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A novel monoclonal antibody against enolase antigen of *Aspergillus fumigatus* protects experimental aspergillosis in mice

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One sentence summary: A monoclonal antibody generated against enolase antigen protects experimental mice challenged with *A. fumigatus*, and may offer novel opportunities towards development of an effective tool for diagnosis and treatment of aspergillosis.

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ABSTRACT

Aspergillus fumigatus is one of the most common opportunistic fungal pathogens responsible for a variety of diseases in human, from allergic bronchopulmonary aspergillosis to chronic pulmonary aspergillosis, mostly in immunocompromized patients. In this study, one monoclonal antibody MAb R-5 (IgM) raised against enolase cell surface protein of *A. fumigatus* exhibited significant inhibition of spore germination in *A. fumigatus* (88.3%), *Aspergillus flavus* (57.4%) and *Aspergillus niger* (30.6%). The MAb R-5 also showed *in vitro* fungicidal activity against these species as follows: *A. fumigatus* (24.1%), *A. flavus* (13.3%) and *A. niger* (8.8%). These findings were supported by the indirect immunofluorescence microscopy, where the antibody showed binding with germinated spores and hyphae of *A. fumigatus* as well as *A. flavus* and *A. niger*. *In vivo* protective effect of MAb R-5 was evaluated in BALB/c mice challenged intravenously with *A. fumigatus* spores, where a significant reduction in CFU (85.9%) was observed in kidney tissue. The mean survival time of mice treated with MAb R-5 (18.5 days) was also enhanced compared to control (6.5 days). These results indicate that MAb R-5 could be valuable in diagnosis as well as in the treatment of broad range of *Aspergillus* infections.

Keywords: *Aspergillus fumigatus*; enolase; hybridoma; monoclonal antibody

INTRODUCTION

Aspergillus fumigatus is a ubiquitous, saprophytic and filamentous fungus, which causes a wide range of fungal infections in immunocompromized individuals including patients treated with immunosuppressive drugs, HIV/AIDS patients, cancer patients, people undergoing chemotherapy, steroid treatments, major surgical treatments, hematologic and solid organ transplantation (Latgé 1999; Dagenais and Keller 2009; Badiie and Hashemizadeh 2014). The number of antifungal drugs compared to antibacterial agents is low, and the available drugs are

not free from side effects (Badiie and Hashemizadeh 2014). The situation is further complicated by the difficulties in early stage diagnosis of invasive aspergillosis (Jantunen et al. 2000). Furthermore, the emerging trend of antifungal resistance makes the situation of more concern (Loeffler and Stevens 2003). Therefore, there is a need of concerted efforts for new strategies that may lead to the discovery and development of safe, effective and broad-spectrum antifungal agents. The development of MAbs against pathogenic fungus is a new approach for diagnosis and treatment of fungal infections, which shows high specificity and

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low toxicity (Gómez et al. 1997; Casadevall and Pirofski 2012). The fungal cell surface antigens play an important role in its pathogenicity and colonization as these proteins are directly involved in the interactions of the fungus with the mammalian host cells (Arana et al. 2009). These proteins are responsible for the activation of various host immune responses and their detailed studies may result in identification of important targets for new drug development (Figueiredo, Carneiro and Bozza 2011). In this study, a mouse monoclonal antibody R-5 (IgM) was generated against the enolase cell surface protein of *A. fumigatus* and characterized for its diagnostic and therapeutic potential.

MATERIAL AND METHODS

Fungal strains, cell line and mice

A clinical isolate of *A. fumigatus* was used in this study. The other pathogenic fungal species used for cross-reactivity tests were clinical isolates of *Aspergillus flavus*, *Sporothrix schenckii*, *Aspergillus niger* (ATCC 6275), *Trichophyton mentagrophytes* (ATCC 9533) and *Candida albicans* (ATCC 10231). These fungi were grown at 37°C on Sabouraud's dextrose agar (SDA) slants for 24 h to 7 days and stored at 4°C.

The mouse myeloma Sp2/O cell line (fusion partner) (ATCC CRL-1581) was maintained in RPMI-1640 with 10% fetal bovine serum (Sigma) in a humidified incubator with 5% CO₂ at 37°C. The BALB/c mice were used for immunization and isolation of spleen cells for hybridoma production. A prior permission was obtained from local animal ethics committee of the institute for this study.

