2008

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ORIGINAL ARTICLE

The Single-Shot GnRH Immunocontraceptive Vaccine (GonaCon™) in White-Tailed Deer: Comparison of Several GnRH Preparations

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Keywords
contraception, gonadotropin-releasing hormone, infertility, *Odocoileus virginianus*, overabundance, wildlife

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Submitted November 5, 2007;
accepted April 14, 2008.

Citation
Miller LA, Gionfriddo JP, Fagerstone KA, Rhyan JC, Killian GJ. The single-shot GnRH immunocontraceptive vaccine (GonaCon™) in white-tailed deer: comparison of several GnRH preparations. Am J Reprod Immunol 2008; 60: 214–223

doi:10.1111/j.1600-0897.2008.00616.x

Problem
An effective, single-injection, multi-year, GnRH contraceptive agent is needed to control reproduction in overabundant white-tailed deer populations.

Method of study
Two GnRH conjugates, GonaCon™ (GnRH–KLH) and GonaCon-B™ (GnRH–blue protein), were prepared in emulsion form as one-injection and two-injection immunocontraceptive vaccine formulations. In addition, the GnRH–KLH protein conjugate was lyophilized and suspended in AdjuVac™ adjuvant to produce a fifth vaccine formulation. Each formulation was administered to a group of five captive adult female white-tailed deer. Reproductive performance of treated female deer was monitored for 5 years to determine the comparative efficacy of the various treatments.

Results
The longevity of the contraceptive response (2–5 years) was strongly influenced by the design of the conjugate antigen, the adjuvant used, and the delivery form of the vaccine.

Conclusion
One-injection and two-injection formulations of GonaCon™ and GonaCon-B™ produced multi-year contraception in adult female white-tailed deer. GonaCon-B™ provided a longer lasting contraceptive effect.

Introduction

Non-lethal tools are needed for the control of overabundant wildlife populations in settings in which traditional management methods, such as regulated sport hunting, cannot be used because of legal prohibitions or safety concerns. The development and use of safe, effective, and humane reproductive inhibitors may provide a partial solution to the problem of overabundant populations of deer and other wildlife in situations in which hunting is not an option. Reproductive inhibitors must be tailored to the physiology of the target species, as no contraceptive agent is effective, practical, and appropriate for use in all species.[1]

GonaCon™ Immunocontraceptive Vaccine is a highly effective contraceptive agent that prevents pregnancy in white-tailed deer (*Odocoileus virginianus*),[2] elk (*Cervus elaphus*),[3] wild horses (*Equus caballus*),[4,5] domestic and feral swine (*Sus scrofa*),[6–8] bison

(*Bison bison*),[9] domestic cats (*Felis catus*),[10] California ground squirrels (*Spermophilus beecheyi*),[11] and Norway rats (*Rattus norvegicus*).[12]

Immunocontraceptive vaccines based on porcine zona pellucida (PZP) and gonadotropin-releasing hormone (GnRH) control fertility by stimulating the production of antibodies that bioneutralize proteins or hormones essential for conception. PZP immunocontraceptive vaccines have been widely tested in white-tailed deer[2,13,14] and other wildlife species.[15,16] Female white-tailed deer vaccinated with PZP may cycle repeatedly and mate several times during the breeding season while still remaining infertile.[17] The multi-cycling problem associated with PZP vaccines prompted investigation into the potential use of GnRH as a contraceptive vaccine.

Gonadotropin-releasing hormone is produced and secreted by the hypothalamus and is responsible for the release of two pituitary hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the subsequent release of hormones that control the functioning of the ovaries and testes. Antibodies to GnRH induce infertility by binding to circulating endogenous GnRH and preventing the GnRH from binding to pituitary receptors, thereby precluding the release of FSH and LH. The severe reduction or absence of FSH and LH in the bloodstream leads to atrophy of the gonads and concomitant infertility in both sexes. Immunoneutralization of endogenous GnRH through the introduction of a GnRH conjugate in a vaccine appears to be highly specific, and does not appear to affect other hypothalamic-releasing hormones.[18]

An ultimate objective of efforts to develop infertility agents such as immunocontraceptive vaccines is the application of these materials to overabundant populations of free-ranging wildlife to slow the growth of such populations. Much of the early research effort to use GnRH immunization as an infertility method was focused on the contraception of males.[19–21] Reduction of population growth via contraception would be most effectively accomplished by targeting females, however, so most of our studies have dealt with the use of GnRH vaccine to induce infertility in females.

In this paper we describe the use of a newly developed GnRH vaccine in captive, adult, female white-tailed deer. We also describe recent improvements to the vaccine including (i) development of a single-injection technology that provides a multi-year contraceptive effect, (ii) development of a new adjuvant to replace Freund's™ adjuvant, and (iii) replacement of keyhole limpet hemocyanin (KLH) as a protein carrier molecule with a more cost-effective mollusk protein ('blue protein') for conjugation with synthetic GnRH peptide. All of these improvements contributed to the development of a more effective and practical contraceptive vaccine for use in wildlife.

