

Vet Pathol 44:716–721 (2007)

Pathology of Inhalational Anthrax Infection in the African Green Monkey

N. A. TWENHAFEL, E. LEFFEL, AND M. L. M. PITT

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

Abstract. There is a critical need for an alternative nonhuman primate model for inhalational anthrax infection because of the increasingly limited supply and cost of the current model. This report describes the pathology in 12 African green monkeys (AGMs) that succumbed to inhalational anthrax after exposure to a low dose (presented dose $200\text{--}2 \times 10^4$ colony-forming units [cfu]) or a high dose (presented dose $2 \times 10^4\text{--}1 \times 10^7$ cfu) of *Bacillus anthracis* (Ames strain) spores. Frequent gross lesions noted in the AGM were hemorrhage and edema in the lung, mediastinum, and mediastinal lymph nodes; pleural and pericardial effusions; meningitis; and gastrointestinal congestion and hemorrhage. Histopathologic findings included necrohemorrhagic lymphadenitis of mediastinal, axillary, inguinal, and mesenteric lymph nodes; mediastinal edema; necrotizing splenitis; meningitis; and congestion, hemorrhage, and edema of the lung, mesentery, mesenteric lymph nodes, gastrointestinal tract, and gonads. Pathologic changes in AGMs were remarkably similar to what has been reported in rhesus macaques and humans that succumbed to inhalational anthrax; thus, AGMs could serve as useful models for inhalation anthrax studies.

Key words: African green monkeys; anthrax; *Bacillus anthracis*; biologic warfare agent; inhalation anthrax disease; rhesus macaques.

Nonhuman primates (NHPs) have been used to study disease caused by *Bacillus anthracis* because the pathogenesis and lesions closely emulate those seen in humans. *B. anthracis* is a 1- to 10- μm , gram-positive, spore-forming bacillus that is easily observed using light microscopy. The rhesus macaque (*Macaca mulatta*) has been used almost exclusively as the NHP model for inhalational anthrax disease for more than 10 years.^{1,4,6} Rhesus macaques are becoming increasingly difficult to obtain, however, because of availability and cost. This dwindling availability has become a limiting factor in performing efficacy studies for candidate vaccines and therapeutics against infectious biothreat agents, such as *B. anthracis*. A well-characterized NHP model is needed as an alternative to the rhesus model.

African green monkeys (AGMs, *Chlorocebus aethiops*) are readily available, tractable, and relatively inexpensive. Unlike rhesus macaques, AGMs are not

carriers for *Cercopithecine herpesvirus 1* (B virus) that can be transmitted from NHPs to humans via scratches or bites. Use of an NHP that is free of B virus can substantially reduce some of the safety concerns associated with NHP research. AGMs have been successfully developed as useful models for a number of human diseases, such as parainfluenza and leishmaniasis.^{2,3} Therefore, AGMs are good candidates for characterization as an alternative NHP model for inhalational anthrax.

In this study, we evaluated the time to death and pathologic changes of AGMs challenged with aerosolized *B. anthracis* (Ames strain) spores. AGMs were exposed to either a low dose (presented dose $200\text{--}2 \times 10^4$ colony-forming units [cfu]) or a high dose (presented dose $2 \times 10^4\text{--}1 \times 10^7$ cfu) of *B. anthracis*. Findings from the AGM were compared in parallel to those from rhesus macaques challenged with the same spore lot

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 01 SEP 2007		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Pathology of inhalational anthrax infection in the african green monkey. Veterinary Pathology 44:716 - 721				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Twenhafel, N Leffel, E Pitt, ML				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD				8. PERFORMING ORGANIZATION REPORT NUMBER RPP-06-042	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES The original document contains color images.					
14. ABSTRACT There is a critical need for an alternative nonhuman primate model for inhalational anthrax infection because of the increasingly limited supply and cost of the current model. This report describes the pathology in 12 African green monkeys (AGMs) that succumbed to inhalational anthrax after exposure to a low dose (presented dose 200-2 x 10(4) colony-forming units [cfu]) or a high dose (presented dose 2 x 10(4)-1 x 10(7) cfu) of Bacillus anthracis (Ames strain) spores. Frequent gross lesions noted in the AGM were hemorrhage and edema in the lung, mediastinum, and mediastinal lymph nodes; pleural and pericardial effusions; meningitis; and gastrointestinal congestion and hemorrhage. Histopathologic findings included necrohemorrhagic lymphadenitis of mediastinal, axillary, inguinal, and mesenteric lymph nodes; mediastinal edema; necrotizing splenitis; meningitis; and congestion, hemorrhage, and edema of the lung, mesentery, mesenteric lymph nodes, gastrointestinal tract, and gonads. Pathologic changes in AGMs were remarkably similar to what has been reported in rhesus macaques and humans that succumbed to inhalational anthrax; thus, AGMs could serve as useful models for inhalation anthrax studies.					
15. SUBJECT TERMS Bacillus anthracis, anthrax, vet med, inhalation, pathology, laboratory animals, nonhuman primates					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

