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A COMPARISON OF FREUND'S COMPLETE AND FREUND'S MODIFIED ADJUVANTS USED WITH A CONTRACEPTIVE VACCINE IN WILD HORSES (*EQUUS CABALLUS*)

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Abstract: Fifteen captive wild mares (*Equus caballus*) were treated with porcine zona pellucida contraceptive vaccin and either Freund's Complete Adjuvant ($n = 7$) or Freund's Modified Adjuvant ($n = 8$). All mares received a booste inoculation of porcine zona pellucida plus Freund's Incomplete Adjuvant a month later. Anti-porcine zona pellucid antibodies were measured over 10 mo following the initial inoculation. There were no significant differences in antibody titers at any point during the 10 mo, and seven of the eight mares in the Freund's Modified Adjuvant group were abov the 60% level at the end of the study, which is considered to be the contraceptive threshold for horses. There were no significant differences in titers between pregnant and nonpregnant horses, nor was there a significant correlation betweer age and titers. One local injection site reaction occurred after booster treatment with Freund's Incomplete Adjuvant and 11 healthy foals were born during the course of the study. These data suggest that Freund's Modified Adjuvant is an acceptable substitute for Freund's Complete Adjuvant in certain free-ranging and captive wildlife species.

Key words: Adjuvants, antibodies, contraception, *Equus caballus*, immunology, porcine zona pellucida, horses.

INTRODUCTION

Porcine zona pellucida (PZP) epitopes have been used effectively as a contraceptive vaccine in a wide variety of captive and free-ranging wildlife species over the past 15 yr.[21] It is the evolutionary conservation of the mammalian sperm receptor, from which the PZP molecule is derived in pigs, that results in this efficacy across many mammalian species, but the homology of the sperm receptor epitope across species has also rendered the PZP vaccine a poor immunogen. Thus, the efficacy of the PZP epitope as an immunocontraceptive depends on the effectiveness of adjuvants with which it is used.

Porcine zona pellucida has been used in captive[23] and free-ranging wild horses (Equus caballus)[15-19,31,33-36] since 1988 with a high degree of efficacy. The adjuvant of choice has been Freund's Complete Adjuvant (FCA) for the initial inoculation and Freund's Incomplete Adjuvant (FIA) for subsequent booster inoculations. The 90% or greater efficacy resulting from the use of PZP with FCA[31] is not surprising because this particular adjuvant is viewed as the "gold standard" among adjuvants.[1] However, the use of FCA has raised concerns because of two potential side effects. The first concern arises from historical data, derived almost exclusively from laboratory animals, that indicate the use of FCA can lead to injection site reactions, including open abscesses.[7,28] The second concern is that the FCA can cause false-positive tuberculosis (TB) test results in treated animals. The primary adjuvant ingredient in FCA is the dried fractionated cell walls of *Mycobacterium tuberculosis*, and although it acts as a powerful nonspecific immune stimulant, it can also cause antibodies against the TB organism.

The issue of FCA-induced injection site reactions and abscesses has been studied in both wild horses and captive exotic species in zoos. Among wild horses on Assateague Island National Seashore, only three abscesses appeared after 381 treatments (0.007%), and one of these appeared after treatment with FIA rather than FCA.[17] In another study,[36] 60 wild mares receiving the standard two-inoculation protocol of PZP plus FCA followed by PZP plus FIA and observed in captivity daily for 1 mo did not form a single abscess. Among zoo animals treated with PZP, 1,185 treatments with either darts or hand injection resulted in a total of 16 abscesses (0.013%) (J. F. Kirkpatrick, unpubl. data). Twelve of those abscesses occurred following inoculations with FCA, three with FIA, and one with a different adjuvant. In contrast, PZP plus FCA inoculations, given in the neck of horses, results in a significantly higher occurrence of abscesses (I. K. Liu, pers. comm.). Thus, it appears that, if the PZP plus FCA treatment is given exclusively in the gluteal or hip muscles of large ungulates, injection site reactions are not a significant problem. Despite these data, U. S. Department of Agriculture (USDA) officials overseeing wild horse safety issues persist in their opposition to the use of FCA.

