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A MURINE MODEL FOR STUDY OF ANTICRYPTOCOCCAL ACTIVITY MEDIATED BY CYTOTOXIC IMMUNE CELLS – ROLE IN IMMUNIZATION AND HUMAN VACCINE STRATEGIES

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NK and T cells play a pivotal role in host defence to Cryptococcus neoformans (C. neoformans) fungus which affects especially hosts with impaired cell mediated immunity. The vaccine against cryptococcosus is not developed yet, thus we established an animal BALB/c mice model to analyse anticryprococcal activity of immune cells. We detected that non-stimulated spleen mononuclear cells (MNC) from non-immunized mice have the capacity to exhibit anticriptococcal activity on the incapsulated C. neoformans strain (ATCC 34873) and this activity can be enhanced by non-adherent cells (NAC). In order to obtained antigen-specific anticryprococcal activity, MNC and NAC were stimulated in vitro with corpuscular (Ag1) or soluble (Ag2) C. neoformans antigen prepared from the acapsular strain Cap67 (ATCC 52817). In vitro stimulation of immune cells with both C. neoformans antigens enhanced the anticryptococcal activity of MNC and NAC. NAC fraction expressed the highest anticryptococcal activity, also in the presence and in the absence of accessory cells (AC). The highest anticryptococcal activity of effector cells was detected after immunization of mice with the same C. neoformans antigens and after additional stimulation of immune cells in vitro with the some antigens. These data demonstrated that growth inhibition of C. neoformans mediated by mice effector cells can be enhanced with corpuscular, as well as soluble antigens. Thus designin an animal model which is simple and reproducible and can be used for further studies and development of immunization strategies against human cryptococcosis.

Key words: animal model, Cryptococcus neoformans, cryptococcal antigens, immunization, NK cells, T cells

INTRODUCTION

Cryptococcus neoformans (*C. neoformans*) is one of the most common fungi which cause invasive fungal infections in patients with compromised cell-

mediated immunity, including AIDS (Coker, 1992). The importance of cell-mediated immunity and CD4+T lymphocytes in host resistance against *C. neoformans* is well documented and is exemplified by the high susceptibility to progressive infection with this pathogen in AIDS patients with reduced CD4+T cells (Mitchell and Perfect, 1995). Although CD4+ cells are critically important for immune responses and host defence to *C. neoformans*, both CD4+ and CD8+ T cells are involved in the generation of the protective immune response (Huffnagle *et al.*, 1991). However, *in vivo* CD8+ cells depletion abrogated host defence to *C. neoformans* in comparison with CD8+ cells undepleted mice (Mody *et al*., 1994) and rats (Arsić, 1993; 1997), suggesting that CD8+ T cells contribute to the protective immune response. This raises the possibility to protect hosts with deficiency of CD4+ cells, such as in AIDS, by designing immunization strategies for stimulating cytotoxic immune cells. Also, some studies have demonstrated that murine NK cells *in vitro* bind to and kill *C. neoformans* (Hidore *et al*., 1991) and that NK cells are effective in the clearance of cryptococci from the tissues of infected mice (Hidore and Murphy, 1986). It is known that anticryptococcal activity of NK cells is constitutively expressed and mediated dominantly by perforin, while CD8+ T cells require prior activation with *C. neoformans* and is mediated with granulysin expression (Ma *et al*., 2004). Recently it was showed that in murine non-adherent cells anticryptococcal cytotoxicity is perforin and non-perforin medated (Petković *et al*., 2010). According to data which demonstrated that pre-immunization with heat-killed *C. neoformans* significantly prolonged the survival time of mice (Kawakami *et al*., 1995) and that mice genetically engineered to lack CD4+ T cells could be successfully vaccinated to develop resistance to *C. neoformans* (Aguirre *et al*., 2004) the aims of this study were to compare the role of corpuscular and soluble *C. neoformans* antigens for immunization and growth inhibition of *C. neoformans* and to design a simple and reproducible animal model for further studies and immunization strategies against human cryptococcosus.

