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Survival in experimental *Candida albicans* infections depends on inoculum growth conditions as well as animal host

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Evidence is presented that the growth medium used to prepare a *Candida albicans* challenge inoculum is a significant factor determining the ability of a fungus strain to gain an initial invasive hold immediately after injection into an animal host, and thus determining gross strain lethality. Three *C. albicans* strains, one known to be attenuated in virulence, were grown in two broth media and injected intravenously at different doses into female NMRI mice and male albino guinea pigs. For each fungus strain and challenge dose, survival was longer from inocula grown in a diluted, buffered peptone-based broth than from inocula grown in Sabouraud glucose broth. When animals were challenged intravenously with yeast doses adjusted to give the same mean survival time regardless of strain or growth medium, the progression of fungus tissue burdens (c.f.u. g^{-1}) in kidneys, lungs, liver, spleen and brain samples was broadly similar for all three *C. albicans* strains but differed between the two animal hosts. The morphological form of *C. albicans* recovered from infected tissues differed at the level of both the fungus strain and the host tissue. Use of survival-standardized inocula provides a means of distinguishing differences in progression of experimental disseminated *Candida* infections that are related to the infecting strain from those related to the animal host.

Keywords: *Candida albicans*, experimental infection, virulence

INTRODUCTION

Much recent research on the pathogenesis of disseminated *Candida albicans* infections has focused on genes encoding putative virulence factors. The criterion for virulence is usually measurement of survival times of intravenously infected mice (Becker *et al*., 1995; Bulawa *et al*., 1995; Buurman *et al*., 1998; Calera *et al*., 1999, 2000; Csank *et al*., 1998; De Bernardis *et al*., 1998; Diez-Orejas *et al*., 1997; Gale *et al*., 1998; Ghannoum, 1998; Ghannoum *et al*., 1995; Hube *et al*., 1997; Jiang *et al*., 1997; Kvaal *et al*., 1997; Lay *et al*., 1998; Leberer *et al*., 1997; Leidich *et al*., 1999; Lo *et al*., 1997; Mio *et al*., 1996; Monge *et al*., 1999; Sanglard *et al*., 1997; Sarthy *et al*., 1997; Timpel *et al*., 1998; Wysong *et al*., 1998; Yaar *et al*., 1997; Yamada-Okabe *et al*., 1999; Zhao *et al*., 1997). This approach determines a strain's gross lethality but takes no account of likely differences in challenge susceptibility of individual mouse strains, where immune responses to *C. albicans* depend on the genetic background of the animal challenged (Ashman & Bolitho, 1993; Ashman *et al*., 1991, 1996, 1997; Fulurija *et al*., 1996). Nor does it distinguish between virulence differences that relate to host–fungus interactions occurring immediately after challenge, when the majority of injected *C. albicans* are cleared from the circulation within minutes (Baine *et al*., 1974; Iannini *et al*., 1977; Jeunet *et al*., 1970; Rink *et al*., 1981; Sawyer *et al*., 1976), and those that arise later in the infectious process as organs become progressively invaded by the fungus.

We were interested in devising an approach to experimental intravenous *C. albicans* infection that facilitated differentiation of pathological effects dependent on host factors from effects related to the infecting strain. We therefore experimented with three *C. albicans* isolates, including one known to be highly attenuated in virulence which formed germ tubes poorly in serum. The fungi were injected intravenously into two animal types, female mice and male guinea pigs, deliberately chosen to maximize any host-related differences in

Abbreviation: MI, morphology index.

progression of the infection. Preliminary experiments have shown that the pattern of infection seen after injection of *C. albicans* intravenously in guinea pigs differed markedly from the pattern in similarly infected outbred mice (Agabian *et al.*, 1994; Buurman *et al.*, 1998). In particular, the organ distributions of *C. albicans* differed with time between the two hosts, and fungal cells recovered from mouse tissues by KOH treatment formed extensive true hyphae, whereas hyphae were seldom seen in guinea pig tissues.

Since growth conditions for *C. albicans* can influence properties such as surface hydrophobicity of a challenge inoculum and hence lethality for animals (Antley & Hazen, 1988), we first investigated whether inocula grown at the same temperature but in two peptone-based broth media of considerably different composition also showed any medium-related difference in lethality. We designed these dose versus medium versus survival time experiments to reveal the maximum extent of quantitative inter-experiment variability in challenge–survival tests. In a second series of experiments, animals were infected with inocula that gave the same mean survival time regardless of inherent lethality differences in the test strains. Under these conditions, the morphological forms of the fungus in deep organs varied between *C. albicans* strains as well as between animal hosts, but the gross fungus burdens recovered from deep organs were mainly host-dependent. The use of survival-standardized inocula provides a means for future experiments to distinguish experimentally between fungus factors that are important for virulence at the time of intravenous challenge and those that relate to later pathogenic events.

