Vaccine 27 (2009) 2138–2143

Contents lists available at ScienceDirect

Vaccine

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

Poly(lactide-co-glycolide) microspheres: A potent oral delivery system to elicit systemic immune response against inactivated rabies virus

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ARTICLE INFO

Article history:
Received 1 October 2008
Received in revised form 23 January 2009
Accepted 29 January 2009
Available online 6 February 2009

Keywords:
Poly(lactide-co-glycolide (PLG)
Rabies
Oral immunization

ABSTRACT

Rabies is an endemic, fatal zoonotic disease in the developing countries. Oral vaccination strategies are suitable for rabies control in developing countries. Studies were performed to investigate the suitability of poly(lactide-co-glycolide) (PLG) microspheres as an oral delivery system for β-propiolactone inactivated concentrated rabies virus (CRV). Immune responses induced by encapsulated (PLG + CRV) and un-encapsulated inactivated rabies virus after oral and intraperitoneal route administrations were compared. The anti-rabies virus IgG antibody titer, virus neutralizing antibody (VNA) titers obtained by mouse neutralization test (MNT) and IgG2a and IgG1 titers of mice group immunized orally with PLG + CRV showed significantly ($p < 0.001$) higher response than the group immunized orally with un-encapsulated CRV. There was no significant difference ($p > 0.05$) between groups inoculated by intraperitoneal route. The stimulation index (SI) obtained by lymphoproliferation assay of PLG + CRV oral group also showed significantly ($p < 0.001$) higher response than the group immunized orally with un-encapsulated CRV, suggesting that oral immunization activates Th1-mediated cellular immunity. Immunized mice of all experimental groups were challenged intracerebrally with a lethal dose of virulent rabies virus Challenge Virus Standard (CVS). The survival rates of mice immunized orally with PLG + CRV and CRV alone were 75% and 50%, respectively, whereas intraperitoneally immunized groups showed 100% protection. The overall results of humoral, cellular immune response and survival rates of mice immunized orally with PLG + CRV were significantly ($p < 0.001$) higher than those of mice immunized orally with CRV alone. These data suggest that the PLG encapsulated inactivated rabies virus can be used for oral immunization against rabies.

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1. Introduction

Rabies, a fatal neuroencephalomyelitis is transmitted through the bite of rabid animals. Human mortality from endemic canine rabies was estimated to be 55,000 deaths per year in Asia and Africa [1]. Although vaccination of domestic and wild animals and removal of stray dogs have been effective in rabies control in developed countries, similar approaches have been difficult to implement in developing countries, primarily because vaccination of animals and humans exposed at risk of rabies is expensive. In addition wild animals which constitute reservoirs for the propagation of the virus are not readily accessible for vaccination and hence an alternative approach to limit the spread of the disease is warranted [2]. Oral immunization which has been successful in control of rabies in wild animals uses live attenuated vectors or attenuated vaccines [3,4]. These technologies may not be readily available for vaccine manufactures in developing countries. However inactivated virus vaccines are of wide use in developing countries. Hence vaccine formulations using inactivated virus are attractive options for alternative (oral/mucosal) immunization strategies. To be viable, oral vaccination may be improved by using adjuvants or gastrointestinal protectants to maintain antigen integrity [5]. A suitable delivery system that minimizes the antigen dose, boosters and offers protection from the stomach acids is needed to control rabies using oral vaccination. Among a variety of inert and biodegradable polymers for controlled release studied so far, poly(lactide-co-glycolide) (PLG) particles made from lactic and glycolic acids holds promise for delivery of variety of antigens to the immune system [6]. PLG microspheres have been used in the delivery of a variety of antigens because of their stability, non-toxicity [7] and ability to induce strong immune response in various animal models [8–12].

This study was under taken to investigate the protective efficacy of orally delivered β-propiolactone (BPL) inactivated rabies

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0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.01.129

virus (RV) encapsulated in PLG microspheres in mice. The immune responses induced by oral vaccination were compared with intraperitoneal route of immunization.

2. Materials and methods

2.1. Cells and viruses

BHK-21 cell line (baby hamster kidney cell line, ATCC, USA) was cultured at 37 °C and 5% CO_2 in a humidified incubator using Dulbecco's modified Minimum Essential Medium (DMEM, GIBCO, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, GIBCO, USA) and antibiotics (100 IU of penicillin/ml and 100 μg of streptomycin/ml).

Rabies virus (PV-11 strain, Institut Pasteur, France) was propagated using BHK-21 C13 cells grown in 150 cm^2 tissue culture flasks [13]. Challenge Virus Standard (CVS) mouse brain strain of rabies virus (Institut Pasteur, France) was used for mouse protection test to determine the vaccine potency and mouse neutralization test (MNT) to determine the virus neutralizing antibody (VNA) titers.

