PATHOGENESIS OF RESPIRATORY ANTHRAX IN *MACACA MULATTA*

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PREVIOUS reports have endeavoured to describe the histopathology of anthrax in *Macaca mulatta* monkeys challenged by the cutaneous (Berdjis, Gleiser, Hartman and Gochenour, 1961) and respiratory routes (Gleiser, Berdjis, Hartman and Gochenour, 1961).

The literature contains many references concerning respiratory anthrax in experimental animals (Vélu, Soulié and Bellocq, 1943; Vélu, Gavaudan and Soulié, 1943; Young, Zelle and Lincoln, 1946; Barnes, 1947; Ross, 1957; Albrink and Goodlow, 1959) and in man (Greenfield, 1882; Eppinger, 1894; Fraenkel, 1925; Albrink, Brooks, Biron and Kopel, 1960), in which attempts were made to determine the site of germination of *Bacillus anthracis* spores and establish the route of dissemination. The pathogenesis of this infection is still obscure.

This paper presents additional data obtained by serial killings in an attempt to clarify this situation and to detect the initial lesion responsible for respiratory anthrax before overwhelming septicaemia is established.

MATERIALS AND METHODS

Twenty-two young, immature, healthy *M. mulatta* monkeys were used in 3 experiments.

Two respiratory exposure experiments were performed with a modified Henderson dynamic aerosol generating device (Henderson, 1952). In both experiments, 2 animals were simultaneously exposed to each cloud generated. The concentration and virulence of the spore suspension aerolized were determined by plate counts on nutrient agar and subcutaneous titration in guinea-pigs. The spore suspension used was Vollum-189 strain of *B. anthracis*, stored in a concentration of 5×10^{10} spores/ml. at 5°. The appropriate dilutions of this material were heat-shocked at 60° for 30 min., 48 hr. prior to use. Respiratory doses presented were estimated by assay of impinger recoveries for each exposure port.

In the first experiment, 14 monkeys were exposed to aerosol clouds of such concentration that each monkey was presented with a dose of approximately 2×10^5 spores of *B. anthracis* (Low Dose). Temperatures were taken twice a day and roentgenograms were obtained daily using a 1 MEV X-ray machine. Two monkeys were killed each day, from day 1 to day 6, except that on day 3, 4 animals were killed. At autopsy quantitative bacteriology was done on hilar and tracheobronchial lymph nodes, lung, spleen and blood. Blood was also inoculated into diphasic media in Castaneda bottles (Castaneda, 1947).

In the second experiment, 8 monkeys were exposed to aerosol clouds of such concentration that each monkey was presented with a dose of approximately 2×10^6 spores of *B. anthracis* (High Dose). Temperatures and X-rays were obtained daily. Two monkeys were killed per day for 3 days starting on day 1. Quantitative bacteriological cultures were made of the blood of each animal at the time that the animal was killed. Two animals died of anthrax on day 2 of this experiment and these 2 animals were excluded from this study.

All animals were autopsied routinely and representative sections of all organs were fixed in 10 per cent formalin. Routine histological procedures were employed. Haematoxylin

and eosin (H and E) stain was regularly employed. The periodic acid Schiff procedure (PAS) (Popper, Paronetto and Barka, 1960), with and without diastase digestion, and the Brown and Brenn stain (B and B) (Brown and Brenn, 1931; Armed Forces Institute of Pathology, 1957), for bacilli, were used frequently. A modified acid-fast stain, after Ross (1957), and a modified Weigert's stain (Armed Forces Institute of Pathology, 1957) were also employed for detection of spores in tissue sections. Blood smears were also examined at time of autopsy.

Technique for lung examination.—The intact lungs and trachea were fixed in formalin. Each lung was then sectioned transversely at regular 0·5 cm. intervals. In most cases, all of the lung tissue was embedded in paraffin for microscopic examination. In a few cases, only representative blocks of lung tissue were embedded. In order to demonstrate all possible solitary lung lesions, serial sections were made of each block of tissue where microscopical examination of the initial section showed any suspicious lesion. In these instances, every 5th–10th section was examined microscopically.

RESULTS

Clinical observations

With the exception of a slightly raised body temperature in an occasional animal, no specific clinical changes were observed during this study (Table I).

TABLE I.—*Maximum Temperatures and Gross Pathology of Monkeys Infected with Anthrax by the Aerosol Route and Serially Killed*

Day of killing	Dose*	Monkey Acc. No.	Maximum temperature °F.	Gross pathology		
				Lymphadenopathy†	Respiratory system	Others
1	Low	912	N‡	NR§	NR	NR
		913	N	NR	NR	NR
	High	893	104·0	NR	NR	NR
		894	104·2	NR	NR	NR
2	Low	914	104·0	Minimal	NR	Mediastinitis, oedema, minimal
		915	N	Moderate	Haemorrhage	Mediastinitis, oedema, moderate
	High	896	N	Moderate	NR	Mediastinitis, oedema
		898	N	Minimal	NR	NR
3	Low	918	104·0	NR	NR	NR
		916	105·2	Minimal	NR	NR
		917	105·4	Minimal	Haemorrhagic nodule	Adrenal haemorrhage
		919	104·2	Minimal	NR	Adrenal haemorrhage
	High	901	N	Minimal	NR	Meningeal injection
		902	104·2	Mild	Haemorrhagic nodule	Mediastinitis, oedema
4	Low	920	104·4	NR	NR	NR
		921	N	NR	NR	NR
5	Low	922	N	NR	Haemorrhage	Mediastinitis, oedema, minimal
		923	N	NR	NR	NR
6	Low	924	N	NR	NR	NR
		925	104·4	NR	NR	NR

