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TECHNICAL MANUSCRIPT 416

PATHOGENESIS OF PLAGUE: II. AN EQUIVALENT OF THE GENERALIZED SHWARTZMAN REACTION IN THE MONKEY

Milton J. Finegold
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OCTOBER 1967

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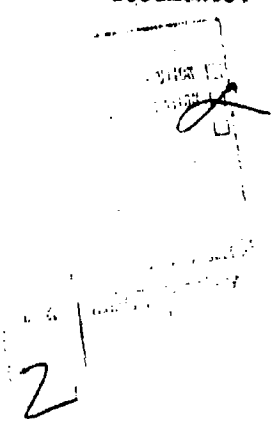

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John J. Petery

Richard F. Berendt

Pathology Division
MEDICAL SCIENCES LABORATORY
and
Experimental Aerobiology Division
AEROBIOLOGY AND EVALUATION LABORATORY

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October 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

Glomerular fibrin thrombi were observed in monkeys with both pneumonic and bubonic plague. Eighty per cent of animals in this study dying of pneumonic plague had glomerular thrombi. These findings are equivalent to those of the generalized Shwartzman reaction and suggest the possibility that endotoxin released from Pasteurella pestis during infection contributes to the pathogenesis of plague.

I. INTRODUCTION

Plague has been a deadly and terrifying disease for at least 2,200 years, yet little is known of the means whereby Pasteurella pestis causes tissue injury and death.[1] With the advent of antibiotic therapy, the infection has been successfully controlled wherever patients have had access to medical facilities. Occasional cases, however, have died of plague despite successful antibiotic sterilization of their blood and tissues.[2] Such observations have supported the concept of toxemia as an important mechanism of lethality.[1]

The nature of the toxicity or of the toxin has not been defined. Vaccines prepared from killed P. pestis have had toxic properties.[3] The murine toxin, whose pathophysiological and biochemical actions have been extensively studied,[4,5] is a protein that affects only mice and rats in doses compatible with an in vivo role. Within the last 16 years, several workers[6-8] have isolated lipopolysaccharides with various degrees of toxicity and lethality from P. pestis. To date, each of these preparations either has differed in some way from typical endotoxins of gram-negative bacteria or has not been fully tested for typical endotoxin activities.

One such test would be the production of the generalized Shwartzman reaction. Although other substances have been used, lipopolysaccharides from gram-negative bacteria given intravenously are the classical agents of the reaction.[9] They act by stimulating intravascular coagulation and production of circulating aggregates of fibrin. As the clots are cleared by the reticuloendothelial system, a temporary blockade of further clearance develops. A second appropriately timed injection of endotoxin results in massive intravascular coagulation with deposition of fibrin thrombi in capillaries throughout the body, particularly in renal glomeruli.[9] The consumption of clotting factors may lead to the apparent paradox of widespread capillary thrombosis and a hemorrhagic diathesis.[10]

Examples of endotoxemia and an equivalent of the generalized Shwartzman reaction in man include several infections with gram-negative bacilli, such as meningococcemia, infantile diarrhea caused by Escherichia coli, pyoderma gangraenosum, Pseudomonas septicemia, and cholera.[11] On the basis of our observations on monkeys with P. pestis infection and a review of the literature, we propose that plague be added to the list. In our preceding paper,[12] studies on blood coagulation during pneumonic plague are presented. The morphological features of the disease are described below.

II. MATERIALS AND METHODS

The isolation, maintenance, and cultural characteristics of the KIM-10 strain of *P. pestis* are described in the preceding paper,[12] as are the methods of aerosol generation, animal exposure and dosimetry.

Monkeys that died were necropsied within 8 hours of death; others were sacrificed with either chloroform vapor or intravenous injections of magnesium sulfate or pentobarbital. Complete gross examinations were performed, and sections of lungs, bronchial lymph nodes, spleen, liver, adrenal, and kidneys were fixed in 10% neutral formalin. Following routine paraffin embedding and sectioning at 5 to 7 microns, sections were stained with hematoxylin and eosin (H&E). Phosphotungstic acid - hematoxylin (PTAH) was used to demonstrate fibrin after mordanting with mercuric chloride.[13] For evaluation of glomerular structure, the alcian blue - periodic acid Schiff (PAS) reaction was most useful. *P. pestis* was easily seen with H&E, but its morphology was best studied with Giemsa's stain. In selected cases, formalin-fixed kidney was post-fixed with osmium tetroxide and embedded in Epon 812 for ultrathin sectioning and examination in the electron microscope. Saturated aqueous uranyl acetate was the stain.

