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ANTIBODY RESPONSE OF ELK IMMUNIZED WITH PORCINE ZONA PELLUCIDA

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ABSTRACT: Immunocontraception using porcine zona pellucida (PZP) vaccines is being explored as a nonlethal method of solving the problems of locally overabundant wildlife populations. This study characterized the immunological response of captive elk (*Cervus elaphus nelsoni*) to PZP challenge using 18 3-yr-old cows and was conducted from 14 September 1994 to 13 December 1995. All animals were given a single PZP inoculation and 1 mo later six of these animals were randomly chosen and received a booster inoculation. Blood samples were drawn from all animals at the time of the initial inoculation and 1, 2, 4, 6, 10, and 15 mo later. Immunological response was assessed by measuring anti-PZP antibody levels in serum. All animals demonstrated a strong immune response with no evidence that the booster enhanced antibody levels. Antibody levels rose from between 0 and 4 at the time of the initial injection to peak levels of 85 to 163 within 2 to 6 mo, followed by a noticeable decline by 15 mo post-vaccination. Limited data suggest that antibody levels >100 may be required to effect contraception. High individual variability in immune response observed in this study suggests it may be difficult to predict the proportion of animals effectively treated. Disruption of seasonal synchrony in calving also could occur if antibody levels in individuals fall below effective levels while animals are still cycling. These results indicate that immunocontraception using PZP vaccines is possible for elk. However, carefully controlled population experiments will be required in order to assess the potential and limitations for management applications of this technique.

Key words: Adjuvants, *Cervus elaphus*, contraception, fertility control, immunocontraception, porcine zona pellucida antibody, reproduction, Rocky Mountain elk, vaccine.

INTRODUCTION

Problems of managing locally overabundant wildlife populations living in close association with high density human populations, parks, and other sanctuaries are becoming increasingly common. Translocation or destruction of excess animals is often difficult or impossible due to excessive costs, lack of release areas, public opposition to lethal management techniques, or legal mandates. A possible alternative to these traditional population control techniques is the control of reproduction through the application of contraceptives (Warren, 1995). A variety of contraceptive techniques have been proposed and are in various stages of development and testing. These include steroid implants, surgical or chemical sterilization, and vaccines. Although all of these techniques could potentially be useful in specific situations, most current research is focused on immunocontraception with porcine zona pellucida (PZP) vaccine. Since the efficacy of PZP depends on the adequate stimulation of the immune system and the consequent production of anti-PZP antibodies, an initial step in evaluating the potential of PZP as a contraceptive tool for managing a wildlife population is to evaluate antibody response to PZP treatment. Such studies have been conducted for a number of species including white-tailed deer (*Odocoileus virginianus*), horses, and a variety of captive exotic species (Liu et al., 1989; Kirkpatrick et al., 1996a; McShea et al., 1997). Results from these studies indicate that immunological response to PZP vac-

cines is not uniform across species, necessitating separate investigations for each species that is being considered for experimental treatment with PZP. The purpose of this study was to characterize serum antibody response of captive Rocky Mountain elk (*Cervus elaphus nelsoni*) to PZP vaccination.

MATERIALS AND METHODS

The study was conducted at the Starkey Research Facility (La Grande, Oregon, USA; 42°13′N, 118°3′W) from 14 September 1994 to 13 December 1995 using 18 3-yr-old cow elk that had been raised in captivity. Research protocols for captive animals were in accordance with approved U.S. Forest Service animal welfare guidelines (Wisdom et al., 1993). During prior experiments animals had been randomly assigned to three 0.10 ha pens, each housing six animals. Approximately 55% of the daily diet of each animal was offered as pelleted rations and 45% as alfalfa hay. Diets of cows in the three pens differed over the course of the study as per the requirements of another study described by Vagnoni et al. (1996). Briefly, cows in one pen were offered a high quality ration at ad libitum levels to provide for good nutritional condition year-round. Pellets offered to these elk averaged 15% crude protein and 72% in vitro dry matter digestibility (IVDMD). Cows in the second pen were offered a reduced quality ration with restricted feeding levels designed to maintain body mass about 10% below that of cows in the first pen. Their pelleted ration averaged 17% crude protein and 53% IVDMD. Cows in the third pen were offered the reduced quality ration with restricted feeding levels in late autumn through late winter, to induce about 10% mass loss in winter, and were offered the high quality ration at ad libitum rates from early spring to mid-autumn. All elk were fed the same hay (15–18% crude protein, 56–58% IVDMD). These feeding regimes were maintained until early spring 1995; by late spring 1995 all elk were fed the high quality ration at ad libitum rates through the breeding season in autumn 1995, such that all cows would be in good to excellent condition when breeding began.

