Contraception in mares heteroimmunized with pig zonae pellucidae

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Summary. Ten fertile feral mares and 6 domestic horses (4 fertile mares, 1 infertile mare, 1 gelding) were immunized with heat-solubilized pig zonae pellucidae by 4 injections equivalent to 2000 or 5000 zonae each at 2–4-week intervals and a booster injection of 20 000 zonae 6–10 months after the last of the initial inoculations. The immune response was reflected by high antibody levels as measured by an enzyme-linked immunosorbent assay (ELISA) using immobilized pig zona antigen. In-vivo inhibition of fertility occurred in 12 (86%) of the 14 fertile mares studied and persisted for a minimum of 7 months. Repeated mating of the fertile domestic mares resulted in conception when anti-pig zona antibody concentrations had decreased from initial peak absorbance ratios ($>1{\cdot}0$) to relatively lower levels (0·64 or less with one exception). An indirect immunofluorescence assay, revealed a considerably lower cross-reactive antibody titre with horse oocytes as compared to pig oocytes. Clinical, endocrinological and histological analyses of the ovaries and their function following regained fertility after immunization revealed no abnormalities. One mare remained infertile.

Keywords: horse; contraceptive vaccine; zona pellucida; antibodies; heteroimmunization.

Introduction

Attempts to reduce fertility in several mammalian species have been reported. Active immunization of female rabbits (Gwatkin *et al.*, 1980; Woods *et al.*, 1981; Skinner *et al.*, 1984), bitches (Shivers *et al.*, 1981; Mahi-Brown *et al.*, 1982) and monkeys (Sacco *et al.*, 1983) with pig zonae pellucidae has resulted in reduced fertility of the treated animals. Similarly, immunocontraception was achieved in mice (Gwatkin *et al.*, 1977) and rabbits (Gwatkin & Williams, 1978) by using hamster and cattle zonae. Other investigators have successfully inhibited fertilization *in vivo* by passive immunization, injecting rodents of several species with antisera against mouse (Shahani *et al.*, 1972; Jilek & Pavlok, 1975), rat and hamster (Tsunoda & Chang, 1976; Oikawa & Yanagimachi, 1975) ovaries.

Natural antibodies directed against the zona pellucida have been reported to occur in mares infertile for unexplained causes (Liu & Shivers, 1982). Further attempts to investigate the role of horse 'auto-antibodies' against the zona demonstrated immunological cross-reactivity of pig and horse zonae and in-vitro reduction of binding of boar spermatozoa to pig oocytes treated with horse zona-positive antiserum (Shivers & Liu, 1982).

According to several reports, however, the use of zona components as the antigen in active immunization results in altered cyclicity and ovarian malfunction. Such side effects have been described for the bitch by Mahi-Brown *et al.* (1982, 1985). A decrease in ovarian weight, marked reduction of follicular development, abnormal follicular differentiation and lack of response to induction of ovulation by human chorionic gonadotrophin were also reported for rabbits after

active immunization (Wood *et al.*, 1981; Skinner *et al.*, 1984). Sacco *et al.* (1983, 1987) have reported that fewer ovulated oocytes were recovered from monkeys after injections of purified pig zona antigen, but ovarian function was reduced only temporarily and returned to normal 7 months after the end of the treatment.

The present study was conducted to determine whether heteroimmunization of mares with crude pig zona antigen would temporarily inhibit fertilization *in vivo* and, if so, to assess the possible causes of the inhibition.

Materials and Methods

Selection of feral mares. Thirty (30) feral mares were captured at a wild horse sanctuary of $\sim 2000 \, km^2$ in Northern California holding approximately 200 horses of different ages (~ 120 mares, 15 stallions and 65 geldings). After 6 months of confinement to a large area without stallions each mare was herded into a restraining chute and the reproductive tract was palpated *per rectum* to determine pregnancy status and potential abnormalities. Ten (10) presumably fertile mares were selected for this study on the basis of (1) a palpable normal reproductive tract, (2) a non-gravid uterus and (3) age (estimated to be 3–10 years old): 4 of the mares appeared to be maiden mares and the remaining 6 had foals by their sides. The individual mares were identified by their colour and markings and the group of mares treated was marked by removing their manes entirely with a pair of scissors. The 10 selected mares remained in a small corral for the period of the initial 4 immunizations, were subsequently released and recaptured 8 months later for assessment of pregnancy status and administration of booster injections.

