

Synthetic luteinizing hormone releasing hormone (LHRH) vaccine for effective androgen deprivation and its application to prostate cancer immunotherapy

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Abstract

We have designed a peptide-based immunotherapeutic vaccine for treatment of androgen-responsive prostate cancer. The vaccine targets the luteinizing hormone-releasing hormone (LHRH) decapeptide that results in an androgen-deprivation immunotherapy. The design elements of the peptide immunogens are the LHRH peptide or B cell epitope synthetically linked to different promiscuous helper T cell (Th) sequences, the UBITH[®] epitopes, derived from four natural pathogens for effective immunogenicity in outbred populations, and in some cases, also linked to an adjuvanting peptide from *Yersinia* invasin (Inv) protein. The UBITH[®] LHRH immunogens are adsorbed on Alhydrogel or formulated as several different oil-based emulsions and tested in rodents, dogs, and a non-human primate, baboons. The immunogens generate an anti-LHRH antibody response specific to the LHRH decapeptide element in contrast to LHRH conjugate-carrier protein vaccines where only a small portion of the antibody response is directed to the target epitope and epitopic suppression is noted. Individual UBITH[®] peptide domains, but not the LHRH and Inv peptide domains, are stimulatory in lymphocyte cultures. The UBITH[®] LHRH immunogens in a clinically applicable formulation, controlled the growth of Dunning R3327-H androgen-responsive prostate tumor cells in rats. The results demonstrate universal responsiveness and long duration of androgen deprivation from three diverse species, and thus vaccine efficacy.

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1. Introduction

A treatment for prostate cancer is described based on a synthetic peptide vaccine that effects hormone-deprivation therapy. The active ingredient of the vaccine is a mixture of entirely synthetic peptide immunogens that direct an immune response against luteinizing hormone-releasing hormone (LHRH). LHRH, also known as gonadotropin releasing hormone (GnRH), is synthesized in the hypothalamus and transported by the hypothalamic-hypophyseal portal system to the anterior pituitary where it acts to effect secretion of gonadotropins, luteinizing hormone (LH)

and follicle stimulating hormone (FSH). LH and FSH bind to receptors in the testis and ovary and regulate gonadal function by promoting sex steroid production and gametogenesis. In males, LHRH induces maturation of the testosterone-secreting interstitial cells of the testis. Testosterone is converted to dihydrotestosterone, the form of the hormone that interacts with androgen receptors on prostatic epithelial cells to control their proliferation and apoptosis. Therapies with LHRH agonists interfere with the action of LHRH and block the effects of this hormonal cascade [1]. The growth of hormone-dependent tumors arising in the prostate gland can also be controlled by removal of the growth-promoting hormone(s) [2] or by blocking the LHRH pathway with immunotherapy [3].

The early landmark studies of Huggins and Hodges [2] established the hormonal dependence of prostate cancer and provided the basis for the use of androgen deprivation in its treatment. Reduction of plasma testosterone to castrate

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levels, either through surgical castration (orchietomy) or use of oral or injectable estrogens, became the standard of therapy for disseminated prostate cancer for the next 40 years. In the early 1980's, LHRH analogues were added as an alternative to achieve reversible pharmacologic castration [1]. Several randomized trials have demonstrated therapeutic equivalence of low dose estrogens, LHRH agonists (e.g. leuprolide, buserelin, goserelin), and orchietomy in rate of response, failure-free survival and overall survival [4,5]. By mid 1990's, an immunological approach, LHRH vaccines, had been designed and tested in men to achieve androgen deprivation as a treatment of prostate cancer [3,6] and in post-menopausal women to test the ability to inhibit gonadotropins [7].

The efficacy of neutralizing LHRH/GnRH action through the involvement of hormone-specific antibodies has been demonstrated in a wide range of animal species including humans. Such studies have involved either passive immunization by infusion of anti-LHRH antibodies [8] or vaccination with LHRH peptide coupled to tetanus or diphtheria toxoid (DT) molecules as carriers [3,6,7,9,10] or LHRH in multiple antigen peptide (MAP) constructs [11]. These approaches are impractical for widespread commercial application since passive immunity is inefficient and expensive and the use of peptide-toxoid conjugates and MAP constructs produce variable results. In addition, the peptide-toxoid conjugates and MAP immunogens are difficult to manufacture, and the use of toxoids can lead to carrier-induced anti-haptenic immunosuppression [12,13]. Recombinant LHRH toxoid fusion molecules have the same disadvantages. These limitations are overcome by the UBITH[®] synthetic peptide immunogens [14–18]. Each UBITH[®] immunogen is a chemically defined, homogenous peptide structure that can be reproducibly synthesized and characterized and is readily adaptable to large scale manufacturing processes.

The basis of our strategy is to induce an “anti-self” immunity to LHRH by altering the target molecule on a synthetic peptide immunogen. Immunization with LHRH peptide immunogens produces the desired hormone neutralization effect or “immunological castration” by eliciting anti-LHRH antibodies to the decapeptide (pEHWSYGLRPG-NH₂). We have developed entirely synthetic LHRH immunogens, in contrast to other groups, who have linked the LHRH decapeptide to carrier proteins [3,6,7,11]. This report describes the design of our synthetic LHRH peptide immunogens and the development of vaccines to effect androgen deprivation in rodents, dogs, and baboons, and as an immunotherapy for androgen-responsive prostate cancer.

2. Materials and methods

2.1. Peptide synthesis

Peptide antigens for immunoassays and peptide immunogens for vaccines were synthesized using automated

solid-phase synthesis with F-moc chemistry using terminus and side chain-protected amino acids [19,20] on Applied Biosystems Peptide Synthesizers, Models 430A or 431. After complete elongation of a desired peptide, the co-polymeric styrene beads (Rink Amide MBHA resin, Cat. No. 01-64-0037; Calbiochem-Novabiochem, San Diego, CA, USA) were treated according to standard procedures with 90% trifluoroacetic acid (TFA) to cleave the peptide from the resin and de-block the functional groups on the amino acid side chains. Each free peptide was washed, lyophilized and dissolved in distilled water to the desired concentration. Peptides were purified (>90%) by reverse phase HPLC with gradient elution consisting of an acetonitrile water mixture with added TFA. Synthetic peptides were characterized for the correct composition by amino acid analysis or by N-terminal sequence analysis using Edman degradation chemistry and matrix-assisted laser desorption time-of-flight mass spectrometry using an Ettan MALDI-ToF mass spectrometer (Amersham Bioscience, Piscataway, NJ, USA). Each purified peptide was stored (lyophilized or in solution) at –20 °C until needed.

2.2. Vaccine formulations

For Sprague–Dawley rat studies, individual peptide immunogens (25–400 µg per 0.5 ml dose) and mixtures of purified peptides (100 µg total peptide per 0.5 ml dose) in equal molar ratios were adsorbed to aluminum hydroxide gel [21] (gift of Dr. E.B. Lindblad, Superfos Biosector a/s, Vedbaek, Denmark) in 0.72% NaCl and 0.066 M sodium phosphate buffer, pH 7.0 by mixing overnight at 4 °C.

For Copenhagen rat studies, UBITH[®] LHRH immunogen Mix #1 containing equal molar ratios of peptides p607, p667, p669, p500 (100 µg total peptide per 0.5 ml dose) was adsorbed on aluminum hydroxide gel (Alhydrogel 85; EM Sergeant Pulp and Chemical Co. Inc., Clifton, NJ, USA) in 0.72% NaCl and 0.066 M sodium phosphate buffer, pH 7.0 by mixing overnight at 4 °C.

For dog studies, UBITH[®] LHRH immunogen Mix #1 (400 µg total peptide per 0.5 ml dose) was either adsorbed on Alhydrogel or formulated in an oil-in-water emulsion of 30% Emulsigen (MVP Laboratories Inc., Ralston, NE, USA) containing 5.93 mg/ml dimethyldioctadecyl ammonium bromide [22] (DDA; Eastman Kodak, Rochester, NY, USA), 0.68% Tween, 4.2% ethanol, 0.68% NaCl. Placebo control formulations contained Alhydrogel alone or the emulsion delivery system without immunogen Mix #1.

For baboon protocol #1, UBITH[®] LHRH immunogen Mix #2 containing equal molar ratios of peptides p607E, p667, p500 (400 µg total peptide in 0.15 M NaCl per 0.5 ml dose) in solution were filtered aseptically and combined with two different water-in-oil emulsions, Montanide[®] ISA51 (50:50 (v/v)) or Montanide[®] ISA720 (70:30 (v/v)), or with a water-in-oil-in-water emulsion, Montanide[®] ISA206 (Seppic Inc., Fairfield, NJ, USA), each containing 100 µg monophosphoryl lipid A [23] in 0.8% squalene. For baboon

protocol #2, UBITH[®] LHRH immunogen Mix #2 (25, 100, or 400 µg total peptide in 0.15 M NaCl per 0.5 ml dose) was combined with Montanide[®] ISA51 containing 0.9% DDA.

2.3. Animals

Groups of male Sprague–Dawley rats (8–12 weeks of age; Taconic, Germantown, NY, USA) were immunized in the hind leg by intramuscular route at 0, 3, 6 weeks with individual peptide immunogens (100–400 µg per dose) or peptide mixtures (25–100 µg per dose) adsorbed on aluminum hydroxide. Testes and prostate glands were removed and weighed at autopsy at the end of the study. The mean average weight in vaccine-treated animals was compared with that of placebo control animals to evaluate organ atrophy.

