MORPHOLOGICAL AND OTHER CHARACTERISTICS OF THE AGENT OF FELINE PNEUMONITIS GROWN IN THE ALLANTOIC CAVITY OF THE CHICK EMBRYO

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Plates 1 and 2

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Examination of smears and sections of tissues stained with Giemsa or by Macchiavello's method led to the description of the developmental cycle of the Chlamydozoaceae (1), or the lymphogranuloma-psittacosis group of agents. However, because of their small size no details of structure could be seen before the development of the electron microscope. By the use of this instrument and the adoption of such methods of preparing material for examination as the shadow-casting technique of Williams and Wyckoff (2), and the Hillier and Baker (3) modification of replica preparation (Schaefer and Harker (4)), it is now possible to study in part the structure of the bodies of the agent. In a previous report (5) we presented electron micrographs of the agent of feline pneumonitis purified from yolk sac suspensions by differential centrifugation. During purification much of the agent was lost, and because of the complex nature of yolk, it was difficult to obtain clear preparations. These difficulties were largely overcome by using heavily infected allantoic fluid.

Growth of the Agent of Feline Pneumonitis in the Allantoic Cavity

Material and Methods.—The passages were initiated by injecting 0.5 ml. of a 20 per cent yolk sac suspension into the allantoic cavity of 10 day old embryos. Eggs were harvested 4 to 5 days after inoculation and 0.5 ml. of either a 30 to 50 per cent suspension of the chorioallantoic membrane ground or shaken with beads in allantoic fluid from the same eggs, or allantoic fluid alone, was used for passage.

Results.—In early passages the number of elementary bodies seen in smears of the allantoic fluid was small. This is in agreement with the results of Francis and Gordon (6) who reported infection of 50 per cent of the eggs injected into the allantoic cavity with feline pneumonitis in the two passages they made. There were no deaths among our eggs until the 8th passage, and, in passages following that, the mortality varied from 0 to 30 per cent, but there was no definite trend toward higher mortality as passages increased, although the amount of agent in the allantoic fluid increased 100-fold from the 13th to the

50th passage (see below). The reason for the failure of the agent growing in the allantoic cavity to cause death consistently is not known; perhaps it is because the agent with its toxic factor does not invade the general circulation, or perhaps the lethal effect per infective unit of agent is decreased on prolonged cultivation in the allantoic sac.

At the 13th passage the amount of agent in the chorioallantoic membrane and in the allantoic fluid was determined by titration in the yolk sac, using 5 eggs for each tenfold dilution. The dose infectious for 50 per cent of the eggs (ID_{50}), determined by the method of Reed and Muench (7), of the chorioallantoic membrane suspended in broth was $10^{-8.7}$, and of the allantoic fluid $10^{-6.6}$.

After 30 serial passages in the allantoic cavity, the amount of agent in smears of the allantoic fluid increased, and often "micro colonies" or clusters of elementary bodies were seen. These were circular in shape, sometimes larger than an oil immersion field and usually contained no recognizable fragments of cellular material.

When the amounts of agent in the chorioallantoic membrane and allantoic fluid were determined at the 50th passage (Table I), it was found that the ID_{50} of the allantoic fluid on the 5th day after inoculation was $10^{-8.6}$ as compared with $10^{-6.6}$ at the 13th passage. The titers of both the membrane and the fluid were highest on the 5th day following inoculation. On the 6th and 7th days the titer of the membrane changed little if at all, while that of the allantoic fluid fell significantly.

Multiplication of the agent in the allantoic cavity was slower than in the yolk sac where the amount of agent increased 4.5 logs between 24 and 48 hours after inoculation with $10^{6.0}$ infectious doses (8). Also a much larger infective dose was required in the allantoic sac in order to obtain maximal titers of agent in 5 days. With inocula containing less than $10^{4.0}$ infective doses, the agent could not be found in smears of allantoic fluid 6 days after infection.

Suspensions of chorioallantoic membranes in allantoic fluid from heavily infected eggs were rapidly fatal to mice when injected intravenously, indicating the presence of the toxic substance already described (9). This was confirmed by a toxin neutralization test using antiserum prepared in rabbits against the feline pneumonitis toxin in yolk sac suspensions (Table II).