Selection of domestic horses. These horses provided the opportunity for frequent serological analysis and continued examination of the reproductive tract for folliculogenesis, ovulation and pregnancy status. The group, housed at the Equine Research Laboratory's breeding facility of the University of California in Davis, consisted of 4 presumably fertile mares, 1 infertile mare with chromosome abnormality (63XO genotype) and 1 gelding. The 4 fertile mares were selected on the basis of (1) a palpable normal reproductive tract and speculum examination, (2) age, (3) history of pregnancy, and (4) normal cyclicity. The fertile mares were between 3 and 8 years old, had a history of never having been mated naturally or by artificial means and had normal physiological and behavioural oestrous cycles. The infertile mare and the gelding served as immunological controls.

Preparation of pig oocytes and zona antigen. Zonae pellucidae were isolated using procedures reported by Gwatkin *et al.* (1980) and Dunbar *et al.* (1980). The frozen–thawed ovaries were sliced in a ganged razor blade apparatus. The oocytes were separated by filtration through a series of screens of 210, 150 and 73 μm in the presence of phosphate-buffered saline (PBS, pH 7·2). The oocytes were counted and ground in a Potter–Elvehjem homogenizer. The zonae were trapped on a 48 μm screen, thoroughly rinsed with PBS and heat-solubilized at 70°C for 30 min in PBS for inoculation (doses of 2000 or 5000 zonae in 0·5 ml, 20 000 zonae in 2 ml), or in 0·1 M-glycine buffer (adjusted to pH 9·5 with NaOH) when used for the ELISA. The protein concentration of the heat-solubilized pig zona antigen was determined by the Automated Protein Assay (Technical Bulletin 1177: Bio-Rad Laboratories, Richmond, CA, U.S.A.) microassay procedure using bovine serum albumin as standard. Doses of 2000, 5000 and 20 000 zonae corresponded to 27·4, 68·5 and 274 μg protein, respectively.

Immunization adjuvants. The two initial inoculations used for all 16 horses consisted of a mixture of 3 parts zona antigen and one part amphogel (aluminium hydroxide gel) as adjuvant. As based on preliminary data obtained in an indirect immunofluorescence assay, this adjuvant seemed to be only moderately effective in most of the horses and was therefore substituted by Freund's adjuvant. Emulsions of equal parts of zona antigen and Freund's (complete for the initial, incomplete for the following injections) adjuvant were used for the remaining inoculations. However, serum analyses performed later by ELISA revealed more satisfactory immunization responses in 13 of the 16 horses after use of amphogel as adjuvant.

Immunization protocol. A series of 4 intramuscular injections of zona antigen was administered to each of the 16 horses at 2–4-week intervals. The doses for the 1st, 3rd and 4th injection consisted of 5000 zonae, the second injection dose consisted of 2000 zonae. At 6–9 months after the last injection, when antibody concentrations in the fertile domestic mares had decreased to levels that allowed conception at absorbance ratios of < 0.64 (one exception), the fertile domestic and feral mares received booster injections of 20 000 zonae each. Serum samples for antibody determination were obtained from all the horses before each inoculation and from the domestic horses monthly (with some variations) throughout the 3·5-year period of this study.

Mating of mares and pregnancy test. After the initial 4 inoculations, the feral mares were returned to their natural habitat with known fertile stallions: 8 months later they were recaptured and examined for pregnancy status by palpation *per rectum*. After immunization, the 4 fertile domestic mares were rectally palpated every other day through each oestrous cycle (every 18–21 days) of the mating season for observation of follicular development and

ovulation. (The natural mating season usually lasts from March through October and is followed by a 4-month anoestrous period.) Through each cycle and following the development of a preovulatory follicle of at least 40 mm in diameter, the mares were inseminated naturally or artificially with semen from known fertile stallions until ovulation. At 16–25 days after ovulation, ultrasonographic scanning was utilized for early pregnancy detection (embryo and heart beat). Additional pregnancy examinations by rectal palpation were performed at 45 and 60 days of gestation. The first pregnancies of the domestic mares that occurred during the study were terminated at 25 days by injection of 10 mg prostaglandin (Lutalyse: Upjohn, Kalamazoo, MI, U.S.A.). At 4 months after delivery of healthy foals in the second pregnancy, to Mares ST and GO, all 4 domestic mares were ovariectomized by colpotomy.

ELISA for determination of zona antibodies. The assay was performed essentially as described by Voller *et al.* (1980). Fox *et al.* (1981) and Drell *et al.* (1984) have measured antibody activity to pig zona antigen by the ELISA procedure. The biotin–avidin bridge method (S. Grimes, personal communication) was chosen for this study because of its low background reactivity.