* Low dose: 1×10^5–2×10^5; high dose: 1×10^6–2×10^6.
† Hilar and tracheobronchial nodes.
‡ N = normal temperature.
§ NR = not remarkable.

Daily roentgenograms revealed no demonstrable lesions in lungs and mediastinum. The bacteriological findings are summarized in Tables II and III.

TABLE II.—*Results of Bacteriological Studies at Autopsy of Monkeys Infected with Anthrax: Low Dose*

Day of killing	Monkey Acc. No.	Culture					Smear	
				Lymph node				
		Blood	Lung	Hilar	Tracheo-bronchial	Spleen	Blood	Spleen
1	912	−	+	−	−	−	−	−
	913	−	+	−	−	−	−	−
2	914	+	+	+	+	+	−	−
	915	+	+	+	+	+	−	+
3	918	−	+	−	−	−	−	−
	916	+	+	+	+	+	+	+
	917	..	..	..	..	..	+	..
	919	..	..	..	..	..	+	..
4	920	+	+	+	+	+	−	−
	921	−	+	−	−	−	−	−
5	922	−	+	+	−	−	−	−
	923	−	+	−	−	−	−	−
6	924	−	+	−	−	−	−	−
	925	−	+	−	−	−	−	−

TABLE III.—*Results of Bacteriological Studies at Autopsy of Monkeys Infected with Anthrax: High Dose*

Day of killing	Monkey Acc. No.	Blood culture	Smear	
			Blood	Spleen
1	893	−	−	..
	894	−	..	..
2	896	+	..	..
	898	+	−	..
3	901	−	−	..
	902	+	−	+

Gross pathology

The summary of the gross changes (Table I) suggests a dose-time-relationship in experimental respiratory anthrax. As early as day 2, lesions were found in the respiratory system and its tributary lymph nodes. These lesions were more severe on day 3. After day 3 and on days 4 to 6, there were no further consistent changes in the gross autopsy findings in the animals who had received the low dose. No animals who had received the high dose remained alive after day 3.

No meningitis or substantial mediastinitis were observed in these animals, differing somewhat from the observations in terminal respiratory anthrax (Gleiser *et al*, 1961). There was no evidence of tracheal or bronchial ulcers, as described by Fraenkel (1925), at the site of inoculation or " primary anthrax lesion," in man.

Special consideration is reserved for the " pulmonary mite lesion," described by Innes, Cotton, Yerich and Smith (1954) and noted in an earlier report (Gleiser

et al., 1961) as an almost constant finding in the lungs of *M. mulatta*. In connection with this lesion, " haemorrhagic parasitic nodules " (Gleiser *et al.*, 1961) were observed on day 3 in both low and high dose animals.

Lung mites, *Pneumonyssus simicola* and related species, have a reported incidence of 80 to 100 per cent in this host (Innes *et al.*, 1954 ; Ruch, 1959). The lesions are readily found at autopsy in all portions of the pulmonary lobes. They appear as subpleural, discrete, moderately depressed, yellowish white lesions, occasionally congested, and varying in size from 1–2 mm. The microscopical picture of the lung mite lesions is that of chronic bronchiolitis with peribronchiolitis. The lesion occurs in bronchioles at the level where cartilage is absent. The area of primary concern is that portion of the respiratory tree which begins with the terminal bronchiole as shown in Fig. 16 ; here it divides into respiratory

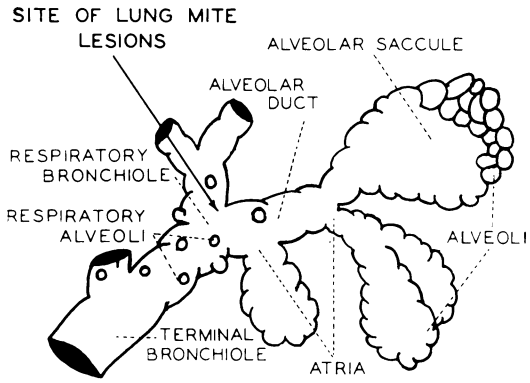


FIG. 16.—Diagram showing principal site of lung mite lesions (*Pneumonyssus simicola*).

bronchioles which give rise to individual alveoli (respiratory alveoli) and terminate in alveolar saccules. Mite lesions must be borne in mind in any consideration of respiratory infections and their pathogenesis since they involve that portion of the respiratory tree most intimately concerned with experimental respiratory infections.