Groups of sexually mature intact male dogs (randomly divided between mongrel and pure bred animals 8–10 months of age; Pfizer Animal Health, Lincoln, NE, USA) were immunized in the hind leg by intramuscular injection at 0, 3, 6 weeks with 400 µg per dose of UBITH[®] LHRH immunogen Mix #1 in Alhydrogel or in Emulsigen + DDA. Placebo control intact male dogs received the adjuvant in the delivery system without peptide immunogens and control dogs were surgically castrated without receiving any vaccine treatment. Testes and prostate glands were removed and weighed at autopsy (end of the study). Organ atrophy in dogs was defined as the percentage change in organ weight per total body weight. All organ weight data are expressed as a percentage of the total body weight to account for size differences in animals comprising both the treated and control groups.

Adult male baboons (*Papio cynocephalus*; 6–14 years of age; Southwest Foundation for Biomedical Research, San Antonio, TX, USA) in protocol #1 were immunized at 0, 3, 6, 16 weeks with 400 µg per dose of UBITH[®] LHRH immunogen Mix #2 in three oil-based emulsions each containing adjuvant or with Mix #2 immunogens containing adjuvant alone. Baboons in protocol #2 were immunized at 0, 4, 34 weeks with 25, 100, or 400 µg per dose of Mix #2 immunogens formulated in Montanide[®] ISA51 containing DDA. Testes length and width were approximated by caliper at 2–4 week intervals and calculated as the mean cross-sectional area in cm².

Toxicity (i.e. pain, redness and swelling) at the intramuscular injection site (upper hind leg for rodents, dogs and baboons) was not observed with UBITH[®] LHRH immunogens in Alhydrogel. Transient redness was noted in some baboons receiving the more potent water-in-oil emulsions.

2.4. Dunning H rat prostate tumor model

Groups of male Copenhagen (COP/N) rats (Frederick Cancer Research and Developmental Center, Frederick, MD, USA) were implanted subcutaneously with 1 mm³ pieces of Dunning R3327-H tumor (gift of Dr. J.T. Isaacs, Johns Hop-

kins Oncology Center, Baltimore, MD) to test the efficacy of UBITH[®] LHRH immunogen Mix #1 at 100 µg total peptide per dose formulated in Alhydrogel to control the growth of the Dunning H androgen-responsive prostate tumor cells.

The original R3327 tumor, discovered by Dunning [24], was a spontaneously occurring prostatic adenocarcinoma in an old inbred male Copenhagen rat. A number of so-called Dunning R3327 prostate tumor lines were derived, including the Dunning H tumor line, which is predominantly androgen-dependent and rarely metastatic, and which grows with a doubling time of 21 ± 6 days when implanted under the skin of intact male Copenhagen rats [25]. The Dunning H subline is a heterogeneous tumor composed of clones of both androgen-dependent and androgen-independent tumor cells and will respond to castration of the host by cessation of growth eventually followed by re-growth as hormone-independent tumor [25]. The androgen-sensitive Dunning H tumor used in this study was received as a frozen piece of tumor tissue which was passaged in intact male Copenhagen rats. Tumor pieces (approximately 1 mm³ each) were implanted subcutaneously into three groups of eight rats each. The sizes of tumors growing in the rats were measured in three dimensions using calipers and tumor volume was approximated by the formula: length (cm) × width (cm) × height (cm) × 0.5236 and expressed as tumor volume in cm³.

2.5. Serological assays

2.5.1. Solid-phase enzyme-linked immunoassay (ELISA) for detection of antibodies to synthetic peptides

LHRH decapeptide, individual T helper (Th) peptide domains (UBITH[®] peptides), or adjuvanting peptide from *Yersinia* invasin protein (Inv peptide) were coated on 96-well microtitre plates at 5 µg/ml and dried overnight. Serum samples were serially diluted 10-fold with a starting serum dilution of 1:100. Briefly, 100 µl samples of diluted sera were incubated in the wells for 60–90 min at 37 °C, washed with phosphate buffered saline and incubated for 60 min at 37 °C with horseradish peroxidase (HRP)-conjugated goat or rabbit species-specific immunoglobulin G. HRP-goat anti-rat IgG (Cappel, ICN Biomedicals Inc., Costa Mesa, CA, USA), HRP-rabbit anti-dog IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and HRP-goat anti-human IgG (Fc) for detection of baboon IgG (Anogen, Division of Yes Biotech Laboratories Ltd., Mississauga, Ontario, Canada) were purchased as indicated. The plates were washed again and incubated with either *O*-phenylenediamine (1.67 mg/ml; Sigma, St. Louis, MO, USA) or 3, 3', 5, 5'-tetramethyl benzidine (0.2 mg/ml; Sigma) plus hydrogen peroxide as substrate for 15 min at 37 °C. The enzyme-substrate reaction was stopped by the addition of stop solution (1.0 M sulphuric acid). ELISA titres were quantitated using an automated plate reader at 492 nm (Dynex Technologies, Chantilly, VA, USA).

2.5.2. Radioimmunoassay (RIA) for detection of antibodies to LHRH

The anti-LHRH titre was determined by incubation of ^{125}I labeled LHRH (DuPont Co., Wilmington, DE, USA) with serum samples from rats and dogs at 1:100 dilution and baboons samples at 1:50 dilution. Briefly, serum samples were diluted with 1% bovine serum albumin, mixed (1:1) with radiolabeled-LHRH, and incubated overnight at room temperature. Bovine gamma-globulin (0.5%) and polyethylene glycol (25%) were added, mixed and the tubes centrifuged at 4000 rpm for 25 min at 4 °C. Supernatant was aspirated from each tube and the radiolabeled-immune complex (pellet) was counted (Packard Cobra II-Auto Gamma Model 5010, Meriden, CT). The results were adjusted to mean experimental values and calculated as nmol/l based on standard curve measurements and serum dilution factors.

2.5.3. Radioimmunoassay (RIA) for detection of serum testosterone

Testosterone levels were determined by a standard RIA kit using ^{125}I labeled testosterone (Cat. No. TKTT-500; Diagnostic Products Corp., Los Angeles, CA, USA) using serum samples from rats, dogs and baboons at 1:100 dilution. Testosterone levels were calculated as nmol/l based on standard curve measurements.

2.6. Lymphocyte proliferation analysis

Peripheral blood mononuclear cells from baboons were isolated by Ficoll-hypaque gradient centrifugation and washed three times with sodium phosphate buffered saline. Cells (2×10^5 per well) were cultured in 96-well plates containing RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum. The cells were cultured alone or with individual peptide immunogens (p607E, p667, p500), individual peptide domains (LHRH decapeptide; UBITH[®]4, UBITH[®]5, UBITH[®]6 peptides; Inv peptide) or an unrelated peptide (p1412). On day 6, 1 μCi of ^3H thymidine (^3H -TdR; New England Nuclear, Boston, MA, USA) was added to each of three replicate wells. After 18 h of incubation, cells were harvested with a multiple well harvesting apparatus, and ^3T -TdR incorporation was determined (Packard Tri-Carb Liquid Scintillation Analyzer, Model 1900TR, Meriden, CT, USA). The stimulation index (SI) was calculated by dividing the mean counts per minutes ^3H -TdR incorporated into cells stimulated with 5 $\mu\text{g}/\text{ml}$ of a peptide or peptide domain by the mean counts per minutes ^3H -TdR incorporated into cells cultured with medium alone.

3. Results

3.1. Design of LHRH synthetic peptide immunogens

A diverse array of chimeric LHRH peptide immunogens, each carrying both the target LHRH decapeptide site and

Table 1

Functional domains of UBITH[®] LHRH peptide immunogens

Peptide function	Sequence identity	Sequence ^a
Target epitope	LHRH _{1–10}	EHWSYGLRPG-NH ²
UBITH [®] 4 ^b	HBsAg _{19–33} Th	FLLLRILTIPQSLD
UBITH [®] 5 ^b	MVF _{288–302} Th	LSEIKGVIVHRLEGV
UBITH [®] 6 ^b	TT _{830–844} Th	QYIKANSKFIGITEL
UBITH [®] 7 ^b	PT _{18–41} Th	GAYARCPNGTRALTVAELRGNAEL
Adjuvanting site ^c	Inv _{718–732}	TAKSKKFPSTATYQF

^a Peptides are synthesized with a UBITH[®] sequence covalently linked to the N-terminus of LHRH decapeptide through a glycine–glycine (GG) spacer, except as noted in Table 2.

^b UBITH[®] epitopes are T helper (Th) cell sequences found in several pediatric vaccines: hepatitis B virus surface antigen (HBsAg) [26], measles virus fusion protein (MVF) [27], tetanus toxin (TT) [28], and pertussis toxin (PT) [29].

^c In some cases the N-terminus of the adjuvanting domain from *Yersinia* invasin (Inv) protein [14,30] is added to the N-termini of the UBITH[®] LHRH immunogens through a GG spacer.

a distinct helper T cell epitope, was produced by United Biomedical Inc. The Th epitopes (designated as UBITH[®]) are covalently linked to the anti-LHRH antibody-eliciting epitope (B cell epitope) by continuous solid-phase synthesis. The UBITH[®] domains are promiscuous Th epitopes that stimulate helper T cells of a broad range of histocompatibility backgrounds. The UBITH[®] epitopes used here range from 15 to 24 residues in length and they correspond to Th sequences found in several pediatric vaccines including hepatitis B virus surface antigen (HBsAg_{19–33}) [26]; measles virus fusion protein (MVF_{288–302}) [27]; tetanus toxin (TT_{830–844}) [28]; or pertussis toxin (PT_{18–41}) [29]. A further modification to some of the UBITH[®] LHRH peptides is the addition to the amino-terminus of a domain from *Yersinia* invasin protein (Inv_{718–732}) [30] which has demonstrated significant adjuvanting activity [14]. The individual functional domains of the UBITH[®] immunogens are separated by glycine–glycine (GG) spacers. Table 1 summarizes the individual peptide segments comprising UBITH[®] LHRH peptide immunogens.

