Minimal Infectious Inoculum of *Histoplasma capsulatum* for Mouse and Chick Embryo. (24110)

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Until Emmons(1) first isolated *Histoplasma capsulatum* from the soil, little was known about its habitat and mode of distribution in nature. Following its isolation from the soil it was hypothecated that the fungus may be carried with dust and that it probably enters the host through the respiratory tract. Later studies by Ajello and Zeidberg(2) Grayston *et al.*(3), Ibach *et al.*(4) have substantiated this hypothesis especially with dust and debris associated with chicken houses, silos and storm cellars. Since the yeast phase has been shown by laboratory studies to be very sensitive to environmental conditions, the fungus is thought to exist in nature in the mycelial phase. Histoplasmosis has been produced in animals by inoculating with the yeast phase or with portions from the entire mycelial phase. Few qualitative or quantitative studies have been made to determine the actual constituents of the mycelial phase used as the inoculum. For these reasons it has been difficult to establish unequivocally the actual fragment portions of the fungus causing infection in laboratory animals. There are many predisposing factors which influence infectivity of the mycelial phase, one of which is the amount of the inoculum. The determination of the minimal inoculum to induce infection in animals has been adequately documented for only the macroconidium of the fungus(5,6). The regularity with which animals can be infected with fragments of the mycelial phase has not been reported. This investigation was undertaken to determine, if possible, the minimal inoculum necessary to induce infection and the regularity with which animals can be infected with this amount of inoculum. A single spore or fragment of the mycelial stage of the fungus was taken as the smallest practical amount of inoculum to use. On this basis animals were injected with single particles as follows: (1) non-germinated tuberculated macroconidia, (2) germinated tuberculated macroconidia, (3) non-germinated microconidia, (4) germinated microconidia, and in addition(5) a non-branching mycelial fragment.

Materials and methods. The strain of the organism used in the first series of experiments was a culture of the Ellis isolate of *H. capsulatum* which had been grown on Sabouraud's glucose agar and allowed to remain on the same medium for 3 months without transfer. In the second series of experiments, in which only chick embryos were inoculated, the Ellis isolate had been grown on corn grains and allowed to remain on the same grains for 3 years. Each of the 5 types of inocula from the mycelial phase was isolated using a Chambers micromanipulator. The mycelial phase of the fungus was suspended in a drop of Sabouraud's broth which contained 40 μg streptomycin and 20 units penicillin per ml. A moisture chamber as described by Richter (7), with addition of 2 small copper capillary tube holders, was used to prevent evaporation of the hanging drop. Each unit of inoculum was introduced into a sterile glass capillary tube which was placed aseptically inside the shaft of a one inch, 21 gauge hypodermic needle. A small ball of semi-firm rubber cement was placed in the needle shank to prevent leakage. The needle was attached to a Leur Loc syringe which contained 40 μg streptomycin and 20 units of penicillin per ml of sterile saline. Each unit of inoculum was observed microscopically when placed in the capillary tube just prior to inoculation. The unit was placed toward the center of the capillary tube to minimize possibility of loss prior to inoculation. After each inoculation the capillary tube was examined microscopically for the presence of the inoculum. Since the inoculum was never found in the capillary tube it was assumed that it had been transferred

to the host and that infection, if any, was the result of the inoculation. Each white Swiss mouse was inoculated intraperitoneally. The capillary tube containing the inoculum was always placed a small distance back of the point of the needle to prevent breakage or loss of the inoculum during the inoculation procedure. Mice dying within 72 hours after inoculation were considered as traumatic deaths and discarded. Any animal dying after the third day was autopsied and the liver, spleen and adrenals were removed and ground separately in a sterile mortar using sterile sand and saline. One ml of each tissue and extract was streaked separately onto one blood agar plate and on each of 2 Sabouraud's agar plates which contained 40 μg streptomycin, 20 units of penicillin, and 1.5 mg of actidione per ml of medium. The remainder of the mice were maintained for 8 weeks, then sacrificed and autopsied. The liver, spleen and adrenals of these mice were treated as above and cultured separately on Sabouraud's agar. The same procedure was followed for the control mice which had been inoculated with sterile saline which contained the antibiotics. The yolk sac of 5-day-old White Leghorn embryos was inoculated aseptically through a perforation in the shell over the air sac. The perforation was sealed with collodion. The eggs were incubated at 33°C in a Bower's humidaire incubator automatically set to rotate every 6 hours. The eggs were candled daily. The eggs containing dead embryos were discarded on the third day. Embryos which died after the third day were triturated and streaked on Sabouraud's and blood agar containing the above listed antibiotics. After 18 days incubation the living embryos were sacrificed, autopsied, and the liver, spleen, and adrenals were triturated and portions of each streaked on modified Sabouraud's agar. When these organs had not developed, the complete embryo was triturated and streaked. A series of embryos was inoculated with sterile saline and antibiotic solution. These embryos were cultured using the same procedure as above and were used as controls. Impression smears were made of various tissues of mice and chick embryos, including yolk sac. These smears were stained with Giemsa and observed under oil immersion for yeast-like type of cells of the fungus. Mouse and chick embryo tissues were incubated at room temperature for 6 weeks on the artificial media and examined periodically for the presence of the fungus.

