International Journal of Biological Macromolecules 81 (2015) 100–111

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Vaccine potential of plasma bead-based dual antigen delivery system against experimental murine candidiasis

Ejaj Ahmad, Qamar Zia, Munazza Tamkeen Fatima, Mohammad Owais*, Mohammed Saleemuddin*

Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India

ARTICLE INFO

Article history:
Received 6 January 2015
Received in revised form 23 July 2015
Accepted 24 July 2015
Available online 29 July 2015

Keywords:
Plasma beads
PLGA microspheres
Prophylactic vaccine
Biodegradable polymer
Candida albicans

ABSTRACT

The development of prophylactic anti-candidal vaccine comprising the *Candida albicans* cytosolic proteins (Cp) as antigen and plasma beads (PB) prepared from plasma as sustained delivery system, is described. The immune-prophylactic potential of various PBs-based dual antigen delivery systems, co-entrapping Cp pre-entrapped in PLGA microspheres were tested in the murine model. Induction of cell mediated immunity was measured by assaying DTH and NO production as well as *in vitro* proliferation of lymphocytes derived from the immunized animals. Expression of surface markers on APCs (CD80, CD86) and T-cells (CD4+, CD8+) was also evaluated. Humoral immune response was studied by measuring circulating anti-Cp antibodies and their subclasses. When the prophylactic efficacy of the vaccines was tested in mice challenged with virulent *C. albicans*, the PB-based formulation (PB-PLGA-Cp vaccine) was found to be most effective in the generation of desirable immune response, in terms of suppression of fungal load and facilitating the survival of the immunized animals.

1. Introduction

In spite of its recognized commensal status, the dimorphic *Candida albicans* turns pathogenic when host immune defenses are compromised, causing a variety of infections ranging from superficial to life threatening disseminated candidiasis. Predisposing factors include severe underlying diseases (i.e. leukemia or AIDS), impaired phagocytic function (granulocytopenia or neutropenia), changes in the host stasis (i.e. aging or treatment with broad spectrum antibiotics), intravenous drug usage, organ transplantation as well as abdominal surgery and trauma [1]. Nosocomial blood stream isolates of *Candida* stand as the fourth most common cause of mortality (of 40–50%) in the hematogeneously disseminated diseases [2,3]. The advent of AIDS and the increasing use of potent immune-suppressive therapies to combat autoimmune diseases, malignancies as well as transplantation rejections revived interest in effective vaccines against *C. albicans*. Besides, lack of an early and accurate diagnostic procedure, the limited chemo-therapeutic agents available to combat the infection and emergence of resistant strains contribute to the high morbidity and mortality associated with *C. albicans* infections.

No licensed vaccine is available as of yet despite the observation that candidiasis occurs in almost 75% of all females during their life time [4]. An anti-candidal vaccine that is preventive, protective and therapeutic is therefore of utmost importance to combat the growing incidences of *C. albicans* and related fungal pathogens. Several immunogenic proteins including a 58-kDa mannoprotein (mp58), heat shock protein 90, a secreted acid proteinase, and phosphomanan enriched cell extract fraction have been purified from *C. albicans* cells and their prophylactic potentials evaluated [5–11].

Pharmaceutical vaccine formulations presently being tested in various experimental and clinical models are of particulate nature. In contrast to most soluble antigens, which are ignored by the immune system, particlulate antigens are recognized as foreign and as danger [12]. Furthermore, soluble proteins are poorly taken up by antigen-presenting cells whereas particulate antigens are avidly ingested. Hence, the starting-point when designing a new vaccine delivery system is often that a particulate structure is required. Moreover, ideal delivery system should release the entrapped antigen in a sustained manner to ensure its extended exposure to the cells of immune system and thereby offer effective prophylaxis against invading pathogens. As an alternative, biodegradable polymeric microspheres system (MS) have been intensively studied for their feasibility as potent delivery vehicles

* Corresponding authors. Tel.: +91 0571 2720388; fax: +91 0571 2721776.
E-mail addresses: owais_lakhnawi@yahoo.com (M. Owais), msaleemuddin@rediffmail.com (M. Saleemuddin).

http://dx.doi.org/10.1016/j.ijbiomac.2015.07.047
0141-8130/

for soluble antigens [13,14]. However, a major problem hindering the progression of MS-based vaccine formulations for human use is the issue of antigen stability during microencapsulation, storage, and release [15–17]. PLGA is a polyester composed of one or more hydroxy acid monomers – D/L-lactic, and/or glycolic acids. PLGA microspheres have been widely investigated for their ability to entrap various therapeutics including proteins/vaccines and deliver them in the animal models. PLGA MS can provide antigen release over weeks and months following continuous or pulsatile kinetics [15,16] leading to the generation of remarkable immune response. Furthermore, PLGA MS are readily recognized and ingested by macrophages and dendritic cells, an important property for stimulating the immune system [18].