Microscopic Pathology—Low Dose

Day 1 (2 *monkeys*)

Lymphatic system.—There was minimal oedema of the lymph nodes associated with dilated lymphatic channels and minimal increase in the cellular debris within the germinal centres. These findings were not clearly related to experimental anthrax. The spleen showed only moderate congestion with increased cellular debris and macrophages. No bacilli were detected.

Other organs.—Passive congestion of various organs was observed. Occasional small localized subpleural haemorrhages, perhaps related to mite lesions, were present. No bacilli were detected. The hepatic cells of the centrolobular portion of the liver were rich in diastase-digestible PAS-positive material (Fig. 1, normal and Fig. 2). The von Küpffer cells showed minimal non-glycogenic PAS-positive material. The parenchymal cells of the liver contained multiple small droplets of fat without other associated morphological changes.

Day 2 (2 *monkeys*)

Lymphatic system.—The architectural pattern of the lymph nodes was well preserved although all of the lymph nodes showed some oedema and congestion.

There was no significant haemorrhage or necrosis. Occasional bacilli were detected only in tracheobronchial lymph nodes. There were increased numbers of macrophages and cellular debris with apparent erythrophagocytosis. Some of the lymph channels contained homogeneous, pale eosinophilic fluid residue. In instances, the lymph channels contained inflammatory cells but bacilli were never observed within these structures. In the spleen the architectural framework was not destroyed. The parenchyma was markedly congested; there were small foci of haemorrhage in isolated and slightly depleted follicles. Cellular debris and macrophages were moderately increased. Occasional bacilli were visible in the parenchyma.

Respiratory system.—These animals showed no pulmonary lesions attributable to anthrax, even though parasitic lesions were present. Minimal oedema, focal extravasation of erythrocytes and a few macrophages were found within the alveoli. No bacilli were detected in multiple sections of lungs.

Liver.—Findings were similar to those of day 1. No bacilli were detected. Fig. 3 illustrates accumulation of diastase-digestible PAS-positive material with centrolobular orientation. A fat stain also demonstrated finely divided particles taking the lipid stain in the central portion of the liver lobules.

Other organs.—The remaining organs showed minimal to moderate congestion and no demonstrable bacilli.

Day 3 (4 monkeys)

Lymphatic system.—The pathological changes observed in the lymph nodes varied from no essential structural alterations in the non-septicaemic animal (Acc. no. 918) to partially haemorrhagic and necrotic nodes in septicaemic monkeys (Acc. nos. 916 and 919). The tracheobronchial and hilar nodes were the most severely damaged. In another septicaemic animal (Acc. no. 917) the architectural pattern showed no significant disorder nor was there lysis (Fig. 4). The lymphatic tissue and lymph channels, although dilated, contained none or very few bacilli as shown in Figs. 5 and 6. This observation is contrasted with the presence of masses of bacilli within the blood vessels in splenic parenchyma (" bag of bacilli "). The findings in the spleens varied from no essential changes to marked disorder and lysis.

Respiratory system.—" Specific pulmonary lesions," consisting of " superinfection " of anthrax on a pre-existing mite lesion, were seen. These lesions consisted of necrotizing bronchiolitis rich in bacilli with concomitant bronchiolectasis. The lumen of such a bronchiole was usually enlarged and filled with pale pinkish material containing numerous bacilli and a few inflammatory and desquamated cells (Fig. 9). The bronchiolar space also contained fibrin strands and a thick layer of hyalinized and necrotic dense material, rich in bacilli. The adjacent pulmonary parenchyma (Figs. 9 and 10) revealed oedema, haemorrhage, varying numbers of bacilli and marked inflammatory infiltrates which were both acute (anthrax) and chronic (parasitic). Remaining lung parenchyma and other mite lesions were noncontributory and unremarkable, even when the bacilli were present in alveolar capillaries (Figs. 7 and 8).

Other organs.—In 2 of 4 animals the adrenal glands showed focal haemorrhage and necrosis with congestion of the cortex. Other organs were not involved. The hepatic parenchyma was essentially unaltered; however, a PAS stain

revealed an increase in non-glycogenic material in prominent von Küpffer cells. These cells contained bacilli (Fig. 11). A fat stain revealed fat droplets in hepatic cells, especially in the centrolobular portion.

Day 4 (2 *monkeys*)

Lymphatic system.—Lymph nodes and spleen showed minimal oedema and moderate congestion with occasional small areas of haemorrhage. No necrosis was detectable. In one animal, occasional bacilli were seen in some lymph nodes ; no bacilli were present in the spleen.

Respiratory system.—Although some parasitic lesions were present, no significant pathological changes were observed in lung parenchyma. No bacilli were seen.

Other organs.—Other organs were unremarkable and unmodified.

Day 5 (2 *monkeys*)

Multiple foci of pulmonary haemorrhage in one animal and moderate oedema and congestion of lymphoid organs were the only findings. No bacilli were seen. Other organs were unmodified.

EXPLANATION OF PLATES

All photomicrographs are taken from slides stained either with haematoxylin and eosin (H and E), with periodic acid-Schiff (PAS) counterstained with haematoxylin, or with Brown and Brenn Gram stain (B and B), as indicated.