METHODS

Fungi and growth conditions. *C. albicans* SC5314 (Buurman *et al.*, 1998; Hube *et al.*, 1997; Lay *et al.*, 1998; Sanglard *et al.*, 1997) is the wild-type parent of a generation of genetically marked strains (CAI-4, CAF4-2 and others), originally described by Fonzi & Irwin (1993), which have been used as the source strain for many gene disruption experiments with *C. albicans* (Lay *et al.*, 1998). *C. albicans* B2630, available as ATCC 44858, was isolated in 1968 from the infected lungs of a parrot: it is the strain that has been used for many years in animal infections involved in the discovery of azole antifungal agents (Fransen *et al.*, 1984). Strain RV4688 was originally isolated from a clinical source by R. Vanbreuseghem, Antwerp, Belgium, and has been deposited as ATCC 28516. This isolate was found to be attenuated in virulence for animals in preliminary experiments (unpublished data).

All three yeast strains were maintained on Sabouraud glucose agar (Oxoid). For the preparation of inocula, a yeast colony was gently sampled with a wet inoculating loop and yeasts were transferred to 5 ml inoculation medium in a sterile, 15 mm glass test tube. Two inoculation media were used: one was Sabouraud broth (mycological peptone, Oxoid, 10 g l^{-1}; glucose, 40 g l^{-1}) and the other was CYGi, a 10-fold dilution of another peptone-glucose medium previously described (Odds, 1991) and comprising pancreatic casein digest (Merck), 3·0 g l^{-1}; yeast extract (Difco Laboratories), 3·0 g l^{-1}; glucose, 6·0 g l^{-1}; MOPS (Acros Organics), 10 g l^{-1}; and Tris (Acros), 3·0 g l^{-1}; pH 7·2, sterilized by autoclaving.

The test tube cultures were incubated in wheels rotating at 20 r.p.m. set at an angle of 5° to the horizontal in an incubator at 30 ± 1 °C (Odds, 1991). For measurement of growth rates, the turbidity of the cultures was determined spectrophotometrically at 570 nm at intervals up to 24 h after inoculation. For preparation of inocula, the cultures were incubated for 18–20 h at 30 °C, then the cell concentration was estimated by means of haemocytometer counts. The morphology of all three strains in both media was spheroidal yeast cells without pseudohyphal or hyphal forms. The yeast suspensions were diluted in sterile physiological saline as required. The suspensions contained >95% viable cells as judged by phase-contrast microscopy. A sample of each suspension used for infection was serially diluted and plated on Sabouraud agar to determine the numbers of c.f.u. ml^{-1}. For comparisons between host species, infecting inocula were expressed as c.f.u. (g body weight)$^{-1}$.

For examination of *C. albicans* morphology *in vitro*, yeasts were grown overnight in CYGi broth at 30 °C with constant rotation and cell suspensions were diluted to $1–5 \times 10^5$ yeast cells ml^{-1} in pre-warmed volumes of foetal calf serum, buffered Eagle's modified essential medium (EMEM; Odds *et al.*, 1985) or modified Sabouraud broth (MSAB; Evans *et al.*, 1975). These cultures were incubated at 37 °C (serum and EMEM) or 40 °C (MSAB). Samples were removed for microscopic observation and scored for morphological form after 2, 4 and 6 h incubation.

Experimental infections. Specific pathogen-free male albino guinea pigs and female NMRI mice were the animal hosts. Pirbright albino guinea pigs (Charles River Associates, Kisslegg, Germany) were housed individually and mice (Charles River Associates, Brussels, Belgium) in groups of up to ten. They were maintained under conditions approved by the Animal Welfare Committee of the Janssen Research Foundation and were fed food and water *ad libitum*. Individual animals were weighed and infected intravenously with *C. albicans* via either the lateral tail vein (mice) or the lateral vein of the penis (guinea pig). In a first series of experiments, animals in groups of five were infected with different concentrations of the three strains of *C. albicans*, grown in Sabouraud or CYGi broth, to establish the relation between infecting dose and mean survival time. To investigate the extent of quantitative variations in survival time, a schedule was devised that involved randomization of dosages, strains and animal hosts so that on each day when an experiment was set up, a subset of combinations of strain, medium, inoculum size and animal host was chosen randomly. In this way, no more than one concentration of any individual *C. albicans* strain from Sabouraud or CYGi medium was inoculated in the same host in the same experimental run. The day of death of each infected animal (or day when animals in moribund condition were killed) was recorded and observations were continued up to 21 d after challenge. The experiments were all set up within a 3-week period and new batches of experimental animals of approximately the same age were purchased each week.