Previously, we have demonstrated that beaded form of blood plasma can easily entrap drug and release in a slow and sustained fashion [19]. These drugs remained in circulation for a longer duration than the antibiotics administered in the free form *in vivo*. Since their preparation does not require additional thrombin or other proteins, it can be readily accomplished by using autologous plasma, thereby minimizing the risk of immunological complications [19]. Taking a step further, we prepared PLGA microspheres containing *C. albicans* cytosolic antigen (Cp), and entrapped it inside PB to further control stability and release. Additionally, we also evaluated the efficacy of the in house prepared formulation in offering protection to Balb/c mice against challenge by the pathogen. Partial purification of *C. albicans* proteins was accomplished by ammonium sulfate fractionation. It was observed that while the Cp encapsulated in PLGA microspheres (PLGA-Cp) successfully suppressed the fungal load in the vital organs and increased the survival of the challenged animals. The formulations when further entrapped into plasma beads (PB-PLGA-Cp) offered far superior prophylactic response.

2. Materials and methods

2.1. Chemicals

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymers, bicinchoninic acid (BCA), IgG, isotypes IgG1, and IgG2a used in the study were purchased from Sigma Chemical Company. IL-4 and IFN-γ cytokine kits, and monoclonal anti-mouse CD4, CD8, CD80, and CD86 and their isotypic controls, were procured from BD Biosciences. Other chemicals used in the study were of analytical grade and procured locally.

2.2. Animals

Inbred female Balb/c mice (4–6 weeks old), weighing 20 ± 2 g were obtained from the animal house facility of CDRI, Lucknow (India). After procurement, the animals were acclimatized with laboratory conditions for a period of 7 days under standard husbandry conditions: room temperature (22 ± 3 °C), relative humidity (65 ± 10%) and 12 h light/dark cycle. The experimental animals had free access to standard dry pellet diet and water ad libitum under strict hygienic conditions. In all experimental procedures, efforts were made to minimize pain and suffering. Experiments involving bleeding, injection, and sacrifice of animals were strictly performed following the mandates approved by the Institutional Animal Ethics Committee constituted as per the recommendations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (http://moef.nic.in/division/committeepurpose-control-and-supervision-experiments-animals-cpcsea-1). As recommended by IAEC, AMU, Aligarh, India, humane endpoints were considered essential for culling of mice which survived at the conclusion of the experiment. Mice were administered an anesthetic cocktail of ketamine and xylazine (ketamine 80 mg/kg and xylazine 12 mg/kg using 26 G needle) intraperitoneally (i.p.) and then euthanized via cervical dislocation.

2.3. Preparation of the cytosolic antigen

C. albicans ATCC 1808 strain was kindly made available by the Jawaharlal Nehru Medical College, AMU, Aligarh. The culture was maintained on YPD Agar. For isolation of cytosolic proteins, cells were collected and washed with normal saline, and mechanically disrupted with mortar and pestle in lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl and 1 mM EDTA) in the presence of protease inhibitor cocktail (Sigma). This was followed by probe sonication (20 s on and 5 s off pulses for 2 min) in the presence of same set of protease inhibitor cocktail to restrict proteolysis. The homogenate was centrifuged at 8720 × *g* for 15 min at 4 °C and the supernatant collected. The lysate was partially purified by ammonium sulphate precipitation. The 0–60% ammonium sulphate fractional precipitate was discarded and the supernatant collected was further subjected to 80% ammonium sulphate precipitation. The pellet was collected by centrifugation, dissolved in PBS and dialyzed extensively against the same buffer. The fraction precipitated between 60–80% ammonium sulphate contained proteins mainly in the range of 25–95 kDa, and was used as the antigen (Cp) in this study.

2.4. Preparation of PLGA microsphere-based Cp antigen

PLGA microspheres were prepared using water in-oil-in water (w/o/w) emulsion by the solvent evaporation technique [20]. Briefly, Cp (15 mg dry weight dissolved in 0.5 ml PBS) was primarily emulsified with PLGA solution (190 mg PLGA dissolved in 1.0 ml of DCM) using a bath type Sonicator. The primary emulsion was further mixed with 10% (w/v) PVA (100 ml) and homogenized using the Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA). The resulting (o/w) emulsion was stirred at 25 °C for 18 h to allow solvent evaporation and microsphere formation. Microspheres thus formed were collected by centrifugation at 10,000 × *g* for 10 min, washed with 20 mM PBS, lyophilized and stored at 4 °C till further use. The SEM of the microspheres was performed, courtesy Electron Microscope Facility, Jawaharlal Nehru University, New Delhi. The PLGA Particle size was measured by Zeta-sizer Nano ZS as well using DTS software (Malvern Instrument Limited, UK). The Zeta average diameter of the Cp loaded microspheres was found to be in the range of 750 ± 150 nm, while zeta-potential was −30 ± 0.45 mV, respectively (Fig. 1).

2.5. Entrapment efficiency

Quantitative estimation of the protein entrapped in the PLGA microsphere was carried out as follows. Briefly, known volumes of sham PLGA microspheres (empty microspheres) and those containing Cp were dissolved in 0.1 N NaOH solution. The released protein was estimated by BCA procedure [21]. The entrapment efficiency of Cp in PLGA microsphere was 30–35%.

2.6. Preparation of fibrin beads

The procedure for the preparation of fibrin beads as described by Ahmad et al. [22] was followed. Briefly, 250 μl of plasma was mixed with 50 μl of Cp (corresponding to 500 μg of protein; present as either free antigen or antigen entrapped in PLGA microsphere) and adequate 0.35 M $CaCl_2$ added to obtain final 40 mM calcium concentration. Aliquots (3.0 μl) of the mixture were transferred with the help of a micropipette as droplets at regular distance over a glass slide covered with Parafilm. The petri dishes were incubated at