During the 1994 breeding season the experimental elk were isolated from bulls, but were allowed to breed in 1995. Protocol used for breeding reflected requirements of another study. During breeding in 1995, the 17 cows (one cow died in July 1995) in this study were combined with an additional 40 cows and randomly assigned to an early breeding group and a late breeding group held in two 1.5 ha pens in size. A bull was placed with the early-breeding group from 5 September through 5 October; this bull was then held with the late-breeding group from 5 October through 5 November.

The PZP vaccine was prepared from porcine ovaries as described by Liu et al. (1989) and stored at −5 C until used in the field. The initial inoculation for all animals in the experiment was prepared as an emulsion of 0.5 cc vaccine (equivalent to approximately 5,000 zonae or 64.3 μg of protein in phosphate buffer) and 0.5 cc of Freund's complete adjuvant (FCA). The two vaccine components were mixed just prior to administration, using 100 plunger strokes of two 5-cc glass syringes joined with a double hubbed needle. The emulsified vaccine-adjuvant mix was then administered to all animals in each pen via intramuscular injection (Liu et al., 1989). Cows were chemically sedated for vaccination with light doses of xylazine hydrochloride (Lloyd Laboratories, Shenahdoah, Iowa, USA; 0.4 mg/kg) administered IM, sedation was reversed after vaccination using yohimbine hydrochloride (Wildlife Pharmaceuticals, Fort Collins, Colorado, USA; 0.1 mg/kg) administered IV. The initial dose of PZP vaccine was administered in the right hind leg on 14 September 1994. Approximately 1 mo later on 13 October 1994 two animals from each pen were randomly selected and given a second injection in the left hind leg using the identical protocol as the initial injection with the exception that Freund's incomplete adjuvant (FIA) was used instead of FCA (Liu et al., 1989).

Blood samples were collected from all animals at the time of the initial vaccination and on six additional dates, 13 October 1994, 23 November 1994, 18 January 1995, 30 March 1995, 7 July 1995, and 13 December 1995, approximately 1, 2, 4, 6, 10, and 15 mo after the initial vaccination. Blood was collected using a 20 ml syringe by jugular venipuncture and transferred to vacutainer tubes (Becton Dickinson, Rutherford, New Jersey, USA) which contained thrombine gel, a clot enhancer. We collected 12 ml of blood and allowed it to clot for 2 hr prior to centrifuging and decanting serum. Serum was stored frozen at −20 C until assayed.

Anti-PZP antibody levels were determined by an enzyme-linked immunosorbent assay (ELISA) on the basis of the method described by Voller et al. (1986), with modifications previously reported for horses (Liu et al., 1989) and additional modifications for Cervidae as described by Kirkpatrick et al. (1996b) and Turner et al. (1996). Fifty μl of 5 μg/ml porcine

zona antigen solution in 0.1 M glycine buffer (pH 9.5) was placed in each well of a flat-bottom ELISA microplate "Sumilon" (low protein binding, cat.# MS-3496, Sumitomo Bakelite Co., distributed by E & K Scientific Products, Saratoga, California, USA) and incubated overnight at 4 C. The plate was washed with PBS-Tween and incubated for 1 hr each at room temperature with 50 μl per well of the following PBS-Tween-diluted reagents and subsequent washings with PBS-Tween: (1) elk serum 1:200, (2) biotinylated rabbit anti-deer IgG 1:250, and (3) alkaline-phosphatase avidin (Zymed Laboratories, San Francisco, CA) 1:500. Finally, (4) 50 μl substrate solution of 1 mg p-nitrophenyl phosphate/ml (5 mg tablets: Sigma Chemical Co., St. Louis, Missouri, USA) in 10% diethanolamine buffer (pH 9.8) was distributed to each well. The plate was scanned for absorbance at 405 nm using an MR 580 Microelisa Auto Reader (Dynatech Laboratories, Alexandria, Virginia, USA) when the absorbance of the positive reference serum had reached a level close to 0.800 after incubation of approximately 20 min.

The experimental elk sera were tested in duplicate and their antibody levels were expressed as percentage of the positive reference serum (mean experimental serum absorbance/mean reference serum absorbance). The positive reference serum consisted of a pool of four white-tailed deer sera that had demonstrated anti-porcine zona antibody levels in the medium to high positive range after multiple immunizations. Four pooled pre-immunization deer sera served as negative control serum. The same control sera were used in all the tests. Reagent dilutions were selected for optimal assay sensitivity within reasonable incubation time, after testing a titration of a medium positive deer serum with a titration of biotinylated anti-IgG at several dilutions of alkaline-phosphatase avidin.

