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Fertility Control in the Bitch by Active Immunization with Porcine Zonae Pellucidae: Use of Different Adjuvants and Patterns of Estradiol and Progesterone Levels in Estrous Cycles[1,2]

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ABSTRACT

To determine the changes in patterns of 17β-estradiol and progesterone levels underlying abnormal cycles in bitches immunized with solubilized crude porcine zonae pellucidae (cPZP), to attempt to circumvent these problems by immunizing with a purified zona fraction (pPZP), and to test the effectiveness of different adjuvants, bitches were immunized with cPZP or pPZP 2–6 times with no adjuvant, Freund's adjuvant, alum adjuvant, or the adjuvant CP-20,961. The bitch immunized without adjuvant had a low titer with a normal cycle and fertility. Immunization with cPZP and adjuvant produced moderate to high titers of antizona antibodies and infertility. Bitches with high titers experienced abnormal estrous cycles. Estradiol rose during proestrus, but instead of falling sharply in early estrus as in controls, it remained elevated. Progesterone did not rise. The moderate-titered bitches had normal cycles and steroid patterns. Bitches immunized with pPZP had moderate titers. Cycles were normal after 3 injections, but after 6 injections one bitch had an abnormal cycle. One pPZP-immunized bitch remained fertile but the others were infertile. Alum was the mildest adjuvant, causing no injection site lesions, but the highest titers occurred with Freund's and CP-20,961 adjuvants. All three adjuvants induced titers sufficient to inhibit fertility.

Infertility in bitches immunized with PZP may be due to prevention of zona penetration, because their antisera inhibited zona penetration of oocytes by spermatozoa in vitro. However, alterations in ovarian function preventing ovulation and luteinization could be involved in high-titered bitches.

INTRODUCTION

The dog is a species with a serious need for better means of birth control (Anonymous, 1976). The steroidal compounds in use today require considerable motivation on the part of the owner because they must be given often and are relatively expensive, especially for a large animal (Burke and Reynolds, 1975; Sokolowski and Geng, 1977). It is known that oocytes treated with antisera against homologous or certain heterologous zonae pellucidae are not penetrable by spermatozoa (Gwatkin et al., 1977; Tsunoda and Sugie, 1977; Mahi and Yanagimachi, 1979; Trounson et al., 1980; Aitken et al., 1982; Mahi-Brown et al., 1982). We thought that immunization of bitches against porcine zonae pellucidae might be a safe and specific means of effecting contraception in the bitch.

In a preliminary study in which we immunized bitches with porcine zonae pellucidae in Freund's adjuvants (Mahi-Brown et al., 1982), the bitches became infertile. Although canine oocytes treated with antisera from the bitches were not fertilizable in vitro, we cannot be sure that this was the only cause of infertility because the bitches also had aberrations in their estrous cycles characterized by prolonged proestrous bleeding or receptivity. Alteration of estrous cycles is not an acceptable side effect

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and must be prevented before antizona pellucida immunization can be considered an appropriate approach to birth control in the bitch. Study of the endocrinology underlying the altered cycles might help in our understanding of the problem.

One hypothesis that might explain the abnormal estrous cycles we observed is that antibodies were produced by the immunized bitches that reacted with ovarian antigens other than those of the zona pellucida. These antibodies could have been produced in response to nonzona ovarian antigens contaminating the zona preparation (Gulyas et al., 1983). In the present study, therefore, we immunized some bitches with a porcine zona pellucida fraction purified by the method described by Yurewicz et al. (1983). This method is reported to yield a fraction enriched in the component of the zona pellucida with an apparent molecular weight of about 60,000 (ZP-3).

In addition, most bitches immunized previously (Mahi-Brown et al., 1982) developed lesions at injection sites varying from mild granulomas to large ulcerated abscesses. Intramuscular injections, in particular, led to lameness in the injected thigh. These lesions were very slow to heal. The effectiveness of this approach can be positively evaluated only if it is without major side effects. Therefore, we determined to attempt immunization without adjuvant or with adjuvants other than Freund's. Two adjuvants were selected for testing. Alum adjuvant (aluminimum hydroxide gel) was successfully used by Gwatkin et al. (1980) to immunize bitches against bovine zonae. They reported no undesirable side effects from the adjuvant. The synthetic lipid amine *N,N*-dioctadecyl-N′-bis[2-hydroxyethyl] propanediamine (CP-20, 961; Pfizer, Groton, CT) has been found to be comparable to Freund's adjuvants in effectiveness and to be well tolerated by dogs (Niblack et al., 1979).

We report here the results of our study in which we immunized bitches with either purified or crude porcine zona pellucida in different adjuvants and assayed gonadal function as expressed in patterns of serum estradiol and progesterone throughout proestrus, estrus, and early metestrus.

MATERIALS AND METHODS

Preparation of Zonae Pellucidae

Zonae pellucidae were collected from porcine ovaries according to methods published previously (Mahi-Brown et al., 1982). Briefly, ovaries that had

TABLE 1. Bitches used in study and their immunizations.

Bitch ID no.	Breed	Age (yr)	Wt. (kg)	Parity (previous litters)	Immunizations		
					Antigen[a] (tot. mg)	Adjuvant	no. & route[b] of injections
0	mixed	1	25	0	C (0.8)	None	3 s.c.
4	mixed	4	23	1	C (0.4)	Freund's	1 s.c.[c]
7	mixed	3	12	2	C (1.2)	CP-20,961	3 i.m.
8	mixed	1	25	0	C (1.2)	CP-20,961	3 i.m.
9	mixed	1	25	1	C (1.2)	Freund's	3 i.m.
10	mixed	1	13	0	C (1.2)	Alum	3 s.c.
11	mixed	1	11	0	C (0.8)	Alum	2 s.c.
12	pointer	4	28	1	P (1.2)	Alum	3 s.c.
13	mixed	1	7	0	– (0)	none	0 –
13	mixed	1	7	0	P (1.6)	CP-20,961	4 i.m.[d]
14	pointer	6	25	3	P (1.2)	CP-20,961	3 i.m.[d]
16	Newfoundland	6	46	1	– (0)	none	0 –

[a] C = crude PZP, P = purified PZP.

