

The Pathology of Experimental Anthrax in Rabbits Exposed by Inhalation and Subcutaneous Inoculation

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● **Objective.**—Although rhesus monkeys are considered to be an appropriate model for inhalational anthrax in humans, an alternative for vaccine and therapeutic efficacy studies is desirable. This study characterized the pathology of lethal anthrax in rabbits challenged by subcutaneous inoculation and aerosol exposure.

Materials and Methods.—New Zealand white rabbits were exposed by subcutaneous inoculation or aerosol to lethal doses of *Bacillus anthracis* spores.

Results.—The pathology of anthrax in rabbits exposed by either route was similar, with principal findings occurring in the spleen, lymph nodes, lungs, gastrointestinal tract, and adrenal glands. The cardinal changes were hemorrhage, edema, and necrosis, with bacilli and limited leukocytic infiltration. Features that depended on the route of exposure included mediastinitis in aerosol-exposed rabbits, a primary dermal lesion after subcutaneous inoculation, and differences in the pattern of lymph node involvement. Lesions observed in rabbits were comparable to those of

inhalational anthrax in humans and rhesus monkeys. Noteworthy differences included the lack of leukocytic infiltration in brain and meningeal lesions, the relatively mild mediastinal lesions, and a lower incidence of anthrax-related pneumonia in rabbits compared with humans. These differences may be attributed to the greater susceptibility of rabbits to anthrax. Increased susceptibility is associated with both reduced leukocytic response to the bacilli and a more rapid progression to death, which further limits development of leukocytic infiltrates in response to the basic lesions of hemorrhage and necrosis. Primary pneumonic foci of inhalational anthrax, which may be influenced by preexisting pulmonary lesions in humans, were not observed in our rabbits, which were free of preexisting pulmonary disease.

Conclusion.—Anthrax in rabbits may provide a useful model for evaluating prophylaxis and therapy against inhalational anthrax in humans.

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Anthrax, caused by the gram-positive, spore-forming bacterium *Bacillus anthracis*, is primarily a disease of domestic herbivores, but it also occurs in humans as an infrequent zoonosis, typically acquired from contact with contaminated wool, hides, or meat. The three major forms of the human disease—cutaneous, inhalational, and gastrointestinal—reflect the route of entry of spores, which can occur through intradermal inoculation, inhalation, or ingestion, respectively.¹ Each form of the disease can progress to fatal systemic anthrax.

B. anthracis has long been recognized as a potential biological warfare or terrorist threat agent, and recent events in the Persian Gulf have further emphasized the need to

develop adequate countermeasures against inhalational anthrax as a weapon of mass destruction.^{2,3} Depending on both proximity to an effective delivery system and environmental factors affecting dispersal of the spores, the potential exists for high-dose inhalational exposure under such a scenario. During the past several years, considerable research has been devoted to the development of pre-exposure and postexposure anthrax prophylaxis by experimental human anthrax vaccines and antibiotic regimens.^{1,4-11} The majority of these efforts use rhesus monkeys or guinea pigs as the animal models of choice for efficacy studies. In addition, the Food and Drug Administration (FDA) requires the guinea pig potency test before release of each lot of the human anthrax vaccine adsorbed currently licensed for use in the United States (21 CFR 620.23). As animal models, however, rhesus monkeys and guinea pigs have a number of disadvantages. Although rhesus monkeys are considered to be an appropriate model for inhalational anthrax in humans,^{12,13} nonhuman primate use is beset by practical considerations, including the monetary investment in individual animals, intensive husbandry requirements, and safety issues incident to handling of the animals. Recent studies indicate that guinea pigs are not an accurate predictor of vaccine efficacy in nonhuman primates.^{4,5,8-10} Guinea pigs, in contrast to rhesus monkeys, are difficult to protect by immunization with anthrax vaccine adsorbed, and they exhibit considerable variation in survival after subsequent challenge by virulent strains of *B. anthracis*. An alternative is needed for

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In conducting this research, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH publication 86-23, revised 1985).

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efficacy studies against inhalational anthrax. Since inhalational anthrax in humans is virtually 100% fatal and treatment is rarely successful, an animal model that is highly sensitive to lethal infection was deemed most appropriate.

Initial studies were conducted to define the subcutaneous and aerosol median lethal doses (LD_{50}) for *B. anthracis* spores of the Ames strain in rabbits. Subsequent studies were performed to determine the efficacy of anthrax vaccine adsorbed and alternative vaccine candidates against anthrax induced in rabbits by inhalation or subcutaneous exposure. Preliminary data suggest that rabbits are similar to nonhuman primates in their ability to be protected by the current FDA-licensed human vaccine. Near-100% protection against lethal aerosol challenge was achieved with use of an abbreviated vaccination schedule in both species (M.L.M.P., unpublished data, 1996). Given preliminary evidence that the human anthrax vaccine appears to be efficacious against inhalational anthrax in rabbits, and in the interest of validating efficacy trials conducted in rabbits, we sought to determine how well the disease pattern in the animal model, as reflected by the terminal pathology, approximates the disease in humans. In this article, we describe the terminal pathology of lethal experimental anthrax developed in rabbits used in LD_{50} and preliminary vaccine efficacy studies, and we compare these findings to those for inhalational anthrax in humans.

MATERIALS AND METHODS

Animals

Necropsy specimens were obtained from lethally infected, nonvaccinated, male and female New Zealand white rabbits (*Oryctolagus cuniculus*) that had died after subcutaneous inoculation with (19 rabbits) or aerosol exposure to (22 rabbits) *B. anthracis* spores of the Ames strain (Table 1). Animals were observed for survival at least twice daily during the daylight phase of the photoperiod, for 21 to 28 days after exposure. Rabbits were identified by cage card and/or by subcutaneously implanted microchips and were housed individually in stainless steel rabbit cages in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Rabbits were fed once daily with commercial rabbit chow. Tap water was provided ad libitum.

Spore Preparation

The virulent Ames strain of *B. anthracis* was obtained from the US Department of Agriculture, Ames, Ia. It was grown in Leighton-Doi medium, and spores were harvested and washed in sterile distilled water as described elsewhere.⁴ The spores were purified by centrifugation through 58% Renografin-76, washed again, resuspended in 1% phenol, and stored at 4°C.

For subcutaneous inoculation, spores were suspended in sterile water for injection and then heat shocked at 60°C for 45 minutes. Appropriate dilutions were prepared, to achieve the desired dose of spores in a final volume of 0.5 mL/dose. Spore dilutions were held on ice until administered. Actual spore counts in the inoculum were verified by quantitative bacterial culture. Rabbits were inoculated with 0.5 mL of the material (dose range, 43 to 1.56×10^6 colony-forming units (CFU); subcutaneous $LD_{50} = 1.56 \times 10^5$ CFU; subcutaneous lethal dose₉₉ (LD_{99}) = 2.83×10^6 CFU) in the dorsal interscapular region.