Chorioallantoic membranes from 7 eggs were pooled, weighed, and shaken with combined allantoic and amniotic fluids from 3 of these eggs, so as to give 1 gm. of tissue to 3 ml. of total suspension. The membranes and fluids were those containing the most agent as shown by examination of smears stained by Macchiavello's method. The suspension was shaken with beads for 20 minutes, centrifuged at 2000 R.P.M. for 15 minutes, and then diluted with yolk and fluids from normal 6 to 8 day old embryonated eggs. In the titration 14 gm. Swiss mice were given 0.5 ml. intravenously of the dilutions of toxin shown in the table.

Serum from rabbit 7-5, bleeding 12/1, was obtained after 3 weeks of immunization with toxin prepared from yolk sacs heavily infected with the agent of feline pneumonitis. All injections were intravenous and the schedule described in the paper of Rake and Jones (10) for the preparation of antisera was followed.

This serum preparation had protected mice against two lethal doses of toxic factor derived from yolk sac at a dilution of 1/800 but not at 1/1000 when last tested 3 years before (9). For the present experiment serum dilutions were prepared in yolk and fluids from normal embryonated eggs so as to give, when mixed with equal parts of 1/3 suspension of toxin, a 1/6 dilution of toxin and twofold dilutions of serum from 1/200 to 1/1600. These mixtures were incubated at room temperature for 2 hours and then 0.5 ml. inoculated intravenously into 14 gm. Swiss mice. The results are shown in Table II.

TABLE I

The Amount of Agent in the Chorioallantoic Membrane and in the Allantoic Fluid

Time after inoculation	ID_{50} of	
	Chorioallantoic membrane	Allantoic fluid
0 hour	$10^{-5.8}$	$10^{-7.2}$
1st day	$10^{-5.9}$	$10^{-6.4}$
2nd day	$10^{-7.9}$	$10^{-7.2}$
3rd day	$10^{-8.5}$	$10^{-7.2}$
4th day	$10^{-8.4}$	$10^{-7.5}$
5th day	$10^{-9.0}$	$10^{-8.6}$
6th day	$10^{-8.5}$	$10^{-7.3}$
7th day	$10^{-8.5}$	$10^{-6.8}$

TABLE II

Toxin from Feline Pneumonitis Grown in the Allantoic Cavity and Its Neutralization by Specific Antitoxin

Dilution of toxin	Dilution of antitoxin	Time of death of mouse No.				
		1	2	3	4	5
		hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*
1/3	—	<18	<18	<18	<18	<18
1/6	—	<18	<18	<18	<18	<18
1/12	—	<18	22	25	25	**<88**
1/24	—	<42	**<88**	**<88**	**<88**	**<112**
1/6	1/200	**<66**	S	S	S	S
1/6	1/400	24	24	<42	S	S
1/6	1/800	<16	20	<42	<42	<42
1/6	1/1600	<16	<16	<16	<16	<20

Figures in bold-face type are deaths due to infection rather than toxin.
S indicates survival for 21 days.

Method of Purification of the Agent for Photography.—It was found that most of the agent was present in the sediment after centrifuging for 30 minutes at 3600 R.P.M. in an angle centrifuge.

The infected allantoic fluid, therefore, was purified by the following process. The first centrifugation, in an ordinary centrifuge at 800 R.P.M. for 10 minutes, removed most of the

cells. (Chilling of the eggs for 5 hours before harvesting the fluid prevented bleeding into the allantoic fluid.) The supernate was then spun at 3600 R.P.M. for 30 minutes in the angle centrifuge, and the sediment from this centrifugation was resuspended in saline or in distilled water and recentrifuged at the same speed for 30 minutes. The sediment was resuspended in a small volume of saline or distilled water, concentrating the agent 5 to 10 times. A small drop of this suspension was dried on the collodion-coated screen, and if saline had been used in the process of purification the screens were washed with distilled water. Some preparations were shadowed with gold (2) under vacuum of the order of 0.00003 mm. Hg in a device designed and built by Dr. H. Sidney Newcomer.

Replicas were made by pouring a solution of 1 per cent collodion in amyl acetate over a dried film of the suspension of agent on a clean glass slide. After carefully removing the collodion film by floating it off in water, screens were dropped onto the film, then removed with the adhering film, dried, and shadowed with gold, utilizing the device and method described in the preceding paragraph. Since the elementary bodies themselves are not exposed to high vacuum this technique does not contribute to the distortion of the agent.