MATERIAL AND METHODS

Animals. A total of 60 pathogen free female BALB/c mice, 6-8 weeks old, 29±6.4 g, were obtained from the animal colony maintained at the Institute of Microbiology and Immunology, Medical faculty, University of Ljubljana, Slovenia. The study protocol was performed according to International Statutes on Animal Experiments and approved by The Ethics Committee of Medical faculty, University of Ljubljana, Slovenia.

Microbial strains. C. *neoformans* strain Cap67, unencapsulated mutant of B3501 (ATCC 52817) was used for the isolation of cryptococcal antigens (Ag1 and Ag2), immunization and *in vitro* challenge study. The encapsulated serotypes D strain B3501 (ATCC 34873), which is the isogenic parent strain of Cap67, can cause human infection via the pulmonary route, was utilized in the *in vitro* growth inhibition assay. The strains were obtained from the American Type Culture Collection (Manassas, VA) and were maintained on Sabourad dextrose agar (SDA) and passed to fresh slants every month, as previously described (Mody *et al*., 1989).

Preparation of C. neoformans antigens. Corpuscular (Ag1) and soluble (Ag2) antigens were prepared for the study. Heat-killed cells of *C. neoformans* Cap67 (Ag1) were prepared as previously described (Muth and Murphy, 1995). Supernatant of heat-killed *C. neoformans* Cap67 (Ag2) was prepared during the same procedure: *C. neoformans* Cap67 was grown for 96 h, washed three times, resuspended at 1 x 10^9/mL and the suspension of cryptococci was heat killed in phosphate-buffered saline (PBS) (pH 7.2) at 80° C for 1 h. The heat-killed *C. neoforman* was concentrated by centrifuging at 2000 x g for 10 min and the supernatant (Ag2) was removed, filtered over a 0.2 μm filter pore and stored at -70° C.

Study design and immunization protocols. First group of mice (n=5) was immunized with *C. neoformans* Ag1 by intraperitoneal (ip) injection of suspension containing 5 x 10^7 *C. neoformans* cells, and the second group (n=5) was immunized with *C. neoformans* Ag2 (0.5 mL) by ip injection. Five days later the first group was boosted by ip injection containing 5 x 10^8 *C. neoformans* Ag1 and the second group was boosted by ip injection containing 1.0 mL *C. neoformans* Ag2. Control mice (n=5) were injected with an equal volume of PBS (pH 7.2) by the same routes. Eight days later the mice were sacrificed, spleens were removed and effector cell prepared.

Effector cells preparations. Immune effector cells were prepared from the spleens which were first mechanically disrupted. The mononuclear cells (MNC) were obtained after centrifugation at 800 x g for 20 minutes over a Ficoll density gradient and suspended in RPMI 1640 (5% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin G, 0.1 mg streptomycin) (Sigma-Aldrich, Austria). To separated non-adherent cells (NAC) the MNC were passed over a nylon wool column and the efficacy of adherence to nylon wool was evaluated by flow cytometry. Stimulation of effector cell populations (5 x 10^5/well) with *C. neoformans* Ag1 or with *C. neoformans* Ag2 was done in 96-wells plates in quadruplicate for 24 h. Non-stimulated or *C. neoformans* Ag1/*C. neoformans* Ag2 stimulated effector cells used for anticryptococcal activity were: (i) MNC, (ii) NAC with accessory cells (AC), (iii) NAC without of AC.

Immunofluorescent labelling and flow cytometry. The efficacy of separation of effector cell population was evaluated by flow cytometry. Monoclonal antibodies (mAb) were used to identify cell populations after the separation on nylon wool. Effector cells population was labelled with FITC-conjugated anti-CD3 and anti-CD86 mAb and with PE-conjugated anti-CD19, anti-CD49b mAb (Becton Dickinson, USA). After 1 h of incubation the labelled cells were washed twice and then analyzed by FACSCalibur flow cytometer and CELLQuest software (Becton Dickinson). NK and T cells are labelled with anti-CD49b and anti-CD3 mAb. B cells and AC such as macrophages or other APC are labelled with anti-CD19 and anti-CD86 mAb.

