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The feasibility of rabies virus-vectored immunocontraception in a mouse model

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ABSTRACT

Immunocontraceptive vaccines may be an alternative to surgical sterilization. Dual rabies vaccination and dog population management is a helpful tool for rabies prevention. A synthetic gonadotropin-releasing hormone (GnRH) peptide coupled to a carrier protein or T cell epitope is efficacious in inducing immunocontraception in a variety of mammals. However, virus-vectored GnRH recombinant vaccines have advantages over the conjugation method. In a previous in vitro study, we were able to insert a GnRH-coding sequence into the rabies virus (RABV) glycoprotein (G) gene, and the recombinant viruses grew to high titers in cells. Here, we further focused on the RABV G in accepting various copy numbers of GnRH. We demonstrated although RABV G protein with up to 4 copies of GnRH was well expressed, the recombinant virus was recovered only when 2 copies of GnRH (20 amino acids) were incorporated into the G, indicating a possible insertion limit in making a full infectious clone. The investigation provides insight into the utility of RABV G as a carrier for small peptides and its suitability for vaccine studies. Following our previous study, we selected ERAg3p/2GnRH and tested the construct in mice. The vaccine induced ⩾80% infertility after three doses without any adjuvant, in live (8 of 10 mice infertility) or inactivated (13 of 14 mice infertility) formulations; while the pregnancy rate was 100% (10 of 10 mice) in the controls. This initial success of immunocontraception in mice is promising, and we are now optimizing the vaccine formulation by using adjuvants and exploring novel delivery methods to minimize the dosage.

Introduction

Global demonstration projects of canine rabies elimination require new tools [1]. Historical prevention of canine rabies relies on vaccination and control of dog populations [2,3]. However, resources are insufficient for surgical sterilization in both developed and developing countries. New non-surgical methods, such as immunocontraception, may have added benefit in the future [4,5]. A vaccine that simultaneously protects against rabies and reduces fertility in both male and female dogs would be a major advance in rabies prevention. Synthetic gonadotropin-releasing hormone (GnRH) peptides coupled to a carrier protein or T cell epitopes is efficacious in inducing immunocontraception [6–9]. However, virus-vectored GnRH recombinants as self-replicative in cell culture have the advantage over the conjugation method in vaccine manufacturing. Here, we demonstrated the feasibility of rabies virus (RABV)-vectored immunocontraception in a mouse model. RABV is a compact negative-sense, single-stranded RNA virus. Rabies vaccine is one of the oldest biologics used in humans for both pre- and post-exposure prophylaxis [10]. Human rabies vaccination does not require adjuvant priming, and long-lasting immune responses are documented [11–13]. More than a century of vaccination practices and the safety record of rabies vaccines have led to the current investigations into using RABV as a vector for recombinant antigens against other infectious diseases, such as Ebola, AIDS, SARS and anthrax [14–17]. A common strategy for RABV engineering is to incorporate a gene of interest into an extra transcriptional unit created in the RABV genome. For a small peptide like GnRH, the traditional strategy may not be successful, because GnRH is a small self-antigen. Our previous in vitro study demonstrated we were able to insert GnRH coding sequence into selected locations in the RABV G gene [18]. Here, we further focused on the RABV G gene in accepting the copy number of GnRH, and demonstrated although RABV G recombinant protein with up to 4 copies of GnRH was well expressed, the recombinant virus was recovered only

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when 2 copies of GnRH (20 amino acids) were incorporated into the G protein, indicating a possible insertion cap in making a full infectious clone. Our investigation provides insight into the RABV G as a carrier for small peptide vaccine studies. Following our previous study, we selected ERAg3p/2GnRH and tested the construct in mice. The vaccine induced ⩾80% infertility after three doses without any adjuvant, in live (8 of 10 mice infertility) or inactivated (13 of 14 mice infertility) formulations; while the pregnancy rate was 100% (10 of 10 mice) in the controls. This initial success of immunocontraception in mice is promising, and we are now optimizing the vaccine formulation by using adjuvants and exploring novel delivery methods to reduce the immunization dosage.

Materials and methods

RABV G gene and mutagenesis

The RABV ERA G gene was cloned into the pcDNA3.1/Zeo (−) plasmid (Invitrogen, Carlsbad, CA), creating pcDNA-G. The GnRH coding sequence was introduced into the G gene as described [19,20]. To evaluate the G gene in accepting multiple copies of GnRH, the GnRH coding sequences were increased gradually from 2 to 20 copies, resulting in constructs of pcDNAG-2GnRH (G gene with 2 inserted GnRH copies), pcDNAG-3GnRH, pcDNAG-4GnRH, pcDNAG-5GnRH, pcDNAG-6GnRH, pcDNAG-7GnRH, pcDNAG-8GnRH, pcDNAG-9GnRH, pcDNAG-10GnRH, pcDNAG-13GnRH and pcDNAG-20GnRH.

