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Active and Passive Immunization with rHyr1p-N Protects Mice against Hematogenously Disseminated Candidiasis

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Abstract

We previously reported that Candida albicans cell surface protein Hyr1 encodes a phagocyte killing resistance factor and active vaccination with a recombinant N-terminus of Hyr1 protein (rHyr1p-N), significantly protects immunocompetent mice from disseminated candidiasis. Here we report the marked efficacy of rHyr1p-N vaccine on improving the survival and reducing the fungal burden of disseminated candidiasis in both immunocompetent and immunocompromised mice using the FDA-approved adjuvant, alum. Importantly, we also show that pooled rabbit anti-Hyr1p polyclonal antibodies raised against 8 different peptide regions of Hyr1p-N protected mice in a hematogenously disseminated candidiasis model, raising the possibility of developing a successful passive immunotherapy strategy to treat this disease. Our data suggest that the rabbit anti-Hyr1p antibodies directly neutralized the Hyr1p virulence function, rather than enhanced opsonophagocytosis for subsequent killing by neutrophil in vitro. Finally, the rHyr1p-N vaccine was protective against non-albicans Candida spp. These preclinical data demonstrate that rHyr1p-N is likely to be a novel target for developing both active and passive immunization strategies against Candida infections.

Introduction

Candida species, the third most common cause of healthcare-associated bloodstream infections [1] causes approximately 60,000 cases of hematogenously disseminated candidiasis per year in the United States [2], resulting in billions of dollars of healthcare expenditures. Notwithstanding current antifungal therapy [3,4,5], mortality remains unacceptably high [6,7,8]. Because of the rising incidence of life-threatening candidiasis and high treatment failure rates, more effective prophylactic and therapeutic strategies are needed.

HYR1 belongs to the IFF gene family of C. albicans, which includes 12 members [9]. It encodes a cell surface glycosylphosphatidylinositol (GPI)-anchored protein that is expressed during hyphal formation [10,11]. In our previous study, we showed that Hry1p mediated C. albicans resistance to phagocyte killing in vitro and contributed higher fungal burden in organs rich in phagocytes (e.g. liver and spleen) [12]. Native HYR1 is positively regulated by transcription factor Bcr1p [13]. We found that autonomous HYR1 expression reversed the hyper-susceptibility to phagocyte-mediated killing of a bcr1 null mutant of C. albicans in vitro [12]. Further, heterologous expression of HYR1 in C. glabrata rendered the organism more resistant to phagocyte killing [12]. Our study also showed that a vaccine based on the recombinant N terminus of Hry1p (rHyr1p-N) markedly improved survival of immunocompetent mice challenged intravenously with C. albicans when mixed with either Freund’s or alum as an adjuvant [12].

The current studies were performed to further define the vaccine efficacy of rHyr1p-N vaccine in both immunocompetent and immunocompromised mice using the FDA-approved alum as an adjuvant. Further, the breadth of protection induced by rHyr1p-N was evaluated by its efficacy against non-albicans Candida species. Finally, we sought to study the potential use of passive immune therapy in disseminated candidiasis using anti-Hyr1p antibodies.

Results

The rHyr1p-N vaccine significantly improved survival and decreased fungal burden in immunocompetent mice challenged intravenously with C. albicans

To determine the most effective dose of the rHyr1p-N immunogen, an approximately 3-fold dose range was evaluated (1 to 33 µg per mouse). Female juvenile BALB/c mice were immunized with rHyr1p-N plus alum (2% Alhydrogel; Brenntag Biosector) or with alum alone. These mice were subsequently infected with a lethal inoculum of C. albicans (7x10^7 blastospores). Vaccinated mice had significant improvements in survival and compared to adjuvant control mice (Figure 1A). All tested doses, except 1 µg, prolonged or improved survival compared to mice.
The rHyr1p-N effectively protected immunocompromised mice against candidiasis

It is known that a significant fraction of immunocompromised patients do respond to a variety of vaccines [14,15,16,17]. We sought to define the potential usage of the rHyr1p-N vaccine to protect neutropenic mice from disseminated candidiasis. Immunized mice were killed twelve days following the boost with 30 µg of rHyr1p-N. Vaccination significantly increased the mouse immune response as determined by detection of increased anti-rHyr1p-N antibody titers ($P = 1.08 \times 10^{-05}$) (Figure 3A). One day after the bleeding, mice were made neutropenic. Vaccination resulted in significant improvements in survival ($P = 0.007$ versus control) (Figure 3B).

