The Gene Encoding Phosphoribosylaminomimidazole Carboxylase (ADE2) Is Essential for Growth of Cryptococcus neoformans in Cerebrospinal Fluid

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A cryptococcal meningitis model in corticosteroid-treated rabbits was used to assess the requirement for the phosphoribosylaminomimidazole gene (ADE2) for virulence of Cryptococcus neoformans. A wild-type strain (H99), an ade2 auxotroph of H99 (M001), and a randomly selected prototrophic transformant of M001 (M001.1c) which had received the cloned ADE2 cDNA copy were inoculated intrathecally into immunosuppressed rabbits. While M001 was avirulent in the central nervous system model, virulence was completely restored to wild-type pathogenicity in the prototrophic transformant. This study identifies the pathogenic importance of an endogenous adenine pathway in this yeast and confirms that purine biosynthesis is a potential target for antifungal therapy. It also demonstrates that the virulence of C. neoformans can be molecularly changed and detected within a clinically relevant animal model.

Cryptococcus neoformans is a common cause of deep-seated mycoses in immunocompromised hosts. With the advent of human immunodeficiency virus infections and an increasing use of corticosteroids, this yeast has become one of the leading causes of life-threatening central nervous system infections. Despite treatment with amphotericin B, flucytosine, and the new triazoles, such as fluconazole and itraconazole, a significant number of treatment failures for cryptococcal meningitis occur (23, 37). In patients with AIDS, the infection can only be controlled with continuous suppressive therapy (4). The continuing search for safe and more effective therapies for cryptococcal meningitis must be encouraged.

In vitro screenings of large numbers of compounds for antimicrobial activity have been used quite successfully for both antibacterial and antifungal drug development. However, a more directed approach that involves targeting an organism’s unique pathobiology may also provide novel strategies and targets for the discovery of new antifungal agents. Since eucaryotic pathogens such as fungi share many metabolic pathways with the host cell, unique and selective targets will be required.

One class of novel antifungal targets could be based on factors that are essential to the pathogenicity of the yeast and yet absent from the host. Both bacteria and fungi have been shown to possess these virulence factors, which include a variety of toxins, enzymes, and tissue adhesins (10). Specifically, several notable virulence factors have been identified in C. neoformans, including polysaccharide production and melanin formation in conjunction with the ability to grow at 37°C (12, 18, 19, 36). Because these phenotypic characteristics for virulence have been shown to be under genetic control (19), the genes and enzymes regulating these phenotypes may represent specific targets for new antifungal agents.

Despite the attention given to virulence factors that allow the parasite to directly cause harm to the host or circumvent the host response, other factors contribute to infection simply by producing certain “housekeeping” or metabolic functions that are essential to the survival and growth of the parasite within the host. In particular, the purine and pyrimidine biosynthetic pathways of an organism have been shown to be linked to its virulence within the host (9, 16, 17, 21). In fact, one antifungal agent, flucytosine, has already exploited the unique enzymes in the pyrimidine pathway of Candida and Cryptococcus spp. (3) to effectively inhibit the growth of these yeasts in vivo. An association between decreased virulence and purine auxotrophy in bacteria and fungi is also well documented. As early as 1950, Bacon et al. (1) reported that Salmonella typhi strains carrying a mutation in the purine biosynthetic pathway were markedly less virulent than the wild-type strain in mice. This decrease in the virulence of purine auxotrophs has also been found in Salmonella typhimurium (21), Salmonella dublin (21), Klebsiella pneumonias (13), Bacillus anthracis (15), Yersinia pestis (5, 40), and Candida albicans (16, 17). The association of decreased virulence and purine or pyrimidine auxotrophy presumably results from the inability of the organism to multiply sufficiently in the host environment, reflecting the limited availability of nutrients or the inability to use exogenous metabolites from the host (2, 38).

In the present study, we examined the importance of an intact purine metabolic pathway in C. neoformans for its ability to produce meningitis in an immunocompromised host. The inactivation of the phosphoribosylaminomimidazole carboxylase gene (ADE2) in C. neoformans by mutagenesis and subsequent restoration by transformation were used to directly correlate the functional activity of this gene with the yeast’s ability to grow within the subarachnoid space.