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The issue of the potential false-positive TB test results after PZP plus FCA treatment is a serious issue with many species, particularly captive exotic species in zoos. However, no reliable test for TB exists in equids, and the use of FCA in these taxa, and wild horses in particular, represents a moot point. Nevertheless, USDA officials persist in raising this issue and even the possibility that one day a reliable test might exist. This problem was solved in zoo animals by substituting Freund's Modifed Adjuvant (FMA) for FCA. Freund's Modified Adjuvant relies on the freeze-dried fractionated cell walls of *Mycobacterium butyricum*, a bacterium commonly found in rancid butter, with no known associated pathologies. As such it cannot cause false-positive TB test results. To date, however, only a single study of FMA that examines actual antibody titers, as well as contraceptive efficacy, has been conducted with fallow deer (*Cervus dama*).[4] In that study, PZP plus FMA followed by a booster inoculation of PZP plus FIA proved as efficacious as an initial treatment of PZP plus FIA followed by two booster inoculations of PZP plus FIA, but no comparison was made with FCA.

The purpose of this study was to compare the effectiveness of FCA with that of FMA in the wild horse, based on antibody titers against PZP. The hypothesis, based on previous work on contraceptive efficacy in zoo animals and fallow deer,[4] was that FMA would not be as effective as FCA in terms of raising anti-PZP antibodies but that the differences would not be significant with regard to contraceptive antibody titers.

METHODS AND MATERIALS

Animals

Fifteen captured wild mares, 6–16 yr of age, were randomly selected from several herd management areas and housed at the Bureau of Land Management holding facility at Palomino Valley, Nevada. They were selected on the basis of (1) good health, and (2) normal reproductive age classes. No determination of pregnancy status was made. Animals were housed in a paddock of approximately 3,300 m^2, which provided for daily exercise and freedom of movement. All mares were wormed before the onset of the PZP treatments and given the normal spectrum of Bureau of Land Management vaccinations, including Strepguard (Bayer, Shawnee Mission, Kansas 66216, USA), Encevac (Bayer, Shawnee Mission, Kansas 66216, USA), FluVac (Wyeth, Madison, New Jersey 07940, USA), and Imrab (Merial, Duluth, Georgia 30096, USA), along with appropriate boosters. Animals were fed daily with alfalfa hay, and a salt/mineral block was available at all times. For initial treatment, booster treatment, and monthly blood collections, the animals were partially immobilized in a hydraulic squeeze chute. A contract veterinarian was on site throughout the trial. Animals were examined daily by a BLM employee.

PZP preparation

The native PZP antigen was prepared at the Science and Conservation Center (SCC) at ZooMontana, in Billings, from porcine ovaries.[5] The PZP antigen was screened for porcine viruses by the USDA laboratory in Ames, Iowa, and for pathogenic bacteria at the SCC. Qualitative analysis was carried out by means of polyacrylamide gel electrophoresis (PAGE), and permanent images of the gels were stored on computer. The antigen was titrated to doses of 100 μg in 0.5 cc phosphate buffer (pH 7.0), stored at −44°C, and transported frozen to Palomino Valley.

Treatment protocol

Seven animals received an initial inoculation of PZP plus FCA (Sigma Chemical, St. Louis, Missouri 63178, USA). The 0.5 ml of PZP was emulsified with 0.5 ml of FCA as described[15] and given intramuscularly in the hip or gluteal muscles by hand injection. Eight mares received an initial inoculation of PZP plus FMA (Calbiochem, Inc., La Jolla, California 92039, USA), prepared and administered in the same manner as described above. The FMA contains 0.85 mg/ml of bacterial cell suspension suspended in 85% Drakel 5 NF and 15% Arlacel A, mannide monooleate oil. Twenty-seven days later all 15 mares received a booster inoculation of 100 μg PZP plus FIA (Sigma Chemical, St. Louis, Missouri 63178, USA), prepared and administered as described above. Each 1 ml of FIA contains 0.85 ml of paraffin oil and 0.15 ml mannide mononucleate. A 5.0-ml venous blood sample was collected in Corvac sterile separation tubes at the time of the initial and booster inoculations. Thereafter, a blood sample was collected every month (at approximate 30-day intervals) for 9 mo. Serum was harvested and stored frozen until shipment to the SCC.

Antibody titer analysis

Anti-PZP antibodies were quantitatively analyzed.[3] Heat-solubilized PZP protein was diluted to a concentration of 130 μg/ml in PBS and then further diluted in coating buffer (0.1 M Na_2CO_3, pH 9.6) using 138 μl of the diluted PZP solution and 22.5 ml coating buffer; 200 ml of this dilution was

placed in each well of a 96-well Nunc plate and incubated overnight at 4°C. Blank wells contained only coating buffer. After incubation, the plates were washed three times with PBS-0.05% Tween buffer. A blocking solution consisting of PBS-0.05% Tween and 1.0% gelatin was placed into each well and incubated 1 hr at 37°C.

