therefore interested to determine whether we could protect animals against systemic ricin intoxication with a single dose of RiVax, and to determine if ID vaccination would be advantageous in this situation. In assessing serum antibody titers and survival following challenge, we observed that ID and IM vaccination was equally effective.

Serum antibody titers in animals receiving a single RiVax dose were significantly higher than in sham vaccinated mice, but also significantly lower than in animals receiving a full 3 dose vaccine regimen. In fact, 2 weeks after a single vaccination, antibody titers in animals were lower

than those observed at 8.5 months after a full 3 dose vaccine regimen. Given these data, we predicted that none of these mice would survive. Surprisingly, we found that in the middle dose groups (50 μg), 50% of animals survived. Even more surprising was that very few animals in the high dose group of 200 μg did not survive. From these initial experiments, it appears that survival following a single administration of RiVax in alum is not dose dependent. One possible explanation for this could be the induction of allergy. It has been know for quite a long time that ricin can induce significant IgE production and allergy. Studies of ricin allergic people working in a castor bean plant showed that they had a significant rate of anti-ricin IgE induction [121]. This led to studies in rats which showed that administration of ovalbumin with ricin induced an increase in ovalbumin specific IgE, but not ovalbumin IgG [122]. Therefore, in our studies, it might be that beyond a certain threshold dose, RiVax may be able to sensitize animals. And upon subsequent challenge, sensitized animals cannot survive the robust allergic response. On the other hand, animals in our low dose group of 10 μg most likely did not develop enough of an immune reaction to RiVax in order to protect from ricin. This idea is in agreement with the fact that we observed a dose dependent antibody response following a single administration of RiVax. This hypothesis would be an important one to follow in order to know if there is a point at which

RiVax may sensitize an individual to ricin. Though even if it did, an allergic response to ricin challenge is likely more easily treated than ricin intoxication itself which was described in Chapter 1.

The majority of studies investigating the administration of a single dose of vaccine have been done in humans and often report the antibody titers following one dose of vaccine; these studies often go on to administer subsequent boosts, so it is difficult to assess the protective value of a single vaccine dose [123-126]. Influenza vaccine is given in a single dose, but it is important to realize that we cannot compare results from these studies to our data because most individuals are vaccinated yearly against influenza. Since influenza vaccines from year to year share antigens they may boost cross reactive memory cells. Therefore, an annual influenza vaccination is really an annual boost. In fact, the first time an individual is immunized against influenza, it is recommended that they receive two doses [127].

PART E. PROTECTION OF MICE FROM INCREASING DOSES OF RICIN

Protection from physiologically relevant doses of toxin/pathogen is an important requirement for any vaccine. Determination of what this dose could be when dealing with an agent of bioterrorism can be very difficult to ascertain and depends on several factors. As mentioned in Chapter 1, toxicity due to ingestion of ricin is likely to be highly variable from individual to individual, due most likely to digestive differences among individuals which could interfere with ricin absorption [24]. In addition, , particle size plays an important role in determining the toxicity of aerosolized ricin, with smaller particles being more toxic due to their ability to travel deeper into the lungs [2]. Lastly, the quality of the ricin preparation is probably the most important determinant of ricin toxicity and this can vary significantly. Therefore, in designing a ricin vaccine, it is important that circulating antibodies are able to neutralize extremely high doses of ricin due to the lack of predictability of exposure. In our experimental model, we selected a 10X LD_{50} of ricin, which is equivalent to an LD_{90}. However, in this study, we chose to test the limits of this protection and determine if ID administration can confer additional protection against a supra-lethal dose of ricin.

While we found ID and IM vaccination to be equivalent in protecting mice against a supra lethal IP injection of ricin, we also found that animals were well protected against up to a 100X LD_{50} of ricin administered IP, with nearly 80% of all animals surviving.

Since protection against high doses of ricin was dose dependent, we predict that additional vaccine boosts and/or higher vaccine doses would protect from even higher doses of ricin. Another possible strategy may be to combine systemic (either ID or IM) vaccinations with oral boosts. Earlier in this Chapter, the importance of mucosal sIgA versus serum IgG was discussed. Serum IgG may be sufficient to protect animals from a 10X LD_{50} of ricin, but perhaps it is not enough to protect from a 300X LD_{50} and could benefit from having more sIgA on board. Given what we know about the induction of mucosal immunity, the best way to increase sIgA would be to vaccinate at the mucosa. Therefore, the best strategy to provide sufficient protection against supra-lethal ricin doses may be to administer the first and second vaccinations *via* the ID route and the third *via* the oral route. Of course oral vaccination with a protein vaccine has its own challenges and would require the optimization of a delivery system that protects the vaccine from degradation.