Materials and methods

Deer Research Center at Pennsylvania State University

White-tailed deer used in these studies were born, raised, and maintained at the Pennsylvania State University (PSU) Deer Research Center, where a captive deer herd has been maintained since 1972. The original source of animals for the PSU deer herd was the wild white-tailed deer population in central Pennsylvania. Supplements to the PSU captive deer herd occurred approximately every 5 years until 1996. Since then, the PSU Deer Research Center has maintained a closed herd. During our study, the PSU deer facility encompassed 22 acres of natural woodland/forest habitat, which were divided into nine separate paddock areas that ranged in size from 0.12 to 1.54 ha (0.3–3.8 acres). Vegetation on the open areas consisted of a mixture of clover and orchard grasses, but most of the land was covered with dense eastern deciduous forest that had little understory vegetation. Deer were kept in outdoor paddocks at a density of 25–37 animals per hectare (10–15 animals per acre). During the non-breeding portion of the year, treated female deer were isolated from males. To test vaccine contraceptive efficacy, however, each year, from the first week of November through the end of the following February, four bucks of proven sire ability in the control herd were confined with the aggregated females from all five vaccine treatment groups. The animal facility at the PSU Deer Research Center was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Our study was approved by the Institutional Animal Care and Use Committee of PSU.

Development of the One-shot GnRH Vaccine

The GonaCon™ Immunocontraceptive Vaccine formulations used in this study consisted of synthetic GnRH peptide conjugated to one of two mollusk hemocyanins: KLH (*Megathura crenulata*) or blue

protein hemocyanin (*Concholepas concholepas*). Each 1.0-mL dose contained 850 or 1000 μg of the GnRH-mollusk hemocyanin conjugate. Four different treatments resulted from combinations of the two amounts of conjugate and the two protein carriers. A fifth treatment consisted of 1000 μg of the GnRH–KLH conjugate that was lyophilized and then suspended in AdjuVac™ adjuvant.

The Peptide (Hapten)

The Gonadotropin-releasing hormone used in this study was synthesized at Global Peptide Services (Fort Collins, CO, USA), with the structure [pE-HWSYGLRPGGC-SH]. The underlined amino acids represent the native GnRH molecule. The 10-amino-acid GnRH peptide hormone was made immunogenic by coupling it to a large carrier protein that contained numerous T-cell epitopes. Immune responses to the GnRH peptide vary with the coupling position of the GnRH molecule.[22–24] A glycine (G) was added at the C terminus as a spacer, followed by a cysteine (C) to ensure consistent alignment of the peptide to the maleimide-activated protein carrier KLH. This GnRH analog, called GnRH-glycys, has a conformation similar to that of native GnRH.[25]

Mollusk Hemocyanin Protein Carrier

GnRH is a small peptide hormone that is not naturally immunogenic. It becomes immunogenic when it is conjugated to a large carrier protein that contains numerous T-cell epitopes. Carrier proteins used in vaccine production include tetanus toxoid,[21] diphtheria toxoid,[26] bovine serum albumin (BSA), ovalbumin, and KLH.[22,25] BSA is not a good carrier for use in cattle or deer because of the phylogenetic proximity between cattle and deer. Our vaccine formulations incorporated the mollusk hemocyanin proteins KLH (Pierce Chemical, Rockford, IL, USA) and 'blue protein' (Biosonda, Santiago, Chile). These mollusk proteins are highly immunogenic in mammals because of their large sizes (molecular weights of 6–8 million), and because the mollusks are phylogenetically distant from the vertebrate target species.

Optimal Conjugate Design

Bachmann et al.[27] showed that the design of the immunogen is a critical factor in obtaining a strong B-cell response to an individual epitope. In a review of studies of immune responses to hepatitis B vaccines, Banatvala et al.[28] reported that the strength of the primary response is influenced by the antigen dose and structure, and that stronger primary responses result in longer lasting immune memory. To make the conjugate used in our vaccine highly immunogenic, we dissolved the hemocyanin proteins in a 7.5-pH, high-salt buffer that kept the large molecules intact so that coupling would occur on their surfaces. The coupling of as many as 300 GnRH peptide molecules on the surface of a mollusk protein mimicked the immune image of many pathogens, including viruses and bacteria that exhibit rigid, highly organized, highly repetitive protein epitopes. This mimicry of the repetitive nature of pathogen epitopes is an important aspect of the GnRH-hemocyanin conjugate design. Consistent alignment of the peptide facilitates the coupling of the immunogenic protein to the sulfhydryl group of the terminal amino acid and leaves the 10-amino-acid GnRH peptide exposed to the immune system.

Before the GnRH peptide can be coupled to the carrier protein, the carrier protein must be activated so that the available maleimide groups on the carrier protein can react with the terminal sulfhydryl-containing peptides on the GnRH peptide. KLH purchased from Pierce Chemical was pre-activated Imject® (Pierce Biotechnology Inc., Rockford, IL, USA) maleimide-activated mcKLH. Our conjugation process followed Pierce Chemical's recommended procedure with minor modifications. Our primary modification reduced the quantity of GnRH from the recommended GnRH:KLH ratio of 50:50 to 30:70, thus reducing the quantity of excess free GnRH remaining in solution after conjugation.

Blue protein was not maleimide activated when purchased from Biosonda. Maleimide activation was accomplished using sulfo-SMCC purchased from Pierce Chemical. To conjugate the GnRH peptide to the maleimide-activated mollusk protein, we followed the Pierce Chemical instructions except that we changed the ratio of blue protein to GnRH just as we did in the GnRH–KLH procedure.