We also evaluated the kidney fungal burden on day 10 post infection. Concordant with our survival result, we found that mice vaccinated with 30 µg of rHyr1p-N had 1.50 log fold decrease in fungal burden compared to kidneys harvested from control mice (Figure 3C, $P = 0.002$).

Passive immunization with anti-Hyr1p IgG prolonged the survival of mice infected with C. albicans

Since some patients might not respond to an active vaccine strategy, we evaluated the possibility of using passive immuno-therapy targeting Hyr1p. We generated polyclonal antibodies by vaccinating rabbits with 8 hydrophilic, highly antigenic 14-mer peptides located within rHyr1p-N region (Table 1). Purified IgG targeting these 8 peptides were pooled and used to treat naïve mice infected with a lethal dose of C. albicans. Mice receiving anti-Hyr1p IgG at either 1 or 3 mg (but not when administered at 0.3 mg) were protected substantially from infection when compared to mice receiving non-specific, rabbit control IgG from commercial source (Figure 4A, 4B and 4C).

To determine if the generated anti-Hyr1p antibodies enhanced phagocyte function by increasing opsonophagocytosis or by neutralizing Hyr1 killing resistance, we isolated and prepared F(ab')$_2$ fragments from pooled IgG raised against the 8 peptides of Hyr1p (conjugated to keyhole limpet hemocyanin or KLH) or from non-specific, rabbit control IgG. These fragments were used in HL-60 derived neutrophil killing assay against C. albicans conditionally overexpressing or suppressing Hyr1p rather than wild-type C. albicans to demonstrate specificity of these fragments to Hyr1p and not to other members of IFF family [9]. Consistent with our previous mouse IgG data [12], we found that F(ab')$_2$ fragments prepared from anti-Hyr1p antibodies but not those prepared from control antibodies were able to restore HL-60 derived neutrophil killing of the HYR1 conditional expressing strain to levels equivalent to that of the suppressing strain (Figure 4D).

To verify that the protection elicited by antibodies was indeed due to anti-Hyr1p antibodies and not due to non-specific protection caused by antibodies reacting to unrelated immunogen such as peptide carrier protein KLH, the purified IgG targeting the 8 hydrophilic rHyr1p-N peptides was absorbed with C. albicans hyphae prior to testing for their protective activity against hematogenously disseminated candidiasis. The absorbed IgG did not stain C. albicans hyphae (Figure 5A), indicating the anti-Hyr1p IgG were successfully eliminated. Furthermore, similar to non-specific, rabbit control IgG, the absorbed IgG did not protect mice from C. albicans infection, whereas the purified, non-absorbed IgG did (Figure 5B, $P = 0.002$).

The rHyr1p-N vaccine substantially reduced tissue fungal burden in BALB/c mice challenged with several non-albicans species of Candida

A vaccine that elicits protection against C. albicans and other non-albicans species is highly desirable because a significant number of patients do respond to a variety of vaccines [14,15,16,17].
The number of Candida infections are caused by non-albicans species. For example, *C. glabrata* represents the second most common cause of candidiasis and *C. krusei* is resistant to azole therapy. Using blast searches we were able to detect Hyr1p like molecules in several Candida species with amino acid similarity ranging between 47–72% in certain areas. Thus, we vaccinated mice with rHyr1p-N as a Candida vaccine.

