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Experimental Study of the Pathogenicity of Aspergilli for Mice

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The relative virulence was determined for 14 species of aspergilli, by inoculating normal mice intravenously with graded doses of spores. Eleven were found to possess some degree of virulence, whereas three others were avirulent. Members of the *Aspergillus flavus* group were the only species that consistently killed mice with doses as low as 10^4 viable spores. When the in vivo fate of spores was compared for a virulent and an avirulent strain of *Aspergillus*, spores of the latter were cleared rapidly from the liver and spleen but grew in the kidneys and brain, producing progressive disease. Mice which inhaled spores did not succumb, but macrophages washed from their lungs contained spores. A relationship of virulence to spore characteristics such as germination time, size, shape, and external markings could not be established. Virulence could not be related to aflatoxin production inasmuch as at least one virulent strain did not produce aflatoxin in vitro.

Aspergilli are world-wide in distribution and the genus now includes 132 recognized species (8). The spores are frequently inhaled by man, but most cases of human aspergillosis are thought to be caused by only a single species, *Aspergillus fumigatus*, with *A. flavus* as the next most important pathogen (3). Much is known about host factors and conditions which predispose to human aspergillosis (e.g., *see* reference 1), yet little is known as to why one species predominates as the most important pathogen, even though many occur commonly in man's environment.

This study was undertaken to compare the relative virulence of several species of aspergilli, in hope of defining those characteristics which might make one species more virulent than another, and to investigate aspects of natural resistance.

MATERIALS AND METHODS

Culture materials. The following fungal strains were used: *A. flavus* 38 (supplied by E. B. Tilden, Animal Hospital of the Chicago Zoological Park, Brookfield, Ill.), 1957 (K. B. Raper and D. I. Fennell, Department of Bacteriology, University of Wisconsin, Madison), and C60 and 16 (both from Mycology Clinic, Tulane University); *A. fumigatus* 105 (E. B. Tilden), C61 (Tulane University), and Eng (Mary English, Bristol Royal Hospital, England); *A. parasiticus* 465, *A. tamarii* 427, *A. oryzae* 447, *A. foetidus* 341, and *A. terreus* 265 (all from K. B. Raper and D. I. Fennell); *A. terreus* 1960 (C. W. Hesseltine, Northern Regional Research Laboratories, Peoria, Ill.); *A. japonicus* 5117 and *A. niger* 326 (K. B. Raper and D. I. Fennell); *A. niger* 2 (M. Hood, Charity Hospital, New Orleans, La.); and *A. phoenicis* 365, *A. ustus* 283, *A. candidus* 312, *A. flavipes* 287, and *A. clavatus* 4 (all C. W. Hesseltine).

The aflatoxin-producing characteristics were as follows: *A. flavus* 38, and *A. parasitucus* were shown to produce aflatoxin B_1 in vitro at both 26 and 37 C, although the amount produced at 37 C was less than that at 26 C. *A. tamarii*, *A. oryzae*, and *A. clavatus* failed to produce aflatoxin, as detectable by chromatographic methods (2), at either temperature when cultured on wheat cereal or Sabouraud agar (6). The other cultures were not tested.

Cultures were grown in petri plates containing Sabouraud Dextrose Agar (Difco) for 4 days at 37 C. Species which grew but did not sporulate well at this temperature were grown at 26 C for 9 days. The matured cultures were dried for an additional 2 days at 37 C by replacing glass covers with porous asbestos tops. Spores from each strain were collected by inverting the cultures and tapping gently. Some of the spores were used in the dry state to produce aerosols for respiratory infection. For a study of virulence by the parenteral route, spores were suspended in phosphate-buffered saline (*p*H 7.2) by shaking with glass beads, then were filtered through glass wool and stored at 4 C while estimation of viable spore concentration was being made. Counting was facilitated by use of pour plates prepared with Littman's medium; rapidly spreading growth was prevented and discrete colonies formed. Maximal counts were

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apparent after 2 days of incubation at 37 C. During this period, the viability of the stored suspensions did not change, as determined by plate counts. Killed spores, used as controls, were obtained by heating a suspension at 80 C in a water bath for 20 min. All suspensions were shown to be free from bacterial contamination by culturing on blood agar. Fresh suspensions were prepared for each experiment.