Adjuvant Design – AdjuVac™

An important component of any vaccine is a non-specific immune stimulant called the adjuvant. A widely used adjuvant is Freund's Complete Adjuvant (FCA; Difco Laboratories, Detroit, MI, USA), which induces strong and long-lasting immunity to a broad

range of antigens.[29] Concerns over FCA's severe injection-site reactions and its potential carcinogenicity, however, prompted NWRC scientists to develop a new adjuvant by modifying the USDA-licensed Johne's vaccine, Mycopar™ (Fort Dodge Animal Health, Fort Dodge, IA, USA). Mycopar is approved for use in food animals, thus mitigating the concern for use in deer that may be hunted for human consumption.

The new adjuvant, called AdjuVac, contains a small quantity of killed *Mycobacterium avium*, a common bacterium found in many species of wildlife and domestic animals throughout the world.[30–32] Because *M. avium* is widely distributed in natural environments,[33–35] many animals have been exposed to it, and for that reason they may respond to the bacterium in AdjuVac as a previously encountered bacterial antigen.

Preparation of the Vaccine Emulsion

The creation of a depot at the vaccine injection site may promote a long-lasting immune response through the slow release of antigen.[36,37] To produce a depot effect, we prepared our vaccine as an emulsion. This was accomplished through the dropwise addition of 0.5 mL of the GnRH-carrier protein conjugate in mollusk-stabilizing buffer (MSB) to 0.5 mL of AdjuVac adjuvant with vigorous vortexing. The emulsion was stiffened by passing it through a 22-gauge needle three times.

Animal Handling, Vaccination, and Sample Collection

Twenty female deer were divided into four treatment groups with five deer per group. Four preparations were tested (i) GnRH–KLH-AdjuVac (850 μg one-shot), (ii) GnRH–KLH-AdjuVac (1000 μg/1000 μg two-shot), (iii) GnRH–blue protein-AdjuVac (1000 μg one-shot), and (iv) GnRH–KLH-AdjuVac (1000-μg lyophilized suspension).

Prior to handling and vaccination, animals were sedated with Xylazine™ (100 mg/mL; Bayer, Leverkusen, North Rhine-Westphalia, Germany), which was administered intramuscularly (i.m.) at 2.2–4.4 mg/kg (depending upon the nervousness of the individual animal). Each deer was then given an i.m., hand-injected dose of 1.0 mL of one of the vaccine preparations. Each injection was delivered to the right hindquarter with a 3 mL syringe and a 2.54 cm (1-inch), 19-gauge, hypodermic needle. The sedative effect was then reversed with Tolazine™ (100 mg/mL; Lloyd Inc., Shenandoah, IA, USA) administered intravenously (i.v.) or i.m. at 4.0 mg/kg.

Blood samples were taken before vaccination in July and then again during September, October and February each year. Approximately 7–10 mL of blood was collected from the jugular vein with 10-mL vacutainer blood collection tubes and 2.54 cm (1-inch), 18-gauge needles (Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were centrifuged and serum was isolated and then stored at −70°C. During the first week of February each year, a blood sample was collected and centrifuged, and the serum was evaluated for progesterone and anti-GnRH antibody titers. At the same time in February, ultrasound examinations were performed to determine if the does were pregnant. Ultimate reproductive efficacy was assessed by evaluating annual fawn production.

Laboratory Testing

Progesterone

The Coat-A-Count Progesterone *In-vitro* Diagnostic Test Kit™ (Diagnostic Products, Los Angeles, CA, USA) was used in these studies according to the manufacturer's recommended procedure. For each test, we used 0.1 mL of serum.

Anti-GnRH antibody titers

The enzyme-linked immunosorbent assay (ELISA) was used to measure anti-GnRH antibody titers. Fifty microlitres of serum was used for each assay. A 96-well plate was prepared by adding 100 ng of BSA-GnRH antigen to each well and then blocking with SeaBlock™ from Pierce Chemical. As either KLH-GnRH or blue protein-GnRH was used in the vaccine, BSA-GnRH was added to the ELISA plate, causing only antibodies to GnRH to be detected.

Deer blood serum was serially diluted from 1:1000 to 1:128,000 in phosphate-buffered saline containing SeaBlock. Antibodies in the deer serum to GnRH on the plate were directed with the following linkages: deer anti-GnRH bound to GnRH on the plate, rabbit anti-deer IgG bound to the deer IgG, and goat anti-rabbit-peroxidase bound to the rabbit IgG. Chromogen tetramethylbenzidine was used to develop the color, and 2 M H_2SO_4 was used to stop the reaction. The color intensity of the sample was read at 450 nm with a Dynatech MR 5000 ELISA plate

reader (Dynatech Laboratories, Alexandria, VA, USA).

Contraceptive efficacy: The mean reproductive success of the entire PSU captive deer herd was used as a control value that was compared with that of the GnRH-treated deer. Deer intentionally bred at the PSU facility during the period 1994–1998 produced an average of 1.7 fawns per year.[2] The deer in the current study were managed at the same facility and were maintained under conditions similar to those during the earlier period. In this study, if a vaccinated doe bore twins, she was physically removed from the treatment group (to reduce animal maintenance costs), but she was retained in our data analysis with the assumption that she would have remained fertile for the remainder of the study. Treated does that bore single fawns remained in the treatment groups.

Results

Group 1: GnRH–KLH-AdjuVac™ (850 μg one-shot)

All five does vaccinated in July 2000 with the formulation of GonaCon™ that contained KLH (850 μg single-shot) were infertile during the 2000–2001 breeding season (Table I). A year later, three of the five does (60%) remained infertile, whereas the remaining two treated does each bore a single fawn. One of the infertile does died in July 2002, leaving four deer remaining in the treatment group. One of the fertile does that produced a fawn in 2002 bore twins in 2003 and was then physically removed from the treatment group. In our analysis, however, we assumed that, in the absence of revaccination, she would have remained fertile throughout the rest of the study. Of the two remaining infertile does, only one produced a fawn (during 2005).