In a second series of experiments, guinea pigs and mice were again infected with the three strains of *C. albicans*, grown in the two different media, but inoculum sizes were judged from the results of the first experiments to give a predicted survival time of 14 d in all cases. This time was chosen to allow for infection to progress in deep organs without distortions of the infection pattern resulting from acute deaths. On days 1, 2, 3, 4, 7 and 14 after challenge, three animals from each experimental group were killed. The left and right kidneys, liver, lungs, spleen and brain were removed under aseptic conditions

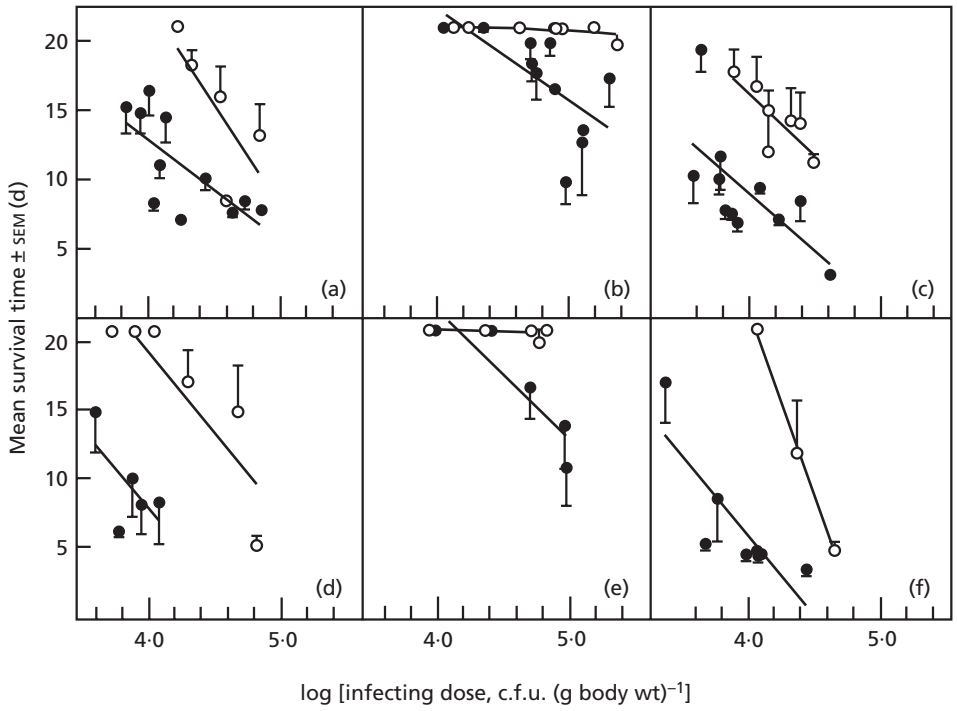

Fig. 1. Mean survival times ±SEM for groups of five mice (a–c) and five guinea pigs (d–f) infected intravenously with *C. albicans* B2630 (a, d), RV4688 (b, e) or SC5314 (c, f). ○, Yeasts grown in CYGi broth; ●, yeasts grown in Sabouraud broth. For each fungus strain and challenge dose, each data point was determined from a separate experiment to reveal the extent of quantitative variation in survival times. Trend lines were determined by logarithmic regression analysis of the means.

and a portion of each organ was weighed and placed in 6 ml sterile physiological saline. These samples were homogenized in an ultra-Turrax-type apparatus (Janke and Kunkel) and serial dilutions were plated on Sabouraud agar for determination of *C. albicans* c.f.u. (g organ)$^{-1}$. A further 2 ml of each homogenate was mixed with 2 ml 60% (w/v) KOH solution to permit recovery of *C. albicans* cells for morphological examination. The mixtures were incubated for 2 h at 30 °C with gyratory shaking at 100 r.p.m., centrifuged at 1500 ***g*** for 10 min, and the supernatant fluid was removed. The pellet was washed in distilled water three times by repeated resuspension and centrifugation and finally resuspended to a volume of approximately 0·5 ml in water for microscopic examination of the morphology of the recovered *C. albicans* cells.

Estimation of *C. albicans* morphological forms *in vitro* and *in vivo*. To estimate the nature and distribution of *C. albicans* cell shapes recovered from infected tissues or grown *in vitro*, an adaptation of the 'morphology index', MI (Merson-Davies & Odds, 1989) was used. Cell units with a spherical or nearly spherical shape were scored 1; cell units with an ovoid shape and a length up to twice the diameter of the cell were scored 2; cell units with a pseudohyphal appearance (obvious constrictions at septal junctions) and with a length more than twice the cell diameter were scored 3; and parallel-sided hyphal cell units with minimal constrictions at septal junctions were scored 4. For each microscopic field examined, the numbers of cells with shape score 1, 2, 3 or 4 were recorded and the process was repeated, when sufficient cells were available for scrutiny, until at least 50 cell units had been examined and scored. When this system was used for *C. albicans* grown *in vitro*, only newly emergent growth forms were assessed; the morphology of parent blastoconidia was not scored; 100 cell units were scored for cultures *in vitro*.

