Journal of Biotechnology 216 (2015) 20–28

Contents lists available at ScienceDirect

Journal of Biotechnology

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

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

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ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 30 July 2015
Accepted 5 October 2015
Available online 9 October 2015

Keywords:
Filamentous phage
Phage display
Gonadotropin releasing hormone
GnRH antibodies
Contraception
Hormone-dependent reproductive cancers

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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http://dx.doi.org/10.1016/j.jbiotec.2015.10.001
0168-1656/

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.

After incubation for 30 min, the columns were washed three times with 400 μl binding buffer, and antibodies were eluted with 400 μl elution buffer. GnRH antibodies were isolated from the total antibodies in the second purification step. Total antibodies (40 μl) were diluted with 160 μl PBS, pH 7.4 and incubated for 30 min at room temperature with magnetic beads carrying GnRH peptides. To prepare the beads, a mixture (1:1) of two synthetic peptides (biotin-EHWSYGLRPG-$CONH_2$ and NH_2-EHWSYGLRPG-Lys-biotin-$CONH_2$) at final concentration 1 mg/ml were immobilized on streptavidin beads (Dynabeads MyOne Streptavidin T1 Kit, Invitrogen, Oslo, Norway) via biotin. Configuration of the peptides allowed both ends of the GnRH peptide to be accessible for antibody binding maximizing isolation of different GnRH antibody clones present in the sera. The incubation was followed by several washes and an elution step as described in the manufacturer's instruction for the beads. Finally, the eluates were dialyzed using Slide-A-Lyzer G2 Dialysis Cassettes, 10 K MWCO (ThermoScientific) in 500 ml TBS, 0.02% Tween-20 (changed 3 times) at 4 °C for 20 h total. GnRH antibodies were measured using ELISA, as described in Section 2.6 below.

2.3. Phage display library

To identify phage clones displaying peptides that bind to GnRH antibodies, f8-8 landscape phage display library was used. The library was constructed by insertion of oligonucleotides encoding for random 8-mer peptides in a phage vector type 8 (Petrenko et al., 1996). This phage vector has one copy of gene 8; therefore, all copies of the phage major coat protein VIII in the library are modified with fusion peptides. Such particles were named "landscape phage" to emphasize the dramatic change in surface architecture caused by thousands of copies of the foreign peptide presented in a dense, repeating pattern along the tubular capsid. General methods of handling the landscape phage, including phage propagation, purification, titering, production of pure phage clones, and preparation of PCR products for phage DNA sequencing were performed as described previously (Brigati et al., 2008).

2.4. Selection of GnRH antibody-binding phage

GnRH antibodies immobilized on magnetic beads were used as targets for selection of the antibody-binding phages. The antibodies included cat and dog GnRH antibodies prepared as in Section 2.2 above and commercial polyclonal rabbit antibodies (Abcam, Cambridge, MA; cat #16,216). For antibody immobilization, 200 μl of GnRH antibodies in TBS containing 0.02% Tween-20 were added to 50 μl magnetic beads (Dynabeads® Protein A, Novex®, Life Technologies, Oslo, Norway) for 30 min incubation at room temperature with gentle rotation. After the incubation, supernatants were removed and beads washed in 200 μl TBS, 0.02% Tween-20. To block nonspecific binding, 400 μl of blocking buffer (TBS, 0.5% BSA) was added to the beads for one hour at room temperature. The blocking buffer was removed and the beads were rinsed with 400 μl TBS, 0.02% Tween-20.

To select GnRH antibody-binding phages, an aliquot (approximately 1×10^{10} cfu) of the phage library was diluted in 200 μl blocking buffer (TBS, 0.5% BSA) and added to the beads coupled with GnRH antibodies (steps involved in selection of GnRH antibody-binding phage are shown schematically in Fig. 1). After one hour incubation at room temperature, the supernatant containing phage not bound to the antibodies was removed and the beads were washed with 200 μl TBS, 0.05% Tween-20. Bound phages were eluted with a low pH buffer (200 mM glycine-HCl pH 2.2, 0.1% BSA). Phages recovered via elution were amplified in *Escherichia coli* K91BluKan host bacteria and used in a subsequent selection round for antibody binding. In each following selection round, freshly

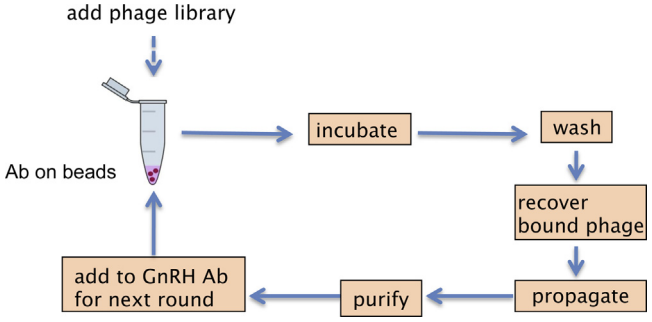

Fig. 1. Schematic presentation of steps involved in selection of GnRH antibody-binding phage from a phage display library. First, an aliquot of phage library (approximately 1×10^{10} cfu) is added to a tube containing GnRH antibodies immobilized on magnetic beads for incubation. After that, unbound phages are removed by washing. Elution buffer is added to release bound phages. Phages in the eluate are amplified in bacteria, purified and used in additional selection rounds on GnRH antibodies (not shown in the figure) to allow enrichment of phage clones specific for binding to GnRH antibodies.