The plates were washed five times with 200 µl of PBS-Tween buffer, each time followed by the addition of the primary antibody. Initial dilutions of reference control and test sera were made with PBS-0.05% Tween-0.1% gelatin and incubated 1.5 hr at 37°C. After the plates were washed five times with PBS-0.05% Tween buffer, a 1:400 solution of antibody, consisting of anti-horse IgG conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland 20879, USA) diluted in PBS-0.05% Tween buffer-0.1% gelatin was added to each well (200 µl/well) and incubated 1.5 hr at 37°C. Following incubation, plates were washed three times with PBS-0.05% Tween-0.05% Tween buffer and then twice with PBS.

An enzyme substrate solution (200 µl/well) of 0.1 M Na_2CO_3, $MgCl_2$, H_2O, and *p*-nitrophenyl phosphate (Sigma Chemical Co, St. Louis, MO 63178, USA) was added to each well and allowed to react for 30–60 min at room temperature with gentle shaking until absorbance of positive reference serum reached an optical density of approximately 1.5. After color development, the plates were read at 404 nm on a Molecular Devices Model Emax spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA 94089, USA) using non-coated wells as reagent blanks.

All test sera were assayed in duplicate and expressed as a percentage of the positive reference sera, which consisted of a pool of sera from horses that had demonstrated anti-PZP titers in the high-positive range (mean of experimental serum absorbance/mean of reference serum absorbance) and had not become pregnant following treatment. The dilutions used in these determinations correspond to the dilution of the reference sera giving 50% maximum binding.

Statistical analysis

Differences in antibody titers between treatments with FCA and with FMA, at each month across the 12 mo of the study, and for differences between pregnant and nonpregnant animals, were tested for significance by Fisher's exact test for contingency tables, by the Tukey-Kramer Multiple Comparison test, and by unpaired *t*-test with Welch's correction applied.[28] Correlations between antibody titers and age were tested for significance by ANOVA.

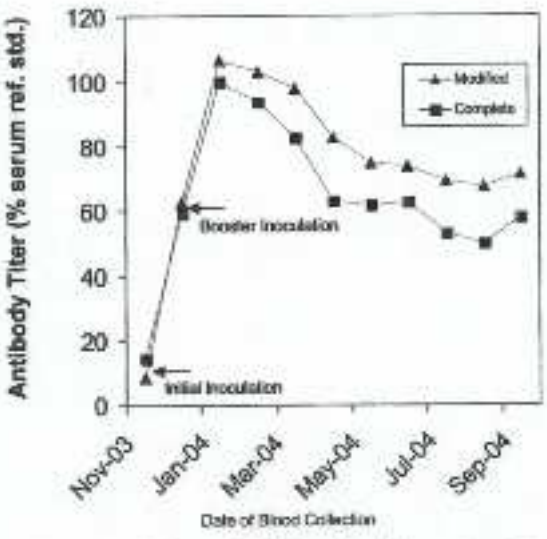

Figure 1. Mean anti-PZP antibody titers in captive wild mares created with PZP plus FCA ($n = 7$) or PZP plus FMA ($n = 8$), over a 10-mo period. There were no significant differences ($P < 0.05$) between FMA- and FCA-treated mean titers at any point in the 10 mo of the study.

RESULTS

Across the 10 mo of the study, one animal (#3779) presented an injection site reaction in the form of an abscess. This abscess was approximately 25 mm in diameter and appeared on 14 January 2004, or 1 mo following the FIA booster inoculation. It drained and healed without incident. Anti-PZP antibody titer values for individual animals are given in Table 1, and the mean antibody titers for the 15 mares and the sequential rise and fall over time are illustrated in Figure 1. Although titers were consistently higher in FMA-treated mares, there were no significant differences ($P < 0.05$) in titers at any time in the 11-mo course of the study, regardless of the statistical test applied. Peak titers in both treatment groups were attained from 30 to 60 days following the FIA booster inoculation and declined thereafter, until the 9-mo postbooster inoculation.

The range for titers in the FMA group at January 2004, the point where highest titers were produced, and October 2004, at the conclusion of the study, were 120% and 77%, and 114% and 20% of the positive reference standard, respectively. The FMA group had one animal that was clearly a poor responder (#4304, age 16 yr), yet the mean titers for the eight FMA animals were not significantly lower than those in the FCA group despite this bias.