FIG. 1.—Liver. Control. Acc. No. 1078. PAS. ×40.

FIG. 2.—Liver, day 1. Centrolobular orientation of numerous droplets and dust-like, diastase-digestible PAS-positive material (dark spots and black dots). Acc. No. 912. PAS. ×40.

FIG. 3.—Liver, day 2. Increased accumulation of PAS-positive material with more orientation. Acc. No. 915. PAS. ×40.

FIG. 4.—Tracheobronchial lymph node, Low Dose, day 3. Fairly well preserved architectural pattern ; no significant haemorrhage or necrosis. H and E. ×25.

FIGS. 5 and 6.—Same, many bacilli in blood vessels and none in lymph channels and subcapsular sinuses. B and B. ×115 and ×230, respectively.

FIG. 7.—Lung, Low Dose, day 3. Common pulmonary mite lesion appearing as chronic bronchiolitis unaffected by anthrax even though alveolar system exhibits many bacilli as shown in Fig. 8. Acc. No. 919. H and E. ×49.

FIG. 8.—Same, showing numerous bacilli in alveolar wall and none at edge of parasitic lesion (shown in upper right corner). B and B. ×220.

FIG. 9.—Lung, Low Dose, day 3. " Specific pulmonary lesion." Necrotizing and ulcerative bronchiolitis with bronchiolectasis ; marked destruction of neighboring tissues by bacillary invasion. Acc. No. 916. H and E. ×49.

FIG. 10.—Same, early invasion of neighboring tissues adjacent to mite lesion (shown in upper right corner) in relatively unharmed alveolar system. B and B. ×220.

FIG. 11.—Liver, Low Dose, day 3. Prominent Küpffer cells phagocytosing numerous bacilli and exhibiting many nonglycogenic PAS-positive particles. Acc. No. 917. PAS. ×848.

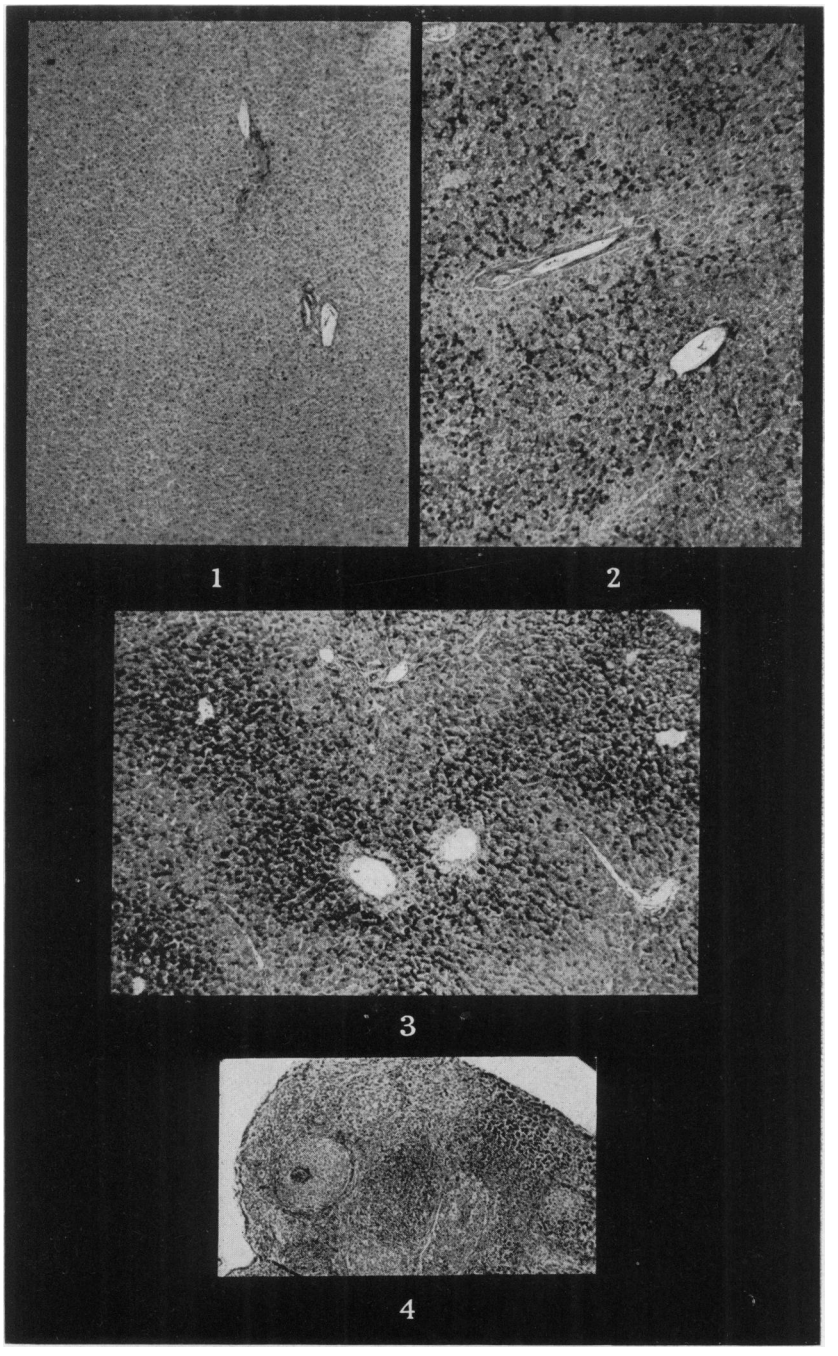
FIGS. 12 through 15.—Lung, High Dose, day 2. " Specific pulmonary lesion ", step sections : 1, 20, 65, and 90, respectively. Acc. No. 898. H and E. ×57.