Growth inhibition assay of C. neoformans. For the growth inhibition assay we used the human pathogen *C. neoformans* encapsulated strain serotype D B3501 (ATCC 34873) which is the isogenic parent strain of Cap 67 (ATCC 52817). *C. neoformans* was maintained on SDA and log-phase yeasts were harvested by suspension in RPMA 1640 medium, washed and then counted on a

hemocytometer to adjust yeast concentration to 1 x 10^4/mL. Immune effector cells (5 x 10^5) were cultivated with encapsulated *C. neoformans* (1 x 10^3) in a total volume of 0.2 mL RPMI 1640 in quadruplicate wells of a flat-bottom 96-well microtitre plates. Control wells contained only 1 x 10^3 cryptococcal target cells in the medium. After 72 h of incubation on 37°C the content of each well was washed and diluted in sterile PBS. The different dilutions were plated in duplicate on SDA and incubated for 3 days at 37°C in order to determine colony-forming units (CFU) of *C. neoformans* by counting colonies. Antifungal activity of effector cells was determined as described previously (Levitz and Dupont, 1993). The percentage of cryptococcal growth inhibition was determined according to the formula:

100/(CFU experimental/CFU control plate) x 100 = % cryptococcal growth inhibition

Statistical analysis. Ex vivo experiments were performed 4 times while *in vitro* study was done in quadruplicates. Values are expressed as the mean +/- standard error of the mean (SEM). The Student's t-test with Boneferroni correlation was used to determine the statistical significance in pair wise comparisons with $p<0.05$ being considered statistically significant.

RESULTS AND DISCUSSION

MNC and NAC from non-immunized mice express anticryptococcal activity after in vitro stimulation with Ag1 or Ag2. In the initial experiments we tested the antifungal activity of spleen MNC and demonstrated that cells from non-immunized mice *in vitro* stimulated with *C. neoformans* Ag1 or with *C. neoformans* Ag2 express antifungal activity (Fig. 1A). To obtain NAC, spleen MNC was treated with nylon wool and the separation efficiency for CD3+ cells was 79.1 +/- 2.3%, while for CD49b+ cells was 7.9 +/-0.5, confirmed with flow cytometry. Anticryptococcal effect of NAC was tested and observed growth inhibition assay of *C. neoformans* was stronger compared to MNC (Fig 1B). Further *in vitro* stimulation of NAC with *C. neoformans* Ag1 or *C. neoformans* Ag2 showed higher anticryptococcal activity compared to non-stimulated NAC (Fig 1C). Data showed that MNC and NAC from non-immunized animals exhibit anticryptococcal activity, and furthermore the NAC are capable to be *in vitro* activated with different *C. neoformans* Ag.

After immunization MNC and NAC express higher anticryptococcal activity. Highest anticryptococcal activity was observed after *in vitro* incubation of effector cells with Ag1 or Ag2 in the presence of APC. In order to determine whether *in vivo* immunization enhances the ability of MNC and NAC anticryptococcal activity, we compared activities of immune cells obtained from Ag1 or Ag2 immunized mice. MNC from immunized mice exerted 2-3 fold higher growth inhibition of *C. neoformans* compared to MNC from non-immunized mice (data not shown). Relative to the results obtained from non-immunized mice (Fig. 1C) further we tested the inhibition capacity of NAC from immunized mice by using the same protocol. Results showed that the anticryptococcal activity mediated by NAC from immunized mice was significantly higher compared to anticryptococcal activity of NAC obtained from non-immunized mice (Fig. 2), and also with presence of AC

less than 1%. In non-immunized animals corpuscular Ag1 showed a better activity ($p>0.05$; not significant). The best anticryptococcal activity was observed with MNC and NAC obtained from immunized mice further stimulated with the same antigens by which the immunization was done ($p<0.05$; significant), as well as with soluble Ag2 (Fig. 2).