Plasmid transfection, RABV G and GnRH expression

The BSR cells (a clone of baby hamster kidney cell line) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). The day before transfection, the cells were split at 50,000 cells/ml, and 2 ml of cell suspensions were seeded into each well of a 6-well-plate (Thermo Fisher Scientific, Waltham, MA). Twenty-four hours after incubation, each well of BSR cells at ~50% confluency were transfected with the above 11 individual pcDNAG-GnRH constructs at 1 μg/well, using the TransIT-LT1 reagent, according to the manufacturer's instructions (Mirusbio, Madison, WI). Forty-eight hours post-transfection, the cells were fixed using 10% PBS-buffered formalin SF100-4 (Thermo Fisher Scientific, Waltham, MA), and reacted with a panel of mouse anti-RABV G monoclonal antibodies (CDC, Atlanta, GA) at 37 °C for 30 min. The cells were washed 3 times in PBS buffer (pH 7.2, 0.01 M), and stained with an anti-mouse IgG AB-FITC conjugate (Millipore Light Diagnostics™, Temecula, CA) at 37 °C for 30 min. After 3 intensive washes in PBS (pH 7.2, 0.01 M), the cells were dried at room temperature and visualized under fluorescent microscopy. Similarly, the GnRH expression was analyzed by reacting with the rabbit anti-GnRH antibodies at a 1:200 dilution (USDA Wildlife Services). The conjugate goat anti-rabbit IgG (H + L)-FITC was used at a 1:200 dilution (Vector Laboratories Inc., Burlingame, CA).

Virus recovery, growth dynamics, purification of virus and recombinant G-2GnRH protein

After RABV G and GnRH expression were verified in plasmid-transfected cells, the hybrid genes G-2GnRH, G-3GnRH, and G-4GnRH from the pcDNA plasmids were cloned, respectively, back into the RABV ERA genome. An established protocol was followed for virus recovery [20]. We measured the growth dynamics between ERAg3p and ERAg3p/2GnRH viruses [18]. Similar to Method 2.2 above, 48 h after virus infection, the G and GnRH expression was analyzed. We purified the RABV ERAg3p virus, the G protein from ERAg3p, the ERAg3p/2GnRH virus, and the G-2GnRH protein from ERAg3p/2GnRH virus, as described [21,22].

Co-expression of G and GnRH on the virus surface

Forty-eight hours after ERAg3p/2GnRH virus infection, BSR cells were fixed using 10% buffered formalin phosphate SF100-4 (Thermo Fisher Scientific, Waltham, MA). The cells were blocked using 10 mM glycine PBS (pH 7.2, 0,01 M) for 1 h, reacted with a mixture of mouse anti-G mAb 507-1 at 1:200 dilution (CDC, Atlanta, GA) and rabbit anti-GnRH antibodies at 1:200 (USDA Wildlife Services), followed by simultaneous staining with a mixture of FITC-labeled goat anti-mouse and Rhodamine-labeled goat anti-rabbit secondary antibodies at a 1:200 dilution (KPL Inc., Gaithersburg, MD). The RABV G and GnRH staining in virus-infected cells were viewed under immunofluorescence microscopy.

SDS–PAGE and western blotting

The purified RABV ERAg3p, ERAg3p/2GnRH virions, G and G-2GnRH proteins were subject to SDS–PAGE, followed by western-blotting. In brief, 10 μl of the materials were loaded to gels, and were transferred to a PVDF membrane (Sigma–Aldrich, St. Louis, MO) after SDS–PAGE was completed. The membrane was blocked in 5% nonfat milk (PBS, pH 7.2, 0.01 M) overnight at 4 °C. For detection of RABV G protein, a linear epitope mAb 63-80-6 (CDC, Atlanta, GA) against the G was added to the membrane. After 30 min incubation at room temperature, the membrane was washed 3 times in PBS-T (pH 7.2, 0.01 M, plus 0.05% Tween-20). The anti-mouse IgG-HRP conjugate at 1:1000 was added (Sigma–Aldrich, St. Louis, MO), and the bands were visualized in tetramethylbenzidine substrate solution (USB, Cleveland, OH). For detection of GnRH, a similar procedure was followed by using rabbit anti-GnRH antibodies at a 1:500 dilution (USDA Wildlife Services) and anti-rabbit IgG-HRP conjugate at a 1:1000 dilution (Sigma–Aldrich, St. Louis, MO).

Mouse vaccination using the ERAg3p/2GnRH virus

The animal protocol #2132FRAMOUC-A3 was established in compliance with CDC Institutional Animal Care and Use Committee. Two month-old CBA/CaJ mice (Charles River Laboratory, Wilmington, MA) were divided into 4 groups and immunized intramuscularly (i.m.) in the quadriceps muscle: group A, control mice: the placebo injection was 50 μl of 10^7 focus forming units (ffu) of ERAg3p virus (10 cages of paired female and male mice, with each cage having 1 male and 1 female); group B, 1 dose of 50 μl at 10^7 ffu of live ERAg3p/2GnRH virus for both sexes (15 cages of paired female and male mice, with each cage having 1 male and 1 female); group C, 3 doses of live ERAg3p/2GnRH vaccine for one sex (10 cages: 5 pairs with 1 vaccinated female and 1 unvaccinated male, and 5 pairs with 1 unvaccinated female and 1 vaccinated male; one pair per cage); group D, 3 doses of inactivated ERAg3p/2GnRH recombinant vaccine (gamma irradiated, CDC, Atlanta, GA) for both sexes (14 cages of paired female and male mice, with each cage having 1 male and 1 female). In the 3-dose groups, the animals received 2 additional boosts at 3-week intervals. Two weeks after immunization or the last boost, mice were matched for breeding. Mice were observed twice daily. Observation was terminated 1 year after the breeding test or when mice became pregnant. At termination, mice were euthanized according to the protocol #2132FRAMOUC-A3.