Culture conditions and isolation of cell surface antigen

Aspergillus fumigatus was grown in Sabouraud's dextrose broth (SDB) at 37°C under shaking condition (180 rpm) for 5 days. The mycelia were isolated, washed thrice with autoclaved triple-distilled water and stored at -80°C until used. The cell surface proteins were extracted as described by VEDIYAPPAN et al. (2000) with slight modifications. Briefly, the frozen mycelia were thawed and mixed with ammonium carbonate buffer containing β-mercaptoethanol (1% V/V), DTT (10 mM) and protease inhibitor (Sigma), left for 4 h on a shaker, then filtered and the supernatant was precipitated with ethanol (1:9). The precipitated proteins were dissolved in buffer containing 8 M Urea/20 mM DTT and quantified by BCA method.

Generation of monoclonal antibodies

Female BALB/c mice (6–8 wk old) were subcutaneously given the cell surface proteins (100 µg) first in Freund's complete adjuvant (1:1) followed by five booster doses in Freund's incomplete adjuvant at an interval of 15 days. The mice were checked for optimum antibodies by ELISA. The splenocytes from immunized mice were fused with Sp2/O myeloma cell line according to a standard protocol (Harlow and Lane 1988). The fused cells were suspended in RPMI 1640-HEPES modified medium containing HAT and 10% fetal bovine serum (Sigma), distributed in 96-well cell culture plates and incubated at 37°C, in 5% CO₂ atmosphere. The plates were checked for hybrid cell colonies after 7 days onwards and checked by ELISA for MAb production. The positive hybridoma cells were subcloned (for single cell cloning) twice by limiting dilution and rechecked by ELISA. The positive clones were further expanded for antibody production in culture flasks

containing serum-free medium (Sigma). The monoclonal antibodies in serum-free medium were then precipitated with 50% ammonium sulfate, dialyzed against DPBS, purified by using IgM purification kit (Pierce) according to the manufacturer's guidelines, quantified and stored at -80°C for further use.

Isotyping

The isotype of monoclonal antibody was determined by using Mouse Antibody Isotyping Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

SDS-PAGE and Western blotting

Cell surface proteins were resolved by SDS-PAGE in a minigel system according to the method of Laemmli (Laemmli 1970) and electro-transferred on to a polyvinylidene difluoride (PVDF) membrane as described earlier (Kumar et al. 2011). The polyvinylidene difluoride membranes were incubated separately in the supernatant of ELISA positive single cell clone for overnight at 4°C. The membranes were then washed thrice with TBST and incubated with secondary antibody (Peroxidase-conjugated anti-mouse IgA, IgG and IgM) for 90 min. After washing, immunoreactive protein bands were developed and visualized with DAB.

Identification of antigen recognized by MAb R-5

The surface proteins were separated by 2-D gel electrophoresis, transferred to polyvinylidene difluoride membrane and immunoblot using MAb R-5 as previously described (Kumar et al. 2011). The proteins were visualized by Coomassie brilliant blue staining. The immunoreactive protein spot of interest (recognized by MAb) was excised from the 2-D gel and characterized by MALDI-TOF-MS analysis. The Mascot search parameters were carbamidomethylation at cysteine residue as fixed modification, methionine oxidation as variable modification, all entries for taxonomy, one missed cleavage site for enzyme trypsin, peptide mass tolerance 1.2 Da and other search parameters were taken as default setting. The experiments were done at least thrice to confirm the results.

Immunofluorescence

The conidia of *A. fumigatus* were grown on Sabouraud's dextrose agar at 37°C for 48 h, washed and suspended in DPBS (1 × 10⁵ cells/ml). This suspension was placed on poly-L-lysine coated glass slides and fixed with 4% paraformaldehyde. The glass slides were treated with 1% BSA and incubated with the MAb R-5 (50 µg/ml in PBS) for 1 h at 37°C. The glass slides were then washed with DPBS and incubated with FITC conjugated anti-mouse antibody diluted 1:100 in DPBS for 1 h at 37°C, and then washed again, mounted and observed under a phase-contrast fluorescence microscope.

