Comparison of Deposition Patterns for Small and Large Particle Aerosolized Toxins and Resulting Disease in Guinea Pigs and African Green Monkeys

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ABSTRACT

The battlefield risk from toxic industrial chemicals, materials, or threat agents is aerosol exposure. In most cases, this is not the natural route of exposure, so we must rely on animal models to predict disease course in humans. Deposition patterns for small (1µm) and large (8µm) particles were easily predicted, but effect on disease course for Ricinus communis (ricin) was unknown. Ricin small particle: Guinea Pig (GP) – histopathologic findings (lung) were absent at 1-hr timepoint, but present by 6-hr. Ricin deposition at 6-hr was less than that at the 1-hr timepoint. After 24 hr, pulmonary changes represented significant progression of disease and amount of ricin in lungs was significantly decreased. After 48 hr, there was advanced progression of disease and amount of ricin in the lungs was comparable to 24-hr timepoint. Ricin large particle: GP – minor changes in large airways represented early pathological effects of aerosol ricin exposure at all timepoints; significantly diminished compared to pathology of small particles. Compared to small particle, abundant ricin antigen was detected in nasal cavity at these timpoints. The cohort of GP exposed to large particle ricin euthanized at 1-hr and 6-hr postchallenge contained abundant ricin antigen in large pulmonary airways and nasal passages. In contrast, only small amounts of ricin antigen were detected in lungs of GP exposed to large particle ricin euthanized at 48-hr; furthermore, immunopositive staining was absent in the nasal cavity and contiguous airway passages. Ricin small particle: African green monkey (AGM) – Lung pathology at 4 or 8-hr post-exposure was normal, but at 16, 24, and 36-hr, progression of changes due to acute ricin intoxication was present. Deposition patterns were similar to small particle in GP. Ricin large particle: three AGM survived without developing clinical signs of illness. Both the GP and AGM were most susceptible and disease progression most severe after inhalational exposure to a 1µm aerosol. Although some GP were susceptible to large particles, there was a delayed time to death, compared to death due to a small particle aerosol. AGM were not affected by an 8µm aerosol. These different outcomes are important for risk assessment of human health factors on the battlefield.

Leffel, E.K.; Hartings, J.M.; Pitt, M.L.M.; Stevens, E. (2007) Comparison of Deposition Patterns for Small and Large Particle Aerosolized Toxins and Resulting Disease in Guinea Pigs and African Green Monkeys. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 11-1 – 11-12). Meeting Proceedings RTO-MP-HFM-149, Paper 11. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

"Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army."

1.0 INTRODUCTION

Well-characterized animal models of disease represent the critical components for relevant *in vivo* evaluation of medical countermeasures against toxin threat agents. The Food and Drug Administration's 'animal rule' requires development of multiple animal models of disease that are comparable to human disease, when exposed by the same route. Particle size of an aerosolized toxin will impact deposition in the animal and may alter the dose inhaled. Particle size will also affect the distribution of the toxin in the animal, potentially changing the severity of resulting disease. Therefore, proper characterization of particle size must be correlated with pathogenesis in order to identify models in which to study therapeutic/vaccine candidates for safety and efficacy.

Currently, there is a lack of aerosol models for some of the toxin agents, and there are virtually no comparative pathogenesis data with respect to exposures involving multi-sized aerosols. Multi-sized aerosols with a distribution of particle sizes in the 1 – 15 micron (μm) range are common in the environment and technically easier to generate in a bioterrorism scenario than are mono-disperse aerosols. Historically, threat assessment, vaccine efficacy, and therapeutic studies have been conducted using highly respirable, small-particle (1μm) aerosols [1-5]. Few aerosol animal models have been developed that comprehensively address the issue of particle size and how it influences deposition, retention, and clearance of the pathogen [6-8]. Because multi-sized aerosols are more realistic in a battlefield scenario or terrorist attack, it is very important to understand the impact of the larger particles. Because the mass of material in a particle is proportional to the third power of the particle radius, just a few large particles in a multi-sized aerosol can carry more biological material than orders of magnitude more smaller particles in the same aerosol. When one considers that a 10 μm particle carries 1000 times more agent than a 1 μm particle originating from the same source, inhalation of just a small number of 'large' particles may profoundly affect the course of disease, even though a larger number of 'smaller' particles are inhaled.