Figure 2. Representative histopathological sections from kidneys were shown. (A) Control mice infected with *C. albicans* had multiple abscesses showing mostly yeast forms with some hyphae and pseudohyphae throughout the kidneys. (B) rHyr1p-N vaccinated mice (33 μg) infected with *C. albicans* had less abscesses with far less fungi visible. (C) Semiquantitative evaluation of the severity of infection indicated significant abscess and Candida cells reduction in vaccinated mice compared to control mice. Sections were stained by PAS. Thirty random fields were examined by a blinded assessor (GL) to assess the number of lesions per field. Number of organisms per lesion was evaluated in 120 lesions in the control unvaccinated mice. The average number of organisms per lesion was determined by dividing the total number of fungal cells by the number of lesions counted. *P* < 0.0001 by Wilcoxon rank sum test.

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Figure 3. rHyr1p-N vaccine prolonged survival and decreased fungal burden in neutropenic mice infected with *C. albicans*. Balb/c mice (n = 20 per arm) were vaccinated with rHyr1p-N mixed with alum or alum alone (control), treated with cyclophosphamide, and then infected with *C. albicans* 15563 at 1 × 10⁵ blastospores. Two days before cyclophosphamide treatment, half of the mice were bled and individually marked for antibody titer using ELISA (A) (rHyr1p-N vaccinated versus control, *P* = 1.08E-05 by Wilcoxon rank-sum test) and survival (B) (rHyr1p-N vaccinated versus control, *P* = 0.007 by log rank test). The other half mice were used for fungal burden (C) *P* = 0.002 by Wilcoxon rank-sum test.

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plus alum as above, then challenged with *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, or *C. tropicalis*. Three days post infection mice were sacrificed and the kidneys harvested for determination of tissue fungal burden through colony counts. Mice vaccinated with rHyr1p-N had 0.65–1.69 log decrease in kidney fungal burden compared to mice vaccinated with alum alone (Figure 6, $P<0.001$).

**Discussion**

*C. albicans* vaccine development has focused on using cell surface components [18,19], peptides derived from cell wall proteins as immunogens [20], or on antibodies targeting cell surface components [21,22]. Our group has been working for decades towards developing immunotherapeutic approaches to prevent or ameliorate disseminated, healthcare-associated fungal infections. These efforts have resulted in the initiation of a Phase I clinical trial of our anti-*Candida* vaccine that targets the Als3p, a known adhesin/invasion [23].

In our efforts to develop additional protective antigens against *Candida*, we have identified properties of the recombinant N-terminus of Hyr1p (rHyr1p-N) [12] that make it highly desirable for further development as both active and passive immunotherapy target. In our previous study, we demonstrated by using indirect immunofluorescence that Hyr1p is expressed on the cell surface of *C. albicans* hyphae [12]. These findings were further confirmed by our comparative indirect immunofluorescence of *C. albicans* using purified IgG raised against 8 hydrophilic peptides of rHyr1p-N pre- and post-absorption with *C. albicans* hyphae and

**Table 1.** Hyr1 peptides used in this study.

| Peptide Number | Sequence     | MW (kDa) | pI   | Purity (%) | Source     |
|----------------|--------------|----------|------|------------|------------|
| 1              | CGPSAPESEDLNTTP | 1.5      | 3.44 | 86.1       | This study |
| 2              | CGNRDHFRFEGYPPDT | 1.9      | 5.69 | 99.4       | This study |
| 3              | CGYDSKLFRIVNSRG | 1.7      | 9.16 | 95.7       | This study |
| 4              | CKIKGTGCVTADEDT | 1.5      | 4.70 | 86.4       | This study |
| 5              | CLINAVYTDGPPVNN | 1.6      | 6.25 | 94.1       | This study |
| 6              | NSKSSTSFSFDGIC | 1.6      | 6.25 | 91.4       | This study |
| 7              | CEPTHNFYLKDSSS | 1.8      | 7.19 | 85.8       | This study |
| 8              | TSRIDRGGIOGFHGC | 1.6      | 8.27 | 91.8       | This study |

Additional cysteine residues on the N- or C-termini were used to conjugate the 14-mer peptide to KLH.