(presented dose of 1×10^3 – 1×10^5 cfu). Furthermore, results were compared with historical data from our laboratory involving infected rhesus macaques.

Adult male and female AGMs and rhesus macaques were obtained from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) colony. Research was conducted in compliance with the Animal Welfare Act and other principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. USAMRIID is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. *B. anthracis* (Ames strain) spores were produced at USAMRIID.¹⁰ The presented dose (cfu) was calculated based on minute-volume, and whole-body plethysmography was performed to determine minute-volume for each animal.¹¹ The aerosol exposure was conducted in a class III biological-safety cabinet, in a head-only chamber.⁸ All exposed animals succumbed to anthrax and are included in this report. Complete necropsies were performed on each animal in a biosafety level 3 necropsy facility. Tissues collected for histopathologic evaluation were immersion-fixed in 10% neutral buffered formalin for 21 days. Sections prepared for examination using light microscopy were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Anthrax bacilli were stained in select tissues using the Gram-Twort staining method. Briefly, deparaffinized sections were immersed in crystal violet for 1 minute and stained with Lugol's iodine and neutral red/fast green.

In AGMs, the time to death was 3 to 17 days in the high-dose group and 7 to 25 days in the low-dose group (Table 1). Gross and histologic pathology findings were similar in the low-dose and high-dose groups. Gross pathologic changes (Figs. 1–4 and Table 1) in the AGMs included edema, congestion, and hemorrhage, sometimes accompanied by necrosis in the lung, mediastinum, meninges and brain, spleen, axillary and/or inguinal lymph nodes, mesentery, mesenteric lymph node, and gastrointestinal tract. Other gross necropsy findings included pleural and/or pericardial serosanguinous effusion, moderate to marked subcutaneous edema, and peritesticular or periovarian hemorrhage. Diffuse edema was a consistent feature in the lung and mediastinum, often accompanied by multifocal congestion and hemorrhage affecting multiple lung lobes. Six AGMs had a serosanguinous pleural and/or pericardial effusion.

Gross mediastinal changes included edema, congestion, and hemorrhage. There was significant widening of the mediastinum by edema in some cases. The mediastinal and tracheobronchial lymph nodes were generally enlarged 2 to 3 times normal and were often congested, edematous, and hemorrhagic.

Meningitis was a consistent finding in the AGMs. The meninges of affected AGMs were typically hemorrhagic but occasionally had a milky or opaque appearance that was confirmed microscopically as meningitis. The meningitis was often widespread over the entire surface

of the cerebrum, cerebellum, and brain stem. The spinal cord was not examined. Gross changes observed in the abdominal cavity consisted primarily of mild splenomegaly and congestion and/or hemorrhage of the mesentery, gastrointestinal tract, adrenal glands, and peritesticular and periovarian adipose tissue.

Histologically, there were features of acute inflammation and necrosis, often together with anthrax bacilli, in multiple tissues (Figs. 5–8). The pulmonary interstitium was expanded by fibrin, edema, and few macrophages and neutrophils. Alveoli were filled with edema, often admixed with fibrin, hemorrhage, macrophages, and neutrophils. Alveolar walls were occasionally disrupted and replaced by cellular and karyorrhectic debris (necrosis). Histologic features common to multiple tissues included congestion, fibrin, edema, hemorrhage, parenchymal loss, necrotic debris, and acute inflammatory cell infiltrates (primarily neutrophils and macrophages).