In-vitro fungicidal activity

The fungicidal activity of MAb R-5 against different fungal strains used was determined as described by Magliani et al. (1997). Briefly, 1 × 10³ fungal cells were added to 100 µl of MAb R-5 (50 µg) and incubated for 18 h at 37°C. An irrelevant MAb (Sigma) was used as a control. The fungal cell suspension (20 µl) were then spread on Sabouraud's dextrose agar plates, incubated at 37°C for 48 h and the CFU were calculated.

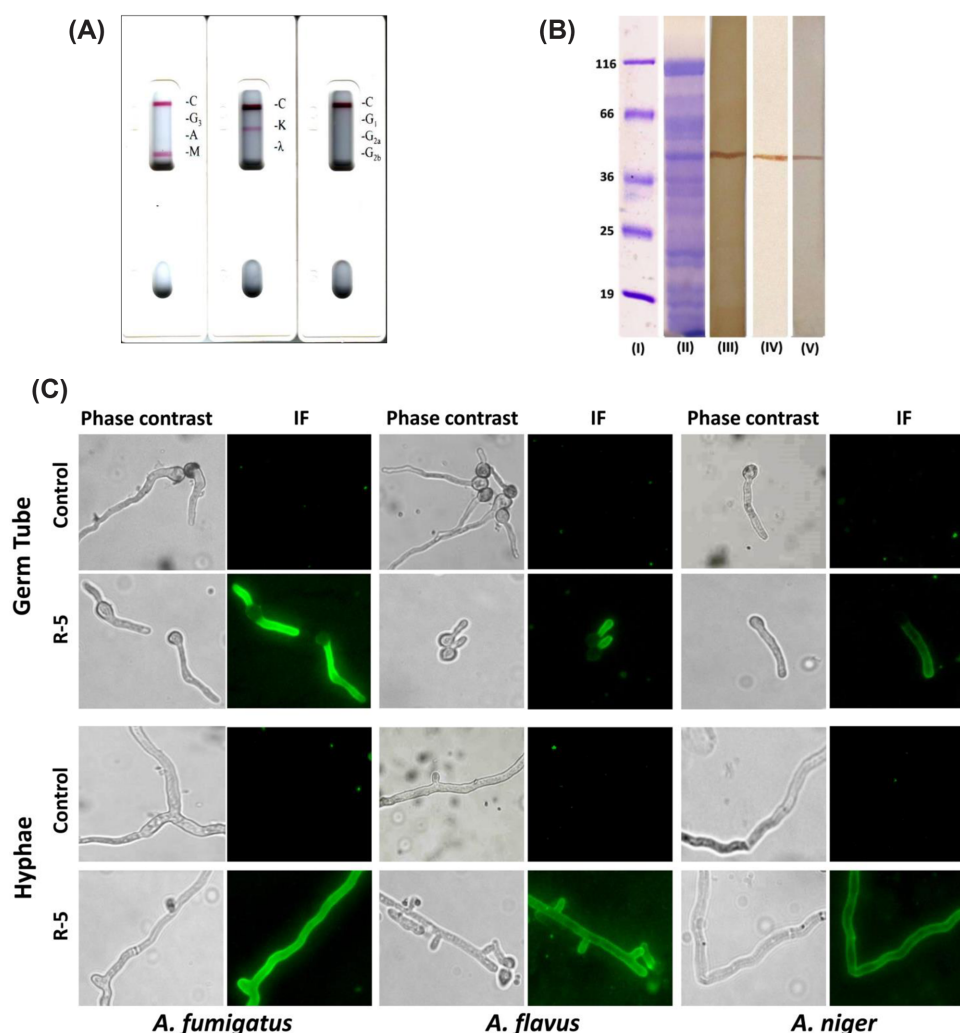


Figure 1. (A) Isotyping of MAb R-5 using isotyping Kit; (B) SDS-PAGE and immunoblot analysis: (I) Molecular weight marker (II) Cell surface protein profile of *A. fumigatus* on 12% gel stained with Coomassie brilliant blue; Immunoblot using MAb R-5 in cell surface proteins of *A. fumigatus* (III), *A. flavus* (IV) and *A. niger* (V); (C) Immunofluorescence and phase contrast photographs of germ tubes and hyphae of *A. fumigatus*, *A. flavus* and *A. niger* treated with MAb R-5 and control (no primary antibody).