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Figure 4. Dose dependent passive immunization with anti-Hyr1p IgG protected against murine hematogenously disseminated candidiasis. Mice were given 0.3 mg (A), 1 mg (B) and 3 mg (C) of anti-Hyr1p IgG by intraperitoneal injection 2 hr before infecting with $6.2 \times 10^5$ blastospores of *Candida albicans* 15563 via the tail vein. Survival of mice ($n = 10$ per group) was monitored twice daily. * $P = .001$ by log-rank test vs. mice receiving non-specific, rabbit control IgG. (D) Effect of vaccinated or control F(ab')2 on blocking HL-60 derived neutrophil killing of *C. albicans*. *C. albicans* overexpressing or suppressing Hyr1p were used in the assay to demonstrate specificity of the F(ab')2 fragments to Hyr1p. Control denotes assay performed either in the absence of F(ab')2 or in the presence of F(ab')2 from non-specific, rabbit control IgG. Data are displayed as median ± interquartile range. * $P = .001$ by Mann-Whitney test.

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control IgG (Figure 5A). We hypothesized that immunotherapies targeting the cell wall Hyr1p would have the dual benefit of the immune system recognizing the fungus and enhancing phagocyte killing of *Candida*. In this study, our data on rHyr1p-N has shown efficacy in animal models at doses 10–30 times less than those used for rAls3p-N (i.e. ~50% survival for 10–33 μg dose for rHyr1p-N vs. 300 μg dose of rAls3p-N) [12,19,24]. Additionally, the mechanism of action appears to be considerably different from that of rAls3p-N. Rabbit polyclonal IgG raised against 8 different 14-mer peptides from regions of rHyr1p-N substantially protects mice from experimental disseminated candidiasis, whereas, our previous studies indicated that the mechanism of action of the rAls3p-N vaccine is dominantly dependent on T cells and anti-rAls3p-N antibodies are not the central mechanism of protection [19,24]. Furthermore, the rHyr1p-N maintained its efficacy in the neutropenic mouse model. These findings suggest that Hyr1p is a promising target for both active and passive immunization.

Tissue fungal burden and histopathological examination of kidneys harvested from mice vaccinated with rHyr1p-N or alum alone further confirmed the efficacy of the rHyr1p-N vaccine. However, it appears that the histopathology difference between the control (Figure 2A) and rHyr1p-N vaccinated mice (Figure 2B) was more prominent than that of tissue fungal burden of the same organs. In this regard, it has been previously reported that colony counting can underestimate the tissue fungal burden in the presence of hyphae and pseudohyphae [25,26], likely because tissue homogenization kills fungal filaments. We found that control mice had significantly more filamentous fungi in kidneys than vaccinated mice which had less abscesses mainly consisting of yeast form fungal elements. Therefore, tissue homogenization likely artificially lowers the colony counts for kidneys harvested from control mice but not from rHyr1p-N-vaccinated mice, making the difference less prominent.

Our results also show a dose response of anti-Hyr1p IgG in protecting mice from disseminated candidiasis. We confirmed that the protection elicited by anti-Hyr1p IgG was specific to Hyr1p since absorbed IgG with *C. albicans* hyphae lost its ability to protect mice against hematogenously disseminated candidiasis (Figure 5B). These results suggest that the mechanism of protection rendered by rHyr1p-N appears to be attributed, at least in part, to protective antibody response. Further studies to elucidate the role of T-cells vs. B-cells in the mechanism of rHyr1p-N protection against disseminated candidiasis are currently under active investigation.

