Vaccine 32 (2014) 918–923

Contents lists available at ScienceDirect

Vaccine

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

Immunotherapeutics to prevent the replication of *Brucella* in a treatment failure mouse model

N. Jain-Gupta[a], A. Contreras-Rodriguez[b], G.P. Smith[a], V.K. Garg[c], S.G. Witonsky[d], S. Isloor[e], R. Vemulapalli[c], S.M. Boyle[a], N. Sriranganathan[a,*]

[a] *Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061-0342, United States*
[b] *Departmento de Microbiologia, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico*
[c] *Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, United States*
[d] *Department of Large Animal Clinical Sciences, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061-0342, United States*
[e] *Department of Veterinary Microbiology, Veterinary College, Karnataka Veterinary, Animal Sciences and Fisheries University, Hebbal, Bangalore 560024, India*

ARTICLE INFO

Article history:
Received 9 October 2013
Received in revised form
12 December 2013
Accepted 18 December 2013
Available online 4 January 2014

Keywords:
Brucellosis
Treatment failure
Immunotherapy
Therapeutic vaccine
Outer membrane vesicles
Cell mediated immunity and humoral immunity

ABSTRACT

Outer membrane vesicles (OMVs) from *Brucella melitensis* and irradiated *Brucella neotomae* have been shown to be effective vaccines against a *B. melitensis* challenge in a mouse model. The present study evaluates the efficacy of these two vaccines as immuno-therapeutics in combination with conventional antibiotics against a *B. melitensis* infection. BALB/c mice chronically infected with *B. melitensis* were treated for 4 weeks with doxycycline and gentamicin and vaccinated twice during the course of therapy. Antibiotics in sub-therapeutic concentrations were chosen in such a way that the treatment would result in a therapeutic failure in mice. Although no additive effect of vaccines and antibiotics was seen on the clearance of *B. melitensis*, mice receiving vaccines along with antibiotics exhibited no *Brucella* replication post-treatment compared to mice treated only with antibiotics. Administration of irradiated *B. neotomae* along with antibiotics led to higher production of IFN-γ *ex vivo* by splenocytes upon stimulation with heat inactivated *B. melitensis* while no such effect was seen by splenocytes from mice vaccinated with OMVs. OMV vaccinated mice developed significantly higher anti-*Brucella* IgG antibody titers at the end of the treatment compared to the mice that received only antibiotics. The mice that received only vaccines did not show any significant clearance of *Brucella* from spleens and livers compared to non-treated control mice. This study suggests that incorporating OMVs or irradiated *B. neotomae* along with conventional antibiotics might be able to improve therapeutic efficacy and control the progression of disease in treatment failure cases.

1. Introduction

Brucellosis is the most common bacterial zoonotic disease worldwide [1]. Treatment of animal brucellosis is economically not feasible and infected animals and their products remain the source of infection for humans [2]. The World Health Organization's recommendation for treatment in humans involves combination antibiotic therapy for 6–8 weeks [3]. Even after prolonged treatment, the rate of treatment failure is 1–5% and the relapse rate remains as high as 5–10% in humans [2]. In the case of humans, treatment failure is defined by the persistence of signs and symptoms at the end of scheduled therapy [4]. Relapse is defined as the reappearance of signs and symptoms or positive blood cultures within one year of treatment. In the case of experimental murine brucellosis, treatment failure is characterized by the presence of *Brucella* in targeted organs like spleen, liver and lung at the end of the therapy. There is no relapse model following treatment of *Brucella* infection in mice. The reasons for such high treatment failure in humans are not completely understood. Patient non-compliance due to the long duration of therapy can be the major cause for treatment failure; thus, shorter therapy duration is desirable. In the case of relapses, isolated *Brucella* usually are sensitive to the same antibiotic treatment, indicating lack of development of drug resistance [5]. Thus, there is a need for an alternative therapeutic option other than conventional antibiotics. This should help to

* Corresponding author at: Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Disease (CMMID), Virginia Polytechnic Institute and State University, 1410 Prices Fork Road, CMMID, Blacksburg, VA 24061-0342, United States. Tel.: +1 540 231 7171; fax: +1 540 231 3426.
E-mail address: nathans@vt.edu (N. Sriranganathan).