Table I Contraceptive Efficacy of Four GnRH Vaccine Formulations Given to Captive Adult Female White-Tailed Deer

	Year post-treatment				
Treatment group	1	2	3	4	5
GnRH–KLH one-shot	100	60	50[a]	50	25
GnRH–KLH two-shot	100	100	80	60	n/a[b]
GnRH–blue one-shot	100	100	80	80	80
GnRH–KLH lyophilized one-shot	40	0	n/a[b]	n/a[b]	n/a[b]

All vaccine formulations were emulsions except the lyophilized formulation, which was a suspension. Table values are the percentages of treated deer that were infertile each year. Five deer were injected with each vaccine formulation.
GnRH, gonadotropin-releasing hormone; KLH, keyhole limpet hemocyanin.
[a]One doe died during previous year, leaving $n = 4$.
[b]Treatment group was removed from study.

Group 2: GnRH–KLH-AdjuVac™ (1000 μg/1000 μg two-shot)

For 2 years, all five does in this treatment group were infertile. During the third year, four does remained infertile, and during the fourth year, three remained infertile (Table I).

Group 3: GnRH–blue protein-AdjuVac™ (1000 μg one-shot)

All five treated does remained infertile through the first 2 years of the study (Table I). During each of the third and fourth years, only one of five does became pregnant. Interestingly, one doe produced a single fawn in the third season, but did not reproduce during the following year. Another doe that had been infertile for the first three breeding seasons produced a single fawn during the fourth season. Although she was then given a booster vaccine dose (same formulation as the primary dose) that caused her to be infertile during the fifth year of the study, we assumed for the purposes of our analysis that she would have remained fertile in year five had she not been revaccinated.

Group 4: GnRH–KLH-AdjuVac™ (1000 μg lyophilized suspension)

The GnRH–KLH vaccine given as a lyophilized suspension did not provide a long-lasting immune response. Only two of the five deer in this treatment group developed a sufficiently strong antibody response to achieve infertility during the first year (Table I). Titers in these two does dropped below contraceptive level (antibody levels ≥1:64,000 generally are associated with infertility in adult white-tailed deer[2]) during the second year, when all five does became pregnant (Fig. 1, Table I). Hence, the trial was terminated.

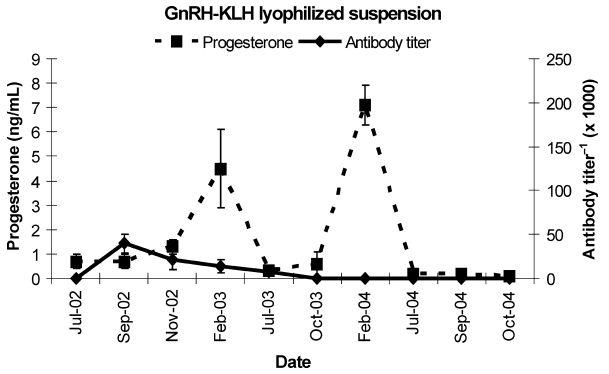

Fig. 1 Gonadotropin-releasing hormone–keyhole limpet hemocyanin lyophilized suspension, one-shot. A single injection (1000 μg) of this GonaCon™ vaccine formulation was given to five captive adult female white-tailed deer during July 2002. The GnRH–KLH conjugate was lyophilized and made into a suspension with the oil-based adjuvant AdjuVac™. A contraceptive effect was induced only in two deer by this vaccine, and it lasted for only 1 year, as reflected in the elevated blood serum progesterone concentrations observed in February 2003 and 2004, as well as in the low anti-GnRH antibody titers, especially after 2002. When vaccine efficacy reached zero during 2004, this trial was terminated.

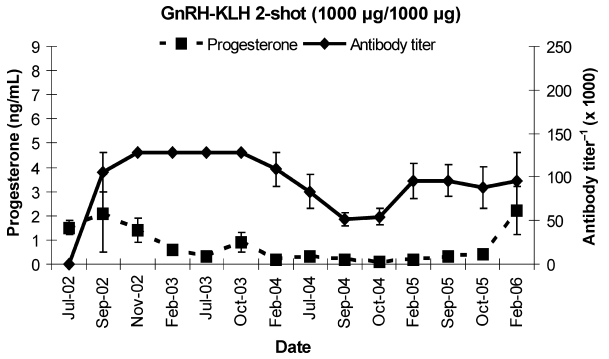

Fig. 3 Gonadotropin-releasing hormone–keyhole limpet hemocyanin emulsion, two-shot. A primary injection (1000 μg) of this GonaCon™ vaccine formulation was given to five captive adult female white-tailed deer during July 2002, and a booster injection (1000 μg) was given during September 2002. The GnRH–KLH conjugate was made into an emulsion with the oil-based adjuvant AdjuVac™. Elevated anti-GnRH antibody titers persisted until 2004, when an apparent self-boosting effect re-elevated the titers. The low blood serum progesterone concentrations observed in deer in this treatment group throughout the 3.5-year trial were associated with a lack of cycling and pregnancy.