The preparations were examined with an RCA electron microscope type EMU.

Morphological Characters

Elementary bodies purified from allantoic fluid (Fig. 1) are similar in appearance to those purified from yolk sac (5). The irregularity of the surface caused by distortion of the body during drying, giving the body the appearance of a wrinkled half pea, is clearly shown in Fig. 2 of gold-shadowed replicas of the elementary bodies, and in Figs. 3 and 4 of the gold-shadowed bodies and the shapes of their shadows. Sometimes the bodies resemble hollow rubber balls with one side punched in (Fig. 5).

These pictures also show that at least two components make up the elementary body, a dense substance, usually centrally located, and a surrounding thinner substance which seems to represent a limiting membrane from which the central mass has shrunken away.

It is possible that these two components exist in the living state in the relation to one another shown in these photographs. However, it is probable that this apparent separation into two components may be due entirely to distortion of the bodies during drying.

Elementary bodies in chains, pairs, and clusters can be seen in Figs. 3, 5, 6, and 7. In clusters, the elementary bodies give the impression of possessing a sticky substance on their surfaces (Fig. 6).

The group of elementary bodies in Fig. 7 appears to be enmeshed in a matrix, composed of strands of material some of which are connected to the elementary bodies. The material in the background is somewhat similar in appearance to the ether-soluble antigen of *Rickettsia* shown in electron micrographs by Shepard and Wyckoff (11).

The average size of the gold-shadowed elementary bodies was calculated from measurements of 92 bodies. The mean diameter is 525 mμ with a standard deviation of $\pm$84 mμ and a standard error of $\pm$5.5 per cent. The size ranges from 303 mμ to 728 mμ, with 73 per cent of the measurements lying between

468 and 572 mμ. This variation in size of the elementary bodies can be seen in Fig. 4. Only a small number of measurements of unshadowed elementary bodies from allantoic fluid were made. From data on 15 elementary bodies, the mean diameter is 479 mμ with a standard deviation of ±44 mμ and a standard error of ±4.88 per cent. This agrees with previous measurements of elementary bodies from yolk sac suspensions, in which the mean diameter was 455 mμ (5).

When the height of the bodies was calculated from the length of the shadows and the angle at which the gold was cast, it varied from 175 mμ to 375 mμ for 23 measurements. This confirms the impression that the agent flattens out on drying and indicates that the figures given here for the mean diameter of the elementary bodies probably differ considerably from the true dimensions in the natural state. In this connection it might be pointed out that another related agent with very similar morphology both with the light and the electron (5) microscopes, *i.e.* that of lymphogranuloma venereum, has been found by direct measurement with a light microscope to be between 200 to 400 mμ in diameter (12, 13).

The impression gained from this study is that the elementary bodies of the agent of feline pneumonitis are either spherical or hemispherical bodies with a limiting membrane. Since they contain a large amount of water, an inference justified by the amount of distortion that occurs during drying, it is difficult to decide, from electron micrographs, what the actual shape of the bodies is.

SUMMARY

The agent of feline pneumonitis has been grown in the allantoic cavity for 50 serial passages. During this time the amount of agent in allantoic fluid increased about 100-fold. The titer of the agent in the allantoic fluid of the individual embryo reached a peak on the 5th day and then declined. Large inocula were required in order to obtain maximal titers. The toxic factor was present in suspensions of chorioallantoic membranes in allantoic fluid from heavily infected eggs, and could be neutralized by the specific antitoxin produced in rabbits by injection of toxin in yolk sac suspensions.

Electron micrographs of the agent of feline pneumonitis grown in the allantoic cavity show that the elementary body is composed of a dense centrally located substance surrounded by a thinner material, part or all of which is the limiting membrane. Separation of these two portions of the body may be due partially or entirely to distortion during drying. The wrinkled surface of the elementary bodies is evidence that such distortion does occur. The average diameter of gold-shadowed bodies is 525 mμ.