3.2. Immunization with UBITH[®] LHRH immunogens blocks testosterone synthesis

Sprague–Dawley male rats were immunized with individual UBITH[®] LHRH immunogens to test their effectiveness to obtain castrate levels of testosterone. Historically, this animal strain has been used to evaluate other testosterone-deprivation therapies [25]. These rats are outbred, express different histocompatibility antigens and are representative of a genetically heterogeneous population.

3.2.1. Rat protocol #1

Four different UBITH[®] LHRH immunogens (p588, p583, p550D, p500) without the *Yersinia* invasin (Inv) adjuvanting peptide and the same four immunogens with the Inv peptide sequence attached (p607, p667, p669, p668) were tested

Table 2
Efficacy study of individual UBITH[®] LHRH immunogens in male rats

UBITH [®] LHRH immunogen ^a	Code name	Animals response per group (n = 5)	
		Antibody level ^b	Testosterone level ^c
Inv-GG ^d -UBITH [®] 4-GG-LHRH	p607 ^e	4/5	3/5
UBITH [®] 4-GG-LHRH	p588	4/5	4/5
Inv-GG-UBITH [®] 5-GG-LHRH	p667	4/5	3/5
UBITH [®] 5-GG-LHRH	p583	0/5	0/5
Inv-GG-UBITH [®] 6-GG-LHRH	p668	1/5	0/5
UBITH [®] 6-LHRH	p500	5/5	3/5
Inv-GG-UBITH [®] 7-GG-LHRH	p669	1/5	1/5
UBITH [®] 7-LHRH	p550D	1/5	1/5
Alhydrogel without peptide (control)	None	0/5	0/5

^a Individual UBITH[®] LHRH immunogens (100 µg per dose) were adsorbed on Alhydrogel and administered at 0, 3, 6 weeks by intramuscular route.

^b Number of animals with anti-LHRH antibody levels above 0.50 nmol/l per group of five animals at 10 weeks following three immunizations.

^c Number of animals with testosterone levels below 0.05 nmol/l per group of five animals at 10 weeks following three immunizations.

^d Some immunogens were synthesized with glycine–glycine (GG) linkers separating the functional domains as indicated.

^e Peptide p607E differs from p607 by a single residue substitution of glutamic acid (E) for aspartic acid (D) in the UBITH[®]4 peptide segment for improved peptide stability.

in groups of five rats to assess the immunopotency of each UBITH[®] LHRH immunogen adsorbed to aluminum hydroxide (Superfos) and administered at 100 µg per dose. The animals were immunized at 0, 3, 6 weeks and serum was collected at 0, 3, 5, 8, 10 weeks for evaluation of anti-LHRH antibody and serum testosterone levels. Six UBITH[®] LHRH immunogens, without or with the Inv peptide, were selected for additional studies based on immunopotency results shown in Table 2. Four UBITH[®] LHRH immunogens (p607, p588, p667 and p500) showed strong immunopotency and two immunogens (p550D, p669) showed anti-LHRH activity in only one of five rats. Two immunogens had minimal or baseline anti-LHRH activity (p583 and p668) and were not evaluated further.

3.2.2. Rat protocol #2

Briefly, groups of five rats were immunized with four UBITH[®] LHRH immunogen mixtures (Mix A, Mix B, Mix C, and Mix #1). Four mixtures of four immunogens, combined in equimolar ratio, were adsorbed on aluminum hydroxide (Superfos) and tested at 25, 50 and 100 µg per dose (Table 3) in order to identify the most immunopotent combination. The animals were immunized at 0, 3, 6 weeks and serum was collected at 0, 3, 5, 8, 10 weeks for evaluation of anti-LHRH antibody and serum testosterone levels. Mix A contained four UBITH[®] LHRH immunogens without the Inv peptide (p588 + p583 + p550D + p500). The composition of the other three mixtures substituted the corresponding peptide covalently linked to the Inv peptide as follows: Mix B substituted p607 for p588; Mix C also substituted p607 and p667 for p583; Mix #1 also substituted p607, p667 and p669 for p550D; and p500 was present in all four mixtures. A two-fold dose escalation permitted the selection of the immunogen mixture with the strongest immunopotency. Specifically, p607 + p667 + p669 + p500 mixture (Mix #1) demonstrated that five of five rats had castrate levels

of testosterone and atrophied testes at all three dose levels and was identified as the preferred mixture. Although neither p550D nor p669 immunogens had striking anti-LHRH titres, when Mix C was compared to Mix #1; it is apparent that p669, with the Inv peptide attached, was preferred to immunogen p550D without Inv. Individual peptide immunogens were ranked for potency based on the number of animals immunocastrated in protocol #1 (Table 2) as well as from additional dose escalation studies of individual LHRH immunogens (data not shown). The immunogen with the strongest anti-LHRH antibody response was p607. The order of anti-LHRH immunopotency for individual immunogens was p607 ≥ p667 > p500 >> p669.

3.2.3. Rat protocol #3

Two groups of 12 rats were also immunized at 0, 3, 6 weeks with UBITH[®] LHRH Mix #2 consisting of three immunogens (p607E, p667, p500) combined in equimolar ratio at 25 and 100 µg per dose and adsorbed on aluminum hydroxide (Alhydrogel). Serum was collected at 0, 3, 5, 8, 11, 16, 20 weeks for evaluation of anti-LHRH antibody and serum testosterone levels. At 11 weeks, all rats had castrate levels of testosterone at both dose levels. At 20 weeks, 11 of 12 rats at 100 µg dose and 9 of 12 rats at 25 µg dose level remained castrated. The p669 immunogen was removed from UBITH[®] LHRH Mix #1 because of poor peptide stability. The Mix #2 immunogens demonstrated improved immunopotency and were used in vaccines for the non-human primate studies.

3.3. Immunization with UBITH[®] LHRH immunogens prevents prostate tumor growth

The Dunning R3327-H rat tumor model was used to test the immunotherapy for efficacy by showing the effect of the LHRH vaccine on the growth of an androgen-dependent

Table 3
Efficacy study of UBITH[®] LHRH peptide immunogen mixtures in male rats

UBITH [®] LHRH immunogen mixtures ^a	Dose (μg)	Number of animals per total animals in group		
		Antibody level ^b	Testosterone level ^c	Testes status ^d
UBITH [®] Mix A p588 + p583 + p550D + p500	100	4/5	3/5	2/5
	50	3/5	1/5	1/5
	25	0/5	0/5	0/5
UBITH [®] Mix B p607 + p583 + p550D + p500	100	5/5	5/5	4/5
	50	5/5	5/5	4/5
	25	3/4	3/4	2/4
UBITH [®] Mix C p607 + p667 + p550D + p500	100	5/5	5/5	5/5
	50	5/5	5/5	3/5
	25	4/5	3/5	3/5
UBITH [®] Mix #1 p607 + p667 + p669 + p500	100	5/5	5/5	5/5
	50	5/5	5/5	5/5
	25	5/5	5/5	5/5
UBITH [®] Mix #2 p607E + p667 + p500	100	12/12	12/12	12/12
	25	12/12	12/12	12/12
None (Alhydrogel only)	0	0/5	0/5	0/5

^a Mixtures of UBITH[®] LHRH immunogens (25–100 μg per dose as indicated) were adsorbed on Alhydrogel and administered at 0, 3, 6 weeks by intramuscular route.

^b Number of animals with anti-LHRH antibody levels above 0.50 nmol/l per total number of animals tested per group ($n = 5$) at 10 weeks following three immunizations.

^c Number of animals with testosterone levels below 0.05 nmol/l per total number of animals tested per group at 10 weeks following three immunizations.

^d Number of animals with atrophied testes at 10 weeks per total number of animals evaluated per group. Atrophied testis weights were less than 25% of the mean weight of the control group. All animals immunized with Mix #2 had atrophied testes at 10 weeks but were followed for an additional 10 weeks to monitor duration of response.

prostate tumor. Copenhagen male rats were each implanted subcutaneously with 1 mm³ fragments of Dunning H tumor at week 0 and they were divided into three groups of eight animals each.

3.3.1. Prophylaxis model

Copenhagen male rats in Group 1 implanted with Dunning H tumors were immunized with 100 μg total peptide per dose of UBITH[®] LHRH immunogen Mix #1 adsorbed on Alhydrogel at 0, 3, 6 weeks and then boosted at 26 and 58 weeks post-tumor implantation when testosterone levels became detectable in serum. Rats in Group 2 implanted with Dunning H tumors were also immunized at 0, 3, 6, 26, 58 weeks with Alhydrogel alone. Blood was collected by tail vein cannulation under light anesthesia at 2 week intervals for assays. Tumor growth was followed with tumor measurements every 2 weeks. Androgen ablation was monitored for serum testosterone and palpation for testes re-growth, and anti-LHRH antibody titres were determined by RIA (data not shown). Tumors did not appear in any of the eight animals receiving the LHRH vaccine (Group 1) when compared to the eight control animals (Group 2) as illustrated in Fig. 1 (left panel). The mean tumor size represents the mean of the volumes of all nodules in all animals in each group. At the end of the study (120+ weeks), approximately 20 weeks after the testosterone levels started to increase in the Group 1 rats, small tumors of palpable size appeared

and continued to grow in two of the remaining five animals. Testosterone levels in the other three animals remained less than 0.5 nmol/l and tumors of palpable size were not noted at the end of the study.

