EXPERIMENTAL INHALATION ANTHRAX IN THE CHIMPANZEE *

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Although anthrax has long been recognized in domestic animals and man, precise techniques have only recently been devised to allow a controlled study of the disease in experimental animals by utilizing a respiratory route of infection.[1] "Woolsorters' disease" and "ragpickers' disease" have been described in man, and a portal of entry by way of the tracheal or bronchial mucosa has been suggested as a basis for the subsequent development of "anthrax pneumonia."[2,3] Another interpretation of the observations in human cases suggests that the lung parenchyma serves as a portal of entry for organisms and that a generalized systemic infection rather than a primary pneumonic process results.[4] Experiments using a variety of animals have tended to support the latter view,[5,6] and recent investigation in guinea pigs has demonstrated the passage of spores from the alveolar spaces to regional lymph nodes with subsequent germination and dissemination of the vegetative forms.[7]

The experiments reported here were designed to correlate the clinical behavior of infected animals with the development of the disease and to determine the tissue alterations in higher primates, with particular reference to the respiratory tree.

Material and Methods

The experimental animals consisted of 4 young, healthy chimpanzees, weighing from 18 to 23 pounds. All but one were classified as *Pan troglodytes* (*Schwarz*); the exception (Bill) belonged to the group *Pan troglodytes troglodytes*. The animals had complete pre-exposure physical examinations, and records were made of blood counts, blood cultures, stool examinations, rectal temperatures and roentgenograms of the chest. All reports indicated that the animals were normal except that occasional hookworm ova were encountered in the stools. Following exposure, the chimpanzees were examined daily, and the blood was cultured. Rectal temperature was taken twice daily, and chest roentgenograms were obtained every other day.

Five 10-fold dilutions in saline were made of each sample of blood. Three plates each of cysteine blood agar and Wilson's peptone agar

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were inoculated with 0.1 ml. of each dilution of blood as well as undiluted blood. The plates were incubated for 24 hours at 37° C., after which colonies were counted. In cases where no colonies were observed at the first reading, the plates were re-incubated for 24 additional hours and then reread. Each of 5 mice received intraperitoneal injections (0.5 ml.) of the first 1:10 dilution of each blood sample. When the mice died, heart blood smears and culture on agar plates substantiated the presence of *Bacillus anthracis.*

The strain of *B. anthracis* used was designated Vollum 1B. The concentrations of spores in the generator were adjusted to be within the limits of 4×10^6 and 6×10^6 spores per ml. of suspension medium (casein acid digest containing one per cent phenol).

Aerosol was generated by means of a University of Chicago Laboratory (UCTL) atomizer, introduced into a modified Reyniers chamber[8] and maintained at a temperature of 25° C. ± 1 degree and a relative humidity of 30 ± 3 per cent. The UCTL atomizer was operated with a total air flow of 100 liters per minute (5 liters of primary air at a pressure of 12 pounds per square inch and 95 liters of secondary or mixing air), and the liquid suspension of *B. anthracis* spores was disseminated from the atomizer by means of a constant feed device at a rate of approximately 0.2 ml. per minute. The aerosol was continuously generated directly into a 120-liter mixing chamber where it was kept turbulent, mixed, and at a constant spore concentration.

At the time of animal exposure, the aerosol from the mixing chamber was drawn through a 16-inch length of 2-inch diameter tubing into a mask fitted tightly to the face of the chimpanzee by a pneumatic gasket. The aerosol flowed over the face of the animal at a rate of 8.7 liters per minute. The chimpanzee, anesthetized with Nembutal,® inhaled the cloud for 5 minutes. A critical-orifice, liquid impinger with a flow rate of 2.5 liters per minute was employed to determine the viable spore concentration of the aerosol. This impinger was situated at a point in the mask directly over the nostrils of the animal and was operated continuously during the entire exposure period. Since the sample was drawn from a cloud at exactly the same level as the chimpanzee's face, it represented the number of viable spores available for inhalation per unit volume (liter) of aerosol.

