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INFECTION OF WHITE SWISS MICE WITH AIRBORNE *CRYPTOCOCCUS NEOFORMANS*

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ABSTRACT

SMITH, C. D. (Communicable Disease Center, Kansas City, Kansas), R. RITTER, H. W. LARSH, AND M. L. FURCOLOW. Infection of white Swiss mice with airborne *Cryptococcus neoformans*. J. Bacteriol. **87**:1364–1368. 1964.—A group of 39 white Swiss male mice were allowed to run 3 days on previously sterilized soil that had been seeded with *Cryptococcus neoformans* 1 year previously. It was determined that the soil contained an average of 1.6×10^6 viable yeast cells per ml. The mice were observed for 24 weeks, at which time the survivors were necropsied. The total mortality rate during this period was 44%; 67% of the mice had positive cultures, including all who had a fatal infection. Two additional experiments were done with the same strain with the use of aerosols produced by a Henderson apparatus. In experiment 1, 39 mice received 11,000 viable cells each; in experiment 2, 40 mice received 14,622 viable cells. Both groups of mice showed similar mortality rates, with a total of 77% dead after 20 weeks in experiment 1, and 80% in experiment 2. All of the mice in experiment 1 had positive cultures, as did 95% of the mice in experiment 2.

The occurrence of *Crytococcus neoformans* in nature is common and widespread; isolations have been reported from many sources throughout the world, including peaches, milk, normal skin, and human gastrointestinal tract (Littman and Zimmerman, 1956). Many of the isolates from nature appear to have the same degree of virulence for mice as do those obtained from fatal cases of cryptococcal meningitis in man (Hasenclever and Emmons, 1963). Recently, frequent isolations were made from weathered pigeon droppings or pigeon nests (Emmons, 1955; Littman and Schneierson, 1959; Kao and Schwarz, 1957; Emmons, 1960; Yamamoto, Ishida, and Soto, 1957). *C. neoformans* was isolated in this laboratory from dried starling droppings accumulated on the metal beams of a steel mill.

The opportunity for human contact with the fungus appears to be readily available, but the number of reported cases is relatively low. The cases that are reported are often not recognized until late in the disease and are often fatal, especially if the meninges are involved. Human infection with *C. neoformans*, therefore, may be more common than is recognized, with the majority of cases being relatively mild and asymptomatic with rapid recovery; or, man may not be as susceptible a host to the fungus as are some laboratory animals, and infections may be rare though exposures are common.

The respiratory tract seems to be the major portal of entry of the *Cryptococcus* under natural conditions. Hence, it would seem that this route of inoculation for infection studies should simulate naturally occurring cryptococcosis. Previous experimental inoculations were done by more artificial routes, such as subcutaneous, intraperitoneal, intravenous, or by intranasal instillation (Wade and Stevenson, 1941; Cox and Tolhurst, 1946; Kligman and Weidman, 1949; Hasenclever and Mitchell, 1960; Ritter and Larsh, 1963; Abrahams and Gilleran, 1960).

Two methods of respiratory exposure were used in this study, one exposing mice to loam soil seeded with *C. neoformans*, and the other exposing mice in a Henderson (1952) apparatus to an aerosol containing the organism.

MATERIALS AND METHODS

Soil exposure. Seeded loam soil cultures were prepared as follows. Soil was spread in a thin layer about 0.25 in. deep in a covered metal pan. On 3 consecutive days, this was autoclaved for 45 min at a pressure of 15 psi. Moisture in the soil was maintained by adding distilled water, but not to the extent of flooding the soil. Sterile soil (100 ml) was placed into each of four 250-ml Erlenmeyer flasks fitted with loose cotton plugs wrapped with gauze. The flasks were placed inside

humidity chambers for growth of the fungus. These chambers consisted of half-gallon, wide-mouth pickle jars with screw-cap lids. To maintain humidity, 100 ml of distilled water were placed in the bottom of each jar before the flasks were inserted. The pickle jars containing water and flasks of soil were autoclaved for 15 min at a pressure of 15 psi.