RESULTS

Growth rates and morphological phenotype of the three *C. albicans* strains *in vitro*

All three strains grew at similar rates in the two broth media. Specific growth rates for B2630, SC5314 and RV4688, respectively, were 0·52, 0·56 and 0·46 h^{-1} in Sabouraud broth and 0·30, 0·36 and 0·32 h^{-1} in the dilute medium.

All three strains formed germ tubes in foetal calf serum at 37 °C. By 4 and 6 h, 100% of B2630 and SC5314 cells appeared as characteristic parallel-sided hyphal outgrowths, mean MI = 4·0. RV4688, by contrast, formed true hyphae less readily in serum. Mean MI ± SD for this strain was 2·2 ± 1·0 at 2 h, 2·8 ± 1·2 at 4 h and 2·7 ± 1·0 at 6 h (n = 100 for each estimation). The proportion of cell outgrowths with MI = 4 was 9% at 2 h, 43% at 4 h and 31% at 6 h. RV4688 was therefore a strain with a highly mixed morphological phenotype in serum. In EMEM and MSAB, only B2630 and SC5314 formed true hyphae, with MI = 4 at 4 h for >95% of new outgrowths, respectively. For RV4688, after 4 h incubation in EMEM or MSAB, there were no true hyphal outgrowths formed: approximately 50% of outgrowths were buds

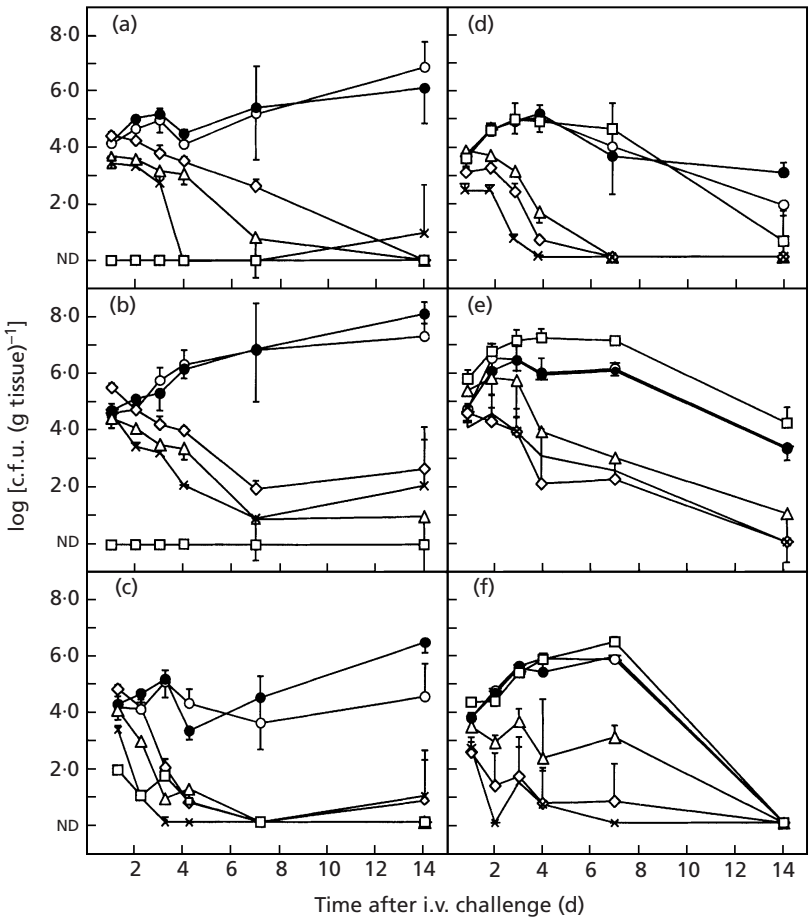

Fig. 2. Organ burdens (c.f.u. g^{-1}) of *C. albicans* in mouse tissues (a–c) and guinea pig tissues (d–f) after intravenous challenge. (a, d) B2630; (b, e) RV4688; (c, f) SC5314. Yeasts were grown in Sabouraud broth and the challenge doses used were standardized to give a mean survival time of 14 d. The data for experiments with yeasts grown in CYGi broth were similar to those shown. ○, Left kidney; ●, right kidney; △, spleen; ◇, liver; ×, lungs; □, brain.

with MI = 1, 40% long buds with MI = 3 and 10% pseudohyphal forms with MI = 3.