MATERIALS AND METHODS

Organisms and media. The wild-type C. neoformans strain was H99, a serotype A clinical isolate which has been shown to induce chronic meningitis in steroid-treated rabbits (31). The ade2 mutant M001 was derived from an auxotrophic screen of H99 after UV irradiation at a dose allowing 10% survival of the original inoculum. The adenine auxotroph M001 was specifically identified by complementation with

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the cloned cryptococcal H99 ADE2 cDNA copy by biolistic techniques for DNA delivery (41). One of the stable prototrophic transformants, M001.1c, which had received the ADE2 cDNA, was chosen for evaluation of its virulence in the rabbit meningitis model. This transformant was shown to be reconstituted by homologous recombination, with insertion of the cDNA copy among the ADE2 cDNA (41). All transformants were maintained on yeast extract-peptone-dextrose (YPED) agar plates at 4°C. Organisms were propagated on either YEPD or Sabouraud agar plates at 30 or 37°C.

Experimental chronic meningitis in rabbits. The wild-type parent strain H99, the ade2 auxotroph M001, and the randomly selected transformant M001.1c were examined for their ability to produce disease in the immunocompromised rabbit meningitis model (31). Organisms were propagated on Sabouraud agar plates for 48 to 72 h at 30°C, collected on cotton swabs, and suspended in phosphate-buffered saline (pH 7.4) at a concentration of 4.5 \times 10^7 cells per ml. Approximately 1 \times 10^5 to 1.5 \times 10^7 viable C. neoformans cells in a volume of 0.3 ml were injected intracisternally into New Zealand White male rabbits that had received an intramuscular injection of 2.5 mg of cortisone acetate per kg (50 mg/ml; Merck, Sharpe, & Dohme, West Point, Pa.) 24 h earlier and then daily for 2 weeks. The infection was monitored for 2 weeks by withdrawing cerebrospinal fluid (CSF) at 2, 4, 7, 11, and 14 days after inoculation and plating 0.1 ml of undiluted and serially diluted aliquots of CSF on Sabouraud plates for quantitative yeast counts. Rabbits were sedated with 8 to 10 mg of xylazine and 100 mg of ketamine given intramuscularly prior to all cisternal inoculations and withdrawals.

In vitro growth kinetics. The mean generation time for in vitro growth at 37°C was determined for the wild-type parent (H99), the ade2 auxotroph (M001), and the prototrophic transformant (M001.1c). YEPD broth that had been warmed to 37°C was inoculated with an overnight YEPD broth culture of each strain of C. neoformans at a final cell density of 10^6/ml and allowed to incubate with shaking at 37°C. At various times (0, 2.5, 5, 8, 14, 24, 32, and 48 h), samples were taken from the broth and diluted onto YEPD agar plates for assessment of viable quantitative yeasts.

Preparation of DNA and Southern blot analysis. DNA was isolated from C. neoformans spheroplasts as described previously (32). Mid- to late-log-phase C. neoformans grown in YEPD were washed in 500 mM sodium chloride–50 mM EDTA, exposed to 1% mercaptoethanol in the sodium chloride-EDTA buffer, treated with 5 mg of lysing enzyme (Trichoderma harzianum) per ml in the presence of 1.0 M sorbitol–0.1 M sodium chloride–0.1 M EDTA, and lysed in 2% sodium dodecyl sulfate. The DNA was extracted with phenol-chloroform, treated with RNase A (10 \mu g/ml), and precipitated with 1% cetyltrimethylammonium bromide in 200 mM sodium chloride. The DNA was dissolved in TE (Tris-EDTA), reextracted with phenol-chloroform, and precipitated in absolute ethanol at −20°C.

