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CONTRACEPTIVE POTENTIAL OF THE PORCINE ZONA PELLUCIDA VACCINE IN THE AFRICAN ELEPHANT (Loxodonta africana)

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ABSTRACT

Immunocontraception has been successful in controlling free-roaming equids; however, what is the potential for the immunocontraceptive control of the African elephant (Loxodonta africana)? The porcine zona pellucida (pZP) glycoproteins share antigenic domains with the African elephant zona pellucida (elZP) glycoproteins, and anti-zona pellucida serum antibodies have been successfully stimulated. To determine the cross-reactivity of the pZP and elZP, immunocytochemistry was evaluated by light and electron microscopy. Specifically, the binding of polyclonal antibodies against total heat-solubilized-porcine zona pellucida to fixed elephant ovary sections was evaluated. The elZP of primary, secondary and tertiary follicles was recognized by the rabbit-anti-pZP serum, but there was no apparent recognition of the primordial follicles. The ability of anti-pZP antibodies to recognize the elZP demonstrates that there is molecular homology between the pZP and elZP glycoproteins. This homology makes the African elephant a candidate for pZP immunocontraception. Three captive elephants were vaccinated with 400 μg pZP with a synthetic trehalose dicorynomycolate (S-TDCM) adjuvant. The elephants received 2 boosters of 600 μg pZP at 4 wk and 10 mo after the primary vaccination. The vaccinated female elephants developed significant ($P<0.05$) titers to pZP over prevaccination levels. These levels persisted for 12 to14 mo after the third vaccination. This preliminary evidence shows that the female elephant can develop significant serum antibody levels to pZP. These levels of antibodies are comparable to those required in horses for successful immunocontraception. Thus, porcine zona pellucida immunocontraception might be used to control elephant populations.
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Key words: immunocontraception, African elephant, zona pellucida

INTRODUCTION

The density and mortality rates of populations of elephants in Africa vary greatly (18). In the Republic of South Africa, a combination of good park management and virtually no poaching has led to a minimum of 5% per annum elephant population growth (4). In 1990, the Convention on International Trade in Endangered Species (CITES) promulgated a ban on ivory trading, causing concerned members of the public to focus world attention on the plight of the African elephant. Public involvement and the inflow of financial support have led to decreased poaching in some African nations (17). The high elephant densities in Kruger National Park have caused concern over habitat destruction. In conjunction with the South African National Parks, an investigation was undertaken to determine if the porcine zona pellucida (pZP) glycoproteins have shared determinants with the elephant zona pellucida (elZP).

To date, pZP has been shown to be a highly versatile immunocontraceptive molecule. Injection of total heat solubilized pZP into horses resulted in contraception (11, 12, 24). The immunocontraceptive effect usually lasts for 12 mo and has been effective in reducing fertility of free-roaming horses (5, 6). The vaccine appears to be devoid of any major side effects (5). The pZP vaccine has been used for more than 8 yr in horses, with no adverse reactions; moreover, the vaccine does not affect the fetus of pregnant mares (5). Further, immunocytochemistry with rabbit anti-pZP polyclonal antibodies exhibited no cross-reaction with the brain, heart, lung, kidney, liver, bladder, stomach, small intestine, large intestine, skeletal muscle, skin, spleen, pancreas or lymph node of horses and dogs.[b] Western blots with anti-pZP polyclonal serum also revealed no detectable cross-reactivity with equine luteinizing hormone (LH) or equine chorionic gonadotropin (eCG).

The pZP has been shown to have cross-reactivity with the zona pellucida of many species, including humans (19), mice (21), rabbits (20, 25), horses (12, 15, 24), dogs (1, 13, 14), squirrels, monkeys (22) and 40 species of zoo animals (5, 8, 9).

The aim of this study was to determine if elZP has shared determinants with the pZP. On the basis of this evidence, the ability of a pZP immunocontraceptive to produce titers in adult female elephants was also explored.

