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Immune Mechanisms and Characterization of Injection Site Reactions Involved in the Multi-Year Contraceptive Effect of the GonaCon™ Vaccine

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Abstract: The term “vaccine” has traditionally been associated with establishing immunity (antibodies) to a disease. This immunity is usually developed following administration of killed microorganisms. Disease vaccines typically require 1 to 3 injections, depending on the antigen design and efficacy of the vaccine. The effectiveness of the disease vaccine depends on the immune response developed by the host following exposure to the disease organism. The immunocontraceptive vaccine GonaCon™ is designed to produce immunity to the “self” hormone (GnRH), which is essential to reproductive activity in the mammal. Antibodies to GnRH reduce its biological activity resulting in infertility of both sexes. GonaCon™’s effectiveness as a single-injection immunocontraceptive wildlife vaccine depends on 4 factors. The first is the use of a large foreign mollusk protein in the GnRH conjugate. Second is the design of mollusk/GnRH protein conjugate that presents the GnRH antigen in a repetitious fashion. This design mimics the “danger signal” found in bacterial pathogens to which the animal has been previously exposed. Third is the addition to the vaccine of micrograms of Mycobacterium avium, which is ubiquitous in the environment and activates memory cells. The fourth factor is use of a water-in-oil emulsion, which provides a depot at the injection site, allowing a slow release of the vaccine. With this formulation, the vaccine is presented to the body as a “chronic infection”, even though it is not infectious. The granuloma that normally develops at the injection site plays a prime role in the host’s defense against this “chronic infection”. A WHO report on the use of the alum adjuvant in human vaccines states that “development of a small granuloma is inevitable with vaccines adjuvanted with aluminum, and is to be considered necessary to the efficacy of the adjuvant.” Researching GonaCon™ for use in companion animals, NWRC has looked at many different adjuvants intended to reduce the injection site reaction while at the same time retaining an effective vaccine. This paper reports on the role of the adjuvant and the injection site on the effectiveness of the vaccine.

Key Words: AdjuVac™, adjuvants, contraception, emulsion, fertility control, GnRH, GonaCon™, immunocontraception, injection site, Mycobacterium avium, wildlife population control

INTRODUCTION

A vaccine is an antigenic preparation used to establish immunity to a disease. The term “vaccine” derives from Edward Jenner’s use of cowpox (vaccia means cow in Latin; cowpox, when administered to humans, provided protection against smallpox), the work that Louis Pasteur used as the basis for his vaccine studies. Jenner realized that milkmaids who had contact with cowpox did not get smallpox. The antigenic material became known as a “vaccine”, and the process of distributing and administrating vaccines became known as “vaccination” (Freund et al. 1948).

The concept for an immuno-contraception vaccine is to take immunogenic material that will produce antibodies to reproductive proteins or hormones and inject it into an animal; the animal’s immune system initiates a reaction to the “foreign manterial”, producing antibodies. The antibodies made against these reproductive proteins or hormones then will interfere with their reproductive function, resulting in infertility within the target animal.

Scientists at the USDA National Wildlife Research Center (NWRC) began the study of GnRH in 1994 as a contraceptive vaccine for white-tailed deer (Odocoileus virginianus) because of the potential concerns with the use of the porcine zona pellucida (PZP) immunocontraceptive vaccine: PZP increases the length of the rut, which increases energy use by deer, and therefore could lead to increased human conflicts, such as car-deer collisions. In contrast, GnRH contraceptive vaccines reduce the length of the rut. Early formulations of the GnRH contraceptive vaccine required multiple injections and used Freund’s adjuvant as an adjuvant (Miller et al. 2000, Curtis et al. 2008). The term “adjuvant” comes from the Latin word adjuvare, “to help”, and the adjuvant is used to increase the immunogenic effect of the vaccine. NWRC scientists continued to improve on the vaccine, producing a single-injection vaccine that is effective for multiple years and that uses a safer adjuvant (AdjuVac™) developed at the NWRC (Miller et al. 2004b).