Mean survival times of mice and guinea pigs with disseminated *C. albicans* infection

Fig. 1 shows the results of experiments in which yeasts of the three strains of *C. albicans*, grown in either Sabouraud or CYGi broth, were injected intravenously at different concentrations. Each mean ± SEM data point in each dose-response curve was the result of a separate experiment. The results indicated considerable quantitative variation between runs (individual data points) and between animals (SEMs for each point), despite which the lethality trends for each strain tested were clear and unequivocal. In both mice and guinea pigs, SC5314 and B2630 showed similar lethality and RV4688 was markedly attenuated in virulence for both animal hosts. The dose–survival time curves for all three strains depended on the culture medium used to grow the yeasts that were injected, with inocula from Sabouraud broth cultures consistently leading to shorter survival times, dose for dose, than inocula from CYGi broth cultures.

From regression analysis of the dose-response data in Fig. 1, the following infecting doses were estimated to result in mean survival times of approximately 14 d in subsequent experiments. Inocula prepared in CYGi: for mice, 17000 c.f.u. g^{-1} (SC5314), 40000 c.f.u. g^{-1} (B2630), 700000 c.f.u. g^{-1} (RV4688); for guinea pigs, 15000 c.f.u. g^{-1} (SC5314), 35000 c.f.u. g^{-1} (B2630), 300000 c.f.u. g^{-1} (RV4688). Inocula prepared in Sabouraud broth: for mice, 4000 c.f.u. g^{-1} (SC5314), 7000 c.f.u. g^{-1} (B2630), 200000 c.f.u. g^{-1} (RV4688); for guinea pigs, 3000 c.f.u. g^{-1} (SC5314), 4000 c.f.u. g^{-1} (B2630), 170000 c.f.u. g^{-1} (RV4688).

Tissue distributions of *C. albicans* and fungal morphology in tissues of animal hosts with disseminated infection

Fig. 2 shows the logarithms of counts of viable *C. albicans* determined for mice and guinea pigs infected with *C. albicans* challenge doses chosen to result in mean survival times of 14 d. The results shown are from experiments where the yeast inoculum was grown in Sabouraud broth. Essentially the same results were obtained when the inoculum was grown in CYGi: when challenge inocula were adjusted to achieve the same levels of infectivity, no differential effects of culture medium were apparent on the distribution of cells in major organs.

In mice, the kidneys were the organs with the highest burdens of *C. albicans* throughout the observation period. All three strains were recovered at concen-

trations of around 10^5 c.f.u. g^{-1} for the first 4 d after infection, rising to 10^6 or 10^7 c.f.u. g^{-1} by day 14 (Fig. 2a–c). (For SC5314, the rise was to 10^5–10^6 c.f.u. g^{-1}.) Tissue burdens in lungs, liver and spleen also followed similar patterns for all three *C. albicans* strains, with initial counts of 10^3–10^5 c.f.u. g^{-1} at 1 d post-challenge falling to <100 c.f.u. g^{-1} by 7 d post-infection. At no time was viable *C. albicans* recovered from the brains of animals infected with B2630 or RV4688; however, mice infected with SC5314 had low but detectable *C. albicans* counts in brain samples on days 1, 2 and 3 after challenge.

In guinea pigs (Fig. 2d–f), the progression of tissue burdens after intravenous challenge with all three *C. albicans* strains differed from those in mice. In this host, c.f.u. recovered from brain tissue were uniformly equally high as those recovered from the right and left kidneys. The highest tissue burdens were determined for RV4688 (Fig. 2e), but the general pattern of changes in tissue burdens with time were essentially similar for all three strains regardless of the growth medium used to prepare the challenge dose. Highest counts of *C. albicans* were recovered from brain and kidney samples on day 4 or day 7 after challenge, with a marked decrease in the c.f.u. g^{-1} in brain and kidney samples by 14 d after challenge. Counts of viable fungi from liver, spleen and lung samples all followed a similar trend, with a tendency towards decreasing c.f.u. g^{-1} from day 3 post-challenge onwards, with the lowest counts recovered in animals still alive at the predicted mean survival time of 14 d.

Morphology of *C. albicans* recovered from infected tissues

In tissue samples where fewer than 10^3 c.f.u. g^{-1} were recovered in culture, too few *C. albicans* cells were seen in the pellets of fungi recovered after KOH treatment to permit estimation of morphology scores on a sample size of at least 50 cells. Only for samples from kidneys (both animal hosts) and brain tissues (guinea pigs) was there a consistently high number of fungal cells available for morphology scoring to permit analysis of fungal morphology at different times after challenge. The data in Fig. 3 show that *C. albicans* true hypha formation in the tissues (percentage of cells with MI 4) varied both with the infecting fungus strain and the animal host but was not dependent on the growth medium used to prepare the inoculum.