MATERIALS AND METHODS

Fixation of the Elephant Ovaries

Elephant ovarian tissues were cut into 3 to 5 mm^3 blocks with a sterile scalpel and placed into each of the following fixatives: Bouin's, neutral-buffered formalin and glutaraldehyde. The fixed samples was processed using the following methods. The tissues fixed in Bouin's and in neutral-buffered formalin were embedded in paraffin, whereas the tissues in glutaraldehyde were embedded in plastic.

Bouin's Fixation and Paraffin Embedding

Tissues were placed into 30 mL of Bouin's fluid (saturated aqueous picric acid, 75 mL; 37% formalin, 25 mL; glacial acetic acid, 5 mL) and fixed for 24 h at 4°C (2). Samples were washed under running water and were then placed for 1 h in 4 sequential 30 mL volumes of 50% ethanol (ETOH) at 4°C until the yellow stain disappeared. Following this, the samples were dehydrated, cleared, infiltrated and embedded in paraffin.

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[b]Fayrer-Hosken, personal communication 1999.

Neutral Buffered Formalin (NBF) Fixation and Paraffin Embedding

Tissues were placed into 30 mL of NBF (20%) and fixed for 24 h at room temperature (10, 23). Next, samples were dehydrated and embedded. All paraffin embedded samples for histology were then sectioned to 3 μm thickness on a Leitz microtome[c] and stained using Gill's hematoxylin and eosin (H&E; 3) using an automated processor.[d]

Glutaraldehyde Fixation and Plastic Embedding

Tissues were sectioned into 1 to 2 mm^3 cubes and fixed in 1% glutaraldehyde[e] in phosphate buffered saline (PBS), pH 7.4, at room temperature for 2 h. The samples were washed 5 times in chilled PBS and stored at 4°C overnight. After refrigeration, the samples were washed with 10 mL each of PBS and deionized water. They were then dehydrated through a series of ETOH solutions (30, 50, 75, and 95%) for 15 min each at 4°C and allowed to come to room temperature. Final dehydration steps included three, 30-min incubations in 100% ETOH at room temperature. Samples were infiltrated and embedded in JB-4®, a glycol methacrylate-based plastic resin,[e] and 1.5 to 2.0-μm sections were obtained using a Sorvall JB-4 Porter-Blum microtome. The sections were placed on glass slides and, after drying, were stained with H&E using routine methods.

Preparation of the Anti-pZP Antibody

Male New Zealand White rabbits were vaccinated with 400 μg of highly purified pZP in Complete Freund's Adjuvant and then boosted twice 2 wk apart with 200 μg of highly purified pZP in Freund's Incomplete Adjuvant. Serum was harvested from the rabbits 2 wk after the last vaccination. To confirm the specificity of the antibodies for pZP, 1-D and 2-D Western blot analyses were performed. In the Western blots, only pZP glycoproteins were stained, and all 3 families of pZP were recognized (pZP1, pZP3α, and pZP3β).

Elephant Oocyte Fixation and Embedment

Graafian follicle elephant oocytes was enrobed in 58% molten noble agar to facilitate handling, then promptly immersed in a fixative containing 1% paraformaldehyde, 1% glutaraldehyde and 0.1% picric acid in 0.1 M cacodylate-HCl buffer, pH 7.2. After several hours of fixation, the fixative was rinsed from the oocytes with several changes of buffer, followed by several rinses of deionized water to remove the buffer from the oocytes. A graded series of ethanol was used to dehydrate the oocytes to 95% ethanol. The oocytes were infiltrated gradually with 95% ethanol and LR White Medium Grade resin, a hydrophilic acrylic resin,[e] and then several long changes of 100% LR White resin. The oocytes were embedded in a gelatin capsule with fresh 100% LR White resin and were allowed to polymerize 24 h in a 58°C oven. The gelatin capsule was trimmed, and 1-μm sections were obtained using a Reichert Ultracut S ultramicrotome[f]. Sections were placed on glass slides and allowed to dry with moderate heat before staining with 1% Toluidine Blue in 1% sodium borate.

