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Varying Virulence in Rabbits Infected with Different Filamentous Types of *Histoplasma capsulatum*

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Histoplasma capsulatum filamentous primary isolates and their subcultures are separable into two distinct colonial types (A and B) having different microscopic characteristics. Yeast forms of the A and B types and the parent (P) strains from which they are derived are microscopically indistinguishable. Critically standardized inocula of living P, A, and B yeasts from one strain of *H. capsulatum* (G-184) were injected intravenously into 12 rabbits. Each type produced progressively debilitating disease, but in varying degrees. Of the 12 animals, 6 died within 2 to 14 weeks. A persisting copious nasal exudate, beginning at or before 1 week, was cultured weekly at 26 C on Mycosel (BBL) agar. Pure cultures of A and B filamentous type colonies were recovered from exudates of animals receiving A and B yeasts, respectively, whereas both filamentous types were isolated from rabbits injected with P yeasts, with B predominating. Only A and B yeasts thus maintained their filamentous integrity during animal passage. It was noted that dissemination of *H. capsulatum* through the nares of infected rabbits represents a possible hazard to laboratory personnel heretofore unrecognized. It is also a possible means of cross-infecting or sensitizing or cross-infecting and sensitizing animals housed in the same room, if A and B yeasts prove not to be antigenically identical.

Histoplasma capsulatum primary isolates and their subcultures can be separated into two distinct mycelial, colonial types (1). Type "A," for albino, consists of white, coarse, broad, aerial hyphae. The few macroconidia produced are smooth, and the microconidia are smooth or spiny. Diffusible pigment is absent until the culture is at least 1 month old. This type eventually becomes sterile on modified Sabouraud agar. Type "B," for brown, is characterized by flat colonies which become light tan to dark brown within a few days. The narrow hyphae are pigmented and produce enormous numbers of "tuberculated" macroconidia, generally regarded as characteristic of this species. An abundant brown pigment rapidly leaches throughout the medium. The original type "P," for parent, from which A and B are derived, is a mixture in which the ratio of A to B is unknown and not constant, since type A eventually overgrows type B. All three types are readily converted to the yeast phase, and in this phase are microscopically and macroscopically indistinguishable.

Pursuant to determining possible differences in their antigen-antibody spectrum, critically standardized inocula of living yeasts of each of the three types were injected intravenously into rabbits. During the first week after inoculation, the animals developed nasal discharges rarely observed in rabbits infected with less critically standardized dosages of "living" yeasts of "stock" strains of *H. capsulatum*. The nasal discharges were cultured at weekly intervals for 14 weeks.

The purpose of this report is to describe the results of these ancillary cultural studies and the kinds of infections in which the nasal discharges were more consistently positive, and to discuss the relevance of these findings in determining whether there are differences in the virulence, pathogenesis, immunological response, and susceptibility to drugs of the A and B yeasts. Distribution of *H. capsulatum* through the nares as a hazard to laboratory personnel is also briefly noted.

MATERIALS AND METHODS

Preparation of inocula. The three mycelial types from *H. capsulatum* strain G-184, a primary human isolate, were converted to the yeast phase on blood agar without antibiotics. The growth curve was determined for each in Trypticase soy broth dialysate (TSB) with continuous gyrotary shaking at 37 C, as

described earlier (4), and was essentially identical for the three types. Growth activity was checked by optical density (4); reduction of a tetrazolium salt (3) and the viability of the cells were checked by vital staining with Janus Green B (2). Cells were harvested at the peak propagative stage of growth, or after approximately 36 hr, for one inoculum, and at the beginning of the stationary phase, or after 120 hr of incubation, for the second inoculum. Both inocula were adjusted to contain 212.5 $\times$ 10^6 living cells/ml of TSB for immediate injection into rabbits.

Animal studies. Each of twelve, 5- to 8-lb female albino rabbits was injected intravenously with 2 ml of the critically standardized inocula (425 $\times$ 10^6 living yeasts). Six rabbits received the 36-hr yeasts, two each of types P, A, and B, respectively, and six received the 120-hr yeasts, also two each of the respective types.

Cultural studies. Discharges from the rabbits' noses were cultured weekly. They were collected on sterile swabs and were streaked directly onto Mycosel agar plates (BBL) which were incubated at room temperature. Colonies usually appeared within 7 to 10 days. No precise method of quantitation was attempted. However, four or five colonies were selected at random from each plate and were pinpoint-inoculated (1) onto Emmons' modified Sabouraud agar (Difco) plates to confirm the filamentous types of colonial growth recovered from the rabbits. After incubation at room temperature for 2 to 3 weeks, the subcultures were examined microscopically to make sure that they were *H. capsulatum* and were photographed in black and white with a Polaroid camera. All plates were held for 6 weeks to ascertain that additional colonial types did not appear.