3.3.2. Therapeutic Model

Copenhagen male rats in Group 3 were implanted subcutaneously with Dunning H tumor pieces (1 mm³ size) and the tumors were permitted to grow until they reached a volume of approximately 0.4 cm³ at which time the animals were immunized with 100 μg total peptide per dose of UBITH[®] LHRH immunogen Mix #1 adsorbed on Alhydrogel at 26, 29, 32 weeks post-tumor implantation, and later boosted at 58 and 82 weeks. Blood was collected for determinations of anti-LHRH and testosterone levels every 2 weeks. Note that tumors started to appear by approximately 20 weeks and by 30 weeks remained at approximately 0.4 cm³ (mean tumor volume) throughout the study period in the eight animals receiving Mix #1 in Alhydrogel in Group 3 in comparison to the rapidly growing tumors in the eight Group 2 control animals receiving the Alhydrogel alone as illustrated in Fig. 1 (right panel). At the end of the study, testosterone levels were permitted to rebound and, as expected, the androgen-sensitive Dunning H tumors started to grow again in 4 of 5 of the remaining animals. Tumor re-growth was not noted in one animal that appeared to remain castrated by the prior LHRH immunization.

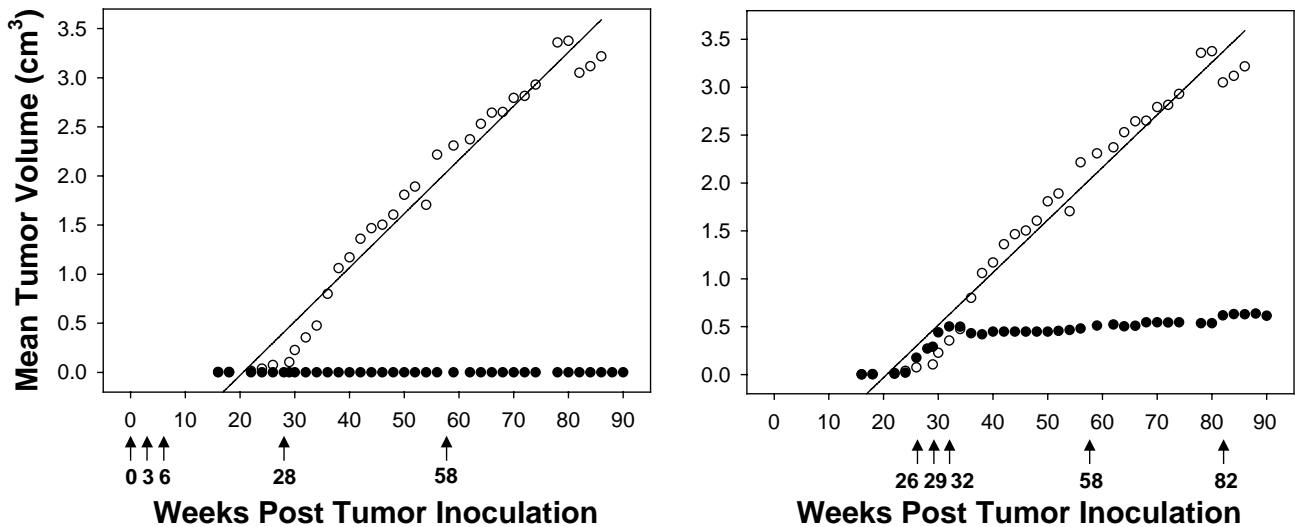


Fig. 1. Proof of concept using Dunning R3327-H rat tumor model. Three groups of Copenhagen male rats ($n = 8$) were implanted subcutaneously with a small fragment of Dunning H tumor (as described in Section 2.4) at week 0. In the Prophylaxis Model (left panel), Group 1 (solid circles) rats were immunized with UBITH[®] LHRH immunogen Mix #1 beginning at 0, 3, 6, 26, 58 weeks after tumor implant and compared with control Group 2 (open circle symbol) rats immunized with Alhydrogel alone at the same schedule. In the Therapeutic Model (right panel), Group 3 (solid circles) rats were immunized with UBITH[®] Mix #1 beginning at 26, 29, 32, 58, 82 weeks after tumor implant and compared with control Group 2 rats. Tumor growth was measured every 2 weeks and mean tumor volume was calculated. The priming and booster immunizations are indicated by arrows.

3.4. Efficacy studies of UBITH[®] LHRH immunogens in sexually mature male dogs

Intact young adult mongrel and pure-bred male dogs were immunized with 400 μg of UBITH[®] LHRH immunogen Mix #1 adsorbed on Alhydrogel at 0, 3, 6 weeks (Group 1) or formulated in Emulsigen containing DDA at 0, 3, 6 weeks (Group 2). Placebo control intact dogs were immunized with either Alhydrogel alone or Emulsigen/DDA delivery system alone (Group 3). Castrated control dogs did not receive any vaccines (Group 4). Blood samples were collected from all animals at 0, 3, 6, 8, 10, 12, 16, 20 weeks for determination of anti-LHRH titres and testosterone levels in serum. By week 6, significant levels of anti-LHRH antibodies were observed in six of seven animals in Group 1 (data not shown) and in all eight animals in Group 2 (Fig. 2). The potent LHRH antibody responses caused a concomitant decrease in serum testosterone in the treated animals. By week 6, serum testosterone was at castration levels (<0.05 nmol/l) in six of seven animals immunized with UBITH[®] LHRH immunogens adsorbed to Alhydrogel (Group 1) and in all eight dogs receiving the Emulsigen/DDA formulation (Group 2) and they remained at this level through at least week 12 after which time testosterone levels started to rebound in some animals in Group 1. The failure to castrate one of seven dogs in Group 1 is attributable to the lack of a sustained LHRH antibody response in this animal. Antibody responses in dogs parallel those observed in rats, where LHRH-specific antibody titres of 0.5 nmol/l must be maintained for several weeks to achieve castration. Castrate levels of testosterone were noted in one of seven dogs in Group 1 and in all

eight dogs in Groups 2 at week 20 (Fig. 2). Intact control dogs (Group 3) and surgically castrated dogs (Group 4) did not have detectable anti-LHRH activity. All animals in the placebo control Group 3 had detectable testosterone levels throughout the study period while the surgically castrated dogs of Group 4 had castrate levels as expected (data not shown).

At week 20, the animals were sacrificed and their relevant reproductive organs (i.e. testes and prostate glands) were dissected and weighed. The mean organ weights of the vaccine-treated dogs were compared to the mean organ weights of the placebo control dogs. Data from the non-responsive animal in Group 1 were not included for the mean weight calculation. To correct for differences in the sizes of the animals, organ weights were expressed as a fraction of the total body weight of the originating animal. The mean organ weights for the placebo control Group 3 was set at 100%. The mean weights of the testes and prostate glands for Group 1 were 43.7 and 30.3%, respectively, and for Group 2 they were 21.8 and 22.5%, respectively. The increased organ size in Group 1 is related to the rebound of testosterone in most animals at 20 weeks when compared to Group 2 where all animals maintained castrate levels of testosterone. The consistent efficacy in the dogs of the Group 2 oil-in-water formulation over the Group 1 Alhydrogel formulation suggested that a more immunopotent vaccine delivery system would be necessary for the UBITH[®] LHRH peptide immunogens to attain efficacy in higher animals. The following non-human primate studies were evaluated with water-in-oil emulsion-based delivery systems.

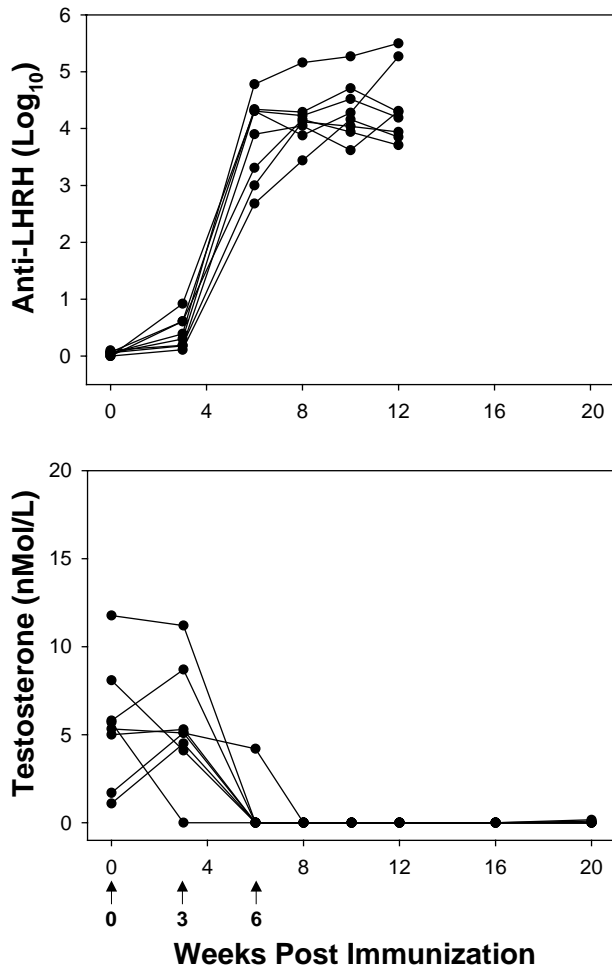


Fig. 2. Efficacy study in intact male dogs. Dogs were immunized at 0, 3, 6 weeks (indicated by arrows) with 400 μ g total peptide per dose of UBITH[®] LHRH immunogen Mix #1 formulated in Emulsigen containing DDA (Group 2). Animals were bled at 0, 3, 6, 8, 10, 12, 16, and 20 weeks and tested for anti-LHRH antibody levels (upper panel) and testosterone levels (lower panel). Each solid black symbol represents an individual dog; the anti-LHRH titres were sufficient to effect androgen deprivation in all dogs at 20 weeks (end of study).