Determination of pathogenicity. Male CF1 mice, 20 to 25 g, were used in all experiments. The usual period of observation was 21 days, although parts of some groups were held 6 months or longer.

Nine aerosol exposure studies were performed by use of 8 to 18 cortisone-pretreated mice in each; no normal mice were exposed. After 20 min of exposure to spore clouds generated (9) from 400 mg of dry *A. fumigatus* C61 spores, three animals per experiment were sacrificed to determine the inhaled dose. The lungs of each mouse were homogenized separately, and pour plates were made. On the basis of these plate counts, a calculated 10^5 to 5×10^6 viable units reached the pulmonary bed. In six of the experiments, the calculated dose was 4×10^6 per mouse. In two experiments, three to six mice were sacrificed 1.5 hr after exposure; macrophages were washed from the lungs, and Giemsa-stained preparations were examined for the presence of spores.

Groups of 20 mice were inoculated via the lateral tail vein with 0.5 ml of suspension, prepared from each culture and containing 10^2, 10^4, or 10^6 viable units per ml. In one experiment, a group of cortisone-pretreated mice were included as a control, otherwise all mice were without prior treatment. In another experiment, controls were inoculated with a heat-killed suspension derived from the suspension containing 10^6 viable units per ml. The number of viable units was determined at the beginning and end of each series of animal inoculations by preparing triplicate pour plates of the highest dilution. All counts agreed within $\pm 5\%$ of the estimated concentration.

In those experiments in which mice were pretreated, 5 mg of cortisone acetate was given subcutaneously 2 days before infection; antibiotic protection was instituted at the same time (9).

To determine the fate of spores after intravenous inoculation, mice were inoculated with 10^4 viable spores of *A. clavatus* (avirulent) or *A. flavus* 38 (virulent) and were sacrificed at intervals during 30 days. The lungs, liver, spleen, kidneys, and brain were homogenized individually, and total viable fungal counts were determined by duplicate pour plates.

To detect possible differences in rates of phagocytosis of spores from strains of differing virulence, animals were inoculated intraperitoneally with *A. clavatus* or *A. flavus* 38. The peritoneal cavities were washed 1 to 3 hr later, and the macrophages were examined after staining with Giemsa.

In vitro spore studies. To determine whether retardation of growth at body temperature could account for lack of virulence, the rate of growth of *A. clavatus* (avirulent species) was determined at 25, 37, and 40 C. *A. flavus* 38, a virulent species, was used as a parallel control. Spores were inoculated onto pinpoint areas of Sabouraud agar plates, and colony size was recorded after 12, 24, and 48 hr of incubation. Spore germination time and the effect of *p*H on germination also were determined at 37 and 40 C, by use of the method of Goos (4).

For all strains, the size of suspended spores was measured with an optical micrometer. Any external markings were noted.

RESULTS

Normal mice proved to be susceptible to most of the species of aspergilli inoculated intravenously (Fig. 1); cortisone pretreatment, although greatly enhancing disease, was not necessary to ensure infection. Variation in virulence was demonstrated best, however, by response to graded doses. All species, except *A. clavatus*, *A. candidus*, and *A. flavipes*, killed mice when 10^6 spores were inoculated intravenously, but *A. parasiticus*, *A. oryzae*, *A. tamarii*, and *A. flavus*, irrespective of strain, proved to be the most virulent inasmuch as only they consistently killed mice at the lower dose level of 10^4 spores. None of the aspergilli caused death when only 10^2 spores were inoculated. *A. clavatus* was further tested in cortisone-pretreated mice and still did not exhibit virulence; the spores often remained viable in the tissues, but there was no evidence of fungal growth.

An experiment with *A. flavus* 38 was repeated several times, and there was always 100% mortality within 5 days for mice inoculated with 10^6 viable spores. With inoculation of 10^4 spores, mortality ranged from 15 to 75%, with 38% as the average in nine separate experiments. The time till death, however, was less variable since the period of greatest mortality after inoculation with 10^4 spores was always between 5 and 10 days after inoculation, irrespective of total deaths. Animals surviving this critical 10-day period usually did not die. A chronic disease was observed, however, in some of the mice held for prolonged observation, since a few deaths occurred several months after inoculation. There was no indication of disease other than aspergillosis.