Results. The percentage of white mice and chick embryos infected by single particles of inoculum from the 3 months' culture of *H. capsulatum* grown on Sabouraud's glucose agar is presented in Table I. The results indicate that infection in mice takes place following injection with a single spore of each type used but not with the non-branching mycelial fragments. Non-germinated tuberculated macroconidia proved the most and non-germinated microconidia the least infective of the spores. In chick embryos the germinated tuberculated macroconidia were 3.6 times more infective than were non-germinated macroconidia, just the reverse of that found in white Swiss mouse.

Results from chick embryos inoculated with single units of inoculum from a 3-year-old culture of fungus grown on corn grains are shown in Table II. Each type of inoculum was capable of causing experimental histoplasmosis.

TABLE I. Percentage of White Mice and Chick Embryos Infected by Single Particle Inoculum of 3-Months-Old Culture of the Ellis Isolate of *Histoplasma capsulatum.*

Units inoculated	White mouse		Chick embryo	
	Infective ratio	% infected	Infective ratio	% infected
Non-germinated tuberculated macroconidia	4/48	8.2	0/40	.0
Non-germinated microconidia	1/47	2.1	3/42	7.1
Germinated tuberculated macroconidia	3/48	6.2	7/51	13.7
Germinated microconidia	1/19	5.3	3/51	5.9
Non-branching mycelial fragments	0/20	.0	4/28	14.3
Total	9/182	5.0	17/212	8.0

x/n = No. of animals infected/No. of animals inoculated.

TABLE II. Percentage of Chick Embryos Infected by Single Particle Inoculum of 3-Year-Old Culture of Ellis Isolate of *Histoplasma capsulatum*.

Units inoculated	Infective ratio	% infected
Non-germinated tuberculated macroconidia	4/73	5.5
Non-germinating microconidia	6/69	8.7
Germinating tuberculated macroconidia	4/65	6.2
Germinating microconidia	6/71	8.5
Non-branching mycelial fragments	9/81	11.1
Total	29/359	8.1

x/n = No. of chick embryos infected/No. of chick embryos inoculated.

The non-germinated tuberculated macroconidia were the least infective of the inocula used. Microconidia proved to be more infective than macroconidia and there was no significant difference between germinated and non-germinated microconidia. In these, as in earlier studies, the mycelial fragments were the most infective in White Leghorn chick embryos.

In addition to white Swiss mouse and leghorn embryos, 2 other series of experiments were completed using a hybrid chick embryo and a C57 strain of black mice. In one instance hybrid fertile eggs were inadvertently supplied and these were inoculated prior to knowledge of the error. This hybrid was a cross between White Leghorn cockerel and New Hampshire Red hen. Although the same technics were followed in inoculating and culturing the hybrid embryos, striking differences in infectivity of the particles occurred. Of the 129 hybrid embryos living 72 hours after inoculation only 4 developed an infection. The mycelial fragment was the only infective particle in that 4 of 28 embryos yielded the fungus on culture. No infection occurred in the C57 strain of black mice inoculated with germinated tuberculated macroconidia. Nineteen of these animals received the inoculum intraperitoneally and 15 by inhalation route. Black mice were inoculated since it has been stated that pigmented animals are more susceptible to certain pathogenic fungi than are the white strains (Personal communication from N. F. Conant).