III. RESULTS

A. PNEUMONIC PLAGUE

Thirty-eight rhesus monkeys were necropsied after exposure to aerosols containing the KIM-10 strain of *P. pestis*. The average inhaled dose was 30,000 organisms per animal. Twenty-five monkeys died from 4 to 7 days after exposure, and the other 13 animals were sacrificed when they appeared to be moribund on days 4 to 8. There was no consistent relationship between dose and time of survival, nor was there a consistent pattern of increasing pathological change with increasing dose or duration of survival. The pathological responses of lungs, bronchial lymph nodes, spleen, liver, and adrenal were similar in all animals. The only difference between groups of animals occurred in the kidneys. Fibrin thrombi (Figs. 1-4) in glomerular capillaries were in 80% of the dying monkeys, but 11 of 13 sacrificed animals receiving similar doses and living as long were negative (Table 1).

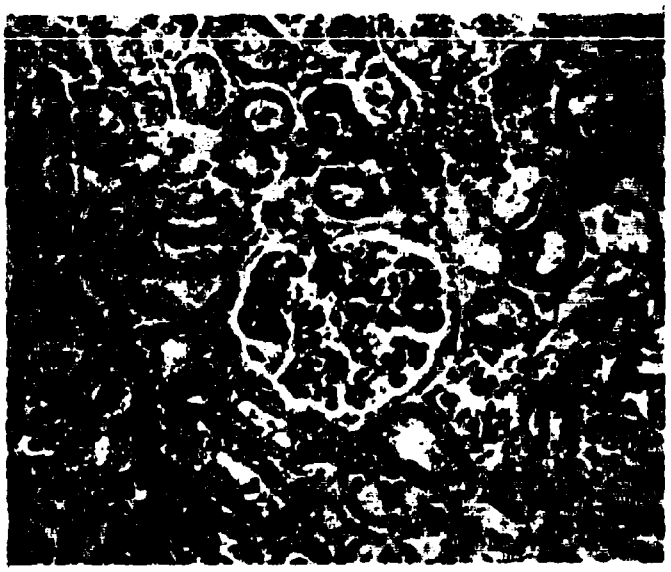

Figure 1. Glomerular Fibrin Thrombi. The afferent arteriole and several capillery loops are occluded by dense, homogeneous, purple-stained material. Phosphotungstic acid hematoxylin. 130X.