prepared beads carrying GnRH antibodies were incubated with phage obtained from the previous round. A total of three selection rounds were performed. To monitor the selection process, the output phage was titered after each round and phage enrichment was calculated as a ratio of output to input phage. Subsequently, phage DNAs were amplified by PCR and sequenced to translate peptides displayed on selected phages. In each selection experiment, at least 80 phage DNAs were sequenced. DNA sequencing was performed at the DNA Core Facility of the Center for Computational and Integrative Biology (Massachusetts General Hospital, Cambridge, MA, USA).

2.5. Immunization of mice

CD-1 outbred 6-week-old male mice (Charles River, Wilmington, MA, USA) were used in immunization experiments in accordance with the protocol approved by the Auburn University Animal Use and Care Committee. Prior to immunization, all phage preparations were purified from endotoxins as described previously (Aida and Pabst, 1990). To optimize the dose and administration route for phage-GnRH constructs, mice were immunized with one of the phage constructs at three different doses, 2×10^{10}, 1×10^{11}, or 5×10^{11} virions (vir) per mouse, using either intramuscular or subcutaneous injections. Once the optimized phage dose and administration route were established, all mice in the following experiments were treated as described below.

Mice (10 per group) were immunized with phage at 5×10^{11} vir in 500 μl PBS (250 μl on each side) per mouse by subcutaneous administration. Five groups of mice were immunized with different individual phage-GnRH constructs. Mice in two negative control groups were injected either with PBS or with the phage vector used to generate phage-GnRH constructs. Blood samples were collected via tail vein puncture on day 0 and then at weeks 3, 5, 7, and 10 and GnRH antibodies detected as in Section 2.6 below. To measure serum testosterone, Mouse and Rat Testosterone ELISA kit (ALPCO, Salem, NH, USA) was used as suggested by the manufacturer.

Additional experiments aimed at enhancing of antibody responses to phage-GnRH constructs and suppression of testosterone were accomplished as follows. One group of mice ($n = 7$) was immunized with phage at a high dose of 2×10^{12} vir in 500 μl PBS (250 μl on each side) per mouse by subcutaneous administration. The second group of mice was immunized with phage mixed with Thermogel AK97 (Polyscitech, West Lafayette, IN). Blood samples were collected via tail vein puncture on day 0 and then at weeks

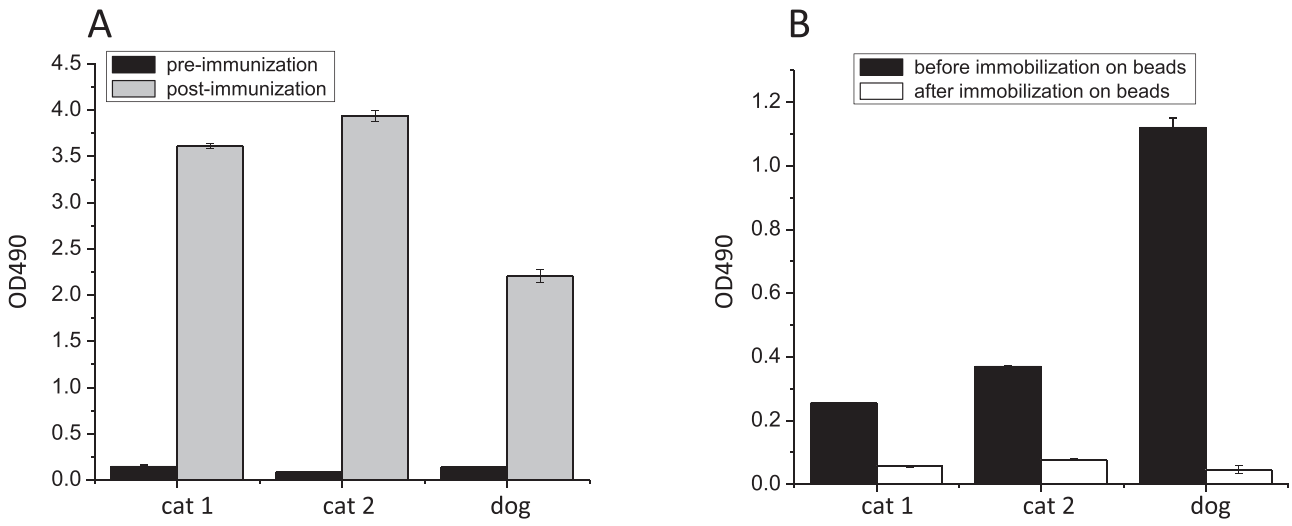

Fig. 2. ELISA detection of GnRH antibodies in preparations used for identification of GnRH-like phage clones. (A) GnRH antibodies in sera collected from two cats and a dog immunized with Canine Gonadotropin Releasing Factor Immunotherapeutic. To assay binding antibodies, sera were diluted 1:25,600. Black columns indicate sera collected from cats and a dog before immunization, grey columns—after immunization. (B) GnRH antibodies in purified antibody preparations before (black columns) and after (white columns) immobilization on magnetic beads. ELISAs were performed at antibody dilution 1:800. Data are shown as OD_{490} means ± SD.