[b] Injections were given at a single site either subcutaneously (s.c.) or intramuscularly (i.m.).

[c] Received 1 injection of 0.4 mg in this phase of the study. Received 4 injections totaling 0.2 mg 2 yr earlier.

[d] Bitches 13 and 14 received an additional 2 injections or 1 injection (0.4 mg each), respectively, between first and second cycles after start of immunization.

been stored frozen were thawed and sliced with ganged razor blades in phosphate-buffered saline with 1 drop/liter of Tween-80 (PBSt). The oocytes that were released from follicles were collected on nylon screens with progressively smaller mesh sizes and treated for 1 h in 0.3% collagenase (Type II; Sigma Chemical Co., St. Louis, MO). They were again collected on a screen and rinsed with PBSt, then washed off the screen into a tissue homogenizer and gently homogenized to rupture the oocytes. The resulting isolated zonae pellucidae were then collected on the screen, rinsed, and washed into 15-ml conical centrifuge tubes. They were washed by centrifugation 4 times and resuspended in 0.5 ml of 0.9% saline. The zonae were then solubilized by heating them to 70°C for 30–60 min (until examination with a dissecting microscope confirmed solubilization). The tubes were finally centrifuged at 1200 × *g* to remove any remaining particulate matter. The supernate was then pooled and stored at −50°C until used.

Purification of Zona Pellucida Preparation

The solubilized zonae were purified by gel filtration with Ultrogel AcA 34 (LKB Instruments, Inc., Rockville, MD) and ion exchange chromatography with DEAE Biogel A (Bio-Rad Labs, Richmond, CA) as described by Yurewicz et al. (1983). Although this is probably still not a pure preparation, it should be considerably less contaminated with ovarian antigens than the crude solubilized solution. We compared the crude zona preparation (cPZP) with the partially purified preparation (pPZP) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and found that a low molecular weight band (M_r about 42,000) observed in the cPZP was absent from the pPZP.

Immunization of Bitches

The 11 bitches used in this study were obtained through donations or were reared in the colony (2 bitches). Table 1 lists their ages, weights, prior breeding histories (parity), whether they received cPZP or pPZP or were untreated, the adjuvant used, and the route of injection. One bitch was given 3 subcutaneous injections of cPZP (0.27 mg each) in PBS without adjuvant at 2-wk intervals (this was 4.5 times more cPZP than bitches immunized earlier; Mahi-Brown et al., 1982). All other bitches received 0.4 mg of cPZP or pPZP in 0.5 ml of 0.9% NaCl or 20 mM Tris buffer (pH 8.0) with an adjuvant (see below and Table 1). Vaccines with alum adjuvant were prepared by mixing 0.5 ml of PZP with an equal volume of sterile aluminum hydroxide gel (Rehsorptar; Reheis Chem. Co., Kankakee, IL). After allowing 5 min at 4°C for adsorption of the antigen to the gel, the mixture was centrifuged at 1800 rpm at 4°C for 15 min, rinsed twice with 0.9% NaCl by centrifugation, then resuspended in 0.9% NaCl to a final volume of 1 ml. The suspension was injected subcutaneously behind the shoulder. Vaccines with the adjuvant CP-20,961 were prepared by emulsifying 0.5 ml of cPZP or pPZP with 1.18 ml of soybean oil vehicle (Intralipid; Cutter Labs, Berkeley, CA), 7.5 μl Tween-80, and 10.5 mg adjuvant dissolved in 75 μl ethanol. The emulsion was injected intramuscularly in the thigh. Vaccines with Freund's adjuvants were prepared as described by Mahi-Brown et al. (1982). Injection sites were examined 1 and 7 days after each injection for evidence of inflammation or abscesses. Bitches were injected at monthly intervals (except for Bitches 4 and 11 and the last 2 injections for Bitch 13) beginning in late metestrus or anestrus, and all but three received 3 injections. Bitch 4 was a carryover from the preliminary phase of the study. She received 4 injections of cPZP (total of 0.2 mg) with Freund's adjuvants, then no more for 20 mo, at which time she was given a booster. Her hormone profile was followed only during the estrous cycle following the booster at 20 mo. Bitch 11 came in season shortly after her second injection, so she was bred instead of being given another injection. Because we wanted to make sure than pPZP would produce fewer side effects than cPZP, we immunized Bitch 13 a total of 6 times, 4 before her first breeding and 2 during her next anestrus.

Examination of Bitches' Cycles and Serum Sampling

Bitches were bled 10–14 days after each injection and sera were prepared by standard techniques. Bitches were examined at least once per week for evidence of proestrus (vulval swelling or sanguineous vaginal discharge); when they were found to be in proestrus, vaginal smears were made at least every 48 h until they entered metestrus. The smears were stained with Wright's stain. Because the bitches were initially examined weekly, the actual duration of proestrus could not be accurately determined. When they came in heat, most bitches were bled at 2-day intervals (as much as possible; some longer intervals occurred) throughout proestrus, estrus, and early metestrus. Bitch 0 was used only for the adjuvant study (receiving no adjuvant) and was not bled for steroid assays. Bitch 11 was difficult to bleed because of her fractious temperament, so we did not collect blood during her heat. Serum was collected from Bitch 8 only sporadically for similar reasons. Also, Bitch 12 had very scant vaginal discharge during proestrus and estrus, so we missed all of proestrus in her first cycle after immunization. Bitch 16 was sampled only a few days into metestrus. All sera were stored frozen at −50°C until used. Bitch 16, who served only as a control for normal steroid patterns, was not bred during this cycle but had been proven fertile previously. Also, Bitch 13 was not bred at her first estrus, which occurred before she was immunized, and therefore served as an additional control. All others were bred three times to one or two fertile stud dogs at 48-h intervals beginning on the first or second day of estrus.

