Virulence of *Coccidioides immitis* Determined By Intracerebral Inoculation in Mice. (20240)

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The low virulence of pathogenic fungi for laboratory animals (as expressed by death rates) is well known. Several authors have investigated the virulence of different fungi, and sought to establish reasonably constant death rates. They accomplished this either by decreasing the resistance of the animals by means of X ray(1), or by incorporating the inoculum in mucin(2-5), by using the intracerebral route of inoculation(6,7), or by using a more susceptible mouse strain(7). But no attempt was made in these studies to calculate the LD_{50} and to compare the virulence of different fungus strains.

For many reasons, it was felt desirable to investigate methods which would measure and compare the virulence of strains of *Coccidioides immitis*. Since death rates are a convenient and simple measure for virulence, computation of the LD_{50} was considered the primary purpose of such work. On the basis of the established LD_{50} the virulence of different strains would then be comparable. Since *C. immitis* inoculated intraperitoneally causes death in mice only if rather concentrated inocula are used, this was judged to be an unsatisfactory way for computing and comparing death rates. Of the virulence enhancing methods the most promising one seemed to be the intracerebral route of inoculation, employed by Howell and Kipkie(6) for obtaining death rates with *Histoplasma capsulatum* in mice. Although pigmented mice were found more susceptible to *Histoplasma*(7) and to *Blastomyces dermatitidis* (8) than albino mice, the former variety seemed less practical because pigmented mice are difficult to obtain, and more expensive. Therefore, the response of albino mice to intracerebral inoculation with *C. immitis* was made the subject of this investigation.

Materials and methods. Two strains of *C. immitis* were used: Strain No. 973 (Tucson, Ariz., 2-13-1946) was isolated from sputum; strain No. 2226 (Tampa, Fla., 6-1-1950) was isolated from a draining sinus. Old cultures of these strains, grown on Sabouraud's glucose agar at room temperature, were examined microscopically for the presence of arthrospores and saline suspensions made without the aid of a dispersing agent. Ten-fold dilutions in saline were made of the original suspension, and 0.2 ml aliquots of each dilution was streaked on Sabouraud's glucose agar plates (5 or more plates for each dilution). Such plates were incubated at 37°C and colony counts made after 2 to 4 days. Depending upon the number of colonies per dilution, 4-fold dilutions of the original suspension were made so that they contained a number estimated to be between 0 and 100 fungus particles per intracerebral inoculum (0.03 ml for young mice, 0.05 ml for old mice). Locally bred albino mice of different ages and both sexes were used in the experiment. Although no difference of susceptibility was noted in different age groups, animals of approximately the same size and age were used in each experiment. The mice were inoculated into the left cerebral hemisphere under anesthesia, usually 5 to 6 animals per dilution, using several 4- or 5-fold dilutions. LD_{50} estimates were obtained by the method of Reed and Muench(9), and the Standard Error $\log \cdot LD_{50}$ was computed as proposed by Pizzi (10). Sabouraud's glucose agar plates were inoculated, using the same syringes and dilutions with the amount of suspension injected into each mouse (0.03 ml or 0.05 ml). Sterile saline (0.2 ml) was added to each plate and the inoculum streaked with a sterile glass rod to insure even distribution of particles. Colony counts were made after the plates were incubated at 37°C for 2 to 4 days and again 1-2 days later. Although the counts were sometimes higher on the second examination, other times they were decreased because larger colonies had fused together. The higher counts were considered final and were used to calculate the regression line (method of least

TABLE I. Statistical Data on Regression and Fiducial Limits of LD_{50} for 2 Strains of *C. immitis*.

Strain	a*	b*	$s_{y \cdot x}{}^2$†	y‡ LD_{50}	y‡ $LD_{50}+2SE$	y‡ $LD_{50}-2SE$	$s_y \cdot t_{95\,pc}$§ $LD_{50}+2SE$	$s_y \cdot t_{95\,pc}$§ $LD_{50}-2SE$	95% fid. limits of yLD_{50}
973	.25	.12	2.71	1.2	2.7	.6	3.57	3.65	1 to 6.3
2226	1.29	.21	5.35	3.0	3.9	2.4	4.98	5.02	1 to 8.9

* Parameters of regression. † Square of stand. error of the estimate. ‡ Plate count, estimated from regression, corresponding to LD_{50}, $LD_{50}+2$ S.E. LD_{50}, and $LD_{50}-2$ S.E. LD_{50}. § pc = %.

TABLE II. Experimental Data of Intracerebral Titration of 2 Strains of *C. immitis* in Albino Mice.