For aerosol exposure, spores were suspended to appropriate starting concentrations in sterile water for injection, and were then heat shocked at 60°C for 45 minutes. Eight-milliliter aliquots of appropriate dilutions of spores were used for aerosol exposure, with a 3-jet Collison nebulizer with a head-only box and muzzle-only exposure used as described elsewhere.^{1,14,15} The concentration of spores in the aerosol (sampled in water in an all-glass

Table 1. Anthrax in Rabbits: Exposure and Survival

Animal Number	Sex	Dose (CFU)*	Dose ($\times LD_{50}$)†	Route‡	Day of Death
1	M	43	0.03	S	3
2	M	43	0.03	S	4
3	M	4300	2.76	S	3
4	M	4300	2.76	S	3
5	M	4300	2.76	S	3
6	F	4300	2.76	S	4
7	M	16 050	10.29	S	3
8	F	16 050	10.29	S	3
9	F	16 050	10.29	S	3
10	F	33 250	21.31	S	2
11	F	33 250	21.31	S	2
12	F	156 000	100.00	S	2
13	F	156 000	100.00	S	2
14	F	156 000	100.00	S	3
15	F	156 000	100.00	S	3
16	M	156 000	100.00	S	3
17	F	156 000	100.00	S	3
18	F	156 000	100.00	S	3
19	M	156 000	100.00	S	3
20	M	83 400	1.52	A	3
21	F	83 400	1.52	A	3
22	M	107 000	1.95	A	2
23	M	114 000	2.07	A	2
24	M	466 000	8.47	A	2
25	F	500 000	9.09	A	3
26	F	606 000	11.02	A	3
27	F	694 000	12.62	A	2
28	F	740 000	13.45	A	3
29	F	874 000	15.89	A	2
30	F	886 000	16.11	A	2
31	F	920 000	16.73	A	3
32	M	4 540 000	43.24	A	3
33	M	6 340 000	60.38	A	2
34	M	6 400 000	60.95	A	2
35	M	6 660 000	63.43	A	2
36	M	7 060 000	67.24	A	3
37	M	7 400 000	70.48	A	2
38	M	8 060 000	76.76	A	2
39	M	8 460 000	80.57	A	2
40	M	8 660 000	82.48	A	2
41	M	10 300 000	98.10	A	2

* CFU indicates colony-forming units.

† LD_{50} indicates median lethal dose.

‡ S indicates subcutaneous; A, aerosol.

impinger) and the aerosol-inhaled dose were determined as described elsewhere.^{1,14,15} The aerosol-inhaled dose ranged from 8.34×10^4 to 1.03×10^7 CFU *B. anthracis* (aerosol $LD_{50} = 1.05 \times 10^5$ CFU; aerosol $LD_{99} = 1.36 \times 10^6$ CFU).

Necropsy

A complete necropsy was performed on each animal included in the pathology study. Gross findings were recorded, and the incidence of each finding was tabulated. Representative tissue specimens were selected and immersion-fixed in 10% neutral buffered formalin. Immediately before immersion fixation, lungs were inflated with 10% neutral buffered formalin.

Histopathology

Formalin-fixed tissue specimens were processed and embedded in paraffin (TissuePrep, Fisher Scientific, Fair Lawn, NJ) according to established procedures.¹⁶ Histology sections were cut at 5 to 6 μ m and stained with Harris' hematoxylin-eosin. Selected tissues were stained with Gram and Giemsa stains. Histopathologic findings were determined by routine light microscopy. Each finding, such as edema, hemorrhage, necrosis, and inflammation, was graded individually on a severity scale of 1 (minimal) to 5 (severe) on the basis of estimates of distribution and extent of

Table 2. Anthrax in Rabbits: Incidence of Principal Gross Pathologic Findings by Route of Exposure

Organ and Finding(s)*	Subcutaneous (n = 19)	Aerosol (n = 22)
Lymph nodes		
Mandibular, HEM	0	13
Axillary, HEM	4	1
Inguinal, HEM	0	3
Splenomegaly	19	18
Sacculus rotundus, HEM	11	5
Cecal appendix, HEM	11	5
Lung, congestion		
ED	8	9
HEM	4	2
Adrenal, HEM	8	12
Ovary, HEM	4 (n = 11)	2 (n = 8)
Stomach, HEM, ED	2	5
Skin, inoculation site		
ED	19	NA
HEM	17	
Ventral cervical ED	3	4
Axillary ED	5	0
Epistaxis	3	10

* HEM indicates hemorrhage; ED, edema.

involvement within examined sections. The incidence of involvement for each organ or tissue was determined as the number of animals that had one or more of the principal histologic findings attributable to anthrax in that tissue. The severity index was calculated as the sum of severity scores for individual findings in an organ, divided by the number of animals in which that organ was examined histologically.

RESULTS

Clinical Observations

Rabbits included in the pathology study died 2 to 4 days after exposure to *B. anthracis* spores, with mean survival times of 2.9 days and 2.4 days for subcutaneously inoculated and aerosol-exposed rabbits, respectively. Although there was a trend for decreased survival time with increasing dose, the effect was minimal (Table 1). Fulminant disease appeared to be an all-or-none response, and no protracted illness was observed, regardless of the dose. Clinical signs were not generally apparent until within 24 hours of death, at which time rabbits became progressively lethargic and weak. Several rabbits, later found to have brain or meningeal lesions, exhibited brief periods of excitation and hyperactivity within hours or minutes before death.

Gross Pathologic Findings

In general, gross findings were similar, regardless of the route of infection. The most significant alterations occurred in the lymph nodes, spleens, lungs, adrenal glands, and gastrointestinal tracts of both groups of rabbits (Table 2). Hemorrhage and edema were the most prevalent changes within affected tissues. The most readily apparent differences between the two groups occurred in the pattern of lymph node involvement, in alterations at the dorsal scapular site of subcutaneous inoculation, and in changes at the axillary region of subcutaneously inoculated rabbits.

In subcutaneously inoculated rabbits, axillary lymph node involvement predominated, whereas mandibular lymph node involvement was most apparent in aerosol-exposed rabbits. Affected lymph nodes varied from mild-

ly reddened to frankly hemorrhagic. Mediastinal nodes were obscured by adipose tissue and generally were not examined grossly. In the majority of rabbits, the spleen was dark red, was congested, was enlarged up to three times normal size, and had rounded edges. Petechiae and ecchymoses were commonly visible through the serosal surface of the sacculus rotundus and cecal appendix in both groups, with increased frequency among subcutaneously inoculated rabbits. Hemorrhage, also commonly observed in the adrenal glands and ovaries of rabbits in both groups, occurred less frequently in the Peyer's patches of the ileum, lungs, and meninges of the brain. The lungs of approximately 40% of rabbits in each group exhibited a lobular pattern of dark red mottling due to congestion and edema throughout all lung lobes. Hydrothorax and mediastinal hemorrhage were observed in only 2 and 1 of 22 aerosol-exposed rabbits, respectively, and were absent in subcutaneously inoculated rabbits. In all subcutaneously inoculated rabbits, the site of inoculation was thickened by dermal and subcutaneous edema, commonly accompanied by hemorrhage. Edema was frequently evident in the axillae of subcutaneously inoculated rabbits, as a gelatinous thickening of subcutaneous tissues. Edema of the ventral cervical to submandibular region was observed in fewer than 20% of the animals in both groups. In a small number of rabbits from each group, the mucosal surface of the stomach was mildly thickened by scattered foci of edema, up to 1 cm in diameter, with central hemorrhage. Multifocal petechiae occurred in the renal cortex of 1 aerosol-exposed rabbit. In addition to the changes noted above, all rabbits exhibited congestion of multiple organs.