FIG. 12.—Small, localized bronchiolar ulceration ; marked peribronchiolar inflammation enlarged lumen containing fibrinous material and desquamation and inflammatory cells with some bacilli.

FIG. 13.—Section 20 : More extensive necrotizing ulceration, prominent inflammatory reaction and numerous bacilli ; section of mite in lumen.

FIG. 14.—Section 65 : Myriads of bacilli in lumen and peribronchial area ; epithelial lining entirely destroyed by superinfection.

FIG. 15.—Section 90 : More destruction and more extension. Several alveolar bronchioles are intercommunicating ; all equally damaged.



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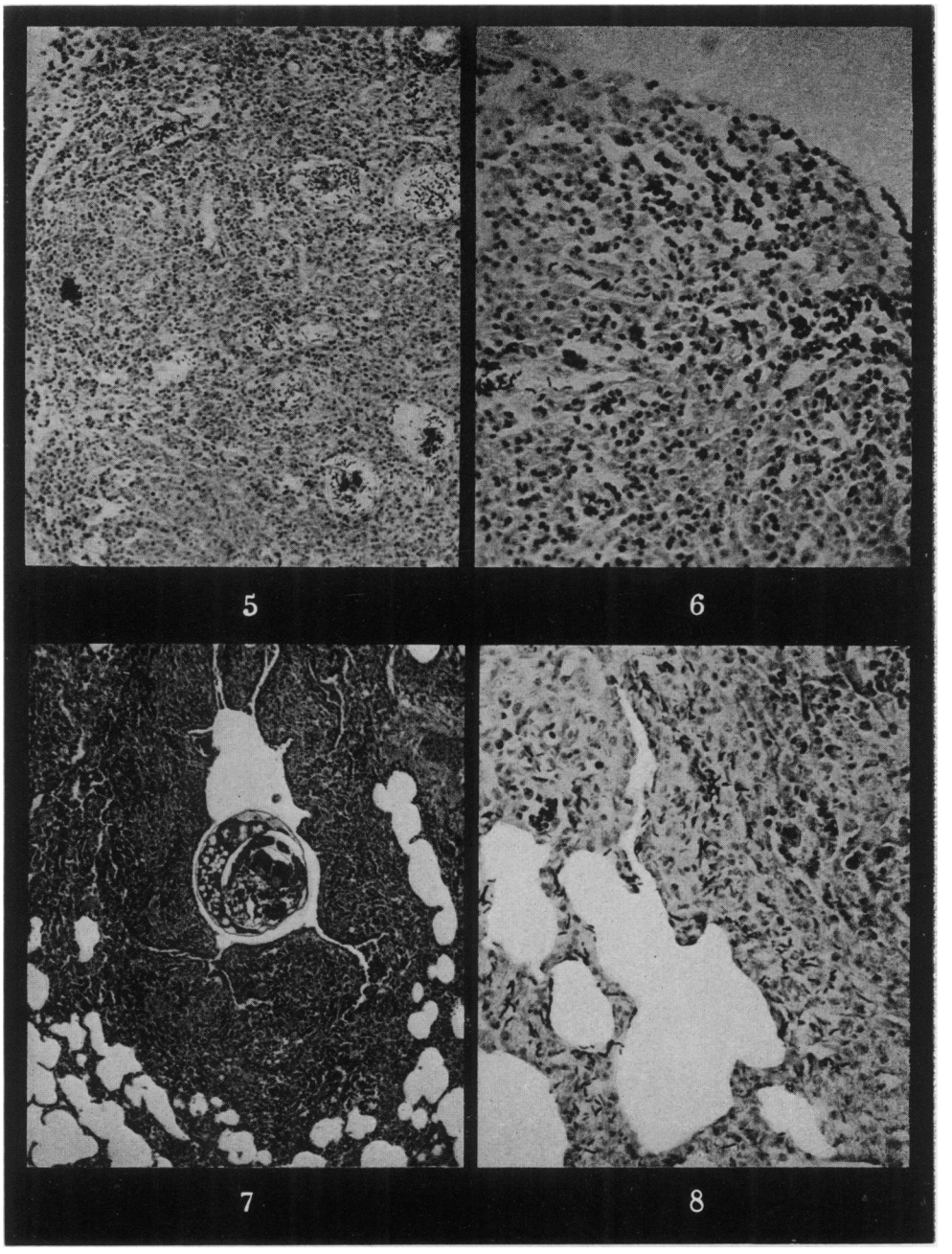

