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A synthetic gonadotropin-releasing hormone (GnRH) vaccine for control of fertility and hormone dependent diseases without any adjuvant

Xu Jinshu [a,c], Liu Jingjing [a,b,*], Peng Duan [a], Zhu Zheng [a], Ming Ding [a], Wu Jie [a], Cao Rongyue [a], Hu Zhuoyi [a], Rouel S. Roque [b]

[a] *The Minigene Pharmacy Laboratory, Biopharmaceutical College, China Pharmaceutical University, Tong Jia Xiang 24, Nanjing 210009, Jiangsu, PR China*
[b] *Department of Cell Biology and Genetics, University of North Texas Health Science Center, Fort Worth, TX, USA*
[c] *School of Pharmacy, Nanjing Medical University, Nanjing, PR China*

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Abstract

Active immunization against self-peptides have gained widespread acceptance inspite of their low immunogenicity. Recent applications involving multiple copies of self-peptides in linear alignment and conjugation with carrier proteins appear to increase the immune response against self-peptides. As with most vaccines, however, immunogens require supplementation with adjuvants to elicit an optimum immune response. In the present study, we prepared a double-chain miniprotein with each chain containing three linear repeats of the self-peptide gonadotropin-releasing hormone (GnRH3), the hinge region of human IgG1 (hinge), and a T-helper epitope from the measles virus protein (MVP). The GnRH3-hinge-MVP miniprotein was conjugated to purified recombinant heat shock protein 65 (Hsp65) of *Mycobacterium bovis* and used to immunize rats primed with subcutaneous injections of Bacillus Calmette-Guerin (BCG) in the absence of adjuvants. The GnRH3-hinge-MVP-Hsp65 stimulated the production of specific anti-GnRH antibodies in the absence of adjuvants and the antibody titer was comparable to that produced in rats immunized with the dimeric miniprotein in the presence of Freund's adjuvant. Moreover, immunization with the adjuvant-free GnRH3-hinge-MVP-Hsp65 induced degeneration of the reproductive organs in both male and female rats unlike those immunized in the absence of Hsp65 or in control animals inoculated with the vehicle only. Histological examination of the affected organs showed atrophy of the seminiferous tubules with dimished spermatogenesis in the testes of male rats. In female rats, the uteri were much smaller in size and the ovaries exhibited reduced follicular development. These findings demonstrated that GnRH3-hinge-MVP-Hsp65 mounted a strong immune response in the absence of conventional adjuvants, and could prove useful in control of fertility and the treatment of conditions/diseases where GnRH ablation is required.

Keywords: Gonadotropin releasing hormone; Hinge protein; T-helper epitope; Heat shock protein; Self-peptide; Carrier-protein; Adjuvants; Vaccine

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide hypothalamic hormone that plays a central role in the regulation of mammalian reproduction. GnRH, also known as luteinizing hormone releasing hormone (LHRH), selectively stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary to promote maturation of ovarian follicles or sper-

Abbreviations: GnRH, gonadotropin releasing hormone; Hsp65, heat shock protein 65 kDa; hinge, hinge region of human IgG1; MVP, measles virus protein (LSEIKGVIVRLEGVAK); BCG, Bacillus Calmette-Guerin (attenuated *Mycobacterium tuberculosis* var. *bovis*); VEGF, vascular endothelial growth factor; HRP-horseradish peroxidase; IPTG, Isopropyl-β-D-1-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; ELISA, Enzyme-linked immunosorbent assay

* Corresponding author. Fax: +86 25 83204240.
E-mail addresses: Liujingj@public1.ptt.js.cn, kingshu2002@yahoo.com (L. Jingjing).

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matogenesis [1–3]. This function has attracted widespread attention to the enormous potential of GnRH inhibitors as anti-fertility agents [4,5]. The high level of expression of GnRH and its receptors or sex steroids and their receptors in a number of malignant human tumors, including cancers of the breast, ovary, endometrium, and prostate [6–8], has also contributed to the large number of studies targeting GnRH for anti-cancer therapy [9–11]. Among the various approaches investigated in controlling GnRH activity, active immunization using peptide-based vaccines has gained widespread acceptance [12–14]. The induction of immune response against self-peptide, such as GnRH, however, has not proven reliable for a variety of reasons. Self-peptides exhibit low immunogenicity, the chemical conjugation using self-peptides have low efficiency, and peptide antigen preparations are highly heterogenous [13]. These problems have been partially obviated by administering immunogens containing multiple copies of the self-peptides in linear alignment [13] or conjugation of peptide vaccines with defined T-helper epitopes [12,15]. Linear repeats of GnRH fused to the receptor-binding domain of pseudomonas exotoxin A generated GnRH-specific high-titer antibodies and promoted degeneration of ovaries in female rabbits [13]. In our laboratory, we have successfully tested a synthetic peptide consisting of three GnRH repeats (GnRH3) and the hinge region of human IgG1 (hinge). The double-chain miniprotein obtained by oxidizing GnRH3-hinge-MVP successfully induced anti-GnRH antibody responses in rats in the presence of Freund's adjuvant [16].