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EXPLANATION OF PLATES

PLATE 1

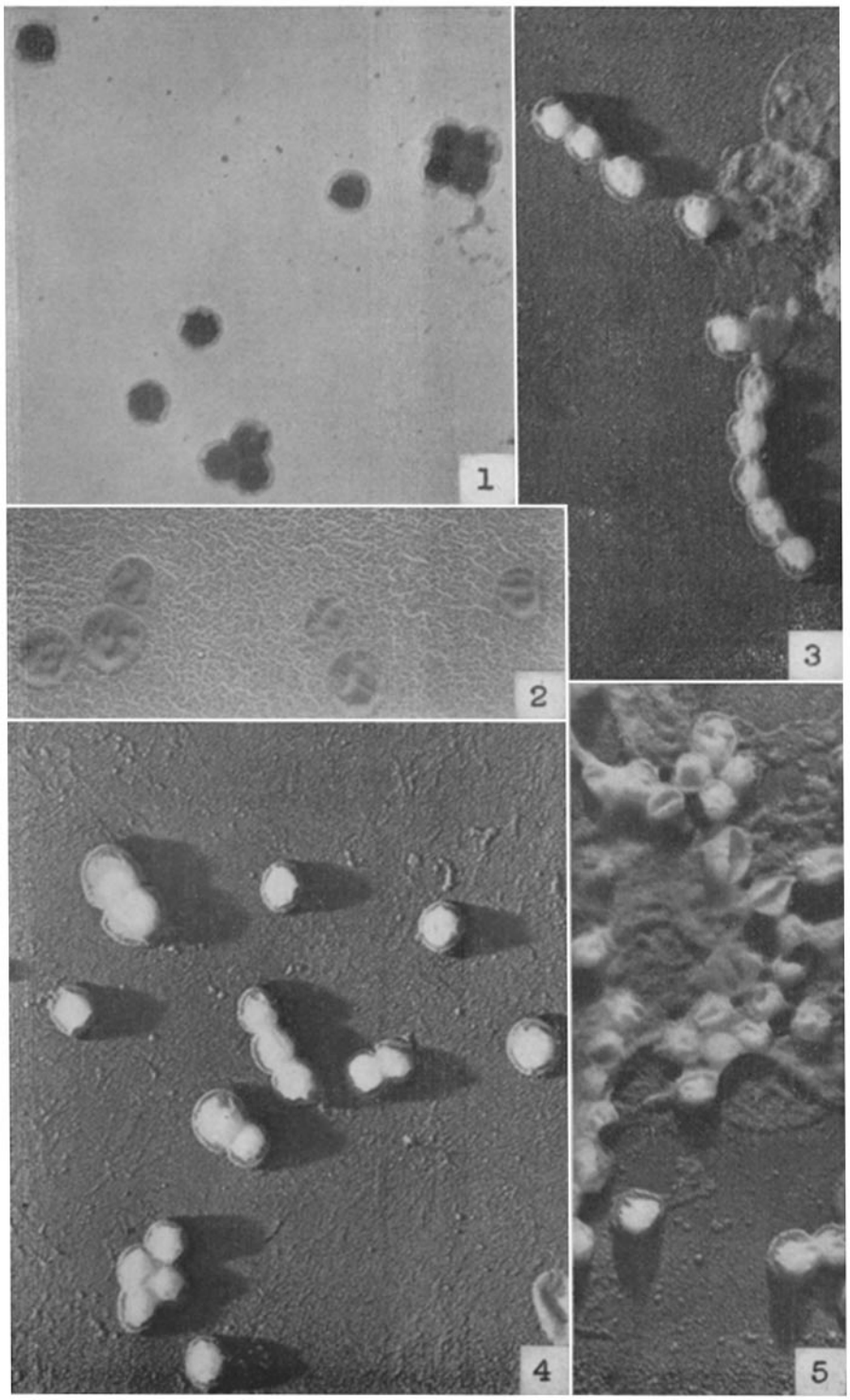
FIG. 1. Elementary bodies not shadowed with gold. × 14,160.

FIG. 2. Replicas of elementary bodies, gold-shadowed, 21.6 mg. of gold, angle 22°, 12 cm. distance. × 14,160.

FIG. 3. Elementary bodies in chains, gold-shadowed, 23.5 mg. of gold, angle 12°, 10 cm. distance. × 14,160.

FIG. 4. Elementary bodies gold-shadowed, 23.5 mg. of gold, angle 12°, 10 cm. distance. × 14,160.

FIG. 5. A group of elementary bodies gold-shadowed, 23.5 mg. of gold, angle 12°, 10 cm. distance. × 14,160.