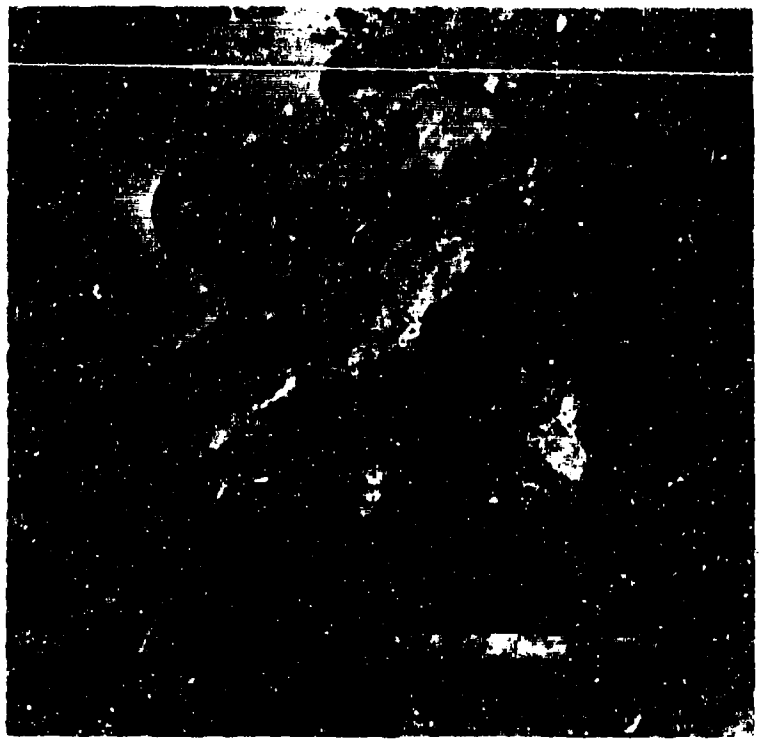

Figure 2. Glomerular Fibrin Thrombus. An electron photomicrograph of a capillary reveals masses of fibrillar material (F) in dense packets within the lumen. Also present are portions of erythrocytes (e) and cell debris. The urinary space (U) and epithelial cell with foot processes (arrows) are also seen. The state of preservation is only fair because the tissue was prepared for electron microscopy from large blocks fixed for routine light microscopy in 10% phosphate-buffered neutral formalin. Uranyl acetate. 5,400X. Insert shows a higher magnification of the fibrillar material in the capillary. The fibers are cross-striated with a regular periodicity of approximately 187 A. Uranyl acetate. 41,000X.

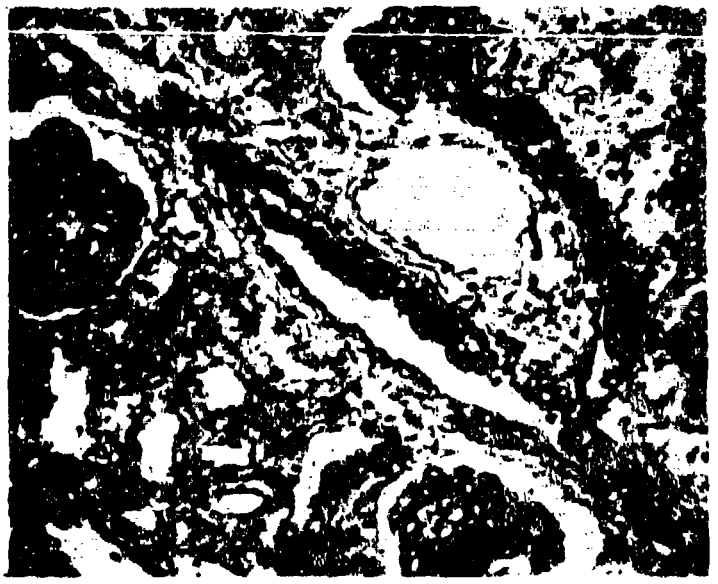

Figure 3. Mucopolysaccharides in the Glomerular Thrombi. Section was incubated in 0.05% trypsin solution for 3 hours prior to staining with aldehyde fuchsin for 10 minutes. The thrombi are intensely stained, as is the elastic tissue of the arterioles. Aldehyde fuchsin. 130X.

Figure 4. Control Section for Figure 3. Section from the same kidney was incubated for 3 hours in a phosphate buffer solution lacking trypsin prior to aldehyde fuchsin staining. Now only the elastic fibers of the vessels stain and the thrombi are unstained. Trypsin digestion of the protein component of the thrombus unmasks the sulfated mucopolysaccharide. Aldehyde fuchsin. 130X.

TABLE 1. GLOMERULAR FIBRIN THROMBI IN PNEUMONIC PLAGUE

	No. of Monkeys	Thrombi	
		Present	Absent
Died[a/]	25	20	5
Sacrificed[b/]	13	2	11

a. Deaths occurred from 4 to 7 days after exposure.
b. Sacrifices were performed on days 4 to 8, when animals appeared moribund.