The histoanatomic site of deposition of aerosols within the respiratory system depends largely on the size of the particles. Particles of 5 μm or larger will generally be deposited in the nasopharyngeal region and will remain there until removed, by sneezing or coughing. Particles of approximately 2 to 5 μm are predominantly deposited in the tracheobronchiolar region of the respiratory tract. These particles are cleared by ciliary propulsion and either expelled (coughing/sneezing) or swallowed into the gastrointestinal tract. Particles of 1 μm, or smaller, can penetrate the alveolar sacs of the lungs. They will either be absorbed into the blood stream, or be cleared by alveolar macrophages. The inhaled dose of an aerosolized toxin is largely dependent on the interaction between the particle size and the individual's respiratory pattern and anatomy. Because there may be subtle differences between species, the studies presented here will address dose response in both rodent and nonhuman primate models.

Ricin is a toxic 66-kDa protein derived from the bean of the castor plant (*Ricinus communis*). It is a lectin that contains ribosome-inactivating proteins, the key component to its toxicity. The proposed mechanism of action is thought to be depurination of ribosomal RNA after entrance into a target cell via endocytosis [9]. Ricin is highly toxic by a variety of routes of administration, including inhalation [10]. Once in the lung, ricin has the ability to covalently bind with a variety of cell types. Once bound, ribosomal activity is inhibited and rapid cell death ensues. The generalized tissue necrosis observed in lung tissue eventually manifests into a diffuse necrotizing bronchopneumonia [11].

2.0 METHODS

2.1 **Animal Models:** Adult Hartley guinea pigs (GPs) and adult African green monkeys (AGMs) were used in all studies. Animals were maintained and fed according to facility standard operating procedures and were provided water ad libitum. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. USAMRIID is a fully accredited facility by the Association for Assessment and Accreditation of Laboratory Animal Care International. Prior to inclusion in the study, GPs and AGMs were determined to be clinically healthy, acclimated to the facilities, and identified by either subcutaneous microchip or chest tattoo. AGMs were anesthetized by an intramuscular injection of a ketamine-acepromazine-xylazine mixture before plethysmography and aerosol exposure.

2.2 **Aerosol Generation:** Exposure to ricin toxin occurred in a Class-III biological safety cabinet maintained under negative pressure. GPs were exposed in a whole-body chamber and AGMs in a head-only chamber; each exposure time was 10 min. Aerosols were generated by 3-jet collison nebulizer (BGI, Inc. Waltham, MA) (1µm) or spinning top aerosol generator (8 µm), controlled by an Automated Bioaerosol Exposure System [12], and particle size measured by a TSI 3321 aerodynamic particle sizer. In each species, comparable doses were used at 1µm and 8 µm particle sizes. Whole-body plethysmography (Buxco Research Systems) was performed on each AGM to obtain the minute volume, immediately before the exposure, as previously described. Aerosol concentration of ricin toxin was determined by constantly sampling the chamber using an all-glass impinger (AGI; Ace Glass, Vineland, NJ) containing sterile water as the collection medium. Determination of presented dose was calculated using a protein assay.

2.3 **Study Design:** The LD50 (lethal dose of 50% of population) was determined for small particle sizes in both the GP and the AGM. An equivalent number of LD50s was used in each subsequent deposition and retention study. The LD50 for large particles could not be determined for either species so the maximum deliverable aerosol starting concentration was used, without resulting death. For GPs: Four groups were euthanized at the following time points postexposure to either small-particle (n=5/group) or large-particle (n=2 to 4/group) ricin: one, six, 24, or 48 hrs. For AGMs: animals were euthanized after exposure to small particles at four, eight, 16, 24 and 32 hrs. Serial time points were not completed for large- particle exposure because AGMs were not clinically susceptible to a large-particle ricin aerosol. Complete necropsy (on GPs and AGMs) was performed and the following tissues collected for histopathologic evaluation: brain; lungs; heart; thymus; thyroid gland; trachea; esophagus; mediastinum with tracheobronchial lymph node and aorta; liver; gallbladder; spleen; pancreas; stomach; small intestine; colon; cecum; kidney; adrenal gland; urinary bladder; reproductive organs; lymph nodes, inguinal, axillary, and cervical; tongue; larynx; and, brachial plexus (peripheral nerve).