The strain used for the inoculum was isolated from a human case of cryptococcosis (M10-B). The strain was grown on Sabouraud Dextrose Agar slants at 37 C for 3 days, and was then harvested by scraping the cells from the surface of the agar with a wire loop. On the surface of each flask of sterilized soil, 1 ml of a suspension of the cells in saline (comparable in density to a no. 3 McFarland tube) was inoculated. The jars were capped and held at room temperature for 1 year. When the jars were opened, the soil was mixed in the flasks by hand shaking. There was no visible growth present. From each flask 1 ml of soil was removed. Serial dilutions in saline were cultured for viable cells on Sabouraud Dextrose Agar plates incubated for 4 days at 37 C. Each of the four soils was found to contain over 1.5×10^6 viable cells of *C. neoformans* per ml based on colony counts, with an average of 1.6×10^6 per ml of soil. The soil from each flask was placed in a stainless-steel mouse cage, and was allowed to dry for 4 days in a biological safety cabinet at room temperature.

A total of 40 white Swiss male mice, approximately 4 weeks of age, were placed in four cages, 10 to a cage, and were allowed to run on the seeded soil for 4 days. To create optimal conditions for aerosol production, water was withheld from the animals during the first 2 days to keep the soil as dry as possible. The mice were then removed, placed in sterile cages, with 8 mice in each cage, and observed for 24 weeks. All mice which died during the experiment, as well as all the mice remaining at the termination, were necropsied; cultures were made of lung, liver-spleen combined, and brain. These tissues were homogenized by grinding in a mortar, with the aid of sterile sea sand. An approximate 1:10 dilution of the homogenized tissues was made with 0.1% cysteine in physiological saline (pH 6.5); 1 ml of each tissue suspension was plated on a blood-agar base (5% blood) and a Sabouraud Dextrose Agar plate. The plates were incubated for 1 week at 37 C, and the results were recorded.

Henderson aerosol exposure. The aerosols used were produced with a Henderson apparatus. The techniques and the formula used for the determination of the spray factors and suspension concentrations were similar to those outlined by Elberg and Henderson (1948). Porton all-glass short-stem impingers containing cysteine saline were used as the samplers, and a Collison nebulizer was used as the atomizing instrument.

White Swiss male mice, 5 weeks of age and weighing 15 to 18 g, were used in two similar experiments.

The inocula for both Henderson exposures were prepared with the use of the same strain of *C. neoformans* that was used for the soil exposure previously described. Stoppered 250-ml Erlenmeyer flasks containing 35 ml of Sabouraud Dextrose Agar were inoculated with the fungus and incubated at 37 C for 48 hr. The yeast cells were harvested by adding 0.067 M phosphate-buffered saline (pH 7.0) to the flasks and by swirling gently by hand until most of the growth was in suspension. This suspension was washed three times and resuspended in fresh buffered saline.

Experiment 1. The washed suspension of cells was diluted in phosphate-buffered saline to obtain 1.4×10^7 cells per ml by hemocytometer count. A 1-ml sample of this suspension was diluted serially in buffered saline to obtain a suspension of 140 cells per ml; 0.5 ml of this suspension was plated on each of ten Sabouraud Dextrose Agar plates. The plates were incubated for 7 days at 37 C, and colony counts were done for the purpose of determining the viability of the cell suspensions. The counts averaged 77 colonies per plate, instead of the expected 70 colonies per plate. This probably reflects a slight variation in either the cell counts or dilution technique.

The mice were exposed for 20 min in a Henderson apparatus. When the viability results were obtained, calculations showed that each mouse received 11,000 viable cells of *C. neoformans* in the challenge.

One mouse died as a result of mechanical injury during this experiment, and was excluded from the calculations. After exposure, the mice were observed for 20 weeks. Mice that died during this time were necropsied, and cultures were made of the liver and spleen combined and of the brain and lungs separately, with the same technique described previously. After 20 weeks, the remain-

ing mice were necropsied and examined as above. A weekly cumulative mortality rate based on the number of mice that died each week was determined.

Experiment 2. After the results of the first Henderson exposure experiment were obtained, another experiment was performed under similar conditions. The main difference between the two experiments is that in the second experiment each mouse received 14,622 viable cells compared with 11,000 in the first.

RESULTS

Soil exposure. The cumulative per cent mortality of the mice exposed to soil seeded with *C. neoformans* is shown in Fig. 1. The mortality rate was low the first few weeks, reaching only 5% by the 8th week. An accelerated rate begins at the 8th week and appears to level off at the 16th week at 38%. Only 6% died during the last 8 weeks.