3.5. Efficacy studies of UBITH[®] LHRH immunogens in adult male baboons

3.5.1. Baboon protocol #1

Three adult male baboons (*Papio cynocephalus*), 6–14 years of age, were immunized with UBITH[®] LHRH immunogen Mix #2 (p607E + p667 + p500) at 400 μ g total peptide per dose formulated in either of three different water-in-oil emulsion vaccine delivery systems containing monophosphoryl lipid A as adjuvant (Table 4). The immunization schedule was 0, 3, 6, 16 weeks. High titered anti-LHRH antibody was noted to support immunocastration by 6 weeks in the three baboons (#7446, #7663, #8010) receiving emulsion-based formulations. Duration of androgen deprivation was observed for at least 40 to >52 weeks depending on the formulated emulsion used (Fig. 3). One

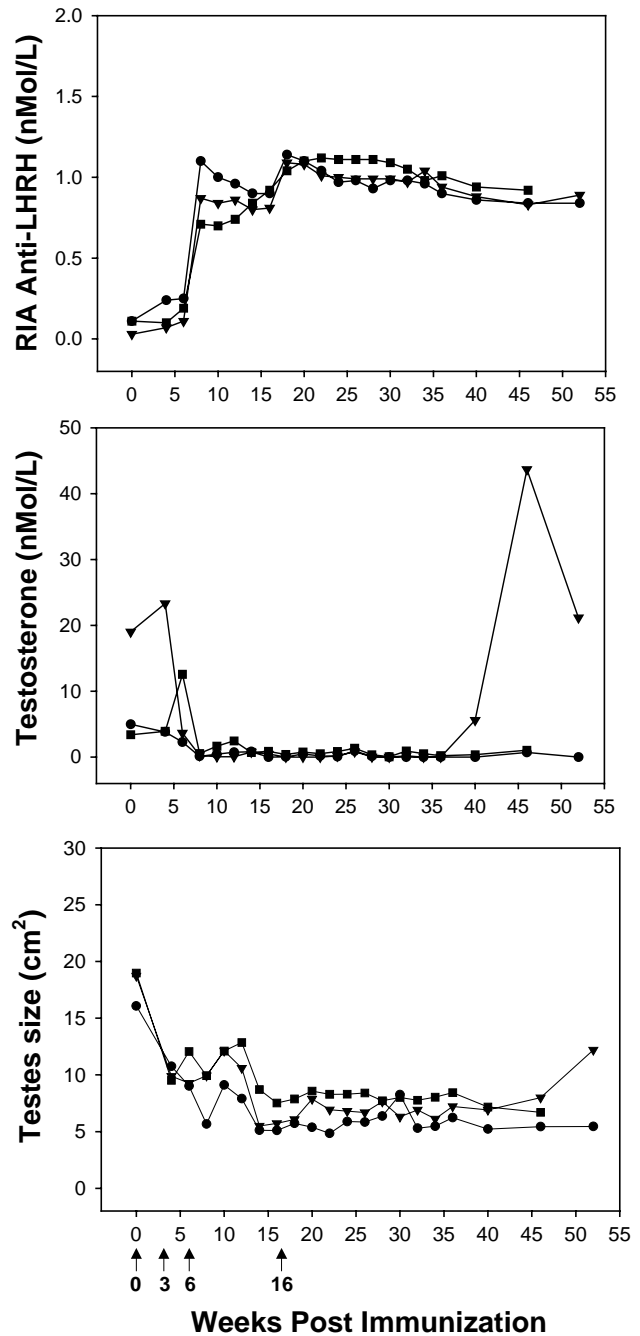


Fig. 3. Efficacy study in adult male baboons. In protocol #1, individual baboons were immunized at 0, 3, 6, 16 weeks (indicated by arrows) with UBITH[®] LHRH immunogen Mix #2 at 400 μ g formulated in Montanide[®] ISA 720 (black circles, baboon #7446), in Montanide[®] ISA 206 (black triangles, baboon #7663) or in Montanide[®] ISA 51 (black squares, baboon #8010), each containing monophospholipid A. Baboons were bled at week 0, bimonthly from 4 to 36 weeks and then at 4–6 week intervals until 52 weeks and tested for anti-LHRH levels by radioimmunoassay (upper panel), serum testosterone levels (middle panel), and testes size measurement (lower panel).

Table 4

Site-directed immunoreactivity of immune baboon serum samples to LHRH immunogen Mix #2^a

LHRH immunogens (400 µg per dose)	Delivery system	Animal no.	Antibody titre (log ₁₀) ^b			Testosterone level (nmol/l) ^c	
			LHRH	UBITH [®] 4	UBITH [®] 5		UBITH [®] 6
UBITH [®] Mix #2 (p607E + p667 + p500)	ISA 720	7446	4.50	0.16	0.14	0.14	0.0
	ISA 206	7663	3.56	0.18	0.18	0.15	0.0
	ISA 51	8010	4.50	0.35	0.38	0.87	0.0
	Squalene	7052	2.33	0.13	0.14	0.15	13.7

^a Solid-phase enzyme-linked immunoassay (ELISA) tests of individual peptide domains are described in Section 2.5. Serum samples at week 10 are taken from baboon protocol #1 and tested at 10-fold serial dilutions from starting dilution 1:100.

^b Results are expressed as log₁₀ ELISA titre of anti-peptide antibody to each domain at 10 weeks. Pre-bleed ELISA titre results at week 0 are between 0.12 and 0.26 for all samples tested. Anti-LHRH antibody titres of >1:10³–1:10⁴ are detected. Anti-UBITH[®] antibody titres are all negligible. Anti-Inv peptide reactivity was negative or weakly positive after three immunizations but returned to background levels (titre <1:10²) within 1 month after the third immunization.

^c Serum testosterone deprivation, measured by radioimmunoassay, is noted at 8 weeks in all three baboons (#7446, #7663, #8010) immunized with Mix #2 immunogens formulated in Montanide[®] oil-based emulsions containing adjuvant but not in the baboon (#7052) immunized with Mix #2 immunogens formulated in squalene with adjuvant.

baboon (#7052), immunized with 400 µg per dose of Mix #2 immunogens in a squalene-based adjuvant suspension, developed low anti-LHRH antibody levels and maintained normal levels of testosterone. In the three baboons with castrate levels of testosterone, three clinical endpoints were monitored: reversibility, duration of androgen deprivation and re-growth of testes. Androgen rebound was noted in one of three baboons at 40 weeks (#7663) and detection of serum testosterone preceded observable re-growth of testes by approximately 10 weeks. Castrate levels of testosterone were maintained in the other two baboons at 52 weeks (end of study).

3.5.2. Baboon protocol #2

Six adult male baboons were immunized with UBITH[®] LHRH immunogen Mix #2 at 25 µg (#8477, #8616), 100 µg (#8617, #8995) or 400 µg (#8518, #9186) per 0.5 ml dose of total peptide formulated in Montanide ISA 51 emulsion containing DDA. The immunization schedule of this dose escalation study was 0, 4, 34 weeks and blood was collected at 2 week intervals to week 52 and then monthly until week 95 (end of study). The baboons were followed for anti-LHRH antibody levels, duration of androgen deprivation and testes involution and re-growth. Results from representative baboons at 100 µg (#8517) and 400 µg (#8518) dose levels through week 95 are shown in Fig. 4. After the two priming doses, the level of anti-LHRH antibody was at least 0.5 nmol/l in all six baboons at 6 weeks and castrate levels of testosterone were noted in all animals. In four of six baboons (#8517, #8518, #8615, #8995), testosterone rebound was observed around 22–24 weeks and testes re-growth was noted at 26–28 weeks. In the other two baboons (#8477, #9186), testosterone rebound was noted following decreases in anti-LHRH levels below 0.5 nmol/l.

At 34 weeks post-initial immunization, the six baboons received booster injections of the UBITH[®] LHRH immunogen Mix #2 in Montanide[®] ISA 51 containing DDA at the respective dose levels. All six baboons had castrate levels of

serum testosterone when tested 2 weeks after the boost. Two baboons showed rebound of testosterone (#8477, #9186) whereas four baboons maintained castrate levels of testosterone at 95 weeks (end of study). Organ re-growth occurred approximately 1 month after the testosterone rebound in two animals. The testes remained atrophied in the other four baboons and were observed to be 24–36% of the original organ sizes as calculated by cross-sectional area.