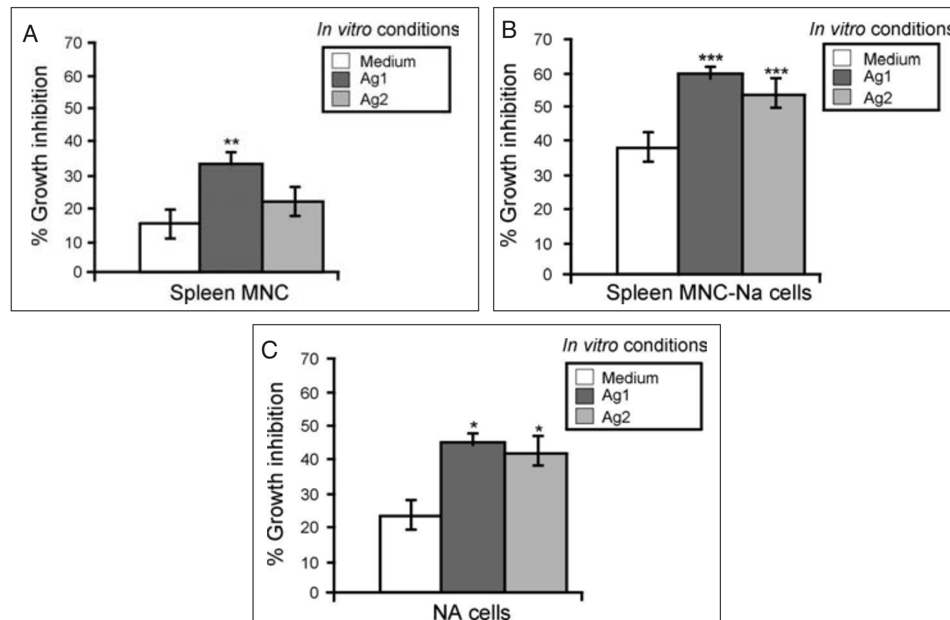

Figure 1. Effector cells from non-immunized mice and growth inhibition of *C. neoformans* mediated by spleen MNC (A), spleen MNC-NA cells (B) and NA cells (C) after *in vitro* stimulation with *C. neoformans* Ag1 or *C. neoformans* Ag2. Data are representative of three experiments performed in quadruplicate. Error bars represent the standard error of the mean (SEM). Significant differences: *($p<0.05$); **($p<0.01$) and ***($p<0.001$) compared to control

Our data is in correlation with previous reports that growth inhibition of *C. neoformans* is mediated by NAC and that anticryptococcal activity is enhanced by immunization with heat-killed *C. neoformans* (Fromtling *et al*., 1983; Levitz *et al.,* 1995; Murphy *et al*., 1998), but we also found that: (i) immunization with soluble antigens (Ag2) can enhance the antifungal activity of MNC and also of NAC which can be activated *in vitro* in the presence or absence of AC, (ii) immunization resulted in enhanced anticryptococcal activity and this effect was highest when immunization and further *in vitro* stimulations were done with the same antigens (iii) the animal model is simple and reproducible and can be useful for further studies and immunization strategies against human cryptococcosis.

It has been known that freshly isolated T cells and NK cells from lymphoid tissues of normal, non-immunized mice have the capacity to bind *C. neoformans*

in vitro and inhibit the growth of *C. neoformans* (Levitz *et al*., 1994). Our results have shown that spleen MNC from non-immunized mice are capable to be activated *in vitro* with either Ag1 or Ag2 and exert enhanced antifungal activity compared to non-activated cells (Fig 1A). After nylon wool depletion of adherent cells the anti-cryptococcal activity mediated by NA cells was higher (Fig 1B). We demonstrated that NA cells from non-immunized mice were activated directly *in vitro* by fungal molecules to enhance antifungal activity (Fig 1C). As shown in Fig. 1 the antifungal activity was enhanced independent of which *C. neoformans* antigens were used. As the NA cells separated by passage over nylon wool were depleted of AC and mice have not been exposed to *C. neoformans* before the experiment, we assume that responding cells for anticryptococcal activity were NK cells and/or naïve cytotoxic T lymphocytes.