Affiniti-purified rabbit anti-deer IgG (H + L) (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA) was biotinylated according to the method described by Bayer et al. (1986) and Gretch et al. (1987). The anti-deer immunoglobulin G (IgG) was adjusted to a concentration of 4.7 μM IgG in PBS and to pH 8.0 with 0.15 M NaOH. Seventy five μl of a 230 μg/ml biotinamidocaproate N-hydroxysuccinimide ester (Sigma) solution in N,N-dimethylformamide (Sigma) were added to 1 ml of IgG solution for an 8:1 mole ratio of ester to antibody. The mixture was stirred for 1 hr, diluted with 1 ml PBS (pH 7.2) and dialysed (12–14,000 MW cut-off) four times against 1 L of PBS (pH 7.2) at 4 C within 48 hr. Since IgG from elk was not available for this study the results of the ELISA assays may have to be considered as only semi-quantitative, but permit comparisons among the various experimental treatments. Serum collected 13 December 1995 was used to diagnose the pregnancy status of all elk at the end of the experiment by use of an radioimmunoassay of serum pregnancy-specific protein-B (PSPB) (Sasser et al., 1986; Noyes et al., 1997). Pregnancy status was confirmed based on births the following spring.

By chance the six elk randomly chosen to receive the booster injection had a higher mean antibody level at the time of the second injection than the other 12 animals, indicating a stronger initial response to the first vaccination. In order to control for this initial difference in the two groups a repeated measures analysis of covariance was carried out using Proc Mixed in SAS (SAS Institute Inc., 1982) with the antibody levels measured 1 mo after the initial injection as covariates. We treated time as a categorical variable and the baseline covariate was incorporated in the model as a time by baseline antibody-time interaction term. This allowed us to determine the effect of the higher baseline antibody values at discrete time steps. Specifically, we modeled the individual antibody levels as a linear combination of the baseline antibody-time interaction effect, a time effect, a treatment protocol effect (one- versus two-injection), and a time-treatment protocol effect. The random error terms were assumed to be normally distributed with means of 0 and a covariance structure which was modeled using the spatial power model in Proc Mixed (SAS Institute Inc., 1982). Time was treated as a continuous variable for the purposes of modeling the covariance structure. Initially, we also incorporated a random effect due to pens, but there was no evidence of a pen effect and we dropped that term from the model. There was also one extremely high baseline antibody level for an animal in the two-injection group and the analysis was run twice, once with that animal included and once without, and the conclusions did not change. The results presented are from the analysis which included that animal. We examined plots of the residuals and saw no indications of serious violations of model assumptions.

RESULTS

All animals demonstrated a strong immune response to the PZP challenge with anti-PZP antibody levels raising from the negative control range between 0 and 4 at the time of the initial injection to peak levels of 85 to 163 within 2 to 6 mo followed

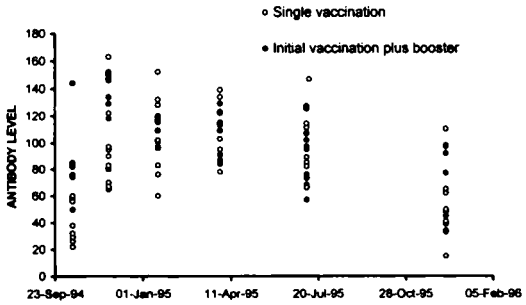

FIGURE 1. Anti-PZP antibody levels in 12 captive female elk treated with a single vaccination and six captive elk given a booster vaccination 1 mo after receiving the initial challenge. Antibody levels were measured by enzyme-linked immunosorbent assay and reported as percent of positive reference serum. Blood samples were drawn at the time of the initial vaccination and approximately 1, 2, 4, 6, 10, and 15 mo after the initial vaccination.

by a noticeable decline by 15 mo post-vaccination (Fig. 1). Although there was strong evidence of a time effect ($P < 0.01$), there was no evidence of a treatment protocol effect ($P = 0.46$) or an interaction between time and treatment protocol ($P = 0.49$). The baseline effect appears to have differed between the one- and two-shot treatment groups only during the first time period, 1 mo after the initial injection ($P < 0.01$), but did not appear to have any effect subsequent to that ($P \geq 0.40$). Immune responses of individual animals treated with identical vaccination protocols were quite variable (Figs. 2, 3). Eleven of 17 animals (one animal in the two-injection group died prior to breeding due to problems unrelated to the PZP vaccinations) became pregnant when bred approximately 13 mo after the initial PZP vaccination. The four animals that maintained the highest antibody levels (92 to 146) during the last two sampling dates, approximately 3 mo pre-breeding and 2 mo post-breeding, failed to become pregnant (Fig. 4).