3.6. Proof of concept for UBITH[®] LHRH immunogen design

The LHRH decapeptide, UBITH[®] peptides, and Inv peptide domains from the three UBITH[®] LHRH immunogens in Mix #2 were synthesized as unlinked peptide segments, purified and then coated onto separate microtitre plate wells. Results by ELISA using serum samples at week 10 from the four baboons in protocol #1 demonstrated that the LHRH decapeptide was highly immunogenic and the T cell helper peptides or UBITH[®] epitopes were not immunoreactive with the baboon antibodies in the three baboons immunized with water-in-oil emulsions (Table 4). The Inv peptide showed a transient low-titred reactivity in serum samples from some animals immediately following the boost injection at 16 wpi in protocol #1 but returned to baseline within 4 weeks of the boost injection. Results from baboon protocol #2 also revealed predominant anti-LHRH peptide reactivity for all six animals immunized with UBITH[®] LHRH immunogen Mix #2 emulsions; the UBITH[®] peptides and Inv peptide were not immunoreactive by ELISA tests when animals were immunized two times at 0 and 4 weeks (data not shown).

To demonstrate specific T helper cell immunogenicity for the UBITH[®] epitope, mononuclear blood cells collected and purified at 0, 4, 6, 12 and 14 weeks from all six animals in baboon protocol #2 were co-cultured with individual peptides (data not shown) or peptide domains of the three UBITH[®] LHRH immunogens in Mix #2 to access lymphocyte stimulation in vitro. Lymphocyte proliferation studies demon-

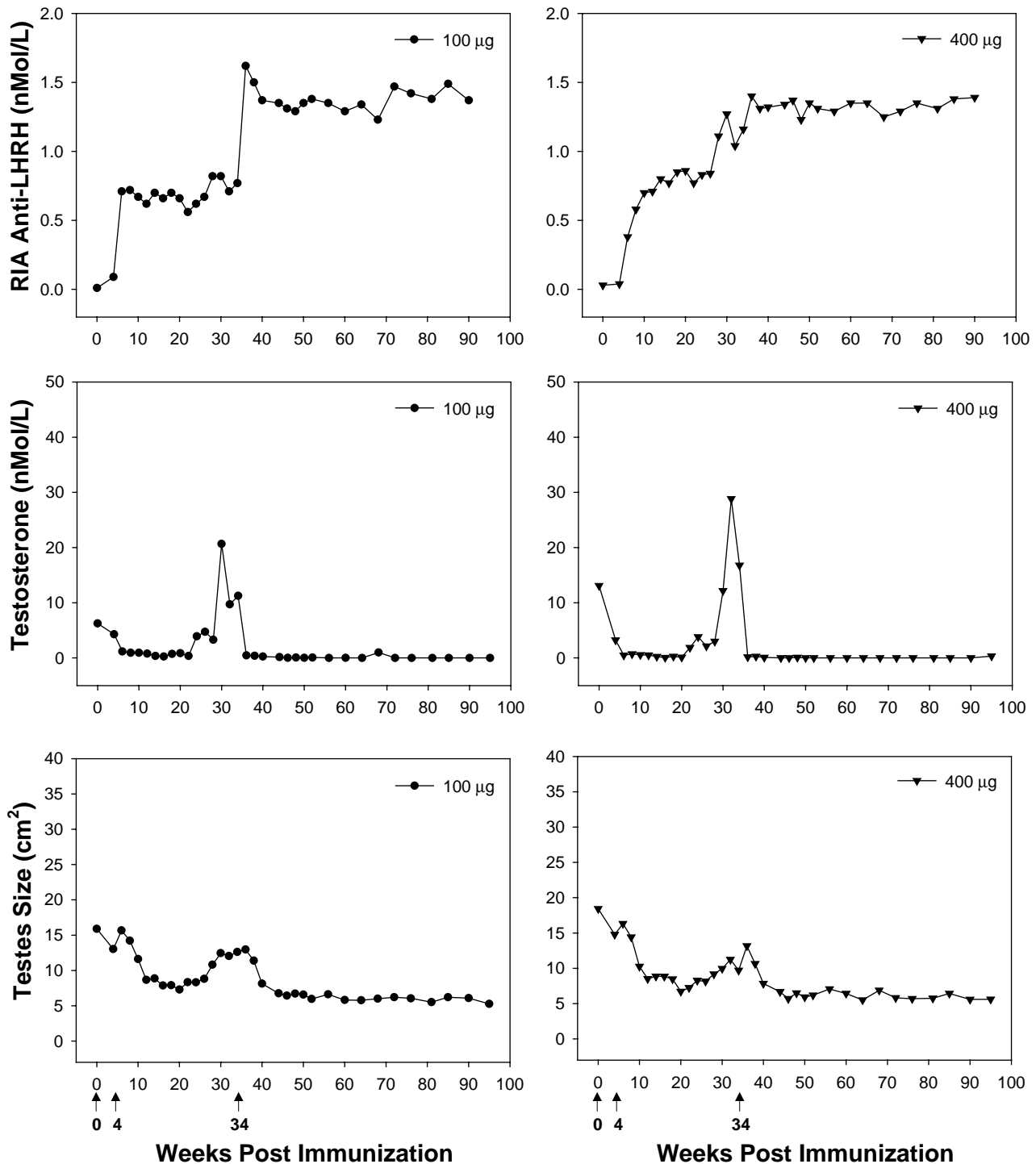


Fig. 4. Efficacy study in adult male baboons. In protocol #2, individual baboons were immunized at 0, 4, 34 weeks (indicated by arrows) with UBITH[®] LHRH immunogen Mix #2 at 100 µg per dose (black circles, baboon #8995, left panels) or at 400 µg per dose (black triangles, baboon #8518, right panels) formulated in Montanide[®] ISA 51 containing DDA. Animals were bled at week 0, bimonthly from weeks 4 to 52 weeks and then monthly to 95 weeks and evaluated for anti-LHRH by radioimmunoassay (upper panels), serum testosterone levels (middle panels) and testes size (lower panels).

strated that specific UBITH[®] T cell helper peptides were each strongly stimulatory in this assay system in baboons receiving immunogens at all three dose levels. Table 5 tabulates the stimulation index when each peptide domain is incubated with lymphocyte cultures from two representative

baboons (#8995, #8518). The LHRH decapeptide, Inv peptide, and an unrelated control peptide did not stimulate any of the baboon lymphocyte cultures regardless of the LHRH immunogen dose used for the immunizations. In contrast, the three UBITH[®] peptide segments were stimulatory in vitro

Table 5
Site-directed lymphocyte stimulation of immune baboon samples by individual peptide domains

LHRH immunogens and delivery system	Animal # (dose)	Weeks post-initial injection ^a	Stimulation index ^b						Testosterone level (nmol/l) ^c
			LHRH	UBITH [®] 4	UBITH [®] 5	UBITH [®] 6	Inv Peptide	Non-relevant Peptide	
UBITH [®] Mix #2 (p607E + p667 + p500) + Montanide [®] ISA51 + DDA	8995 (100 µg)	0*	0.9	1.8	0.7	1.1	1.7	1.9	2.2
		4*	1.0	9.9	11.0	19.2	2.3	1.3	2.8
		6	2.4	5.4	5.9	19.1	1.4	0.4	1.5
		12	1.0	1.8	2.0	12.6	1.3	1.0	0.0
		14	1.6	1.9	1.4	24.7	1.6	0.7	0.0
	8518 (400 µg)	0*	0.0	0.6	0.1	0.1	0.3	0.1	13.1
		4*	1.3	1.5	1.5	4.1	0.6	0.9	3.2
		6	0.4	4.4	2.2	15.4	0.9	0.3	0.0
		12	0.8	5.9	2.8	3.7	2.3	0.8	0.0
		14	0.6	3.7	0.9	7.1	1.6	0.7	0.0

^a Serum samples from baboons immunized at 0, 4 weeks by intramuscular route are indicated by asterisk (*).

^b Lymphocyte stimulation assay is described in Section 2.6. Stimulation index is calculated from mean count values of triplicate cell cultures in the presence of synthetic peptide domains and divided by the mean count values of cultures without peptides added. Positive stimulation index is >3.0 (boldface results).

^c Serum testosterone deprivation, measured by radioimmunoassay, is noted between weeks 6 and 12 in baboons (#8995, #8518) immunized with Mix #2 immunogens formulated in Montanide[®] oil-based emulsions containing DDA.

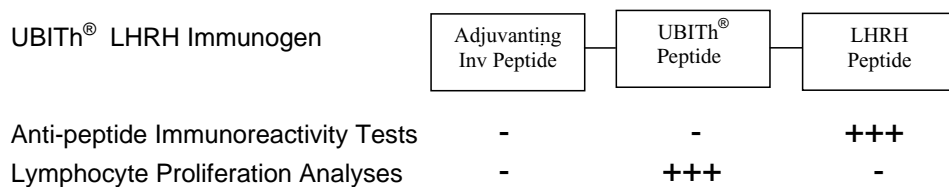


Fig. 5. Prototype UBITH[®] LHRH synthetic peptide immunogen. The LHRH decapeptide is synthesized with a helper T cell (Th) domain (UBITH[®] epitope) covalently linked to the amino terminus of LHRH. These UBITH[®] epitopes mimic epitopes found in several pediatric vaccines. A further modification to some UBITH[®] LHRH immunogens is the addition to the amino-terminus of a domain from *Yersinia* invasin protein. High levels of anti-LHRH antibodies were detected in immune sera from samples collected at week 10 but no appreciable levels of anti-UBITH[®] antibodies (Table 4). Conversely, UBITH[®] peptides stimulated proliferation of cultured T lymphocytes from samples collected at 4, 6, 12, 14 weeks after immunizations at 0 and 4 weeks but not the LHRH decapeptide antibody target or the immunostimulatory Inv peptide (Table 5).

and lymphocyte cultures from each baboon displayed different patterns of response. Cultures from all six baboons were strongly stimulated by UBITH[®]6 (TT_{830–844}) peptide; they were also moderately or weakly stimulated by either UBITH[®]4 (HBsAg_{19–33}) or UBITH[®]5 (MVF_{288–302}) peptides or both peptides. Review of the clinical history of the baboons at Southwest Foundation indicated that these animals had not received prior TT vaccines. Fig. 5 schematically summarizes these observations and the proof of concept design for UBITH[®] LHRH immunogens.