The hyphal damage caused by MAb R-5 was studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, 100 μ l cell suspension (1×10^4 cells/ml) was incubated with 50 μ g of MAb R-5 for 18 h at 37°C. After centrifugation at 600 x g for 10 min, the supernatant was removed, and 100 μ l of MTT (0.5 mg/ml in RPMI) was added and incubated for 3 h at 37°C. The supernatants were removed after centrifugation at 600 x g for 10 min, to it 100 μ l of dimethyl sulfoxide (Sigma) was added to dissolve the formazan product and the absorbance was recorded at 550 nm. An irrelevant monoclonal antibody (Sigma) was used as a control. The experiment was performed in triplicate, and the result represents the mean value of three independent assays performed in triplicates.

In vivo evaluation

The immunotherapeutic ability of MAb R-5 was assessed in 6–8 wk old female BALB/c mice (6 mice in each group). Two groups of mice were injected intravenously 50 μ g of MAb R-5 and irrelevant MAb, respectively, 2 h before intravenous challenge with *A. fumigatus* (1×10^5 spores/mouse). The control group mice were

injected with DPBS and all the mice were kept under observation and deaths recorded for 24 days.

In another set of experiment, the effect of MAb on the fungal burden in kidney tissue was determined in separate groups of experimental BALB/c mice intravenously challenged with the spores of *A. fumigatus*. After 7 days of the challenge, mice were sacrificed, their kidneys removed, homogenized, serially diluted (10 fold) and then cultured on Sabouraud's dextrose agar plates for determination of CFU/gram tissue.

RESULTS

The fusion experiment resulted in 17 hybridoma cell lines in which 4 hybridoma clones were producing MAbs against cell surface proteins of *A. fumigatus*. The MAb producing hybridoma cells were subjected to single cell cloning to obtain single hybridoma cell clones. The ELISA, Western blotting and indirect immunofluorescence study revealed that one monoclonal antibody (MAb R-5) exhibited high binding capacity to surface antigen of *A. fumigatus* and was selected for further studies (Fig. 1B and C). The monoclonal antibody R-5 (MAb R-5) was found to

