MYCOLOGY

Virulence of *Cryptococcus neoformans* serotypes A, B, C and D for four mouse strains

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Summary. The relative virulence of *Cryptococcus neoformans* serotypes A, B, C and D in four mouse strains was assessed by measuring their migration from the foot-pad of the animals to the spleen, lungs and brain in 6-week-old DBA/2, BALB/c, A/J and a hybrid mouse strain by re-isolating yeasts from the internal organs. Comparable doses of each *C. neoformans* serotype were inoculated into the foot-pads of the mice. *C. neoformans* var *neoformans* strains A68, D52, A-(IN) and D-(IN) were more virulent than *C. neoformans* var *gatti* strains B112 and C18. However, the differences in the relative virulence of the var *neoformans* and the var *gatti* serotypes for the mouse strains were not significant ($p > 0.05$). Re-isolation of yeasts from mice showed that the BALB/c mice, in particular, and the DBA/2 mice were more susceptible to disseminated *C. neoformans* infection. The virulence of *C. neoformans* serotypes through foot-pad inoculation of mice was established.

Introduction

Cryptococcosis is commonly acquired by inhalation and the capsule of *Cryptococcus neoformans* has been identified as a virulence factor.^{1,2} Other possible routes of infection have been assessed and one such route is the alimentary tract in immunodeficient hosts.³ Mucosal surfaces can be colonised by the capsulate strains of *C. neoformans*, from where they may cause systemic cryptococcosis.³ Asymptomatic infection in man and animals is common; the clinical manifestations become apparent in conditions of stress and immunosuppression.⁴ Four serotypes of this yeast are known—A, B, C and D. All can cause systemic disease. Most infections in immunodeficient patients with AIDS are believed to be caused by *C. neoformans* var *neoformans* (serotypes A, D).⁵ Dissemination of infection becomes enhanced in cases associated with hypogammaglobulinaemia.⁶

Previous attempts to induce resistance in mice experimentally through different routes of inoculation with live and killed, virulent and sometimes avirulent strains, have been unsatisfactory.^{7,8} On the other hand, survival in mice may be enhanced by immunisation with anti-*C. neoformans* capsular polysaccharide or monoclonal antibodies.^{9,10} Furthermore, it is not known whether cutaneous inoculation does result in immunisation. Cryptococcal lesions produced in the

lung, as a result of host defence mechanisms against invading cryptococci, are determined by both the immunological competence of the host and the virulence of the invading yeast.¹¹ Cryptococcosis may be fatal, depending on the inoculum size, when the route of inoculation is intravenous or intraperitoneal.^{12,13} Although skin cryptococcomas may arise, as demonstrated in guinea-pigs inoculated intraperitoneally,¹² there are doubts as to whether systemic infection occurs when viable *C. neoformans* are implanted cutaneously or subcutaneously, in spite of the partial success reported earlier.^{8,14} The present study examined the susceptibility of four mouse strains to the relative virulence of *Cryptococcus neoformans* serotypes A, B, C and D inoculated into the footpad.

Materials and methods

Experimental animals

Two hundred and twenty-four 6-week-old mice belonging to four strains—DBA/2, BALB/c, A/J and a hybrid (crossed from DBA/2 and CBA)—were used. All the mice were inbred at the National Institute for Trypanosomiasis Research, Vom, Nigeria.

Organisms

Seven strains of *C. neoformans* were used; they included all four serotypes designated *C. neoformans* var *neoformans* (strains A68, A551, D52) and *C.*

neoformans var *gatti* (B112 and C18); these were provided by Dr J. E. Bennett, NIH, Bethesda, MD, USA, and Dr R. Ikeda, Department of Microbiology, Meiji College of Pharmacy, Tokyo, Japan. Two further strains, *C. neoformans* var *neoformans* of serotypes designated as A(IN) and D(IN) were isolated from bird droppings in Nigeria. All the *C. neoformans* strains except *C. neoformans* var *neoformans* A551 were capsulated.

Preparation of yeast inocula

Viable yeast cells were harvested from 48-h Sabouraud agar cultures into sterile phosphate-buffered saline (PBS) and the concentrations were adjusted to 10^7 organisms/0.02 ml. The concentration of the yeast in the inoculum was confirmed by viable counts in replicate pour-plates.