2.4 **Ricin Detection – Immunochemistry:** An immunoperoxidase assay (ABC-PO) was completed on selected slides using a goat-anti-ricin polyclonal antibody. Normal monkey lung served the negative control for the AGM immunohistochemistry study, and normal guinea pig lung was used for the GP study. Positive control tissue was lung from a monkey and a guinea pig exposed to a lethal dose of aerosolized ricin for each AGM and GP group, respectively. For the immunohistochemistry study, unstained sections were deparaffinized, rehydrated in ethanol, subjected to methanol-hydrogen peroxide block for 30 min at room temperature, rinsed in water and PBS, and heat pretreated in TRIS

/ EDTA for 30 min at 97ºC. Slides were rinsed, and a serum-free protein block (DAKO) plus 5% normal rabbit serum and avidin was applied for 30 min. The polyclonal antibody at a dilution of 1:200 plus biotin was then applied to all slides and incubated overnight (12 hrs) at room temperature. Slides were rinsed, and biotinylated rabbit anti-goat IgG (1:200) applied for 30 min, and rinsed again. The ABC-HRP complex was applied to slides for 30 min at room temperature and then rinsed in PBS. All sections were exposed to DAB permanent chromagen for ~ 5 min, rinsed, counter-stained with hematoxylin, dehydrated, and cover-slipped with Permount.

2.5 Ricin Detection – Electrochemiluminescence (ECL) Assay: At time of necropsy, tissue samples were obtained, weighed, and frozen at -70ºC. At time of assay, samples were homogenized in PBS/0.3% Tween and used in a sandwich immunosassay (Bioveris Corp.). Briefly, a biotin-labelled antibody was used in conjunction with a streptavidin-coated magnetic bead that was incubated with the tissue sample. When binding occurred, a light signal was recorded and ricin concentrations were quantitated as nanograms (ng) ricin per gram (g) of tissue.

3.0 RESULTS

3.1 Small particle – Guinea Pig

One hour after exposure to aerosolized small particle ricin
Abundant ricin immunostaining was present in the examined sections of lung, but significant histologic lesions were not observed. The mean concentration of ricin in the whole lung was 35.9 ± 8.2 ng/g.

6 hours after exposure to aerosolized small particle ricin
Several histopathologic changes in the lungs were attributed to the acute effects of ricin, including bronchiolar epithelial degeneration and necrosis, histiocytic and heterophilic pneumonia, and acute heterophilic bronchitis and bronchiolitis. The pulmonary changes were acute, mild and associated with ricin immunopositive staining; the most notable changes were at the level of small and terminal bronchioles, alveolar ducts, and contiguous alveoli. Compared to GPs killed at 1 hr postexposure, the overall amount and intensity of immunopositive staining in this group was less; most notably, extracellular intraluminal immunostaining in bronchioles was weaker and less abundant, and immunostaining was absent in the intrapulmonary bronchi. The mean concentration of ricin in the whole lung was 31.1 ± 18.0 ng/g.

24 hours after exposure to aerosolized small particle ricin
The amount and intensity of the ricin immunostaining in the lungs of GPs in this group was significantly decreased. The majority of the histologic lesions in the lungs were attributed to the progressive pathologic effects of aerosolized ricin exposure. Significant progression of disease was observed, including pneumonia, hemorrhage, necrosis, mesothelial hypertrophy, and peribronchovascular and alveolar edema. These histologic lesions would have significantly hampered respiration and oxygen exchange at the level of the alveolus. The mean concentration of ricin in the whole lung was 20.0 ± 10.5 ng/g.