SC5314. In guinea pigs, true *C. albicans* hyphae (MI score 4) predominated in kidney and brain samples over the first 4 d after challenge (Fig. 3a, b). With progression of infection, the morphology of SC5314 cells recovered from guinea pig kidneys (7 and 14 d after challenge) changed to a more pseudohyphal appearance, with a decrease in cells with MI 4 (Fig. 3a) and an increase in percentage of forms scored as MI 2 and 3 (not shown). In brain samples, too few fungal cells were recovered at 14 d to allow analysis. In the kidneys of mice infected with SC5314, early samples (up to day 3 post-challenge)

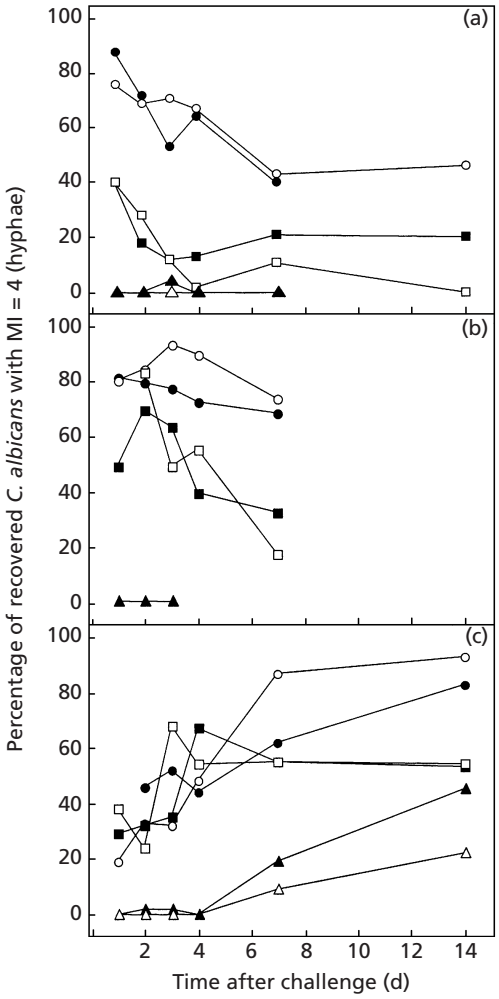

Fig. 3. Percentage of hyphal forms (MI = 4) among *C. albicans* cells recovered by KOH treatment of infected tissues of mice and guinea pigs at different times after infection with *C. albicans* SC5413 (circles), B2630 (squares) and RV4688 (triangles). Data are presented for fungi recovered from guinea pig kidneys (a), guinea pig brains (b) and mouse kidneys (c). The challenge inocula were standardized to give a mean survival time of 14 d and were grown in either SAB broth (filled symbols) or CYGi broth (open symbols). The percentages were determined from microscopic counts of true hyphal cell units in samples where at least 50 fungal cell elements were obtained from two or three animals.

contained fungal cells with mixed morphologies (Fig. 3c). From day 4 to day 14, the fungus morphology altered from predominantly spheroidal yeast forms (MI = 1) on days 1 and 2 to predominantly hyphal forms (MI = 4) on days 7 and 14.

B2630. In samples of guinea pig kidney tissue, mixed fungal morphologies were found at all times after i.v. challenge, and hyphal forms became rare from 3 d after challenge (Fig. 3a). Elongated yeast cells (MI = 2) and pseudohyphal forms (MI = 3) predominated in samples from day 4 onwards, which is why the proportion of cells with MI 4 in Fig. 3 falls below 50%. In guinea pig

brain samples, the distribution of morphologies showed a preponderance of hyphal forms on days 1–3 (Fig. 3b), with a decreasing proportion of such forms later in the course of infection. Among mice infected with B2630, kidney samples showed increasing hyphal predominance among fungal morphologies recovered over the course of infection, similar to the results obtained with SC5314 (Fig. 3c).

RV4688. The predominant morphological form in guinea pig kidneys and brain and in mouse kidneys from animals infected with this *C. albicans* strain was the spheroidal yeast cell. In kidney and brain samples from guinea pigs, true hyphal forms (MI = 4) were recorded for only four fungus cells out of more than 4000 sampled (Fig. 3). However, in samples from kidneys of mice infected with RV4688, significant proportions of true hyphal forms were seen on days 7 and 14 after challenge (Fig. 3c), even though cells with morphology score 1 remained predominant.

