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STUDIES ON INHALATION ANTHRAX:
III. MORPHOLOGIC STUDIES

Frederic G. Dalldorf
Arnold Kaufmann

APRIL 1966

UNITED STATES ARMY
BIOLOGICAL CENTER
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TECHNICAL MANUSCRIPT 290

STUDIES ON INHALATION ANTHRAX:
III. MORPHOLOGIC STUDIES

Frederic G. Dalldorf
Arnold Kaufmann

Pathology Department
MEDICAL SCIENCES LABORATORY

Project 1C014501B71A
April 1966
In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

FOREWORD

Arnold Kaufmann is associated with the U.S. Public Health Service, Communicable Disease Center, Atlanta, Georgia.
A morphologic study was made of tissues from 23 cynomolgus monkeys that contracted inhalation anthrax following prolonged exposure to dust from goat hair known to be contaminated with anthrax spores. Twenty monkeys died of anthrax, in one monkey anthrax was considered a contributing cause of death, and in two monkeys sacrificed at the end of the experiment early anthrax infection was found incidentally. The morphologic features of the infection in these 23 monkeys support the concept that, in inhalation anthrax, spores are carried to the hilar lymph nodes of the lung where they germinate to produce a local primary lesion and septicemia. Electron microscopic studies of the lungs of two monkeys revealed that many pulmonary capillaries were occluded with fibrin bacilli and leukocytes; these indicate that anthrax causes death in this species by producing widespread pulmonary capillary thrombosis.
I. INTRODUCTION

Other papers in this series describe the experimental techniques and the epidemiologic and bacteriologic results of a long-term exposure of monkeys to air laden with dust from goat hair known to contain anthrax spores.* This report presents a morphologic study of tissues from the infected monkeys. Light and electron microscopic findings will be presented that bear on the early pathogenesis of respiratory anthrax and on the cause of death in anthrax septicemia.

Some investigators are of the opinion that infection in inhalation anthrax begins with a primary lesion located in the mucosa of the respiratory tract. Others believe that the inhaled spores are carried directly into the pulmonary lymphatics and that infection is first established in the regional hilar lymph nodes draining the lungs. The first theory is based largely on early accounts of autopsy material from humans dying of respiratory anthrax, in which morphologists described what they considered to be primary anthrax lesions in various parts of the respiratory tract.† Further evidence for the concept of an initial respiratory infection in inhalation anthrax can be found in the experimental work of Berdjis.‡

In that experiment rhesus monkeys were exposed to aerosols containing large numbers of anthrax spores and a morphologic study was made of the lungs of monkeys sacrificed at varying intervals during the development of the disease. His study showed that the bronchiectatic lesions caused by lung mites, so frequently encountered in this species,§ were infected early in the disease with large numbers of anthrax bacilli. Other investigators are of the opinion that in inhalation anthrax the respiratory tract is not involved initially but that spores are carried to the hilar lymph nodes, where they germinate to produce a primary lymphatic anthrax lesion.¶ This concept is based on morphologic studies of patients dying of respiratory anthrax and on experimental studies of chimpanzees and guinea pigs exposed to anthrax spores via the respiratory route.

Over the years there has been considerable study of the cause of death in anthrax septicemia. The various theories have been proposed but none has received complete acceptance. Recent clinical and electron microscopic studies of rats, guinea pigs, and rabbits dying of anthrax have shown that the respiratory failure in the terminal phase of anthrax septicemia is caused by widespread pulmonary capillary thrombosis.** Similar changes

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were also found in the lungs of rats injected intravenously with anthrax toxin. An electron microscopic examination of the lungs of two infected monkeys in this study was carried out in an effort to see if similar thrombosed pulmonary capillaries could be demonstrated.

II. MATERIALS AND METHODS

Ninety-one cynomolgus monkeys were exposed to goat hair dust containing anthrax spores for varying periods of time ranging from 3 to 45 days. The monkeys were housed, two or three to a cage, in a trailer beside the sorting room of the mill processing contaminated goat hair. Dust-laden air was piped from the work area in the mill into the exposure chamber and then back into the factory exhaust system. The all-glass impingers with the British preimpingers were used to sample for particles smaller than 5 microns in diameter, and periodic samples were taken from the exposure chamber to estimate the dose of spores received.* The monkeys were checked at least three times a day for illness or death. When dead monkeys were found, necropsies were performed in the laboratory portion of the trailer. At the conclusion of each of the three experiments the surviving monkeys were sacrificed with an intravenous injection of Nembutal and were necropsied. Samples of blood, spleen, and hilar lymph nodes were cultured for the presence of *B. anthracis*. The larynx, trachea, lungs, and hilar lymph nodes were removed in toto, perfused through the trachea with 10% formalin and placed in additional formalin. The entire brain was removed and placed in formalin, together with pieces of the spleen, liver, kidneys, intestines, adrenal glands, abdominal lymph nodes, and any skin lesions. All the formalin-fixed tissues were sent to Fort Detrick for final processing and morphologic evaluation. After gross examination, routine sections were prepared from all organs received. The entire respiratory tract was examined carefully for the presence of any mucosal lesions. Most of the larynx and the entire trachea were submitted for sectioning. The numerous transverse sections of the trachea included all adjacent cervical and mediastinal lymph nodes. Longitudinal sections of the main bronchi from each lobe of the lungs were also prepared. The cut tissue blocks were processed in the usual manner and sections were stained with hematoxylin and eosin and by the Giemsa method. In many cases Brown-Brenn stains for gram-positive microorganisms were also obtained.