In this study, we show that pooled IgG raised against 8 Hyr1 peptides directly neutralized the function of Hyr1p in resisting phagocyte killing rather than enhanced opsonophagocytosis. This
is evident by the ability of F(ab\')2 fragments (prepared from anti- 
rHyr1p-N antibodies) to restore phagocyte killing of *C. albicans*
overexpressing Hyr1p to levels equivalent to that of the
suppressing strain (Figure 4D). However, the rHyr1p-N vaccine
maintained its efficacy in neutropenic mice. This can be explained
by the fact that cyclophosphamide induces leukopenia in mice
with minimal effect on tissue phagocytes. Further experimentation
is necessary to determine specific peptide(s) by which antibodies
are generated to protect the host against disseminated candidiasis.

In summary, the rHyr1p-N vaccine is a promising candidate for
further development. The vaccine is efficacious in both immuno-
competent and immunocompromised mice, when mixed with
alum as an adjuvant, against multiple clinical isolated strains of
*C. albicans* [12], and against several non- *albicans* Candida species.

**Materials and Methods**

**Candida strains and growth conditions**

*C. albicans* 15663, *C. glabrata* 31028, *C. parapsilosis* 22019 and *C.
tropicalis* 4243 are clinical bloodstream isolates collected from
Harbor-UCLA Medical Center. *C. krusei* 91-1159 was generously
provided by Michael Rinaldi, San Antonio, TX. *C. albicans* strains
CAAH-31 and THE31 were engineered as described in our
previously described [12]. In brief, juvenile (10–12 week) Balb/C mice
were vaccinated subcutaneously with 30 μg of rHyr1p-N mixed with
alum (2% Alhydrogel; Bremntag Biosector, Frederikssund, Denmark) as
an adjuvant in phosphate buffered saline (PBS) on day 0, boosted
with the same dose on day 21, then infected via the tail vein on day 35
[27]. Control mice were vaccinated with alum alone.

To test the efficacy of the vaccine in immunocompromised
mice, we injected 107 *C. albicans* blastospores (1×10⁷) intraperitoneally
with 200 mg/kg of cyclophosphamide on day −2 followed by another
dose of 100 mg/kg on day +7 relative to infection. This regimen results
in approximately 10 days of leukopenia with reduction in neutrophil,
lymphocyte and monocyte counts, as described previously [28,29,30]. For both immunocompetent and neutro-
penic mice differences in survival between vaccinated and
immunocompromised mice were compared by the Log Rank test.

For passive immunization, immune IgG was administered intraperitoneally to naive mice 2 hr before infecting intravenously with
*C. albicans*. Control mice were given non-specific, rabbit IgG
(Immunex Corporation, Seattle, WA.) IgG doses were repeated 3 days after
infection, and survival of mice was monitored twice daily.

Quantitative culturing of kidneys from vaccinated or control
mice infected with different species of *Candida* was performed as
previously described [31]. In brief, mice were infected through tail
veins. Kidneys were harvested 3 days post infection, homogenized,
serially diluted in 0.85% saline, and quantitatively cultured on
YPD that contained 50 μg/ml chloramphenicol. Colonies were

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**Figure 6. rHyr1p-N vaccine reduces tissue fungal burden in BALB/c mice infected with non-albicans species of Candida.**

BALB/c mice (n = 10 per group) were vaccinated with alum or alum plus
rHyr1p-N (30 μg) and boosted three weeks later. Two weeks after
the boost, mice were challenged via the tail vein with *C. glabrata* (3.2×10⁹),
*C. krusei* (3.4×10⁹), *C. parapsilosis* (9.6×10⁹), or *C. tropicalis* (3.2×10⁹).
Kidney fungal burden was determined on day 3 post infection. The y
axis reflects the lower limit of detection of the assay. * P<0.001 versus
adjuvant control by the Mann-Whitney U test.

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counted after incubation of the plates at 37°C for 24 to 48 hr, and results were expressed as log CFU per gram of infected organ. Concomitant with the fungal burden experiment, kidneys were removed aseptically from two mice per group for histopathological examination. Kidneys were immersed in zinc formalin fixative until examination. Fixed organs were dehydrated in graded alcohol solutions, embedded in paraffin, and cut into 6-μm-thick sections. Mounted sections were stained with Gomori methenamine silver and examined by light microscopy [32].