DISCUSSION

Many studies have now been published in which *C. albicans* strains with specific disruptions of genes encoding putative virulence factors have been shown to be attenuated in terms of lethality for mice by intravenous challenge (Becker *et al*., 1995; Bulawa *et al*., 1995; Buurman *et al*., 1998; Calera *et al*., 1999, 2000; Csank *et al*., 1998; De Bernardis *et al*., 1998; Diez-Orejas *et al*., 1997; Gale *et al*., 1998; Ghannoum, 1998; Ghannoum *et al*., 1995; Hube *et al*., 1997; Jiang *et al*., 1997; Kvaal *et al*., 1997; Lay *et al*., 1998; Leberer *et al*., 1997; Leidich *et al*., 1999; Lo *et al*., 1997; Mio *et al*., 1996; Monge *et al*., 1999; Sanglard *et al*., 1997; Sarthy *et al*., 1997; Timpel *et al*., 1998; Wysong *et al*., 1998; Yaar *et al*., 1997; Yamada-Okabe *et al*., 1999; Zhao *et al*., 1997). In these studies, the culture media used to generate the challenge inoculum, when specified at all, indicate a considerable diversity of conditions. Antley & Hazen (1988) showed that inocula grown at room temperature had a higher surface hydrophobicity and were more lethal to mice than inocula with lower surface hydrophobicity, grown at 37 °C. Our data show that, even when the growth temperature is kept constant, the lethality of challenge inocula differed with the medium on which the inoculum was grown. Both of the media used were complex and based on peptone-glucose combinations: however, one was unbuffered and the other was buffered at pH 7·0 and was more dilute. We did not explore the physiological differences between the yeast cells produced in the two broths, but differences in surface hydrophobicity or in expression of switching systems in the fungus (Kvaal *et al*., 1997) may have been altered by growth conditions and led to differences in gross challenge lethality. Rieg *et al*. (1999) have shown a direct association between mouse survival times after challenge and growth rates of *C. albicans* strains *in vitro*. However, growth rates of our three strains were similar in each of the broths, so that growth rate differences *in vitro* are unlikely to explain the gross lethality differences *in vivo*.

Our results in animal lethality tests (Fig. 1) show that, even though gross differences in virulence between strains can be demonstrated by challenge experiments with inocula prepared in a single medium, the quantitative variation in survival rates is considerable both between animals and between experiments. This suggests that the reproducibility of lethality differences measured between parent and mutant strains of *C. albicans* may be more reliably established from experiments with randomized challenge dose designs, similar to that used in the present study, rather than in single experiments, especially when those differences are not substantial.

This is the first study in which differences in lethality have been broken down into (a) effects related to the preparation of the inoculum, (b) the challenge dose normalized for inherent differences in the virulence of individual strains and (c) two different host species. This approach facilitated the distinction between progression of infection as related to individual host tissues and infective effects related specifically to the infecting strain. While the growth medium influenced mean survival times in simple dose–survival titration experiments, when the challenge inocula were normalized to equalize survival times, no influence of growth medium was evident in terms of organ burdens of *C. albicans* or in fungal morphologies in infected tissues at various times after challenge. These findings suggest that although the growth conditions for a *C. albicans* infecting inoculum are an important factor in determining a strain's ability to gain an initial invasive hold immediately after injection into an animal host, they are unimportant in the subsequent development of infection.

The quantitative *C. albicans* organ burdens measured at times after challenge were similar for all three strains of the fungus, but differed between the two animal hosts tested (Fig. 2). This suggests that host factors play a more important role in determining quantitative tissue burdens than any virulence factors in the fungus, once infection has been established in tissues by administration of a suitably large inoculum. Several prior studies have shown that, in a murine host, the genetic background of the mouse strain determines not just overall susceptibility to intravenous *C. albicans* challenge, but also the susceptibility of individual host tissues to infection by circulating fungus cells (Ashman & Bolitho, 1993; Ashman *et al*., 1991, 1996, 1997; Fulurija *et al*., 1996). The mouse gene products that regulate the susceptibility of individual tissues to the fungus have been designated Carg1p and Carg2p (Ashman *et al*., 1997). Gender is a further significant influence on susceptibilities of mice of *C. albicans* challenge (Ashman *et al*., 1991). In our experiments, the hosts used were of different genders, as well as different species, a deliberate choice to emphasize the considerable differences effected by host factors in progression of experimental disseminated *C. albicans* infections. Ashman *et al*. (1997) observed extensive fungal colonization of brain tissue and severe brain lesions only in four of 15 recombinant inbred mouse strains derived

from two progenitor strains. Neutrophil responses have been shown to influence differences in susceptibility of mouse brains and kidneys to *C. albicans* infection (Fulurija *et al*., 1996). Our finding that brain burdens of *C. albicans* matched those of kidneys in guinea pigs but not in mice is consistent with these previous reports of differences in animal host susceptibility to cerebral infection with the fungus.