Determination of Antibody Titers in Sera

Indirect immunofluorescence (IIF) was used to determine the titers of antibodies that cross-reacted with canine zonae pellucidae in the sera collected after each injection. IIF was chosen because it was used in our earlier study and we wanted to have direct comparisons of present titers with former ones (for details of this procedure, see Mahi-Brown et al., 1982). Briefly, canine ovarian oocytes freed from the corona radiata by vortexing them in 75 mM sodium citrate (pH 7.8) were treated for 1 h at room temperature (25°C) with serial dilutions (10-fold first, then twofold from the highest positive 10-fold dilution) of antisera (in PBS with 1% polyvinyl pyrrolidone, PBSP), preimmune

sera, or PBSP alone. After they were rinsed twice with the buffer, they were treated with 30–50 μl of fluorescein isothiocyanate-conjugated Protein A (0.1 mg/ml; Sigma Chemical Co.) for an additional hour. They were then rinsed 4 times with the buffer, mounted on slides, and examined with a fluorescence microscope. The titer of antibody in each serum was expressed as the greatest dilution of the serum at which fluorescence greater than that of the preimmune control could be detected clearly. Undiluted sera were also tested for their ability to prevent zona penetration of canine ovarian oocytes by spermatozoa in vitro (for details of the procedure see Mahi and Yanagimachi, 1978, 1979; Mahi-Brown et al., 1982).

Radioimmunoassay of Estradiol and Progesterone in Sera

The assay we used for 17β-estradiol was described by Nelson et al. (1978). This assay and the assay for progesterone were very similar to those described and validated for the dog by Nett et al. (1975). Briefly, estradiol was extracted from 500 μl of serum with 10 ml of diethyl ether and progesterone from 250 μl of serum with petroleum ether. The extracted hormones were dried and resuspended in 500 μl of PBS-gelatin. Duplicate 200-μl aliquots were then assayed. Bound and unbound hormones were separated by use of charcoal and dextran and counting was performed with a Packard Tri-Carb Liquid Scintillation Spectrometer. Sera were extracted and assayed twice if the volume permitted, and results were averaged. In a few cases, hormone concentrations were so high that the assays had to be repeated with greater dilutions of sera. All values were corrected for recovery, which averaged 90.5% for estradiol and 82.9% for progesterone. The sensitivities of the assays were 5 pg/tube for estradiol and 25 pg/tube for progesterone. Interassay and intraassay variations were 13% and 5%, respectively, for estradiol and 10% and 6%, respectively, for progesterone. Reagents used were: tritiated 17β-estradiol and progesterone (New England Nuclear; Boston, MA), estradiol and progesterone standards (Steraloids, Inc., Wilton, NH), antiestradiol antiserum (Dr. G. D. Niswender, University of Colorado), and antiprogesterone antiserum (Dr. Walter Morishige, University of Hawaii).

RESULTS

The titers of antibodies that bound to canine zonae in sera of bitches immunized with porcine zonae are listed in Table 2. To allow easier comparison from bitch to bitch, only the third antiserum from each bitch is listed (except for Bitches 4 and 11, who were immunized differently). The first two antisera were consist-

TABLE 2. Antifertility effects of immunizing bitches with porcine zona pellucida.

Bitch ID no.	Antizona antibody titer[a]	% Zona penetration by sperm (no. eggs insem.)[b]	Estrous cycles	Fertility of bitch[c]
Control cycles				
13	0	100 (46)	normal	not bred
16	0	100 (28)	normal	not bred
Immunized with cPZP without adjuvant				
0	1:100	not tested	normal	fertile
Immunized with cPZP and developing high titers				
4	1:4,000[d]	0 (53)	abnormal	infertile
8	1:10,000	13.3 (60)	abnormal	infertile
9	1:20,000	8.0 (60)	abnormal	infertile
Immunized with cPZP and developing moderate titers				
7	1:2,000	0 (52)	normal	infertile
10	1:2,000	36.8 (57)	normal	infertile
11	1:1,000	1.9 (52)	normal	infertile
Immunized with pPZP				
12	1:4,000	1.8 (55)	normal	infertile
13	1:2,000	25.0 (32)	normal[e]	infertile
14	1:1,000	11.5 (61)	normal	fertile

[a] By indirect immunofluorescence using antiserum prepared after third immunization except for Bitches 4 (5th) and 11 (2nd).

[b] Fresh canine oocytes pretreated for 1 h with antiserum collected after third (5th for Bitch 4, 2nd for Bitch 11) immunization.

[c] At first cycle after immunization.

[d] All data are for current immunization. This bitch had a titer of 1:10,000 2 yr earlier in her initial series of immunizations.