After exposure, the collecting fluid (tryptase saline) of the impinger was suitably diluted and plated on Wilson's peptone agar. By knowing the volume of aerosol sampled and by determining from plate counts the concentration of viable spores in the collecting fluid, it was possible to calculate the number of viable spores per liter of aerosol. Prior to exposure, the volume of air inhaled by the animal was estimated by a

direct measurement with a dry test meter. Also, as a check on the direct measurement of inhaled volume, an additional estimate was made during the actual exposure by measuring the amplitude recorded on a Sanborn recorder connected to a strain gauge which had been calibrated with a dry test meter immediately prior to the experiment. The strain gauge was taped to the sternum of the chimpanzee. In this way a reasonably reliable estimate of the minute-volume was achieved, and the strain gauge measurements could be used as a continuous check of the respiratory volume while the animal was in the exposure apparatus and direct measurements were impossible. The total number of viable spores inhaled by the chimpanzee was calculated by multiplying the number of viable spores in a liter of aerosol by the minute-volume (liters) and the duration of exposure (minutes).

Direct microscopic measurements were made on aerosol particles collected by means of a millipore filter and on glass slides in a settlement chamber. The number median diameter of these particles collected at the face mask was about 1.3 μ and the volume median diameter, 8.5 μ.

RESULTS

CLINICAL OBSERVATIONS

The pertinent data are summarized in Table I.

The first two animals (John and Melvin) exhibited no positive physical disorder after their initial exposure and survived despite the fact that organisms were demonstrated in the blood of one on the second through the tenth days and of the other from the third through the eleventh days. The animals maintained their appetites and their vigorous protestations to physical examination in unabated manner. Although a bacteremia was apparent in each, it was of low grade and exhibited no progression. The temperature varied little from normal (100° to 101° F.).

In the second pair of animals exposed (Bill and Grove), blood cultures were positive for *B. anthracis* on the second and third days, and the bacteremia progressed rapidly as determined by plate counts. No abnormalities of behavior and no abnormalities in physical examination were apparent until the morning of the day of death. At that time, both animals exhibited marked tenderness on palpation in the region of the spleen, an observation which represented the first physical sign of illness in either. They were observed at 4:00 p.m., and both seemed to be normal to casual inspection at that time. When next checked at 8:00 p.m., one (Bill) was dead, and 2 hours later the other (Grove) was noted to be in some distress. A careful physical examination was performed. Temperature was subnormal (97.8° F.); the animal appeared

TABLE I

Colonies of B. anthracis in the Blood, and Mortality Rate in Challenged Mice on Successive Days after Exposure

	First exposure				Second exposure	
	Melvin	John	Grove	Bill	Melvin	John
Dry test meter dose	32,800	34,350	39,700	66,500	90,300	112,000
Aerosol, median particle diameter (μ)	1.4	1.4	1.2	1.05	1.4	1.4
Fate of chimpanzee	Survived	Survived	Died	Died	Survived	Died
Days after exposure:						
First						
Colonies*	0	0	—	0	0	0
Mort. in mice†	—‡	—	0/5	1/5	0/5	0/5
Second						
Colonies	3	—	3	3	0	0
Mort. in mice	—	—	1/5	1/5	0/5	0/5
Third						
Colonies	30	18	43	70	0	0
Mort. in mice	—	—	5/5	2/5	0/5	0/5
Fourth						
Colonies	18	13	380	506	23	68
Mort. in mice	—	—	5/5	5/5	0/5	4/5
Fifth						
Colonies	—	12	810	660	40	1,630
Mort. in mice	—	—	5/5	5/5	1/5	5/5
Sixth						
Colonies	0	0	13,000 117,000§	90,300	0	13,500
Mort. in mice	4/5	5/5	5/5	5/5	0/5	5/5
Seventh						
Colonies	0	13			0	2.89×10^5
Mort. in mice	—	—			0/5	5/5
Eighth						
Colonies	6	10			0	4.3×10^8 (at necropsy)
Mort. in mice	5/5	5/5			0/5	5/5
Ninth						
Colonies	—	—				
Mort. in mice	—	—				
Tenth						
Colonies	0	0				
Mort. in mice	3/5	3/5				
Eleventh						
Colonies	0	0				
Mort. in mice	—‖	2/5‖				

