Evaluation of the Experimental Pathogenicity of Some *Cryptococcus* Species in Normal and Cyclophosphamide-Immunodepressed Mice

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Abstract The pathogenic potential of distinct *Cryptococcus* species has been evaluated in mice rendered leukopenic by one or two injections of the potent immunosuppressive drug cyclophosphamide (Cy). Pathogenicity assessment included enumeration of viable cryptococcal cells in animal organs and histopathological observations. It was found that putatively non-pathogenic species of *Cryptococcus*, in particular *C. cereanus* and *C. albidus*, showed significant lethality for Cy-treated mice. In Cy-immunodepressed mice, challenged with the infectious cryptococcal cells two days after pharmacological treatment, a significant decrease of LD50 (equivalent to at least one order of magnitude) was observed for all *Cryptococcus* species. However, the pathogenicity enhancement due to Cy immunodepression was greater with *C. neoformans*. In all cases, brain and kidney were the most invaded tissues as also evidenced by histopathological examination, which showed the typical cystic lesion. All the observations made point to the conclusion that the pathogenic potential, for the immunomodulated host, of *Cryptococci* other than *C. neoformans* is significant being quantitatively and not qualitatively different from that of *C. neoformans*, as evidenced by a similar organotropism and similar type of histological lesions in the target organs (brain and kidney).

While *Cryptococcus neoformans* is generally considered to be the only pathogenic species of the genus *Cryptococcus*, other species have been isolated from clinical specimens in man and animals. For instance, *C. albidus*, *C. laurentii*, *C. lactactivorus*, *C. terreus*, and *C. uniguttulatus* have occasionally been isolated from sputum, pharyngeal swab, bronchial washing, lung biopsy, cerebrospinal fluid and urine of immunodepressed patients (13, 21, 26); some of the species above have also been isolated from cases of bovine mastitis. *Cryptococcus lactactivorus* has been shown to be able to reproduce experimental infection of mammary glands (22).

Three cases of meningitis due to *C. albidus* have also been reported (5, 17, 27). One of these affected a patient under corticosteroid therapy (17). The authors claimed the possibility of infection by other cryptococcal species. We have isolated
a strain of *C. albidus* from various organs in a case of bovine abortion (6).

Despite the intrinsic importance of these isolations, studies addressing the experimental pathogenicity of these minor species of *Cryptococcus* have not been carried out, so that the pathogenic role of these species is not at all clear. To our knowledge, studies on pathogenicity of *Cryptococcus* in normal and immuno-modified animals have been conducted only with *C. neoformans* (8, 9).

To contribute to this field we have studied the pathogenic potential of several species of *Cryptococcus* in comparison with *C. neoformans*. Pathogenicity has been assessed both in normal and in cyclophosphamide-immunodepressed mice with studies involving organ distribution and histopathological observations.

**MATERIALS AND METHODS**

*Microorganism source and growth conditions.* The yeasts (and their source) used throughout this study were: *C. neoformans* var. *neoformans* (4710/Istituto Superiore di Sanità; I.S.S., Rome, Italy) isolated from pharyngeal swab; *C. albidus* var. *diffluens* (17 AB/1.S.S.), isolated from bovine abortion; *C. laurentii* (4687/1.S.S.), isolated from expectorate of tuberculous patient; *C. terreus* (1895/C.B.S.); *C. lactactivorus* (now transferred to the ascomycetous yeast *Sporopachydermia lactactivora*) (5771 C.B.S.); *C. Cereanus* (now transferred to the ascomycetous yeast *Sporopachydermia cereana*) obtained from Prof. D.E. Phaff, University of California, Davis, Calif., U.S.A. All species used had morphological and biochemical characteristics conforming to the standard description of the species (15) and all of them are maintained in recognized microbial type collections (I.S.S. and C.B.S., Centralbureau voor Schimmelcultures, Delft, Netherlands, as indicated). The strains were routinely maintained in malt extract agar (malt extract Difco 2%; (w/v) glucose 2%; (w/v) peptone 1%; (w/v) agar 2%).