Histopathologic Findings

A summary of incidence and relative severity of the principal histopathologic findings is presented in Table 3. After subcutaneous inoculation with or aerosol exposure to *B. anthracis* spores, the most consistent histopathologic findings occurred in lymphoid tissues, including the lymph nodes, spleen, and gut-associated lymphoid tissues of the sacculus rotundus and cecal appendix.

Lymph node lesions generally presented as hemorrhagic lymphadenitis (Figures 1 and 2). The principal morphologic features were lymphoid necrosis and depletion, bacilli within sinuses and depleted cortical and paracortical areas, hemorrhage, fibrinoid necrosis of vessels, edema, and variable infiltration by heterophils. In more-severely affected lymph nodes, the normal architecture was nearly obliterated by necrosis and hemorrhage. Although the morphologic characteristics of lymph node lesions were essentially the same in both groups of rabbits, the pattern of lymph node involvement and the relative severity of lesions depended on the route of exposure. Mediastinal nodes were the most frequently affected lymph nodes in both groups of rabbits; however, the mean lesion severity was greater for aerosol-exposed rabbits. Axillary node involvement occurred with greater incidence and severity among subcutaneously inoculated rabbits, whereas submandibular node involvement was more prominent among those exposed by aerosol. Mesenteric node involvement was similar between the two groups.

Acute fibrinous splenitis, seen in all rabbits, was characterized by numerous heterophils, extremely large numbers of bacilli, and fibrin deposition throughout the red

Table 3. Anthrax in Rabbits: Principal Histopathologic Findings by Route of Exposure

Organ and Finding(s)*	Subcutaneous†		Aerosol‡	
	IN	SI	IN	SI
Lymph nodes				
Mediastinal, NEC, INF, HEM	17/18	6.33	22/22	8.09
Mandibular, NEC, INF, HEM	10/15	1.73	18/22	3.18
Mesenteric, NEC, INF, HEM	9/15	1.67	13/22	2.82
Axillary, NEC, INF, HEM	14/18	5.56	9/21	1.05
Inguinal, NEC, INF, HEM	6/18	0.56	7/22	0.82
Spleen, NEC, INF, HEM	19/19	9.00	22/22	7.91
Sacculus rotundus, NEC, HEM	14/19	3.89	9/22	1.54
Cecal appendix, NEC, HEM	13/18	4.22	11/21	1.76
Peyer's patches, NEC, HEM	6/12	1.75	2/13	0.23
Thymus, NEC	5/19	0.74	3/22	0.27
Lung, ED, ALV, INF	18/19	5.47	21/22	5.36
Mediastinum, INF, HEM	0/19	0.00	8/22	0.95
Adrenal, HEM	18/19	3.00	16/22	2.27
Kidney, tubular NEC, HEM	16/19	1.63	12/22	0.95
Ovary, HEM	4/10	1.40	5/8	1.62
Stomach, HEM, ED	4/19	0.95	4/22	0.59
Brain/meninges, HEM	5/19	1.32	4/22	0.45
Bone marrow, INF	13/19	1.68	9/22	0.64
Circulatory CON, LEUK, FIB, BAC	19/19	NG	22/22	NG
Inoculation site, INF, ED, HEM	10/11	8.82	NA	

* NEC indicates necrosis; INF, inflammation; HEM, hemorrhage; ED, edema; ALV, alveolar flooding; CON, congestion; LEUK, leukocytosis; FIB, fibrin; and BAC, bacillemia.

† IN indicates incidence (number of rabbits with one or more of the findings listed for an organ, divided by the number of rabbits in which the organ was examined histologically); SI, severity index (sum of the lesion severity scores, divided by n); NG, not graded; and NA, not applicable.

pulp; necrosis and depletion of the white pulp; multifocal hemorrhage; and diffuse congestion (Figure 3).

Lesions within other lymphoid tissues were comparable between the two groups, although a slight reduction in incidence and severity of changes in the sacculus rotundus and cecal appendix occurred after aerosol exposure. Changes in lymphoid follicles of the sacculus rotundus and cecal appendix (Figure 4) were similar to those occurring in lymph nodes. Peyer's patches of the ileum were also similarly affected, although with decreased incidence and severity. Changes in the thymus were relatively mild and included small scattered foci of lymphoid necrosis and depletion, with bacilli, edema, and minimal to mild hemorrhage. Necrosis of bronchus-associated lymphoid tissue occurred in 1 rabbit in each group.

We observed significant pulmonary changes in both groups of rabbits, and—with the exceptions of interstitial pneumonia in 2 of 22 aerosol-exposed rabbits and pleuritis in 1 of 19 subcutaneously inoculated rabbits—these changes were the same between the two groups. Typically, there was distention of alveolar capillaries by congestion and large numbers of bacilli, large masses of bacilli enmeshed in fibrin within larger pulmonary vessels, interstitial edema, flooding of alveolar spaces by eosinophilic edema fluid, and minimal to mild perivascular and peribronchiolar infiltrates of heterophils (Figure 5). Pulmonary hemorrhage was confirmed histologically in 1 aerosol-exposed rabbit but was not found in any subcutaneously inoculated rabbits. Acute interstitial pneumonia occurred in 2 of the 22 aerosol-exposed rabbits but in none of the subcutaneously inoculated rabbits. In the rabbits with pneumonia, alveolar septa were mildly thickened by infiltrates of heterophils, in addition to the typical pulmonary changes noted above. Acute pleuritis, observed in only 1 subcutaneously inoculated rabbit, was characterized by mild expansion of the visceral pleura by infiltrates of heterophils, bacilli, and edema.

Acute mediastinitis was observed occasionally in aerosol-exposed rabbits and, when present, was always accompanied by severe changes within associated mediastinal lymph nodes (Figure 6). In the most severely affected animals, there was infiltration of mediastinal connective tissues by moderate numbers of heterophils, with hemorrhage, fibrin, edema, and bacilli. We occasionally noted edema, hemorrhage, and bacilli within tissues immediately surrounding affected lymph nodes of subcutaneously inoculated rabbits, but changes limited to the lymph node capsule without extension into surrounding fibroadipose tissue, as was seen in aerosol-exposed rabbits, were not considered sufficient to warrant a diagnosis of mediastinitis.