The single-shot GnRH contraceptive, GonaCon™, is the end result of many years of formulation development. The vaccine has proved to be an effective contraceptive in many species (Miller et al. 2004b), including deer (Gionfriddo et al. 2006), domestic and feral pigs as well as wild boar (Sus scrofa; Miller et al. 2004a, Killian et al. 2003. Massei et al. 2008), wild horses (Equus caballus; Killian et al. 2006a) bison (Bison bison; Miller et al. 2004c), feral cats (Felis catus; Levy et al. 2004), and California ground squirrels (Spermophilus beecheyi; Nash et al. 2004), as well as other mammalian species.

There are several key components of the vaccine that are important in the single-injection multi-year effectiveness:

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1) The use of a large foreign mollusk protein in the GnRH conjugate.

2) The design of the mollusk/GnRH conjugate which presents the GnRH antigen in a repetitive fashion. This design mimics the “danger signal” presented by bacterial pathogens to which the animal has been previously exposed.

3) Use of an emulsion consisting of water in a mineral oil, which provides a depot at the injection site, allowing a slow release of the vaccine.

4) The addition to the vaccine of micrograms of Mycobacterium avium, which is ubiquitous in the environment and activates memory cells to enhance the immune response.

**Unique Conjugate Design in GonaCon™**

The injection of a small (10 amino acid 1,500 MW) GnRH molecule as a vaccine would not produce an immune response, first because it is “too small” and second because the body would recognize it as “self”. To increase the size of the molecule and make it appear foreign, the GnRH peptide is coupled to a large foreign molecule, such as a mollusk hemocyanin protein.

Up to 300 GnRH peptide molecules are coupled to the surface of the 6-8 million-MW mollusk hemocyanin protein; this structure mimics the immune image of many pathogens to the host target animal, 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. By keeping the mollusk protein intact with a stabilizing buffer, the GnRH peptide couples to the surface of the molecule. The immune response of the target animal is then highly directed to the surface-repeating GnRH peptides. This design provides a high antibody response to a normally small non-immunogenic peptide. However, injection of this conjugate would create only a short-term immune response, as the protein would be released immediately from the injection site and destroyed by proteolytic enzymes.

**AdjuVac™ in a Water-in-Oil Emulsion**

The longevity of the contraceptive response is related to the delivery of the antigen in an emulsion form. GonaCon™ is formulated as a water-in-oil emulsion with the adjuvant AdjuVac™. The oil is a mineral oil and the antigen is the GnRH-hemocyanin conjugate mentioned above. To develop an emulsion, the water-soluble antigen is slowly mixed into the oil-based AdjuVac™ while the oil is being vortexed. The oil-based AdjuVac™ contains a surfactant. The surfactant is similar to a dishwashing detergent, with a polar and non-polar end. This design allows the two non-miscible solutions, water and oil, to combine. A properly prepared emulsion is stable at 4°C for months to years; the emulsion will begin to separate within weeks if it is not prepared properly. A poor emulsification will reduce the long-term effectiveness of the vaccine.

The stable water-in-oil emulsion provides a depot effect when injected into tissue, resulting in a slow release of the vaccine and protecting the antigen from a rapid degradation by enzymes. In addition, a micro-diffusion of oil droplets containing the antigen moves down by gravity into a draining lymph node. The antigen is protected in these lymph nodes by Follicular Dendritic Cells (FDC), which provide a slow release of the antigen over months and years (Burton et al. 1994). Thus, AdjuVac™ in a water-in-oil emulsion is essential in order to obtain the desired single-injection, multi-year response.

