Experimental Parasitology xxx (2017) 1–7

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Production and characterization of monoclonal antibodies against cathepsin B and cathepsin B-Like proteins of *Naegleria fowleri*

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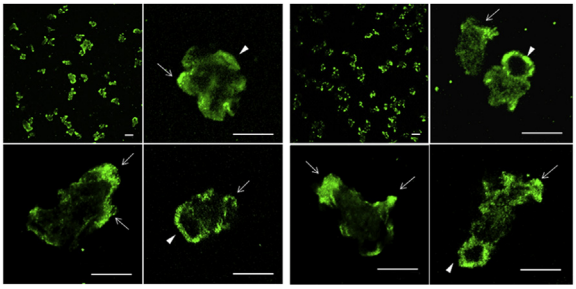
HIGHLIGHTS

- We produced anti-NfCPB and anti-NfCPB-L McAbs using a cell fusion technique.
- High antigenicity of 2C9(NfCPB) and 1C8(NfCPB-L) McAbs selected for use.
- 2C9 and 1C8 proteins were localized on cytoplasm, pseudopodia and amoebastomes.
- Two McAbs will be useful for the pathoimmunological studies of PAM.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 July 2017
Received in revised form
18 August 2017
Accepted 11 September 2017
Available online xxx

Keywords:
Naegleria fowleri
Cathepsin B protein
Cathepsin B-Like protein
Cell fusion
Monoclonal antibody

ABSTRACT

Naegleria fowleri causes fatal primary amoebic meningoencephalitis (PAM) in humans and experimental animals. In previous studies, *cathepsin B* (*nfcpb*) and *cathepsin B-like* (*nfcpb-L*) genes of *N. fowleri* were cloned, and it was suggested that refolding rNfCPB and rNfCPB-L proteins could play important roles in host tissue invasion, immune response evasion and nutrient uptake. In this study, we produced anti-NfCPB and anti-NfCPB-L monoclonal antibodies (McAb) using a cell fusion technique, and observed their immunological characteristics. Seven hybridoma cells secreting rNfCPB McAbs and three hybridoma cells secreting rNfCPB-L McAbs were produced. Among these, 2C9 (monoclone for rNfCPB) and 1C8 (monoclone for rNfCPB-L) McAb showed high antibody titres and were finally selected for use. As determined by western blotting, 2C9 McAb bound to *N. fowleri* lysates, specifically the rNfCPB protein, which had bands of 28 kDa and 38.4 kDa. 1C8 McAb reacted with *N. fowleri* lysates, specifically the rNfCPB-L protein, which had bands of 24 kDa and 34 kDa. 2C9 and 1C8 monoclonal antibodies did not bind to lysates of other amoebae, such as *N. gruberi*, *Acanthamoeba castellanii* and *A. polyphaga* in western blot analyses. Immuno-cytochemistry analysis detected NfCPB and NfCPB-L proteins in the cytoplasm of *N. fowleri* trophozoites, particularly in the pseudopodia and food-cup. These results suggest that monoclonal antibodies produced against rNfCPB and rNfCPB-L proteins may be useful for further immunological study of PAM.

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http://dx.doi.org/10.1016/j.exppara.2017.09.004
0014-4894/

1. Introduction

Free-living pathogenic *Naegleria fowleri,* a causal agent of primary amoebic meningoencephalitis (PAM) in humans, inhabits natural environments such as ponds, rivers, hot springs and swimming pools, where they can occur in three different forms, trophozoite, cyst and occasionally flagellate cell (Culbertson, 1970; Ma et al., 1990; Visvesvara et al., 2007). Increasingly *N. fowleri*, the "brain-eating amoeba", is becoming recognized as an important pathogenic microorganism of concern in aquatic recreation activities (Heggie, 2010; Sifuentes et al., 2014). In the United States, Australia and Europe, more than 300 cases of PAM have been reported (Budge et al., 2013; De Jonckheere, 2011; Yoder et al., 2010). Furthermore, death due to PAM, as a neglected tropical disease (NTD), has been increasing in Asian countries such as Pakistan, India, Thailand and Vietnam (Gupta et al., 2009; Shakoor et al., 2011; Siddiqui and Khan, 2014; Siripanth, 2005).

The symptoms of PAM, due to infection of *N. fowleri* via the nasal cavity from warm water contaminated with amoebic trophozoites, are headache, nausea, vomiting, fever and meningitis (Schuster and Visvesvara, 2004; Shin and Im, 2004). Because the clinical symptoms of PAM are similar to acute purulent encephalitis and bacterial meningitis, early clinical diagnosis is very difficult. Thus, PAM diagnosis depends mainly on finding trophozoites in tissue biopsies or cultivation of CSF (central spinal fluid). But the practice is not easy. Recently developed diagnostic methods depend on primer-based PCR (polymerase chain reaction) analysis post tissue biopsy or cultivation of CSF (Kang et al., 2015; Ma et al., 1990; Qvarnstrom et al., 2006; Reveiller et al., 2002; Schuster and Visvesvara, 2004).

Two possible pathogenic mechanisms of *N. fowleri* causing PAM are recognized. The contact-dependent mechanism is concerned with the formation of amoebastomes, or food-cups, activated by *nfa1* and *nf-action* genes. A contact-independent mechanism, with the secretion of phospholipase A, neuraminidase, elastase, perforin-like protein and cysteine protease, also has been reported (Kang et al., 2005; Kim et al., 2008; Marciano-Cabral and Cabral, 2007; Schuster and Visvesvara, 2004; Shin et al., 2001; Sohn et al., 2010).