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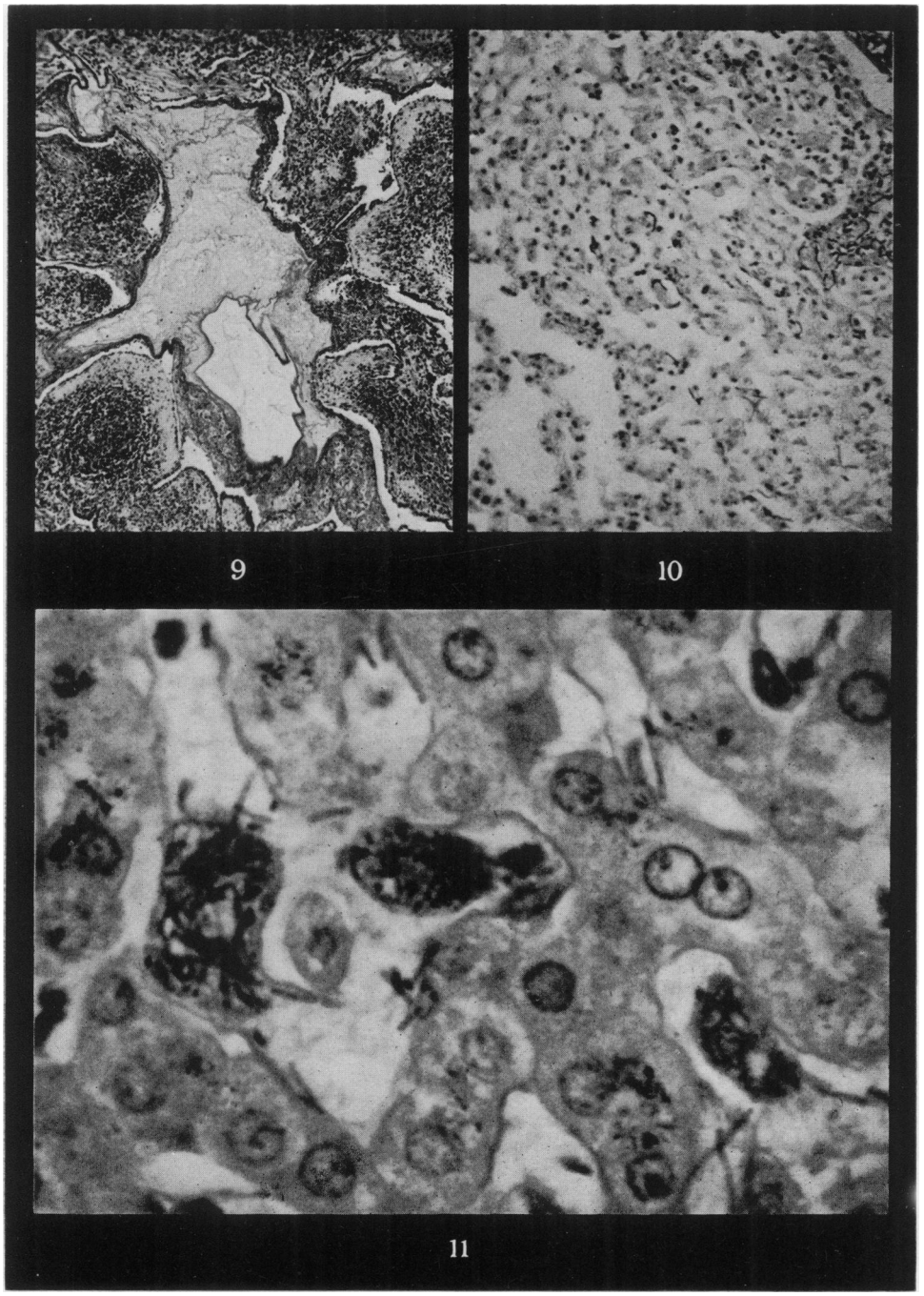