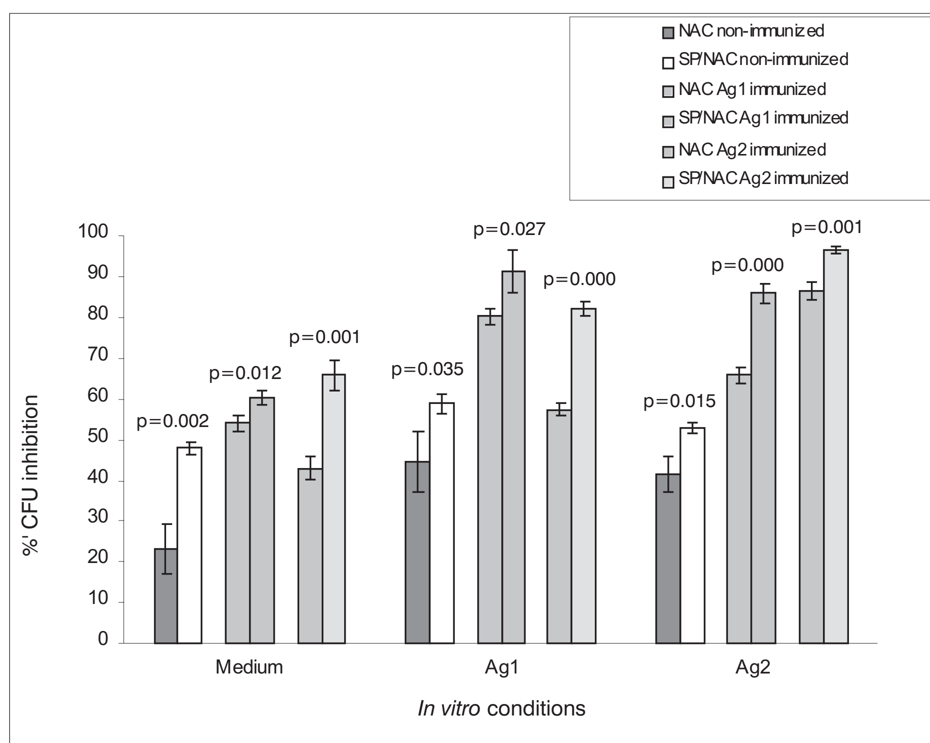

Figure 2. Growth inhibition of *C. neoformans* and different effectors cell populations from non-immunized and immunized mice in medium or incubated with Ag1 or Ag2 *in vitro*. Data are representative of three experiments performed in quadruplicate. Error bars represent the standard error of the mean (SEM)

Previous investigations demonstrated that both cell populations can inhibit *in vitro* growth of *C. neoformans* (Hidore *et al*., 1990). It has been shown that *C.*

neoformans contains a mitogen able to stimulate naive T cells (Mody *et al*., 1999) and also that murine NK cells, human primary NK cells and the human NK cell line constitutively expressed anticryptococcal activity (Ma *et al*., 2004). Therefore, T lymphocytes and NK cells can be activated directly by Ag1 and Ag2 *C. neoformans* and subsequently exert antifungal activity.

Further, we demonstrated that growth inhibition of *C. neoformans* is enhanced by immunization either with corpuscular or soluble antigens (Fig 2). Antifungal activity of spleen MNC was additionally enhanced after *in vitro* stimulation. The highest inhibition of fungal growth was detected when cells were *in vitro* stimulated with the same antigens that we used for immunization. Heat-killed cells of the unencapsulated mutant of B3501 (Cap 67) of *C. neoformans* (Ag1) (Muth and Murphy, 1995) or supernatant of heat-killed Cap 67 (Ag2) were used for immunization of BALB/c mice and *in vitro* cultivated with NA cells. The anticryptococcal activity of NA cells was tested for *in vitro* growth inhibition assay for *C. neoformans*. Besides, the well known role of heat-killed corpuscular antigen (Ag1) in immunization and stimulation of effectors cells showed that the supernatant of heat killed *C. neoformans* (Ag2) can stimulate the NA cells mediated anticryptococcal response. It was known that there are two major antigen fractions of *C. neoformans*: glucuronoxylomannan (GXM) and mannoproteins (MPs). GXM is a primary component of the polysaccharide capsule and is the major virulence factor which has an immunosuppressive role during the immune response (Yauch *et al*., 2006; Villena *et al*., 2008). In contrast, MPs are present in the unencapsulated strain and have been identified as the key antigen that stimulate T-cell responses (Wozniak and Levitz, 2009). In accordance with this we prepared Ag1 and Ag2 from an unencapsulated Cap67 mutant of *C. neoformans* to stimulate NA cells in our model. Alongside this, the encapsulated strain serotype D (B3501) was utilised in the growth inhibition assay as encapsulated strains cause human infection.