Synthetic or recombinant vaccines against a wide variety of diseases are not sufficiently immunogenic when administered in the absence of carrier proteins and/or adjuvants [17–20]. However, adjuvants appropriate for human use, such as aluminium hydroxide, have limited use with respect to the type of antigen employed and the stability of the final vaccine preparation [21]. Freund's complete adjuvant, while useful in animal immunization, is not acceptable for clinical use due to contamination with non-metabolizable oil and mycobacteria and its toxicity [22].

Heat shock proteins (Hsp) are a family of approximately 25 highly conserved proteins that increase in expression in response to various forms of stress [23]. Hsp65, a member of the Hsp superfamily, exhibits high binding efficiency to large protein substrates, thereby, increasing their stability. Because of this characteristic, Hsp65 has been used as a carrier protein and facilitated immune responses to conjugated vaccines in animals [17,24–27]. Recently, conjugation of mycobacterial Hsp70 to GnRH-analogue has successfully induced a anti-GnRH antibody response, and effected the reproductive system of male mice [28]. Inspite of these advantages, Hsp65 has not been tested as a carrier protein for anti-GnRH peptide vaccines. Therefore, in this study, we investigated the immunogenicity of Hsp65-conjugated double-chain GnRH3-hinge-MVP antigen in the absence of Freund's adjuvant.

2. Materials and methods

2.1. Preparation of double-chain GnRH3-hinge-MVP miniprotein

A DNA fragment coding for the C-terminal fragment of *L. asparaginase* (ansB-C) and an acid-labile aspartyl-prolyl linker was generated by PCR amplification using *E. coli* chromosome DNA as template. Amplification was performed using the forward primer (5′-CCCCATGGATACGCCATTCGATGTCTA-3′) located at 663 to 683 of asparaginase, and the reverse primer at downstream of the termination signal (5′-GCC AATCGGATCCGCGTACTGGTTGAAGATCTTGG-3′) [29]. PCR reactions were carried out in 100 μl volumes using 100 ng of DNA template and 1 μM primers in the standard reaction buffer (dNTP's 0.2 mM; 50 mM KCl; 10 mM Tris–HCl, pH 9.0; 0.1% Triton X-100). Typically, the reaction was taken through 30 cycles of 30 s denaturation at 94 °C, 2 min annealing at 55 °C and a 2 min extension at 72 °C. The resulting DNA fragment was digested with restriction endonucleases *Nco*I and *Hind*III, and subsequently inserted downstream of the T7φ10 or T7 promotor of the expression vector pET28a (Novagen, Germany) resulting in the pED plasmid.

The partial coding sequence of the GnRH-hinge-MVP was amplified by PCR using the oligonucleotides: 5′-GGTCTGCGTCCGGGTGGTACCTGCCCGCCGTGCCCTGCTCCGCTG-3′ and 5′-ATGCACGATAAC ACCTTTGATTTCAGACAGCGGAGCAGGGCACGG-3′ and cloned into pCR2.1 vector (Novagen, Germany). The resulting plasmid was used as DNA template for amplification of the full coding sequence of GnRH-hinge-MVP using the following oligonucleotides: 5′-GGGGGATCCGACTCAGCACTGGTCT TACGGTCTGCGTCCGGGTGGT-3′ and 5′-GGCCAAGCTTATTTAGCAACACCCTCCAGACGATGCAC GATAACACCTTT-3′. The resulting PCR fragment with *Bam*HI site (located at the 5′ end penultimate to the coding sequence of GnRH-hinge-MVP) and *Hind*III site (located just after the 3′ end to the termination codon TAA), was digested with *Bam*HI and *Hind*III, and inserted into pED linearized with *Bam*HI and *Hind*III. The resulting plasmid pED-GnRH expressing GnRH-hinge-MVP was used to transform *E. coli* BL21.

The recombinant plasmid pED-antiGnRH3 expressing GnRH3-hinge-MVP was constructed from pED-GnRH by extending N-terminal part of GnRH-hinge-MVP with two GnRH peptides in series using add-PCR. Add-PCR amplification was performed using pED-GnRH as DNA template and the 5′ first step primer (5′-CCGGGTGAACACTGGTCTTACGGCCTGCGCCCGGGTGAACACTGGAGCTACGGTCTGC GT-3′) and a 3′ common primer (5′-GGCCAAGCTTATTTAGCAACACCCTCCAGACGATGCACGATA ACACCTTT-3′). After the reaction was taken through 10 cycles, the second step PCR was initiated with 5′ second step primer (5′-GGGGATCCGGAACATTGGAGCTACGGTCTGCGTCCGGGTGAACACTGGTCT -3′)