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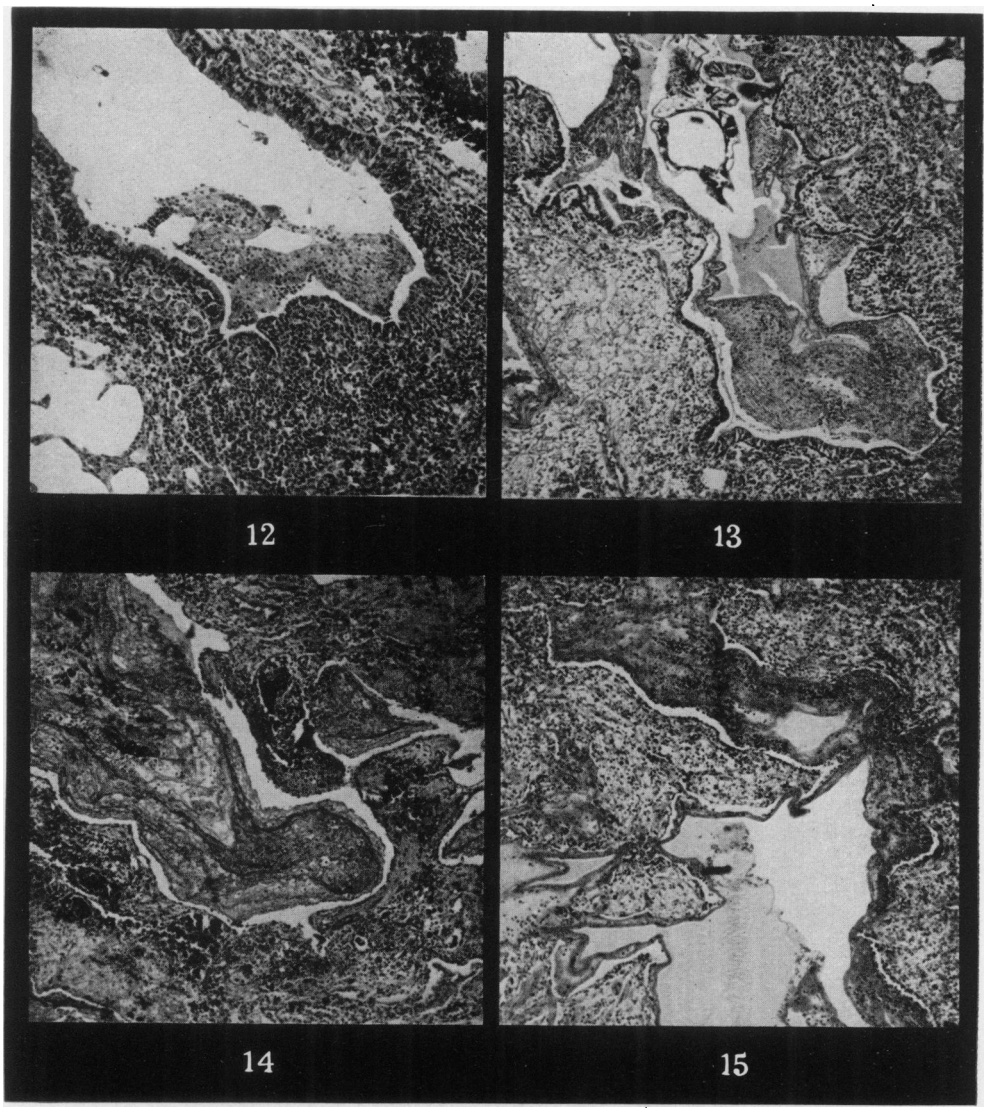

12 13

14 15

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Day 6 (2 *monkeys*)

No significant pathological changes attributable to anthrax were seen. No bacilli were observed.

Microscopic Pathology—High Dose

Day 1 (2 *monkeys*)

No significant pathological changes or bacilli were seen in any organ.

Day 2 (2 *monkeys*)

Lymphatic system.—Lymph nodes were damaged slightly more than those of Low Dose animals. Although a number of nodes were congested and oedematous, the architectural pattern was well preserved. Occasionally there were dilated sinuses, increased cellular debris and macrophages, and varying degrees of depletion, especially in tracheobronchial nodes. These revealed haemorrhage and necrosis with numerous bacilli. The architectural pattern of the splenic parenchyma was markedly disturbed by diffuse necrosis. Most of the follicles were depopulated and exhibited necrotic centres with minimal peripheral haemorrhage. Bacilli were especially numerous in the necrotic areas.

Respiratory system.—Necrotizing ulcerative bronchiolitis (" specific pulmonary lesion ") was observed in one animal (Acc. no. 898). Fig. 12–15 illustrate this finding in step sections at 4 levels. The neighbouring tissue was involved and disclosed myriads of bacilli, numerous inflammatory cells and diffuse haemorrhage. This resulted in complete destruction of underlying parenchyma within the focus of involvement. Elsewhere the pulmonary parenchyma was unremarkable.

Liver.—Widespread central and midzonal early hepatocellular degeneration and marked cloudy swelling were present. No frank necrosis or haemorrhage were present. The cloudy swelling was accompanied by multiple small droplets of fat, especially in the centrolobular portion. The sinusoids were dilated and contained varying numbers of bacilli. Prominent von Küpffer cells also contained many bacilli as shown previously and illustrated in Fig. 12. There was also an increase in the PAS-positive material in the von Küpffer cells.

Other organs.—Apart from small, focal haemorrhages in the adrenal gland, no other significant pathological changes were seen.

Day 3 (2 *monkeys*)

Lymphatic system.—Most lymph nodes and spleens revealed no disturbance of architectural pattern, although they contained bacilli. Varying amounts of oedema, congestion, erythrophagocytosis and cellular debris were observed in all lymph nodes. Only the tracheobronchial nodes revealed moderate to marked destruction of parenchyma by haemorrhage with or without necrosis.

Respiratory system.—One " specific pulmonary lesion " was found in the lungs of each animal.

Liver.—There was a moderate amount of hepatocellular degeneration and cloudy swelling, accompanied in one animal by small foci of necrosis. No bacilli were seen and no significant inflammatory response was observed. There were prominent phagocytic von Küpffer cells containing numerous bacilli and non-glycogenic PAS-positive material as illustrated previously (Fig. 11).