RESULTS

The results of these ancillary studies are summarized in Table 1. Only filamentous type A colonies (Fig. 6) were isolated from rabbits infected with type A yeasts (rabbit no. 4, 8–10), and only filamentous type B colonies (Fig. 2) were isolated from animals infected with type B yeasts (rabbit no. 3, 5, 7, and 11). Both A and B filamentous colonies were recovered from rabbits infected with P or parent yeasts (rabbit no 1. 2, and 6), with B predominating (Fig. 3 and 4). The original type P filamentous colony, from which A and B were derived, was not recovered from any of the animals, not even from those infected with P yeasts.

Also, as shown in Table 1, the severity of infections produced divided rather sharply into two groups. Animals in group I developed rapidly progressive respiratory distress, weight loss, and general emaciation. In addition, there was extensive eye involvement. The eyelids swelled, became full of nodules, and eventually closed from granular-like lesions. Nasal discharges from these animals were also particularly copious and persisting, and, with few exceptions, consistently positive for *H. capsulatum*. One of these infected animals (rabbit no. 5) died during the 10th week and two animals (rabbit no. 1 and 3) died during the 13th week. A fourth animal (rabbit no. 4) was sacrificed for comparative histopathological studies with animals 1 and 3; a fifth animal (rab-

TABLE 1. *Cultural studies of nasal discharges from rabbits infected with type P, A, or B yeasts of Histoplasma capsulatum strain G-184*

Group	Rabbit no.	Type injected	Inoculum (hr)	Weeks after inoculation														Type(s) isolated
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	
I[a]	1	P	36	−	+	+	+	+	+	+	+	+	+	+	+	+	D[b]	A and B
	2	P	36	−	+	+	+	+	+	−	−	+	+	+	+	+	R[c]	A and B
	3	B	36	−	+	+	+	+	+	+	+	+	+	+	+	+	D	B
	4	A	36	−	+	+	+	+	+	+	+	+	−	+	+	−	S[d]	A
	5	B	120	−	+	+	+	+	+	−	−	+	D					B
II[e]	6	P	120	−	+	+	+	+	+	−	−	−	−	−	−	−	R	A and B
	7	B	120	−	+	+	+	−	+	−	−	−	−	−	+	−	R	B
	8	A	36	−	+	+	+	−	−	−	−	−	−	−	−	−	R	A
	9	A	120	−	+	+	+	+	−	−	−	−	−	−	−	−	R	A
	10	A	120	−	−	+	+	+	−	−	−	−	−	−	−	−	R	A
III	11	B	36	+	D													B
	12	P	120	−	D													None

[a] Infections characterized by rapidly progressive respiratory distress, weight loss, and general emaciation; copious persisting nasal discharge; and extensive eye involvement.
[b] Died.
[c] Recovered.
[d] Sacrificed.
[e] Symptoms much less severe and transitory; eye involvement mild or absent.

bit no. 2), despite an equally severe infection, slowly recovered over the next 6 months. Four of the five animals received 36-hr inocula. Animals in group II experienced much less severe respiratory distress, weight loss, and nasal discharge, and essentially no eye involvement. All symptoms were transitory. Although the nasal discharges were positive as early as in group I, they became negative, with one sporadic exception (rabbit no. 7), during the fifth to seventh weeks. Four of the five animals in group II received 120-hr inocula.

Two animals died during the second week, before extensive visible systemic symptoms developed (group III). However, only the nasal discharge of animal no. 11 yielded *H. capsulatum* during the first week after inoculation.

DISCUSSION

The principal finding in this study was that the A and B yeasts maintained their filamentous colonial and microscopic integrity during animal passage, whereas the P or original strain from which A and B were derived did not. A possible explanation for this is that the P mycelium is multinucleate and heterokaryotic (1), whereas the yeasts derived from it are uninucleate and presumably homozygous for the colonial genotype. From this and other studies in this laboratory, it is obvious that there is little to be gained from further comparative studies of yeasts from type P filamentous strains in experimental animals. The ratio of A to B is unknown even in the primary isolate. Since A rapidly overgrows B, all unmonitored filamentous primary isolates soon consist of type A only. Most strains in "stock" culture collections probably consist of this type and have not become "pleomorphic" as is generally thought. In fact, B-type filamentous colonies could not be isolated from the G-184 strain used in this study after only 1 year as an "unmonitored" "stock" culture. In contrast, both A and B types, separated from the original isolate at the time of isolation from the patient, maintained their original integrity, microscopically and macroscopically, during storage at 4 C over the same period.