[e] Normal first cycle, abnormal second cycle.

ently of lower titer than the third. Bitch 0, immunized without adjuvant, developed a low titer (1:100) and remained fertile. Bitches 8 and 9 developed high titers (greater than 1:10,000) against cPZP, whereas Bitches 7, 10, and 11 developed more moderate titers (1:1000–1:2000). Bitch 4 had a titer of 1:4000 at this point, but 2 years earlier her titer had been high (1:10,000), so she is included with the high-titered bitches in discussions below. Bitches 12, 13, and 14, immunized with pPZP, also developed moderate titers. Zonae of oocytes treated with antisera from Bitches 4 and 7 were not penetrable by spermatozoa in vitro. Antisera from Bitches 8, 9, 11, 12, and 14 were almost as effective, and those from Bitches 10 and 13 were the least effective, but still allowed penetration of only 36.8% and 25.0% of the zonae, respectively. Neither the preimmune sera from any of the bitches nor sera from the control bitches had any effect on zona penetration in vitro (Table 2).

The levels of steroids in proestrus, estrus, and metestrus of the controls (Fig. 1) were typical for bitches as recorded in the literature (Concannon et al., 1975; Olson et al., 1982). Estradiol levels rose during proestrus and peaked at the end of proestrus (>105 pg/ml), after which they fell sharply. Progesterone rose gradually at the end of proestrus, then sharply at the beginning of estrus (to 24 ng/ml by Day 6) as the estradiol levels fell. Progesterone then remained elevated for several weeks while estradiol rose only a little throughout metestrus.

Bitches with moderate titers against cPZP (Bitches 7, 10, and 11) had apparently normal estrous cycles, but were infertile. The steroid profiles (Fig. 2) resembled those of the controls except that Bitch 7 had a somewhat lower elevation of progesterone (7 ng/ml) and her estradiol rose to 75 pg/ml during metestrus. Interestrous intervals in this bitch before and during this study were 7 and 6 mo, respectively. Only Bitch 10 was retained for follow-

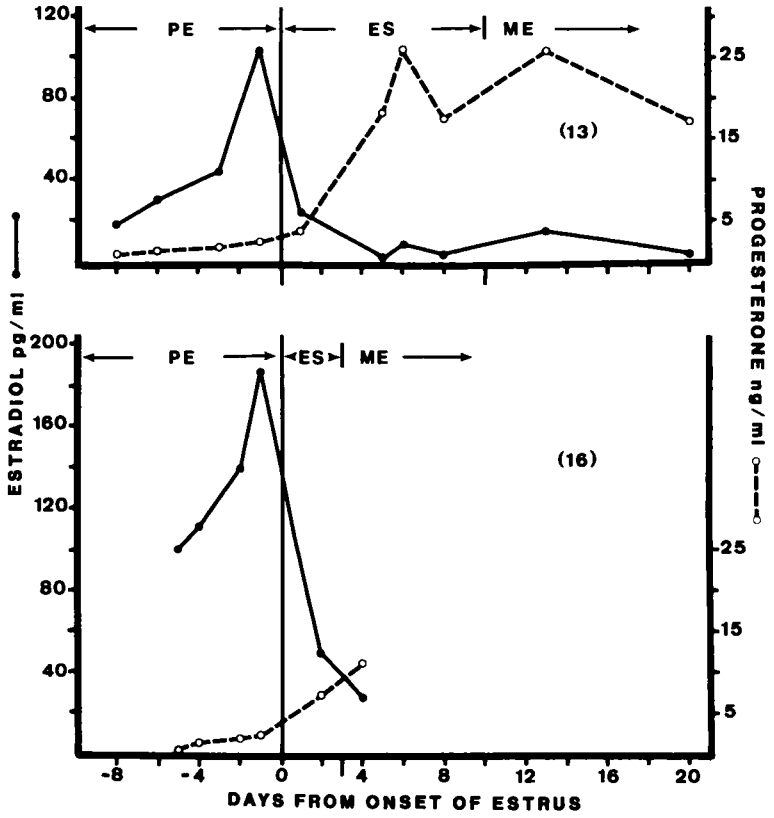

FIG. 1. 17β-Estradiol and progesterone patterns in control Bitches 13 and 16, which were unimmunized and unbred. Day 0 is the day of onset of estrus. *PE*, proestrus; *ES*, estrus, *ME*, metestrus.

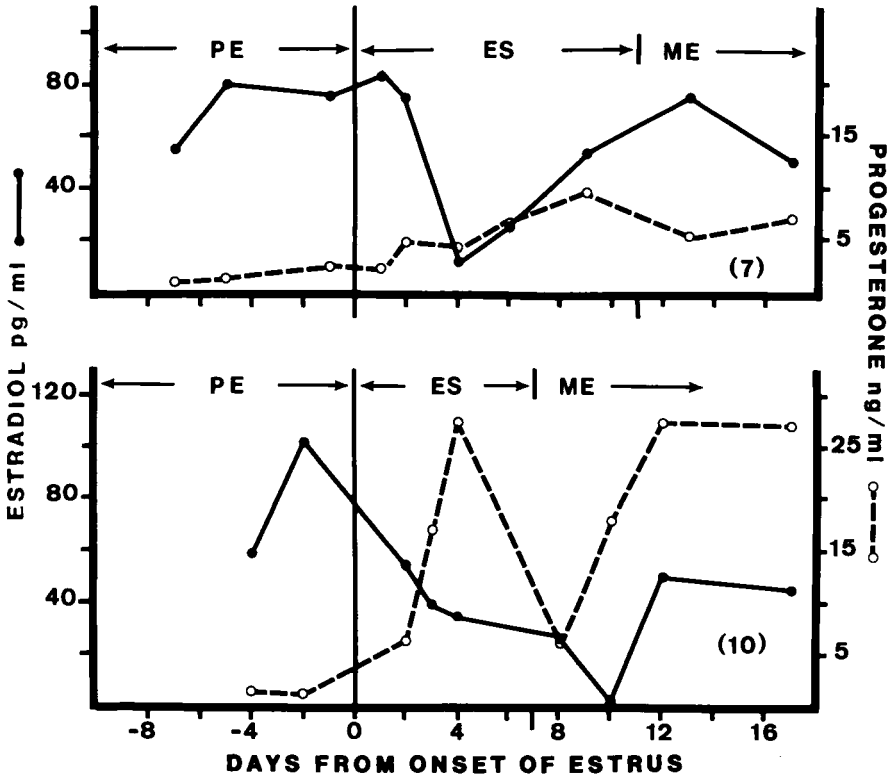

FIG. 2. Estradiol and progesterone patterns in Bitches 7 and 10, which were treated with crude porcine zona pellucida and developed moderate antizona titers. Day 0 is the day of onset of estrus. *PE*, proestrus; *ES*, estrus; *ME*, metestrus.

up. Seven mo after her first estrus, she again had a normal heat and was again infertile.

