Efficacy of a Genetically Engineered *Candida albicans* tet-NRG1 Strain as an Experimental Live Attenuated Vaccine against Hematogenously Disseminated Candidiasis

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We report on the efficacy of the genetically engineered *Candida albicans* tet-NRG1 strain as an experimental live, attenuated vaccine against disseminated candidiasis in both immunocompetent and immunodeficient mice mostly dependent on T-cell immunity. This experimental vaccination model may represent an important tool to unravel the mechanisms of protective immunity during candidiasis.

*Candida albicans* remains the most common cause of nosocomial bloodstream-derived fungal infections (3, 4). Morbidity and mortality associated with hematogenously disseminated candidiasis, a major problem for an expanding population of immunosuppressed patients, most notably neutropenic patients, remain unacceptably high due mainly to the lack of early and accurate diagnostic tools, the limited arsenal of antifungal drugs, their toxicity, and the emergence of resistance (9, 13, 16, 21, 22). Thus, many studies over the years have investigated the mechanisms of protective host immunity against this organism to try to develop alternative immune-based strategies to combat candidiasis. Vaccine development is a major priority (6, 7, 11). Experimental vaccination strategies used by different groups of investigators vary and range from using heat-killed or attenuated organisms to cell wall extracts and recombinant proteins, also with different levels of efficacy (for a review, see reference 11). However, despite these efforts, currently there are no licensed vaccines against any human mycoses, including candidiasis.

In previous studies, we have described the construction of a genetically engineered *C. albicans* strain (SSY50-B) in which *NRG1* (a negative regulator of filamentation) was placed under the control of a tetracycline-regulatable promoter so that morphology and virulence could be manipulated in vivo by adding or omitting doxycycline (DOX) from the animal’s drinking water (19). Using a murine model of hematogenously disseminated candidiasis, we demonstrated that infection with this strain when maintained in the yeast form (in the absence of DOX) led to 100% survival, even at very high infectivity levels, both for immunocompetent mice and for mice with specific immune defects (18, 19). This high level of survival was observed despite the fact that yeast cells reached and proliferated in target organs and were able to maintain significant levels of fungal burden during relatively long periods of time (17–19). These results encouraged us to examine whether prior exposure to this strain when maintained in the yeast form, thus somewhat recreating a “carrier” or “commensal” state, would confer resistance to re-infection by a fully virulent wild-type strain of *C. albicans*. If so, this genetically engineered strain would represent a candidate for an experimental live, attenuated vaccine against systemic candidiasis that could possibly be used as a tool to further decipher mechanisms of protective immunity during candidiasis. This could be particularly helpful in improving the validity of the mouse as an experimental model of candidiasis, since humans are normally exposed to this fungus as a commensal but laboratory mice are not.

For these experiments, we utilized a murine model of hematogenously disseminated candidiasis that is routinely used in our laboratory, with some modifications (17–19). Briefly, cultures of the *C. albicans* tet-NRG1 strain (SSY50-B) for injection were grown overnight at 25°C in yeast extract-peptone-dextrose without DOX. Yeast cells were harvested by centrifugation and washed three times in sterile pyrogen-free saline. After cells were counted using a hemocytometer, dilutions were made to allow the appropriate number of yeast cells (in this case, 1.7 × 10^6 CFU) to be injected in a final volume of 200 μl into the lateral tail veins of 6- to 8-week-old female mice (five mice per group). Unvaccinated control (naïve) mice received a 200-μl injection of saline only. Fourteen days later, all mice received a secondary infection with a lethal dose of 5.2 × 10^5 CFU of the fully virulent *C. albicans* CAF-2 strain (8). Mortality was monitored for 16 days after secondary challenge. For statistical analyses, survival data and differences between groups were analyzed using the Kaplan-Meier and log rank tests. All animal experiments were performed in accordance with institutional regulations, and mice were allowed a 1-week acclimatization period before the start of the experiments.

A first series of vaccination experiments was performed using immunocompetent BALB/c mice, obtained from the National Cancer Institute (NCI; Bethesda, MD). As shown in Fig. 1, prior exposure to the *C. albicans* tet-NRG1 strain kept in the yeast form led to high levels of protection against a secondary infection with a lethal dose of the fully virulent CAF-2 strain, as assessed by survival proportions, with 100% of vaccinated mice versus 0% of naïve (unvaccinated) mice surviving the
infection. Of note, the challenge with the C. albicans CAF-2 strain caused 100% mortality after only 3 days postinfection in the unvaccinated control group, thus demonstrating the very aggressive nature of the infection caused by this infecting dose, which vaccinated animals survived.