The percentage of positive cultures obtained from the mice that were exposed to soil is shown in Table 1. All of the mice that died, and nine additional mice, yielded positive cultures of *C. neoformans*. Thus, although only 44% had fatal infections, 67% had positive cultures.

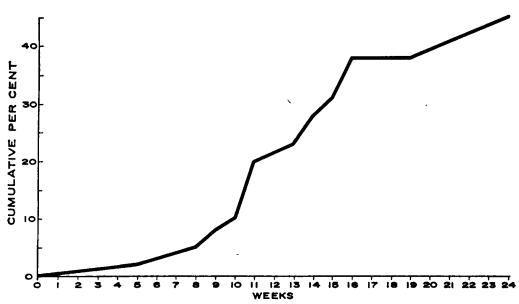

FIG. 1. *Cumulative per cent mortality of mice exposed on sterilized soil inoculated with Cryptococcus neoformans.*

TABLE 1. *Comparison of percentage of positive cultures of Cryptococcus neoformans obtained from organs of mice exposed to seeded soil containing an average of 1.6 × 10⁶ viable cells per ml*

Total no.	Brain		Liver-spleen		Lung		Total positive	
	No.	%	No.	%	No.	%	No.	%
39	21	54	22	56	23	59	26	67

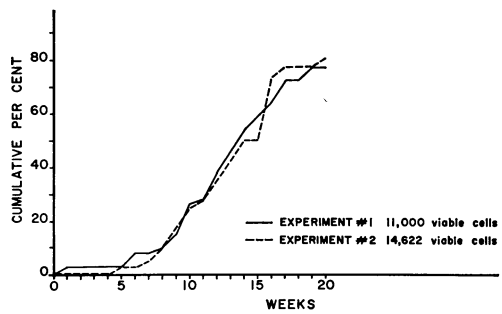

FIG. 2. *Cumulative per cent mortality of mice inoculated with Cryptococcus neoformans by the respiratory route with the use of a Henderson apparatus.*

TABLE 2. *Comparison of percentage of positive cultures of Cryptococcus neoformans obtained from organs of mice infected by aerosols produced with a Henderson apparatus*

Expt	Viable cells	Total no.	Brain		Liver-spleen		Lung		Total positive	
			No.	%	No.	%	No.	%	No.	%
1	11,000	39	31	79	33	85	39	100	39	100
2	14,622	40	31	78	35	88	38	95	38	95

Henderson exposure. The cumulative per cent mortality for both Henderson exposure groups of mice is shown in Fig. 2. Both experiments had similar mortality rates, which were less than 10% up to the 7th week, then climbed rapidly until the 16th or 17th week, and gradually tapered off toward the end of the experiments at 20 weeks. By the end of the 20th week, 77% of the mice in experiment 1, and 80% in experiment 2, were dead.

The number and per cent of positive cultures obtained from the mice in each experiment were also similar (Table 2). The largest number of isolations of *C. neoformans* was obtained from the lungs in both experiments. In experiment 1, all of the mice had positive lung cultures for *C. neoformans;* 95% in experiment 2 also had positive lung cultures.

Liver and spleen cultures yielded *C. neoformans* in 85% of the mice in experiment 1 and 88% in experiment 2. *C. neoformans* was cultured from

brain tissues in 79% of the mice in experiment 1 and 78% in experiment 2.

DISCUSSION

The two methods of exposure of mice to airborne *C. neoformans* showed that the mouse is very susceptible to infection when the fungi are inhaled under simulated natural conditions.

The mortality rates of the mice were similar in both methods of exposure. The mortality of the soil exposure group accelerated about the 8th week and leveled off at the 16th week. Similarly, the mice in the Henderson exposure group began a rapid rise in mortality rate at the 8th week which lasted through the 16th week.

The soil exposure group showed a total mortality of 44% by the 24th week. However, if one considered that only 67% of the mice were in-infected, as shown by positive cultures, then the mortality for the infected mice would be 66%.

The first Henderson aerosol exposure group showed 77% mortality of the mice, and all the remaining animals showed positive cultures. The second Henderson aerosol exposure group had 80% total mortality, and all but two of the remaining mice showed positive cultures.

There was little difference noted in experiment 1 compared with experiment 2 in the mortality rate or positive cultures obtained among the two groups, although the mice in experiment 2 received 3,622 more viable cells of *C. neoformans*.