PART F. ANTIGEN TRAFFICKING TO THE DRAINING LN (DLN) FOLLOWING ID OR IM ADMINISTRATION OF RIVAX +/- ALUM

Studies of DCs in the muscle are very rare and normally investigate specific pathology. Even more rare are studies comparing the immunostimulatory capacity of DCs in the muscle to DCs in the skin. In these studies we have compared the rate at which antigens traffic to the DLN following either ID or IM administration of fluorescently labeled RiVax. Although our studies did not show statistically significant differences between ID and IM delivery of antigen, there were clear trends. Most significantly, without alum, ID delivery resulted in greater migration of RiVax to the DLN, although this did not correlate with the results of our survival studies. With the addition of alum, ID *vs.* IM vaccination resulted in more rapid and massive antigen migration. Increased antigen migration to the DLN is consistent with the fact that there are more DCs (Langerhan's cells) in the skin than there are in the muscle. Hence there are more DCs available to carry antigen to the DLN. The more rapid migration of antigen to the DLN following ID injection is likely due to the fact that lymphatic drainage from the skin is 10-fold greater than lymphatic drainage from the muscle [128]. This finding could provide one explanation for the superior survival seen in ID, but not IM,

vaccinated mice when RiVax is administered on alum. In comparing antigen trafficking with and without alum, there is a clear difference in kinetics, with alum causing more rapid antigen uptake. Previous studies have suggested that alum has significant influence on DCs and increases their ability to carry antigen [42]. It is possible that differential antigen kinetics is one of many mechanisms by which alum boosts immune responses.

It is interesting to consider which cell types may be carrying the Alexa-488 RiVax in these studies. Common knowledge would assume that they are skin DCs that have migrated to the DLN, but without staining for DC markers we don't know if that is the case. Knowing that lymph traffic from the skin is 10 fold faster than from the muscle, it is possible that the observed $RiVax^{+}$ cells were LN resident APCs that picked up antigen carried into the LN by the lymphatics. These could be macrophages, B cells or DCs. Knowing that DCs are responsible for the initiation of a primary immune response, it will be critical to determine the numbers of DCs in the DLN carrying antigen following ID or IM vaccination. Then further evaluation of the types and origins of DCs carrying the antigen in both cases must have to be done before we can corroborate the hypothesis that the increased number of DCs in the skin allow for dose

sparing following ID vaccination. Alternatively, it could be that increased uptake of antigen by resident B cells and macrophages may be allowing for more effective boosting following ID *vs* IM vaccination therefore allowing for dose sparing following ID vaccination.

There are some data showing the time course of antigen trafficking to the DLN, though this was not the primary goal of these papers so it is difficult to directly compare their results to those presented here. In a paper by Delamarre et al. the group was interested in differential degradation of proteins and how that impacted on subsequent immune responses. In preliminary experiments the group injected fluorescently labeled RNase-A and RNase-S intradermally into mice then harvested draining lymph nodes and carried out FACS analysis to determine the time point at which antigen delivery peaked. Unlike RiVax, RNase-A and RNase-S are ~15kDa, and the group found that it was most prevalent in the draining node after 2.5 hours and was cleared by 16 hours. It is important to note that these investigators injected animals on the belly and then removed inguinal nodes. Therefore it would be expected that their migration time would be much shorter than what we see with a flank injection. Nevertheless, the time course they observe is similar to what we observed with RiVax and they too did not see antigen in the non-draining lymph

nodes (Delamarre, personal communication). The caveat to both of these studies is the limit of detection; it is possible that more effective labeling may yield different results [81].

Another paper that indirectly relates to this work is one by Sugita et al. where increased DC migration from the skin to the draining LN is explained as a response to inducible nitric oxide synthase (iNOS) inhibitor; this is thought to play a role in contact hypersensitivity. The animals received IP injections of an iNOS inhibitor and 24 hours after the last injection the bellies of the animals were shaven and FITC was painted onto them. The draining inguinal and axillary LNs were harvested 24, 48 and 72 hours later and cells were analyzed by FACS for $FITC^+$ cells. This group observed the peak of their FITC migration at the 24 hour time point, though given the data presented in Figure 3.16, it is possible that their peak was actually within the first 24 hours. The group did not present any data at time points before 24 hours. What is consistent with their work and the work done with RiVax is the significant decline in antigen in the DLN after 24 hours. This group was able to detect signal after 24 hours while we were not; this is most likely due to differences in the sensitivities of the two systems. While they were able to apply copious amounts of FITC onto

the belly of the mouse, we were limited by the degree to which RiVax could be labeled [129].