The renal thrombi were located primarily in glomerular capillaries and, in cases with extensive deposits, some afferent arterioles and intertubular and medullary capillaries were also occluded. Cortical necrosis was seen in some of the kidneys with extensive thrombosis. With fewer thrombi, the parenchymal changes were limited to focal glomerular hypercellularity and vacuolar degeneration of proximal tubular epithelium. Pale eosinophilic fluid was occasionally present in Bowman's space and the proximal tubular lumen. Intratubular hemorrhages were rare. The fibrin was readily seen in H&E-stained sections as homogeneous hyaline material. In Giemsa stains, some thrombi contained bacilli, but most were devoid of organisms. Bacilli were also seen in patent vessels. PTAH stain and electron microscopy[14] confirmed the presence of fibrin. There were focal differences in the PTAH staining of the thrombi. Where the occlusions were complete and the deposits extensive, the material was densely and uniformly purple. Where there was only partial occlusion by smaller amounts of thrombus or at the periphery of the larger masses, thread-like strands of orange staining were present, as described for fibrinoid.[15] The thrombi were PAS-positive. The alcian blue - PAS combination showed that, in the absence of necrosis, the glomerular basement membrane was unaltered. Following the technique of Horn and Spicer,[16] trypsin digestion of kidney sections permitted the demonstration of sulfated mucopolysaccharide in the thrombi with an aldehyde fuchsin stain.

The pathological findings in the other organs were similar to classical descriptions of pneumonic plague.[3,17] They will be briefly summarized.

B. LUNGS

All stages of pneumonia were seen within the lungs of all animals. Generally, a focus of lobular consolidation was found in the peripheral portion of one lobe, most often a lower lobe. It consisted of an area 0.5 to 3 cm in diameter of firm, purple tissue between the central

bronchovascular trunk and the pleura. The pleural surface overlying the lesion was dull and dry. On section, there was hepatization, occasionally with central liquefaction and hemorrhage. The surrounding tissue was edematous, often for a greater area than the consolidated portion. Edema fluid was sometimes present in a second lobe, and, rarely, a second focus of consolidation was found in the same or another lobe. At most, no more than 40% of pulmonary tissue was involved. Microscopically, advanced lesions consisted of a central abscess. Hemorrhages were common and inflamed vessels frequent in such areas. Lesser reactions consisted of alveolar consolidation by polymorphonuclear leukocytes with intact septa. At the margins of the pneumonia, alveoli were filled with edema fluid teeming with bacilli having the bipolar staining typical of P. pestis. Similar areas of alveolar edema without leukocytic infiltration were common in secondary lesions in less involved lobes, presumably arising from intrapulmonary dissemination. Dilatation of lymphatics around bronchi and vessels was common in areas of edematous alveoli. The lymphatics were also heavily colonized by bacteria. Fibrin was notably absent from the exudate at all stages, although intravascular thrombi were sometimes seen.

C. LYMPH NODES

Bronchial lymph nodes draining the infected lobes displayed extensive liquefactive necrosis in all animals. Enormous numbers of bacilli were present. Hemorrhages were numerous.

D. SPLEEN

A reduction in cellularity of the red pulp was common, with hemorrhages and foci of necrosis in some cases. Usually bacilli were abundant, and, sometimes, fibrin was found in the sinusoids. There were a few instances of increased numbers of polymorphonuclear leukocytes. The lymphoid tissue was generally unaffected.

E. LIVER

Lesions were generally minimal and included one or more of the following: sinusoidal congestion, periportal or diffuse sinusoidal leukocytosis, and mild fatty change. Bacilli and fibrin were occasionally found in sinusoids. Infrequently, foci of coagulation necrosis were present, with no anatomical predilection.

F. ADRENALS

There were occasional focal hemorrhages. Fibrin thrombi were notably rare, even in those animals having extensive renal deposits.

There were no large vessel thromboses in any animals.

G. BUBONIC PLAGUE

Because the finding of glomerular fibrin with the KIM-10 strain of P. pestis after aerosol exposure is the first reported observation of intravascular coagulation in plague in many years, the possibility that these observations are limited to the specific strain or route of inoculation had to be considered. Sections from a previous experiment[18] were re-examined. Seven monkeys (Macaca mulatta) had been given 3 x 10^9 bacilli of the Alexander strain subcutaneously. They lived an average of 85.6 hours and displayed typical bubonic plague, with local ulceration and liquefactive necrosis of the subcutaneous tissue at the site of inoculation, inguinal buboes consisting of necrotic lymph nodes, septicemia with abundant intravascular bacilli in all organs, and multiple hemorrhages. Fibrin thrombi were extensive in the glomerular capillaries of six of seven animals.