* Colonies: Number of colonies per ml. of blood.
† Mort. in mice: Mortality rate in challenged mice.
‡ Minus sign indicates test not done.
§ One and a half hours before death.
‖ Negative on subsequent tests.

lethargic and was hard to arouse. His lips and mucous membranes were cyanotic, the pulse was rapid and weak, and respirations were shallow and labored. Tubular breath sounds were apparent on auscultation, and percussion of the chest yielded dullness. He expired 1½ hours later.

The 2 animals which survived initial exposure were rechallenged with a larger dose approximately 6 weeks after the blood cultures became negative. Organisms were demonstrable in the blood of Melvin on the fourth and fifth post-exposure days, but he exhibited no symptoms and thereafter maintained a negative blood culture and had an uneventful recovery. John also developed a positive blood culture on the fourth post-exposure day, and at this time exhibited splenic tenderness. His blood, however, showed a rapidly increasing bacteremia, and he died early on the eighth day after re-exposure. Examination approximately 4 hours before death revealed a temperature of 97° F. and an enlarged spleen, although the overall clinical impression was one of marked improvement.

NECROPSY EXAMINATION

Gross Observations

Bill. Each pleural cavity contained approximately 500 ml. of straw-colored, slightly turbid fluid. The pericardial and peritoneal cavities contained a slight excess of similar fluid. The mediastinal adipose and connective tissues were incorporated into a translucent, edematous mass of gelatinous consistency. The parietal pleura, particularly on the right side, had a similar appearance. The visceral pleura over most of both lungs was thickened with edema fluid. The lungs were mottled pink and dark red in color. When the pulmonary substance was cut, frothy fluid emerged from the pink area, and the dark red regions were hemorrhagic and airless. The trachea and major bronchi contained frothy pink fluid, and the mucosa contained small bright red hemorrhages but no erosions or ulcerations. The lymph nodes on the posterior aspect of the tracheal bifurcation were greatly enlarged and hemorrhagic, with complete loss of grossly recognizable architecture (Fig. 1). This mass of nodes was connected to a smaller, less hemorrhagic group adjacent to the ascending limb of the aortic arch by means of a dark red, beaded lymphatic vessel (Fig. 2). There was a similar enlarged hemorrhagic node adjacent to the thymus, and comparable single nodes were found in the pancreatic chain, as well as in a group draining a dilated loop of duodenum covered by congested but not hemorrhagic serosa. The spleen was enlarged and soft, and its cut surface appeared uniformly dark red in color. The liver was markedly congested. The

meningeal vessels were congested, and the brain was edematous, but neither hemorrhage nor exudate were noted. The heart, pancreas, kidneys, adrenals, testis, thyroid, pituitary and urinary bladder showed no gross lesions. The colon at the hepatic flexure exhibited a large abscess attributable to a parasitic worm. Except for this and the dilated loop of duodenum mentioned previously, there were no gross lesions of the gastrointestinal tract.

Grove. The gross observations were qualitatively similar to those described for Bill.

John. Although this animal exhibited the large soft spleen and gelatinous mediastinal edema seen in the first two, he did not have pleural effusion, hemorrhagic tracheobronchial lymph nodes or massive pulmonary edema and hemorrhage. Edema of the tracheal mucosa was prominent, but no hemorrhage was evident. The only extensive hemorrhages were found in the cortex and medulla of both adrenal glands.