All three bitches with high titers against cPZP had abnormal cycles and were infertile. Bitch 4 first had an abnormal estrous cycle following her initial series of injections (Mahi-Brown et al., 1982). Her proestrus and estrus were normal, but she continued to have a bloody vaginal discharge throughout metestrus. Except for the presence of erythrocytes, however, the smear was typical of metestrus. Her next two cycles, at intervals of 12 and 5 mo, were normal in appearance but infertile. After she was given a booster and 5 mo after the previous estrus, she again had a cycle in which erythrocytes appeared in the metestrus smear. Bitch 9 entered proestrus (7 mo after her first estrus) and remained in proestrus (with a vaginal smear typical of late proestrus) without entering estrus for 30 days. She was euthanized at 30 days because she was in poor condition (as a result of infected abscesses at her injection sites). Although she could not be bred, her persistent proestrus was considered incompatible with fertility. Bitch 8 also had an abnormal estrous cycle (her first cycle). Her proestrus was normal (9 days), but she then entered prolonged estrus as indicated by fully cornified vaginal smear with vulval swelling and discharge. She did not willingly allow a male to mount, but was nevertheless held and bred three times. She continued to have a vaginal smear and discharge characteristic of estrus for 70 days, at which time her ovaries and uterus were removed. Each ovary was found to contain a large cyst.

In striking contrast to the control and moderate-titered bitches, those with high titers of antibodies against cPZP had unusual serum steroid profiles (Fig. 3). Estradiol rose during proestrus as in the controls, but instead of falling markedly at the start of estrus, it remained elevated (70–110 pg/ml) for several weeks. Progesterone remained close to the detectable level or reached only a maximum of 2 ng/ml. The absence of a rise in progesterone suggests that these bitches

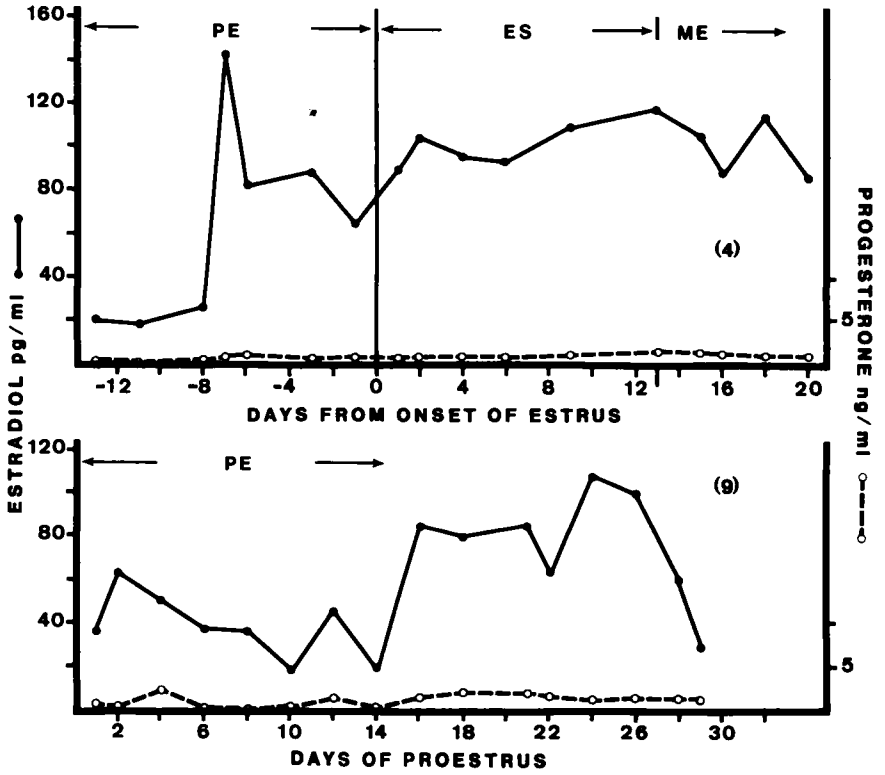

FIG. 3. Estradiol and progesterone patterns in Bitches 4 and 9, which were immunized with crude porcine zona pellucida and developed high antizona titers. For Bitch 4, day 0 is the day of onset of estrus. Bitch 9 did not enter estrus so the abscissa is days of proestrus. Note absence of progesterone rise in both bitches. *PE*, proestrus; *ES*, estrus; *ME*, metestrus.

failed to ovulate. As illustrated in Fig. 3, Bitch 4 had a sustained elevation of estradiol whereas Bitch 9 was more erratic. Bitch 8 was in estrus for 10 wk before she was ovariectomized. When the serum samples collected sporadically during this period were assayed, it was found that she also had erratic elevation of estradiol and no rise in progesterone (data not shown).