For Southern blot analysis, 1 to 3 \mu g of genomic DNA from H99, M001, and M001.1c, and several randomly selected colonies isolated from the CSF of rabbits inoculated with M001.1c were digested with HindIII and separated on a 0.7% agarose gel. After the gel was processed as described by Reed and Mann (35), the DNA was transferred to a Nytran nylon membrane (Schleicher & Schuell, Keene, N.H.) and cross-linked by UV irradiation (UV Stratalinker; Stratagene, La Jolla, Calif.). The DNA probe, a 980-bp HindIII-EcoRV restriction fragment of the ADE2 gene, was prepared by oligolabelling (Pharmacia, Piscataway, N.J.) with [32P]dCTP (New England Nuclear, Boston, Mass.). Hybridization was carried out at 65°C overnight in a hybridizer chamber (Techne, Princeton, N.J.). The nonhomologous label was removed by washing the blot in 2x SSC (0.3 M sodium chloride, 0.3 M sodium citrate) at room temperature for 10 min, once in 2x SSC at 65°C for 1 h, and once in 0.1x SSC-0.1% sodium dodecyl sulfate at 65°C for 1 h. Autoradiography was performed with Kodak-XAR 5 film.

Statistical analysis. Yeast counts in each group during infection were compared with a t test for unpaired means.

RESULTS

In vitro growth kinetics and characteristics. Figure 1 represents the growth of the three strains (H99, M001, and M001.1c) in YEPD broth at 37°C. No detectable differences in the growth curve were noted among wild-type (H99), the ade2 auxotroph (M001), and the transformant (M001.1c). When all three strains were examined under India ink after in vitro growth, no apparent difference in capsular size was observed. All three strains were also plated on caffeic acid agar and observed for phenoloxidase activity. The colonies from each strain turned brown at the same daily rate. Only two phenotypic differences were noted among the three strains: (i) M001 accumulated a red pigment on YEPD plates, whereas strains H99 and M001.1c were white, and (ii) M001 did not grow on adenine-depleted plates, whereas the other strains grew well on these plates. Therefore, the only apparent in vitro difference among the three strains with respect to growth or phenoloxidase or capsule production resides in the mutant’s inability to grow without an exogenous source of adenine.

Determination of virulence. C. neoformans has been shown to produce a chronic progressive meningitis in steroid-treated rabbits that is usually fatal within 4 weeks after intracisternal inoculation (31). However, the infection can be easily monitored for quantitative yeast counts in the CSF, and thus survival as an endpoint can be avoided. As shown in Fig. 2, intrathecal inoculation with 10^7 H99 cells produced a persistent meningitis, as assessed by the persistence of 10^5 to 10^7 CFU of C. neoformans per ml in the CSF throughout the 14-day evaluation. On the other hand, the C. neoformans ade2 auxotroph M001 was not able to replicate in the subarachnoid space. Intrathecal inoculation with 10^7 M001
cells in these immunocompromised rabbits did not produce persistent infection. Fewer yeasts were found in the CSF by day 4 of infection than were found after inoculation with H99. By day 7, the number of yeasts of M001 in the CSF continued to decrease and was significantly lower than that of H99 (P < 0.01, Fig. 2). In fact, the CSF cultures were sterile for all rabbits infected with M001.

With the ability to restore the ADE2 gene in M001, which carried the H99 genetic background, we could assess the virulence of the ade2 auxotroph by transforming it back to adenine prototrophy. Therefore, a randomly selected stable transformant for adenine restoration, M001.1c, was tested for its ability to infect within the subarachnoid space. The transformant received a cloned ADE2 cDNA copy and was shown to harbor the new integrated ADE2 gene along with vector sequences at the homologous site in a tandem repeat (41). As shown in Fig. 2, the prototrophic transformant MOO1.1c was fully virulent and completely restored to the parental (H99) in vivo growth pattern, as determined by the high number of yeasts (10⁶/ml) in the CSF during the 14 days of evaluation.

Figure 3 represents a Southern blot of DNA from strains H99, M001, and M001.1c and two colonies isolated from the subarachnoid space at day 4 after M001.1c inoculation. The genomic DNA was digested with HindIII and probed with the 980-bp ADE2 gene fragment. The ADE2 gene was shown to reside on a 9.4-kb HindIII fragment in H99 and M001. However, because the vector, which carried a HindIII site, integrated at the ADE2-homologous site in M001.1c, two smaller HindIII fragments containing the new ADE2 gene were observed (8.0 and 6.4 kb). The 4.8-kb fragment was consistent with insertion of a tandem repeat of gene and vector. Examination of DNA from colonies during infection with M001.1c confirmed the recombinant nature of the strain producing the infection and excluded any reversion or contamination with wild-type (H99) colonies within the subarachnoid space.