*Infectious challenge.* For the infectious challenge, the microorganisms were grown in glucose nutrient broth (glucose 1%; NaCl 0.5%; (w/v) beef extract Difco 1%; (w/v) peptone 1%) for 48 hr at 27°C on a shaker, at 200 rpm (New Brunswick Scientific Co., In., U.S.A.). The microorganisms were counted, suitably diluted in physiological saline and inoculated intravenously (i.v.) into outbred male Swiss mice, 18–21 g, obtained from Charles River (Calco, Italy).

*Pharmacological treatment.* Cyclophosphamide (Cy, Cytoxan, Sigma Chemical Co., Mo., U.S.A.) was dissolved in 0.85% physiological saline immediately before use and injected intraperitoneally at any of the following concentrations: 100, 150, 200, 300 mg/kg, in 0.2 ml final volume.

The immunodepressive effect of Cy treatment were evaluated by following total and differential leukocytes modifications. For leukocyte counts, the animals were bled from the retro-orbital sinus. Total leukocytes were determined by using a Coulter (Coulter Electronics Ltd., Harpenden Herts, U.K.). Differential leukocyte counts were performed on blood smears after May-Grunwald-Giemsa staining.

*Pathogenicity assessment.* *LD*$_{50}$. The 50% lethal doses (*LD*$_{50}$) were determined by injecting i.v. groups of 10 untreated or Cy-treated animals with graded doses (in
the range of $3.125 \times 10^6$–$10^8$ cells), in a final volume of 0.5 ml. The animals were followed for 40 days and the LD$_{50}$ values were calculated by the method of Spearman and Karber (4).

**Organ invasion.** Growth of the fungus in mouse organs was followed by injecting groups of untreated and Cy-treated mice with either $5 \times 10^6$ cells of *C. neoformans* or $1.5 \times 10^7$ cells of all other species of *Cryptococcus*.

At 4, 8, 24 hr and 2, 3, 4, 5, 7, 14 days, 2 mice per group were sacrificed by cervical dislocation; kidney and brain were removed aseptically, homogenized and diluted with sterile saline solution. Each dilution was then placed on malt extract agar and after 48 hr of incubation at 27 C the developed colonies were counted and expressed as number of colony-forming units (c.f.u.) per gram of organ.

**Histopathology.** Histopathological examination of several organs (kidney, brain, liver, lung, heart, and spleen) of animals challenged with *Cryptococcus* species were performed both during the experiments of mortality assessment and during experiments of yeasts growth in vivo.

The organs were removed aseptically from dead or sacrificed animals and immediately fixed with 10% formaldehyde. After paraffin inclusion the slices of organs were dehydrated and stained with hematoxylin-eosin, PAS-van Gieson, and Grocott methods.

**Statistical evaluation.** The statistical significance of differences in leukocyte counts and c.f.u. of organs between untreated and cyclophosphamide-(Cy) treated animals were evaluated by Student's $t$-test.

**RESULTS**

The Effect of Cyclophosphamide Treatment on Peripheral Blood Leukocyte Counts

Following Cy treatment, total peripheral blood leukocyte counts were seen to diminish at all concentrations of the drug. Representative responses are shown in Fig. 1. Starting from the first day after Cy treatment, the number of leukocytes was significantly lower, as compared to that of untreated animals and the duration of this depression depended on Cy dose. At doses of 200–300 mg/kg, the number of leukocytes returned to nearly normal levels only on days 7–8. At lower Cy doses (100–150 mg/kg) the leukopenic period was short (2, 3 days), returning to normal levels after 3–4 days, after which the usual characteristic effect of increased leukocyte counts (rebound), was observed, as described by others (25).