Other organs.—Occasional small foci of necrosis with and without haemorrhage were found in adrenal gland and bone marrow. The central nervous system showed oedema, congestion, and an early meningeal reaction.

Table IV summarizes the significant microscopical findings.

TABLE IV.—*Summary of Microscopic Pathology of Serially Killed Monkeys Infected with Anthrax by the Respiratory Route*

Organs	Day of killing*	Bacilli		Oedema		Dilated lymphatic channels		Haemorrhage		Necrosis		Inflammatory infiltrate	
		L	H†	L	H	L	H	L	H	L	H	L	H
Lung	1	−	−	±	±	±	±	Focal, minimal		Focal, minimal		−	−
	2	−	+	+	+	+	+	(+)‡	(+)	−	+§	+	(+)
	3	+	+	+	+	+	+	+	+	+§	+§	+	+
Lymph nodes	1	−	−	±	±	+	+	Focal, minimal		Minimal		−	−
	2	(+)	+	+	+	+	+	−	(+)	−	(+)	±	+
	3	+	+	+	+	+	+	(+)	(+)	(+)	(+)	+	+
Spleen	1	−	−	±	±	±	±	−	−	−	−	−	−
	2	(+)	+	+	+	±	+	(+)	+	−	+	+	+
	3	+	+	+	+	+	+	+	+	+	+	+	+
								Hepatocellular degeneration					
Liver	1	−	−	−	±	..	..	−	−	−	−	−	−
	2	−	+	±	+	..	..	±	+	−	−	−	−
	3	+	−	+	+	..	..	+	+	−	+	±	±
Adrenal	1	−	−	−	−	..	..	−	−	−	−	−	−
	2	−	+	−	−	..	..	(+)	+	(+)	+	−	−
	3	+	+	−	−	..	..	+	+	+	+	−	−

* The findings on days 4, 5 and 6 are inconsistent and inconclusive.
† L = low doses; H = high doses.
‡ ( ) = focal.
§ Necrotizing and ulcerative bronchiolitis with bronchiolectasis.

DISCUSSION

The findings at day 1, in both the High and Low dose experiments, were inconstant. Whether the changes, such as increased cellular debris, minimal haemorrhage, and necrosis of an occasional lymph node, were due to experimental anthrax could not be proved. There was an accumulation of PAS-positive material and fat particles in liver with a centrolobular orientation (Fig. 2 and 3). Inasmuch as bacilli were not demonstrable and blood cultures were negative, it was not clear whether these changes were related to anthrax infection. This PAS-positivity, fat accumulation and hepatocellular degeneration, even in the absence of bacilli, needs further investigation.

The first overt anthrax lesion appeared in the lung on day 2 in High Dose monkeys; Low Dose monkeys showed no specific lesions. Despite positive blood cultures in both groups, no bacilli were seen in lungs of Low Dose monkeys and only a few were present in tracheobronchial lymph nodes. In High Dose animals bacilli were conspicuous and numerous at these sites. One is thus forced to conclude that entry of *B. anthracis* into the blood stream may precede the appearance of any recognizable lesion in monkey lungs.

Yet *M. mulatta* frequently does develop a lesion in pulmonary parenchyma, a necrotizing bronchiolitis superinfected on a pre-existing parasitic involvement. Selected step sections of suspicious areas of lung revealed only one such lesion rich in bacilli; all the others were virtually devoid of organisms although the animal was septicaemic. This combination strongly suggests that the lesion was the result of organisms implanted directly by inhalation, followed by germination and multiplication.

These two observations can be reconciled at the present time only by assuming that organisms can reach the hilar nodes and/or the blood stream from the monkey lung in a manner comparable to that described by Ross (1957) for the guinea pig. The present studies do not confirm or deny the existence of such a mechanism. This initial process may continue as an overt septicaemia or, as described by Albrink and Goodlow (1959) in the chimpanzee, may be controlled by the host. In addition, in *M. mulatta*, organisms impinging on damaged lungs may germinate at the site and thus produce a continuing flow of organisms, either to lymph nodes or directly into the blood stream. The result is to place this susceptible animal in double jeopardy and either or both routes may be operative. In theory in "resistant" animals the local lung lesion would be of considerable importance, thus insuring a continued source of blood stream or lymph node involvement. Findings in one human case at autopsy (U.S. Army Medical Unit, unpublished) conform to this hypothesis.

Perhaps the injured bronchiole may serve to facilitate the initial "direct" entry of *B. anthracis*, this being somewhat comparable to the additive effect described by Vélu *et al.* (1943*a*, *b*) for chlorine as a "pre-existing" injurious agent.

SUMMARY

One of the sites of initiation of infection in respiratory anthrax in *M. mulatta* is the bronchiolar wall providing a pre-existing parasitic lesion is present. When anthrax is superimposed, there results a necrotizing and ulcerative bronchiolitis with focal bronchiolectasis, the "specific pulmonary lesion."

The mechanisms by which anthrax becomes disseminated are discussed. Evidence is presented that dissemination may result either by lymphatic drainage or by the blood stream.

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