Hemorrhage occurred in multiple tissues, in addition to those described above, in both groups of rabbits. Hemorrhages were not accompanied by hemosiderosis or inflammation, although fibrinoid vascular necrosis was occasionally present. The adrenal cortex, renal cortical tubules and/or glomeruli, ovaries, and subcutaneous inoculation site were frequently affected. Within the kidneys, glomerular capillaries were often distended by masses of bacilli. Multifocal hemorrhages into uriniferous spaces appeared to have drained into associated renal tubules, which contained blood and small numbers of bacilli and were lined by degenerate to necrotic epithelial cells (Figure 7). Sporadic hemorrhagic foci were present in numerous other tissues, including the neuropil, meninges, and ventricles of the brain (Figure 8); superficial gastric mucosa; ocular structures (ciliary body, iris, and optic nerve); and myocardium.

Within the femoral bone marrow, there were small foci of depletion of hematopoietic elements with infiltration by low numbers of heterophils and aggregates of bacilli (Figure 9). In addition to the rabbits with myelitis, 1 rabbit in each group exhibited aggregates of bacilli in the marrow, without an apparent leukocytic response.

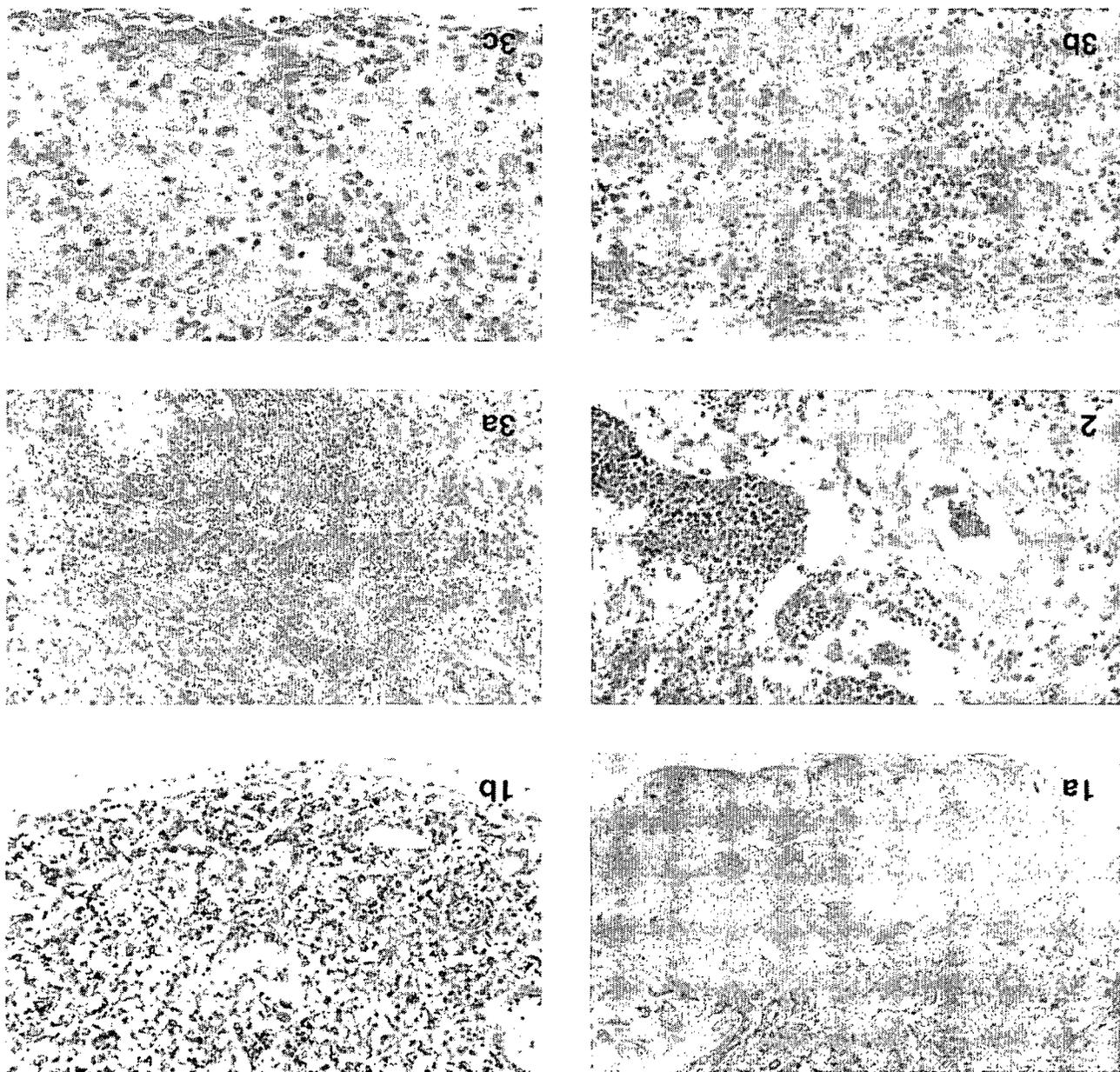
All animals in both groups developed bacillemia, intravascular aggregates of fibrin, congestion of multiple organs, and peripheral heterophilic leukocytosis. The pattern of organ involvement was the same, regardless of the route of exposure. We observed variable numbers of bacilli, often enmeshed in fibrin, within the vasculature of nearly all tissues examined. We saw increased numbers of

circulating heterophils, including immature stages and occasional mitotic figures, in many tissues, which we interpreted as peripheral heterophilic leukocytosis with a left shift (Figure 10). Acute dermatitis and/or panniculitis was a consistent feature at the inoculation sites (Figure 11). Lesions were characterized by infiltration of the dermis and panniculus

Figure 3. Spleen of a subcutaneously inoculated rabbit. A, Note lymphoid necrosis, depletion, and hemorrhage of the lymphoid follicle. There are (B) aggregates of fibrin and (C) numerous bacilli within the red pulp (hematoxylin-eosin, original magnification $\times 132$ [A], $\times 132$ [B], and $\times 264$ [C]).

Figure 2. Axillary lymph node of a subcutaneously inoculated rabbit, showing fibrinoid vascular necrosis, lymphoid necrosis, depletion, and hemorrhage within medullary cords; and bacilli within medullary sinuses (hematoxylin-eosin, original magnification $\times 132$).

Figure 1. Mediastinal lymph node of a subcutaneously inoculated rabbit. A, Note severe depletion of lymphoid tissue, with multifocal hemorrhage (hematoxylin-eosin, original magnification $\times 13.2$). B, Massive numbers of bacilli are evident within the subcapsular sinus and depleted cortical areas (Giemsa, original magnification $\times 132$).



by large numbers of heterophils, numerous bacilli, marked expansion of the dermis and panniculus by edema, multifocal hemorrhage and necrosis, occasional fibrinoid necrosis of the vasculature, and rare thrombosis. Epidermal vesiculation, ulceration, and eschar formation characteristic of cutaneous anthrax in humans were not observed.