**M. avium in AdjuVac™ is Essential**

Freund’s complete adjuvant (Freund et al. 1937) contains milligram quantities of a mycobacterium per injection, resulting in large and painful injection site reactions in many species. The NWRC developed an adjuvant, AdjuVac™, which contains a microgram quantity of killed Mycobacterium avium, a common species of bacteria found in many wildlife and domestic animals worldwide. M. avium subsp. avium and M. avium subsp. paratuberculosis are very stable, closely related bacteria. These bacteria have been isolated in both domestic and wild ruminant and non-ruminant mammals (Beard et al. 2001, Motiwala et al. 2004). M. avium has also been isolated from free-ranging birds (Corn et al. 2005). M. avium has been isolated in river runoff from contaminated pastures in South Wales, United Kingdom, causing a concern for animal and human exposure (Pickup et al. 2006). Because of this exposure in free-ranging birds, wildlife, domestic animals, and water drainage areas, M. avium is ubiquitous in nature, and thus most animals respond to the bacterium in AdjuVac™ as a previously encountered bacterial antigen.

In all of the contraceptive studies conducted with GonaCon™ in several countries, pre-injection blood was tested for antibodies to M. avium (Miller et al. 2004b). Most animals had some antibody to M. avium, as indicated by an IDEXX ELISA assay performed by the Colorado Rocky Mountain Regional Laboratory (Denver, CO). There was found to be a large variation in M. avium titers in animals, within a treatment group and between species of animals. These titers increased after the GonaCon™ vaccination, however, there was no correlation between initial M. avium titer, resulting antibody to GnRH, and subsequent contraception in the individual animal (Perry et al. 2008).

In a study to determine the amount of M. avium needed for GonaCon™ vaccine to be successful as a single-shot vaccine, a study was initiated in rabbits (Oryctolagus cuniculus) using GonaCon™ with 0 μg, 50 μg, 100 μg, 200 μg, and 400 μg of M. avium per dose. The results in Figure 1 suggest that 100 μg is needed to provide the maximum immune response, using a single injection. In a contraceptive study of black-tailed deer using several GnRH vaccine preparations (Perry et al. 2006, 2008), it was determined that M. avium was needed in the single-shot GonaCon™ vaccine for effective long-term contraception.

As the results of these studies demonstrate, the current vaccine, made into an emulsion with the oil-based AdjuVac™ containing 175 μg of M. avium, acts as a mixture of a new highly-immunogenic antigen (mollusk-GnRH conjugate). As most target animals have had a previously exposure to M. avium bacteria, the first injection results in a good immune response. As the immune system responds to M. avium, because of the close association in the vaccine, there is also an immune response to the mollusk-GnRH conjugate.
Injection Site Reactions

A water-in-oil (W/O) emulsion is needed in the GonaCon™ formulation for development of a long-term immune response. It is generally accepted that some local reaction at the injection site is common in W/O emulsions, including granulomas, sterile abscesses, or cysts (WHO 2005). NWRC scientists are working with an international adjuvant company, Seppic of Parie Cedex, France (Aucouturier et al. 2001), to understand the mechanism involved in the injection site reaction, and to reduce the reaction while maintaining the long-term effectiveness of the vaccine.

Over the last 7 years, the final form of the GnRH vaccine (GonaCon™) has been tested in over 400 animals in 9 different animal species by 10 leading scientists in the United States, England, Australia, New Zealand, and South Africa. In general, the scientists are pleased with the general good health of the animals vaccinated and the small size of the injection-site reaction. The most common report is that there is no visible injection site reaction, or that there is a non-visible but palpable lump underneath the skin at the site of the injection. Scientists who have found this lump have been asked to look for any reaction, otherwise they would not generally have noticed it.

In three species, white-tailed deer, elk (Cervus canadensis), and wallaby (Petrogale penicillata) scientists report an occasional visible injection site reaction (unpubl. data, various pers. commun.). One of 29 elk demonstrated large injection site reaction, and 1 of 72 wallabies demonstrated a large injection reaction. Based on previous coyote (Canis latrans; Miller et al. 2006) and the horse studies (pers. commun.), it appears that if a visible reaction occurs, it is because there has been a previous T cell response to a killed bacteria in a vaccine, or the animal has a bacterial infection which can cause the T cell response to our vaccine. In two field studies with white-tailed deer, there were no visible injection site reactions on the surface; however, when the injection site is dissected, there was considerable injection-site reaction deep in the muscle (Gionfriddo 2006). This is in contrast to the target safety study at Penn State (Killian et al. 2006b), where there was no visible injection-site reaction and the granulomas at the dissected injection site were minimal. It is possible that the increased injection-site reaction in the free-range deer is related to increased exposure to infectious organisms in the wild.