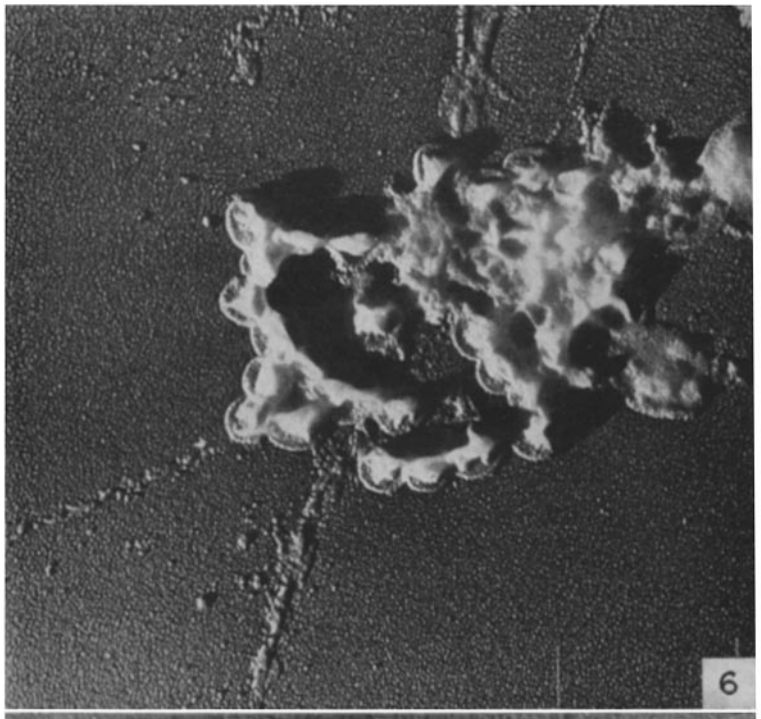


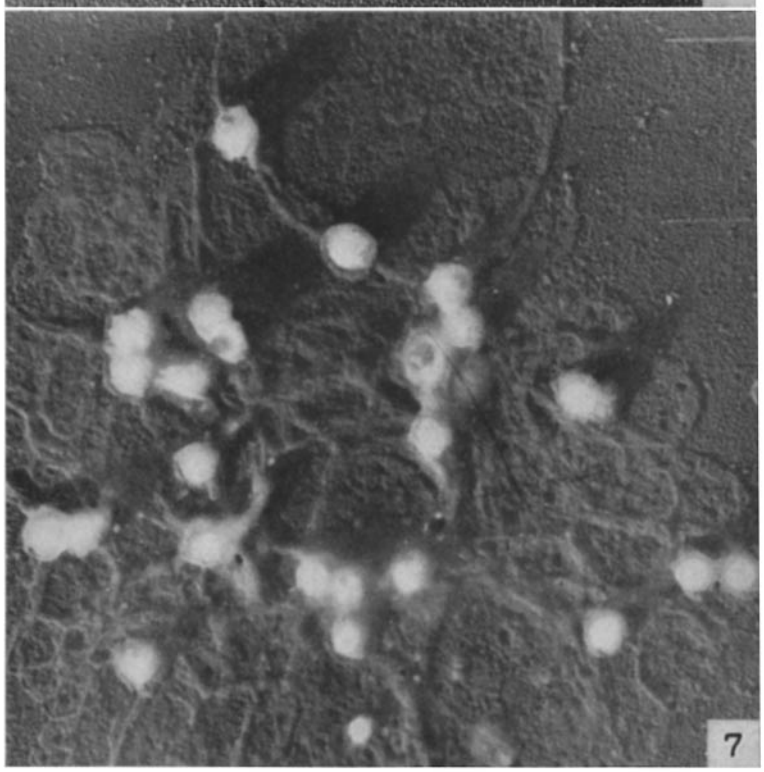
(Hamre, Rake, and Rake: Agent of feline pneumonitis)

PLATE 2

FIG. 6. Group of elementary bodies gold-shadowed, 21.7 mg. of gold, angle 11°. 10 cm. distance. × 14,160.

FIG. 7. Elementary bodies enmeshed in a matrix, gold-shadowed, 23.5 mg. of gold, angle 12°, 10 cm. distance. × 14,160.





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