COMMENT

Similarities in the pathology of inhalational anthrax in rabbits, humans, and rhesus monkeys were readily apparent. Subcutaneous exposure of rabbits also resulted in rapidly fatal septicemic disease, rather than the characteristic skin lesions and low mortality typical of cutaneous anthrax in humans. In rabbits exposed by either route, the most pathologically significant and consistent findings occurred in the spleen, lymph nodes, lungs, gastrointestinal tract, and adrenal glands. Additional lesions occurred in the mediastinum (aerosol exposure only), brain, bone marrow, kidney, thymus, heart, and ovaries. Lesions were necrotizing and hemorrhagic, generally accompanied by little local leukocytic response. The lesions of inhalational anthrax in humans are also characterized as hemorrhagic to necrohemorrhagic, with little cellular inflammatory response.^{17,18} The spleen, lymph nodes, mediastinum, lungs, gastrointestinal tract, and brain are principal sites of involvement.^{13,18-26} Rhesus monkeys inoculated intradermally or exposed to aerosolized spores of *B. anthracis* also develop necrotizing and hemorrhagic lesions similar to those seen in our rabbits.^{12,27-29}

With few exceptions, the pathology of lethal infection by the Ames strain of *B. anthracis* in rabbits was the same whether animals were exposed subcutaneously or by aerosol. Except for the presence of a primary dermal lesion after subcutaneous inoculation and the lack of a corresponding primary pneumonic focus after aerosol exposure, the pathogenesis of anthrax induced by either route appears similar. Ross³⁰ demonstrated that after inhalational exposure, spores are taken up by alveolar macrophages for transport, by way of lymphatics, to intrathoracic lymph nodes. Infection begins with proliferation of the organisms in the lymph nodes rather than as a primary focus in the lungs, thus explaining the rarity of pneumonia after aerosol exposure. Studies by Lincoln et al³¹ further demonstrated the role of lymphatics and intrathoracic lymph nodes in the establishment of systemic inhalational anthrax. The appearance of organisms in the lymphatics draining the lungs and the establishment of infection in the intrathoracic lymph nodes always precedes the development of bacteremia after aerosol exposure. As the phagocytic capacity of the lymph node is overwhelmed, vegetative organisms pass through efferent lymphatics, infect successive nodes, and ultimately enter the blood stream through the thoracic duct. Lincoln et al³¹ demonstrated a similar sequence of events in the establishment of systemic anthrax induced by intradermal inoculation. Regional lymph nodes draining the site of inoculation are infected initially. Shortly before the onset of bacteremia, organisms can be cultured from thoracic lymph, and, ultimately, they enter the blood stream through the thoracic duct. Once bacteremia is established, the pathogenesis is the same, regardless of the route of initial exposure.

Major differences between anthrax induced by the two routes of exposure, in rabbits, were the pattern of lymph node involvement, the development of mediastinitis exclusively in aerosol-exposed rabbits, and the dermal lesion

seen after subcutaneous inoculation. Similarly, Gleiser et al²⁹ reported that the basic nature of the lesions of inhalational anthrax was no different from that seen after intradermal inoculation in rhesus monkeys, although lesion distribution exhibited several distinguishing features. Aerosol-exposed monkeys exhibited a high incidence of mediastinitis accompanied by a high incidence of hemorrhagic meningitis, hemorrhagic pulmonary lesions, and intrathoracic lymphadenopathy. Fritze et al¹² reported a high incidence of mesenteric as well as tracheobronchial lymph node involvement in inhalational anthrax in rhesus monkeys. Intrathoracic lymphadenitis and mediastinitis are considered hallmarks of inhalational anthrax in humans and are believed to represent the primary focus of infection.^{13,17,19,23,32} A low incidence of mesenteric node involvement was also reported in humans with inhalational anthrax.^{19,33} In contrast to the findings for inhalational anthrax, Berdjis et al²⁸ described a low incidence of mediastinitis and hemorrhagic meningitis, with cellulitis at the inoculation site, and primarily axillary lymph node involvement in monkeys inoculated intradermally in the forearm.

Differences in the pattern of regional lymph node involvement reflect normal lymphatic drainage from the subcutaneous or pulmonary site of exposure. In contrast to findings in humans and rhesus monkeys, however, we observed significant mediastinal lymph node involvement in rabbits, regardless of the route of exposure. A possible explanation lies in the fact that the mediastinal lymph nodes in various species can receive afferent lymphatics from muscles of the dorsal thoracic wall and scapula, which was the site of inoculation in our rabbits, in addition to efferent lymphatics originating from the intercostal, tracheobronchial, and bronchopulmonary nodes.³⁴⁻³⁶ Greater lesion severity with extension into mediastinal tissues of aerosol-exposed rabbits can be attributed to earlier, more direct involvement of intrathoracic nodes during the course of aerosol infection. The greater incidence and severity of axillary node involvement among subcutaneously inoculated rabbits may be similarly regarded as a consequence of lymphatic drainage from the site of inoculation, resulting in early involvement of the axillary nodes. The increased incidence and severity of submandibular node involvement occurring in aerosol-exposed rabbits may be the consequence of either direct oropharyngeal deposition or mucociliary clearance of a portion of the aerosol from distal respiratory tissues.

The mediastinal lesions we observed in aerosol-exposed rabbits were similar, although less severe than those described in humans. The decreased incidence and severity of the lesions in rabbits were most likely due to the rapid progression of systemic changes, resulting in relatively short survival times compared to survival times for humans or monkeys. In aerosol-exposed rabbits, the mean survival time was 2.4 days after exposure, with only a minimal effect of decreasing dose on prolonging survival. Specific dates of exposure are rarely known for human cases, but estimates for 41 cases from the Sverdlovsk outbreak place mean survival time at 18.5 days after exposure.^{19,37} The clinical course of infection was also modified through extensive medical intervention in those cases. A more protracted course of clinical disease would allow greater opportunity for extension of the primary focus of infection from the intrathoracic nodes into adjacent mediastinal tissues.

The effect of prolonging the time course of infection on the severity of mediastinal lesions was demonstrated experimentally in rhesus monkeys. Gleiser et al³⁸ found that mediastinal edema and some hemorrhage were frequent findings in aerosol-exposed monkeys, but massive hemorrhagic mediastinitis was limited to (1) an animal that died 11 days after exposure to a low aerosol dose and (2) those animals in which the course of the disease was prolonged through the use of antibiotics. In some monkeys that died within 2 to 5 days after exposure to high doses of aerosolized spores, mediastinitis was not seen and intrathoracic lymph node involvement was the only gross finding.