48 hours after exposure to aerosolized small particle ricin
Compared to the GPs killed at 1 hr, six and 24 hours postexposure, the histopathologic changes in these GPs represented significant progression of disease. The lungs contained similar histologic lesions as those euthanized at 24 hrs, including pneumonia, bronchiolar epithelial degeneration and

necrosis, heterophilic bronchiolitis, peribronchovascular and alveolar edema, and mesothelial hypertrophy. Additional lesions representing progression of disease included deposition of fibrin in alveoli and in areas of alveolar septal necrosis and type II pneumocyte hypertrophy. The histologic lesions would have significantly hampered respiration and oxygen exchange. The mean concentration of ricin in the lung was 3.1 ± 1.2 ng/g. The liver, spleen, and kidney from two animals were negative for ricin by the ECL assay.

3.2 Large particle – Guinea Pig

1 hour after exposure to aerosolized large particle ricin
Detection of moderate to abundant ricin antigen primarily in the conducting airways of the lungs suggested that at least some of the acute changes in the bronchi and bronchioles were due to the early pathologic effects of aerosol ricin exposure. Abundant ricin antigen was detected in the nasal cavity. In the lungs ricin immunostaining was more abundant and intense in the upper airways, such as the intrapulmonary bronchi and large bronchioles, and less intense and abundant in the terminal bronchioles and alveolar ducts.

6 hours after exposure to aerosolized large particle ricin
Acute degeneration and sloughing of the airway epithelium was present in the bronchi and bronchioles. Abundant ricin antigen was present in the nasal passages and larger conducting airways of the lungs; very little ricin antigen was detected in the alveoli or alveolar ducts in the lungs. Abundant ricin antigen was detected in the nasal cavity, primarily within the ventral meatus.

24 hours after exposure to aerosolized large particle ricin
Small amounts of ricin antigen was detected within a few large bronchioles in the lungs, but the pulmonary changes were relatively mild. Peribronchovascular edema, mild acute inflammation in the intrapulmonary bronchi and bronchioles, and degenerative changes in the epithelium of large pulmonary airways were observed and may have resulted from aerosolized large-particle ricin. The severity of the histologic lesions and amount of immunopositive staining were minimal when compared to the small-particle ricin experimental cohorts. Ricin antigen was not detected in terminal bronchioles, alveolar ducts, or alveoli. Additionally, ricin was not detected in the nasal passages, vomeronasal organ, or nasopharyngeal duct.

48 hours after exposure to aerosolized large particle ricin
Bronchopneumonia, epithelial degeneration in the bronchi and bronchioles, and peribronchovascular and alveolar edema were observed and attributed to aerosolized ricin. The trachea was immunonegative for ricin. While small amounts of ricin antigen were detected in a few intrapulmonary bronchi, immunopositive staining was not observed in the remaining sections of lung in these guinea pigs, including the bronchioles, alveolar ducts, and alveoli. Although bronchopneumonia was present, the pulmonary lesions in these GPs were significantly less severe than those of its cohorts exposed to small particle ricin sacrificed at 48 hrs and probably would not have significantly hampered respiration and oxygen exchange at the level of the alveolus.

Table 1: Summary of Gross Pathology and Histopathology Pulmonary Findings in the GP, small particle exposure

1 hr n = 4	6 hr n = 5	24 hrs n = 5	48 hrs n = 5
no gross lesions no significant histo.	bronchial epithelial necrosis pneumonia bronchitis and bronchiolitis	marked pneumonia / necrosis hemorrhage aveolar edema mesothelial hypertrophy	severe pneumonia fibrin in alveoli aveolar septal necrosis pneumocyte hypertrophy

Table 2: Summary of Immunohistochemistry Pulmonary Findings in the GP, small particle exposure

1 hr n = 4	6 hr n = 5	24 hrs n = 5	48 hrs n = 5
abundant in bronchi abundant in bronchioles moderate in alveoli weak/moderate in trachea weak/moderate in larynx	abundant in bronchi abundant in bronchioles abundant in alveoli weak in trachea	weak in bronchioles weak in alveolar structures	weak in bronchioles