Previously, Lee et al. (2014) cloned *N. fowleri* cathepsin B (NfCPB) and cathepsin B-Like (NfCPB-L) proteins consisting of 1.038bp (assumed molecular weight 38.4 kDa) and 939bp (34 kDa) fragments, respectively. The refolding recombinant cathepsin B (rNfCPB) and cathepsin B-like (rNfCPB-L) proteins play important roles in the proteolytic activities on immunoglobulin, collagen, fibronectin, hemoglobin and albumin (Lee et al., 2014).

In the present study, in order to develop useful monoclonal antibodies essential for further patho-immunological studies, we produced anti-NfCPB and anti-NfCPB-L monoclonal antibodies (rNfCPB McAb and rNfCPB-L McAb) using the cell fusion technique and observed their immunological characteristics.

2. Materials and methods

2.1. Amoebae culture and preparation of lysate and excretory-secretory protein

Pathogenic *N. fowleri* (Carter Nf69 strain, ATCC 30215) and non-pathogenic *N. gruberi* (Schardinger strain; ATCC 30960) were axenically cultured at 37 °C in Nelson's medium and PYNFH medium, respectively, with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) according to methods previously described (Kang et al., 2015). *Acanthamoeba castellanii* and *A. polyphaga* were axenically cultured at 25 °C in PYG medium according to previously reported methods (Sohn et al., 2017). Amoebic lysates (Nf-lysate, Ng-lysate, Ac-lysate and Ap-lysate) were prepared by the freezing-thawing method, and after ultracentrifugation at 12,000 rpm at 4 °C for 2hr, the aqueous supernatants were collected (Kim et al., 2016). Preparation of *N. fowleri* excretory-secretory protein (Nf-ESP) was performed using the previously reported method (Kim et al., 2009). Briefly, *N. fowleri* trophozoites were harvested in 50 ml tubes and incubated in PBS (pH 7.4) at 37 °C for 1 h. After centrifugation at 800 × g for 5min, the collected supernatant was used as the Nf-ESP.

2.2. Recombinat NfCPB and NfCPB-L protein preparation

To obtain the rNfCPB and rNfCPB-L proteins, the *nfcpb* and *nfcpb-L* genes were cloned into the pExp5-TOPO TA vector (Invitrogen, USA); pExp5-TOPO-*nfcpb* and pExp5-TOPO-*nfcpb-L* were then transformed into *Escherichia coli* strain BL21 (DE3) according to the method previously described (Lee et al., 2014). Following inoculation into Luria-Bertani medium containing 100 mg/ml of ampicillin, *E. coli* was incubated with 200 rpm rotation at 37 °C until 0.4–0.6 OD_{600} was reached. After the addition of 1 mM isopropylβ-D-thiogalactopyranoside (IPTG, Goldbio, USA), *E. coli* was incubated with 200 rpm rotation at 37 °C for 4 h and pelleted down. The pellet was resuspended in 8M urea lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01M Tris-HCl) and pelleted down again. The collected supernatant was subjected to purification with nickel-nitrilotriacetic acid (Ni-NTA) chromatography (QIAGEN, USA) and concentration by Amicon® Ultradevice (cut-off 10 kDa; Millipore, USA). Purified rNfCPB and rNfCPB-L proteins were stored at −20 °C until use.

2.3. rNfCPB and rNfCPB-L immunization

Immunization methods previously described (Lee et al., 2007) were employed. Each 50 μg of rNfCPB and rNfCPB-L proteins was mixed with 100 μl of Freund's complete adjuvant (Sigma, USA) and intraperitoneally injected into 6-week-old female BALB/c mouse. Next, the mice were intraperitoneally boosted twice with 25 μg of each protein mixed with 100 μl of Freund's incomplete adjuvant (Sigma, USA) at two-week intervals. Polyclonal antibody formation was evaluated from caudal vein bloods by ELISA (antigen, 0.1 μg/100μl/96-well; secondary antibody, 1:10,000 diluted goat anti-mouse IgG conjugated alkaline phosphatase). At 4 days before cell fusion, a final boosting with 25 μg of each protein suspended in PBS (pH 7.4) was administered.

2.4. Cell fusion and monoclonal antibody production

The cell fusion technique used was a slight modification of the methods of Lee and Kim (Kim et al., 2012; Lee et al., 2007). Briefly, the splenocytes isolated from immunized mouse under anesthesia with Ketamin-Rompun solution was fused with myeloma cells (F/o cell line) cultured in complete DMEM (Dulbeco's Modified Eagle Medium, Gibco, USA) medium by adding PEG (polyethylene glycol) solution. The mixture was cultured in 96-well plate with HAT (hypoxanthine-aminopterin-thymidine, Sigma) medium at 37 °C, in a 5% CO_2 incubator for 3 days. Next, the cultivating medium in each 96-well culture plate was replaced with HT (hypoxanthine-thymidine, Sigma) medium. After the identification of hybridoma cells from each well, the secretion of antibody from hybridoma cells was evaluated by ELISA. The hybridoma cells producing antibodies were scaled up in 24-well plates and consequently 75-cm^2 culture flask (Nunc, USA) by culturing at 37 °C, in a 5% CO_2 incubator. Selection of monoclones producing monoclonal antibodies was made using the limiting dilution method (Kim et al., 2012). Massive production of monoclonal antibodies was obtained from ascites of mice injected with monoclone cells. Then, antibody immunoglobulins were purified in a Protein A column (proA™, KOREA), concentrated by Amicon® Ultradevice, isotypes determined with

Please cite this article in press as: Seong, G.-S., et al., Production and characterization of monoclonal antibodies against cathepsin B and cathepsin B-Like proteins of *Naegleria fowleri*, Experimental Parasitology (2017), http://dx.doi.org/10.1016/j.exppara.2017.09.004