2.2. Preparation of concentrated BPL-inactivated rabies virus (CRV)

The rabies virus harvests obtained from infected BHK-21 cells grown in tissue culture flasks was inactivated with BPL (1:4000) and the degree of inactivation was determined by mouse inoculating test [14]. The concentrated, inactivated rabies virus was prepared as described elsewhere [15,16]. The pellet was resuspended in small volume of STE buffer (0.15 M NaCl, 0.01 M Tris and 0.001 M EDTA, pH 7.5) and protein was estimated using bicinchoninic acid (BCA) protein assay kit (Bangalore Genei, India). The purified rabies viral proteins were visualized on 10% SDS-PAGE after staining with Coomassie brilliant blue stain. For the detection of rabies viral proteins the concentrated rabies virus was resolved over10% SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). The blot was probed with rabbit anti-rabies hyperimmune serum (1:500). Binding of specific antibodies to CRV was detected using anti-rabbit IgG-HRPO conjugate (1:5000, Bangalore Genei, India).

2.3. Preparation and characterization of PLG microspheres

PLG microsphere (Sigma, USA) containing purified CRV was prepared as described by Rosas et al. [17]. Briefly 6% (W/V) of PLG (50:50) was dissolved in 5 ml of dichloromethane (Merck, India) and emulsified with 5 mg of purified rabies virus antigen by high-speed homogenization for 2–4 min. The primary emulsion (W/O) was mixed with 25 ml of 1 mM HEPES buffer, pH 7.5 containing 8% polyvinyl alcohol (MW 30,000–70,000; Sigma, USA) and emulsified by high-speed homogenization for 5 min in order to form double emulsion (W/O/W). Finally, 50 ml of 2% isopropanol solution was added and stirred for 1 h. The microspheres were collected by centrifugation at 10,000 × *g* for 15 min and washed thrice with distilled water and stored at −20 °C after freeze drying. PLG microspheres treated similarly but without antigen were used as control in animal immunization experiments.

The size and surface morphology of prepared microspheres were visualized by scanning electron microscopy (SEM). Briefly, a small pinch of the lyophilized microspheres were coated on the metallic slub by ion sputtering or gold coating and then observed under different magnifications in a scanning electron microscope (JEOL JSM 840X, Japan) and the images captured. Viral antigen load in the microspheres was determined by measuring the protein content using BCA method after known quantity (20 mg) of microspheres were hydrolyzed by using 3 ml of 5% (W/V) SDS in 0.1 M NaOH solution and stirring overnight at room temperature. The mixture was centrifuged and the protein content of supernatant (hydrolysate) was determined. Antigenicity of the CRV released from microspheres was determined by Dot-ELISA using rabies virus specific rabbit polyclonal serum and compared with results obtained using pre-encapsulated CRV [18].

Table 1
Experimental groups of mice used in this study. The mice were inoculated with appropriate vaccine as indicated.

Sl. no.	Groups	No. of mice	Route	Antigen dose/mice (μg)
1	CRV	15	Oral	50
2	PLG + CRV	15	Oral	50
3	PLG[a]	15	Oral	–
4	CRV	15	IP	50
5	PLG + CRV	15	IP	50
6	PLG[a]	15	IP	–
7	Naive	15	–	–

IP: intraperitoneal; PLG: poly(lactide-co-glycolide); μg: microgram.
[a] Control groups inoculated with equivalent quantity of empty microspheres.

2.4. Immunization and sample collection

Swiss albino mice (3–4 weeks old; $n = 105$) were used in this study. The animals were maintained and used according to the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experiment groups are shown in Table 1. Mice were randomly assigned to each experimental group. Mice were inoculated with respective antigen in adequate quantity of microspheres suspended in PBS. Blood samples were obtained on day 0, 7, 14, 21, 28, 35 and 42 post-immunization. Splenocytes from vaccinated and control Swiss albino mice were isolated after lysis of erythrocytes using RBC lysis buffer (Sigma, USA). The viability of splenocytes was >95% as determined by trypan blue dye exclusion method.

2.5. ELISA and determination of rabies virus specific Ig titers

Serum IgG, IgG1 and IgG2a antibody responses were determined by ELISA. Ninety-six-well Maxisorp immunoplates (Nunc, Denmark) were coated with appropriately diluted CRV (100 μl) in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After removal of unbound antigens, the un-reacted sites were blocked using 200 μl of 3% BSA by incubation at 37 °C for 1 h. The test sera (100 μl) and control sera were subjected to serial two-fold dilutions with phosphate-buffered saline–Tween 20 (PBS containing 0.05% Tween 20, V/V; PBST) as a diluent and incubated at 37 °C for 1 h. After washing thrice with PBST, 100 μl/well of appropriately diluted HRP-conjugated IgG raised against mouse IgG, IgG1and IgG2a (Bangalore Genei, India) were added to the plates and incubated at 37 °C for 1 h. After removal of unbound conjugate, positive binding was detected by adding substrate (OPD/H_2O_2) and incubation for 15 min at 37 °C in dark. The enzyme–substrate reaction was stopped by adding 100 μl/well of 1.25 M sulphuric acid and absorbance read at 492 nm. Rabies virus specific antibody titers were expressed as the reciprocal of the highest serum dilution that showed an OD_{492} value above the cut-off value, which was defined as the average OD_{492} value of seven non-immunized sera ± 3 standard deviations.

2.6. Mouse neutralization test

MNT was carried out essentially as described [19]. Briefly, the heat inactivated sera samples were serially diluted and incubated with 50 LD_{50} of CVS at 37 °C for 90 min. The presence of un-neutralized virus in the mixture was checked by intracerebral inoculation of mice ($n = 10$) for each serum dilution. Control groups

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