Serum Progesterone Concentrations and Ultrasound Examinations

Except in the deer treated with the lyophilized suspension (group 4), serum progesterone concentrations were generally low in treated deer throughout the study (Figs 2–4). Progesterone levels assayed on

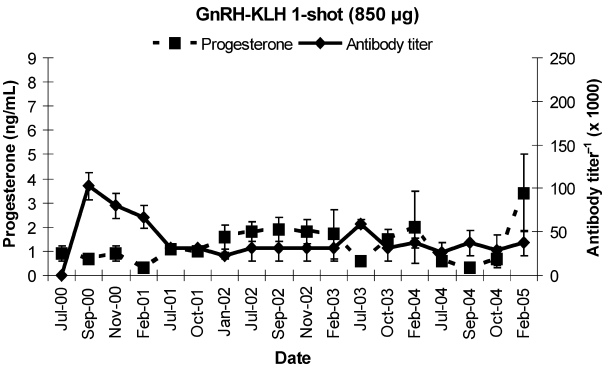

Fig. 2 Gonadotropin-releasing hormone–keyhole limpet hemocyanin emulsion, one-shot. A single injection (850 μg) of this GonaCon™ vaccine formulation was given to five captive adult female white-tailed deer during July 2000. The GnRH–KLH conjugate was made into an emulsion with the oil-based adjuvant AdjuVac™. An initially strong immune response, as indicated by high anti-GnRH antibody titers, resulted in the contraception of all five deer during the first year after vaccination. The immune response declined during the second year and then remained fairly stable throughout the remainder of the 4.5-year trial.

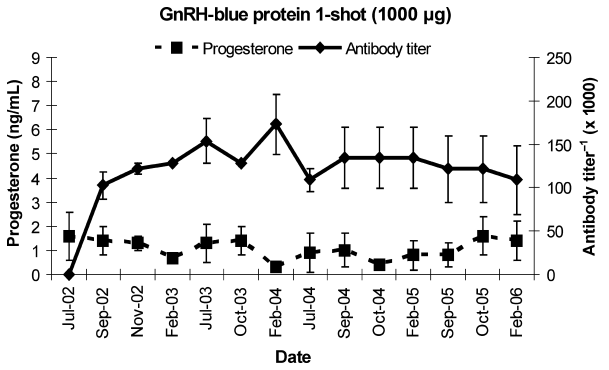

Fig. 4 Gonadotropin-releasing hormone–blue protein emulsion, one-shot. A single injection (1000 μg) of this GonaCon-B™ vaccine formulation was given to five captive adult female white-tailed deer during July 2002. The GnRH–blue protein conjugate was made into an emulsion with the oil-based adjuvant AdjuVac™. Elevated anti-GnRH antibody titers persisted throughout the 3.5-year trial, during which several apparent self-boosting effects occurred. The low blood serum progesterone concentrations observed in deer in this treatment group throughout the trial were associated with a lack of cycling and pregnancy.

serum drawn each February had a strong, positive correlation with spring fawning. Multiple studies have demonstrated that GnRH contracepted deer have serum progesterone concentrations of <1.5 ng/mL in the month of February while controls and deer that are no longer contracepted by GnRH have concentrations >3.0 ng/mL. In this study using

progesterone concentrations of >3.0 ng/mL as a positive indicator of pregnancy, we found that the correlation between elevated progesterone and spring fawning was 0.90 ($r^2 = 0.95$, $P < 0.0001$). Ultrasound performed at the same time also had a high correlation with spring fawning ($r^2 = 0.95$, $P < 0.0001$).

Anti-GnRH Antibody Titers

An examination of the anti-GnRH antibody titers measured in deer in the various treatment groups (Figs 1–4) and the corresponding contraceptive effects (Table I) indicated that these factors were directly related. The 850 μg, single-shot KLH treatment produced initially high antibody titers and contraception in all five does during the first year after vaccination (Fig. 2). The decline in antibody levels during the second and subsequent years, however, corresponded with a decline in the contraceptive rate. In vaccine trial 2, the 1000 μg/1000 μg KLH primary and booster doses provided a much longer lasting antibody response (Fig. 3), which produced longer lasting contraceptive effects (Table I). The replacement of KLH by the blue protein carrier produced an immune response with a single injection that was stronger than that observed in response to the KLH two-shot treatment (Figs 3 and 4). This strong antibody response produced a 5-year contraceptive effect in four of five blue protein-treated does (Table I). On the other hand, the use of 1000 μg of GnRH–KLH conjugate, lyophilized and suspended in AdjuVac, provided only a weak, short-term immune response (Fig. 1). Only two of five does were infertile during the first year after vaccination, and, as antibody titers declined during the second year, all five does became pregnant (Fig. 1, Table I).

Discussion

GonaCon™ Immunocontraceptive Vaccine suppresses reproductive activity in female white-tailed deer, and may be effective in reducing undesirable behavior associated with the autumn breeding period or rut. PZP vaccines, on the other hand, may actually increase the incidence of such behavior by extending the duration of the breeding season.[17,38,39] By lengthening the rut, PZP vaccines are likely to increase rut-associated movements of deer, thereby increasing the occurrence of deer–vehicle collisions. On the other hand, two-shot formulations of GnRH-based vaccines such as GonaCon™ have limited potential for practical application as wildlife contraceptive agents, and the Freund's™ adjuvant used in early formulations of GnRH vaccines caused large injection-site granulomas.[40] The development of a non-Freund's, single-injection immunocontraceptive vaccine represents an important breakthrough in wildlife contraception.