4. Discussion

An effective and practical synthetic peptide-based immunotherapeutic vaccine for treatment of androgen-responsive prostate cancer has been designed and tested in rats, dogs and baboons. The mode of action for the immunotherapeutic is androgen deprivation of the androgen-dependent prostate cancer [2]. The LHRH/GnRH immunotherapeutic comprises a mixture of UBITH[®] LHRH peptide immuno-

gens either adsorbed on aluminum hydroxide (Alhydrogel) or formulated with adjuvants as water-in-oil emulsions. The studies in rodents and dogs used four different UBITH[®] LHRH immunogens (Mix #1) and studies in adult male baboons used an improved mixture of three UBITH[®] LHRH immunogens (Mix #2). The design elements of each immunogen include the LHRH decapeptide as the B cell target epitope, a UBITH[®] epitope for T cell help, and in some cases, an adjuvanting Inv peptide from *Yersinia* invasin protein. The LHRH immunogens are formulated as mixtures in order to maximize the immune response in individual animals. These studies demonstrated efficacy, universal responsiveness, and long duration of castrate levels of testosterone as well as proof of concept for the immunogen design.

Individual UBITH[®] LHRH immunogens, each carrying a distinct T helper cell epitope from protein antigens found in pediatric vaccines, have been identified and shown to be effective in generating anti-LHRH antibody levels sufficiently high (>0.5 nmol/l) to effect androgen deprivation in rats, dogs and baboons as illustrated in this report. Efficacy was readily noted in rodents with our individual UBITH[®]

LHRH peptide immunogens adsorbed on aluminum hydroxide (Tables 2 and 3). However, formulation of synthetic peptides in a safe and effective delivery system for human application has been challenging. Results presented in the dog protocol illustrate that mineral salt-based formulations (e.g. Alhydrogel) were transiently effective but not durable enough to sustain an anti-LHRH response whereas, addition of an adjuvant (e.g. DDA) to oil-based emulsions were 100% effective in dogs for at least 20 weeks (end of study). In baboon protocol #1, emulsion-based formulations enhanced the effectiveness of the anti-LHRH response (Table 4). In baboon protocol #2, the effective UBITH[®] LHRH immunogen dose was decreased by including the DDA adjuvant in the emulsion-based formulations. These studies demonstrate that emulsions can effectively support long-term duration of a specific anti-LHRH peptide response and also specifically stimulate responsiveness to UBITH[®] helper T cell epitopes (Table 5). These results are proof of efficacy for the product design.

The Dunning R3327-H tumor model for androgen-responsive prostate cancer in Copenhagen rats has permitted the efficacy of the UBITH[®] LHRH immunogens for hormone-deprivation therapy to be tested and validated. The immunotherapy was 100% effective in preventing development of the androgen-dependent Dunning H tumors in the Prophylaxis Model and in arresting the growth of established tumors of palpable size in the Therapeutic Model as long as testosterone levels were suppressed to castrate levels by anti-LHRH antibody at serum levels that were sufficient to neutralize circulating LHRH. The Dunning H rat tumor experiment extended over 2 years in duration. It is of particular relevance to clinical applications that the Dunning H tumors did not grow in the androgen-deficient environment evoked by the immunotherapy. This is in contrast to early observations of Isaacs et al. [25] who measured the effects of surgical castration on the growth of Dunning H tumors and observed delayed growth of tumor implants in castrated animals when compared to control animals; tumors continued to grow in the androgen-independent environment with a time delay. These results suggested the presence of a local LHRH loop within the rat prostate and Dunning H tumors, in addition to the LHRH loop of the pituitary–gonadal axis. Additional studies support a local LHRH loop in the prostate. Reddy et al. [31] reported direct inhibitory action of LHRH on the accessory organs in the rat. Srkalovic et al. [32] demonstrated LHRH receptors and their modulation by LHRH antagonists on Dunning H tumor cells. Fuerst et al. [33] demonstrated that active immunization with an LHRH-diphtheria toxoid conjugated vaccine leads to down-regulation of gonadotropins and testosterone and atrophy of the testes and prostate glands. Growth inhibition of the androgen-sensitive Dunning R3327-PAP tumor by this LHRH–DT conjugated vaccine was shown to be caused by suppression of cell division rather than increase in tumor cell death [33].

In our study, control of tumor growth of the androgen-dependent tumor cell population was controlled for over 2

years by anti-LHRH antibody that effectively suppressed testosterone synthesis. When anti-LHRH antibody levels were sufficiently diminished and a testosterone rebound was noted, this was followed by re-growth of quiescent Dunning H tumor cell populations. At the end of the study, small tumors became evident in two of five animals remaining in the early LHRH-immunized Group 1 and continued tumor cell growth was notable in the established tumors in four of five animals remaining in the late LHRH-immunized Group 3. These observations confirm viability of the tumor implants after remaining quiescent in vivo for over 2 years. The results of these rodent studies also have important implications for the clinical situation where conventional androgen-deprivation therapy suppresses prostate cancer growth rather than destroying tumor cells.

The hormone deprivation action of the UBITH[®] LHRH immunogen vaccines in blocking testosterone synthesis has several applications. In a clinical setting, LHRH immunotherapy is a treatment option for androgen-responsive prostate cancer or for other hormone-responsive benign tumors (e.g. endometriosis, uterine fibroids, etc.) presently treated by LHRH agonists [34]. In companion animals, LHRH immunotherapy can be used for immunocontraception [9], for reversible behavior modification or for treatment of benign prostatic hyperplasia (BPH) or androgen-dependent prostatic intraepithelial neoplasia (PIN) in elderly sexually intact dogs whose owners do not elect to surgically castrate [35]. The prevalence of BPH or PIN in dogs is apparently influenced by age and presence of testicular androgens as in humans. In the swine industry [36,37], the LHRH vaccine can be used for removal of androstenediones or boar taint, known to cause off-flavor of the pork from boars, as well as for growth promotion.

The LHRH secreted from the hypothalamus stimulates the anterior pituitary to secrete LH and FSH that is required for normal spermatogenesis. Prolonged treatment with LHRH agonists desensitizes the LHRH receptors and results in suppression of gonadotropins and spermatogenesis [38,39]. In our two baboon protocols, castrate levels of testosterone were maintained for at least 1 year in six of nine baboons. The reason for continued androgen deprivation in these animals is not known but could be due to suppression of LHRH receptors or loss of Leydig cells, as reported after extended use of LHRH agonists in non-human primates [40] and after 2 years of LHRH agonist therapy in humans [41], or continued high levels of effective anti-LHRH antibody. However, androgen rebound was observed in three of nine animals despite the presence of elevated anti-LHRH antibody in serum. In protocol #1, hormonal rebound was noted after 34 weeks of castration in baboon #7663 and in protocol #2, after the 34 week booster immunization, rebound was noted after another 36 weeks in baboon #8477 and after only 16 weeks in baboon #9186. Several reasons may account for the hormonal rebound noted, including immunogen dose, emulsion formulation, reduced anti-LHRH antibody level and/or younger age. Experiments in baboons (*Papio hamadryas*), ranging in

age from 6 to 15 years, showed that animal to animal variation in response to hormonal stimuli differed in relation to the basal LH concentrations and to the pituitary–gonadal response to administration of LHRH agonists rather than to the age of the animal, although degenerative changes were noted in testes in aging baboons [42].

The LHRH/GnRH immunotherapy for androgen-responsive prostate cancer has a definable endpoint (testosterone level) to evaluate vaccine efficacy, which is distinct from the markers to assess cancer progression (e.g. PSA level). In one candidate LHRH/GnRH vaccine, the LHRH decapeptide was modified by replacement of glycine at position 6 with D-lysine linked through a spacer to diphtheria toxoid and adsorbed on Alhydrogel [3]. The immunotherapy was delivered by intramuscular route at 0 and 1 month later. The authors did not indicate how many patients generated sufficiently high antibody titres to benefit clinically by androgen deprivation and a fall in the level of prostate-specific antigen (PSA). In another candidate LHRH/GnRH immunotherapy that has achieved variable success in a phase 1 clinical trial, the active ingredient, D17DT, is an immunogen consisting of a GnRH decapeptide linked through its amino-terminus via a spacer to DT and formulated as a water-in-oil emulsion [6]. The authors reported clinical response was related quantitatively to antibody production. The four patients out of the group of 11 with significant anti-GnRH responses also had the best results in terms of suppression of both testosterone and PSA levels and the duration of androgen deprivation was 6 months or longer. The authors proposed that one reason for lack of response in some patients may be due to epitopic suppression when the host is exposed to an immunogenic dose of a recall carrier antigen (e.g. DT) and ineffectual immunization may occur. In our studies, the UBITH[®] LHRH immunogen Mix #2 was formulated in water-in-oil emulsions and shown to be fully immunogenic and efficacious in a non-human primate, the baboons (Figs. 3 and 4). Most recently, the UBITH[®] LHRH immunogen Mix #2 has been reformulated in a proprietary immunostimulatory complex adsorbed on Alhydrogel and in addition, has a milder adjuvanting system for clinical use. Recent studies have shown the new immunogen Mix #2 formulation to be efficacious and non-reactogenic in baboons (unpublished observations). The advantages of the UBITH[®] LHRH immunotherapy are: (1) the antibody response is site-specific, directed to the LHRH decapeptide and not to the UBITH[®] and Inv peptide domains, and (2) the use of multiple promiscuous T cell helper epitopes avoids the consequences of epitopic suppression and will elicit a more universal response as shown by our studies in primates.