Eleven of the mares were pregnant at the time of

Table 1. Anti-PZP antibody titers for wild mares treated with PZP plus Freund's Complete Adjuvant (C) or Freund's Modified Adjuvant (M).*

Horse #	4115	4308	4313	4327	4328	4433	4590	3662	3779	4304	4305	4595	4672	4781	4787
Age	12	13	16	12	16	16	8	12	7	16	16	6	8	8	8
Adjuvant	C	C	C	C	C	C	C	M	M	M	M	M	M	M	M
Date															
11/13/03	3	19	12	11	18	27	11	3	4	4	5	8	19	16	10
12/10/03	25	59	68	69	37	64	93	88	71	44	39	44	56	88	73
1/12/04	97	104	92	102	98	113	93	110	117	77	106	112	94	117	120
2/17/04	97	86	93	80	96	110	94	107	125	71	95	102	109	111	106
3/17/04	93	65	89	64	86	103	79	106	120	48	95	109	109	101	96
4/15/04	69	60	62	44	72	74	61	85	104	28	76	103	100	98	65
5/13/04	67	53	50	44	65	87	65	77	89	22	61	121	88	81	60
6/15/04	71	48	46	60	72	87	55	78	77	19	49	106	115	85	61
7/15/04	69	50	43	42	66	59	42	75	63	21	45	103	112	70	66
8/12/04	61	41	39	53	58	56	42	63	70	20	52	99	110	71	56
9/15/04	79	40	43	58	59	67	60	67	67	20	63	108	114	64	68

* Antibody titer values are given as a percentage of a positive reference standard.

initial inoculation, including four in the FMA grou and seven in the FCA group; and all 11 mares gav birth to healthy foals from 4 to 6 mo followin initial treatment, and all foals were weaned in Oc tober 2004. Antibody titers at 1 mo post–boost inoculation, when titers were the highest, for th four pregnant mares treated with FMA ranged fro 97% to 64.7%; those for the four FMA-treated non pregnant mares were 110% to 78%, and the diffe ence between the two groups was not significantl different ($P = 0.64$). Nor were titers significantl different between the four nonpregnant mares trea ed with FMA and the seven pregnant mares treate with FCA ($P = 0.107$). There was no significan correlation between antibody titers and age at eithe 1 mo post–booster inoculation ($r = -0.22$) or a the termination of the study, at 9 mo post–booste inoculation ($r = -0.77$).

DISCUSSION

The single injection site reaction for the mar treated with FMA plus FIA is consistent with pre vious data for FCA in wild horses[36] and for FM/ in fallow deer[4] and other zoo animals.[21] This is no surprising, considering that almost all literature re porting injection site reactions for FCA has bee confined to small laboratory animals.[29]

The pattern of temporal changes in antibody ti ters follows patterns seen previously in a numbe of species treated with PZP plus FCA, includin horses,[25,35,38] white-tailed deer (*Odocoileus virgini anus*),[25,32] and several species of exotic cervids,[1] with peak antibody titers occurring 1–2 mo follow ing booster inoculations.

The duration of contraceptive titers considere adequate for contraception is of interest to wildlif managers, particularly in the case of free-rangin wildlife or large expansive game parks, when physical access to animals is limited and booste inoculations are often difficult to give. The "con traceptive threshold" for most species is no known, but it has been generally accepted that it i approximately 60% of serum reference standards i horses.[25] Seven of eight animals treated with FM/ maintained concentrations of antibodies at or abov this level throughout the 10 mo of the study, an three of seven animals in the FCA group were a or above the 60% level at 10 mo. This is also con sistent with results from a variety of field studie with wild horses with PZP plus FCA, where suc cessful reproduction was the endpoint and the in oculations are given by or after November.[15,25] Th FMA group contained one poor immune responde (mare #4304), yet this did not bias the overall out

come of the study, attesting to the efficacy of the FMA.

The antibody titer values resulting from this study also demonstrate the individual differences among individual animals within treatment groups. The 90% efficacy seen in other studies with wild horses[15-18,23,32,34,36] reflects a variety of technical problems associated with delivering the PZP vaccine, including poor mixing procedures and ineffective dart delivery, but individual variation in the immune response to the vaccine is clearly a biological factor too. Although most treated animals will still maintain contraceptive antibody titers, despite the differences, a few animals will always represent poor immune responders, and titers will fall below contraceptive levels.

The birth of seven healthy foals from mares treated with FCA and four from mares treated with FMA during the pregnancy is consistent with earlier results with wild horses[17,19,20] and the birth of healthy young in a variety of zoo species treated with FMA during pregnancy.[3] These results are not unexpected, considering the proposed mechanism of contraceptive action for PZP, but these data support the current idea that FMA has no effect on the health or progress of the pregnancy either. With wild horses, any other free-ranging species, and animals in game parks, diagnosis of pregnancy is not always possible before treatment; thus, safety issues regarding administration during pregnancy are of importance.