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cDarmstadt, GDR.

dHacker Instruments Inc, Fairfield, NJ.

ePolysciences Inc, Warrington, PA.

fLeica Inc, Deerfield, IL.

Immunocytochemistry for Light Microscopy

All three fixation methods were evaluated for the tissue-fixation ability and the ability to preserve tissue antigenicity. The paraffin blocks were sectioned and the sections placed on slides. The slides were deparaffinized and blocked overnight in TRIS-buffered saline (TBS) containing 3% bovine serum albumin (BSA). Slides were washed and incubated with the primary antibody (rabbit-anti-pZP, 1:100) in TBST (TBS with Tween 20) for 2 h. After incubation, the slides were washed with TBS. The slides were then incubated with the secondary antibody (protein A-10 nm gold, 1:100) in TBST for 1 h. After this step, the slides were washed in TBS, followed by triple distilled water. The staining of the slides was enhanced using standard silver enhancement,[g] dried, and permanently mounted.

The JB-4 blocks were sectioned and placed on slides. After the sections were allowed to dry, the slides were blocked in 3% BSA in TBST overnight in a wet chamber at 4°C. After blocking for 24 h, the sections were washed in TBST and incubated in primary antibody, anti-pZP, at 1:500 in TBST for 2 h at room temperature in a wet chamber. After washing in TBST, the slides were then incubated in the secondary antibody (protein-A conjugated to 10 nm colloidal gold[g]) at 1:10 in TBST for 1 h at room temperature in a wet chamber. Next, the slides were jet washed in TBS and deionized water for 1 min each. The sections were enhanced using the silver-enhancement technique, and the reaction was stopped with deionized water after color development was observed. The sections were allowed to air dry, and coverslips were mounted with Flo-Texx®[h] prior to evaluation.

Immunocytochemistry for Transmission Electron Microscopy

Thin sections of the oocyte were obtained and mounted on Formvar carbon-coated nickel grids. The grids were blocked for 30 min in a TBS-BSA buffer solution (0.2 M Tris-buffered saline, pH 8.2 with 1% globulin-free BSA) with Tween 20 added (1 drop/mL buffer). After washing in buffer, the grids were incubated in the primary antibody (rabbit-anti-pZP) at 1:16,000 in TBS-BSA overnight in a wet chamber at 4°C. The grids were washed in buffer before incubating in the secondary antibody, Protein-A conjugated to 10 nm colloidal gold, diluted 1:20 in TBS-BSA, 30 min at room temperature in a wet chamber. The grids were washed in buffer and then deionized water before continuing with a 10-min silver enhancement. After enhancement, the grids were washed in deionized water and poststained with 5% methanolic uranyl acetate and Reynold's lead citrate. The grids were observed with the JEM-1210 transmission electron microscope.[i]

Vaccination of Female Elephants .

As a result of significant cooperation with zoos, 3 elephants were vaccinated with the pZP vaccine. A 30-yr-old African elephant (Elephant 1) at the Riverbanks Zoological Park and Botanical Garden, in Columbia, South Carolina; a 25-yr-old African elephant (Elephant 2) at the Greenville Zoo in Greenville, South Carolina; and a 20-yr-old Asian elephant (Elephant 3) at the Calgary Zoo, Calgary, BC, were vaccinated. Elephants 1, 2 and 3 were initially vaccinated with a 10% oil-in-water emulsion containing 400 μg of pZP and 5 mg of synthetic trehalose dicorynomycolate[j] (S-TDCM). Elephants 1 and 3 were initially boosted with 400 μg of pZP.

Elephant 2, however, was boosted with 600 µg of pZP based on the initial serum antibody levels. For the subsequent boosters, all elephants received 600 µg of pZP in a 10% oil-in-water emulsion with 5 mg of S-TDCM adjuvant. Booster administrations were given after 4 wk and again 10 mo later.