The importance of NK cells for immunization effects and anticryptococcal activity has already been demonstrated (Marr *et al*., 2009), but enhanced anticryptococcal activity in our model can be ascribed to both NK cells and to mitogenic activity of *C. neoformans* on naïve T cells. Therefore, the antifungal activity of *in vitro* stimulated NA cells was dependent on the presence of expanded cytotoxic cells (Petković *et al*., 2010).

The described animal model is more simple and reproducible then models of *in vivo* infections with viable *C. neoformans* (Arsić *et al*., 1993; 1997). Taken together, our data suggest that immunization could hold promise in the setting of cryptococcosis, and that efficacy could depend on immunomodulation of the natural immune response of the host. In this study we employed an animal model to investigate the efficacy of immunization on anticryptococcal activity mediated with NA cells. Further investigations are needed to establish the function of the recapitulation of natural immunity and a better understanding of natural resistance to cryptococcosis. If the anticryptococcal mechanisms mediated with cytotoxic immune cells could be enhanced, it may be possible to create human vaccines and/or therapy strategies in the protection against *C. neoformans* in patients with a reduced number of CD4+ lymphocytes.

In summary, the data presented here demonstrated that soluble antigens also possess the capacity to enhance anticryptococcal activity. This is confirmed by the simple and reproducible animal model which can be useful for further evaluation of immunization and cellular immune response.

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EKSPERIMENTALNI MODEL ZA ISPITIVANJE ANTIKRIPTOKOKNE AKTIVNOSTI POSREDOVANE MIŠJIM CITOTOKSIČNIM ĆELIJAMA – ULOGA IMUNIZACIJE I STRATEGIJA ZA HUMANU VAKCINU

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SADRŽAJ

Ključnu ulogu u odbrani domaćina od gljive *Cryptococcus neoformans* (*C. neoformans*) koja dominantno izaziva infekcije kod osoba sa deficitom celularne imunosti imaju NK i T ćelije. Antikriptokokna vakcina za prevenciju kriptokoze je

još uvek u fazi razvoja zbog čega je cilj ovog rada bio da razvije animalni model za analiziranje antikriptokone aktivnosti imunskih ćelija na BALB/c miševima.

Utvrđeno je da nestimulisane mononuklearne ćelije (MNĆ) slezine neimunizovanih miševa ispoljavaju antikriptokoknu aktivnost u testu inhibicije rasta inkapsuliranog soja *C. neoformans* (ATCC 34873) i ona je povećana izdvajanjem neadherentnih ćelija (NAĆ). Za analiziranje antigen indukovanog ćelijskog imunskog odgovora, MNĆ i NAĆ su *in vitro* stimulisane korpuskularnim (Ag1) ili solubilnim (Ag2) antigenima *C. neoformans* koji su dobijeni od akapsularnog soja Cap67 (ATCC 52817). *In vitro* stimulacija imunskih ćelija sa oba *C. neoformans* antigena dovodi do pojačanja antikriptokokne aktivnosti i MNĆ i NAĆ. Inhibicija rasta *C. neoformans* je bila značajno veća kada su kao efektorske ćelije korišćene samo NAĆ u prisustvu akcesornih ćelija (AĆ), ali je bila prisutna i u njihovom odsustvu. Najveći stepen antikriptokokne aktivnosti su ispoljile efektorske ćelije imunizovanih miševa kada su dodatno stimulisane *in vitro*, istim antigenom sa kojim je izvršena imunizacija. Dobijeni rezultati su ukazali da je povećanje antikriptokokne aktivnosti moguće primenom i korpuskularnih i solubilnih antigena. Prikazan animalni model je jednostavan i reproducibilan i može se primenjivati za dalja istraživanja u cilju izrade vakcine za prevenciju kriptokokoze ljudi.