Cultures and direct microscopic examination of various organs from the animals that died within 10 days showed fungal proliferation only in the kidneys and brains. Mice that died several months after inoculation usually had one diseased kidney, but other organs, including brains, were always free from fungus.

When the fate of intravenously inoculated spores from a virulent and an avirulent aspergillus was compared, the rate of clearance was found to vary. Spores of avirulent *A. clavatus* were cleared rapidly from all organs except the livers and spleens (Table 1); in these two organs the counts remained relatively unchanged. This

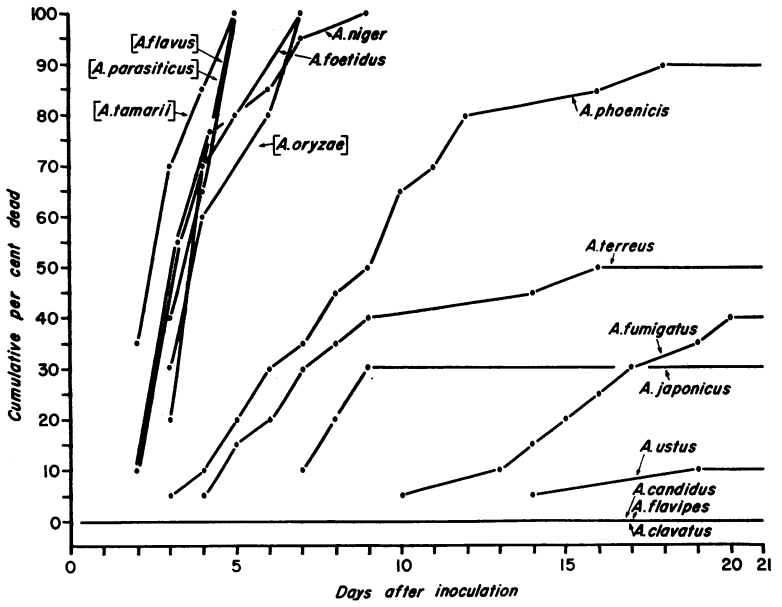

FIG. 1. *Cumulative mortality rates of mice inoculated intravenously with 10^6 viable spores from various species of Aspergillus. The species enclosed by brackets were the most virulent, consistently killing mice with a lower spore dose of 10^4. All are members of Raper's A. flavus group. The curves of A. flavus, A. fumigatus, A. terreus and A. niger represent strains 38, 105, 265, and 326, respectively; studies with other strains of these species showed comparable pathogenicity.*

TABLE 1. *Fungal population in organs of mice sacrificed at intervals after inoculation intravenously with 10^4 viable spores of Aspergillus clavatus, avirulent species*[a]

Organ	Mouse no.	Time after inoculation that mice were sacrificed								
		4 hr	24 hr	2 days	5 days	10 days	15 days	20 days	25 days	30 days
Kidneys	1	214	36	20	12	10	0	4	2	2
	2	242	42	26	6	0	2	6	14	0
	3	92	24	14	6	0	2	0	0	2
Lungs	1	505	48	10	2	0	10	0	0	0
	2	250	32	0	4	0	4	0	0	0
	3	295	18	6	2	0	0	0	0	0
Liver	1	7,300	4,350	6,550	5,950	5,500	4,250	3,300	2,335	3,000
	2	7,600	7,050	7,900	4,250	5,700	4,200	3,325	3,660	4,050
	3	8,150	6,650	6,600	6,100	4,500	5,600	3,615	2,415	3,450
Spleen	1	340	344	618	300	242	180	162	12	290
	2	258	408	364	404	136	270	52	174	252
	3	350	372	336	282	382	248	212	164	254
Heart	1	23	26	6	0	0	0	0	0	0
	2	26	6	0	0	0	0	0	0	0
	3	40	18	12	0	0	0	0	0	0
Brain	1	0	0	2	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0
	3	4	0	0	0	0	0	0	0	0

[a] Observations were confirmed by repeated experiments, and the data shown here are representative examples.

persistence of spores was not seen with virulent *A. flavus*, for which a decrease in fungal counts of livers and spleens was recorded at 5 days after inoculation (Table 2). The target organs for progressive disease, however, were the brains and kidneys as indicated by the increase in their fungal counts at 5 and 10 days after inoculation with the virulent isolate. The lungs were efficient in clearing both avirulent and virulent spores, and disease was never observed grossly or microscopically at this site. These observations were confirmed by three separate experiments for each of the two organisms. Five to 20 mice were sacrificed at each interval of time.