Enzyme Linked Immunosorbent Assay (ELISA) Detection of Anti-pZP IgG Levels in Elephants

For all assays, each data point was performed in triplicate, and each plate had a positive control (rabbit anti-pZP) and two negative controls (normal rabbit serum and no pZP in the well). Each well of the ELISA plates[k] was coated with 2 µg/well of pZP. The pZP was reconstituted from a lyophilized pZP stock to a concentration of 1 mg/mL in 0.02 M TRIS at pH 8.2. Fifty microliters of the stock solution (2 µg pZP) was added to the wells. The plates were incubated for 6 h at 4°C. The plates were then washed 3 times with TBS. To block the plates, 200 µL of TRIS buffered saline and TBST with 5% BSA[l] were added to each well. The covered plate was incubated at 4°C for 12 ho. The plates were removed from the refrigerator and allowed to warm to room temperature. The TBST was removed, and the wells were washed 3 times with TBS. To each well, 50 µL of the elephant serum at a dilution of 1:500 or 1:1,000 were added and incubated for 4 ho. The elephant serum was poured off, and the plates were washed 3 times with TBS. The secondary antibody, rabbit anti-elephant IgG[m], was added at 50 µL/well (1:1,000 dilution in TBST) and incubated for 2 ho. Finally, 50 µL/well of the tertiary antibody (goat anti-rabbit with alkaline phosphatase) were added at a 1:2,000 dilution in TBST. The plates were then incubated for 2 h at room temperature. The plates were rinsed 3 times with TBS and dried. At this time, 200 µL of carbonate buffer were added to each well to adjust the pH to 9.8. After 5 min, the buffer was removed, and the plate was dried on paper towels. Finally, 50 µL/well of p-nitrophenyl phosphate in carbonate buffer at pH 9.8 were added. The color reaction was allowed to proceed for 30 min, after which it was stopped by adding 50 µL/well of 3 M NaOH. The plate was read at 405 and 492 nm.

Statistical Analysis

Mean serum antibody levels were compared statistically using an analysis of variance (ANOVA) and a Tukey's test to perform a pairwise comparison of the means. This was performed using computer software SAS[n] (version 6.21). Statistical significance was accepted at $P<0.05$.

RESULTS

Normal Histology

Primary (Figure 1A), secondary (Figure 2A), and tertiary follicles were easily identified in all fixed samples of ovarian tissue. Bouin's fixation provided the best preservation of the cellular detail. The primary follicles (Figure 1A) consisted of a single layer of granulosa cells surrounding the oocyte. The oocyte did not have a clearly visible zona pellucida at x 1,000 magnification. The secondary follicles (Figure 2A) had 2 to 3 layers of cuboidal granulosa cells, and the zona pellucida was clearly visible surrounding the oocyte. In the tertiary follicles, there were multiple layers of cuboidal to columnar granulosa cells, and the zona was clearly visible.

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[k]Immulon 96 well, Chantilly, VA.

[l]Fraction V, Sigma, St Louis, MO.

[m]Courtesy of Dr. Henk Bertschinger.
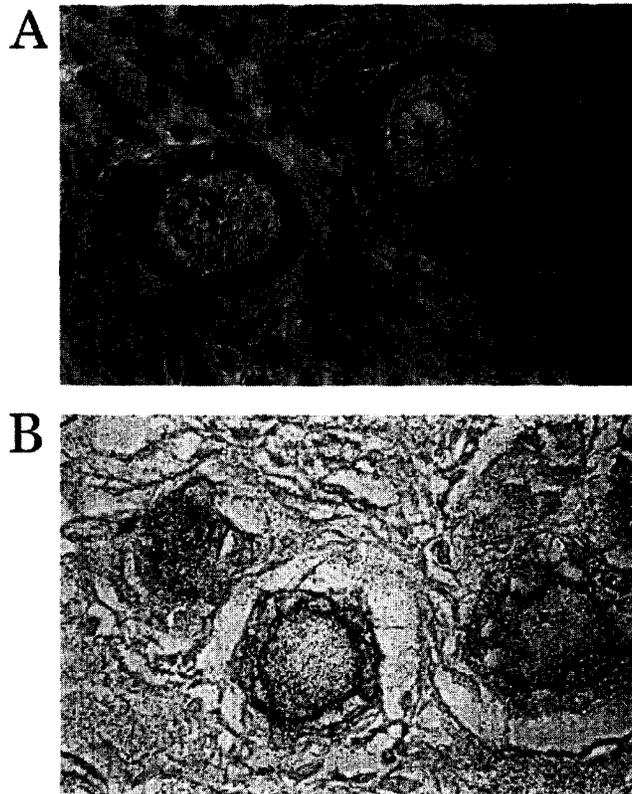
[n]SAS Institute, Cary, NC.