It is noteworthy that 2 of 10 animals in the intradermal study by Berdjis et al²⁸ developed mediastinitis. Although the distribution of lymph node involvement was not presented in detail, beyond citing axillary nodes as a specific site, the development of mediastinitis in these monkeys suggests that mediastinal node involvement may occur after intradermal and aerosol exposure in rhesus monkeys, as occurred in our rabbits. In the study of inhalational anthrax in rhesus monkeys, by Gleiser et al,²⁹ mediastinitis was interpreted to be an extension of lesions originating in the mediastinal lymph nodes. Mediastinal lesions were most intense around intrathoracic nodes, which were frequently necrotic and hemorrhagic; and, as indicated above, some monkeys dying rapidly after exposure to high doses of aerosolized spores developed intrathoracic lymph node involvement without mediastinitis. In a more recent study of inhalational anthrax in rhesus monkeys with a mean survival time of 5.5 days after exposure, Fritz et al¹² observed a gross incidence of thoracic node involvement in 46% and mediastinitis in only 30% of monkeys at necropsy. Such findings suggest that intrathoracic lymph node involvement precedes the development of mediastinitis and that mediastinitis is the result of direct extension of lesions from the lymph nodes into adjacent tissues. A similar pathogenesis for mediastinal lesions may be involved in the Berdjis study²⁸ of anthrax in monkeys exposed by intradermal inoculation. The mediastinitis of inhalational anthrax in humans is also described as having a perinodal distribution and was interpreted to have arisen as an extension of primary lymph node lesions.³⁹ Mediastinal lymph node involvement would be expected to occur early in the course of the disease after aerosol exposure. This could account for the greater severity of mediastinal node lesions we observed in our aerosol-exposed rabbits, in addition to providing increased opportunity for extension into surrounding mediastinal tissues.

The lesions we observed in the gastrointestinal tract were comparable among rabbits exposed by aerosol and subcutaneous inoculation. Gut-associated lymphoid tissues of the sacculus rotundus, cecal appendix, and ileum were primarily affected. We also noted sporadic foci of hemorrhage with bacilli and edema in the stomach, small intestine, colon, and esophagus. In humans, inhalational anthrax is also associated with gastrointestinal hemorrhage, necrosis, and edema involving the stomach, small intestine, and colon.^{18-20,33,39} However, Abramova et al¹⁹ noted that, among the Sverdlovsk cases, the intestinal lesions did not involve Peyer's patches. In contrast, other investigators reported that intestinal lesions in humans with inhalational anthrax were sometimes the result of bacilli multiplying in the gut-associated lymphoid tissues.²⁵

One might attribute the presence of lesions in the gas-

trointestinal mucosa, gut-associated lymphoid tissues, and mesenteric lymph nodes, after aerosol exposure, to primary gastrointestinal anthrax. It is known that 65% to 70% of inhaled *B. anthracis* spores are ultimately coughed up, are swallowed, and pass into the stomach within a few hours of exposure.⁴⁰ However, the presence of identical gastrointestinal findings in subcutaneously inoculated rabbits, where ingestion of spores is unlikely, suggests that these lesions occur secondary to lymphatic or hematogenous dissemination of the bacilli. Gastrointestinal lesions were typically accompanied by distension of associated blood vessels by numerous bacilli, also consistent with hematogenous origin. Investigators who examined cases from the Sverdlovsk outbreak believed that the gastrointestinal lesions were mainly, or possibly wholly, of hematogenous origin, and they emphasized that, in 90% of all human anthrax cases involving gastrointestinal lesions, the enteric pathology occurs secondary to hematogenous dissemination from a cutaneous or respiratory site of exposure.¹⁹ Similarly, hematogenous dissemination was considered to be the most likely origin for gastrointestinal lesions in aerosol-exposed rhesus monkeys.¹² A study by Lincoln et al³¹ showed that rhesus monkeys are extremely resistant to gastrointestinal exposure to *B. anthracis*, providing additional evidence that the gastrointestinal lesions were not likely due to primary infection by ingestion.

Pulmonary lesions were observed in nearly all rabbits, regardless of the route of exposure. An interesting finding among the aerosol-exposed rabbits was the occurrence of 2 cases of acute interstitial pneumonia. Whether the pneumonia represented primary pneumonic anthrax or developed secondary to bacteremia could not be determined definitively. However, the interstitial pattern was most consistent with secondary hematogenous origin, as opposed to bacterial pneumonia of inhalational origin, which typically presents as a bronchopneumonia.⁴¹ Fritz et al¹² reported anthrax-related pneumonia in 2 of 13 rhesus monkeys and interstitial pneumonia in a third monkey after exposure to aerosolized spores. The two cases of anthrax-related pneumonia were thought to be secondary to bacilli occluding and disrupting alveolar septal capillaries. The single case of interstitial pneumonia was thought to represent an early event in the development of anthrax-related pneumonia. The presence of pneumonitis in 3 of 10 intradermally inoculated monkeys studied by Berdjis et al²⁸ also supports a hematogenous route as a viable pathogenesis for the development of pneumonic anthrax. A similar pathogenesis appears likely for the development of pneumonia in our rabbits, and it may account for many of the pneumonias cited in cases of anthrax in humans.

There is evidence suggesting that the greater incidence of pneumonia among humans with inhalational anthrax might be influenced by the presence of preexisting pulmonary lesions. Case reports made before the Sverdlovsk outbreak of 1979 suggest that primary pneumonic anthrax did not occur in the absence of preexisting pulmonary disease.^{13,20,42} In the Sverdlovsk outbreak, however, large-focal hemorrhagic and necrotizing pneumonia interpreted as primary bronchopneumonia was reported for 11 of 42 cases.^{17,19,39} The incidence of preexisting pulmonary lesions was not specifically addressed in reports of the Sverdlovsk cases. However, epidemiologic data reported by Meselson et al¹⁷ suggest that a significant segment of the affected population engaged in activities associated with impaired pulmonary function that may have rendered them at in-

