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Humoral immune responses against gonadotropin releasing hormone elicited by immunization with phage-peptide constructs obtained via phage display

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ABSTRACT

Phage display is based on genetic engineering of phage coat proteins resulting in fusion peptides displayed on the surface of phage particles. The technology is widely used for generation of phages with novel characteristics for numerous applications in biomedicine and far beyond. The focus of this study was on development of phage-peptide constructs that stimulate production of antibodies against gonadotropin releasing hormone (GnRH). Phage-peptide constructs that elicit production of neutralizing GnRH antibodies can be used for anti-fertility and anti-cancer applications. Phage-GnRH constructs were generated via selection from a phage display library using several types of GnRH antibodies as selection targets. Such phage constructs were characterized for sequence similarities to GnRH peptide and frequency of their occurrence in the selection rounds. Five of the constructs with suitable characteristics were tested in mice as a single dose 5×10^{11} virions (vir) vaccine and were found to be able to stimulate production of GnRH-specific antibodies, but not to suppress testosterone (indirect indicator of GnRH antibody neutralizing properties). Next, one of the constructs was tested at a higher dose of 2×10^{12} vir per mouse in combination with a poly(lactide-*co*-glycolide) (PLGA)-based adjuvant. This resulted in multifold increase in GnRH antibody production and significant reduction of serum testosterone, indicating that antibodies produced in response to the phage-GnRH immunization possess neutralizing properties. To achieve optimal immune responses for desired applications, phage-GnRH constructs can be modified with respect to flanking sequences of GnRH-like peptides displayed on phage. Anticipated therapeutic effects also might be attained using optimized phage doses, a combination of several constructs in a single treatment, or application of adjuvants and advanced phage delivery systems.

1. Introduction

Gonadotropin releasing hormone (GnRH) is a ten amino acid long peptide, which acts as a master reproductive hormone via regulation of the release of major gonadotrophic hormones. The sequence of GnRH peptide is identical in the vast majority of mammalian species, except guinea pig (Jimenez-Liñan et al., 1997). GnRH-based antigenic preparations can stimulate production of neutralizing GnRH antibodies that inactivate endogenous GnRH. This leads to the reduced release of gonadotropic hormones and subsequent suppression of gonadal function, including related production of sex hormones. Accordingly, GnRH-based preparations with GnRH neutralizing properties hold great potential in appli-

Abbreviations: cfu, colony forming unit; FSHR, follicle-stimulating hormone receptor; GnRH, gonadotropin releasing hormone; GnRHR, gonadotropin releasing hormone receptor; PLGA, poly(lactide-*co*-glycolide); PEG, polyethylene glycol; vir, virion.

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cations where suppression of gonadal hormones is required. For example, this can be a tool for fertility control and prevention of undesired sexual behavior in feral and wild animals (Jung et al., 2005; Levy et al., 2004; Miller et al., 2008). GnRH immunization is also proposed as a way to improve growth performance and meat quality in cattle and to prevent the occurrence of boar taint in male pigs (Bonneau and Enright, 1995; Dunshea et al., 2001; Thompson, 2000). In humans, GnRH-based vaccines are regarded as effective candidates for treatment of prostate and other hormone-sensitive reproductive cancers (Jinshu et al., 2005; Simms et al., 2000; Wang et al., 2010).

Generation of effective vaccines based on GnRH is challenging for two major reasons: (1) GnRH is a small decapeptide with very low immunogenicity, and (2) GnRH is naturally present in the body and, therefore, is recognized by the immune system as a "self" protein with no to very low antibody response when administered to an animal. To increase immunogenicity, various strategies have been employed, including coupling of GnRH to carrier proteins such as heat shock protein 65 (Xu et al., 2008) and keyhole limpet hemocyanin (Miller et al., 2008) and to strong T helper cell epitopes (Jung et al., 2005; Zeng et al., 2005), the use of effective adjuvants (Ferro et al., 2004) and advanced nanoparticle delivery systems (Gebril et al., 2014). However, while these efforts resulted in good progress in the field, low GnRH immunogenicity remains a persistent difficulty. In addition, production of recombinant fusion proteins or synthetic peptides encapsulated in nanoparticles is costly and their shelf lifetimes are limited.