Several factors were involved in the production of this single-injection, multi-year vaccine. It has been known for more than 20 years that to produce antibody to GnRH, the peptide must be coupled to a larger protein. Many different proteins and coupling techniques have been used (as discussed in the *Materials and methods* section), but in many cases, much of the resultant antibody production targeted the carrier protein and not the GnRH peptide. Therefore the vaccines required a primary and several booster doses to produce a 6-month-long response.[21,26,41]

In our conjugate, the mollusk protein remained intact because of the presence of a stabilizing buffer, and the GnRH peptide coupled to the surface of the carrier molecule. The immune response of the target animal was highly directed to the repeating GnRH peptides on the surface. This directed response provided a strong antibody response to a normally small, non-immunogenic peptide.

Factors Essential for a Multi-year Response to a Single Vaccine Injection

Emulsion form of delivery

The longevity of the contraceptive response appears to be related to the delivery of the antigen in an emulsion form. A properly prepared emulsion is stable at 4°C for months to years; if the emulsion is not prepared properly, it may begin to separate within weeks.[42] A poor emulsification would preclude the long-term effectiveness of the vaccine. The need to produce a stable emulsion is probably related to the need for a depot effect at the injection site to provide a slow release of the antigen. Although lyophilization of the GnRH-mollusk hemocyanin conjugate and addition of the lyophilized antigen to the AdjuVac™ adjuvant in a suspension form did not provide a long-term, single-injection contraceptive effect (Table I), lyophilized delivery may be effective in a two-shot form.

Presence of Mycobacterium avium in AdjuVac

GonaCon™ vaccine is a mixture of a new, highly immunogenic antigen (GnRH-mollusk hemocyanin) with a previously experienced *M. avium* bacterium. In a GnRH vaccine study with black-tailed deer (*Odocoileus hemionus columbianus*), Perry et al.[43] injected a group of deer with GnRH vaccine in which *M. avium* had been replaced by DD-Dextran in the adjuvant. The first-year response to the DD-Dextran was 14% contraception ($n = 14$). The deer were revaccinated during the following year, resulting in a 90% contraception rate. In the same study, deer ($n = 8$) given a single injection of GonaCon™ vaccine that contained *M. avium* exhibited contraception rates of 79% during the first year after vaccination, and 75% during the second year.[43]

Self boosting: follicular dendritic cells provide continued presence of antigen

The antibody titers illustrated in Figs 2–4 indicate that self-boosting of the GnRH antibody is essential for the single-injection, multi-year contraceptive effect. Follicular dendritic cells (FDC) in the lymph node that drains the site of injection may provide the basis for the long-term immune response associated with some antigens. An immunoglobulin G (IgG) antibody needed for an immunocontraceptive response has a half-life of 23 days.[44] Although antigen-specific memory B-cells are produced with an initial immunization, the induction of immunocontraception that persists for months or years requires a repeated stimulation of these B-cells by the injected antigen.

The long-term immune response is thought to be dependent on the formation of immune-complexes (ICs). In the two-dose paradigm, specific antibodies produced by the primary dose are present at the time of injection of the second dose of antigen, resulting in the formation of ICs. The second dose of antigen selects for the higher affinity antibody-producing B-cells. The higher affinity antibodies are more likely to bind to the target antigen. Affinity maturation of the antibody occurs gradually as new memory B-cells are selected by exposure to small (picogram) quantities of retained antigen.

To be effective in producing long-lasting antibodies, antigen administered in a single dose must be retained in the body long enough to produce specific antibodies that will bind with the antigen to form ICs. These ICs could then bind FDCs, protecting the antigen from macrophage and liver degradation. ICs may begin to form within 7–14 days after a primary injection, or within minutes after a booster injection.[45]

The presence of a specific antibody is the significant difference between primary and secondary antibody responses, as the specific antibody from the primary response combines with the antigen to form immune complexes for transport into draining secondary lymphoid tissues. Many of these immune complexes are eliminated by phagocytic cells, but some are trapped on the surface of FDC for months to years.[27,46]

In the absence of specific antibody, soluble antigen freely diffuses through the draining lymph nodes and interacts with the appropriate immune cells.[45] Initial clearance of antigen is because of catabolism.[45] By the fourth day, there is enough specific antibody to begin a second phase of immune clearance, in which antigen complexes with specific antibody are cleared by macrophages in the afferent and efferent sinuses of the lymph nodes, the sinusoids of the liver, and the marginal sinuses of the spleen.[46,47] FDC trap immune complexes and retain picogram amounts of antigen on their surfaces for long periods.[45]

Antigen availability to the immune system is highly regulated throughout this process. Low levels of specific antigen induce antigen release from FDC, and high antibody concentration inhibits the release of antigen.[45] High concentrations of antibody interact with the antigen on FDC processes and crosslink them so that the antigen is not available to the surrounding cells.[45] In addition, it appears that the antigen released from FDC is significantly more immunogenic than free antigen, such that it may take 1000 pg of free antigen to elicit antibody production, whereas it takes only 10 pg of FDC-retained antigen to elicit the same response.[48] This greater immunogenicity may also be caused by the proximity of the antigen-containing FDC and the B-cells in the draining lymph nodes.