Blocks of tissue from the lungs of three monkeys were also prepared for electron microscopy. One-mm pieces of tissue were cut from the formalin-fixed, perfused lungs and placed in Millonig's buffer containing 2% osmium tetroxide for 30 minutes at room temperature. The blocks were washed, dehydrated, and embedded in Epon 812. Ultra-thin sections were cut on a Porter Blum ultramicrotome with a diamond knife, stained with uranyl acetate and examined with a RCA EMU-3C electron microscope.

III. RESULTS

Twenty-three of the 91 monkeys exposed to the dust from the contaminated goat hair had morphologic evidence of anthrax infection. Twenty monkeys died of anthrax, in another monkey anthrax was considered a contributing cause of death (No. 40), and in two monkeys sacrificed at the end of the study there was evidence of early anthrax infection of the mediastinal lymph nodes (No. 50 and 51). No attempt was made to record the clinical features of the illness in the sick monkeys because of the sporadic time of infection, the relative lack of signs of illness, and the brevity of the terminal symptomatic phase of the disease. In most cases the first evidence of anthrax was the discovery of a dead monkey. The morphologic features of respiratory anthrax observed here were similar to those previously described by others in humans and experimental animals. A summary of the gross and microscopic features of the disease is presented in Table 1.

The most common gross findings in the infected monkeys were edema of mediastinal tissues, pleural effusion, and hemorrhagic necrosis of the mediastinal lymph nodes. There were varying amounts of fluid in the pleural cavities and the lungs of some of the monkeys were moderately edematous. In two monkeys, one or two lobes of the lungs were hemorrhagic and consolidated. The spleens were usually enlarged and friable. The leptomeninges covering the brain of some of the monkeys dying of anthrax were hemorrhagic, and in one monkey there was a large subarachnoid hemorrhage measuring 2 cm in diameter. There were no grossly discernible lesions in the mucosa of the trachea or major bronchi. There was no gross evidence of primary cutaneous or gastrointestinal anthrax.

Histologic examination of the tissues showed that the infection was largely limited to the circulatory and lymphatic systems. At death there was also widespread dissemination of numerous bacilli throughout all the blood vessels and the sinusoids of the liver, spleen, and mediastinal lymph nodes. There was very little leukocytic reaction to these bacilli, and the only apparent morphologic response to the infection was edema, hemorrhage, and necrosis.
<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Cumulative Days after Exposure Began</th>
<th>Cultures for B. anthracis</th>
<th>Mediastinal Edema</th>
<th>Mediastinal Pleural Effusion</th>
<th>Mediastinal Lymph Nodes*</th>
<th>Cervical Lymph Nodes*</th>
<th>Splenic Necrosis*</th>
<th>Meningitis</th>
<th>Diagnosis</th>
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<td>-</td>
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<td>+</td>
<td>-</td>
<td>Staphylococcal septicemia, cachexia and anthrax</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>0</td>
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<td>0</td>
<td>Anthrax in one mediastinal lymph node, sacrificed.</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Anthrax</td>
</tr>
</tbody>
</table>

a. Key for lymph node morphology
  + = anthrax bacilli with follicular necrosis
  ++ = bacilli with necrosis and edema
  +++ = bacilli with marked necrosis, edema and hemorrhage
  0 = no tissue submitted

b. Key for splenic morphology
  + = sinusoids engorged with neutrophiles and bacilli
  ++ = neutrophiles and bacilli in sinusoids with central necrosis of lymph follicles
  +++ = necrosis of red and white pulp with hemorrhage
  0 = no tissue submitted
The mediastinal lymph nodes were always heavily infected with anthrax bacilli. The first observable changes consisted of invasion of the sinusoids by bacilli and central necrosis of the lymph follicles. As the infection progressed, bacilli were more numerous and there was increasing evidence of necrosis of other parts of the lymph nodes, with pronounced edema and hemorrhage. Finally, the lymph nodes were greatly enlarged and hemorrhagic. The most advanced necrotic lesions found always involved the mediastinal lymph nodes. In some monkeys the cervical lymph nodes adjacent to the trachea were also infected but always to a lesser degree than the mediastinal lymph nodes. The spleen was frequently infected with anthrax bacilli. The first observable changes were the accumulation of large numbers of neutrophils and bacilli in the splenic sinusoids. As the number of bacilli increased, central necrosis of the lymph follicles became prominent; finally, there was widespread necrosis of the entire organ with hemorrhage.