Microscopic Observations

The first two animals which succumbed (Bill and Grove) presented essentially similar histologic alterations, differing only in degree of involvement. The extent and severity substantiated the impressions derived from the gross observations. Since Bill presented the more accentuated changes, the lesions in him will be described. The pleura was thickened as the result of separation of the connective tissues by edema fluid devoid of vegetative anthrax bacilli or cellular exudate (Fig. 3). The pulmonary vessels were markedly distended with blood and a myriad of anthrax bacilli (Fig. 4). The alveoli contained edema fluid in some regions and were filled with blood and bacilli in others. Scanty cellular exudate and few bacilli were found in the alveoli free of hemorrhage. The bronchioles and small bronchi contained variable quantities of blood, mucus, and organisms. The enlarged, congested lymph nodes at the tracheal bifurcation and adjacent to the thymus, pancreas, and duodenum, exhibited almost complete replacement of recognizable architecture by massive hemorrhage (Fig. 5) and accumulations of neutrophils. Elsewhere, the nodes showed marked dilatation of sinusoids, a dearth of lymphocytes, and small, follicular, germinal centers. Some of the latter were necrotic. The spleen retained recognizable architecture, but germinal centers were surrounded by zones of hemorrhage and many organisms were distributed throughout (Fig. 6). The liver was not remarkable except for extreme acute passive congestion and numerous bacilli in the sinusoids. The blood vessels of the kidneys were congested and contained many bacilli. Some of the glomerular spaces and many of the proximal convoluted tubules con-

tained granular, pink-staining, noncellular substance. The proximal tubules were slightly dilated, and the tubular epithelium was somewhat swollen. The intestine of the worm found in the colonic abscess contained organisms morphologically similar to *B. anthracis*. The dilated loop of duodenum mentioned in the gross description was the seat of serosal congestion, but no other pathologic alterations were noted. The gelatinous swelling of fat and connective tissue observed in the mediastinum of all 3 animals and about the kidneys and adrenals of Grove and John revealed edema but little cellular exudation or hemorrhage. The other tissues examined (heart, pancreas, adrenal, testis, urinary bladder, thyroid, tonsil, pituitary, and brain) contained anthrax bacilli in blood vessels but no lesions.

The animal dying following a second exposure (John) exhibited few histologic lesions. The lungs showed minimal edema and focal congestion. The tracheal and bronchial mucosa were edematous, but no cellular exudate was apparent. The spleen resembled the spleens of the other two animals. Some of the lymph nodes contained dilated sinusoids and exhibited a paucity of lymphocytes, but the architecture was maintained, and there was no massive hemorrhage. The mediastinal and perirenal tissues were identical in histologic appearance to their counterparts in the other animals. There was hemorrhage in the cortex and medulla of both adrenal glands. No other significant microscopic features were observed.

Discussion

These experiments indicate that the chimpanzee is susceptible to *B. anthracis* when it is administered in aerosols of small particle size via the respiratory route. At a calculated inhaled dose of 32,000 to 66,500 spores, bacteremia appeared on the second or third day following exposure, and 2 of the 4 animals succumbed on the sixth day. It is noteworthy that in the presence of a bacteremia of considerable magnitude, the 2 animals that died exhibited no objective findings, and until a few hours before death the only evidence of illness was tenderness in the splenic region. The rapidity with which death ensued in apparently healthy animals was dramatic.

In the absence of therapy, the development of positive blood cultures following infection with *B. anthracis* by any route has been considered a grave prognostic sign. It is of interest that the 2 survivors developed a bacteremia on the second or third day following exposure. The bacteremia continued at a low level for 8 days. It is of further interest that one of these animals, on re-exposure to a larger inoculum, developed a delayed bacteremia on the fourth day which persisted for only 2 days and was followed by complete recovery of the animal. It

would appear that the presence of a bacteremia was not necessarily significant as to outcome. A rapid progression in numbers of organisms in the blood, on the other hand, is ominous.