There was no difference in the rate of phagocytosis of virulent and avirulent spores after intraperitoneal inoculation; both were readily phagocytized.

None of the mice exposed to aerosols died of aspergillosis. Macrophages washed from the lungs of sacrificed animals revealed phagocytized spores. In a sample experiment, 64% of 50 macrophages counted contained an average of 7 spores per cell, with some cells having as high as 20.

There was no apparent retardation of germination or growth of *A. clavatus* (avirulent) at 37 C or on a medium with *p*H 3.5, 5.2, or 7.2. Both *A. flavus* (virulent) and *A. clavatus* grew better at 37 than at 25 C. The germination of *A. clavatus* spores at 37 C was a few hours slower than that of *A. flavus* spores, but, within a short time, germination of both was complete (Table 3). The growth of each was relatively rapid, and, at 2 days, there was no difference in colony size. However, *A. clavatus* spores did not germinate at 40 C, whereas those of *A. flavus* did. One observation was consistently made, viz., spores of both isolates increased approximately twofold in diameter before germinating, and their cytoplasms stained when lactophenol cotton blue was added.

The size, shape, and markings of spore walls of the 14 species agreed well with those stated in the monograph of Raper and Fennell (8). Spores of the most virulent species, *A. flavus*, *A. parasiticus*, *A. tamarii*, *A. oryzae*, *A. foetidus*, *A. niger*, and *A. phoenicis* (listed in descending order of virulence), were either rough or echinulate. All other species had smooth spores, including the avirulent species of *A. candidus*, *A. flavipes*, and *A. clavatus*. In exception to this pattern, two species of relatively low virulence (*A. japonicus* and *A. ustus*) had rough to echinulate walls.

TABLE 2. *Fungal population in organs of mice sacrificed at intervals after inoculation intravenously with 10^4 viable spores of Aspergillus flavus, virulent species*[a]

Organ	Mouse no.	Time after inoculation that mice were sacrificed								
		4 hr	24 hr	2 days	5 days	10 days	15 days	20 days	25 days	30 days
Kidneys	1	465	38	30	242	870	0	0	0	0
	2	515	58	4	0	555	0	12,750	0	0
	3	545	30	12	0	0	0	0	0	0
Lungs	1	1,080	42	38	0	0	0	0	0	0
	2	1,210	94	0	0	0	0	0	0	0
	3	1,085	72	4	0	0	0	0	0	0
Liver	1	4,050	1,800	1,960	280	0	2	0	0	0
	2	5,550	2,900	370	465	72	4	0	0	0
	3	5,900	4,350	1,565	720	42	4	0	0	0
Spleen	1	216	64	180	2	0	0	0	0	0
	2	204	138	12	16	0	0	0	0	0
	3	146	92	30	10	0	0	0	0	0
Heart	1	174	40	4	0	0	0	0	0	0
	2	152	50	32	0	0	0	0	0	0
	3	172	50	2	0	0	0	0	0	0
Brain	1	18	8	8	1,870	8	0	0	0	0
	2	22	12	8	500	24	0	0	0	0
	3	20	22	56	180	350	0	0	0	0

[a] Observations were confirmed by repeated experiments, and the data shown here are representative examples.

TABLE 3. *Comparison of spore germination rate at 37 and 40 C for a virulent (Aspergillus flavus) and an avirulent (A. clavatus) species*

Time (hr)	Virulent spores		Avirulent spores	
	37 C	40 C	37 C	40 C
	%	%	%	%
3	0	0	0	0
4	3.7	3.0	0	0
5	41.3	7.0	0	0
6	80.0	51.0	0	0
7	94.6	92.0	10.0	0
8	—[a]	—	50.3	0
9	—	—	86.7	0
10	—	—	97.3	0

[a] The presence of long hyphal elements precluded further observation.