We also evaluated the effect of two Cy injections at various day intervals, using three different concentrations of the drug. The results are shown in Fig. 2. As expected, a more marked leukopenia was evident in mice given two Cy doses than in animals treated only once with Cy (Fig. 1) and this effect was dose-independent. Moreover, the length of leukopenic period was inversely related to the distance between the two inoculations. In all cases, there was no mortality of mice after single or double Cy administration over the experimental period of 60 days.

The differential analyses of leukocytes in Cy-treated animals showed that lymphocytes were mostly reduced in number on days 2–3, when maximum Cy-
induced immunodepression was seen. This reduction ranged from 50\% to 80\%, depending on Cy dose.

**Mortality of Mice Challenged with Cryptococcus Species**

LD$_{50}$ values were determined in untreated and Cy-treated mice following challenge with the distinct species of *Cryptococcus*. The animals were treated once or twice with 3 different concentrations of the drug. In two different experiments the LD$_{50}$ values of Cy-untreated animals were $38 \times 10^6$-$42 \times 10^6$ for *C. neoformans*; $62 \times 10^6$-$67 \times 10^6$ for *C. cereanus*; $67 \times 10^6$-$71 \times 10^6$ for *C. albidus*. In animals treated with Cy a common pattern of increased lethality was observed with all species studied. Significant decrease in LD$_{50}$ was in particular observed when the animals were pretreated with a single dose of 200 or 250 mg/kg of Cy and injected with the microbial cells two days after the pharmacological treatment. Still increased mortality was detected when the animals were given two doses of Cy—the first two days before and the second two days after the challenge. In this latter case the animals showed differences in survival time depending on the species used for challenge. The minimal survival was found in mice challenged with *C. neoformans*.

Figure 3 shows the data of a typical experiment performed with a dose of 200 mg/kg of Cy and demonstrating the effect of a single or double drug treatment on pathogenicity of the species of *Cryptococcus* examined. The statistical significance of the differences is shown.

**Organ Distribution of Cryptococcus Species**

Organ invasion and growth of the different species of *Cryptococcus* in kidney
Fig. 2. Effect of two injections of cyclophosphamide (Cy) on total blood leukocyte counts. Groups of 6 animals were given a first injection of Cy (■, 150 mg/kg, or ▲, 200 mg/kg, or ●, 250 mg/kg) on day 0 and a second injection of the same dose of the drug on day 3 (panel a) or day 4 (panel b), or day 5 (panel c), or day 6 (panel d) following the first administration. At different day intervals the total number of peripheral blood leukocytes were counted as reported in the legend of Fig. 1. The total leukocyte counts of untreated animals were similar to those reported in Fig. 1, ranging from 6,700 to 8,100.

Fig. 3. Effect of time of cyclophosphamide (Cy) administration on mortality of mice challenged with different Cryptococcus spp. The abscissa indicates the day of Cy administration (200 mg/kg) with respect to the day of Cryptococcus challenge, taken as 0 day. The hatched column indicates the mortality of mice treated with Cy both before (day -2) and after (day +2) infectious challenge. White columns are for animals treated only once with the drug at the indicated day. The dotted column gives the LD50 of Cy-untreated (control) mice. LD50 was evaluated using 10 animals per single dose of Cryptococcus challenge. For other details see text and the legends to Figs. 1 and 2. The bar indicates ±2 standard error (95% confidence interval).
Fig. 4. Colony-forming units (c.f.u.) of different *Cryptococcus* spp. in kidney of untreated (●) and Cy-treated mice given a single (▲, two days before infection) or two (■, two days before and two days after infection) administration of 200 mg/kg doses of cyclophosphamide. The challenging *Cryptococcus* species is indicated in each panel. The challenging infectious doses were $5 \times 10^6$ c.f.u. for *C. neoformans* and $1.5 \times 10^7$ for all other species. The asterisk denotes a statistically significant ($P<0.05$) difference between untreated and Cy-treated mice.
and brain of normal and immunodepressed mice were also investigated and the results reported in Figs. 4 and 5. All *Cryptococcus* species tested were able to multiply in the brain and kidney under all conditions, despite remarkable differences in the invasion. Efficient organ colonizations were noted particularly in animals treated twice with Cy. With *C. neoformans* as challenger, the number of c.f.u. was not evidently increased (with respect to Cy-untreated animals) by a single Cy dose. At least with *C. cereanus*, different organ colonizations between normal and immunodepressed mice were also evident given one single administration of Cy.