While our data show a strong animal host-dependency of the fungal burdens in specific tissues, the morphological forms of the fungi recovered from the tissues by KOH treatment were clearly a function of the characteristics of the infecting strain. Two of our three *C. albicans* strains formed true hyphae readily under different, well-characterized environments known to favour filamentous growth *in vitro*, whereas RV4688 formed true hyphae only rarely and reluctantly *in vitro*. In the kidneys of both animal species and the brains of guinea pigs, each of the three *C. albicans* strains appeared to follow an individual course of morphological development, but with characteristics that also indicated at least a partial influence from the host tissue micro-environment (Fig. 3). In mouse kidneys, the morphological trend for all three strains was for the proportion of true hyphal cells to increase with time. In guinea pig kidneys, by contrast, proportions of hyphal cells were high soon after challenge (except for RV4688) and they fell over the course of infection. These trends therefore appeared to be attributable to the host tissues. RV4688 hardly ever formed hyphae *in vivo*, behaviour consistent with its hypha formation *in vitro*: it started to form measurable proportions of true hyphae only in mouse kidney tissues by days 7 and 14 after challenge.

In general, true mycelial forms proliferated in mouse kidneys while elongated yeasts and pseudohyphal forms predominated in guinea pig kidneys. Morphological differences of this nature were noted en passant but not specifically reported by Winblad (1975) in his comparative histopathological study of *C. albicans* infection in the kidneys of mice and guinea pigs. The figure legends in Winblad's paper describe the fungi as 'mainly in the mycelial phase' in mouse kidney sections and as 'both yeast and mycelial phase' in guinea pig kidney sections. The histopathological forms illustrated by Fransen *et al*. (1984) in guinea pig tissues similarly suggest a predominance of yeast forms and pseudohyphae, rather than true hyphae, in most *C. albicans*-infected tissues. De Bernardis *et al*. (1993) described a *C. albicans* strain, CA-2, which formed pseudohyphae *in vivo* in a rat vaginal model of infection, but was unable to form germ tubes *in vitro*. Those observations, plus those of the present study, indicate that morphogenesis of *C. albicans* is a highly complex response of the fungal cells to their micro-environment, with no obligate correlation between a strain's ability to form filaments *in vivo* and *in vitro*.

These observations on morphological development of the three strains *in vivo* generally support the traditional view of *C. albicans* which implicates formation of hyphae as a property conducive to pathogenicity (Kobayashi & Cutler, 1998; Lo *et al*., 1997; Odds, 1988; Brown & Gow, 1999) since the strain that poorly formed hyphae was the least virulent in terms of gross lethality per challenge dose. However, in the present experiments when the strain reluctant to form hyphae was injected at a concentration aimed to give the same mean survival time as in the animals infected with the prolific hypha-formers, its tissue burdens after infection were as high as or even higher than those of the other two strains (Fig. 2) and they followed the same course of changes with time. The pathological factors that ultimately led to death of animals infected with *C. albicans* are unknown, but they were clearly not absolutely dependent on the capacity of the infecting strain to form hyphae: however, the attenuated lethality of RV4688, dose for dose, may indicate that an ability to form hyphae enhances the initial ability of a strain to achieve a pathologically significant hold on host tissues at or soon after the time of intravenous challenge.

Older studies have shown clearance of almost all *C. albicans* cells from the blood of experimental animal hosts within only minutes of intravenous injection (Baine *et al*., 1974; Iannini *et al*., 1977; Jeunet *et al*., 1970; Rink *et al*., 1981; Sawyer *et al*., 1976). These reports indicate that immediate, nonspecific phagocytosis of the fungus cells by the reticulo-endothelial system is a potent and – for the fungus – critical encounter between host and pathogen in models that involve direct intravenous challenge. If overwhelming numbers of a weakly virulent strain are injected to facilitate survival of yeast cells that are not inherently well-equipped to resist immediate host clearance mechanisms, as in our experiments with RV4688, then the surviving yeasts are evidently capable of expressing sufficient virulence to set up pathologically significant tissue burdens, a process that our data show is independent of hypha formation.

The number of studies investigating the influence of specific gene deletions on virulence in *C. albicans* has grown extensively in recent years: most of these publications base their conclusions on the gross lethal outcome of intravenous challenge experiments in mice. The present study adds weight to existing experimental evidence that shows that the intrinsic virulence of *C. albicans* is modulated by both the strain of animal host chosen for lethality testing and the conditions used to generate yeast challenge inocula. Despite the existence of such prior evidence, the lethality testing of 'gene knockouts' in *C. albicans* has been done in a diverse range of mouse strains and the medium, temperature and time used to prepare each inoculum differ from study to study: these variables have not even been detailed in several such publications.

Our study has confirmed and extended the evidence that *C. albicans* intravenous virulence *in vivo* is dependent on the animal species challenged and the growth conditions used for the challenge inoculum, as well as on differences between fungal strains. Our use of survival-standardized inocula offers a novel approach for future investigations to differentiate early- and late-stage host

and pathogen factors involved in the infectious disease process with this fungus. We hope that future experiments on the pathogenesis of experimental *C. albicans* infections will take greater account of the sources of variability we have highlighted in our work, as well as extending to include models that take account of the mucosal barriers to dissemination of infection usually found in the human setting.

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