PART G. CONCLUSIONS

There have been many attempts to develop a prophylactic ricin vaccine, using different preparations of the ricin toxin with or without various adjuvants [62, 66, 70]. But none of these has been as extensively studied as RiVax and none have looked at the ID vaccination route. Since it is likely that a ricin vaccine would be most useful for military personnel, the ease of ID vaccination with lower vaccine doses is important.

Here we have done extensive studies to compare numerous aspects of vaccine efficacy following both IM and ID administration of RiVax both with and without alum. In all of these comparisons, we have observed that ID administration is completely equivalent to, and at low doses in the presence of alum, superior to IM administration. This is especially important given that the primary utility of ID vaccination is the ability to administer lower doses of vaccine while still conferring protection. In our study, we investigated three dose levels of vaccine over a two log range. Due to the wide range of doses that we tested, we were unable to identify the minimum dose that could be administered ID in order to achieve protection which was equivalent to that of an IM administration. Prior studies comparing seroconversion following ID *vs.* IM administration of

adjuvanted protein vaccines have revealed that approximately a 20% dose given ID is equivalent to a 100% dose given IM [85-86]. Therefore, we were not surprised that our low ID dose levels did not confer protection that was equal to our middle and high IM dose. However, this will be an important avenue of research for future studies.

In addition to systemic protection, we found that systemic administration of RiVax (either ID or IM) conferred mucosal protection and maintains normal lung function following exposure to aerosolized ricin. This is clear proof that systemic administration of RiVax with or without alum confers mucosal immunity. This mucosal protection was significantly better following ID administration of RiVax on alum. In light of passive protection and vaccine studies in IgA knockout mice [89, 130], it is likely that improved mucosal immunity following systemic vaccine administration is the result of increased serum specific-antibodies that are able to exudate to mucosal surfaces.

We also observed that a single dose of RiVax, given either ID or IM, on alum induces production of serum specific antibodies while conferring some protection. Further investigation of this issue will be needed before we can administer a single effective dose of RiVax.

In studying the minimum lethal ricin dose in a vaccinated animal, we observed that we were able to protect mice from higher doses of ricin than we had previously tested. Here we found that RiVax vaccinated animals challenged with ricin doses up to a 100X LD_{50} do not die, and that both ID and IM vaccination routes were equally effective.

Lastly, in an attempt to determine why ID vaccination as compared to IM vaccination may facilitate dose sparing, we investigated the kinetics of antigen trafficking to the DLN following either vaccination route. Here we saw noticeable differences in both the kinetics and the amount of antigen migrating to the DLN following ID and IM vaccination. With high variability in the data, more extensive studies must be carried out before these observations can be utilized in vaccine design.

Rivax as compared to other Ricin Vaccines

The group that did the most extensive work in developing a ricin vaccine was Griffiths et al. whose studies focused on a ricin toxoid vaccine, which provided sufficient protection, but did not protect from lung damage [58]. To improve this they used a liposomal adjuvant, which showed qualitative

improvements in lung function and significant increases in BAL IgA which correlated with survival. They did evaluate serum titers following a single vaccine dose, but did not challenge after a single dose [59]. Lastly, Griffiths compared their toxoid vaccine to an RTA vaccine and found the former to be superior in eliciting total antibody, IgA in the BAL and in preventing lung damage [60] [61].

There are a number of interesting comparisons that can be made between the work of Griffiths et al [62]and the work presented here. First and foremost, Griffiths utilized extremely high doses of vaccine; we used many multiples less. Secondly, they did not evaluate lung function, such as histopathology or measurement of impaired breathing. Lastly, Griffiths found their toxoid to be most effective (rather than RTA) and there are concerns that toxoids have the ability to revert to their active form. Griffiths' finding that ricin toxoid made a better vaccine than RTA, which is similar to our vaccine, is not surprising. Toxoid has far more epitopes, and these epitopes are more likely to resemble the epitopes of ricin. RiVax only presents epitopes present in RTA and those that are normally "hidden" by RTB binding; therefore it is likely that we elicit far fewer antibody clones than a toxoid vaccine. Overall, a number of factors prove RiVax to be a better vaccine that Griffiths' toxoid vaccine, specifically: the

safety of RiVax, the significantly lower administration doses, successful protection from ricin morbidity and lung damage. Another disadvantage to the Griffiths' vaccine was the induction of abnormally high IgE; we have not looked at serum IgE levels in RiVax vaccinated mice, but in light of Griffiths' work this would be an interesting area to investigate.