Animal inoculation

Eight mice from each strain were inoculated with each *C. neoformans* serotype strain. Each group of eight mice contained an equal number of males and females. The yeasts were injected into the left hind foot-pad of the mice. Fourteen mice from each strain were used as controls and were inoculated with 0.02 ml of sterile PBS.

Re-isolation of yeast

The experiments were terminated 2 weeks after foot-pad inoculation. Feed was withheld from the mice for 18 h before the animals were killed; CO₂ gas was piped into polythene bags containing the mice. At death, they were examined for skin infection, rough fur coats and for foot-pad lesions. Tissues from the site of inoculation, spleen, lung and brain were removed and examined for lesions and a qualitative examination of each of the organs for the presence of the various *C. neoformans* serotype strains was made by culture.

Culture

Skin covering the foot-pads was removed and the dermis was smeared on the surface of Sabouraud and malt-extract agar. Spleen, lung and brain tissues minced and homogenised in PBS were filtered through clean sterile muslin and the filtrate was centrifuged at 2000 *g* for 5 min. The pellets were used to inoculate Sabouraud and malt-extract agar containing chloramphenicol 0.05 mg/ml. Duplicate cultures were incubated at 25°C and 37°C for 24–120 h. Isolates were re-identified by standard procedures.^{15,16} All the data generated from this investigation were analysed statistically by the analysis of variance (ANOVA) at the 5% level.

Results

At post-mortem examination, lesions characteristic of *C. neoformans* infection in the foot-pad, spleen and lung were observed in some of the infected mice but

Table 1. Isolation history, clinical signs and post-mortem findings

Mouse strains	Age (weeks)	Number of mice	Inoculation route	<i>C. neoformans</i> serotype strains	Clinical autopsy finding/number of mice examined					
					Necrotic foot-pad	Inflamed spleen	Granulomatous lung	Lesions in the brain	Rough hair coats	Dermatropism
DBA/2	6	72	Foot-pad	A68	2	1*	2*†	—	—	—
				B112	1	—	—	—	—	—
				AIN	2	—	—	—	—	—
BALB/c	6	72	Foot-pad	A68	2	1†	1	—	6	—
				B112	—	—	—	—	—	—
				C18	—	—	—	—	—	—
A/J	6	72	Foot-pad	D52	—	—	—	—	—	—
				AIN	1	1†	—	—	—	—
				A68	2	—	—	—	—	—
Hybrid	6	72	Foot-pad	C18	1	1	—	—	—	—
				AIN	3	—	—	—	—	—
				B112	—	—	—	—	—	—
Controls	6	72	Foot-pad	AIN	1	—	—	—	—	—
				—	2	—	—	—	—	—

Lesions observed in the *spleen, lung and foot-pad, in the †spleen and footpad, and in the ‡lung and footpad of the same mouse.

not in the controls, Necrotic foot-pads, inflamed spleens and granulomatous lungs were seen in 23 of the mice inoculated with three *C. neoformans* var *neoformans* serotype strains A68, D52 and A(IN), and two *C. neoformans* var *gatti* serotype strains B112 and C18. Of the *C. neoformans* serotype strains used, strain A68 caused more lesions than the other strains in the foot-pads, spleens and lungs of the DBA/2, BALB/c and A/J mice. The local isolate, *C. neoformans* var *neoformans* A(IN) caused lesions in all four mouse strains but with less involvement of the lungs. Only one BALB/c mouse inoculated with *C. neoformans* var *neoformans* D52 had an inflamed spleen. Lesions were caused in the BALB/c mice by *C. neoformans* serotype strains A68, B112, C18 D52 and A(IN)), and in DBA/2 and A/J mice by *C. neoformans* serotype strain A68, B112, A(IN), and A68, C18 and A(IN), respectively. Lesions in the hybrid mice were restricted to the foot-pads only and were caused by *C. neoformans* var *neoformans* A(IN) and *C. neoformans* var *gatti* B112. Six of the BALB/c mice inoculated with *C. neoformans* var *neoformans* A68 showed rough hair coats. Brain tissues from none of the mouse strains manifested any observable lesions and there were no signs of dermatropism in any of the mice. Two deaths were recorded among the BALB/c mice, one each on the fourth and ninth day respectively after inoculation. The results are summarised in table I.