The above-described results using mice with the BALB/c background clearly demonstrate the protective effect of this experimental vaccination strategy using the tet-NRG1 strain. A caveat for these experiments is that, unlike humans, experimental mice do not normally contain C. albicans as part of their normal microbiota, and we reasoned that the establishment of a “carrier” or “commensal” state in mice with the C. albicans tet-NRG1 strain (since we have previously demonstrated that a significant organ fungal burden remains with the tet-NRG1 strain, even a few weeks after infection) could exert a nonspecific protective effect against a secondary infection due to simple competition for binding sites for fungal cells in deep tissues. However, it is also possible that the protection observed using this vaccination strategy is mediated by specific immune mechanisms. We have previously shown that mice with specific immune deficiencies are able to survive a primary infection with the C. albicans tet-NRG1 strain in the absence of DOX when kept as a yeast (18), thus also allowing the evaluation of the efficacy of this vaccination strategy in different types of immunocompromised mice and thereby providing insights into the mechanisms responsible for protective immunity in this model. To this end, we repeated the above-mentioned vaccination/challenge protocol with B-cell-deficient (bearing a homozygous deletion of the igh locus; C.129B6-IgH-Jh/Jh<sup>Tm1Dhu</sup>), nude (T-cell-deficient mice; CANn.Cg-Foxn1<sup>nu/Crl</sup>), and DBA/2N mice, which are C5 deficient (a component of the complement pathway) and are considered to have impaired neutrophil activity. Nude and DBA/2N mice were obtained from the NCI, while B-cell-deficient mice were obtained from Taconic Farms (Germantown, NY). Both the nude and B-cell-deficient mice used in these experiments are in the BALB/c background. As seen in Fig. 2, as for BALB/c mice, vaccination with the C. albicans tet-NRG1 strain fully protected B-cell-deficient and DBA/2N mouse strains against a secondary infection with CAF-2 (100% survival in vaccinated animals versus 100% mortality in naive mice). However, the same vaccination strategy completely failed to protect T-cell-deficient, nude mice, in which we observed 100% mortality for both naive and vaccinated animals, although nude mice preexposed to the tet-NRG1 strain survived slightly longer than their naive counterparts (Fig. 2C).

Results from this series of experiments using different mouse strains with defects in specific components of the immune system allow us to gain some insight into the mechanisms responsible for protection. Thus, from these data, it is clear that the nature of the protective response in this model is mostly immune based and not due merely to a nonspecific steric hindrance caused by the nonpathogenic tet-NRG1 cells occupying and possibly saturating binding sites in tissues.
(However, it is possible that the slight increase in survival observed with vaccinated versus that of naïve nude mice may be attributable to this “nonspecific” effect.) The results also suggest that neither B cells nor functional neutrophils have any role to play in the protective effect exerted by vaccination with the \textit{C. albicans} \textit{tet-NRG1} strain. In addition, the high levels of protection achieved in B-cell-deficient mice seem to rule out the contribution of antibodies to protection, at least in this specific experimental model. In contrast, the lack of protection observed in the case of T-cell-deficient nude mice strongly indicates a likely role for a cell-mediated adaptive immune response in protection against reinfection, even when the time between primary and secondary infection is relatively short. These observations are similar to those previously reported for the recombinant Als vaccine (10, 20). Perhaps the most significant observation, however, with potentially important clinical implications for vaccine development, is the fact that by using this vaccination strategy we were able to effectively protect DBA/2N mice, which are normally exquisitely sensitive to systemic candidiasis because of their impaired neutrophil activity due to complement C5 deficiency (1, 2). This indicates that vaccine approaches may be feasible for use in patients with neutrophil deficiencies, a major patient population at risk for candidiasis. Our results are also in stark contrast to previous reports by Romani et al., in which their live, attenuated vaccine strain killed DBA/2 mice even at very low doses, highlighting the advantages of using genetically engineered and well-characterized strains rather than attenuated strains with ill-defined genetic mutations (14, 15).

To the best of our knowledge, these are the highest levels of protection described for an anti-\textit{C. albicans} vaccine, particularly in the case of immunodeficient mice and, more specifically, in mice with neutrophil deficiencies, which reinforces the efficacy of our \textit{C. albicans} \textit{tet-NRG1} strain as an experimental live, attenuated vaccine against disseminated candidiasis in mice. However, we are fully cognizant of some limitations of our study, particularly in regard to the development of live, attenuated vaccines for human use (5, 12). Certainly, the use of live, attenuated vaccines carries important risks, particularly if used in immunosuppressed patients, and the majority of at-risk patients for candidiasis fall into this category (12). For example, we have previously demonstrated that the \textit{C. albicans} \textit{tet-NRG1} strain, even in the absence of DOX when kept as a yeast, leads to 100% mortality in mice that have been severely immunosuppressed by pharmacological treatment with cyclophosphamide and cortisone acetate (18). Under similar conditions, we have also observed occasional deaths in DBA/2N mice, particularly if very high levels of infecting inocula of the \textit{C. albicans} \textit{tet-NRG1} strain are used (S. P. Saville and J. L. Lopez-Ribot, unpublished results). Despite these caveats, we feel that the \textit{C. albicans} \textit{tet-NRG1} strain and its use in this infection model represent an important tool in our aim to further understand protective immunity during candidiasis, particularly in light of the fact that \textit{C. albicans} is a normal commensal of humans but not mice, with the ultimate goal of developing safe, effective, and most likely subunit vaccines against candidiasis. Current experiments in our laboratory are aimed at elucidating the specific immunological mechanisms responsible for the induction of the protective immunity seen in this model.

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REFERENCES