Briefly, 50 µl of a 5 µg/ml zona antigen solution in 0·1 M-glycine buffer was placed in each well of a flat-bottom Microelisa Immulon 2 plate (Dynatech Laboratories, Alexandria, VA, U.S.A.) and incubated overnight at 4°C. The plate was washed once and incubated with 200 µl PBS–Tween for 30 min to block unspecific binding sites. After 2 more washes (PBS–Tween) the treatment of the plate consisted of subsequent 1-h incubations with 50 µl/well of PBS–Tween-diluted reagents used in the following order with 3 washes each in between: (1) test serum 1:1000, (2) biotinylated goat anti-horse IgG (Zymed Laboratories, San Francisco, CA, U.S.A.) 1:1000, (3) alkaline phosphatase avidin (Zymed) 1:2000. Finally, 50 µl substrate solution of 1 mg *p*-nitrophenyl phosphate/ml (5 mg tablets: Sigma Chemical Co., St Louis, MO, U.S.A.) in 10% diethanolamine buffer (pH 9·8) were added to each well, and the plate was scanned for absorbance at 405 nm (A_{405}) by an MR 580 Microelisa Auto Reader (Dynatech) when the absorbance of the positive reference serum had reached a level between 0·80 and 1·00 after incubation for 15–20 min.

Optimal concentrations of biotinylated goat anti-horse IgG and alkaline phosphatase avidin were selected after 'checkerboard' experiments in which, in an antigen–antibody (reference serum)-coated plate, doubling dilutions of one reagent are tested against doubling dilutions of the other reagent. The working dilution of the experimental sera (1:1000) was chosen from within the straight segment of the sigmoidal curve obtained after plotting various dilutions of the positive reference serum against their absorbances. Control reagents included PBS–Tween, 3 positive sera (weak, medium, strong) and 2 negative sera. The medium positive serum consisted of a pool of 8 different pre-tested serum samples and served as reference serum. The negative control pools were derived from 12 non-immunized random horses and from the pre-immunization samples of the 6 domestic horses in this study.

The experimental sera were tested in duplicate and their results are expressed as means ± s.e.m. of the ratios of the duplicate experimental absorbances divided by the mean absorbance of the reference serum tested on the same plate (EA_{405}/RA_{405}).

Indirect immunofluorescence assay with horse and pig oocytes. Frozen horse ovaries (obtained from an abattoir) were thawed and minced in ice-cold PBS and the mixture was filtered through gauze and a 73 µm screen. The oocytes were aspirated by micropipette from the cellular material trapped on the screen under a stereo microscope. The difficulty in obtaining horse ovaries and the poor recovery of oocytes from them (an average of 4 per ovary) did not permit any large scale usage as needed in the ELISA. Horse and pig (prepared as described earlier) oocytes (5 of each) were incubated for 30 min with 300 µl serum dilution in PBS on separate Boerner slides inside a humid chamber. They were washed 3 times by transfer to watchglasses holding PBS. After a 30-min incubation with 100 µl fluorescein isothiocyanate-conjugated rabbit anti-horse IgG (Miles-Yeda Ltd, Rehovot, Israel), diluted 1:10, the oocytes were washed and scored for surface zona fluorescence using an Olympus B2-2 microscope.

In-vitro assessment of sperm binding. Pig oocytes ($n = 200$) were incubated with 600 µl horse anti-pig zona serum dilution and washed 3 times by centrifugation. The oocyte pellet was resuspended in 30 µl PBS and transferred to a 5-ml sterile culture tube containing 0·4 ml boar sperm suspension at a concentration of 5×10^6/ml in basal medium (BM) with 0·5% bovine serum albumin (BSA) at pH 7·7. The fresh semen had been previously centrifuged on Percoll and the spermatozoa capacitated by incubation in BM with BSA and in an humidified incubator with 5% CO_2 at 39°C for at least 3 h (Berger & Horton, 1988). The oocytes were gently mixed with the spermatozoa and incubated overnight in the same incubator. The stop–fix technique of Saling *et al.* (1978) for recovering spermatozoa tightly bound to the zona was modified for treatment of the oocyte–sperm mixture. The mixture was carefully layered on discontinuous dextran (mol. wt 500 000: Sigma) gradients (1·8% over 2·25%) in BM using a 1-ml Fisher tube, and centrifuged in a Fisher microfuge at 100 *g* for 2 min. The pellet was resuspended in 25 µl 2·5% glutaraldehyde in PBS and transferred on a slide for microscopic examination. An average of 85 oocytes was counted per slide. The various degrees of sperm binding were classified as: (1) negative = no or less than 5 spermatozoa, (2) intermediate = 5–20 spermatozoa, (3) heavy = >20 spermatozoa bound per oocyte.

Enzyme immunoassay for progesterone. Luteal response was determined by progesterone analysis of plasma by an enzyme immunoassay (Munro & Stabenfeldt, 1984).

Preparation of ovarian tissue for histological examination. Ovarian tissue from the 4 fertile domestic mares was fixed in 10% formalin, embedded in paraffin wax, sectioned at 6 µm and stained with haematoxylin and eosin.