4, 8, and 10 and GnRH antibodies detected as in Section 2.6 below. Testosterone was measured using ELISA kit as above.

2.6. GnRH antibody ELISA

Streptavidin was diluted in TBS at 25 μg/ml and added to 96-well plates (80 μl/well) for 3-h incubation at room temperature. Following the incubation, streptavidin was removed and the wells were washed three times with 150 μl/well TBS. Synthetic GnRH peptide biotinylated either on N- or C-terminus (biotin-EHWSYGLRPG-$CONH_2$ or NH_2-EHWSYGLRPG-Lys-biotin-$CONH_2$) at a final concentration of 6.25 μg/ml in TBS was added to streptavidin-coated wells (80 μl/well) for overnight immobilization at 4° C. After that, the peptide was removed and 150 μl of 1% BSA in TBS were added to the wells for one hour at 37° C to block non-specific binding. Subsequently, the blocking solution was removed and the wells were washed three times with TBS containing 1% BSA and 0.05% Tween-20.

Sera collected from immunized mice were separated from blood cells by centrifugation at 14,800 × *g* for 5 min at room temperature using LWS-M24 Micro-Hematocrit Centrifuge (LW Scientific, Inc., Lawrenceville, GA, USA). The sera were diluted and added to the peptide-coated wells for two hours at 37° C. The wells were then washed three times with TBS containing 0.05% Tween-20. Peroxidase-conjugated goat anti-mouse IgG (H + L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:2000 in TBS, 1% BSA, 0.05% Tween-20 was added to the wells for one hour incubation at room temperature.

The wells were washed as previously, reacted with *o*-phenylene diamine substrate (Acros Organics via VWR, Atlanta, GA) solution, and absorbance measured at 490 nm using Synergy HT reader (BioTek, Winooski, VT, USA). Antibody responses were expressed as means of OD_{490} ± SE at serum dilutions shown in figure legends.

ELISA tests in experiments involving cat and dog sera were performed as for mouse serum above with the exception that anti-cat (peroxidase-conjugated goat anti-cat IgG (H + L); Jackson ImmunoResearch Laboratories, Inc.) or anti-dog (peroxidase-conjugated rabbit anti-dog IgG (H + L); Jackson ImmunoResearch Laboratories) secondary antibodies were used.

2.7. Statistics

GnRH antibody and testosterone measurements were performed in duplicate and mean values ± SE are presented. Mouse treatment groups were compared using one-way ANOVA at a significance value of $P \leq 0.05$ using Origin 7.5 data analysis and graphing software (OriginLab, Northampton, MA, USA).

3. Results

3.1. Generation of phage-GnRH constructs

Phage constructs displaying GnRH-like peptides were generated via selection from a phage display library using GnRH antibodies as selection targets. The following factors were taken into consideration when choosing GnRH antibodies for selection experiments. The major criterion was their biological relevance, namely, their ability to suppress production of testosterone in vivo. Also, antibodies raised in different mammalian species were chosen as several reports indicated that the contraceptive role of individual amino acids/epitopes in the GnRH sequence varies depending on species (Turkstra et al., 2001; Zeng et al., 2007), in spite of the fact that GnRH peptide has the same sequence in all mammals. The antibodies were obtained from different sources, including experimental GnRH antibodies isolated from anti-sera of two cats and one dog immunized with GnRH-based immunotherapeutic and a commercial rabbit GnRH antibody. The anti-sera collected from immunized cats and a dog were confirmed to have GnRH antibodies. Elevated ELISA signals in post-immunization sera as compared to pre-immunization sera indicated the presence of GnRH antibodies in post-immunization samples (Fig. 2A). These GnRH antibody-containing sera had no detectable testosterone (not shown) demonstrating neutralizing capabilities of the antibodies. The commercial rabbit GnRH antibodies were characterized for neutralizing activity via passive immunization of mice. Reduction of testosterone from 3.459 ± 0.02 ng/ml in pre-immunization mouse sera to 0.322 ± 0.159 ng/ml in post-immunization sera was found. Suppression of serum testosterone in the immunized mice served as an indirect indicator of the antibody biological activity. For selection of GnRH antibody-binding phage, individual antibodies were immobilized on magnetic beads. Successful antibody immobilization was confirmed via quantitation of GnRH antibodies