Two groups at USAMRIID have together produced the greatest number of papers about ricin vaccine development, though they have employed different strategies. In studying their ricin anti-idiotype vaccine, this group was the only one to test vaccine efficacy against increasing doses of ricin. They evaluated survival following SC challenge with 20, 35 and 50 µg toxin/kg body weight and found that their vaccine only protected against a 20 µg/kg challenge; this correlates to a 20X LD_{50} *via* the IP or gavage route [63]. In our studies we found that we were able to protect against significantly greater doses of ricin, with as much as 100% survival against a 100X LD_{50} in animals treated with a 3 dose regimen of ID administered RiVax.

The next strategy employed by USAMRIID was to develop a toxoid vaccine and administer it with a unique adjuvant, namely PLGA and PLA. Unlike Griffths' work with a toxoid vaccine, these studies used far lower

doses (50μg given once or twice) give IN, though it was still 50 fold higher than the doses of RiVax that we gave with adjuvant. The group found that just one dose of their vaccine protected 70% of animals and just two doses protected 100% of animals from ricin morbidity for up to one year post vaccination, though they did not evaluate lung function in these animals. These single dose results were superior to what was presented in this study, especially given the doses they were using. We were unable to achieve 70% protection following a single dose with either 200 or 50 μg of RiVax. In the group's next study though, they were able to achieve 100% protection following a single SC administration of their vaccine, though their elaborate oral vaccination schedule was far less successful. While the authors do not focus on it, the utility of a single dose vaccine would be incredible. Unfortunately, none of these studies evaluate lung function following an aerosol challenge, nor do they evaluate the toxicity of their unique adjuvant. And again, just as with Griffiths' vaccine, toxoids do have the potential to revert, while RiVax does not [65] [64].

The next strategy employed by USAMRIID used a dgRTA, which would have minimal toxicity due to its inability to enter cells, but it does retain its VLS inducing site which RiVax does not have. In these works, the group was able to administer as little as two vaccinations of 8 μg of vaccine with

one of two adjuvants, LTR72 or LTR63. This dose is much lower than what other groups have administered, but is still significantly higher than what we have administered with adjuvant. Here the group did histopathology to determine the degree of lung damage in vaccinated animals following challenge and they found that their vaccine and adjuvant did not protect from lung damage. While we did not see any functional decrease in lung function in RiVax vaccinated animals following challenge, it is possible that we may have seen some pathology had we done histopathological analsysis.

The last of USAMRIID's vaccine strategies was to use a truncated RTA administered by IM injection either with or without alum and they found their vaccine to be effective in protecting animals from an aerosolized ricin challenge [68]. Their vaccine was found to be extremely stable and they developed a vaccine formulation that allowed for optimum alum adsorption and storage [69]. The major strength to this strategy compared to their last two was that it did not involve experimental adjuvants, nor did it utilize a toxoid. As compared to RiVax, they were still administering 10 fold higher doses in alum and their vaccine still contained the VLS-inducing site.

Overall, in comparing this study to work that has previously been done there are a number of observations that can be made. First, administration of RiVax in alum utilizes far less antigen than any other ricin vaccine candidate; the studies presented here suggest that even lower doses may be used when the ID route is used. This is the first time that a dose sparing ID regimen has been investigated with a ricin vaccine. Second, RiVax has been shown to protect against aerosol, gavage and IP ricin challenges; no other group has studied a gastric gavage model of challenge following prophylactic vaccination. Third, this study represents the first time that an extensive ricin dose escalation has been done, showing that RiVax can protect from significant ricin doses. Fourth, we have seen here that RiVax is not as successful as other vaccines in its ability to protect in a single dose; perhaps novel adjuvants will be needed to achieve this goal. Fifth, while others have compared different administration routes, none have attempted to understand the mechanistic differences between various vaccination routes, this study is unique in that feature. Lastly, RiVax is the only ricin vaccine to have been studied in clinical trials.

RiVax as Compared to Anthrax Vaccines

In comparing RiVax to other vaccines, the best comparisons arise from other protein vaccines against a toxin because they must protect by a similar mechanism as RiVax. Vaccines that protect from live pathogens are improved by $CD8^+$ T cell responses which are not helpful when protecting from a toxin. There are numerous pathogens that kill *via* their toxin rather than infection, these include anthrax, botulinum toxin, diphtheria, pertusis, and cholera. All of these have either approved vaccines or vaccines that are under development. In order to make the most direct comparisons, I will focus on anthrax since it is produced for a similar population, produced in a recombinant form and is in a similar state of development as RiVax.