Epitopes on M. avium Cross-React

We have had 3 species that have given us consistent injection site reactions, including captive-reared coyotes in Logan, Utah pen studies, dogs (Canis familiaris) in Auburn University laboratory studies (Griffin et al. 2004), and horses in a study in Europe (unpubl., pers. commun.). In each case, we found that these animals previously had been given vaccines containing a killed bacterium. The dog and the domestic coyote were given killed Leptospira in multiple puppy shots to prevent leptospirosis, and the horses were given killed Streptococcus equi, a vaccine to prevent strangles. The injection site reaction appears to be due to antibodies produced to epitopes on the surface of the killed bacterium in each vaccine, which cross-react with epitopes on the surface of the killed M. avium in the AdjuVac™. In the case of the coyotes, the reaction at the injection site was reduced to a small lump under the skin if the Leptospira-containing vaccine had not been given for 2 years. In the Nevada horses, there was no injection-site reaction in horses that did not receive the strangles vaccine (Killian et al. 2006a).

A Balance between Toxicity and Adjuvanticity

In addition to the unique mollusk/GnRH design, the adjuvant should be a water-in-oil (W/O) design. Placing the vaccine in a water-in-oil emulsion is essential for a long-term immune response; however, the W/O emulsion is generally associated with some injection site reaction (Gupta et al. 1993).

Fukanoki et al. (2000a) tested the adjuvanticity and inflammatory response for a water-in-oil emulsion bovine serum albumin (BSA) vaccine using straight-chain hydrocarbons of carbon length from 12 to 18, as compared to branched-chain hydrocarbons. In comparing hydrocarbons with the same carbon chain size, straight-chain hydrocarbons induced significantly higher antibody titers than did branched-chain hydrocarbons. He also found that carbon lengths C12 to C14 resulted in a greater inflammatory response after injection than did C16 and C18 (Figure 2). The inflammatory response of the shorter-chain...
hydrocarbons is thought to be the result of severe tissue destruction due to lipid solvent action, and consequent disruption of cell membranes (Gupta et al. 1993). Fuknoki et al. (2000a) concluded that C16 and C18 hydrocarbons induced elevated and sustained immune responses without inducing severe reactions. Fuknoki et al. (2000b) also found that a stiff emulsion resulted in a slow release of the BSA in the water portion of the vaccine. The slow release of the BSA resulted in a higher antibody titer against BSA, compared to the rapid release that occurs with a less stiff emulsion.

Because of the encouraging results of these studies, NWRC scientists will be testing a revised GonaCon™ with a long-carbon-chain mineral oil in 2008 deer studies, using captive deer at Penn State University.

**Mineral Oil vs. Vegetable Oil (Squalene)**

It has long been suggested that the granuloma formed at the injection site when using mineral oil is related to the fact the mineral oil cannot be metabolized by the animal. Powers et al. (2007) attempted to answer this question by comparing vaccines made of mineral oil as compared to vegetable oil (squalene). They found that there was little difference in the injection site reaction between the two forms of oil. Julius Freund, who developed the first water-in-oil emulsion (Freund et al. 1948), tried to use peanut oil but found that it did not produce the high immune response that resulted from using mineral oil. NWRC studies (unpublished) found that the immune response from squalene adjuvant did not last as long at that found with mineral oil.