Conclusions

GonaCon™ Immunocontraceptive Vaccine is a highly effective, single-injection, multi-year contraceptive agent based on conjugated GnRH–KLH or GnRH–blue protein. Although both formulations of the vaccine caused infertility in female white-tailed deer, GonaCon-B™, which incorporates the GnRH–blue protein conjugate, was a more effective, longer lasting, and more economical contraceptive agent than the GnRH–KLH formulation. Both conjugate formulations of GonaCon™ vaccine used the adjuvant

AdjuVac™ in an emulsion that was injected into the hind limb musculature of the target animal, and both were effective as a single-injection contraceptive agent in captive adult female white-tailed deer. Vaccine delivered as a lyophilized suspension is not as effective when compared to the emulsion form of delivery.

References

1 Fagerstone KA, Miller LA, Bynum KS, Eisemann JD, Yoder C: When, where and for what wildlife species will contraception be a useful management approach? *Vert Pest Conf* 2006; 22:45–54.

2 Miller LA, Johns BE, Killian GJ: Immunocontraception of white-tailed deer with GnRH vaccine. *Am J Reprod Immunol* 2000; 44:266–274.

3 Killian G, Fagerstone K, Kreeger T, Miller L, Rhyan J: Management strategies for addressing wildlife disease transmission: Case for fertility control. *Wildl Damage Manage Conf* 2008; 12:265–271.

4 Killian GJ, Miller LA, Diehl NK, Rhyan JC, Thain D: Evaluation of three contraceptive approaches for population control of wild horses. *Vert Pest Conf* 2004; 21:263–268.

5 Killian GJ, Diehl NK, Miller LA, Rhyan JC, Thain D: Long-term efficacy of three contraceptive approaches for population control of wild horses. *Proc Vert Pest Conf* 2006; 22:67–71.

6 Killian GJ, Miller LA, Rhyan JC, Dees T, Doten H: Evaluation of GnRH contraceptive vaccine in captive feral swine in Florida. *Wildl Damage Manage Conf* 2004; 20:128–133.

7 Killian GJ, Miller LA, Rhyan JC, Doten H: Immunocontraception of Florida feral swine with a single-dose GnRH vaccine. *Am J Reprod Immunol* 2006; 55:378–384.

8 Miller LA, Rhyan JC, Killian GJ: Evaluation of GnRH contraceptive vaccine using domestic swine as a model for feral hogs. *Wildl Damage Manage Conf* 2004; 10:120–127.

9 Miller LA, Rhyan JC, Drew M: Contraception of bison by GnRH vaccine: a possible means of decreasing transmission of brucellosis in bison. *J Wildl Dis* 2004; 40:725–730.

10 Levy JK, Miller LA, Crawford PC, Ross MK, Fagerstone KA, Jordan HL: GnRH immunocontraception of male cats. *Theriogenology* 2004; 62:1116–1130.

11 Nash PB, James DK, Hui L, Miller LA: Fertility control of California ground squirrels using GnRH immunocontraception. *Proc Vert Pest Conf* 2004; 21:274–278.

12 Miller LA, Johns BE, Elias DJ, Crane KA: Comparative efficacy of two immunocontraceptive vaccines. *Vaccine* 1997; 15:1858–1862.

13 Turner JW Jr, Liu IKM, Kirkpatrick JF: Remotely delivered immunocontraception in captive white-tailed deer. *J Wildl Manage* 1992; 56:154–157.

14 Rudolph BA, Porter WF, Underwood HB: Evaluating immunocontraception for managing suburban white-tailed deer in Irondequoit, New York. *J Wildl Manage* 2000; 64:463–473.

15 Kirkpatrick JF, Turner JW Jr, Liu IKM, Fayrer-Hosken R: Applications of pig zona pellucida immunocontraception to wildlife fertility control. *J Reprod Fertil Suppl* 1996; 50:183–189.

16 Kirkpatrick JF, Turner JW Jr, Liu IKM, Fayrer-Hosken R, Rutberg AT: Case studies in wildlife immunocontraception: Wild and feral equids and white-tailed deer. *Reprod Fertil Develop* 1997; 9:105–110.

17 Miller LA, Crane KA, Gaddis S, Killian GJ: Porcine zona pellucida immunocontraception: long-term health effects on white-tailed deer. *J Wildl Manage* 2001; 65:941–945.

18 Awoniyi CA, Reece MS, Hurst BS, Faber KA, Chandrashekar V, Schlaff WD: Maintenance of sexual function with testosterone in the gonadotropin-releasing hormone immunized hypogonadotropic infertile male rat. *Biol Reprod* 1993; 49:1170–1176.

19 Robertson IS, Fraser HM, Innes GM, Jones AS: Effect of immunological castration on sexual and production characteristics in male cattle. *Vet Record* 1982; 111:529–531.

20 Ladd A, Prabhu G, Tsong YY, Probst T, Chung W, Thau RB: Active immunization against gonadotropin-releasing hormone combined with androgen supplementation is a promising antifertility vaccine for males. *Am J Reprod Immunol Microbiol* 1988; 17:121–127.

21 Ladd A, Tsong YY, Walfield AM, Thau RB: Development of an antifertility vaccine for pets based on active immunization against luteinizing hormone-releasing hormone. *Biol Reprod* 1994; 51:1076–1083.

22 Talwar GP: Immunobiology of gonadotropin-releasing hormone. *J Steroid Biochem* 1985; 23:795–800.

23 Ladd A, Tsong YY, Lok J, Thau RB: Active immunization against LHRH: I. Effects of conjugation site and dose. *Am J Reprod Immunol* 1990; 22:56–63.

24 Beekman NJCM, Schaaper WMM, Turkstra JA, Meloen RH: Highly immunogenic and fully synthetic peptide-carrier constructs targeting GnRH. *Vaccine* 1999; 17:2043–2050.