There are two licensed anthrax vaccines on the market (one in the US and one in the UK) which are made of the protective antigen in anthrax which is known to be critical to the pathogenicity of *Baccilus anthrax*. These vaccines are known to require a very extensive vaccination schedule and have been associated with numerous side effects. Therefore, there has been extensive work to make a new recombinant anthrax protective antigen (rPA) vaccine which provides a good comparison to RiVax.

Studies looking at IM vaccination with rPA have shown that it effectively causes seroconversion. Additionally, in mice, guinea pigs and rabbits, it has been found to be protective. Similar to RiVax, following a 3 dose vaccine regimen, doses of rPA as low at 2.5 μg with alum were shown to protect guinea pigs from aerosolized anthrax spores [131]. In rabbits it was found that a single high dose of IM administered vaccine in alum could protect 93.3% of animals from aerosolized anthrax spores. And if two doses were given, the protective dose fell by 10 fold from 100 μg to 10 μg per vaccination [132]. Additionally, IM rPA has been compared to ID rPA and the latter was found to induce significantly more seroconversion after one vaccine of 10 μg, but once a complete 3 dose vaccine regimen was administered and animals were challenged, it was found that the two administration routes provided equivalent protection [133].

Overall, the rPA anthrax vaccine compared well with RiVax and there are a number of observations that translate from one to the other. First, it appears that similar doses are found to be protective, approximately 1 – 3 μg with alum, administered 3 times. The systemic administration of rPA also protects from mucosal challenge, further supporting the idea that we do not need mucosal protein vaccines for these toxins. Interestingly, here

too it was found that ID vaccination may accelerate immune responses, but not augment them.

The Future of RiVax

The studies presented here represent a novel delivery mechanism for a ricin vaccine and have covered essential aspects of vaccine protection that had previously not been explored with RiVax, such as single dose administration and the minimum lethal dose in a vaccinated animal.

In the future, as RiVax and vaccines in general move forward, it will be essential that we gain a better understanding of mucosal protection. Since the mucosa is the primary route of exposure to pathogens and toxins, we must understand all the mechanisms involved in its protection. This would include understanding the extent to which locally produced antibody is responsible for protection as opposed to systemically derived antibody. Passive protection experiments in an $IgA^{-/-}$ mouse would be one way to investigate this mechanism. We also need to acquire a better understanding of the dynamics of an immune response following vaccination by different routes. Experiments looking at the time course of an antibody response to either an ID or IM vaccination would be a good

place to start understanding these differences. This work has definitively shown that vaccination at the mucosa is not necessary for mucosal protection; determining the specific mechanism of this phenomenon is important to future vaccine development. Again, passive protection experiments with an $IgA^{-/-}$ mouse would help delineate the differences between the two types of immune responses.

Determination of the kinetics of an immune response following an ID *vs* an IM vaccination will be of importance not only in the context of RiVax. If indeed ID vaccination provides an accelerated immune response this can be of significant importance not only in military settings, but also in the setting of an emergent pandemic as was feared last year with swine flu. In order to curb the immediate outbreak of an infectious disease, ID vaccination may be far more efficient than IM vaccination.

This work has also added to the body of literature showing that ID vaccination allows for effective dose sparing. This is again most important in emergency situations where vaccine stores are sparse or unexpectedly unavailable. Again, in the situation of an emergent pandemic, any mechanism that allows the effectual growth of vaccine stores would be

invaluable (ie the number of vaccine doses available would increase by 5 fold, if dose sparing ID vaccination was implemented).

In the context of developing the most effective ricin vaccine for military personnel, this work in combination with the fact that RiVax is stable for at least one year as a lyophilized formulation [80], indicates that we have now improved the conditions for storage and optimized the utilization of this vaccine.

Overall, this work has given insight into the improvement of a ricin vaccine and also into general vaccine strategies. Phase I clinical trials of RiVax without alum have already been completed, as mentioned in Chapter 1, and a Phase I trial of RiVax with alum is underway. The next step in the progress of RiVax must be the initiation of Phase II Clinical trials. Withregard to vaccinology in general, ID administration of vaccines needs to be revisited as a safe, cost effective strategy for mass immunization. It should also be considered as a method to accelerate vaccine response time and as a reliable means to induce mucosal protection.

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