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References

- [1] Labrie F, Dupont A, Belanger A, St-Arnaud R, Giguere M, Lacourciere Y, et al. Treatment of prostate cancer with gonadotropin-releasing hormone agonists. *Endocrinol Rev* 1986;7:67–74.
- [2] Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* 1941;1:293–7.
- [3] Talwar GP. Vaccines for control of fertility and hormone-dependent cancers. *Immunol Cell Biol* 1997;75:184–9.
- [4] Gittes RF. Carcinoma of the prostate. *N Eng J Med* 1991;324:236–45.
- [5] Leuprolide Study Group. Leuprolide versus diethylstilbestrol for metastatic prostate cancer. *N Eng J Med* 1984;311:1281–6.
- [6] Simm MS, Scholfield DP, Jacobs E, Michaeli D, Broome P, Humphreys JE, et al. Anti-GnRH antibodies can induce castrate levels of testosterone in patients with advanced prostate cancer. *Br J Cancer* 2000;83:443–6.
- [7] Gual C, Garza-Flores J, Menjivar M, Gutierrez-Najar A, Pal R, Talwar GP. Ability of an anti-luteinizing hormone-releasing hormone vaccine to inhibit gonadotropins in postmenopausal women. *Fertil Steril* 1997;67:404–7.
- [8] Silversides DW, Murphy BD, Misra V, Mapletoft RJ. Monoclonal antibodies against LHRH: development and immunoactivity in vivo and in vitro. *J Reprod Immunol* 1995;7:171–84.
- [9] Ladd A, Tsong Y-Y, Walfield AM, Thau R. Development of an antifertility vaccine for pets based on active immunization against luteinizing hormone-releasing hormone. *Biol Reprod* 1994;51:1076–83.
- [10] Ladd A, Walfield AM, Tsong Y-Y, Thau R. Active immunization against LHRH alone or combined with LHRH-analogue treatment impedes growth of androgen-dependent prostatic carcinoma. *Am J Reprod Immunol* 1995;34:200–6.
- [11] Beekman NJ, Schaaper WM, Turkstra JA, Meloen RH. Highly immunogenic and fully synthetic peptide-carrier constructs targeting GnRH. *Vaccine* 1999;17:2043–50.
- [12] Schutze M-P, Leclerc C, Jolivet M, Audibert F, Chedid L. Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. *J Immunol* 1985;135:2319–22.
- [13] Etlinger HM, Gillessen D, Lahm HW, Matile H, Schönfeld HJ, Trzeciak A. Use of prior vaccinations for the development of new vaccines. *Science* 1990;249:423–5.
- [14] Ladd AE, Wang CY, Zamb TJ. Immunogenic LHRH peptide constructs and synthetic universal immune stimulators for vaccines. US Patent No. 5,759,551 (1998).
- [15] Wang CY. Artificial T helper cell epitopes as immune stimulators for synthetic peptide immunogens including LHRH peptides. US Patent No. 6,025,468 (2000).
- [16] Wang CY, Chang TY, Walfield AM, Ye J, Shen M, Chen SP, et al. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. *Vaccine* 2002;20:2603–10.

- [17] Wang CY, Shen M, Tam G, Fang XD, Ye J, Shen F, et al. Synthetic AIDS vaccine by targeting HIV receptor. *Vaccine* 2002;21:89–97.
- [18] Wang CY, Walfield AM, Fang X, Hammerberg B, Ye J, Li ML, et al. Synthetic IgE peptide vaccine for immunotherapy of allergy. *Vaccine* 2003;21:1580–90.
- [19] Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J Am Chem Soc* 1963;85:2149–54.
- [20] Merrifield RB. Solid phase synthesis. *Science* 1986;232:341–7.
- [21] Lindblad EE. Chapter two. Aluminum adjuvants. In: Stewart-Tull DES, editor. *The theory and practical application of adjuvants*. New York: Wiley; 1995. p. 21–35.
- [22] Hilgers LAT, Snippe H. DDA as an immunological adjuvant. *Res Immunol* 1992;143:494–503 [574–576].
- [23] Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. *Pharm Biotechnol* 1995;6:495–524.
- [24] Dunning WF. Prostate cancer in the rat. *Natl Cancer Inst Monograph* 1963;12:351–69.
- [25] Isaacs JT, Isaacs WB, Feitz WF, Scheres J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancer. *Prostate* 1986;9:261–81.
- [26] Greenstein JL, Schad VC, Goodwin WH, Brauer AB, Bollinger BK, Chin RD, et al. A universal T cell epitope-containing peptide from hepatitis B surface antigen can enhance antibody specific for HIV gp120. *J Immunol* 1992;148:3970–7.
- [27] Partidos CD, Stanley CM, Steward MW. Immune responses in mice following immunization with chimeric synthetic peptides representing B and T cell epitopes of measles virus proteins. *J Gen Virol* 1991;72:1293–9.
- [28] Demotz S, Lanzavecchia A, Eisel U, Niemann H, Widmann C, Corradin G. Delineation of several DR-restricted tetanus toxin T cell epitopes. *J Immunol* 1989;142:394–402.
- [29] Chong P, Zobrist G, Sia C, Loosmore S, Klein M. Identification of T- and B-cell epitopes of the S2 and S3 subunits of pertussis toxin by use of synthetic peptides. *Infect Immun* 1992;60:4640–7.
- [30] Brett SJ, Mazurov AV, Charles IG, Tite JP. The invasins protein of *Yersinia* spp. provides co-stimulatory activity to human T cells through interaction with β 1 integrins. *Eur J Immunol* 1993;23:1608–14.
- [31] Reddy PR, Rao IM, Raju VS, Rukmini V, Reddy RL. Direct inhibitory actions of GnRH on accessory reproductive organs of rat. *J Steroid Biochem* 1985;23:819–22.
- [32] Srkalovic G, Bokser L, Radulovic S, Korkut E, Schally AV. Receptors for luteinizing hormone-releasing hormone (LHRH) in Dunning R3327 prostate cancers and rat anterior pituitaries after treatment with a sustained delivery system of LHRH antagonist SB-75. *Endocrinology* 1990;127:3052–60.
- [33] Fuerst J, Fiebiger E, Jungwirth A, Mack D, Talwar PG, Frick J, et al. Effect of active immunization against luteinizing hormone-releasing hormone on the androgen-sensitive Dunning R3327-PAP and androgen-independent Dunning R3227-AT2.1 prostate cancer sublines. *Prostate* 1997;32:77–84.
- [34] Ferro VA, Stimson WH. Anti-gonadotropic releasing hormone vaccines and their potential use in the treatment of hormone-responsive cancer. *BioDrugs* 1999;12:1–12.
- [35] Waters DJ, Sakr WA, Hayden DW, Lang CW, McKinney L, Murphy GP, et al. Workgroup 4: Spontaneous prostate carcinoma in dogs and nonhuman primates. *The Prostate* 1998;36:64–7.
- [36] Oonk HB, Turkstra JA, Schaaper WM, Erkins JH, Schuitemaker-deWeerd MH, van Nes A, et al. New GnRH-like peptide construct to optimize efficient immunocastration of male pigs by immunoneutralization of GnRH. *Vaccine* 1998;16:1074–82.
- [37] Dunshea FR, Colantoni C, Howard K, McCauley I, Jackson P, Long KA, et al. Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increases growth performance. *J Anim Sci* 2001;79:2524–35.
- [38] Behre HM, Nashan D, Hubert W, Nieschlag E. Depot gonadotropin releasing hormone agonist blunts the androgen-induced suppression of spermatogenesis in a clinical trial of male contraception. *J Clin Endocrinol Metab* 1992;74:84–90.
- [39] Genazzani AD, Massolo F, Ferrari E, Gandolfi A, Petraglia F, Genazzani AR. Long term GnRH-agonist administration revealed a GnRH-independent mechanism stimulating FSH discharge in humans. *Eur J Endocrinol* 1996;134:77–83.
- [40] Wickings EJ, Marshall GR, Nieschlag E. Endocrine regulation of male reproduction. In: Dukelow WR, Erwin J, editors. *Comparative primate biology, reproduction and development*, vol. 3. New York: Liss; 1986. p. 149–70.
- [41] Carroll PR, Kantoff PW, Balk SP, Brown MA, D'amico AV, George DJ, et al. Overview consensus statement. Newer approaches to androgen deprivation therapy in prostate cancer. *Urology* 2002;60:1–6.
- [42] Goncharova ND, Lapin BA. Changes of hormonal function of the adrenal and gonadal glands in baboons of different age groups. *J Med Primatol* 2000;29:26–35.