Cultivation of *Coccidioides immitis* in the Developing Hen's Egg. (19787)

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The tissue, or sporangial, phase of *Coccidioides immitis* has been reported capable of forming in developing hen's eggs. The first report described the appearance of the tissue phase after inoculation of the mycelial phase onto the chorioallantoic membrane(4). The formation of the sporangia in embryo, albumen, yolk and embryonic fluids was also reported(3). Brueck and Buddingh(2) were unable to repeat the above work, but noted the ready formation of the tissue phase in the yolk of developing chick embryos.

In view of these contradictory results, investigations were initiated to determine under what conditions maximum numbers of sporangia developed in the fertile hen's egg. In this paper, experiments are described relating to the optimum age for inoculation of the fertile hen's eggs, the death rate of the inoculated eggs, and the formation of the tissue phase.

Methods. Three- to 12-day-old embryos were chosen and inoculated via either the yolk sac, allantoic sac, or the chorioallantoic membrane routes. The methods employed were essentially those described by Beveridge and Burnet(1). The yolk sac inoculations were made through a small hole punched in the center of the broad end of the egg. Through this a 1¼-inch, 22 gauge, hypodermic needle was thrust past the air sac into the yolk sac. The allantoic sac inoculations were made with a similar needle and through a similar hole punched in the side of the broad end of the egg, along the margin of the air sac. The needle was inserted only a short distance as compared with the full length insertion for the yolk sac inoculation. The inoculum for both routes of inoculation consisted of a 0.1 ml suspension of *C. immitis* spores and hyphal elements in physiologic saline. The chorion side of the chorioallantois was inoculated, using the dropped membrane technic and the same inoculum (0.1 ml) as above. The openings were sealed with a collodion-iodine mixture and the eggs incubated at 96° or 98°F. The eggs were candled twice daily. Physiologic saline suspensions of mature spore-forming cultures of *C. immitis*, strain No. 2150, received from Dr. Norman F. Conant, were used throughout the project.

Results. Effect of embryo age on mortality after infection. The mortality of the inoculated eggs depends more upon the age of the embryo than the route of inoculation (Table I). In developing eggs inoculated via the yolk sac, allantoic sac, or chorioallantoic membrane, 3-, 5-, 7-, and 8-day-old eggs showed an especially high mortality. By the end of the first 5 days of incubation all the embryos in the 3-day series were dead. Dead, also, were 91% of the 5-day eggs, 80% of the 7-day eggs, and 73% of the 8-day eggs. Since smears and cultures were made of eggs from all series, it was possible to rule out secondary bacterial infections as being of importance in these mortality figures. In contrast to 3- to 8-day eggs, the 9- to 12-day eggs showed a low mortality. Of the 9- to 12-day eggs examined during the same 5-day period after inoculation, only 24, 19, 15, and 7%, respectively, died (Table I). Throughout this 5-day period several live eggs were removed for examination. It should be realized then, that these percentages are approximate, and possibly would have been different had these living eggs not been selected for study.

In yolk sac inoculations, the number of deaths for eggs of all ages was low on the first day after infection, ranging from 0 to 12%. This may represent partly nonspecific deaths. Thereafter, the number of deaths increased markedly, reaching a peak on the third day in the 3-, 5-, 7-, and 8-day-old eggs. A much lower peak was noted on the second day, maintained through the third day in 9-day-old eggs.

In the series of eggs inoculated via the

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TABLE I. Mortality of 3- to 12-Day-Old Developing Hens' Eggs after Inoculation with Standard Spore Suspension of *C. immitis* via One of Several Routes.

Age of eggs, days	No. inoculated and route	No. eggs dying 1st-5th day					Total No. dead	% dead end of 5 days' incubation
		1st	2nd	3rd	4th	5th		
3	22 YS*	0	8	13	1		22/22†	100
5	45 "	1	6	33	1		41/45	91
7	56 "	2	7	27	7	2	45/56	80
	25 AS	0	2	7	10	1	20/25	80
8	275 YS	27	44	85	41	4	201/275	73
	60 C-A m	11	8	10	13	2	44/60	73
9	49 YS	2	4	4	2		12/49	24
10	26 "	3	1	0	1		5/26	19
11	44 "	3	1	2	1		7/44	15
12	28 "	1	0	0	1		2/28	7
Total	630							

* YS = yolk sac. AS = allantoic sac. m = membrane.
† Numerator indicates total number of eggs dead; denominator, total number of eggs inoculated.

allantoic cavity no deaths were evident on the first day after infection; whereas, in the series inoculated onto the chorioallantoic membrane 18% deaths were noted. There is some evidence in these 2 series of eggs that the greatest number of deaths occurred on the fourth day, in contrast to the third day in yolk sac inoculations. However, the overall mortality in the 5-day incubation period did not differ appreciably from that of the yolk sac group.