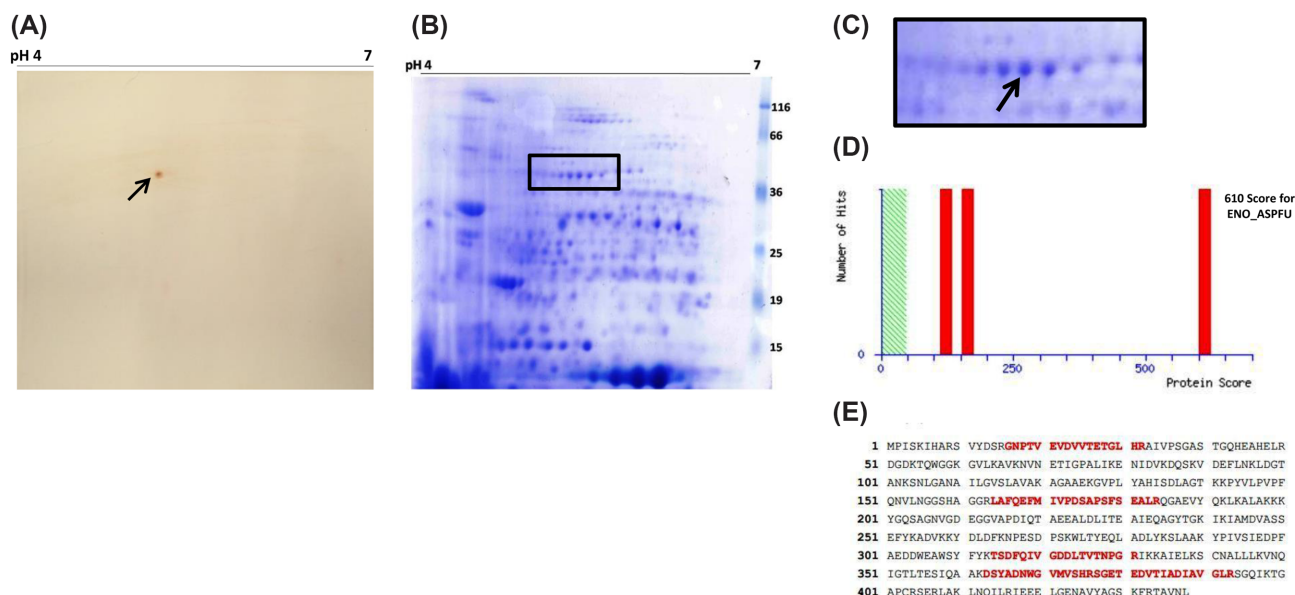


Figure 2. (A) The protein spot recognized by MAb R-5 on 2-D immunoblot; (B) 2D gel electrophoresis of surface proteins extracted from *A. fumigatus*; (C) Arrow indicates the protein corresponding to the spot recognized in the immunoblot; (D) Mascot Score Histogram: Ions score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores >46 indicate identity or extensive homology ($P < 0.05$); (E) Protein sequence coverage (19%) and matched peptides (bold red) with enolase of *A. fumigatus*.

belong to IgM class of antibody containing kappa light chain as determined by the isotyping kit (Fig. 1A).

Immunoblot analysis and identification of protein

Immunoblot analysis showed that MAb R-5 binds with nearly 48 kDa protein present in *A. fumigatus* as well as *A. flavus* and *A. niger* (Fig. 1B). This MAb did not show cross-reactivity with cell surface proteins of *C. albicans*, *S. schenckii* and *T. mentagrophytes* (data not shown).

The peptide mass fingerprinting data obtained from MALDI-TOF experiment were analyzed with the help of Mascot software (Matrix Science) online, *Aspergillus* Genome Database and SWISS-PROT protein sequence database, which revealed that the protein recognized by MAb R-5 was 47.39 kDa enolase allergen from *A. fumigatus* (Asp f 22) (Fig. 2D and E). These results were further confirmed by immunoprecipitation of the above protein and its analysis (data not shown).

Biological activities of MAb R-5

The indirect immunofluorescence study showed that MAb R-5 binds to the surface of germinated conidia and hyphal morphologic stages of *A. fumigatus* as well as *A. flavus* and *A. niger* indicating the presence of the antigen on the surface of hyphae and spores (Fig. 1C). The expression of this surface protein antigen was more in case of *A. fumigatus* as compared to *A. flavus* and *A. niger*.

The *in vitro* fungicidal activity of MAb R-5 was calculated by the reduction in the number of CFU compared to the control with an irrelevant MAb against *Aspergillus* and other fungal species, which revealed that MAb R-5 had a fungicidal effect against *Aspergillus* species, viz. *A. fumigatus* (88.3%), *A. flavus* (57.4%) and *A. niger* (30.6%) (Fig. 3A), and was negligible against *C. albicans*, *T. mentagrophytes* and *S. schenckii*. The MTT reduction assay compared to the control with an irrelevant MAb also confirmed fungicidal activity of MAb R-5, where hyphal damage against *A.*

fumigatus, *A. flavus* and *A. niger* was found to be 24.1%, 13.3% and 8.8%, respectively (Fig. 3B). The MAb R-5 showed insignificant hyphal damage in *C. albicans*, *S. schenckii* and *T. mentagrophytes*.

The *in vivo* protective ability of MAb R-5 was evaluated in experimental BALB/c mice challenged with *A. fumigatus* spores. The kidney tissues of mice that received MAb R-5 had 85.9% reduction in CFU as compared to control mice that received DPBS (Fig. 4A). The mean survival time of the mice treated with MAb R-5 had significantly increased (18.5 days) compared to the mice receiving irrelevant antibody (7 days) and DPBS (6.5 days) (Fig. 4B).