In addition to these changes, the mediastinal and tracheobronchial lymph nodes and the spleen had pronounced lymphoid depletion, lymphocytolysis, occasionally necrotizing vasculitis, and fibrin thrombi. The mediastinum surrounding these lymph nodes was expanded by edema and only rarely contained acute inflammatory cell infiltrates. Edema, fibrin, hemorrhage, neutrophils, and frequently myriad bacilli expanded the meninges of the cerebrum, cerebellum, and optic nerve. Occasionally, neuronal necrosis, spongiosis, gliosis, hemorrhage, neutrophils, and edema were present in the cerebrum and cerebellum.

The gross and histologic changes noted in the rhesus macaques in this study were similar to those for the AGMs. The time to death range in rhesus macaques was 3 to 18 days. Pathologic changes in the rhesus macaques included pulmonary and mediastinal congestion, edema, and hemorrhage; splenitis; lymphadenitis of the mediastinal, tracheobronchial, mandibular, axillary, inguinal, and mesenteric lymph nodes; meningitis; and occasional mesenteric, gastrointestinal, adrenal, and periovarian/peritesticular congestion and hemorrhage.

In this study, the gross and histologic changes in AGMs with fatal inhalational anthrax were similar to those described in humans and rhesus macaques and demonstrate the potential value of AGMs as models of inhalational anthrax.^{1,4,5,7} The most frequent gross lesions of AGM and rhesus macaques in our study were hemorrhage and edema in the lung, mediastinum, and mediastinal lymph nodes. Several AGMs in our study had pleural and/or pericardial effusion, a feature not seen in the rhesus macaques, but one that is similar to features of human inhalational anthrax, in which pleural effusion (hydrothorax) is a significant finding.⁷ However, edema of the mediastinum and lung was seen in both AGMs and rhesus macaques in our study. Mediastinal edema is seen in human anthrax, in which radiographic evidence of a widened mediastinum is considered a diagnostic hallmark.⁷ Hemorrhagic meningitis was seen in both AGMs and rhesus macaques in this study and is a distinct feature of anthrax in humans;