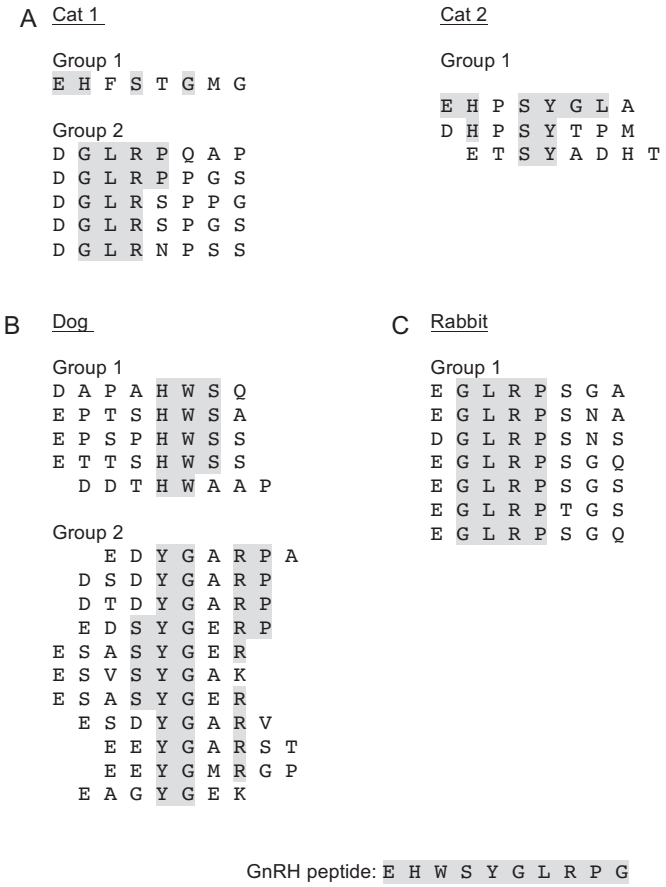

Fig. 3. Peptide sequences identified through selections from f8-8 landscape phage display library for binding to GnRH antibodies. Selections were performed on GnRH antibodies from several different sources, including experimental antibodies isolated from anti-sera of two cats and one dog immunized with Canine Gonadotropin Releasing Factor Immunotherapeutic, and commercial rabbit antibodies. Cat and dog antibodies were affinity purified on a column with GnRH peptide. Rabbit antibodies were used as supplied. Sequences of peptides displayed on phage are shown. The peptides are grouped based on the species of antibody origin, (A)—cat, (B)—dog, (C)—rabbit, and sequence similarities to GnRH peptide. (Note: amino acid residues that are identical to those in GnRH peptide sequence are shaded). GnRH peptide sequence is shown at the bottom of the figure. The sequence of GnRH peptide is identical in the majority of mammalian species, including human, mouse, cat, dog, and rabbit.

in pre- and post-immobilization samples. Significantly less GnRH antibodies were detected in the supernatant samples collected after antibody incubation with the beads (Fig. 2B).

To generate phage-GnRH constructs, four independent phage selection experiments were performed. In each experiment, a different GnRH antibody selection target was used: GnRH antibodies purified from serum of cat 1, cat 2, or dog serum, or commercial rabbit GnRH antibodies. Three selection rounds were conducted in each experiment and at least 80 phage DNAs were sequenced. Enrichment in phage binding to GnRH antibodies was detected after each selection round in all four experiments. The overall enrichment in three rounds on rabbit antibodies was the lowest (35 fold), followed by enrichment obtained using cat 1 and cat 2 antibodies (90 and 82 fold, respectively). The highest phage enrichment was obtained while selecting on dog antibodies, 1500 fold.

Selection on cat 1 GnRH antibodies led to the identification of phage clones that displayed two different GnRH peptide epitopes and, therefore, were placed in two groups (Fig. 3A; amino acid residues that are identical to those in GnRH peptide in specific positions are highlighted). All phage clones in Group 1 were identical and displayed peptides **EH**F**S**T**G**MG spanning from N-terminus to mid portion of GnRH peptide. The second group comprised of five different phage clones with the consensus sequence that included mid portion and C-terminus of GnRH peptide. In this group, two phage clones D**GLRP**QAP and D**GLRP**PGS had the most similarity to GnRH peptide (four amino acids in specific positions). Phage clone D**GLR**SPPG was the most prevalent in the group. Phage clones in Group 1 represented 28% of all clones sequenced. Phage clones in Group 2 represented 46% of all clones sequenced. Selection on cat 2 GnRH antibodies revealed that the majority (69%) of phage clones were identical and displayed **EH**PSYGLA peptide with six amino acids in specific positions identical to GnRH peptide (Fig. 3A).

Selection on dog GnRH antibodies allowed identification of clones with peptides representing the N-terminus as well as peptides spanning from mid portion to the C-terminus of GnRH peptide (Fig. 3B). Based on sequence similarities, the peptides were placed in two groups. Phage clones in Group 1 represented 23% of all clones sequenced. Phage clones in Group 2 represented 73% of all clones sequenced. Phage clones in Group 1 had a consensus sequence **HWS**, which represented amino acids in positions 2–4 of GnRH peptide (N-terminus). Notably, no such clones were identified using cat antibodies. The second group included 11 different phage clones with the consensus sequence from mid portion to the C-terminus of GnRH peptide. Phage clone ED**YGA**G**RP**A had the most sequence similarity with GnRH peptide (four amino acids identical to GnRH in specific positions).