Bacteriophages (phages) are bacterial viruses. They do not infect mammalian cells and therefore are not harmful for animals. Filamentous phages fd (Ff class) used in this study are particles of about 1 μm long and 7 nm in diameter. They are composed of a protein coat that encloses genetic material of the virion—a single-stranded circular DNA. Phage coat proteins can be re-engineered using standard recombinant DNA techniques, thus creating phage particles with unique surface architecture and novel properties. Such particles can be utilized as carriers for delivery of peptides fused to the phage coat proteins with desired immunogenic characteristics to serve as vaccines. Experimental phage-based vaccines were proposed for use in diverse biomedical applications (reviewed in Clark and March, 2006; Manoutcharian, 2011; Prisco and De Berardinis, 2012); among them are treatment of melanoma (Eriksson et al., 2009), HIV (De Berardinis et al., 2003), Alzheimer's disease (Frenkel et al., 2003), candidiasis (Wang et al., 2006), rabies (Houimel and Dellagi, 2009), fasciolosis (Villa-Mancera et al., 2014), and others. Our group has developed phages carrying peptides with contraceptive potentials that were capable of stimulating production of anti-sperm antibodies in pigs (Samoylova et al., 2012a). Anti-sera collected from the immunized pigs were shown to inhibit sperm-oocyte interactions and events associated with embryogenesis in in vitro fertilization system (unpublished data).

It should be noted for vaccine development that phages are known to have natural immunogenicity due to their particulate nature, size, shape, well-defined surface structure of the virion, and possible adjuvant effect of their single-stranded DNA. In several studies, vaccine preparations based on filamentous phages appeared to be effective immunogens even without adjuvants (De Berardinis and Haigwood, 2004; Sartorius et al., 2011). Also important, phage coat proteins (self and fusions) are processed into major histocompatibility complex class I and class II bound peptides, thus capable of stimulating both humoral and cell-mediated immune responses (Gaubin et al., 2003; Hashemi et al., 2010; Ulivieri et al., 2008; Wan et al., 2005).

Several advantages of filamentous phages over other vaccine platforms should be emphasized. Phage is nonpathogenic for animals and does not replicate when administered into animals, including humans. Filamentous phage was approved by the U.S. Food and Drug Administration for experimental use in humans (Krag et al., 2006). No adverse reactions observed in the study indicated the safety of phage preparations. Most recently in Germany, filamentous phage particles were used as immunological carriers in a clinical phase I/II trial in patients with multiple myeloma (Roehnisch et al., 2014). In this study, phage-based preparations produced potent anti-tumor responses and were shown to be safe. Experiments in different mammalian species (mice, dogs, pigs) involving phage inoculations were performed in our laboratory. No side effects, either local or systemic, were observed even with repeated phage administrations (Samoylova et al., 2012a,b). Importantly, unlike vaccines vectored in mammalian viruses constructed for transfection of mammalian cells, phage-based vaccines can be used in deactivated (killed) form since phage particles act as a carrier protein and are not required to be viable. Phages inactivated via UV irradiation were shown to preserve their antigenicity in mice (Samoylova et al., 2012b). From an applied perspective, bacteriophages can be obtained in large quantities from bacterial cultures and at low cost compared to other vectors and synthetic peptides. Also important, phage-based vaccines may be produced safely and without any specialized techniques, equipment or facilities. Phage preparations were shown to be very thermostable (Brigati and Petrenko, 2005), adding to the list of their practical benefits.

The main objective of the present work was the generation of antigenic constructs displaying GnRH-like peptides on the surface of filamentous phage particles. This was accomplished via selection of GnRH antibody-binding phages from a phage display library. The generated phage-peptide constructs were used to immunize mice to characterize their ability to induce production of specific anti-GnRH antibody responses and suppress testosterone. These experiments allowed identification of phage-GnRH constructs that stimulated production of neutralizing GnRH antibodies with high titer and prolonged persistence in blood. Such phage-GnRH constructs may have broad applications in reproductive immunology, including development of anti-fertility and anti-cancer vaccines.

2. Materials and methods

2.1. Cat and dog sera

Samples of cat and dog sera used in this study were obtained in independent experiments performed previously in the laboratory of Dr. Michelle Kutzler. Animals were used in accordance with protocols approved by Animal Care Committees of Oregon State University (Corvallis, OR). Two cats and a dog received one milliliter of Canine Gonadotropin Releasing Factor Immunotherapeutic® (Pfizer Animal Health, Exton, PA, USA) twice. This immunotheraphy was also tested in multiple mammalian species (horses, donkeys, sheep, pigs, cats, dogs, alpacas, llamas, and reindeer) and was shown to be effective in testosterone reduction indicating its ability to stimulate GnRH antibodies with neutralizing properties (Michelle Kutzler, unpublished data). GnRH antibodies in sera of immunized animals were measured using ELISA as described in Section 2.6 (below). Testosterone in cat and dog sera was detected by Coat-A-Count® Total Testosterone radioimmunoassay (Diagnostics Products Corporation, Los Angeles, CA, USA).

2.2. Purification of GnRH antibodies from cat and dog sera

GnRH antibody purification from cat and dog sera was performed in two steps. First, total antibodies were isolated from sera using Nab Protein A Plus Kit (ThermoScientific, Rockford, IL, USA). Fifty microliters of serum collected from individual animals was mixed with 350 μl binding buffer and applied to Protein A columns.