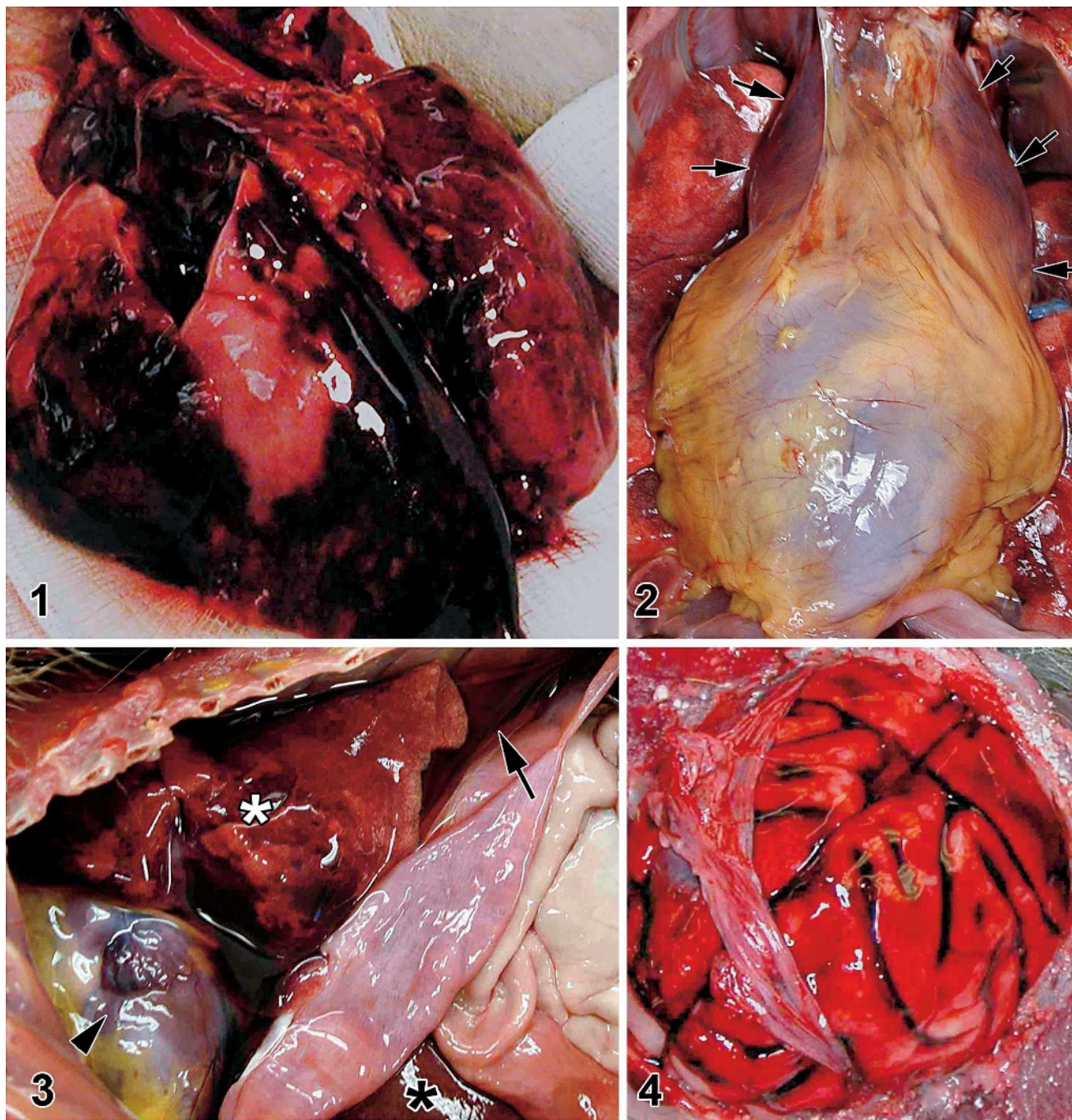


Fig. 1. Lung; AGM W166. Within each lung lobe, there is acute multifocal to coalescing congestion and hemorrhage with diffuse noncollapsing lobes indicating edema.

Fig. 2. Mediastinum; AGM V460. There is edema causing gross expansion of the cranial mediastinum and pericardial fat (arrows).

Fig. 3. Thoracic cavity; AGM V520. Note the serosanguinous pleural effusion (arrow) and diffusely “wet” appearance to the lung (white asterisk). Additionally, the heart (arrowhead) is surrounded by moderate pericardial fluid trapped within the pericardial sac. Liver is identified by black asterisk.

Fig. 4. Brain, meninges; AGM V516. Note the diffusely red meninges, consistent with hemorrhagic meningitis (“cardinal’s cap”).

Table 1. Gross pathologic findings in AGM that succumbed to a challenge of aerosolized *B. anthracis* (Ames strain) spores.*

Animal ID	Low-Dose Group 200–2 × 10 ⁴ (cfu)						High-Dose Group 2 × 10 ⁴ –1 × 10 ⁷ (cfu)					
	V444	W166	U983	V460	V523	V516	V551	W152	U995	V581	V520	05059
Presented dose (cfu)	204	2.2 × 10 ³	3.2 × 10 ³	4.9 × 10 ³	5.5 × 10 ³	9.8 × 10 ³	2.2 × 10 ⁴	2.6 × 10 ⁴	2.8 × 10 ⁴	3.5 × 10 ⁴	9.8 × 10 ⁶	1.0 × 10 ⁷
Days to death	7	13	18	8	25	15	17	10	3	6	5	5
Tissue:												
Adrenal gland	W	R	R	R	W	W	W	W	W	W	W	W
Gastrointestinal tract	W	RE	RE	R	R	RE	W	RE	W	W	R	W
Lung	RE	RE	RE	RE	R	RE	E	RE	RE	RE	RE	RE
Axillary/inguinal lymph node	RE	RES	W	RE	R	W	W	RE	W	W	RE	RES
Mediastinal lymph node	RES	RES	RES	RES	RES	W	ES	RES	W	RE	RES	RE
Mesenteric lymph node	W	W	W	RES	W	W	W	W	W	W	W	W
Mediastinum	RE	RE	RE	RE	RE	RE	RE	RE	RE	RE	RE	RE
Meninges	W	R	REO	REO	W	RE	W	W	W	W	REO	REO
Mesentery	R	W	RO	W	W	W	W	W	W	W	W	W
Pleural and/or pericardial effusion	–	–	+	+	–	+	–	–	+	+	+	–
Spleen	W	W	W	W	W	W	W	W	W	W	S	W

*R = red with congestion and/or hemorrhage, E = edema, S = size increased from normal, O = opaque (inflammation), W = within normal limits.