No relevant differences in the capacity of organ colonization were noted among the other species of *Cryptococcus*. The invasion facilitating effect of Cy was equally evident for brain and kidney although significant invasion and more prolonged persistence was noted in the brain than in the kidney, irrespective of the *Cryptococcus* species used. Later on during infection (day 14) an increased clearance of Cryptococcal cells (mostly evident in *C. laurentii* for kidney and *C. cereanus* and *C. albidus* for brain) was observed in Cy-treated animals. The reason for this behavior
probably resides in the well-known immune-rebounding effect of the drug (2) and in the fact that less than 1 LD$_{50}$ was used as infectious inoculum (see also "MATERIALS AND METHODS").

**Histological Examination**

The most characteristic lesion observed in the organs of Cy-untreated animals infected with *C. neoformans* was of cystic type. The cysts contained numerous cryptococcal cells in a gelatinous capsular material with low or no cellular response.
They were mostly detected in the brain and in the liver of animals, starting from the first week of infection (Fig. 6, a and d). In the brain, cystic reaction increased subsequently to coalescence of individual cysts, alteration and disruption of parenchyma and infiltration in the meningeal, subarachnoidal space. Areas of albuminoid degeneration were also present in the liver. Lungs were also found to be remarkably involved as they showed yeast cells in the alveoli and in alveolar septa and, sometimes, cystic foci. Granulomatous reaction, consisting chiefly of mononuclear cells, were rarely observed during cryptococcal invasion.

In the kidney, cryptococcal cells were recognized in the glomerular capillaries and in the tubuli. Some degeneration in the epithelial cells of the tubuli were observed. In the heart, single yeast cells or few micro-cysts were noted. In the spleen, granulomatous lesions, mostly in the hyperplastic follicles appeared (data not shown).

The histopathological examination of organs of mice infected with C. cereanus and C. albidus revealed lesions of the type, localization, and general features similar to those detected in C. neoformans-infected animals (Fig. 6, b, c, e, and f). The number and extent of these lesions were, however, less in these animals; of still lower entity were the lesions produced in mice challenged with the other species of Cryptococcus studied. The Cy-immunodepressed mice showed more intense organ lesions as compared to Cy-untreated animals, but a similar type of lesion (cystic and degenerative processes largely prevailing over inflammatory ones) were evidenced (Fig. 7, a and b).

DISCUSSION

Recently, it has become increasingly evident that innate resistance mechanisms are involved in the host-parasite relationship in cryptococcosis. Therefore, such natural effectors as polymorphonuclear leukocytes, macrophages, and natural killer (NK) lymphocytes have been shown to kill, or inhibit growth of C. neoformans (7, 14, 19, 20). It is well known that isolates of C. neoformans may widely differ in their
experimental virulence and that mouse strains differ in their natural susceptibility to cryptococcal infection.

In this study, an isolate of \textit{C. neoformans} of low virulence (LD$_{50}$ $4 \times 10^7$ /kg for the outbred Swiss mice) was used in order to test in a significant way the effect of the immunosuppressive drug, cyclophosphamide, in cryptococcal infection. This also served to have a meaningful comparison with all other species of \textit{Cryptococcus}, which, as shown in "RESULTS," require animal immunodepression to show significant pathogenicity. A more virulent \textit{C. neoformans} isolate was also tested (LD$_{50}$ $\sim 10^6$ /kg) but in this case, all mice died within 2–3 days and the effect of cyclophosphamide could not be followed in a sufficiently wide time-schedule (data not shown).