**M. avium-Free, Water-Soluble Adjuvants**

It is known that killed bacteria in the adjuvant have increased the injection site reaction (Powers et al. 2007). We have decreased the amount of killed bacteria in the AdjuVac™ adjuvant to reduce its negative effect. In species that have had consistent injection reactions, we have removed the M. avium from the vaccine and the reaction was eliminated. However, the vaccine is not effective as a single shot without the M. avium. In an attempt to develop an effective adjuvant without M. avium, we have prepared a DEAE-dextran (DD) adjuvant, which is a polycationic derivative of dextran. The degree of substitution corresponds to approximately 1 DEAE-substituent per 3 glucose units. The mean molecular weight is approximately 500,000 MW. DEAE-dextran (DD) (Sigma D-9885; Sigma Corp., St. Louis, MO) was combined with the blue-mollusk-GnRH conjugate, using the repeating positive charges of the DEAE to bind to the repeating negative charges (COO) on the blue protein. The positive charges are tied up by coupling to the GnRH peptide (Figure 3). This water-soluble GnRH vaccine, designated DD-GnRH-B, elicited the best response when compared to 3 other adjuvants (Figure 4). We have shown in a study with black-tailed deer (Perry et al. 2006) that DD as a water-soluble adjuvant does not make an effective single-shot vaccine, but is quite effective when used in a 2-shot vaccine. Therefore, all of the water-soluble adjuvants in this study were tested in a 2-shot regime.

The water-soluble adjuvants have both hydrophilic and hydrophobic regions. The hydrophilic portion is represented by the positive charges on the nitrogen, which bind the polymer to the mollusk-GnRH conjugate. The 8-carbon side chain is introduced to increase the hydrophobic (lipophilic) nature of the adjuvant. This lipophilic nature improves the attraction of the adjuvant to the immune cells and thereby improves the immune response.

All 4 of our water-soluble adjuvants were effective as 2-shot vaccines. DEAE-dextran (DD) provided the best immune response, followed by E8+ (Figure 4). E8+ adjuvant is a modification of a German enteric coating formulation called EUDRAGIT EPO. It is a polyacrylate dimethyl amino that has been modified by our formulation chemist to contain multiple positive charges. The + form indicates the adjuvant is methylated to make the positive charge more stable in acid and base conditions. The DEAE-dextran binding with KLH-GnRH conjugate the E8+ adjuvant demonstrated a good response after a single injection (Figure 4).
Dual Adjuvant Formulation

The water-soluble DD-GnRH-B can be mixed with an incomplete AdjuVac™ (without M. avium) into a dual adjuvant formulation. In this emulsion form of the vaccine, the DD has been used to replace the M. avium in our standard GonaCon™ formulation. DD-GnRH-B with incomplete adjuvant is designated DD-GonaCon™-B. In Figure 5, the DD-GonaCon™-B is compared, as a single shot, to our standard GonaCon™ formulation containing M. avium. Both formulations are effective as a single shot, with our standard GonaCon™ formulation performing slightly better.

Fukanoki et al. (2000b) found that a stronger and more prolonged immune response of oil adjuvanted vaccine was achieved by a slow release of antigen from the emulsion. This requires a stable emulsion. We have found that producing the emulsion with a Microfluidizer (Microfluidics, Newton, MA) produce smaller, more homogeneous micro-particles. This emulsion, which has the consistency of heavy hand cream, was found to be very stable.

CONCLUSION

GonaCon™ has been designed to perform as a single-shot, multi-year vaccine. The adjuvant, AdjuVac™, a mineral oil/surfactant, is mixed with the water-soluble mollusk-GnRH conjugate in a water-in-oil formulation. A new instrument, Microfluidizer, provides a stiff emulsion needed for a stable depot at the injection site. AdjuVac™ also contains microgram quantities of killed M. avium. The presence of microgram quantities of M. avium is necessary for single-shot effectiveness.

The use of water-in-oil emulsion is generally associated with a granuloma and sterile abscess at the injection site. Although we cannot get away from the water-in-oil emulsion, scientists at NWRC are looking for ways to reduce the injection site reaction while keeping the long-term effectiveness of the vaccine.

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