Table 3: Summary of gross pathology and histopathology pulmonary findings in the GP, large-particle exposure

1 hr n = 4	6 hr n = 2	24 hrs n = 2	48 hrs n = 2
sloughed epithelial cells in bronchi and bronchioles mild eosinophilic and heterophilic tracheitis	minimal to mild pneumonia sloughed cells in bronchi sloughed cells in bronchioles tracheitis	sloughed cells in bronchi sloughed cells in bronchioles alveolar edema tracheitis	mild pneumonia bronchial lymphatics dilated trachea epithelial necrosis

Table 4: Summary of immunohistochemistry pulmonary findings in the GP, large-particle exposure

1 hr n = 4	6 hr n = 2	24 hrs n = 2	48 hrs n = 2
moderate in bronchi moderate in bronchioles moderate in alveoli weak/moderate in trachea strong in nasal turbinates	moderate in bronchi moderate in bronchioles weak in alveoli weak in trachea strong/moderate in nasal turbinates	weak in bronchioles neg. in turbinates neg. in esophagus	weak in bronchioles

3.3 Small particle – African green monkey

4 hours after exposure to aerosolized small particle ricin

The histopathologic changes and immunohistochemistry findings were attributed to the acute effects of aerosol ricin intoxication. The histologic findings in the lungs indicated that significant pulmonary lesions from small particle aerosol ricin exposure occurred in the bronchiolar and bronchial epithelium and included 1) acute degeneration and necrosis of respiratory epithelial cells with attenuation, erosion, and sloughing of cells into the airway lumen; 2) concurrent acute neutrophilic inflammation in the airways and lining epithelium; and 3) subepithelial interstitial edema and inflammation.

Immunohistochemistry demonstrated moderate amounts of intense positive immunostaining for ricin multifocally in the lungs. The positive immunostaining was both cell- and non-cell-associated, and was present most often in medium to small (terminal) bronchioles and adjacent alveoli. Small amounts of positive immunostaining were also observed associated with neutrophils and in extracellular mucus within the lumens of a few large bronchi. In alveoli, strong immunostaining was present in alveolar macrophages and neutrophils; moderate to weak staining was observed in hypertrophied alveolar pneumocytes and in extracellular proteinaceous debris.

These microscopic pulmonary lesions appeared to be 1) degeneration and necrosis of the respiratory epithelium in bronchioles and bronchi; 2) accompanying acute neutrophilic inflammation in the affected airways; 3) an extension of low numbers of inflammatory cells, cellular debris, and ricin toxin from terminal bronchioles into adjacent alveoli, with reactive hypertrophy of alveolar pneumocytes; and 4) neutrophilic inflammation of the bronchial glands and abundant mucus in the bronchial lumens. Tissue immunohistochemistry demonstrated ricin antigen in association with the histologic changes in the lungs. The histopathologic and immunohistochemistry findings indicated the most significant pulmonary lesions occurred at the level of the bronchioles and to a lesser degree in bronchi; and the lesions were directly attributable to the effects of inhaled toxin. The pathologic process appeared to extend from the from the terminal bronchioles into adjacent alveoli.

8 hours following exposure to aerosolized small particle ricin:

The histopathologic changes and immunohistochemistry findings at this timepoint also were attributed to the acute effects of aerosol ricin intoxication. The histologic lesions in the lungs indicated a significant progression of pulmonary disease compared to the animal at four hours post challenge. Similar to the animal at four hours, bronchial and bronchiolar epithelial degeneration and necrosis were evident in the eight hour animal and ricin antigen was detected by immunohistochemistry in areas

containing lesions. Pulmonary changes in the eight hour AGM included: 1) alveolar edema; 2) perivascular interstitial edema and lymphangiectasis; 3) alveolar septal necrosis; and 4) hemorrhage. These changes were not observed in the four hour AGM. Additionally, the degree and distribution of the neutrophilic inflammation in bronchi, bronchioles, and adjacent alveoli were more severe compared to the four hour cohort.