The pathologic observations at necropsy resembled those previously described in the guinea pig, mouse, and monkey. These were characterized by widespread edema and hemorrhage, most prominent in the spleen, lungs, and lymph nodes, and by the absence of a pronounced cellular inflammatory exudate in the lungs. This confirms the impressions of others that *B. anthracis* introduced by the respiratory route into susceptible animals results in a fulminating septicemia rather than a primary pulmonary infection. No tracheal or bronchial lesions comparable to those described in "woolsorters' disease" in man were observed.

The fact that the intraperitoneal injection of blood in mice was followed by death of the animal and cultural recovery of the organism, whereas the inoculation of conventional cultures failed to show growth, indicates that mouse injection is a valuable ancillary diagnostic technique.

It is tempting to speculate on the development of a degree of resistance in the previously exposed animal. This may have accounted for the delay in developing bacteremia on re-exposure, the greater number of organisms in the blood before death, and the less severe edema and hemorrhage observed at necropsy. Although these features were striking, the experience with so few animals is far too limited to permit conclusions.

Summary

Four chimpanzees were exposed to *B. anthracis* via the respiratory route, utilizing small particulate aerosols. Each developed bacteremia within 2 to 3 days of exposure. Two animals survived and 2 died. On re-exposure to larger doses, both survivors of the first experiment developed delayed bacteremia, and one died. The clinical course of the disease was not discernible except by the occurrence of bacteremia.

Necropsy observations were comparable to those described for other relatively susceptible laboratory animals. They indicated that *B. anthracis* administered by the respiratory route caused a fulminating septicemia rather than a localized pulmonary disease.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Posterior view of chimpanzee's (Bill) lungs, illustrating pulmonary hemorrhages and large hemorrhagic mediastinal lymph nodes. (U.S. Army photograph.)

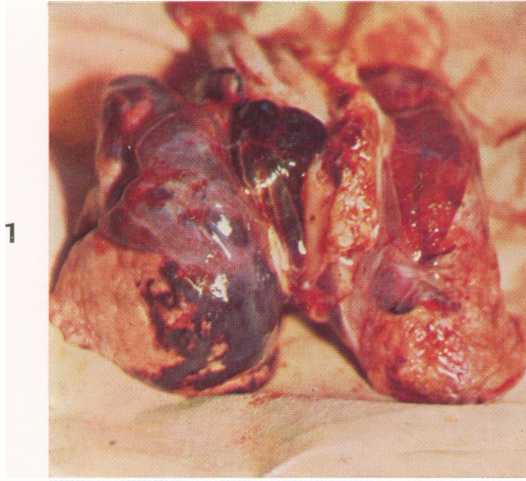
FIG. 2. Posterior oblique view of chimpanzee's (Bill) lungs, demonstrating a markedly dilated beaded lymphatic vessel connecting hemorrhagic nodes on the right with smaller ones on the left. (U.S. Army photograph.)

FIG. 3. Section through chimpanzee's (Bill) lung, illustrating the edematous pleura and hemorrhage into alveolar spaces. Giemsa stain. × 42.5.

FIG. 4. An area of lung (Bill) devoid of hemorrhage, showing partial collapse and congested blood vessels containing many *B. anthracis* organisms. Giemsa stain. × 460.

FIG. 5. Lymph node with architecture essentially obliterated by hemorrhage. The connective tissue about the adjacent thymus is markedly edematous. Giemsa stain. × 6.5.