Tissue impression smears from autopsied animals were made with the hope of determining the fate of the inoculated particles. In many of the stained smears, especially the yolk sac preparations, yeast-like cells were observed using the oil immersion lens. These findings indicate that the mycelial phase does not lay dormant in the animals only to germinate when tissues were cultured on artificial media. Similar observations were made in our earlier study(6) in which yeast-like cells were photographed in tissue smears and sections.

Non-germinated tuberculated macroconidia were individually placed on artificial media to check rate of germination. Quadrant plates were used and presence of spores was ascertained by microscopical observation. More than 150 single macroconidia were studied and in no case did one of these spores germinate. These results indicate that the germination rate of *H. capsulatum* single spores is extremely low.

Discussion. Few studies have been made using a single spore or a fragment of the hypha of *H. capsulatum* to infect animals. Ajello and Runyon(5) reported implantation of single tuberculated macroconidia on agar plugs into the peritoneal cavity of mice. This method proved highly successful in that nearly 100% of the mice were infected. No statement was made by authors as to whether macroconidia were germinated or non-germinated. In another study Larsh *et al.*(6) reported successful infection of chick embryos with a small number of spores as determined by hemacytometer and viability counts. These infection rates were correlated with viability of the inoculum on potato dextrose agar plates. An infectivity rate as high as 41% was reported when 2 spores were used. These investigators made no attempt to produce infection in animals by injection of a single tuberculated macroconidium.

The results reported herein give the first experimental evidence of infectivity of *H. capsulatum* single units of inoculum other than tuberculated macroconidia. To eliminate the inherent criticism of establishing unequivocally the size of the inoculum for these infectivity studies, a Chambers micromanipulator

was used. It was feasible to isolate single fungal elements with this instrument and to place them in sterile glass capillary tubes for inoculation. Presence of the inoculum in the tube was verified in each instance by microscopical observations. In addition, post-inoculation, the capillary was examined microscopically to ascertain presence or absence of the inoculum. This procedure proved tedious, time-consuming, and painstaking one but resulted in establishing the fact that a single spore or fragment of hypha would bring about infection.

Although inoculation of mice and chick embryos was done using aseptic technic, to aid in preventing bacterial contamination of the inoculum, streptomycin and penicillin were included in the collecting and inoculating fluids. This procedure was followed since the content, especially of yolk sacs, was a good medium for growth of contaminating organisms. In the control series it was learned that these antibiotics did not have any apparent deleterious effect upon embryos, white mouse, or growth of fungus. No deaths of animals could be attributed to contaminating organisms. The inoculation procedure proved highly successful and the method did not offer any physical protection to the infectious agents. The use of extraneous material in the inoculum may in part account for the extremely high infectivity rates reported by other investigators.

The macroconidia were more infective than microconidia in White Swiss mice. The differences in infectivity rate of the 2 types of macroconidia were not too significant but the germinated microconidia were 2.5 times more infective than were non-germinated microconidia. In all mouse series the number of infected animals was small and no trend of enhanced invasiveness of any unit was apparent.

It is of particular interest that in the 2 series of chick embryos, inocula of different ages were used, nevertheless, total infectivity in each instance was strikingly similar. The sources of fertile eggs were different but were all from White Leghorn hens. In both chick embryo series the non-branching mycelial fragments produced highest infectivity rate. In the case of 3-year-old corn grain culture this finding would seem to be of significance and may indicate what happens to the fungus in nature.

Summary. (1) Individual infectious particles were isolated from the mycelial phase of *H. capsulatum* using Chambers micromanipulator. The particles consisted of germinated and non-germinated tuberculated macroconidia, germinated and non-germinated microconidia and non-branching mycelial fragments. Each particle was placed into a sterile capillary tube inserted into a 21 gauge sterile inoculating needle. Aseptic technics were followed, in addition, streptomycin and penicillin were added to the collecting and inoculating fluids. Microscopical examination of each capillary tube was made prior to and immediately after inoculation procedure to ascertain the disposition of the particle. Fertile Leghorn eggs and the white Swiss mouse were the experimental animals. (2) Infection resulted from all particles with one exception, mycelial fragments, in the mouse. The total percentage of infectivity in the 182 inoculated mice was 5%. Of the 571 inoculated chick embryos approximately 8% became infected. The most infectious particle was the mycelial fragment. (3) Inoculation of single particles from the mycelial phase of *H. capsulatum* proved that the minimal infection inoculum can be units other than the macroconidium. In each instance the infectivity rate was relatively small but no physical protection was given the inoculated particle.

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