The first cycle after immunization was normal, according to vaginal smears, behavior, and steroid patterns (Fig. 4), in Bitches 12 and 14, immunized with pPZP. The progesterone levels during metestrus rose very high in these bitches, which were large English pointers. Bitch 14 is the only one that conceived when bred. She whelped an apparently normal litter of 7 puppies. She was given another injection of pPZP in alum to test the effects of adjuvant (see below). This raised her antibody titer slightly to 1:2000 and a subsequent cycle was reported to be infertile by the principal investigator of another study, to whom she was given. In Bitch 13, progesterone rose in the first estrus after immunization but did not remain elevated as metestrus proceeded (13*a* in Fig. 4). Bitches 12 and 13 were retained and bred again (5 and 7 mo after the first postimmunization cycles; the cycles designated 12*b* and 13*b* in Fig. 4). Bitch 12 was found at 7 wk gestation to have 2 apparently normal fetuses. However, her ovaries had 7 corpora lutea, suggesting that she still had partial impairment of fertility. Bitch 13, who had received a total of 6 injections of pPZP by this time, remained infertile. Her second cycle was characterized by somewhat prolonged estrus (19 days), and her steroid pattern (13*b* in Fig. 4) was similar to those of the three bitches with high titers against cPZP (Fig. 3). Estradiol levels rose in proestrus and then remained high, whereas progesterone reached a level of only 2.3 ng/ml, which is higher than in the high-titered bitches but not as high as in control cycles.

All three adjuvants used allowed production of antizona titers sufficient to cause infertility. Although use of alum adjuvant did not result in

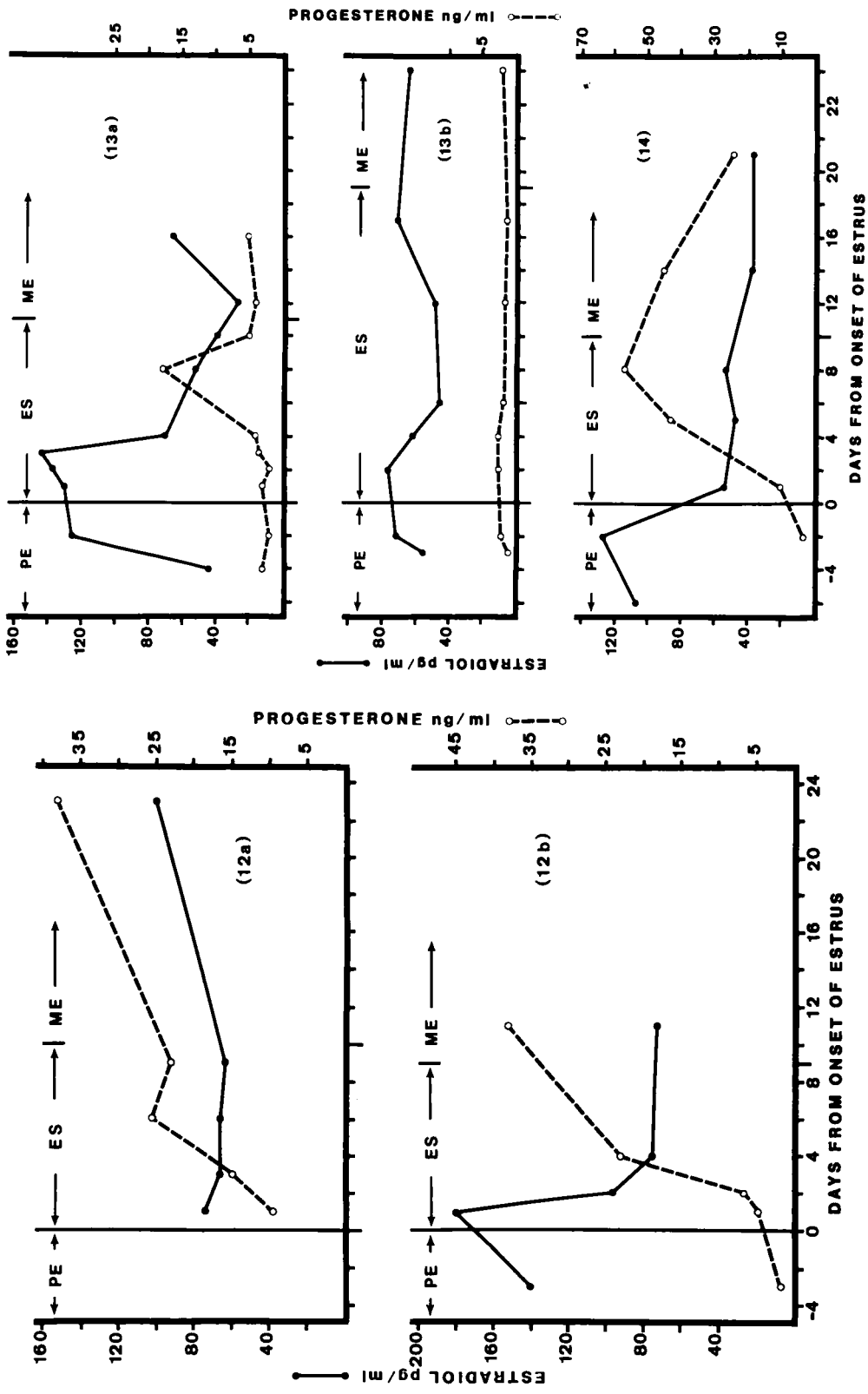

FIG. 4. Estradiol and progesterone patterns in bitches immunized with purified porcine zona pellucida and developing moderate antizona titers. Two cycles (*a* and *b*) of Bitches 12 and 13 and one cycle of Bitch 14 are shown. During the second cycle Bitch 12 (12*b*) is pregnant. Note the abnormal pattern in the second cycle for Bitch 13 (13*b*). Day 0 is the day of onset of estrus. *PE*, proestrus; *ES*, estrus; *ME*, metestrus.