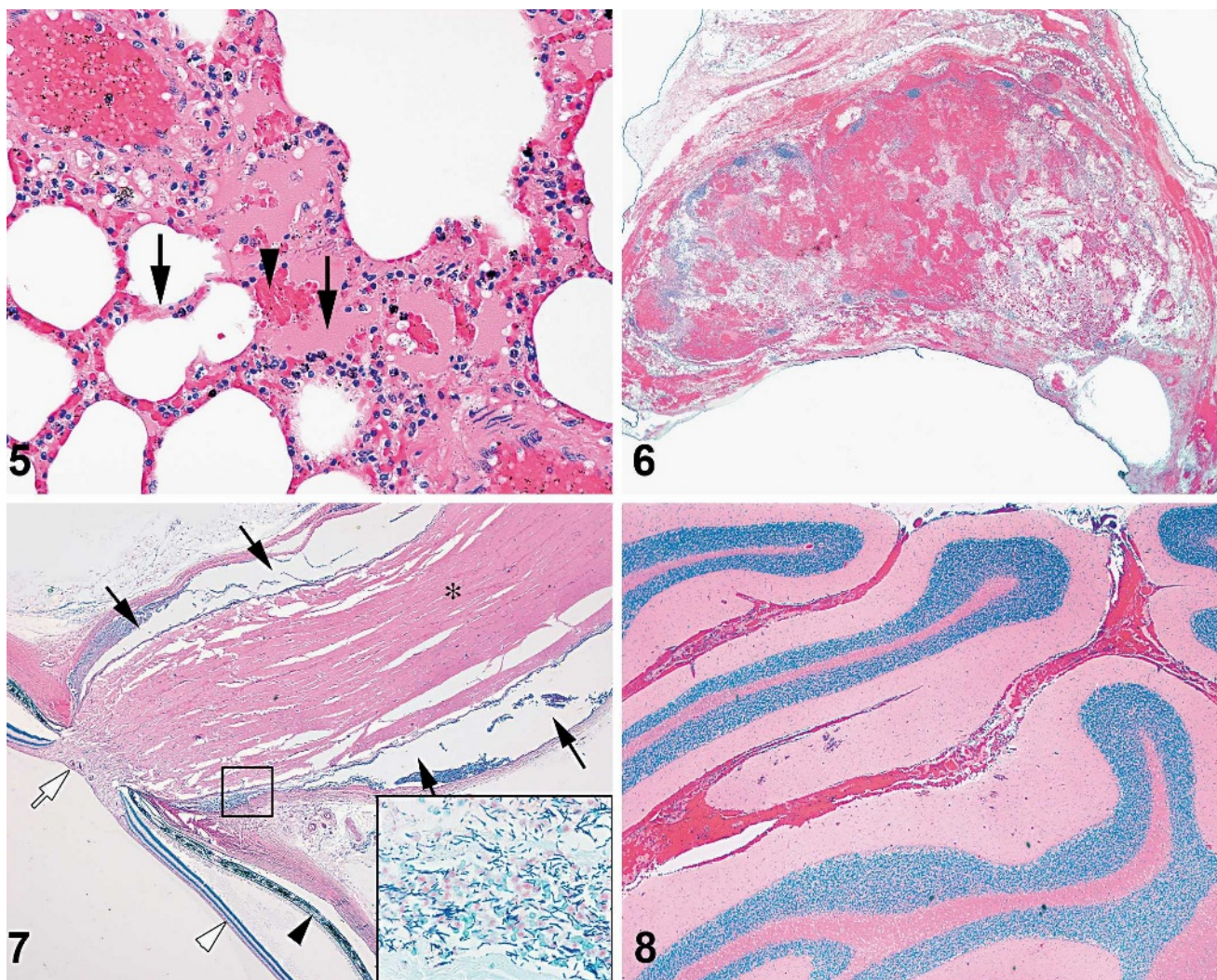


Fig. 5. Lung; AGM W166. Note the interstitial and alveolar edema (arrows) with multifocal hemorrhage (arrowhead). HE.

Fig. 6. Mediastinal lymph node; AGM V581. There is profound loss of lymphocytes and replacement by hemorrhage, edema, fibrin, and scant inflammatory infiltrates. The surrounding connective tissue is expanded by hemorrhage and edema. HE.

Fig. 7. Eye, optic nerve; AGM 05059. Retina (open arrowhead), optic cup (open arrow), choroid (closed arrowhead), sclera, optic nerve (asterisk). The meninges of the optic nerve (solid arrows) are expanded by cellular debris, clear space, and myriad Gram-positive bacilli (box insert). HE and Gram-Twort (box insert).