Selection on commercial rabbit GnRH antibodies also identified a group of clones with similarities to GnRH peptide sequence (Fig. 3C). All of them had the same consensus sequence **GLRP** in positions 2–5, but different flanking sequences. This consensus sequence represents amino acids 6 through 9 in GnRH peptide. The presence of common amino acids in specific positions of peptides

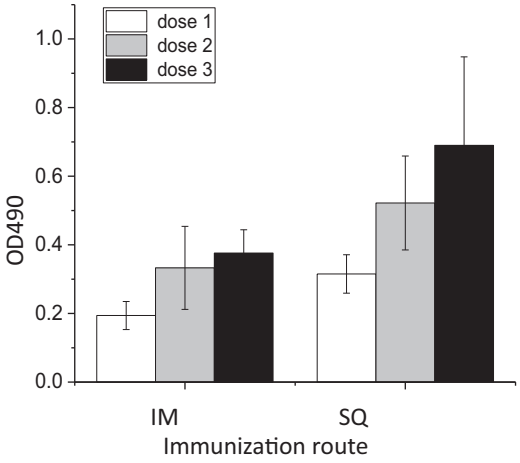

Fig. 4. GnRH antibody responses in mice immunized with D**GLRP**QAP phage construct for optimization of administration route and phage dose. Mice were immunized via either intramuscular (IM) or subcutaneous (SQ) administration at three different doses: dose 1 was 2×10^{10}, dose 2 was 1×10^{11}, and dose 3 was 5×10^{11} vir/mouse. Blood samples were collected at week 5 after immunization and GnRH antibody detected by ELISA. The assay was performed at serum dilution 1:1600. Data are presented as OD_{490} means for each treatment group (6 mice/group) ± SE.

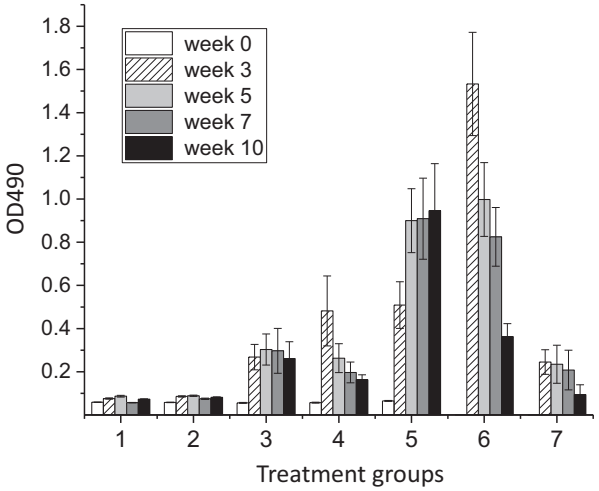

Fig. 5. GnRH antibody responses in mice immunized with different phage-GnRH constructs (by ELISA). The following immunization groups are shown: (1) PBS (negative control), (2) phage vector control without fusion peptide, (3) phage displaying E**GLRP**SGQ peptide, (4) phage displaying D**GLRP**QAP peptide, (5) phage displaying **EH**P**SYGL**A peptide, (6) phage displaying EPTS**HWS**A peptide, and (7) phage displaying DAPA**HWS**Q peptide. (Note: amino acid residues that are identical to those in GnRH peptide sequence are shown in bold). Blood was collected prior to immunization (week 0) and at weeks 3, 5, 7, and 10. ELISA was performed at serum dilution 1:1600. Data are presented as means for each treatment group (10 mice/group) ± SE. OD values in control groups injected with PBS or phage vector (used for generation of phage-GnRH constructs) as well as values in treatment groups before immunization (week 0) were ≤0.1.

displayed on phage (Fig.3A–C) provided indirect evidence of the specificity of the selection process.

3.2. Characterization of phage-GnRH constructs for their ability to stimulate production of GnRH antibodies in vivo in mice

The ability of GnRH antibody-binding phages to stimulate production of anti-GnRH antibodies was evaluated in mice. First, phage dose per mouse and administration route were optimized using phage displaying D**GLRP**QAP peptide. This phage was injected either intramuscularly or subcutaneously at three different doses of 2×10^{10}, 1×10^{11}, and 5×10^{11} vir/mouse and GnRH antibodies measured. As a trend, increased antibody responses were stimulated with the increased phage doses. The highest antibody response was produced by the highest dose of 5×10^{11} vir per mouse. Additionally, subcutaneous phage administration showed a tendency to produce greater GnRH antibody responses than intramuscular immunizations (Fig. 4). Based on these observations, all the following immunizations of mice were subcutaneous and at a dose of 5×10^{11} vir per mouse.