any high titers, all bitches were infertile for at least one cycle and there were no injection site lesions. Bitches immunized with CP-20,961 had a more variable response, with titers ranging from 1:1000 to 1:10,000. The bitch with the lowest titer was the only bitch in the study immunized using any of the adjuvants that conceived at the first cycle after immunization. This same bitch had inflammation and lameness in the thigh in which she was immunized each time. The duration of the inflammation was less than 24 h, however. In order to ascertain whether the antigen or the adjuvant was responsible for this reaction, we waited until she had weaned her litter of puppies and then injected her intramuscularly in the thigh with saline and CP-20,961 without zonae. At the same time we injected her subcutaneously in the shoulder area with pPZP adsorbed to alum. Both injection sites were observed for the next several days for signs of local reaction. Within 24 h, the site at which CP-20,961 had been injected developed a golf ball-sized swelling and the bitch was lame in that leg. The lymph node immediately above the injection site was also enlarged. The swelling and lameness persisted less than 24 h. The site at which pPZP and alum were injected remained normal. It appears, therefore, that this bitch had an inflammatory response to the adjuvant and not the antigen. None of the other three bitches had any noticeable reaction at the injection sites.

In contrast, both bitches immunized with Freund's adjuvants developed abscesses at their injection sites and in one bitch immunized i.m. the lesion drained and became infected. In spite of antibiotic therapy, her condition deteriorated to the point that she was euthanized for humane reasons.

DISCUSSION

According to our results, immunization with PZP is not effective unless an adjuvant is used. Fortunately, adjuvants less traumatic than Freund's can induce titers sufficient to produce infertility. Of the 7 bitches immunized with adjuvants other than Freund's, only in one, immunized with CP-20,961, was there any injection site lesion.

The data reported above help define the underlying etiology of the abnormal estrous cycles and infertility of the bitches with high titers against cPZP. It is somewhat surprising that these three bitches had different aberrations in their estrous cycles yet the changes in their steroid profiles were so similar. For instance, Bitches 4 and 8 entered estrus, according to their vaginal smears, whereas Bitch 9 failed to do so. The infertility of these bitches is not surprising. Clearly they could not establish a pregnancy in the absence of progesterone even if they had ovulated and their ova had been fertilized. Furthermore, the absence of progesterone strongly suggests but does not prove that these bitches failed to ovulate. Preliminary histologic examination has revealed no recent corpora lutea in the ovaries of Bitches 4, 8, or 9, nor were any preovulatory follicles found, even though Bitches 4 and 9 were in proestrus and Bitch 8 in estrus at the time the ovaries were recovered (Mahi-Brown et al., 1984). This confirms the failure of ovulation.

The normal estrous behavior of Bitch 4 in the absence of a rise in progesterone is somewhat surprising. The literature links the onset of estrous behavior in the bitch to elevation in progesterone following estrogen priming (Amoroso, 1949; Concannon et al., 1975, 1977, 1979; Wildt et al., 1979, 1981). The only time the progesterone level in Bitch 4 even slightly exceeded 1ng/ml was after she entered metestrus. The behavior of Bitches 8 and 9 was more predictable, since they had vaginal changes characteristic of proestrus or estrus but did not express estrous behavior.

The bitches with lower titers against cPZP (Bitches 7, 10, and 11) seem to have ovulated, as indicated by the elevated progesterone levels in Bitches 7 and 10. Bitch 7 is somewhat borderline in this regard because her progesterone did not rise nearly so high as in the control bitches. Infertility was probably not due to failure of these bitches to ovulate (except possibly in Bitch 7). Antisera from Bitches 7 and 11 were quite effective in preventing penetration of spermatozoa in vitro, suggesting that this could have been the mechanism producing infertility in vivo. However, it is also possible that the eggs could have been penetrated in vivo but failed to implant. Dudkiewicz et al. (1975) demonstrated that hamster eggs treated with antisera against hamster ovaries failed to hatch from the zona pellucida at the blastocyst stage, with the result that implantation was prevented. Either of these mechanisms is acceptable for contraception in the bitch. The antiserum from Bitch 10 was not nearly as effective in preventing zona penetration in vitro, yet she has been infertile for 2 cycles since she received her last booster

of cPZP. For the in vitro penetration assay, eggs are treated for 1 h with antiserum, then exposed to spermatozoa. In vivo, oocytes are exposed continuously to circulating immunoglobulins, so there may be more thorough coating of their zonae pellucidae. Mahi-Brown et al. (1982) reported that oocytes recovered from the ovary of one bitch shortly after she received her second injection of cPZP were not penetrable by spermatozoa, whereas antiserum from this bitch prepared at the same time did not prevent sperm penetration of other oocytes treated with the antiserum. Thus bitches immunized with cPZP do not have to have extremely high titers against cPZP in order for their eggs to be impenetrable.

None of the three bitches immunized with pPZP (Bitches 12, 13, and 14) developed titers higher than 1:4000, even though they were given the same amount of protein per injection as the bitches receiving cPZP. Yurewicz et al. (1983) noted that pPZP had slightly lower zona antigen activity per mg protein than a less pure preparation of solubilized PZP. The steroid profiles of these three bitches at their first cycles after immunization with pPZP are similar to those of the controls. One of these bitches (Bitch 14) became pregnant and whelped 7 normal puppies at her first cycle after immunization, so clearly her reproductive system was operating normally. Although her titer was the lowest of these three bitches, her fertility was surprising because her antiserum prevented penetration of all but 11.5% of treated oocytes in vitro. The fact that she failed to conceive at a subsequent cycle after receiving a 4th injection to test whether adjuvant or antigen caused injection site lesions suggests that her titer may not have been high enough at the first cycle. This is especially true since her titer rose slightly after this 4th injection. However, it is worth noting in this regard that Bitch 11 had a titer as low as Bitch 14 and yet she was infertile. Perhaps the antibodies produced against the more complex cPZP given Bitch 11 are more potent in inducing infertility. It is also possible that the adjuvant itself was important. None of the bitches receiving alum adjuvant initially (Bitches 10, 11, and 12) conceived at their first estrus. The 4th injection Bitch 14 received was also in alum, in contrast to her first three injections.