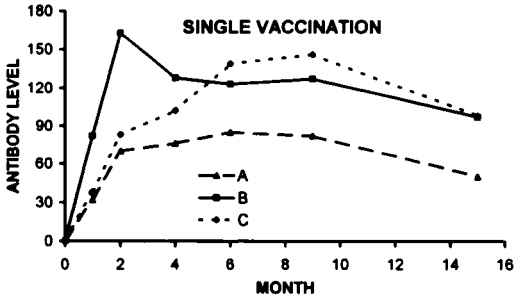

FIGURE 2. Example of individual variation in immune response of three captive female elk that received a single PZP vaccination (animals A, B, and C).

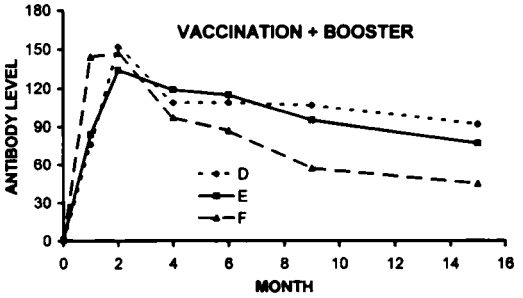

FIGURE 3. Example of individual variation in immune response of three captive female elk that were given a booster vaccination 1 mo after receiving the initial challenge (animals D, E, and F).

DISCUSSION

Results of this experiment demonstrate that the immune system of elk is quite sensitive to challenge with PZP vaccine, with the most striking result being our failure to detect any measurable increase in antibody levels for animals that received a booster vaccination 1 mo after the initial

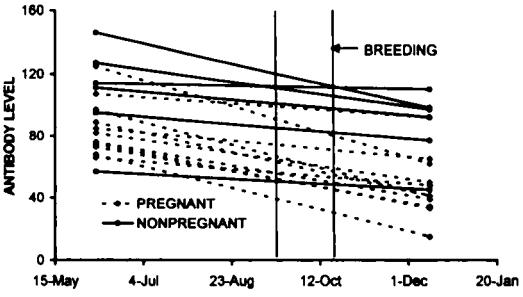

FIGURE 4. Pre- and post-breeding anti-PZP antibody levels in 17 captive female elk that had been vaccinated approximately 13 mo prior to breeding. Solid lines represent animals that failed to conceive and dashed lines represent animals that became pregnant.

challenge. Although the standard protocol for effective contraception using PZP vaccines has been multiple injections (Liu et al., 1989; Kirkpatrick et al., 1990; Turner et al., 1992, 1996), actual tests of the effects of single versus multiple inoculation treatments on antibody levels have only been reported for white-tailed deer. Turner et al. (1996) found antibody levels for animals given a one-inoculation microsphere protocol were only 20% of those of deer receiving a two-inoculation treatment. McShea et al. (1997), using a treatment protocol similar to that employed in this study, found the one-inoculation protocol produced antibody levels approximately one-half that of deer receiving the two-inoculation protocol. In both of these experiments most animals receiving a single inoculation became pregnant during the first breeding season after treatment.

For an effective immune response the PZP antigen must be administered with an adjuvant. The most widely used combination of Freund's complete and Freund's incomplete adjuvant, as described by Freund (1947, 1951), is believed to produce the strongest antibody response. However, FCA, containing heat-killed *Mycobacterium tuberculosis* or *M. butyricum* as the highly antigenic component, can cause severe systemic reactions, chronic pain, and abscesses at the injection site (Amyx, 1987; Jennings, 1995). This problem appears to be considered less serious in wild animals as compared to domestic animals (Kirkpatrick et al., 1992; 1996b). Yet another unwelcome side effect of FCA administration can be the resulting tuberculosis-positive serum reactions. Thus, widespread future use of the PZP vaccine in wildlife species must rest upon the development and use of other adjuvants.

Although the study was not designed to address the question of what level of anti-PZP antibodies is required for effective contraception, data from the last two sampling times (3 mo pre-breeding and 2 mo post-breeding) are useful as a first approximation (Fig. 4). Most animals in this study became pregnant when bred approximately 13 to 14 mo after receiving their first PZP vaccination. These animals had antibody levels in the 40 to 90 range at the time of breeding, which indicates these levels were inadequate to effect contraception. In contrast, the four animals with the highest antibody levels, ≥ 100, failed to become pregnant, suggesting an antibody level of 100 may provide an estimate of the minimum level required to prevent pregnancy. This estimate should be considered an approximation because no blood samples were drawn at the time of breeding, hence, we had to assume a linear trend in antibody levels during the 5 mo between the two sampling dates. The suggested contraceptive threshold for anti-PZP levels in elk is considerably higher than the 50 to 65 documented for horses (Liu et al., 1989) and white-tailed deer (Turner et al., 1996). Similar species-specific differences in the response to the PZP vaccine have been documented by Kirkpatrick et al. (1996b).