Re-isolation of *C. neoformans* serotype strains

A positive virulence test in this investigation was based on the re-isolation of the *C. neoformans* strains from one or more tissues other than the foot-pad. Migration of the yeasts from the foot-pad to the spleen, lungs and brain occurred in 108 (48.2%) of the mice. Culture showed that migration was more common in the DBA/2 and BALB/c mice than in the A/J and hybrid strains. However, there was a difference in the number of positive cultures recorded from the tissues of the DBA/2 and BALB/c mice for the different *C. neoformans* strains; more isolates were

made from tissues of the BALB/c mice. However, the results did not differ statistically ($p > 0.05$) except for results obtained with mouse strains DBA/2 and BALB/c inoculated with *C. neoformans* var *neoformans* A551. Significant differences in virulence for the mice, as recorded from the number of isolates, were found between the DBA/2 and A/J mice and between DBA/2 and hybrid mice ($p < 0.05$). Similar results were obtained when the virulence of the various *C. neoformans* serotypes in the BALB/c mice was compared with that in the A/J and hybrid mice. Only one hybrid mouse yielded a positive brain culture of *C. neoformans* var *neoformans* A68, whereas four isolates—one of strain A68 and three of strain A(IN)—were made from the brains of the A/J mice (table II).

The results showed that *C. neoformans* var *neoformans* strains A68, A(IN) and D52 were more invasive than *C. neoformans* var *gatti* strains B112, and C18. Overall, *C. neoformans* var *neoformans* strain A68 was more virulent, being re-isolated to a greater extent from the spleens, lungs and brains of the mice. With the exception of the foot-pads, the lungs remained the most frequently infected organ whereas re-isolation of the yeasts was least from the brain: 99 isolations of the seven yeasts were made from lung specimens, 85 from spleen and 64 from brain. Positive brain cultures were obtained in 13 instances with *C. neoformans* var *neoformans* A68, on 11 occasions with *C. neoformans* var *neoformans* D52 and A(IN), and on 10 occasions each with *C. neoformans* var *gatti* C18 and *C. neoformans* var *neoformans* D(IN). Nine isolates of strain B112 were made from brain specimens but no isolate of *C. neoformans* var *neoformans* A551.

Discussion

Common symptoms in mice inoculated or infected with *C. neoformans* include listlessness, rough hair coat, hydrocephalus-like bulging of the cranium and,

Table II. Organs from which *C. neoformans* serotype strains were recovered according to mouse strains*

<i>C. neoformans</i> serotype strain	Number of mice from which <i>C. neoformans</i> was recovered															
	DBA/2				BALB/c				A/J				Hybrid			
	Fp	Sp	Ln	Bn	Fp	Sp	Ln	Bn	Fp	Sp	Ln	Bn	Fp	Sp	Ln	Bn
A68	8	7	6	6	8	7	8	5	5	2	3	1	6	1	2	1
A551	8	0	0	0	7	2	1	0	8	0	2	0	6	2	0	0
B112	8	3	5	3	8	4	7	6	8	1	3	0	7	1	0	0
C18	8	5	7	6	8	3	5	4	8	0	0	0	7	0	2	0
D52	8	6	7	6	8	6	6	5	7	2	3	0	8	2	2	0
A(IN)	8	3	5	3	7	7	5	5	7	6	5	3	7	1	1	0
D(IN)	7	6	6	4	8	7	7	6	4	1	0	0	6	0	1	0
Controls	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	55	30	36	28	54	36	39	31	47	12	16	4	47	7	8	1

Fp, foot-pad; Sp, spleen; Ln, lung; Bn, brain.

*Eight mice of each strain were inoculated with each serotype.

in terminal conditions, marked signs of central nervous system disturbance. In the present study, rough hair coats were seen in six BALB/c mice inoculated with *C. neoformans* var *neoformans* strain A68.