For immunogenicity tests, the following five phage-GnRH constructs were administered into mice: E**GLRP**SGQ, D**GLRP**QAP, **EH**P**SYGL**A, EPTS**HWS**A, and DAPA**HWS**Q. They were chosen based on similarities to GnRH peptide sequence (major criterion), their frequencies in the selection experiments, and animal species used for GnRH antibody production. Additionally, two groups of mice, one injected with PBS and the other with phage vector ("empty" phage not displaying fusion peptides), were used as negative controls. Importantly, each mouse received a single injection and no adjuvants were used to boost immunological responses. In each treatment group, the ELISA signal obtained from post-immunization serum was significantly higher than that of baseline signal generated from pre-immunization serum, suggesting that the constructs stimulated production of anti-GnRH antibodies (Fig. 5). While all of the phage-GnRH constructs stimulated production of specific GnRH antibodies, their titer and duration of the presence in anti-sera were different in individual treatment groups. The highest antibody responses were in mice injected with phages bearing EPTS**HWS**A and **EH**P**SYGL**A peptides. The most long lasting antibody responses (no titer decrease in 10 weeks, duration of experiment) were detected in mice injected with phage carrying **EH**P**SYGL**A peptide. Intriguingly, phage constructs displaying identical consensus sequences (EPTS**HWS**A and DAPA**HWS**Q) stimulated significantly different immune responses, most likely due to the different flanking amino acid residues present in the sequences. While all phage-GnRH constructs stimulated production of specific GnRH antibodies, no significant changes in serum testosterone were observed (not shown). This pointed towards a possibility that levels of GnRH antibodies stimulated by phage-GnRH constructs were not sufficient to induce hormonal effects.

3.3. Enhancing antibody responses to phage-GnRH constructs for testosterone suppression

Two approaches aimed at enhancing antibody responses to phage-GnRH constructs were tested: (1) using phage-GnRH constructs at a high phage dose and (2) employing PLGA-PEG-PLGA polymer as an adjuvant/delivery system. In the experiments, mice were immunized with phage displaying **EH**P**SYGL**A peptide. One group of mice was immunized with a dose of 2×10^{12} vir/mouse and the second group was immunized with phage combined with a commercially available product called Thermogel. Thermogel is PLGA-PEG-PLGA co-polymer designed to be liquid at 4 °C, but solid at 37 °C. Thus, it solidifies after in vivo administration and provides slow release effect as the polymer degrades. Moreover, PLGA is known to act as adjuvant.

Immunization of mice with a dose of 2×10^{12} vir/mouse resulted in multifold increase in serum GnRH antibodies (Fig. 6A) as compared to sera of mice inoculated with 5×10^{11} vir/mouse in experiments shown in Fig. 5. Testosterone in post-immunization sera was significantly lower than in pre-immunization samples (Fig. 6B) suggesting that antibodies produced in response to phage-GnRH immunization are neutralizing. Importantly, administration of phage into mice produced no visible adverse effects, either local or systemic, for the duration of the experiment independently of phage dose or antigen composition.

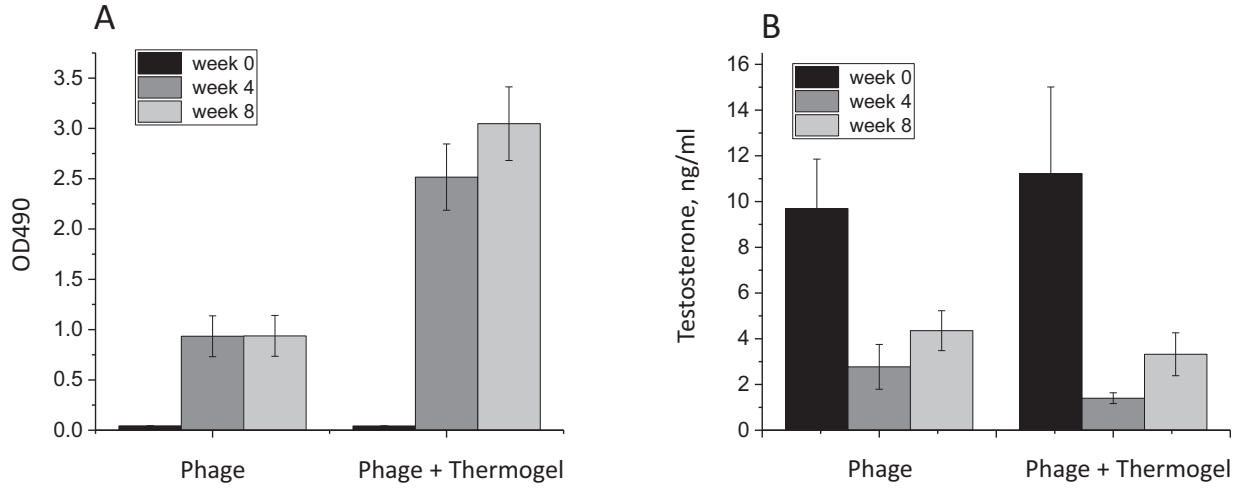

Fig. 6. GnRH antibody (A) and serum testosterone (B) in mice immunized with **EHPSYGL**A phage at high phage dose or phage in Thermogel. One group of mice was immunized with a dose of 2×10^{12} vir/mouse and the second group was immunized with phage mixed with Thermogel. Thermogel is PLGA-PEG-PLGA co-polymer that solidifies at the body temperature and provides slow release effect as the polymer degrades. ELISA was performed at serum dilution 1:6400. Data are presented as means for each treatment group (7 mice/group) ± SE.