**DISCUSSION**

Knowledge of the molecular biology of *C. neoformans* has expanded over the last several years. Molecular karyotyping (32) and phylogenetic analysis (44) have been performed along with various strategies for molecularly typing strains of *C. neoformans* (6, 39, 43). Genes have been cloned and sequenced by a variety of strategies (8, 20). There are now two usable transformation systems, electroporation (8) and biolistics (20), with both having certain advantages. In this study, we continued to merge molecular manipulation of this yeast with in vivo evaluations of its growth and correlations with its pathobiology. This encapsulated pathogenic yeast, which is an increasing cause of disease, has the potential to be a prototype pathogen for the study of yeast virulence factors. Its genetics and virulence phenotypes, its simple growth characteristics, the availability of reproducible and clinically relevant animal models, and the present molecular biology foundation contribute to its significant potential in this area.

In the study of virulence factors, it is essential to have a reproducible and clinically relevant animal model to study the effects of gene manipulation on pathogenesis. We have used the corticosteroid-treated rabbit model of cryptococcal meningitis for the study of various central nervous system immune effects and for evaluation of antifungal agent regimens (25–31, 33, 34, 45, 46). It is our contention that this
model represents an environment similar to that found for yeasts in human cryptococcal meningitis. In particular, the histopathology in rabbits with severe CSF leukopenia mimics that found in AIDS patients with cryptococcal meningitis (31). The model has also predicted treatment findings confirmed by several recent clinical trials. For example, the model demonstrated more rapid sterilization of the CSF after treatment with amphotericin B than after fluconazole treatment (24), which was subsequently confirmed in a human trial. Also, the antifungal activity of iraconazole against cryptococcal meningitis, despite unmeasurable drug levels in the CSF during meningitis, was first shown in this model (33) and later in clinical studies (7). Therefore, we believe that this model has direct relevance to the pathobiology of C. neoformans infection within the central nervous system of humans. However, it should be emphasized that this animal model only examines the interaction of the yeast and host responses at a particular site of infection; it does not examine the pathobiology of the initial pulmonary infection or reactivation within the lung or the innate ability of this organism to invade the central nervous system. We have used this model previously to show the inability of a hypcapsular clone (C3D) of H99 to cause infection (14). Therefore, we anticipated that the model could detect differences in the ability of different C. neoformans strains to grow within the subarachnoid space when certain important characteristics were altered.

In this set of experiments, we have been able to specifically identify a gene within C. neoformans which is essential for its maintenance within the CSF of immunocompromised rabbits. The MOO1 (ade2 mutant) isolate was produced by UV irradiation, and although its in vitro growth in fully supplemented medium was not affected, there was the potential, with this nonselective method, for the elimination of other genes that contributed to the mutant’s lack of growth within the CSF. By replacement of the defective ADE2 gene and restoration of prototrophy, the virulence of MOO1 in the animal model was completely restored.

Several important observations were generated from these results. First, the transformation process, which can be potentially mutagenic, did not appear to affect other important genes linked to virulence in MOO1. Therefore, it is reassuring that C. neoformans can be molecularly manipulated for virulence studies by transformation. Still, all investigators will need to control for nonselective factors which could be introduced by the transforming process, influencing virulence in gene disruption or replacement studies. Second, the biologic transformation system can be used to study other potential virulence genes.