Figure 1. A) Hematoxylin and eosin stained section of an elephant ovary at x 1000
magnification. The section shows 2 primary follicles that are located centrally
surrounded by a single layer of granulosa cells. B) Immunogold staining using a
polyclonal rabbit antibody against heat-solubilized porcine zona pellucida in a section
of an elephant ovary at x 1000 magnification. This section shows 3 primary follicles
surrounded by immunogold staining of the early elephant zona pellucida.

Immunocytochemistry for Light Microscopy

In the primary follicles (Figure 1B), immunogold deposits were concentrated at the oocyte-
granulosa cell junctions. This formed a thin layer of gold staining at the cell junctions.
Furthermore, here was diffuse staining of the primary oocyte cytoplasm, which exceeded that of
the background and control sections. In the secondary (Figure 2B) and tertiary follicles, there was
definitive staining of the zona pellucida; however, there was also some staining of intercorona
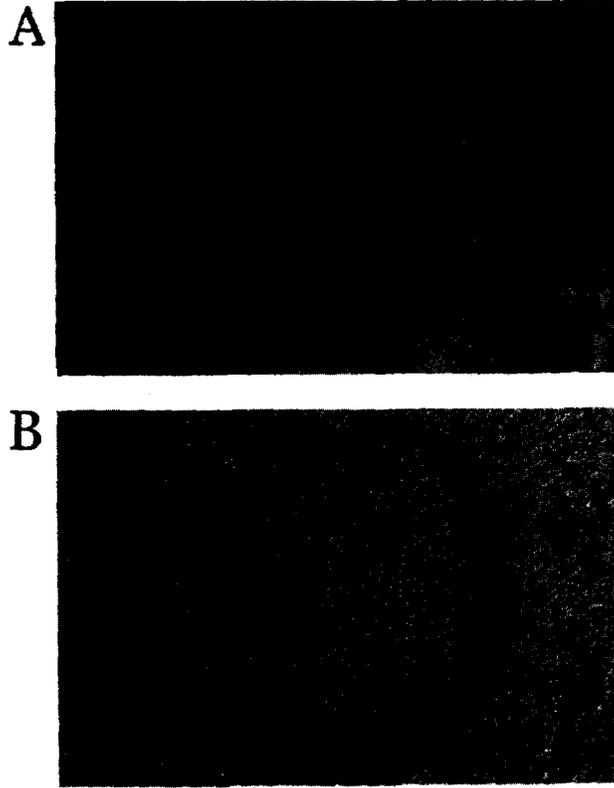radiata cell spaces.

Figure 2.A) Hematoxylin and eosin stained section of an elephant ovary at x 1000 magnification. The section shows a secondary follicle surrounded by layers of granulosa cells. The zona pellucida is clearly visible between the oocyte and granulosa cells. B) Immunogold staining using a polyclonal rabbit antibody against heat-solubilized porcine zona pellucida in a section of an elephant ovary at x 1000 magnification showing a secondary follicle. The follicle is surrounded by immunogold staining of the elephant zona pellucida.