4. Discussion

The objective of this study was to generate and characterize phage-GnRH peptide antigenic constructs that elucidate production of specific GnRH antibodies and can be used potentially for immunocontraception or as immunotherapy for treatment of hormone-dependent reproductive cancers. Theoretically, such phage constructs can be obtained either via cloning of oligonucleotides encoding for exact or rationally modified GnRH peptide in a phage display vector or they can be selected against anti-GnRH antibodies from a library of phages displaying random peptides.

Both of the approaches, cloning in a phage vector or selecting from a random phage display library, were used in the past successfully for generation of phages that modify animal reproductive functions. To develop a contraceptive, Abdennebi et al. (1999) produced recombinant filamentous phages displaying three overlapping N-terminal decapeptides of the follicle-stimulating hormone receptor (FSHR). The constructs were tested in several mammalian species and in animal groups of different sexual maturity. When injected in animals, they induced anti-FSHR immunity capable of inhibiting follicle-stimulating hormone binding. The vaccinations were shown to impair fertility in adult mice and inhibit ovulation rates in ewes and to induce infertility in adult male bonnet monkeys (Abdennebi et al., 1999; Rao et al., 2004). The same constructs were employed for immunization of male mice and goats at the prepubertal stage (Abdennebi et al., 2003). This delayed the acquisition of fecundity in mice by one week. Once sexually mature, litter sizes produced by immunized males and untreated females were reduced by up to 60%. In goats, phage-FSHR vaccines were able to maintain testosterone at low prepubertal levels for several months, suggesting their ability to delay the onset of puberty.

While effective in some applications, generation of individual phage-peptide fusions via cloning in a phage vector have limitations defined by the type of the phage vector and phage biology. Such limitations include length and sequence composition of peptides displayed on phage. Our efforts in construction of phage fusions via cloning of GnRH-like peptides in phage vector type 8 were not successful. The recombinant phages appeared to be either genetically unstable (mutated after several rounds of amplification) or had low propagation rates. This can be explained by specific design of the phage vector type 8. This vector has only one copy of gene 8, thus, all copies of the major coat protein VIII are modified with the fusion peptide. Since phage particles are generated via self-assembly, fusion peptides (depending on their sequence) might interfere with phage assembly as they are located close to each other and interact. On the other hand, this particular type of phage has significant advantages for immunological applications. Each phage particle created with the use of type 8 vector displays 4000 copies of a fusion peptide that drastically increases peptide surface presentation. Additionally, such high-density epitopes are presented in a highly organized manner and are properly spaced for binding to B cell receptors. Such repetitive highly organized epitope patterns usually permit a cross-linking activation of B-cell receptors, which provides robust, long-lasting immune responses.

Alternatively, phage clones displaying desired peptides can be selected from phage display libraries. Phage display libraries are mixtures of billions of different phage clones, each displaying different fusion peptides. In the present study, phage-GnRH constructs were generated via selection from an eight-mer landscape phage display library (Petrenko et al., 1996) generated with the use of type 8 phage display vector. Owing to the constraints applied in the construction process, phage clones that are present in the library are genetically stable and propagate well. The library is composed of approximately 2×10^9 different phage clones (close to the record high complexity for phage display libraries available currently), providing incredible diversity for finding phages displaying peptides that resemble GnRH. Each phage particle in this library displays 4000 copies of a fusion peptide available for recognition by B cells and subsequent production of antibodies against the displayed peptides. Here, phage-GnRH constructs were identified in succeeding selection rounds using different types of GnRH antibodies as selection targets, including antibodies raised in dog, cat, or rabbit. Interestingly, the core sequence XXXX**HWS**X of peptides identified using dog antibodies were very similar to the one, X**HWS**XXLXXX, selected on dog GnRH antibodies by others (Yu et al., 2005). Also noteworthy is the fact that several peptide sequences isolated in the present work using cat or rabbit antibodies contained amino acid core **GLRP** that is very similar to a GnRH-based peptide **GLRPG** shown to possess contraceptive properties in mice (Zeng et al., 2007).

To test antigenicity of the obtained phage-GnRH constructs, they were injected in mice as whole phage particles. It was show that increased phage doses enhance production of GnRH antibod-

ies. Immunization of mice with the maximum dose of 2×10^{12} vir/mouse resulted in suppression of serum testosterone. This indicated that antibodies produced in response to phage-GnRH immunization possessed neutralizing capabilities. Roehnisch et al. (2013) also showed that anti-phage antibody responses increase with the phage dose. Three phage doses were studied with the maximum phage dose of 5×10^{11} vir/mouse demonstrating the most pronounced immune effects. Others demonstrated functional immune responses with no observed side effects when animals were injected with significantly higher phage doses. Goats were inoculated with 1×10^{13} vir/animal of phage displaying cathepsin L1 mimotopes (Villa-Mancera et al., 2014). Reported safe doses in piglets are 2.5×10^{13} per immunization, in mice -1×10^{12} (Segura-Velázquez et al., 2013). No serious adverse events were observed in human patients vaccinated with 1×10^{11} to 2.5×10^{12} bacteriophages (Roehnisch et al., 2014).