In view of the well-known effects of cyclophosphamide on natural immune resistance (1, 16, 23–25), it could be easily anticipated that treatment of experimental animals with an appropriate amount of this drug results in increased susceptibility to disease. This has been aptly verified in various host-parasite models and recently, both in infections sustained by \textit{Candida} spp (2, 3, 18) and in those caused by \textit{C. neoformans} (8, 9). The Cy-immunodepressed mouse seems particularly useful to reveal low fungal pathogenicity potential and it has been shown that those \textit{Candida} species with low but detectable virulence for normal host, are dramatically boosted in their pathogenicity expression in Cy-pretreated mice (3). In a recent study, Duke and Fromtling (8) reported enhanced resistance to \textit{C. neoformans} in mice challenged one day after a single injection of Cy (200 mg/kg) but increased susceptibility when several consecutive daily doses of the drug were administered to the animal after the infectious challenge. These authors did not show what was the impact of their particular Cy regimens on peripheral blood cells or other parameters of the immune system. In this paper we have shown that a marked leukopenia, as reported in other studies (2, 11, 18), is manifested for several days after a single i.p. injection of Cy in normal mouse. Mostly lymphocytes were strongly reduced in number, in substantial agreement with a recent report by Hidore and Murphy (11) demonstrating that a dramatic lymphopenia is characteristic of Cy-treatment in mouse models. This lymphopenic pattern was more severe if a second injection was given by one week after the first one. Under this experimental situation, the animals given either one or two Cy-treatments ranging from 150 to 300 mg/kg became more susceptible to infection by a moderately virulent strain of \textit{C. neoformans}, as shown by marked reduction in LD$_{50}$ on intravenous challenge, counts of live yeasts in the organs, and histopathological observations. This state of increased susceptibility to infection was manifested since the first day after Cy-treatment, peaked after 2 or 3 days and lasted for an additional 3–4 days at the end of which the animals returned to their basic susceptibility to the fungus. Even higher susceptibility to \textit{Cryptococcus} infection was achieved when a booster dose of Cy was injected after the infectious challenge in an apparent correlation with more severe leukopenia. This pattern of Cy-modulated mice susceptibility to \textit{Cryptococcus} infection bears close analogies to the one observed in experimental infections sustained by \textit{C. albicans} (2, 12, 18), suggesting that a similar natural resistance mechanism could be operating in the two infections.
In this paper, the experimental pathogenicity of several species of Cryptococcus other than C. neoformans has also been evaluated both in normal and in Cy-immunomodulated mice. At variance with C. neoformans, these species are generally regarded as “non-pathogenic” ones, although they have been occasionally isolated from diseased humans or animals (5, 6, 13, 17, 21, 22, 26, 27). In the animal model used here, these species showed low but significant pathogenicity at least to an extent comparable to that of a low-virulence strain of C. neoformans. In particular, treatment with Cy, mostly when the drug was administered both before and after the infectious challenge, did always result in a significant lethality whatever the species used for challenge. While C. neoformans ranked first in pathogenicity potential (both in normal and Cy-immunodepressed mice), the differences among all other species of Cryptococcus investigated in this model were not large and for some of them irrelevant. The increase in lethality in Cy-treated animals was genuine since it was paralleled by increased recovery of live yeast cells from various organs and more extensive lesions of target tissues, mostly the brain, confirming the relationship between cultural and histopathological findings already noted, with C. neoformans, by Grosse et al (10). One additional interest of this study is the report that the putatively “non pathogenic” species of Cryptococcus like C. cereanus and C. albidus are able to induce lesions of the type (cystic and or granulomatous) identical to those evidenced with a C. neoformans (10). This suggests that these species do not substantially differ from the frank pathogenic C. neoformans in the mechanism of invasion and in the kind of host tissues damage, once the host’s immune defense mechanism has been sufficiently impaired. Comparable organ tropism among all species examined also suggests that we are, in all cases, dealing with a similar pathogenicity mechanism although possessed to a very dissimilar extent by different Cryptococcus species and strains.

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REFERENCES


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