Although the cited contraceptive thresholds are based on ELISA results originating from the same laboratory, their comparison should not be given too much value for the following reasons. The selection of the positive reference serum pool in the ELISA for each species was usually based on the first batch of sera (sometimes from less than 20 animals) submitted to the laboratory. This allowed comparison of all the results obtained for that species within the laboratory, but cannot be considered a valid standardization of the ELISA for interspecies comparison. The fact that anti-deer IgG had to be used because specific anti-elk IgG was not available, also plays a role, although the strong reactions obtained in this study indicate a high degree of structural similarity between deer and elk immunoglobulin. However, even with optimal standardization of the positive reference serum and specific anti-IgG for each species, when comparing the contraceptive threshold, it always should be kept in mind that the anti-PZP antibody level is

only indirectly responsible for contraception. Instead, it is the cross-reacting part of the anti-PZP antibody that binds to the zona pellucida on the oocytes of the treated species and, thus, blocks sperm binding. For example, a mare serum of an anti-PZP level of 94 (ELISA) and an antibody titer of 1:800 when tested against pig oocytes in immunoflorescence, elicited a cross-reacting antibody titer of only 1:20 against mare oocytes (Liu et al., 1989). Moreover, it has to be expected that the titer correlation between the anti-PZP antibody and its associated cross-reacting antibody varies among the different mammalian species, depending on the degree of structural similarity between porcine and their own zona pellucida. Additionally, individual immune systems among the same species may not always recognize the same PZP structures as antigenic, which could result in variation of the titer correlation between the primary and the associated cross-reacting antibody within the same species.

Determination of the contraceptive threshold is an important tool for controlling immunization procedures within the respective species, with the understanding that there will always be immunological high and low responders among the individuals. Although the antibody level required for effective contraception may actually vary from one animal to the next, it is useful to consider our best estimate of the minimum effective antibody level in order to explore the consequences of the substantial variation observed in the immune response of the individual animals challenged with PZP vaccine in this experiment (Figs. 2, 3). Time required for individual animal immune systems to produce the required antibody level necessary for effective contraception varied from 1 mo after the initial vaccination for a single animal, 2 mo for eight animals, 4 mo for six animals, a single animal required 6 mo, while two animals never reached the threshold. Of the 15 animals attaining threshold levels that were sampled through 15 mo post-injection, 4, 1, 3, 1, 2, 4 animals maintained antibody levels ≥ 100 for 1, 2, 3, 5, 7, 9 mo, respectively.

This variability in immune response with respect to effecting contraception could have important consequences when attempting to manage free-ranging populations. Elk, as well as most other wildlife species being considered for treatment with PZP vaccines, are polyestrous and may continue cycling if they fail to conceive (Haigh and Hudson, 1993). It is uncertain how long estrus cycling would continue in free-ranging animals, but data from captive horses (Plotka et al., 1989), white-tailed deer (Plotka et al., 1977), and elk (Haigh and Hudson, 1993) suggest cycling could continue for 3 to 8 mo beyond the normal breeding season and evidence of recurrent cycling has been documented in PZP experiments involving all three species (Liu et al., 1989; McShea et al., 1997; Heilmann et al., 1997). The high individual variability in the time required for antibody titers to attain effective levels and duration of effectively high antibody titers demonstrated in this study suggests that there will be considerable uncertainty in the consequences of population-level treatment programs. It will be difficult to predict when vaccination programs should be implemented with respect to the onset of breeding and what proportion of the treated animals will be effectively contracepted at the time of breeding. Perhaps an even more important concern is the possibility that antibody levels in individuals are sufficiently high to effect contraception for one or more estrus cycles, but then drop below the effective level while the animal is still cycling. This could result in an unpredictable proportion of the treated animals possibly conceiving outside the normal breeding season and birthing beyond the normal spring parturition period. The higher the individual variability in immune response to challenge with PZP the higher the probability that such treatments will result in asynchrony of seasonal reproductive events in a population, similar to

that suggested through computer simulations by Garrott and Siniff (1992) for male-oriented contraceptive techniques. It is possible that individual variability in immune response decreases as animals are repeatedly treated with the PZP vaccine, thus diminishing this potential problem; however, no experimental data are yet available.

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