DISCUSSION

The cell surface proteins of pathogenic fungi are known to be involved in the initial fungal adhesion to the host tissue surfaces, and these proteins also directly participate in the host-fungal interactions. Antibodies against fungal cell wall proteins have been demonstrated to be useful in the inhibition of fungal adhesion and have been reported to exhibit potent antifungal properties (Moragues *et al.* 2003; Upadhyay *et al.* 2009). Antibodies developed against cell surface hydrophobic proteins of *C. albicans* inhibited its attachment to extracellular matrix (Masuoka *et al.* 1999). The passive immunization with an antibody developed against 70-kDa antigen present on the surface of *S. schenckii* has shown a protective effect in mice (Nascimento *et al.* 2008). In another study, passively administered MABs developed against a cell surface histone-like protein protected mice against lethal infections with *Histoplasma capsulatum* (Nosanchuk *et al.* 2003). These studies suggest that antibodies developed against surface proteins of pathogenic fungus could be valuable for the treatment of disease.

In the present study, MAb R-5 was successfully generated against the surface protein of *A. fumigatus* by conventional hybridoma technology. The results of western blotting and MALDI-TOF analyzes confirmed that the binding target of MAB R-5 was a surface protein, enolase (Fig. 2). The isotype of MAB R-5

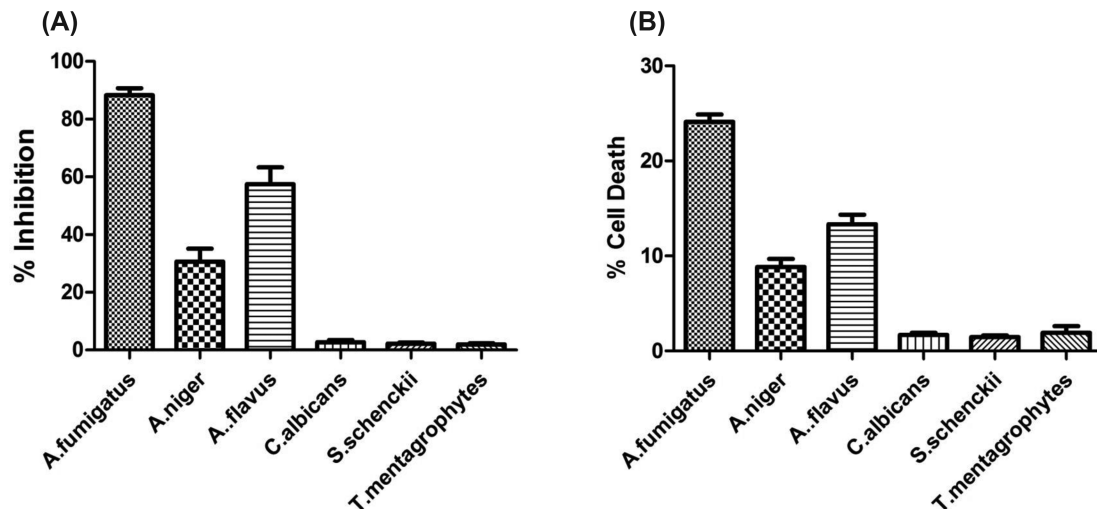


Figure 3. In-vitro fungicidal activity of MAb R-5: (A) The reduction in the number of CFU compared to the control with an irrelevant MAb was calculated against *A. fumigatus*, *A. flavus*, *A. niger*, *C. albicans*, *S. schenckii* and *T. mentagrophytes*; (B) The hyphal damage activity against *A. fumigatus*, *A. flavus*, *A. niger*, *C. albicans*, *S. schenckii* and *T. mentagrophytes* calculated by MTT reduction assay compared to the control with an irrelevant MAb. Values are means of triplicate determinations standard error of the mean. Data are shown as mean $\pm$ S.E.M. ($n = 3$).