IV. DISCUSSION

Glomerular capillary occlusions by fibrin thrombi in plague were first described by Herzog in 1904.[19] He saw them in seven of 20 cases in a Philippine epidemic. They were also found in two of 25 cases of pneumonic plague from the Manchurian epidemic of 1910 to 1911[20] and in approximately 40% of 75 cases of bubonic plague in Manila from 1912 to 1914.[21] Such thrombi were not seen in further studies of human plague in Manchuria[17] or Los Angeles[22] in the 1920's by pathologists acquainted with Herzog's report. For the past 30 years, the finding has been overlooked by students of plague.[3,4,23,24]

The significance of glomerular fibrin thrombi in plague is not clear. Our observation of remarkable differences in the incidence of the phenomenon between animals that died and those sacrificed when apparently moribund at the same time after infection suggests that the thrombi formed terminally. Indeed, renal function, as measured by blood urea nitrogen content, is only occasionally diminished in experimental plague.*[25] The renal lesion appears to be of little consequence in animals already ill with necrotizing pneumonia and septicemia. Yet the mechanism of death in plague does not seem adequately explained by the amount of inflamed tissue found at autopsy or by the mere presence of bacilli in the circulation.[26]

Although the thrombi may not be important for their effects on renal circulation, they may be regarded as the major tissue manifestation of intravascular coagulation. As such, they serve to corroborate the data

* Petery, J.J., unpublished data.

on blood clotting reported in the accompanying paper.[1a] A spectrum of tests designed to monitor various phases of the coagulation process was performed, including the whole blood clotting time, partial thromboplastin time, plasma prothrombin time, platelet count, and fibrinogen concentration. In these tests, significant differences were established by 72 to 120 hours after infection, compared with base line values. These differences were apparent 24 to 72 hours prior to death. Tests for the presence of a fibrinolysin or a circulating anticoagulant were negative. Both the early onset of changes indicating the consumption of plasma clotting factors[11] and the uniformity of these defects among the infected monkeys provide strong evidence for the involvement of intravascular coagulation in the pathogenesis of plague. Consumption of the clotting factors could explain the hemorrhagic diathesis that is frequently observed.[1,3] The absence of thrombi in most of the sacrificed animals may have been because of successful clearance or lysis of circulating fibrin aggregates until the development of terminal shock.

The hypothesis that glomerular fibrin thrombi might be caused by the action of a toxin in plague was proposed by Herzog[19] in discussing his discovery of the thrombi. He noted the absence of a primary vascular lesion in the kidney and the frequent failure to find organisms either in the thrombi or in the multiple hemorrhages throughout the body. Lysed P. pestis were used to produce the local Shwartzman reaction in 1934,[27] but, to date, no material from P. pestis has been used to produce the generalized Shwartzman phenomenon. The murine toxin of P. pestis appears to be a protein that differs from typical exotoxins of gram-positive bacteria in its resistance to protease digestion and its tenacious link to the bacterial cell.[3,24] This material is innocuous for monkeys, among other animals.[5] A polysaccharide fraction was first obtained from culture filtrates and disrupted P. pestis by Seal,[6] who did not describe any toxicologic studies. Davies[7] isolated a lipopolysaccharide by phenol extraction of acetone-dried bacilli. It was strongly pyrogenic for rabbits, but was toxic for rodents only in doses far exceeding those toxins obtained from the usual gram-negative bacilli. Further purification of phenol-extracted endotoxin yielded material with an LD_{50} of 3 mg for mice and 25 mg for guinea pigs, which are enormous doses.[8] The pathological responses of both species to this material, and to a comparable toxin obtained by grinding of acetone-dried bacilli and "Pronase" digestion, consisted of multiple petechiae and acute renal tubular necrosis. There was no mention of thromboses or a study of blood coagulation.[8]

Thus, it appears that some progress is being made in isolating a lipopolysaccharide toxin from P. pestis. If such an extract can be used to reproduce the changes in blood coagulation and glomerular fibrin thrombi observed during infection, the hypothesis that endotoxemia is a significant feature of plague will be strongly supported.

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13. ABSTRACT

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14. Key Words
*Plague
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