A second finding of note was the unexpectedly rapid onset and progressively severe nature of the disease, including extensive eye involvement, in four of six rabbits infected with equal numbers of living yeasts in the peak propagative stage (36 hr) of growth (group I, Table 1). The possibility of even more rapidly fulminating histoplasmosis cannot be discounted in animal no. 11 (group III), because of the very early positive nasal discharge. Despite the small number of animals in this study, the differences in the severity of the disease in animals infected with 36- versus 120-hr inocula were sufficiently striking to suggest that the LD_{50} should not only be established independently for A and B yeasts, but that such determinations should be based on results with inoculating suspensions in which the state of growth activity is as critically controlled as the number of organisms in that particular state. For example, the diverse responses to 36-hr A inoculum (rabbit no. 4 and 8, Table 1) could have been as representative of LD_{50} of the peak propagative stage of A yeasts as of variations in individual host response. The much milder infections (rabbit no. 9 and 10, Table 1) to the same number of living A yeasts in the stationary phase supports this possibility. Similar considerations should not be overlooked in evaluating the responses to 36- and 120-hr B yeasts. In this instance, the 120-hr yeasts appeared to be closer to the LD_{50} for B yeasts in this stage of growth. The same number of B yeasts in the peak propagative stage produced lethal disease in one animal (no. 3, Table 1), and possibly even more rapidly fulminating disease and early death in another animal (no. 11, Table 1). The most critical determinations of LD_{50} values would therefore appear to be essential to establishing whether there are differences between A and B yeasts, particularly with regard to virulence, pathogenesis, immunological response, and susceptibility to drugs in experimental animals. As noted above, the response of the host is directed not to *H. capsulatum* yeasts, but to A or B or A and B yeasts of individual strains in varying and unknown ratios. Now that it is known that the two types exist and are separable, it is very important to ascertain, by the most exacting techniques and methods possible, whether differences exist between them.

The discovery that the nasal discharges were teeming with *H. capsulatum* yeasts, however, also represents a heretofore unrecognized hazard to laboratory personnel handling or caring for infected animals. Although the discharges were collected with swabs for the cultural studies, the organisms could also be isolated in substantial numbers by holding the plates within 6 inches (15.24 cm) of the rabbits' noses. Air in the animal room was thus heavily and continuously seeded with *H. capsulatum* for at least 14 weeks and doubtless longer. For this reason, wearing of appropriate face masks, shields, and glasses by personnel and the continuous flooding of rooms with high-intensity ultraviolet radiation are recommended, in addition to careful handling and disposal of bedding and excreta.