Immunohistochemistry demonstrated moderate amounts of intense positive immunostaining for ricin multifocally in the lungs. The positive immunostaining was both cell and non-cell associated, and was present most often in medium to small (terminal) bronchioles and adjacent alveoli. In bronchioles, immunopositive staining was present most often associated with degenerate neutrophils and necrotic epithelial cells in airway lumens, and along the surface of the lining epithelium.

Similar to 4-hr postexposure animal, the histologic findings in the lungs suggested that bronchial and bronchiolar epithelial degeneration and necrosis were early, significant pathologic events in small-particle aerosol ricin exposure. The pulmonary changes also indicated that alveolar and perivascular edema, hemorrhage, and alveolar septal necrosis were early events in the pathogenesis and progression of disease, although they appeared to occur after acute injury to the bronchial and bronchiolar epithelium. Furthermore, the histologic changes in the 8-hr animal indicated progression of an acute, neutrophilic inflammatory process in the affected airways and bronchial glands, with extension of the inflammatory process and toxin into adjacent alveoli. While small amounts of polymerized fibrin were demonstrated with PTAH staining in the lungs, organized fibrin aggregates were not apparent, and fibrin deposition did not appear to be significant at this stage of the disease progression.

16 hours after exposure to aerosolized small particle ricin

Again, histopathologic changes and immunohistochemistry findings were attributed to the acute effects of aerosol ricin intoxication. The histologic lesions in the lungs indicated further progression of the pulmonary disease compared to its cohorts euthanized at 4-hr and 8-hr postexposure.

Immunohistochemistry demonstrated moderate amounts of intense positive immunostaining for ricin multifocally in the lungs. The positive immunostaining at this time point was also cell- and non-cell-associated, and was present most often in medium to small (terminal) bronchioles and adjacent alveoli. In bronchioles, immunopositive staining was present most often associated with degenerate neutrophils and necrotic epithelial cells in airway lumens, and along the surface of the lining epithelium. In alveoli, lesser amounts of staining were present in alveolar macrophages, necrotic alveolar pneumocytes, degenerate neutrophils, and in the proteinaceous edema fluid. Very small amounts of positive immunostaining were observed in bronchi and found associated with intraluminal degenerate neutrophils and along the surface of degenerate epithelial cells lining the airway.

Similar to the animals euthanized at the earlier time points, the histologic findings in the lungs of this animal indicated that bronchial and bronchiolar epithelial degeneration and necrosis, pulmonary edema and hemorrhage, and alveolar septal necrosis were significant pathologic events in small particle aerosol ricin exposure. The histomorphology of the lesions in this animal suggested acute pulmonary microvascular injury, which resulted from either primary injury to the vascular endothelium of the alveolar septa or damage to alveolar epithelial cells (pneumocytes), with secondary microvascular injury. Acute pulmonary microvascular injury resulted in: 1) leakage of fluids and proteins into the interstitial space and alveoli (alveolar flooding); 2) neutrophilic inflammation; and 3) fibrin deposition. The injury resulted in a loss of diffusion capacity and respiratory insufficiency occurred due to impaired oxygen exchange in alveoli.

24 hours after exposure to aerosolized small particle ricin
Diffusely affecting the alveoli of the lungs there was: 1) massive flooding with abundant edema; 2) smaller amounts of fibrin admixed with the edema; and 3) and many foamy macrophages and degenerate neutrophils. Multifocally in the lungs : 1) alveolar septa were necrotic and replaced with hyalinized fibrin, cellular debris, and hemorrhage; 2) bronchioles were filled with abundant edema and varying numbers of degenerate neutrophils; and 3) the bronchiolar epithelium was degenerate, necrotic or frequently lost or sloughed into the lumen of the airway. There was diffuse, moderate to marked perivascular, peribronchial and peribronchiolar interstitial edema and lymphatic vessels were moderately dilated and filled with protein-rich edema, especially surrounding large airways and vessels. There was diffuse pulmonary congestion, and the endothelium of many small vessels was hypertrophied with prominent nuclei.