Enzyme-linked immunosorbent assay (ELISA)

To test if the rHyr1p-N vaccine induced an immune response, antibody titers of serum samples collected from vaccinated and control mice were determined by ELISA in 96-well plates as previously described [27]. Wells were coated at 100 μl per well with rHyr1p-N at 5 μg/ml in PBS. Mouse sera were incubated for 1 hr at room temperature following a blocking step with Tris-buffered saline (TBS; 0.01 M Tris HCl [pH 7.4], 0.15 M NaCl) containing 3% bovine serum albumin. The wells were washed three times with TBS containing 0.05% Tween 20, followed by another three washes with TBS. Goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Sigma) was added at a final dilution of 1:5000, and the plate was further incubated for 1 hr at room temperature. Wells were washed with TBS and incubated with substrate containing 0.1 M citrate buffer (pH 5.0), 50 mg of o-phenylenediamine (Sigma), and 10 μl of 30% H2O2. The color was allowed to develop for 30 min, after which the reaction was terminated by addition of 10% H2SO4 and the optical density (OD) at 490 nm was determined in a microtiter plate reader. Negative control wells received only diluent, and background absorbance was subtracted from the test wells to obtain final OD readings. The ELISA titer was taken as the reciprocal of the last serum dilution that gave a positive OD reading (i.e., more than the mean OD of negative control samples plus 2 standard deviations).

F(ab')2 blocking assay

To study the mechanism of protection mediated by anti-Hyr1p antibodies in phagocyte-mediated killing of C. albicans, HL-60 cells that have been differentiated to neutrophil-like phenotype were used [12]. Killing assay was conducted in the presence of anti-Hyr1p IgG or F(ab')2 fragments as described before [12]. In brief, HL-60 cells were induced with 2.5 μM of retinoic acid and 1.3% DMSO for 3 days at 37°C with 5% CO2. Immune anti-Hyr1 peptides (Table 1) sera were pooled and total IgG was isolated using protein A agarose (Thermo Scientific). Serum collected from the same rabbits prior to immunization with the peptides served as control serum. The F(ab')2 fragments from immune or control IgG were purified with Pierce F(ab')2 Preparation Kit according to the manufacturer’s instruction. SDS-PAGE analysis indicated >95% of Fc fragment was digested by this kit (data not shown). Next, C. albicans cells overexpressing or suppressing Hyr1p [12] were incubated with 50 μg/ml of vaccinated or control F(ab')2 fragments on ice for 45 min. C. albicans cocultured with the F(ab')2 fragments were incubated with HL-60 derived neutrophils for 1 hr at 37°C with 5% CO2 prior to sonication and quantitative culturing on YPD plates. % killing was calculated by dividing the number of CFU after coculturing with HL-60 derived neutrophils by the number of CFU from C. albicans incubated with media without HL-60 derived neutrophils.

Statistical analysis

The nonparametric log rank test was used to determine differences in the survival times of the mice. Neutrophil killing assay, titers of antibody, and tissue fungal burden were compared by the Mann-Whitney U test or Wilcoxon rank sum test for unpaired comparisons. Correlations were calculated with the Spearman rank sum test. P values of <0.05 were considered significant.

All procedures involving mice were approved by the Los Angeles Biomedical Research Institute animal use and care committee for the project 11672-03 specifically to this vaccine study, following the National Institutes of Health guidelines for animal housing and care. The institute has a US Public Health Service approved animal welfare assurance number A3330-01.

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Author Contributions

Conceived and designed the experiments: GL ASI JEE YF. Performed the experiments: GL ASI SWF YF. Analyzed the data: GL ASI SWF JEE YF. Wrote the paper: GL ASI JEE YF.

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