Fig. 8. Cerebellum; AGM V520. Hemorrhagic meningitis. HE.

historically, this finding is referred to as the “cardinal’s cap.”^{1,4}

Based on our findings, AGMs could provide a useful model for inhalational anthrax. The pathologic similarities between AGMs, rhesus macaques, and humans infected with *B. anthracis* by aerosol suggest that AGMs with anthrax may resemble humans in additional ways, such as the pathophysiologic and immunologic responses to infection. Assessment of these parameters in AGMs with inhalational anthrax should be the focus of future studies to more fully characterize the animal model. AGMs have the potential to serve as an

economical and safe animal model for studies that could include testing the efficacy of novel vaccines or improved therapeutics.

Acknowledgements

Special thanks to the technical staff, Jeff Brubaker, Neil Davis, Gale Krietz, and Chris Mech. We thank Larry Ostby for visual assistance. We also thank Lieutenant Colonel Tom Larsen, DVM, Diplomate ACVP, and Michael C. Babin, DVM, PhD, for their detailed review of the manuscript.

References

- 1 Berdjis CC, Gleiser CA, Hartman HA, Kuehne RW, Gochenour WS: Pathogenesis of respiratory anthrax in *Macaca mulatta*. *Br J Exp Pathol* **43**:515–524, 1962
- 2 Binhazim AA, Shin SS, Chapman WL Jr, Olobo J: Comparative susceptibility of African green monkeys (*Cercopithecus aethiops*) to experimental infection with *Leishmania leishmania donovani* and *Leishmania leishmania infantum*. *Lab Anim Sci* **43**(1):37–47, 1993
- 3 Durbin AP, Cho CJ, Elkins WR, Wyatt LS, Moss B, Murphy BR: Comparison of the immunogenicity and efficacy of a replication-defective vaccinia virus expressing antigens of human parainfluenza virus type 3 (HPIV3) with those of a live attenuated HPIV3 vaccine candidate in rhesus monkeys passively immunized with PIV3 antibodies. *J Infect Dis* **179**:1345–1351, 1999
- 4 Fritz DL, Jaax NK, Lawrence WB, Davis KJ, Pitt MLM, Ezzell JW, Friedlander AM: Pathology of experimental inhalation anthrax in the rhesus monkey. *Lab Invest* **73**:691–702, 1995
- 5 Gleiser CA, Berdjis CC, Hartman JA, Gochenour WS: Pathology of anthrax infection in animal hosts. *Fed Proc* **26**:1518–1521, 1967
- 6 Gleiser CA, Berdjis CC, Hartman JA, Gochenour WS: Pathology of experimental respiratory anthrax in *Macaca mulatta*. *Br J Exp Pathol* **44**:416–426, 1963
- 7 Guarner J, Jernigan JA, Shieh W, Tatti K, Flannagan LM, Stephens DS, Popovic T, Ashford DA, Perkins BA, Zaki SR, Inhalational Anthrax Pathology Working Group: Pathology and pathogenesis of bioterrorism-related inhalational anthrax. *Am J Pathol* **163**:701–709, 2003
- 8 Hartings JM, Roy CJ: The automated bioaerosol exposure system: Preclinical platform development and a respiratory dosimetry application with non-human primates. *J Pharmacol Toxicol Methods* **49**:39–55, 2004
- 9 Ivins BE, Welkos SL, Knudson GB, Little SF: Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce protective antigen. *Infect Immun* **58**:303–308, 1990
- 10 Olobo JO, Gicheru MM, Anjili CO: The African green monkey model for cutaneous and visceral leishmaniasis. *Trends Parasitol* **17**(12):588–592, 2001
- 11 Reed DS, Lind CM, Lackemeyer MG, Sullivan LJ, Pratt WD, Parker MD: Genetically engineered, live, attenuated vaccines protect nonhuman primates against aerosol challenge with a virulent IE strain of Venezuelan equine encephalitis virus. *Vaccine* **23**:3139–3147, 2005

Request reprints from Dr. Nancy A. Twenhafel, U.S. Army Medical Research Institute of Infectious Diseases, Pathology Division, 1425 Porter Street, Fort Detrick, MD 21702-5011 (USA). E-mail: nancy.twenhafel@us.army.mil.