C. neoformans kills 6-week-old Swiss white mice inoculated intracerebrally with a 0.02–0.03-ml inoculum between 4 and 14 days.¹⁷ An inoculum of 10^6 – 10^7 cells/mouse given intravenously or intraperitoneally also yields an LD50 of 1.4×10^3 cells/mouse with an atypical H140 strain.¹³ Findings in this study have differed from an earlier report on subcutaneous implantation of viable *C. neoformans* in mice which resulted in the death of two animals 80 days after inoculation.⁸ Two BALB/c mice died between the fourth and ninth day after foot-pad inoculation. However, these deaths could not be traced to infection with *C. neoformans* at autopsy and from culture of the internal organs. It may have been possible to record some deaths due to cryptococcosis during this investigation if the duration of infection had been allowed to proceed beyond 2 weeks. The route of inoculation may contribute to the speed of spread of the inoculated *C. neoformans*; the cerebral, intravenous, intraperitoneal and subcutaneous routes have all resulted in lethal infections.

This study concentrated on demonstrating the relative susceptibility of four mouse strains to the spread of *C. neoformans* from the foot-pad to the spleen, lung and brain within 2 weeks. Therefore, the number of yeast cells recovered from the cultured organs were not estimated by viable counts, but colony counts for each positive culture ranged from 22 to > 150, with the brain cultures always yielding lower numbers whilst the foot-pads yielded higher counts.

Cryptococcosis has been elicited experimentally through different routes with the mouse model. Such routes include the intravenous, subcutaneous and intraperitoneal routes, although cutaneous cryptococcosis may resolve spontaneously.^{8, 13, 18, 19} No sick animals were observed throughout the 2-week period of this study. Again, this time period may be responsible for the lack of apparent physical clinical signs: isolation from the brains of the mice suggests that if a longer incubation period was allowed, some clinical signs associated with CNS disease could result. No attempts were made to demonstrate antibodies in the sera of mice used, but the yeasts were demonstrated by culture in 48.2% of the animals' internal organs. This suggests that migration from the site of inoculation to other organs had occurred. This is comparable to reports in which mice were inoculated intravenously through the tail with *C. neoformans*.⁸

The mice used in this investigation can be regarded as immunologically competent considering the number that manifested lesions in the lungs.¹¹ Therefore, the six BALB/c mice inoculated with *C. neoformans* var *neoformans* A68 that showed rough hair coats, a common symptom in mice infected with *C. neoformans*, further suggests that there was some degree of greater virulence of the yeasts for these mice.

Not all the tissues examined at autopsy revealed recognisable lesions. As expected, lesions were more evident in the foot-pads (table I). Seibold and colleagues have reported that no gross lesions were seen in the brain of an infected dog but the clinical signs of meningitis and encephalitis were seen.²⁰ Although it may not be adequate to compare clinical signs due to cryptococcosis in a dog's brain with that of mice, findings in this study suggest that the visual recognition of lesions arising from cryptococcal infection in the brains of mice is difficult. This apparent lack of lesions due to *C. neoformans* in the brain may be attributed to the relatively low numbers of viable *C. neoformans* cells recovered on culture.

Virulence can be defined as an organism's capacity to spread beyond the drainage lymph node and so cause disease. The *C. neoformans* serotypes used in this study can be considered relatively virulent, having been re-isolated from the spleens, lungs and brains of the inoculated mice. Apart from *C. neoformans* var *neoformans* strain A551, whose capsule size before inoculation was small, there were no major differences in the degree of invasiveness of the *C. neoformans* serotypes A, B, C and D used. Migration of the yeasts from the foot-pad through to the brain was greater in the BALB/c and DBA/2 mice and this was due more to the *C. neoformans* var *neoformans* serotypes (strains A68, AIN, D52 and DIN). The ability of *C. neoformans* var *neoformans* strains A68, AIN and D52 to invade the mouse tissues more frequently than the var *gatti* strains confirms the epidemiological report that the serotype A, in particular, may be a more common cause of clinical disease.²¹ The other serotypes (strains B112 and C18) also showed considerable virulence in the same mouse strains (BALB/c and DBA/2). Therefore, the difference in the relative virulence of the *C. neoformans* serotypes for the different mouse strains suggests that BALB/c mice, in particular, and the DBA/2 mice are more susceptible to *C. neoformans*. The hybrid strain crossed from the DBA/2 and CBA mice showed little susceptibility to *C. neoformans*. Thus, this observation makes the usefulness of the hybrid mice for immunological studies doubtful.

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