In most monkeys the lungs were not markedly changed and showed no evidence of localized infection. There was usually some minimal diffuse pulmonary edema and the pulmonary capillaries were always dilated and filled with bacilli, but bacilli were rarely found in the alveoli. In two monkeys (No. 69 and 101) there was marked edema and hemorrhage of one or two lobes of the lung with many anthrax bacilli in the alveolar spaces. A third monkey (No. 71) had a typical lobular pneumonia; the edematous alveoli adjacent to the consolidated areas of pulmonary parenchyma contained many typical anthrax bacilli. In all three cases there was infection and necrosis of the mediastinal lymph nodes and anthrax septicemia. The pulmonary lesions in these three monkeys were difficult to interpret pathogenically, but it was felt that they probably represented secondary infection late in the course of the disease rather than primary anthrax pneumonia. None of the histologic sections of the trachea and bronchi contained anything that could be interpreted as a primary anthrax lesion. Most of the monkeys had pulmonary acarasis with typical lung mite lesions, but none of these lesions was infected with anthrax bacilli.

Nine of the 20 monkeys dying of anthrax had extensive edema and hemorrhage of the leptomeninges; the large subarachnoid hemorrhages were occasionally found in the adrenal glands and ovaries. The livers of some monkeys had central lobular necrosis of the parenchymal cells. The remaining viable cells often had prominent cytoplasmic vacuoles. The renal glomeruli were filled with bacilli but the sections of the kidneys were otherwise unremarkable.

Two monkeys that had been sacrificed at the end of the 50-day exposure period had an early anthrax infection involving only the mediastinal lymph nodes (No. 50 and 51). The tissues of these two monkeys were examined extensively to try to find a primary anthrax lesion in the respiratory tract. All of the tissues from the trachea and both lungs were reduced to blocks and Brown-Brenn stains were prepared on all sections to facilitate
the identification of the bacilli. Many lung mite lesions were found and studied. Nowhere could we find any evidence of infection except in the mediastinal lymph nodes draining the lungs.

The lungs of two monkeys dying of anthrax (No. 86 and 92) and one uninfected monkey that was sacrificed at the end of the exposure period were found to be free of autolysis and were selected for electron microscopic study. A comparison of the light microscopic sections of lungs of the normal and infected monkeys showed that the pulmonary capillaries in monkeys dead of anthrax were dilated and filled with bacilli and leukocytes. The electron micrographs of the pulmonary capillaries of the uninfected monkey were essentially normal (Fig. 1). Each capillary was lined by a thin cytoplasmic extension of an endothelial cell (end) and was filled with pale granular plasma. The alveoli (alv) were lined by thin cytoplasmic extensions of epithelial cells (epi). The walls of the capillaries were supported by a thick basement membrane (bm), which appeared in this preparation as a homogenous electron-dense band between the endothelial and epithelial cell membranes. The alveolar septa in the monkey lungs contained many thick bundles of collagen (col).

The electron micrographs of the pulmonary capillaries of the infected monkeys were markedly altered. In some capillaries (Fig. 2) the lumen was filled with homogenous granular fibrin (f) containing many bacilli and occasional leukocytes and erythrocytes. The capillary wall and the endothelial cell membranes were intact. In other capillaries (Fig. 3), the lumen contained granular fibrin (f), dense strands of fibrillar fibrin, bacilli, and leukocytes (leu). The capillary walls were intact. Occasional endothelial cells had large clear areas in their cytoplasm (Fig. 2), but the significance of these was not apparent. The lumen of many pulmonary veins from infected monkeys contained clusters of fibrin, numerous bacilli, leukocytes, and erythrocytes. These masses of fibrin apparently did not occlude the lumen of these larger vessels.