There has been concern that a purine- or pyrimidine-complementing system would not be useful for virulence studies. In fact, the first transformation system in C. neoformans, using the ura3 gene and ura3 auxotrophs in a serotype D genetic background, found that the auxotrophs were avirulent for mice and the ura3-positive transformants continued to be avirulent (42). However, it is now possible to interrupt other genes important to central nervous system pathobiology by inserting the ADE2 gene within the gene of interest and using the biologic system to deliver the disrupted gene (carrying ADE2) to the homologous site. The transformed prototrophic MOO1 strain would retain its full complement of virulence genes for establishment of infection unless the interrupted gene of interest is important. Third, the model and this gene can be used to select promoters responding to in vivo signals and then to identify specific genes and their importance to the organism’s virulence. In elegant studies reported recently by Mahan et al. (20), they used the restoration of virulence in vivo of adenine auxotrophs to select transformants containing in vivo-expressed promoters driving the adenine gene. From these promoters, genes were then identified which were important to virulence (20). It is apparent to us that a similar strategy can now be used in our system to identify site-specific genes which may be related to virulence in C. neoformans. Fourth, we have identified a specific gene and its enzyme that are required for maintaining C. neoformans infection in the CSF. Hence, this gene or its product could be potential antifungal agent targets.

There are several examples of these blocks on purine and pyrimidine biosynthesis as strategies for therapy. The use of 5-flucytosine to selectively block the pyrimidine metabolic pathway of C. albicans and C. neoformans has been well documented (22). The antifungal activity of flucytosine uses the fungal enzyme cytosine deaminase, which is absent in mammalian cells, as a differential selective mechanism against yeast cells (22). The deaminated product, 5-fluorouracil, can replace uracil in the fungal RNA or be converted to 5-fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthetase. Purine metabolism has also been used to demonstrate its importance to virulence. Specifically, Kirsch and Whitney (16) found that a functioning ADE2 locus was required for the virulence of C. albicans in a murine model. Our observations now extend this finding to C. neoformans. The strong association between virulence and purine metabolism observed in many organisms supports the utilization of the adenine pathway as a target for many medically important yeasts.

It is likely that this block in C. neoformans metabolism in vivo represents an important negative factor in growth maintenance of this organism in the CSF rather than a specific interaction with host immune functions. The concentration of free adenine in the CSF is likely to be extremely low. For example, hypoxanthine concentrations in the CSF are 3 to 28 μmol/liter (11), and if free adenine has similar concentrations in the CSF, these levels are much lower than the concentrations required for C. neoformans growth from exogenous sources. Exogenous adenosine would also be a potential source of adenine in the CSF, but reports show that C. albicans cannot use adenosine (38), and even if C. neoformans could use this metabolite, concentrations in the CSF are in the range of 5 to 30 nmol/liter (11), which is unlikely to support growth within the subarachnoid space. Therefore, a block of this pathway in C. neoformans and a local environment which does not support large amounts of exogenous adenine should work synergistically to produce a selective loss of the yeast’s ability to grow and produce disease at this site. It remains unclear whether this strategy would produce only a fungistatic versus a fungicidal effect until inhibitors of the gene or enzyme are made and experiments can be performed. However, it is encouraging that the immunocompromised rabbit was able to completely eliminate the adenine auxotroph, suggesting that a fungicidal effect did occur within the CSF in this model.

If the ADE2 gene for purine metabolism is to be explored as a target for antifungal therapy, it will need to be different from the human gene for purine metabolism. In fact, we have sequence data for the Cryptococcus ADE2 gene which show a probable bifunctional domain for this gene, versus a unifunctional human ADE2 gene. Further structural and functional studies and then development of specific inhibitors will be needed to confirm these differences in gene arrangement and its usefulness. It is also important to
emphasize that there is a significant turnover and hepatic synthesis of purines and pyrimidines in humans. Mammalian cells have the potential to use exogenous nucleosides without a requirement for specific synthesis of purines and pyrimidines. It is apparent from our study that C. neoformans cannot use these exogenous nucleosides in the CSF for growth, and there is no redundancy in the biochemical system of these yeasts to allow growth. Therefore, this gene could be a unique therapeutic target for C. neoformans within a mammalian host.

In summary, this study has identified a specific gene, that for phosphoribosylaminomimidazole carboxylase, in C. neoformans which is essential for its survival in the subarachnoid space of immunocompromised rabbits. This particular biochemical site has the potential to be a target for antifungal therapy.

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