Strain	Dilution by vol.	Mice*	Survival of mice (days after inoc.)	Plate counts
973	1: 64000	3/3	14, 15, 15	11, 9, 8, 3
	1: 256000	2/5	14, 19	5, 3, 2, 2, 1
	1:1024000	3/5	12, 17, 18	1, 1, 1, 1, 0
	1:4096000	0/3		0, 0, 0, 0, 0
	LD_{50}† = 1:512000.			
	S.E. log LD_{50}‡ = ±0.200.			
	Probable No. of particles§ representing LD_{50} = 1 to 6.			
2226	1: 1600	5/5	10, 14, 15, 15, 16	19, 16, 14, 13, 10
	1: 6400	4/5	12, 15, 16, 18	9, 8, 6, 6, 4
	1: 25600	1/5	14	3, 2, 1, 0, 0
	1: 102400	0/5		2, 1, 1, 0, 0
	LD_{50}† = 1:12800.			
	S.E. log LD_{50}‡ = ±0.095.			
	Probable No. of particles§ representing LD_{50} = 1 to 9.			

* Numerator = dead mice, denominator = total No. of mice.
† Method Reed and Muench(9). ‡ Method Pizzi(10). § From Table I, last column.

squares). On the abscissa the doses were plotted, using code numbers 1, 4, 16, and 64 instead of the dilution fractions. On the ordinate the corresponding plate counts were plotted. The count y corresponding to LD_{50} ± 2 S.E. LD_{50} was estimated from the formula of the line, and the corresponding $s_y{}^2$ calculated.* Fiducial 95% limits of plate counts (y) corresponding to LD_{50} ± 2 S.E. LD_{50} were then determined.† The 95% fiducial limits of the plate count representing LD_{50} were considered to be not less than 1, and to lie between $y_{(LD_{50}-2\,S.E.\,LD_{50})} - s_y \cdot t_{95\,per\,cent}$ and $y_{(LD_{50}+2\,S.E.\,LD_{50})} \mp s_y \cdot t_{95\,per\,cent}$. The statistical data as calculated from the experimental data of Table II are summarized in Table I.

Results. The experimental data of 2 titrations are summarized in Table II. It will be seen in this table that each strain had a high degree of virulence, *i.e.*, the LD_{50} was found to correspond to 1 to 6 and 1 to 9 fungus particles for strains No. 973 and No. 2226, respectively.

The survival time of inoculated mice increased with decreasing doses but the animals succumbed well within the 30-day period established by Howell and Kipkie(6) for *H. capsulatum*. With doses below 100 fungus particles the survival time ranged between 10 and 20 days. Where larger doses ranging from 1:400 to 1:1600 by volume were used (experiment not recorded in this paper) death occurred in as few as 3 days.

Early signs of infection included ruffled hair, hunched back and an attempt to hide the head. Later symptoms included the whole scale of nervous signs such as partial or complete paralysis, tonic and clonic convulsions, and vestibular disturbances. Frequently, a bulging of the parietal skull suggested increased intracranial pressure. No animal showing clinical symptoms of infection survived the experiment.

Cultures of the brain were made on Sabou-

* $s_y{}^2 = s_{y \cdot x}{}^2 \cdot \left(1 + \frac{1}{n} + \frac{(x-\bar{x})^2}{\Sigma (x-\bar{x})^2}\right)$.

† $y \pm s_y \cdot t_{95\,per\,cent}$

raud's glucose agar at the time of death. All cultures were positive and sections made from a small number of specimens showed the tissue phase (spherule) of *C. immitis* within the brain substance and the meninges. Occasionally, a localized abscess was seen over the bone of the left hemisphere which corresponded to the inoculation site. Such areas also were positive on culture.

Cultures of the brains of some of the *surviving* mice were negative even though the entire left half of the brain was used as inoculum. Other surviving mice were reinoculated intracerebrally with a massive dose of the fungus. All such mice died with typical symptoms.

Discussion. Ophuls(11) and Stewart and Meyer(12) suggested that mycelial fragments devoid of arthrospores obtained from cultures of *C. immitis* were non-infectious. Since both mycelial fragments and arthrospores may germinate to give colony counts of a suspension made from a culture of this fungus, such a colony count may not be indicative of the exact number of infectious particles present in the suspension. Therefore, although the 2 strains of *C. immitis* used in the present investigation were known to produce abundant arthrospores in old cultures, the exact number of infectious particles (arthrospores) injected in each dose may not have corresponded exactly with the plate counts.

The experiments have shown, however, that large colony counts from suspensions were not necessary to produce symptoms and death when such suspensions were inoculated intracerebrally into mice. Rather, the LD_{50} and MLD counts were found to be of such a low order as to suggest that the prepared inoculum contained a majority of infectious arthrospores with few non-infectious mycelial fragments.

The results of the above experiments do not permit the conclusion that there is a real difference of virulence in strains No. 973 and No. 2226, at least if the LD_{50} is estimated with 95% fiducial limits. In these experiments, however, only a few dilutions of inoculum, and small numbers of mice and plates were used. If these were to be increased, a statistical evaluation would give more narrow limits for the LD_{50} whereby small differences in virulence could be detected.

The intracerebral route of infection, therefore, seems to provide an useful means for comparing the virulence of different strains of *C. immitis*.

Summary. The virulence of 2 strains of *Coccidioides immitis* was evaluated by intracerebral inoculation in albino mice. The LD_{50} for the 2 strains was found to be between 1 to 6 and 1 to 9 fungus particles.

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