Bitch 13 had a normal cycle after receiving 4 injections of pPZP, with nearly normal steroid profiles, but she did not conceive. By the time of her second cycle after immunization she had received 6 injections, and now her cycle was abnormal with an abnormal steroid profile similar to that of the high-titered bitches immunized with cPZP. Apparently, then, this method of purifying PZP is not sufficient to completely prevent occurrence of abnormal cycles. Either there are still nonzona ovarian antigens contaminating the preparation or the antibodies against PZP itself cause alterations in ovarian function. It did take more injections to produce the abnormal cycle, so perhaps further purification would still solve this problem. The preparation we used consists largely of the lowest molecular weight component of the zona pellucida (Yurewicz et al., 1983). It is possible that use of one of the other two antigens of the zona pellucida would be as effective in blocking fertility of bitches without producing these alterations in the estrous cycles.

Although the altered steroid patterns in the high-titered bitches explain the abnormal estrous cycles, we do not yet have an explanation for the altered steroid patterns. Wood et al. (1981) found that rabbits immunized with PZP failed to ovulate in response to injected hCG and the ovaries of the immunized rabbits were significantly smaller than those of control rabbits. These workers (Skinner et al., 1983) subsequently found that 6 wk after the last injection, there was a decrease in primary, secondary, and tertiary follicles, and by 20 wk there were few or no growing follicles. Furthermore, no progesterone was secreted in response to injected hCG, in contrast to unimmunized controls. Skinner et al. suggested that anti-PZP antibodies acted on the cells responsible for synthesis of the zona pellucida. Gulyas et al. (1983) found that cynomolgus monkeys injected with PZP similar to the cPZP we used also failed to ovulate and had depressed levels of estradiol and progesterone. The ovaries of some of the monkeys suffered massive loss of follicles, especially large antral follicles, which Gulyas et al. attributed to contaminants in the zona pellucida preparation used for immunization. These results are in contrast to our results in that we got elevated estradiol in our bitches with high titers of antibodies against cPZP and in the bitch immunized repeatedly with pPZP. However, like Gulyas et al., we also observed depression of progesterone secretion and probably failure of ovulation. Immunization of bitches with PZP was not as potent in this regard in bitches as in rabbits, because more than one

injection was required to develop a high titer and abnormal cycles occurred only in some of the bitches, not all. Neither Gwatkin et al. (1980) nor Shivers et al. (1981) reported any alterations in estrous cycles in the bitches they immunized with bovine and porcine zonae pellucidae, respectively, even though the methods they used to prepare their immunizations were similar to ours.

None of the above studies used purified antigen. However, Sacco et al. (1983) immunized squirrel monkeys with a PZP protein purified by gel filtration, ion exchange chromatography, and affinity chromatography with *Ricinus communis* agglutinin I-agarose resin. The protein finally eluted from the column had an average molecular weight of 60,000 and was free of the other two major zona pellucida proteins. In spite of these purification steps, the monkeys they immunized ovulated far fewer oocytes than the controls and their ovaries were smaller. These observations strongly suggest that the altered estrous cycles observed in cynomolgus monkeys (Gulyas et al., 1983) and bitches (present study) and the failure of rabbits to ovulate in response to hCG (Wood et al., 1981) may be due to direct effects of the immunization and binding of antibodies to the zona pellucida itself and not to antibodies against contaminating ovarian antigens.

One way antibodies specific to the zona pellucida could alter function of follicles is by interfering with communication of the oocyte with its surrounding corona radiata cells. It has been shown that nutrients used by ovarian oocytes are passed to them through the follicle cells that are in intimate contact with the oocytes via gap junctions where the follicle cell processes contact the oocyte plasma membrane (Herlands and Schultz, 1984), so interference with this communication could result in death of the oocytes. However, Shivers et al. (1981) found no evidence to this effect in electron micrographs of ovaries of immunized bitches. Another possibility is that an immune attack on the oocytes and/or follicle cells themselves by the antizona antibodies leads to their destruction. There is recent evidence both that the zona antigens are synthesized by the oocyte alone (Bousquet et al., 1981) and that they are synthesized by the oocyte and follicle cells (Hedrick and Fry, 1980; Tesoriero, 1983). Binding of immunoglobulins to zona antigens associated during secretion with the oolemma of an immature oocyte could cause destruction of the oocyte. If follicle cells secrete or phagocytize zona material at any time during follicle-oocyte growth, they too could become vulnerable to antizona antibodies. Their death could then lead to death of the oocyte. The only direct evidence for any cytotoxic mechanism related to zona immunization is that of Gwatkin et al. (1977), who, using an immunofluorescent technique, localized complement in the zonae of ovulated oocytes from mice immunized with hamster zonae.

Regardless of the mechanism, it is apparent that active immunization against zonae pellucidae has potent antifertility effects. However, alterations in estrous cycles with underlying changes in steroid profiles encourage caution in applying this approach as a contraceptive until the reasons for the changes can be determined and prevented. The elevated estradiol seen in several of our immunized bitches suggests that the mechanism operating in bitches may not be quite the same as that in rabbits or monkeys.

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