Within the mediastinal lymph node, there was diffuse lymphoid depletion with scattered lymphocytolysis (lymphocyte apoptosis), draining neutrophilic and histiocytic inflammation, congestion, and parenchymal and perinodal interstitial edema (immunonegative for ricin).

Immunohistochemistry demonstrated scattered, small amounts of moderately intense positive immunostaining for ricin multifocally in the examined sections of lung; staining was primarily non-cell-associated. Ricin was present most often in small bronchioles and adjacent alveoli associated with cellular debris, fibrin, degenerate neutrophils and necrotic epithelial cells; rare cell-associated staining was present in few alveolar macrophages.

Histochemistry (PTAH for polymerized fibrin): Moderate amounts of fibrin were scattered diffusely throughout many alveoli in the examined sections of lung. Much of the fibrin was present in edematous alveoli and was found as disorganized, loosely arranged, haphazard, fine, fibrillar structures. Multifocally, smaller amounts of fibrin occurred as tangled, dense, organized mesh works of thick, coarse, fibrils; the organized aggregates of fibrin frequently contained entrapped erythrocytes, necrotic epithelial cells, degenerate inflammatory cells, and cellular debris. The organized fibrin aggregates occurred most often in alveoli adjacent to terminal bronchioles. Small amounts of fibrin were also found in the lumens of larger bronchioles.

32 hours after exposure to aerosolized small particle ricin
Histopathologic evaluation of the lungs demonstrated that: 1) alveoli were diffusely filled with abundant edema admixed with fibrin and moderate numbers of viable and degenerate neutrophils and macrophages; 2) there was multifocal necrosis and loss of alveolar septa, and replacement with abundant fibrin, hemorrhage, and degenerate neutrophils; 3) intact, alveolar septa were moderately expanded by congestion, edema, fibrin, and moderate numbers of neutrophils, macrophages, and activated lymphocytes; 4) the epithelium in bronchioles was degenerate and necrotic, and often absent or sloughed into the lumen; 5) some bronchioles contained degenerate neutrophils, and variable amounts of edema, fibrin, and cellular debris; 6) the peribronchiolar interstitium was infiltrated by neutrophils, macrophages, and activated lymphocytes; 7) there was marked perivascular and peribronchiolar interstitial edema; 8) lymphatics were moderately dilated, especially surrounding large airways and vessels; 9) the pleura was mildly expanded multifocally by small amounts of fibrin and variable numbers of viable and degenerate neutrophils; and 10) there was diffuse pulmonary congestion and the endothelium of many small vessels was hypertrophied with prominent nuclei.

Multifocally in the trachea mucosa there was epithelial degeneration with scattered single-cell necrosis (apoptosis) and subacute inflammation (immunonegative for ricin). In the cortex of both adrenal

glands, there was mild acute degeneration and necrosis of adrenocortical cells scattered throughout the zona fasciculata. Small groups of up to 15-20 adrenocortical cells were vacuolated and swollen and contained hypertrophied nuclei (degeneration), or were hypereosinophilic and had fragmented cell borders and shrunken, hyperchromatic nuclei (necrosis). Within the mediastinal lymph node there was diffuse lymphoid depletion with abundant lymphocytolysis (lymphocyte apoptosis), draining hemorrhage, fibrin, neutrophilic inflammation, and perinodal interstitial edema and congestion (immunonegative for ricin).

Immunohistochemistry demonstrated scattered, small amounts of moderately intense positive immunostaining for ricin in the lungs. The immunostaining was primarily non-cell-associated, and was observed most often in the cellular debris, fibrin, and remnant necrotic epithelial cells in small and terminal bronchioles. Scattered immunopositive staining was also present in alveoli adjacent to terminal bronchioles in association with cellular debris, necrotic pneumocytes, and rarely in alveolar macrophages.