From the data of Table I, it seems that 3- to 8-day old embryos were more susceptible to infection by *C. immitis* than 9- to 12-day-old embryos, in that a higher mortality occurred in shorter periods of time. For studies of the formation of the tissue phase, however, it may be disadvantageous to have a host with a short survival time after inoculation, especially if more than 5 days are required for ready observations of the sporangia. However, it was increasingly difficult to observe the fungus in the older (9- to 12-day) eggs. Hence, studies of the tissue phase were largely but not entirely limited to 8-day-old eggs.‡

Distribution of C. immitis in eggs. The yolk sac, allantoic sac, and chorioallantoic membrane were inoculated with suspensions of spores and short hyphal elements in physiologic saline. The resulting infection was followed by examining fresh smears of the yolk, allantoic fluid, amniotic fluid, and when indicated, albumen. Both living and dead eggs of all available eggs were studied daily.

As studied in smears, in the yolk sac inoculations the mycelial phase increased during the first 3-4 days after infection. On the third day, 30% of the living eggs, and an even slightly larger number of the dead eggs, showed mycelium in the yolk fluid. This percentage decreased after the fourth day, and it was often difficult to demonstrate *C. immitis* mycelium on the fifth, sixth, and seventh days after infection. Some smears of the fourth and later days showed mycelium which appeared to be degenerated.

In the 2 series of eggs inoculated in the allantois and onto the chorioallantois, it was unusual to find mycelium at the site of inoculation after the second day of incubation. However, if the air sac became infected mycelium was more likely to be found in the allantoic fluid. One striking feature of the allantoic and chorioallantoic inoculations was that mycelium was often found in the yolk by the third day, a phenomenon which indicated that the fungus could penetrate the extra-embryonic membranes and yolk sac, and also that the yolk fluid was probably a preferable medium in the egg.

Regardless which of the 3 routes of inocu-

‡ In a recent paper, published since the completion of this study, R. A. Vogel and N. F. Conant have shown that sporangia could be obtained in abundance by serial inoculation of the yolk sac of 10-day-old embryos. PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 544.

lation was used, mycelium often could be found growing on the air sac membrane, and was usually present on both surfaces. Such colonies might have arisen as the result of contamination in the case of yolk sac inoculations and from extension in the case of allantois and chorioallantoic-membrane inoculations.

Formation of sporangia. Sporangia, or sporangia-like, bodies have been found to be produced by some *C. immitis* mycelium during the third, fourth, and fifth days of incubation. Whether these bodies were formed at a later period is not known, for few infected older eggs were available for study. The sporangia-like bodies occurred in yolk fluid both of living and of dead eggs. In a few instances, slides which were prepared of positive (mycelium) yolk were found to contain some sporangial initials after being sealed and placed in a slide box for 2-7 days. These sporangial initials were formed after the slide mount was prepared. No sporangia were observed in positive yolk fluid transferred to test tubes, layered with mineral oil and incubated at 37°C and room temperature.

Most of the sporangia-like bodies observed were formed by the mycelium. A few, unattached to the mycelium, may have arisen from spores or short hyphal filaments. It was often difficult to be certain of the identity of these free cells, since some of the normal components of yolk fluid resemble them so closely. The sporangial initials were formed intercalarily, laterally, and terminally. They began as swellings, often uneven in appearance, which contained a thin granular cytoplasm. Vacuoles were formed in this cytoplasm early in the development of the cell and could be seen to move about, increase and diminish in size. The cell wall appeared to remain plastic for a considerable period of time, and was thin-walled compared with that of the sporangia formed in animal tissue. Eventually, cross walls were developed to demarcate the sporangial initial from the mycelium. What has been interpreted as cleavage was a rapid process requiring but a few minutes. The cleaved cytoplasm rounded into endospore-like bodies which usually were of similar size; these increased somewhat in diameter. Mature organisms varied greatly in size, but most averaged about 10-15 μ in diameter.

Discussion. It seems that in developing hen's eggs, yolk is a more satisfactory medium for the formation of the tissue phase than allantoic fluid, amniotic fluid or albumen. As demonstrated by smears, sporangia are formed in some eggs by the end of the fifth day of incubation; however, these sporangia are often few in number and sometimes difficult to differentiate from the normal components of the yolk.