Immunocytochemistry for transmission electron microscopy (TEM)

The zona of the control sections (x 8,000) had no gold staining (Figure 3A) of the zona pellucida, the ooplasm, or the perizonal area. In the sections treated (x 12,000) with anti-pZP (Figure 3B), the gold labeling can be clearly seen only over the zona pellucida. Some gold granules can be seen in the cytoplasm especially within dilated portions of the smooth endoplasmic reticulum.
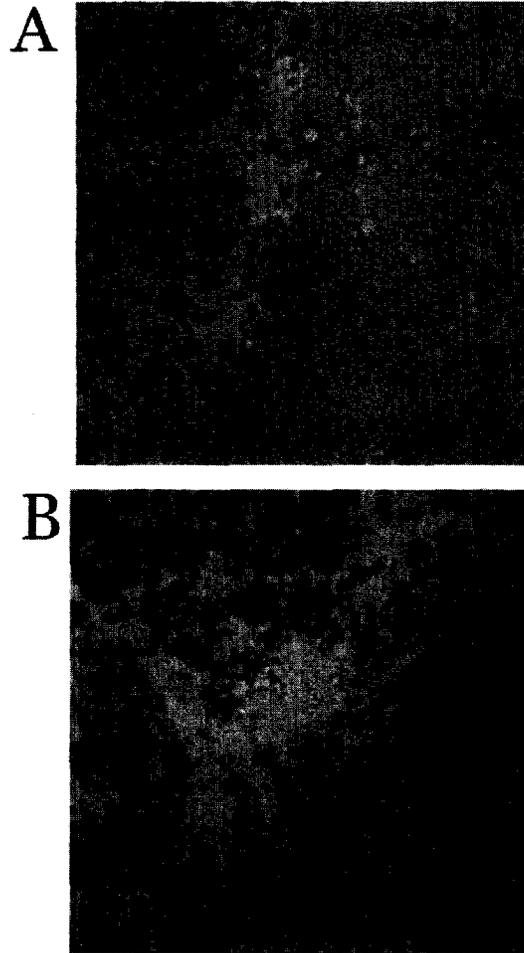
Figure 3. A. Immunogold staining using of a control elephant oocyte at x 8,000 magnification. Neither the ooplasm, the zona pellucida, or the space external to the zona pellucida show any immunogold staining. B. Immunogold staining using a polyclonal rabbit antibody against heat-solubilized porcine zona pellucida in an elephant oocyte at x 12,000 magnification. The elephant zona pellucida is clearly labeled with the immunogold staining. In addition, there is labeling of the ooplasm.

Serum Anti-pZP Levels

The serum levels of anti-pZP IgG rose with each successive vaccination (Figure 4). However, it was not until after the second booster that the levels remained elevated for at least 12 mo. The levels of anti-pZP IgG in Elephant 2 did not attain the levels of Elephant 1. Elephant 3, an Asian elephant, did not develop sustained levels until after the second booster.
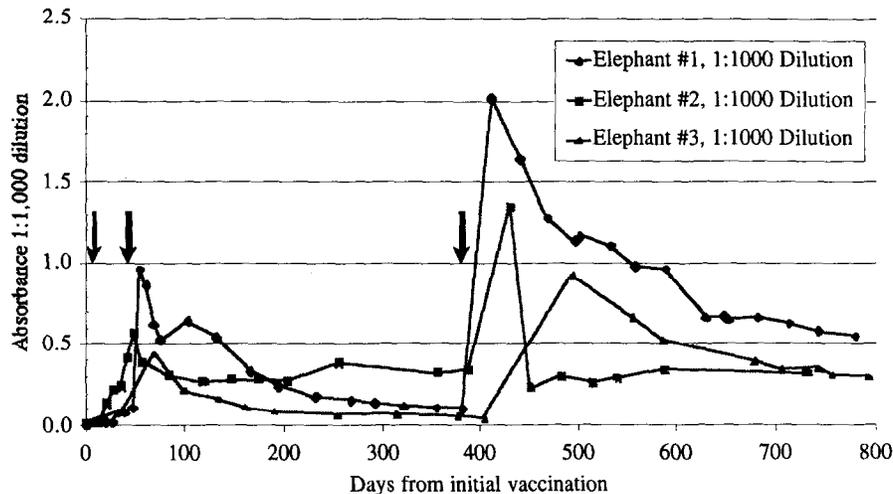
Figure 4. Serum levels of anti-pZP IgG in 3 captive elephants. The levels rose with each successive vaccination, but it rose the most and was sustained the longest after the second booster.