DISCUSSION

The most interesting observation in these studies was the difference in capacity of different organs to eliminate spores of varying virulence. The lung rapidly disposed of spores, regardless of the pathogenic potential of the fungal isolate and regardless of the route of exposure. In the case of liver and spleen, however, only spores of virulent isolates were removed; the host seemed unable to rid these tissues of avirulent spores. Spores of an avirulent aspergillus, although lacking capacity to germinate in liver and spleen, were able to survive, viable but dormant, for long periods of time. This failure to germinate may actually have served as the mechanism of spore survival. It was found that, before germination on agar, spores increased approximately twofold in diameter, presumably by absorption of liquid from the surrounding milieu. If, then, only the virulent species tried to germinate in vivo, and if germination were accompanied by increased swelling and permeability, such spores might, especially if phagocytized, absorb substances destructive to the fungus. It was this thinking that led to our in vitro studies on germination at low *p*H, for it has been shown that phagocytized particles are often enclosed within a cell vacuole which is strongly acidic (7). Germinating spores, however, irrespective of virulence, were not killed by low *p*H. These negative results do not invalidate the hypothesis that avirulent spores may have been protected from in vivo destructive forces by their intact spore walls and, conversely, that spores of virulent isolates became vulnerable during the process of germination because killing could have been effected by other than *p*H changes. However, regardless of the mechanism of defense of liver, lung, and spleen against spores of a virulent species, this defense was not operative at other sites, viz., kidney and brain, for there was extensive proliferation, resulting in eventual death of the animal.

It is tempting to relate roughness of spore wall with virulence, for most of the virulent strains had echinulate walls, whereas most of the avirulent ones had smooth walls. However, echinulation cannot be the sole factor related to virulence, since two species of relatively low virulence had rough to echinulate spores. Ability to grow at various temperatures also was considered in relation to virulence, but no correlation could be found. All species used in these studies grew well at 37 C. *A. fumigatus*, on the other hand, which has been shown by others (8) to grow at temperatures of 40 C and above, was of relatively low mouse virulence.

Toxin production also warrants consideration as a factor determining the pathogenicity of aspergilli. Symmers (10) stated that "diffusion necrosis" often seen in pulmonary aspergillosis is possibly caused by toxic products of the fungus. Also, the role of diffusible toxic metabolites of aspergilli in tissue necrosis and vascular damage was stressed by Gowing and Hamlin (5). Furthermore, it is known that toxic substances can be extracted from the mycelia of certain species. This latter aspect was not explored in our study, although it is to be noted that two of the virulent strains used, *A. flavus* 38 and *A. fumigatus* 105, were found by others (11) to have extractable toxic material. Thus, it is not possible to eliminate the role of such substances in pathogenicity.

Several other toxic metabolites are produced by *A. flavus*, e.g., oxalic acid, kojic acid, tremorgenic substance, β-nitro-propionic acid, and aflatoxins, all of which have been reviewed by Wilson (12) and Wogan (13). It was determined that at least one of the strains of *A. flavus* used in our study produces aflatoxin in vitro at 37 C. However, two other virulent members of the *A. flavus* group, *A. tamarii* and *A. oryzae*, failed to produce aflatoxins. Attempts were made to determine whether aflatoxins were produced in situ in our experimental animals, but the results were uniformly negative. Admittedly, further work is needed before definite conclusions can be made, but there are points which count against aflatoxin having a significant role in experimental murine aspergillosis. First, as stated above, two virulent aspergilli failed to produce aflatoxin in vitro, and this observation is strengthened by the work of Hesseltine et al. (6), who reported that none of 53 isolates of *A. oryzae* produced afla-

toxin. Also, we did not observe liver pathology characteristic of aflatoxin poisoning.

No conclusion can be drawn from this study regarding the characteristic virulence of any single species because insufficient strains were used. Nevertheless, a wide spectrum of virulence within the genus was demonstrated. The singular feature, however, was that cultures most virulent for mice were all members of Raper's *A. flavus* group, most species of which have been found infrequently or not at all in human disease.

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