25 Ferro VA, Stimson WH: Investigation into suitable carrier molecules for use in an anti-gonadotrophin

releasing hormone vaccine. *Vaccine* 1998; 16:1095–1102.
26 Sad S, Talwar GP, Raghupathy R: Influence of the genetic background and carrier protein on the antibody response to GnRH. *J Reprod Immunol* 1991; 19:197–207.
27 Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM: The influence of antigen organization on B cell responsiveness. *Science* 1993; 262:1448–1451.
28 Banatvala J, Van Damme P, Oehen S: Lifelong protection against hepatitis B: the role of vaccine immunogenicity in immune memory. *Vaccine* 2001; 19:877–885.
29 Leenaars PPAM, Koedam MA, Wester PW, Baumans V, Claassen E, Hendriksen CFM: Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. *Lab Anim* 1997; 32:387–406.
30 Beard PM, Daniels MJ, Henderson D, Pirie A, Rudge K, Buxton D, Rhind S, Greig A, Hutchings MR, McKendrick I, Stevenson K, Sharp JM: Paratuberculosis infection of nonruminant wildlife in Scotland. *J Clin Microbiol* 2001; 39:1517–1521.
31 Corn JL, Manning EJB, Sreevatsan S, Fischer JR: Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging birds and mammals on livestock premises. *Appl Environ Microbiol* 2005; 71:6963–6967.
32 Anderson JL, Meece JK, Koziczkowski JJ, Clark DL Jr, Radcliff RP, Nolden CA, Samuel MD, Ellingson JLE: *Mycobacterium avium* subsp. *paratuberculosis* in scavenging mammals in Wisconsin. *J Wildl Dis* 2007; 43:302–308.
33 Motiwala AS, Amonsin A, Strother M, Manning EJB, Kapur V, Sreevatsan S: Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates recovered from wild animal species. *J Clin Microbiol* 2004; 42:1703–1712.
34 Raizman EA, Wells SJ, Jordan PA, DelGiudice GD, Bey RR: *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging deer and rabbits surrounding Minnesota dairy herds. *Can J Vet Res* 2005; 69:32–38.
35 Pickup RW, Rhodes G, Bull TJ, Arnott S, Sidi-Boumedine K, Hurley M, Hermon-Taylor J: *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: diverse opportunities for environmental cycling and human exposure. *Appl Environ Microbiol* 2006; 72:4067–4077.
36 Morein B, Villacrés-Eriksson M, Sjölander A, Lövgren Bengtsson K: Novel adjuvants and vaccine delivery systems. *Vet Immunol Immunopathol* 1996; 54:373–384.
37 Fukanoki S, Matsumoto K, Mori H, Takeda R: Relation between antigen release and immune response of oil adjuvanted vaccines in chickens. *J Vet Med Sci* 2000; 62:571–574.
38 Miller LA, Johns BE, Killian GJ: Long-term effects of PZP immunization on reproduction in white-tailed deer. *Vaccine* 2000; 18:568–574.
39 Curtis PD, Pooler RL, Richmond ME, Miller LA, Mattfeld GF, Quimby FW: Comparative effects of GnRH and porcine zona pellucida (PZP) immunocontraceptive vaccines for controlling reproduction in white-tailed deer (*Odocoileus virginianus*). *Reprod Suppl* 2002; 60:131–141.
40 Curtis PD, Richmond ME, Miller LA, Quimby FW: Pathophysiology of white-tailed deer vaccinated with porcine zona pellucida immunocontraceptive. *Vaccine* 2007; 25:4623–4630.
41 Rabb MH, Thompson DL Jr, Barry BE, Colborn DR, Hehnke KE, Garza F Jr: Effects of active immunization against GnRH on LH, FSH and prolactin storage, secretion and response to their secretagogues in pony geldings. *J Anim Sci* 1990; 68:3322.
42 Herbert WJ: Mineral-oil adjuvants and the immunization of laboratory animals. *In* Handbook of Experimental Immunology in Three Volumes. Volume 3: Application of Immunological Methods, 3rd edn, DM Weir (ed.). Oxford, Blackwell Scientific, 1978, pp A3.1–A3.15.
43 Perry KR, Arjo WM, Bynum KS, Miller LA: GnRH single-injection immunocontraception of black-tailed deer. *Proc Vert Pest Conf* 2006; 22:72–77.
44 Buckley RH: IgG subclass deficiency. *Clin Focus Prim Immune Defic* 1998; 1:1–4.
45 Burton GF, Szakal AK, Kapasi ZF, Tew JG: The generation and maintenance of antibody and B cell memory: the role of retained antigen and follicular dendritic cells. *In* Strategies in Vaccine Design, GL Ada (ed.). Austin, Texas, R. G. Landes, 1994, pp 35–50.
46 Mandel TE, Phipps RP, Abbot A, Tew JG: The follicular dendritic cell: long term antigen retention during immunity. *Immunol Rev* 1980; 53:29–59.
47 Tew JG, Wu J, Qin D, Heim S, Burton GF, Szakal AK: Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol Rev* 1997; 156:39–52.
48 Burton GF, Maeda K, Szakal AK, Tew JG: Immunogenicity of follicular dendritic cell (FDC) associated antigen. *In* Dendritic Cells in Lymphoid Tissues, Y Imai, JG Tew, ECM Hoefsmit (eds). New York, Elsevier Science, 1991, pp 149–156.