Enhanced antibody responses were also obtained when phage-GnRH construct **EH**P**SYGL**A was combined with a PLGA-based polymer called Thermogel. PLGA is a copolymer of lactic and glycolic acids. PLGA nanoparticles have been used as controlled release drug delivery systems for many years and are FDA-approved (Jiang et al., 2005; Shive and Anderson, 1997). PLGA was also used experimentally for delivery of various types of vaccines since it was shown to be an effective adjuvant capable of eliciting B and T cell responses (Audran et al., 2003; Gupta et al., 1998; Maloy et al., 1994). Thermogels are prepared using PLGA and polyethylene glycol (PEG), thus the final material is PLGA-PEG-PLGA triblock copolymer. Incorporation of PEG in PLGA enhances the biocompatibility of the polymer as it becomes more hydrophilic. Thermogels are polymers that are liquid when refrigerated and become solid at the body temperature. They continuously degrade in a body releasing their content. Thermogels are widely utilized for various biomedical applications, including insulin therapy and anti-cancer drug delivery. They are also used for delivery of peptides, proteins, and nanoparticles (reviewed by Alexander et al., 2013). Another compound that has potential to enhance phage immunogenicity is Quil A (Villa-Mancera et al., 2014). This adjuvant is a mixture of triterpenoid saponins isolated from the bark of *Quillaja saponaria* that stimulates both humoral and cellular responses against co-administered antigens.

Additional approaches can be explored to increase immunogenicity/effectiveness of the phage-peptide fusions to achieve desired therapeutic effects. If a target-specific phage is generated via selection from a phage display library and position of the consensus amino acid residues within the displayed peptide is known, the flanking residues can be optimized for obtaining phage-peptide fusions with greater affinity and specificity. This can be achieved via construction of a second-generation phage display library with consensus amino acid residues in fixed positions flanked by random amino acids. Selection of such a library against the initial target might result in identification of peptides with increased affinity. This process is called affinity maturation of peptides displayed on phage. Fleming et al. (2005) employed this approach to identify high affinity peptides that bind B lymphocyte stimulator (BLyS), a member of a tumor necrosis factor family. Peptides selected in their study from a primary library were found to bind BLyS with K_D values of 1–3 mM. To improve the binding affinity, a second-generation library composed of phage clones bearing peptides with the key residues identified through selections from the first-generation library flanking by random amino acids was created. Selections from this second-generation (affinity maturation) library resulted in identification of peptides which demonstrated K_D values of 26 nM that is roughly 100-fold better than that of the first-generation peptides. Investigating affinity of synthetic peptides derived from selection by phage display Ferrières et al. (2000) concluded that sequences flanking the core motif carry determining factors necessary for high affinity. They also highlighted the fact that flanking amino acids are critical for the maintenance of the antigenically reactive structure of peptides displayed on phage. In the recent studies, effectiveness of the affinity maturation of phage display derived peptides through modification of the flanking sequences was shown for a variety of applications (Fujii et al., 2013; Larimer et al., 2014). The role of flanking sequences in stimulation of specific immune reactions was obvious in the present study as well. Two phage constructs having identical consensus sequences (EPTS**HWS**A and DAPA**HWS**Q peptides) produced drastically different antibody amounts when used under the same experimental conditions.

To conclude, phage-GnRH constructs were generated in the present study via selection from a library of phages displaying random octapeptides. Sequences of the selected peptides displayed on phages resembled different GnRH epitopes, including N- and C-terminus as well as the mid-portion of the GnRH peptide. These phage-GnRH constructs stimulated production of specific GnRH antibodies of different strength and duration in mice. At least one of the constructs, when tested at high dose and in combination with PLGA-based adjuvant, generated biologically relevant GnRH antibodies based on significant reduction of serum testosterone in immunized animals. Further optimization of such phage-based constructs as to their affinity to GnRH antibodies and ability to stimulate strong and long lasting humoral immunity can be pursued to achieve desired therapeutic effects in specific biomedical applications, including contraception of animals and treatment of hormone sensitive cancers of reproductive organs.

Acknowledgments

This study was funded by the Found Animals Foundation, Michelson Grant in Reproductive Biology D0910-S10, Animal Health and Disease Research program and the Scott-Ritchey Research Center (SRRC), College of Veterinary Medicine, Auburn University, Auburn, AL, USA. T.S. thanks Nancy Cox (SRRC) for constructive discussions in the course of the study. Dr. Nancy Cox lost her battle to cancer in August of 2014. T.S. also thanks Nancy Morrison (SRRC) for proof reading the article.

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