Table 5: Summary of Gross Pathology and Histopathology Pulmonary Findings in the AGM, small particle exposure

4 hr n = 1	8 hr n = 1	16 hrs n = 1	24 hrs n = 1	32 hrs n = 1
necrosis of respiratory epithelial cells neutrophilic inflammation interstitial edema	alveolar edema alveolar septal necrosis hemorrhage	progression of pulmonary disease	edema pulmonary congestion necrotic alveolar septa necrotic bronchiolar epithelium	edema pulmonary congestion necrotic alveolar septa necrotic bronchiolar epithelium

Table 6: Summary of Immunohistochemistry Pulmonary Findings in the AGM, small particle exposure

4 hr n = 1	8 hr n = 1	16 hr n = 1	24 hrs n = 1	32 hrs n = 1
moderate in bronchioles and alveoli strong in alveolar macrophages	moderate in medium to small (terminal) bronchioles and adjacent alveoli	intense in degenerate neutrophils and necrotic epithelial cells small in alveolar macrophages, small in bronchi	small to moderare in bronchi and alveoli	small to moderare in bronchi and alveoli

3.4 Large particle – AGM

A total of three AGMs were exposed to ricin toxin, in increasing doses, until a maximum deliverable dose was obtained. No animals developed clinical signs of disease after observation for 28 days. There were no signs of abnormal pathology and no detectable ricin antigen in any tissue.

4.0 DISCUSSION

In the GP, the pulmonary pathology was much more extensive and severe in the small-particle group than in the large-particle group, especially at later time points. Although this may seem counterintuitive because of the possibility of larger particle aerosols delivering higher doses, it can be reasoned that the smaller particle aerosol delivered more toxin to the lower airways where resulting damage was lethal. The larger particles remained predominantly in the upper airways as expected. The large particles may have been cleared by local immune mechanisms in the mucosa, and then cleared by the mucociliary processes and exhaled (sneezing) or digested when swallowed, or the protein simply degraded before significant damage to the respiratory system occurred. Ricin antigen was detected by immunohistochemistry at very early time points for the small particles, mostly in the terminal bronchioles, alveolar ducts, and adjacent alveoli. Detection decreased by 24 and 48 hrs, probably due to a dilution effect from edema and fibrin that began to develop in the oxygen exchange airways. In contrast to the small-particle immunostaining results, abundant large-particle deposition was found in the intrapulmonary bronchi and large bronchioles, with only small amounts in the alveolar ducts and alveoli. Like small particle aerosols, the amounts of detectable ricin following large particle delivery decreased over time. Most interestingly, in the large-particle study, significant amounts of ricin antigen were found in the nasal cavities beginning at the 1 hr time point, but none was detected in the small-particle study at any time point. This observation provides convincing evidence that larger particles impacted in the upper airways and were probably trapped/cleared by ciliated respiratory epithelial cells, thereby abrogating pulmonary pathology found in the small-particle aerosol GP study. There was little significant pathology in the mucosal lining of the respiratory passages, suggesting that a large-particle aerosol of ricin toxin is not lethal or incapacitating in the GP model, even at these highest deliverable doses. The pathologic findings are consistent with the observations in the survival of the LD50 study.

In the AGM, pathology for small-particle-exposed animals was similar to observations in the GP. In both the 4- and 8-hr timepoints, the histologic findings in the lungs indicated that bronchial and bronchiolar epithelial degeneration and necrosis occurred early and were significant acute pathologic events. The pulmonary changes also suggested that alveolar and perivascular edema, hemorrhage, and alveolar septal necrosis were early events in the pathogenesis and progression of disease, although they appeared to occur after acute injury to the bronchial and bronchiolar epithelium. Furthermore, the histologic changes from the 4- to 8-hr timepoint indicated progression of the acute, neutrophilic inflammatory process in affected bronchioles with extension of the inflammatory process and toxin into adjacent alveoli. Histologic findings not observed in the early timepoints, but found in cohorts euthanized at later time points (especially at 24- and 32-hrs postexposure) included fibrinous pleuritis and abundant fibrin deposition in alveoli. Pathologic studies from large-particle exposures in the AGM were not performed at early timepoints because the monkeys were not susceptible to highest presented doses, even after 28 days.

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