In the soil-exposure mice that yielded positive cultures, isolations were obtained from all of the organs cultured, with the exception of seven animals. Three were positive from lungs only, two from lungs and liver and spleen, one from lungs and brain, and one from the brain tissue only.

In both experiments with the Henderson aerosol-exposed mice that yielded positive cultures, the majority of isolations were also obtained from all organs cultured. The exceptions in experiment 1 are as follows: five mice with lung infections only; three with lung and liver and spleen; and one with lung and brain infection. In experiment 2, four mice had lung infections only; and four had lungs and liver and spleen infected.

It is impossible to determine how many cells from the soil were actually aerosolized. One might have expected that the soil-exposure mice would have received a larger dose of inhaled cells than did the Henderson group, considering the great number of viable cells in the soil. However, the smaller number of mice that were infected suggests a smaller inhaled dose or unknown factors involved, such as a decrease in virulence of *C. neoformans* after growing in soil medium for 1 year.

When the results of the Henderson aerosol-exposure experiment 1 are compared with a recent report of intranasal instillation (Ritter and Larsh, 1963), there is a contrast in the mortality rate of the animals. Both groups were given doses of 11,000 and 10,000 viable *C. neoformans* cells, respectively, and the same isolate was employed in both. The intranasally infected mice had a rapid rise in the mortality rate starting around the 4th week, and, at the 12th week, 50% were dead. The mortality rate in the Henderson aerosol-infected mice did not accelerate until about the 7th to 8th week, and, at the 12th week, only 38% were dead. In the same report (Ritter and Larsh, 1963), when an equal dose (10,000) was given intraperitoneally, the initial rise in the mortality rate occurred during the third week, and an additional rise began about the seventh week. At the 12th week, over 80% of the mice were dead.

LITERATURE CITED

ABRAHAMS, I., AND T. G. GILLERAN. 1960. Studies on actively acquired resistance to experimental cryptococcosis in mice. J. Immunol. **85**:629–635.

COX, L. B., AND J. C. TOLHURST. 1946. Human torulosis; a clinical, pathological, and microbiological study with a report of thirteen cases. Melbourne University Press, Melbourne, Australia.

ELBERG, S. S., AND D. W. HENDERSON. 1948. Respiratory pathogenicity of Brucella. J. Infect. Diseases **82**:302–306.

EMMONS, C. W. 1955. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (*Columba livia*). Am. J. Hyg. **62**:227–232.

EMMONS, C. W. 1960. Prevalence of *Cryptococcus neoformans* in pigeon habitats. Public Health Rept. U.S. **75**:362–364.

HASENCLEVER, H. F., AND C. W. EMMONS. 1963. The prevalence and mouse virulence of *Cryptococcus neoformans* strains isolated from urban areas. Am. J. Hyg. **78**:227–231.

HASENCLEVER, H. F., AND W. O. MITCHELL. 1960. Virulence and growth rates of *Cryptococcus*

neoformans in mice. Ann. N.Y. Acad. Sci. **89**:156-162.

HENDERSON, D. W. 1952. An apparatus for the study of airborne infection. J. Hyg. **50**:53-68.

KAO, C. F., AND J. SCHWARZ. 1957. The isolation of *Cryptococcus neoformans* from pigeon nests. Am. J. Clin. Pathol. **27**:652-663.

KLIGMAN, A. M., AND F. D. WEIDMAN. 1949. Experimental studies on treatment of human torulosis. Arch. Dermatol. Syphilol. **60**:726-741.

LITTMAN, M. L., AND S. S. SCHNEIERSON. 1959. *Cryptococcus neoformans* in pigeon excreta in New York City. Am. J. Hyg. **69**:49-59.

LITTMAN, M. L., AND L. E. ZIMMERMAN. 1956. Cryptococcosis (Torulosis or European blastomycosis). Grune and Stratton, New York.

RITTER, R. C., AND H. W. LARSH. 1963. The infection of white mice following an intranasal instillation of *Cryptococcus neoformans*. Am. J. Hyg. **78**:241-246.

WADE, L. J., AND L. D. STEVENSON. 1941. Torula infection. Yale J. Biol. Med. **13**:467-476.

YAMAMOTO, S., K. ISHIDA, AND A. SOTO. 1957. Isolation of *Cryptococcus neoformans* from pulmonary granuloma of a cat and from pigeon droppings. Japan. J. Vet. Sci. **19**:179-189.