0264-410X/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.12.058

reduce the duration of therapy and minimize side effects, as well as prevent therapeutic failures and relapses. Activation of IFN-γ producing CD4+ Th1 immune is necessary for the control of *Brucella* infection [6]. However, during chronic infection in humans the immune system shifts from Th1 to Th2 type and thus cannot eliminate infected cells or intracellular *Brucella* [7,8]. *Brucella* infected BALB/c mice show a severe disruption of spleen morphology and depletion in CD4+ and CD8+ lymphocytes that are required to eliminate the infected cells [9]. In both situations there is a clear case for immunotherapy in the treatment of chronic brucellosis that would stimulate the immune system against *Brucella*. The choice of such immuno-stimulants is critical in that they should not create non-specific immune stimulation and should be well tolerated by the infected host. It has been shown previously that DNA vaccines for *Mycobacterium tuberculosis* when used in combination with conventional antibiotics switched the immune response in chronically infected mice from Th2 to Th1 and enhanced clearance of pathogen and prevented relapse [10,11]. Use of immuno-stimulants, like levamisole and IFN-α 2a for the treatment of human brucellosis have been reported but with mixed results [12,13]. Most of the approved vaccines to control *Brucella* infections in animals are live, attenuated strains and cannot be used in infected hosts because of safety concerns. Moreover, it has been found that live, attenuated *B. abortus* strain RB51 given to *B. abortus*-infected mice cleared faster compared to its clearance from non-infected mice and failed to provide clearance of *B. abortus* [14]. There is a need for a non-infectious vaccine candidate that can provide a specific immuno-stimulation to treat *Brucella* infections. Therefore, we tested the potential of outer membrane vesicles (OMVs) obtained from *B. melitensis* and irradiated *B. neotomae* as immuno-stimulants to treat *B. melitensis* infection in mice. OMVs are double membrane structures that are naturally released by Gram-negative bacteria [15]. OMVs are mainly comprised of the outer membrane proteins, periplasmic proteins and lipopolysaccharide (LPS) [16]. We have shown previously that OMVs provide protection against *B. melitensis* challenge in mice [17] and that protection can be further enhanced by adding pluronic P85 to OMVs [18]. Pluronics or polaxamers are non-ionic block copolymers of poly(propylene oxide) (PPO) and poly(eythylene oxide) (PEO) [19] and have been shown to activate pro-inflammatory signaling pathways and immune responses. *B. neotomae* was first isolated in 1957 from desert wood rats in the western United States [20]. It is not known to cause disease in humans or animals. Recently, we have shown that gamma-irradiated *B. neotomae* (IBN) protects against three virulent *Brucella* species in a mouse model [21]. Both these vaccine candidates are considered safer than live-attenuated vaccines that have the risk of reverting back to wild type. In the present work our goal was to determine if treatment with *Brucella* OMVs or irradiated *B. neotomae* could aid in clearing *B. melitensis* from chronically infected mice or could prevent replication of *Brucella* and progression of disease in case of treatment failures.

2. Materials and methods

2.1. Ethics statement

All the mice experiments were done in our AAALAC approved facility and the mice experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) (protocol # CVM-10-048) at Virginia Tech, which follows protocols approved by the American Veterinary Medical Association (AVMA). For retro-orbital bleeding, mice were anaesthetized under isofluorane using Vet Equip Mobile Laboratory Animal Anesthesia System. Mice were euthanized using an overdose of carbon dioxide followed by cervical dislocation.

2.2. Preparation and characterization of vaccines

OMVs were obtained from smooth strain *B. melitensis* 16M and rough mutant *B. melitensis* VTRM1 as previously described [17]. *B. neotomae* was grown in trypic soy broth (TSB) to mid-log phase and harvested by centrifugation and aliquots containing 5×10^{11} colony forming units (CFUs)/mL were stored at −80 °C until use. Two to three weeks before immunization, aliquots of the vaccines were exposed to 350 krads of gamma irradiation using a ^{60}Co source irradiator (Gammacell 220 Irradiator). The inability of the irradiated bacteria to replicate was confirmed by plating on tryptic soy agar (TSA) and incubating for at least 7 days. The irradiated bacteria were stored at 4 °C until used for immunization. Metabolic activity of irradiated *B. neotomae* (IBN) was assessed using Alamar blue as previously described [21].

2.3. Mouse infection and treatment

Approximately 6–8 weeks old female BALB/c mice were infected intraperitoneally (i.p.) with *B. melitensis* 16M (5×10^4 CFUs/mouse) (Fig. 1). Seven weeks post-infection, the mice ($n = 18$) were treated daily for 4 weeks, once daily with antibiotics. Doxycycline and gentamicin (DG) were given at the dose of 3 mg/kg body weight and 5 mg/kg body weight respectively once daily by i.p. injection. A total of 100 μL of PBS containing 5 μg (protein) of OMVs and 0.03% Pluronic P85 (as an adjuvant, kindly provided by BASF, NJ, USA) was administered intramuscularly (i.m.). Approximately, 5×10^8 CFUs equivalent of irradiated *B. neotomae*/mouse was administered i.p. Negative control group of mice were treated with saline administered i.p. Groups of mice ($n = 5$) were euthanized either (1) after 2 weeks of treatment, (2) after 4 weeks of treatment or (3) 4 weeks after the end of the treatment. Spleens and livers were collected and homogenized and serial dilutions were made in TSB. The diluted suspensions were spread on TSA plates and *Brucella* CFUs were determined after incubating the plates at 37 °C for 3–4 days. At the end of the treatment three mice per group were euthanized to perform splenocyte proliferation and cytokine production assays as described below.

2.4. Splenocyte culture and cytokine determination

At the end of the treatment splenocytes from treated mice (3 mice per group) were obtained as previously described [22]. Approximately, 5×10^5 splenocytes/well were seeded in flat bottom 96-well plates and stimulated with heat inactivated *B. melitensis* 16M (MOI of 1:1, 1:10 and 1:100). Splenocytes were stimulated with medium alone as negative control while Concavalin A (1 μg/mL) was used as positive control. After incubating cells for 5 days at 37 °C, supernatants were collected and the concentration of IFN-γ was determined using cytokine ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

2.5. Antibody titers

An indirect ELISA was performed to measure the levels of *B. melitensis* specific antibodies in the plasma of the mice ($n = 5$). Briefly, heat inactivated *B. melitensis* 16M (stock culture was heated at 80 °C for 1 h, inactivation was confirmed by sterility of the culture after 48 h) at the protein concentration of 1.0 μg/mL was adsorbed to wells of polystyrene plates (Nunc Maxisorp) in 50 μL of bicarbonate buffer (pH 9.6). After incubating overnight at 4 °C, plates were washed 4 times with phosphate buffer saline (PBS) containing 0.05% Tween-20. Then the wells were blocked with 2% bovine serum albumin in PBS and incubated for 2 h at room temperature; the plates were washed 4 times as before. A series of dilutions of plasma samples were prepared and added in duplicate to the wells