It appears that the mortality of the inoculated eggs is more dependent upon the age of the embryo than on the route of inoculation. This is well brought out by the figures in Table I, which indicates a striking decrease in mortality with corresponding increase in age of inoculated embryos. The percentage deaths of 7- and 8-day-old embryos in eggs inoculated via the allantoic and chorioallantoic membrane was almost identical with the percentage of deaths of the same age groups inoculated via the yolk sac.

The basis for the marked decrease in mortality and apparent susceptibility to infection of eggs 9 days of age and older has not been investigated. The 70%, or greater, mortality of 3- to 8-day eggs in a 5-day period is decidedly higher than the mortality for 9- to 12-day-old eggs, which is less than 25%. The high mortality rate of the 3- to 8-day eggs may make this age group most suitable for testing in chemotherapeutic studies of coccidioidomycosis.

Summary. 1. Six hundred and thirty 3- to 12-day chick embryos were inoculated with a suspension of the mycelial phase of *C. immitis* in physiologic saline, via the yolk sac, allantoic sac, or chorioallantoic membrane routes. 2. The mortality of the inoculated eggs depended more upon the age of the embryo than the route of inoculation: 3- to 8-day embryos showed a mortality of 100-73% in the 5-day period of incubation; whereas, 9- to 12-day embryos showed a mortality of only 24-7%. 3. The distribution of *C. immitis* in inoculated eggs was studied and the formation of sporangia, or sporangia-like, bodies noted. These

bodies are few in number and difficult to interpret.

1. Beveridge, W. I. B., and Burnet, F. M., 1946, Special Rep. Series 256, London, His Majesty's Stationery Office, 92 p.
2. Brueck, J. W., and Buddingh, C. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 258.
3. Burke, R. C., *Trans. N. Y. Acad. Sci.*, Ser. II, 1950, v12, 188.
4. Moore, M., *Am. J. Path.*, 1951, v17, 103.

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Antigenic Similarity Between the First and Later Egg Passage Strains of Freshly Recovered Influenza A Viruses. (19788)

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Analysis of the significance of antigenic variation of influenza viruses in man depends in part on the validity of the assumption that the antigenic properties of strains recovered in the laboratory in unnatural hosts correspond to those of the agents infecting human beings. From this point of view, the use of mice for the recovery or serial passage of influenza viruses appears to be highly undesirable for it is known(1,2) that these agents may show unpredictable changes in antigenic composition when maintained in this host. On the other hand, there is evidence(3) that recently recovered influenza virus strains may be maintained for 6-12 serial passages in the chick embryo without undergoing antigenic variation. The possibility that a change in antigenic composition may occur immediately after recovery during the first few passages in the chick embryo has not been investigated previously.

The present study was undertaken in order to determine whether, after serial embryo passage, influenza virus strains are antigenically similar to or different from the same strains in the 1st egg passage on recovery from throat washings.

Materials and methods. Recovery and serial passage of viruses. During the 1951 epidemic in New York, a number of influenza A virus strains were recovered by inoculation of chick embryos in this laboratory. On the basis of the extent of multiplication obtained during the 1st embryo passage, throat washings from 2 patients (NA and RA) with influenza A were chosen for further study. On 4 different attempts with each throat washing, infection was induced in a considerable proportion of embryos inoculated. The strains of each virus used in the experiments described below were obtained as follows:

NA. 10-day-old chick embryos were inoculated amniotically with 0.2 ml of throat washings from patient NA which contained penicillin, 250 μ, and streptomycin, 2500 μg/ml. After incubation at 35°C for 70 hours, followed by chilling at 4°C overnight, allantoic and amniotic fluids were harvested separately. Agglutination tests with 1% guinea pig RBC (0.5% final) were carried out with fluids from individual eggs employing a final dilution of 1:4. Of 57 amniotic fluids, 12 were positive. All allantoic fluids from these eggs were negative. The hemagglutination titer of the pool of positive amniotic fluids at 24.5°C was 1:64 with guinea pig RBC and 1:8 with chicken RBC. For the preparation of antisera, the pool of positive amniotic fluids was diluted with 5 volumes of amniotic fluids from the same 1st passage eggs which had given negative hemagglutination reactions. 7 serial allantoic passages were carried out in 10- to 11-day chick embryos, starting with a 10^{-1} dilution of the 1st passage amniotic fluid pool and continuing with a 10^{-3} dilution of allantoic fluid at each subsequent passage. Eggs were incubated at 35°C for 48 hours, chilled at 4°C overnight and the allantoic fluids were harvested, pooled, and stored at –65°C.

RA. Primary recovery of this strain from