FIG. 6. Lymphoid nodule of the spleen with necrosis of cells in germinal center and surrounding hemorrhage containing many *B. anthracis*. Giemsa stain. × 290.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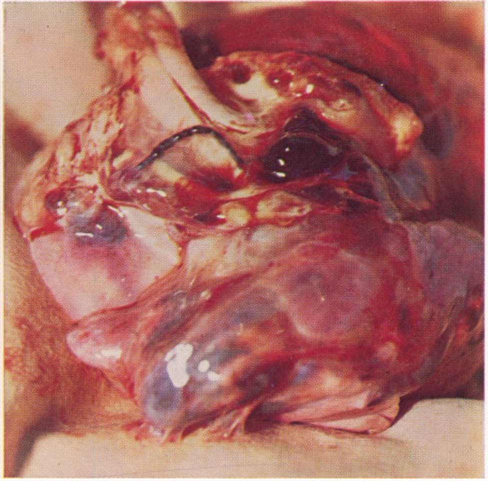
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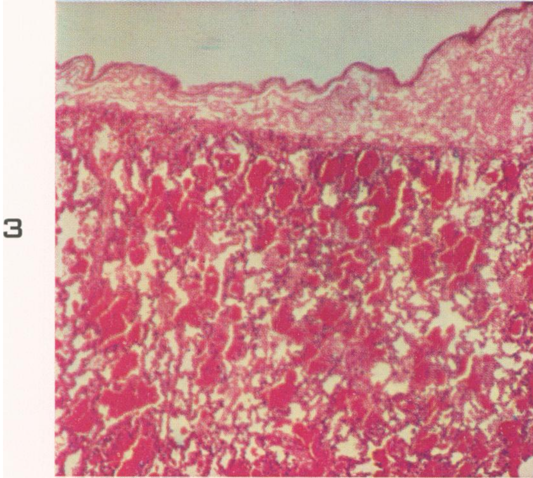
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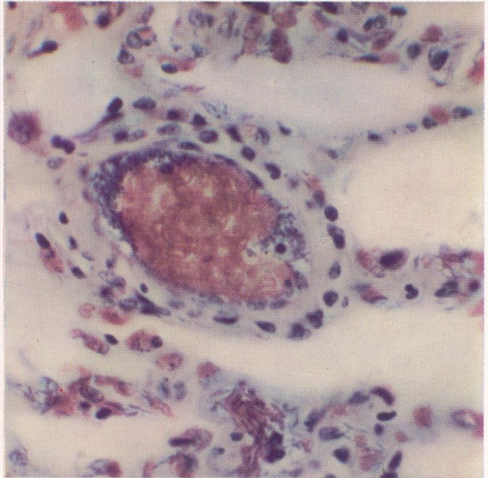
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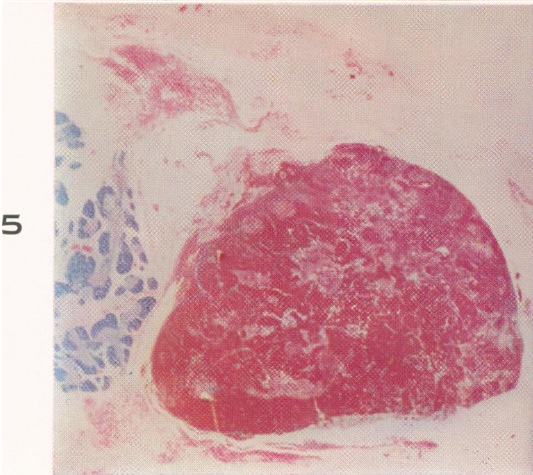
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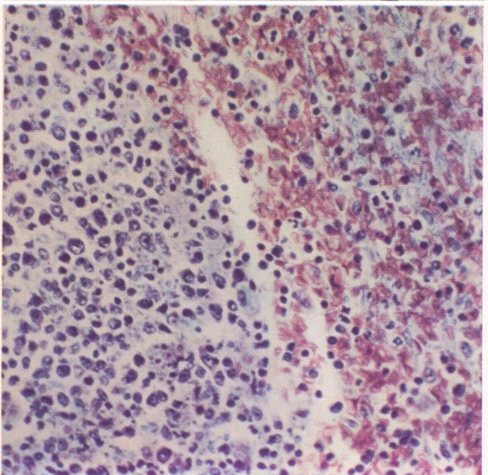
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