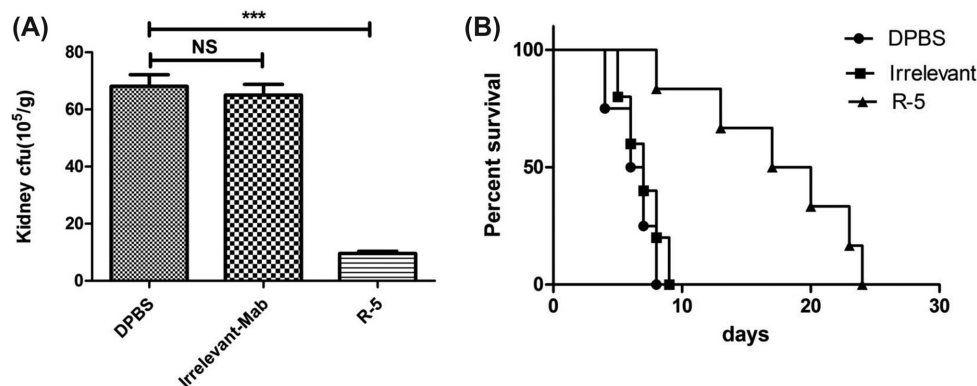


Figure 4. The evaluation of in vivo protective ability of MAb R-5: (A) determination of the CFU reduction in kidney tissue of experimental BALB/c mice challenged with *A. fumigatus* (1.0×10^5 cells per mouse). Mice that received MAb R-5 had 85.9% lower CFU ($P < 0.001$) in kidney tissue than mice that received DPBS; (B) Effect of MAb R-5 on the survival curve for mice infected intravenously with *A. fumigatus* (1×10^5 cells/mouse). The survival curve of mice treated with MAb R-5 was significantly different than for the control mice that given irrelevant antibody ($P < 0.05$) as assessed by the log-rank test.

was determined to be IgM with kappa (κ) light chain. The indirect immunofluorescence analysis showed the presence of enolase on the entire surface of germinating conidia and hyphae. The presence of enolase on the surface of both conidia and mycelia make it an important target for immunodiagnosis as well as immunotherapy.

Enolase is a well-known enzyme, which catalyzes the reversible conversion of 2-phospho-D-glycerate (PGA) and phosphoenolpyruvate (PEP) in glycolysis and gluconeogenesis metabolic pathways (Donofrio et al. 2009; Marcos et al. 2012). The enzyme enolase not only acts in intracellular metabolism, but also interacts with the host immune system and contributes to the pathogenesis in various organisms (Liu and Shih 2007; Avilan et al. 2011; Marcos et al. 2012). Recent studies have shown that it is an important allergen present on the surface of various medically important fungal species (Funk et al. 2016). The ability of the pathogenic fungi to interact with host surfaces and components of the extracellular matrix is essential for their further invasion, growth, colonization and the establishment of the disease (Khan et al. 2010). The surface localization of enolase implies its potential role in the interaction with the host immune system and

tissue invasion process during fungal infections. The surface-expressed enolase has been reported as a plasminogen binding protein used by different pathogenic fungi to promote the host cell-fungus interaction and to bind extracellular matrix components (Fox and Smulian 2001; Stie, Bruni and Fox 2009). The specific antibody responses against enolase of *C. albicans* and the usefulness of these antibodies for the diagnosis of candidiasis have also been demonstrated in different studies (Pitarch et al. 2008; Pitarch, Nombela and Gil 2014). Lai et al. identified the enzyme enolase as an important allergen of *A. fumigatus* (Lai et al. 2002). These studies support the view that specific antibody developed against enolase surface protein of *A. fumigatus* could be useful for the treatment of *Aspergillus* infections.

The in vitro fungicidal effect of MAb R-5 was demonstrated by the reduction in CFU and MTT reduction assay. The fungicidal effect was observed against *A. fumigatus* followed by *A. flavus* and *A. niger*, but no effect on other clinically relevant fungi (*C. albicans*, *S. schenckii* and *T. mentagrophytes*), which signified the specificity of MAb R-5 for *Aspergillus* species. The MAb R-5 was able to reduce the CFU in the kidney tissue of experimental BALB/c mice challenged with *A. fumigatus* and also significantly increased the

mean survival time as compared to control animals. In conclusion, MAb R-5 generated against enolase surface antigen binds specifically on the surface of *Aspergillus* species that has a potential to protect the host from *Aspergillus* infection and increased survival time by reducing fungal load. MAb R-5 may offer novel opportunities for the development of a new and effective tool for diagnosis and treatment of a wide range of *Aspergillus* infections.

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Conflicts of interest. None declared.

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