DISCUSSION

The immunocytochemistry results reported here establish that there are shared epitopes between the elephant and pZP. This was demonstrated at the light microscopy level by immunogold staining of the zona pellucida of primary, secondary and tertiary oocytes, and then confirmed with TEM, which demonstrated that there was significant immunogold staining of the elZP. Moreover, the elZP might originate from the oocyte since immunogold staining of the dilated portions of the smooth endoplasmic reticulum was clearly visible, whereas it is not visible in any of surrounding cells. Hence, antibodies directed against the pZP would also recognize elZP. This data thus provides support for the hypothesis that the elephant vaccinated with pZP could be contracepted and it provides additional evidence that the pZP has significant homology with the zona pellucidae of many mammalian species.

For the vaccination trials, all elephants responded with significant (P<0.05) increases in blood levels of anti-pZP antibodies, specifically that of IgG. The magnitude of the antibody increases were equivalent to those of vaccinated immunocontracepted mares. There were, however, differences in serum IgG levels among elephants, and especially in the lower sustained levels in Elephants 2 and 3 prior to the second booster. However, in Elephant 2 these lower levels might have a nutritional origin, as her diet temporarily had low vitamin E levels. Vitamin E levels have been correlated with immune competence and specifically immunoglobulin levels. Once vitamin E supplementation was initiated, the antibody levels of Elephant 2 increased and were equivalent to those of Elephants 1 and 3. Elephant 3 was an Asian elephant, and this may also have contributed to the different response dynamics in the phase prior to the second booster. Initial indications were that the titers after the first booster might be sustained for a sufficient time to serve as an acceptable immunocontraceptive agent. But only once the profile was completed did it

become clear that a second booster would be needed for sustained levels. Hence the final quantity of serum IgG, after 2 boosters, appears to be sufficient for effective contraception in the elephant, but this needs to be proven in a free-roaming population.

The need for population management of African elephants will become a reality during the next century; however, it is also evident that culling will be increasingly unacceptable to the world community. Thus, alternative strategies must be provided to park and habitat managers in order to control elephant populations. These initial studies are pivotal in determining the potential role of the pZP immunocontraceptive vaccine.

The delivery of pZP vaccine and an adjuvant to free-roaming equids has already been shown to be a practical and economic reality (5). In addition, the pZP vaccine and adjuvant mixtures that have been reported in horses (5, 24) have had very few side effects such as lameness, swelling and abscesses. With the increasing availability of pZP for immunocontraception, there will be more widespread use of this technology for vaccinating animals. However, it is not clear if a remotely delivered pZP vaccine can control the number of free-roaming animals. The initial data from Assateague (5) Island indicate that a free-roaming population of animals can be controlled by remotely delivered pZP immunocontraception. The population of the Assateague horses is now kept at a constant by regular vaccination with pZP. Hence, the pZP technology is probably capable of affecting population control in the African elephant of South Africa.

What are the effects on herd dynamics and social behavior of immunocontraception on the free-roaming African elephant? This is an especially important question in this species (African elephant), whose social interactions may be among the most advanced in the animal kingdom. The potential impact of reduced numbers of calves on herds of the elephant are not known, but this is an important consideration to take into account, since the elephant is known to have highly evolved emotions and can comprehend the concept of death. Elephants will investigate dead relatives and have even been seen carrying the bones of dead relatives. Their bonds are so strong that they will attempt to assist sick, injured, and even dying elephants; the loss of group members results in changes in family cohesion and social bonding (17). Immunocontraception may also supplant the need for culling, thus nullifying its effects on the elephants. Reports by Moss (16) describe that entire family units are seldom culled, and the survivors of these herds are severely traumatized by the disruption of the matriarchal family units. Of the available population control options, it would seem that immunocontraception and reduced calf numbers would be the most acceptable. The potential use of immunocontraception in controlling free-roaming African elephants is very encouraging, and it may provide an important tool for ecology and conservation of both the species and the habitat.

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