Distribution of A and B yeasts through the

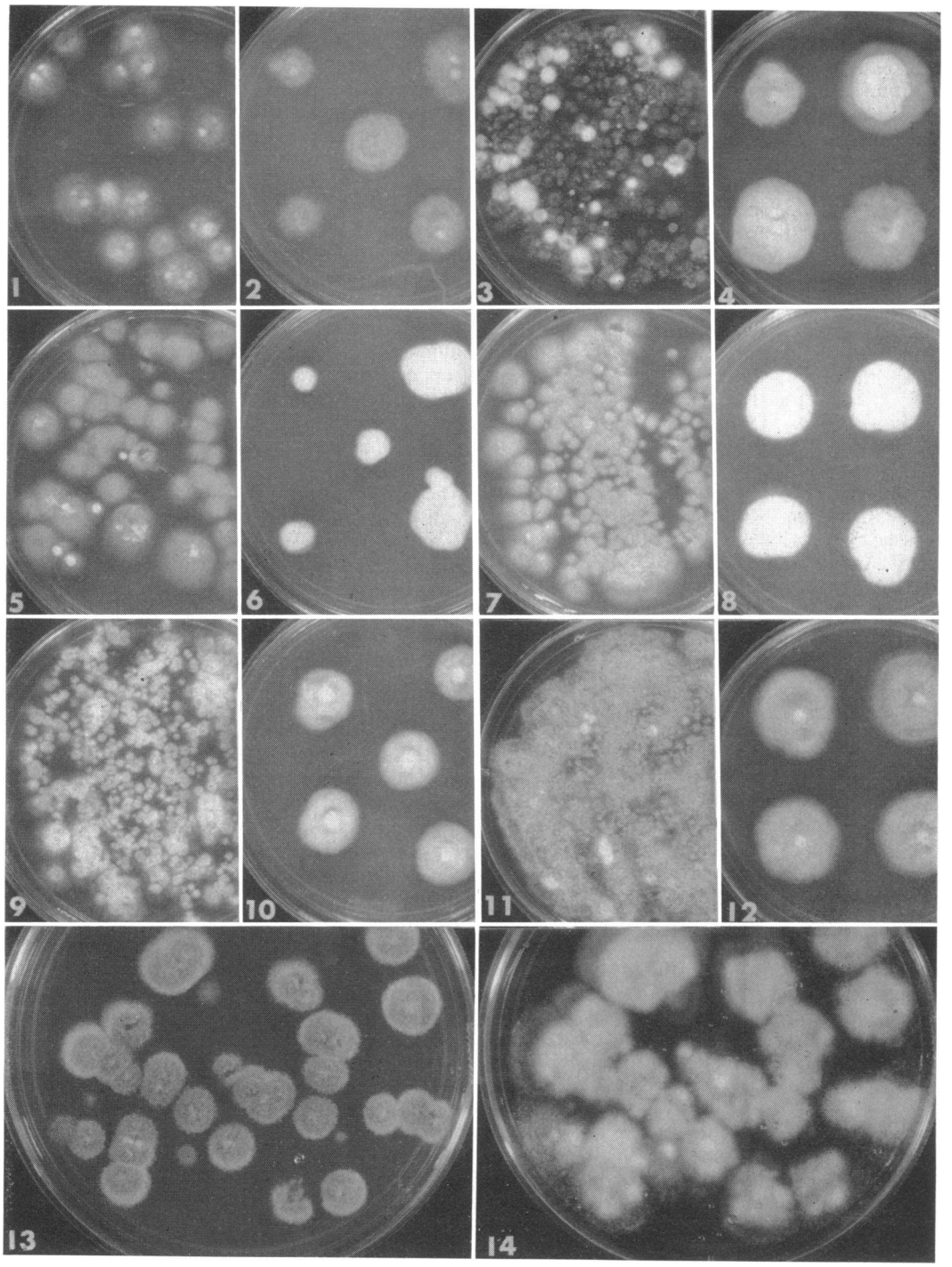

FIG. 1. *Mycosel agar plate 5 weeks after being inoculated with nasal exudate from rabbit no. 1, inoculated for 3 weeks with a 36-hr culture of G-184P. ×0.5.*

FIG. 2. *Subculture on modified Sabouraud agar of five colonies from the plate in Fig. 1, showing typical B type, tan, sparse flat colonies. ×0.5.*

nares could also be a means of cross-infecting or sensitizing animals housed in the same room. It is thus not unreasonable to suggest that the two yeast types be treated as separate organisms until immunological differences have been shown not to exist.

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FIG. 3. *Same as Fig. 1, 11 weeks after infection.* ×*0.5.*

FIG. 4. *Subculture on modified Sabouraud agar of four colonies from the plate in Fig. 3, showing mixed colonial types of A and B.* ×*0.5.*

FIG. 5. *Mycosel agar plate 5 weeks after being inoculated with nasal exudate from rabbit no. 9, inoculated for 3 weeks with a 36-hr culture of G-184A.* ×*0.5.*

FIG. 6. *Subculture on modified Sabouraud agar of five colonies from plate in Fig. 5, showing typical A type, white, fluffy, aerial colonies.* ×*0.5.*

FIG. 7. *Same as Fig. 5, 9 weeks after infection.* ×*0.5.*

FIG. 8. *Subculture on modified Sabouraud agar of four colonies from the plate in Fig. 7.* ×*0.5.*

FIG. 9. *Mycosel agar plate 5 weeks after being inoculated with nasal exudate from rabbit no. 6, inoculated for 3 weeks with a 36-hr culture of G-184B.* ×*0.5.*

FIG. 10. *Subculture on modified Sabouraud agar of five colonies in Fig. 9.* ×*0.5.*

FIG. 11. *Same as Fig. 9, 10 weeks after infection.* ×*0.5.*

FIG. 12. *Subculture on modified Sabouraud agar of four colonies from plate in Fig. 11.* ×*0.5.*

FIG. 13. *Mycosel agar plate 4 weeks after being inoculated with nasal exudate from rabbit no. 8, inoculated for 9 weeks with a 120-hr culture of G-184B.* ×*0.7.*

FIG. 14. *Same plate as Fig. 13, 7 weeks after inoculation, showing the A type overgrowing the B type.* ×*0.7.*