IV. DISCUSSION

Our morphologic studies of the tissues of the monkeys in this experiment support the concept that the initial focus of infection in respiratory anthrax occurs in the lymph nodes draining the lungs and not in the mucosa of the respiratory tract. This conclusion is based on the fact that all the infected monkeys had advanced anthrax lesions of the lower mediastinal lymph nodes and that no primary anthrax lesions were found in the trachea or bronchi of these monkeys despite extensive study. In particular, two monkeys that were sacrificed at the end of the exposure period were found to have early anthrax infections limited entirely to the mediastinal lymph node, despite complete histologic examination of the pulmonary parenchyma.
The only possible exceptions to this theory of initial lymphatic infection occurred in two monkeys that had large areas of consolidated, hemorrhagic pulmonary parenchyma containing many anthrax bacilli (No. 61 and 101) and in one monkey that had a typical lobular bacterial pneumonia surrounded by alveoli containing edema fluid and anthrax bacilli (No. 66). The pulmonary lesions of these three monkeys were associated with advanced anthrax septicemia and infection of the mediastinal lymph nodes and were interpreted as representing secondary infections of edematous lung tissue.

We were unable to find any lung mite lesions infected with anthrax as described by Seldjes in his monkeys exposed to laboratory aerosols containing spores. This discrepancy between his observations and ours is probably a result of the marked difference in the dose of spores given and the duration of the exposure. Our monkeys were exposed to low concentrations of spores for long periods of time; in the studies using laboratory aerosols, animals were exposed to large numbers of spores for a short period of time. In this latter case, the pulmonary macrophages and lymphatic drainage of the lungs may be overwhelmed and spores may germinate in areas other than the regional lymph nodes.

Earlier electron microscopic studies in our laboratory showed that intravenously injected anthrax toxin caused the rapid death of Fischer 344 rats by producing diffuse pulmonary edema that was associated with an elevation of the thin cytoplasmic processes of the endothelial cells lining the pulmonary capillaries from the underlying basement membranes. The increased capillary permeability was followed by widespread pulmonary capillary thrombosis and was associated with signs of respiratory failure. In later studies of rats, guinea pigs, and rabbits infected with anthrax, it was found that pulmonary edema was not a prominent feature of the terminal illness and that the endothelial cell membranes were unaltered. However, there was widespread pulmonary capillary thrombosis associated with signs of respiratory failure.* The electron microscopic studies of the pulmonary capillaries in the infected rats, guinea pigs, and rabbits showed many pulmonary capillary thrombi composed of platelets and fibrin. In the electron microscopic studies of the lungs of the two infected monkeys in this study the pulmonary capillaries were found to contain large clusters of fibrin, many bacilli, and leukocytes. These findings are interpreted as representing occluded pulmonary capillaries and indicate that anthrax septicemia following respiratory infection causes the death of cynomolgus monkeys by producing widespread pulmonary capillary thrombosis.

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Figure 1. Pulmonary Capillary from Normal Monkey. The capillary is lined by thin cytoplasmic extensions from two endothelial cells (end). The lumen is filled with coagulated plasma that appears as finely granular material. The alveoli (alv) are lined by a thin cytoplasmic extension of epithelial cells (epi), the nuclei of which are not included in this photograph. The wall of the capillary is supported by the basement membrane (bm), which appears in this preparation as a thick, homogenous, electron-dense layer between the endothelial and epithelial cell layers. Uranyl acetate, X 30,000.

Figure 2. Pulmonary Capillaries from Monkey No. 86. The capillaries are dilated and filled with granular fibrin (f) and bacilli that appear in cross sections as large dark round structures with outer dense membranes. The thin cytoplasmic membranes of the endothelial cells (end) lining the capillaries are intact. The cytoplasm of two endothelial cells in this photograph have large clear spaces (s), one of which contains a strand of dense fibrillar fibrin. Uranyl acetate, X 15,000.

Figure 3. Pulmonary Capillary from Monkey No. 92. The lumen of this capillary is filled with granular fibrin (f) together with many strands of dense fibrillar fibrin, bacilli and a portion of a leukocyte (leu). The wall of the capillary is unaltered. Uranyl acetate, X 24,000.
LITERATURE CITED


A morphologic study was made of tissues from 23 cynomolgus monkeys that contracted inhalation anthrax following prolonged exposure to dust from goat hair known to be contaminated with anthrax spores. Twenty monkeys died of anthrax, in one monkey anthrax was considered a contributing cause of death, and in two monkeys sacrificed at the end of the experiment early anthrax infection was found incidentally. The morphologic features of the infection in these 23 monkeys support the concept that, in inhalation anthrax, spores are carried to the hilar lymph nodes of the lung where they germinate to produce a local primary lesion and septicemia. Electron microscopic studies of the lungs of two monkeys revealed that many pulmonary capillaries were occluded with fibrin bacilli and leukocytes; these indicate that anthrax causes death in this species by producing widespread pulmonary capillary thrombosis.