The most surprising aspect of the study was the consistently higher titers for the FMA group despite a lack of statistical significance. Dogma within the field of immunology generally views FCA as the most effective adjuvant available; thus, the similar performance of FMA in this study was not predicted. This is advantageous because the cell walls from *M. butyricum*, the active ingredient in FMA, are derived from an organism with no identifiable pathologies associated with it in the published literature. This small statistically insignificant difference may be an artifact of the limited number of animals in the study, but it appears clear that FMA will produce contraceptive antibody titers as well as FCA.

The hazards of various adjuvants have been reviewed,[30] and the characteristics of the "ideal" adjuvant have been discussed elsewhere[9] and include a lack of local and systemic reactions and also the ability to elicit significant immune responses with weak antigens, such as the nonmicrobial PZP glycoprotein. It would also lack carcinogenicity. The current study indicates that FMA meets the first two of these characteristics. The 1995 National Beef Quality Audit reported that 11% of cattle inoculated with FDA- or USDA-approved vaccines produced injection site reactions, including long-lasting lesions and abscesses[29]; however, they are not considered to be a threat to food safety.[12,27] In another study the incidence of injection site reactions in fed cattle ranged from 3.2% to 21.6%, and in nonfed cattle from 28.9% to 40.9%.[12] Neither FCA nor FMA has been associated with rates that high in either captive exotic species or wild horses. The single abscess appearing in this study occurred near the injection site for the series of routine prophylactic vaccinations given just before the PZP inoculations, confounding the cause of that abscess. One serious consequence of abscess formation is that the antigen will become encapsulated and become protected from recognition by the immune process. However, the animal with the abscess produced antibody titers that remained well above contraceptive levels throughout the study.

Still another concern with any adjuvant is the possibility of causing autoimmune diseases, which have been detected after inoculation of dogs with commercially available vaccines for canine distemper, rabies, and parovirus.[24] However, it has previously been shown that PZP does not cross-react with somatic tissues or protein hormones in equids[21]; thus, the issue is a moot point for either adjuvant in this study when it is used with PZP. Finally, the issue of injection site reactions must be viewed with a concern for adverse reactions but also placed within the framework of benefits versus hazards. Considering the problems associated with excessive animal populations in either zoos or on wild horse ranges, the issue appears to be of minor concern.

The third issue, that of carcinogenicity, requires longer-term studies but must also be understood in terms of nonspecific adjuvants. Adjuvants, in general, may cause lesions that become metastatic; however, it is the general inflammatory response that leads to the lesion[2] and not the adjuvant per se. Currently, no specific USDA- or FDA-approved animal vaccines, regardless of the adjuvant used, appear to be associated with sarcomas.[27]

In the case of FCA, inflammatory responses are reduced by lowering the concentration of the mycobacterial concentration from 0.1 to 0.05 mg/ml.[10] Vaccine-associated sarcomas are also often species-related, as in the case of felids,[5,22] or associated with repeated vaccinations at the same site.[7] In the case of FMA, the concentration of *M. butyricum* is 0.1 mg/ml, but after emulsification with the PZP, the actual vaccine contains only 0.05 mg/ml, a level

that is thought to avoid or significantly reduce the incidence of inflammatory responses with FCA.

The quality of the PZP–FMA emulsion is vital to the success of the vaccination. Oil-based adjuvants, such as FMA, provide a vehicle for the transportation of the vaccine to the spleen and lymph nodes and throughout the lymphatic system, thereby enhancing the immune response. Additionally, oil-based adjuvants promote the formation and number of mononuclear cells that are responsible for the production of antibodies.[11] Collectively, these characteristics of FMA may explain the unusually good immune response seen in this study.

CONCLUSIONS

The anti-PZP antibody titers produced in this study help to explain the success of PZP in many species of zoo animals when treated with FMA as the adjuvant, based on contraceptive results alone. The use of FMA as the adjuvant of choice with PZP also appears to be without risk to pregnant animals or to be affected by pregnancies with regard to contraceptive efficacy. The lack of possibility of positive TB test results from animals treated with FMA make this an acceptable adjuvant for captive exotic species as well as for a variety of free-ranging wildlife species. Finally, although the TB test issue is not relevant to equids, this adjuvant is clearly an acceptable alternative to FCA for application of PZP to wild horses.

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