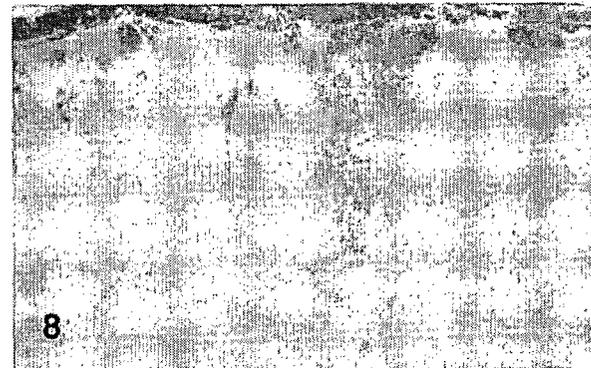
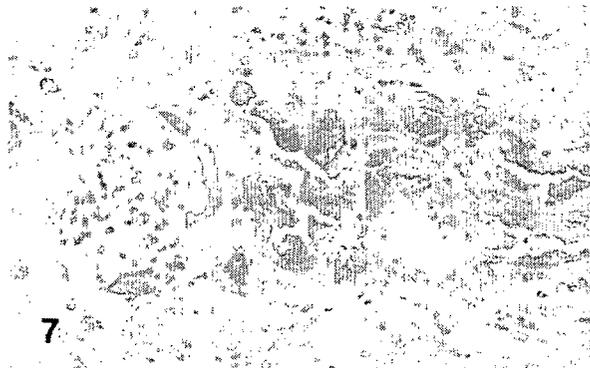
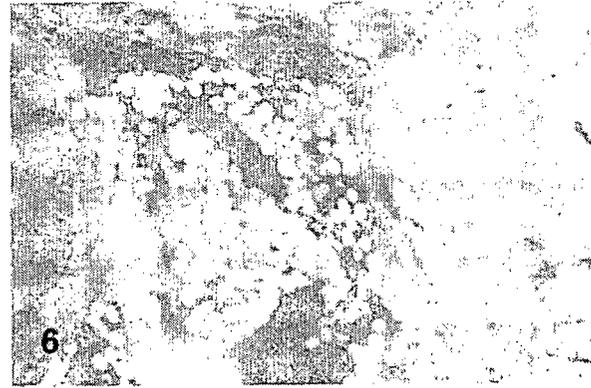
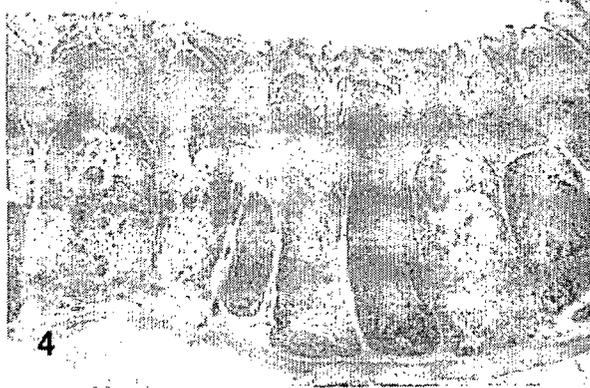


Figure 4. Cecal appendix of a subcutaneously inoculated rabbit. There is severe necrosis and depletion of individual lymphoid follicles, with hemorrhage. Numerous bacilli infiltrate depleted foci, similar to findings illustrated in lymph nodes (Figure 1, B) (hematoxylin-eosin, original magnification $\times 13.2$).

Figure 5. Lung of a subcutaneously inoculated rabbit. A, Note flooding of alveolar spaces with edema fluid and B, severe bacillemia evident in alveolar septal capillaries. Cellular inflammatory infiltrates (ie, pneumonic foci) are not apparent (hematoxylin-eosin, original magnification $\times 13.2$ [A], and Giemsa, original magnification $\times 264$ [B]).

Figure 6. Mediastinum of an aerosol-exposed rabbit. Note multifocal hemorrhage, edema, and necrosis extending into mediastinal fibroadipose tissue adjacent to a necrohemorrhagic mediastinal lymph node (hematoxylin-eosin, original magnification $\times 26.4$).

Figure 7. Kidney of a subcutaneously inoculated rabbit. Note hemorrhage within the uriniferous space and lumina of associated convoluted tubules. Affected tubules are lined by degenerate to necrotic epithelium. Glomerular capillaries are distended by aggregates of bacilli (hematoxylin-eosin, original magnification $\times 13.2$).

Figure 8. Cerebral cortex of a subcutaneously inoculated rabbit. Note hemorrhagic meninges and neuropil and the absence of any apparent cellular inflammatory infiltrates (hematoxylin-eosin, original magnification $\times 26.4$).

increased risk for the development of primary pneumonic foci of anthrax. Information available on 33 of 55 tabulated cases in males indicated that 60% were moderate to heavy smokers. In addition, the most commonly reported occupation of those affected at the time of the outbreak was "welder" (7 of 35 reported). Early investigations of inhalational anthrax in rhesus monkeys described the development of necrotizing bronchopneumonitis believed to represent a superinfection of preexisting lung mite nodules by *B. anthracis*.^{27,29} Similar lesions were not observed in later studies of monkeys free of lung mites.¹² Our studies, performed in rabbits free of significant preexisting pulmonary lesions, and earlier studies by Budner and Barnes^{12,43} also failed to reveal necrohemorrhagic pneumonic foci resembling those described in the Sverdlovsk outbreak.

An additional factor that may have influenced the low incidence of pneumonia and mediastinitis in rabbits compared with that in humans is relative host susceptibility. Anthrax studies in a variety of host species suggest that the degree of leukocytic response may be related to relative host susceptibility, with highly susceptible hosts developing a mild response, in contrast to the more intense response in humans.

All rabbits developed a histologically evident bacterial pneumonia, intravascular aggregates of fibrin, congestion of multiple organs, and peripheral heterophilic leukocytosis. Leukocytosis with a left shift has been reported for human cases.¹³ The level of terminal bacteremia is also considered to be high in humans.⁴⁷ Morphologic changes consistent with shock, frequently noted in human cases, inconsistent with shock, frequently noted in human cases, in-
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Figure 9. Femoral bone marrow of a subcutaneously inoculated rabbit. There is depletion of hematopoietic elements, with infiltration by heterophilic granulocytes and myriad bacilli (hematoxylin-eosin, original magnification $\times 132$).

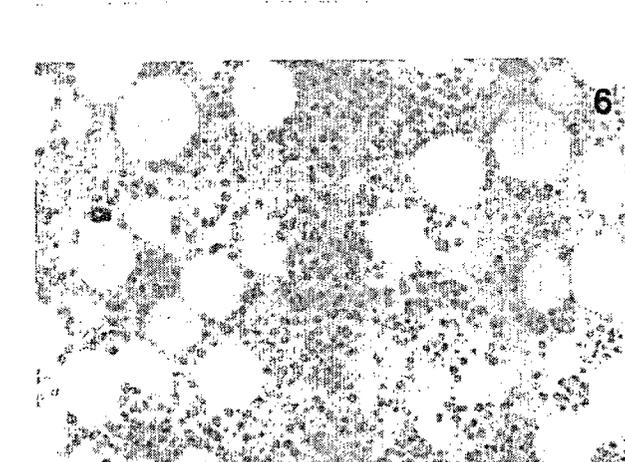
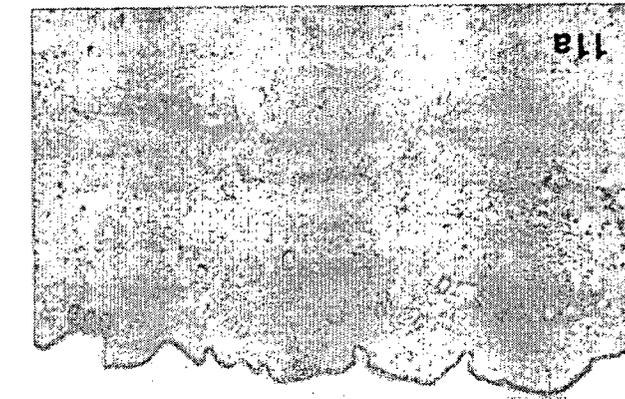


Figure 10. Pulmonary arteriole of a subcutaneously inoculated rabbit. Note the extreme bacteremia and numerous heterophilic granulocytes in various stages of maturation (Giemsa, original magnification $\times 264$).

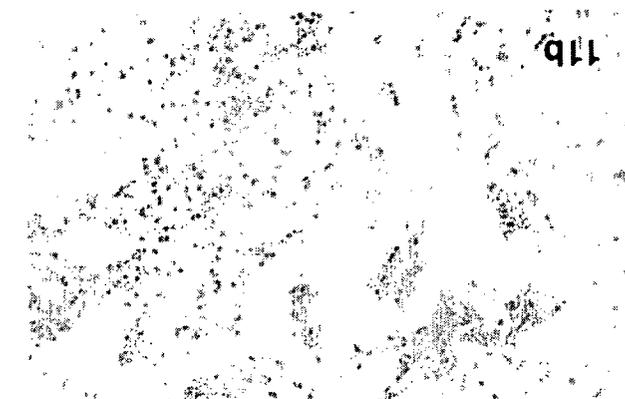


Figure 11. Inoculation site of a subcutaneously inoculated rabbit. The dermis is markedly expanded by edema, hemorrhage, and necrosis. A, Note the absence of epidermal vesiculation or ulceration (hematoxylin-eosin, original magnification $\times 264$). B, Dermal collagen bundles are separated by infiltrates of heterophilic granulocytes admixed with edema, fibrin, and numerous bacilli (Giemsa, original magnification $\times 132$).

Figure 11. Inoculation site of a subcutaneously inoculated rabbit. The dermis is markedly expanded by edema, hemorrhage, and necrosis. A, Note the absence of epidermal vesiculation or ulceration (hematoxylin-eosin, original magnification $\times 264$). B, Dermal collagen bundles are separated by infiltrates of heterophilic granulocytes admixed with edema, fibrin, and numerous bacilli (Giemsa, original magnification $\times 132$).

clude congestion of multiple organs, stasis of erythrocytes in venules and capillaries, and pulmonary edema.¹⁷ The congestion suggests terminal hypotensive shock, consistent with cytokine-induced pathophysiologic events currently believed to contribute to the pathogenesis of anthrax. At high doses, anthrax lethal toxin is lytic to macrophages⁴⁸; however, at sublytic doses, the toxin induces macrophages to express interleukin 1 and tumor necrosis factor.⁴⁹ At high levels, interleukin 1 and tumor necrosis factor can mediate a cascade of physiologic events culminating in fatal shock,⁵⁰⁻⁵² similar to that seen at the terminal stage of systemic anthrax. Intravascular aggregates of fibrin may be due to rapid postmortem clot formation peculiar to anthrax infection and the extreme bacillemia, or they may occur secondary to toxin- or cytokine-induced endothelial alterations favoring a procoagulant state.⁵³⁻⁵⁶ Bacterial thrombi and leukocytosis are also observed in anthrax in rhesus monkeys exposed intradermally or by aerosol.^{28,29,57}

Hemorrhages involving multiple tissues were common among both groups of rabbits and have been described for anthrax in humans^{20,22,33,58,59} and rhesus monkeys^{26,29} as well. In the majority of affected tissues in rabbits, the hemorrhage was accompanied by large numbers of bacilli. In the adrenal glands, however, few bacilli were apparent histologically. Hemorrhage in the adrenal glands varied from minimal to complete obliteration of the cortex and may have been a manifestation of Waterhouse-Friderichsen syndrome. Renal hemorrhages in our rabbits were accompanied by changes that have not been described for anthrax in humans or in nonhuman primates. The appearance of the lesion suggests that hemorrhage occurred through disruption of glomerular capillaries, with subsequent drainage from the uriniferous space into associated convoluted tubules. This resulted in tubular degeneration and necrosis, possibly hemoglobinuric nephrosis. In our study, the renal lesion was minimal to mild in severity, with only low numbers of widely scattered nephrons involved. Significant renal lesions were not a feature of anthrax in intradermally-exposed rhesus monkeys, whereas renal tubular degeneration and tubular casts were reported for inhalational anthrax in that species.²⁹ Significant renal pathology does not appear to be a feature of inhalational anthrax in humans, although there is a limited account of tubular degeneration and necrosis.³⁹

Hemorrhagic meningitis with intense neutrophilic inflammatory infiltrates is frequently associated with inhalational anthrax in humans and nonhuman primates.^{12,13,19,20,29,36} It was also reported to occur in 1 of 10 rhesus monkeys exposed intradermally.²⁸ However, of the 21 cases of meningeal anthrax observed in the Sverdlovsk outbreak, 8 were described as a serous leptomeningitis characterized primarily by edema of the pia mater, with only insignificant infiltration by erythrocytes, mononuclear cells, and neutrophils.³⁹ A low incidence of hemorrhage with bacilli occurred in the brain and meninges of subcutaneously inoculated and aerosol-exposed rabbits. The lesion in rabbits differed from that seen most often in humans or in nonhuman primates in that it was devoid of any accompanying leukocytic infiltrate. The degree of leukocytic response may have been influenced by relative host susceptibility, as we have suggested for the mediastinal, pulmonary, and dermal lesions. In addition, our rabbits were infected with the Ames strain of *B. anthracis*, whereas most earlier studies were of anthrax in nonhuman primates infected with the Vollum-189 or Vollum 1B strains.

Finally, and perhaps most importantly, the rapid progression of fatal systemic changes in rabbits may have limited the opportunity for development of the leukocytic response. In this regard, rhesus monkeys with meningeal anthrax that die soon after exposure have a significantly lower cellular inflammatory response in the central nervous system than do those with longer survival times (G.M.Z., unpublished data, 1997).

Our results indicate that rabbits are extremely sensitive to lethal infection by *B. anthracis*, as evidenced by the fulminant nature of the disease and disseminated pathologic findings. The rapidly fatal course of anthrax induced by high-dose aerosol or subcutaneous exposure in rabbits could be considered disadvantageous in that products efficacious against the more protracted human illness might go unrecognized in such an animal model. However, inhalational anthrax in humans is essentially 100% fatal if left untreated, and inhaled doses in biological warfare or terrorist scenarios might well exceed those of historical occupational exposures. It would be prudent to err on the side of safety by demonstrating efficacy in such a sensitive model.

CONCLUSION

The principal lesions of anthrax were similar in rabbits after subcutaneous injection or aerosol exposure. Major differences were the pattern of lymph node involvement, the presence of mediastinitis exclusively in aerosol-exposed rabbits, and dermal lesions in subcutaneously inoculated rabbits. Although the disease is characterized by a more rapid progression in rabbits, the end-stage pathology of anthrax in the rabbit model appears remarkably similar to that of inhalational anthrax in humans, and it supports the use of rabbits as an appropriate animal model. Furthermore, the more fulminant nature of the disease in rabbits could be considered advantageous in that it provides a rigorous test of candidate products, useful in the development of vaccines and therapeutic regimens against inhalational anthrax in humans.

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