Results

Immune responses of feral mares

The anti-pig zona antibody responses of the 10 feral mares to the zona inoculations, as tested at 5 different time points, are summarized in Fig. 1. The antibody levels of all the mares were similar to one another at the first 4 test times as indicated by low standard errors. Pre-immune sera resulted in mean absorbance ratios of 0·04 and 0·05. The initial low response gradually increased with each inoculation and peaked after the 3rd, measuring a mean absorbance ratio of 1·03. Antibody responses of the feral mares to the 4th injection were not examined since the mares were released after the last inoculation. When 9 of the 10 treated feral mares were recaptured and tested 8 months later (Month 10·5) their results clearly fell into two groups: 7 mares still had remarkably high antibody levels (mean absorbance ratio = 0·82), and no antibodies could be detected in the 2 remaining mares (mean absorbance ratio = 0·06).
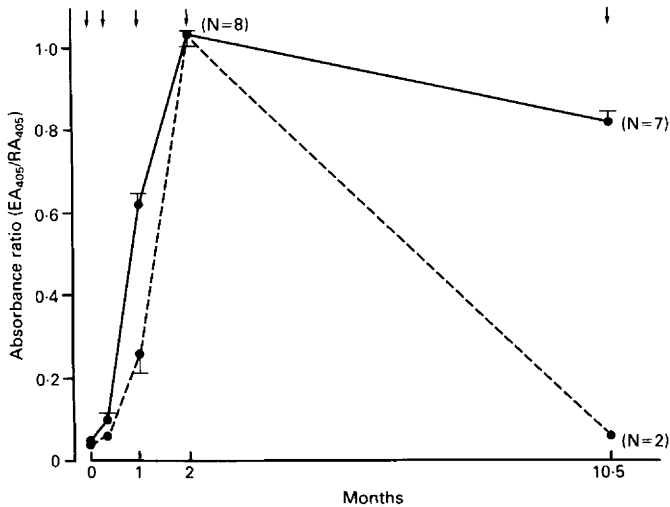

Fig. 1. Immune response to pig zona vaccinations (arrows) in 10 feral mares as measured by ELISA using pig zona antigen. Results of 7 mares (●———●), which had high antibody levels at Month 10·5 of the study, and results of 2 mares (●– – –●) with negative antibody levels at Month 10·5. One mare was not tested at Month 10·5. The mean absorbance ratios ± s.e.m. were calculated from the original duplicate absorbance ratios obtained per individual. Errors of <0·010 are not shown.

Immune responses of domestic horses

The antibody responses of the 6 domestic horses to the zona inoculations were monitored over a total period of 22–41 months. The results, as found in the ELISA using pig zonae as antigen (Fig. 2), are presented individually for each horse because of treatment variability. The 4 initial inoculations gradually increased the antibody concentrations of all 6 horses to peak levels expressed by absorbance ratios between 1·00 and 1·20. When antibody levels of 3 (ST, AP, GO) of the 4 fertile mares had declined to absorbance ratios of ∼0·50 at 7, 10 and 6 months (respectively) after the 4th inoculation, all 4 fertile mares received booster injections. As a result, their antibody levels peaked as before with the exception of Mare GO, which had developed an abscess at the injection site and

therefore received a second booster 4 months later. Antibody levels of the 4 fertile mares were monitored monthly for most of the period of 24–32 months following the booster injection. In 2 mares (ST, GO) antibody concentrations declined to lower levels (absorbance ratios 0·56, 0·42, respectively) 7 and 6 months after the booster. In Mare AP antibody levels remained above absorbance ratios of 0·82 for 23 months. Mare SH, however, maintained the high antibody level it had reached after the booster for 27 months (absorbance ratio at 27th month = 1·03). This originally healthy cycling mare developed bilateral blindness about 18 months after the initial vaccination. The mare's ovaries subsequently remained inactive, causing permanent acyclicity.

The infertile (XO) Mare SQ and the Gelding AL served as immunological control horses and were considered to be immunologically naive to zona components. Because they lacked antigenic exposure and subsequent antibody absorption from the circulation, higher and more persistent antibody levels were expected to result from immunization as compared to normal mares. However, the immune response actually reached by these control horses rather resembled some of the patterns observed in the healthy mares of this study and did not compare to the plateaued pattern of the Mare SH.

Antibody titre of horse versus pig oocytes

Because of the small numbers of available horse oocytes the indirect immunofluorescence assay was used instead of ELISA for antibody titre comparison of horse and pig oocytes. An antiserum of medium–strong reactivity against pig zona (Mare AP, after 3rd inoculation, absorbance ratio 0·94) was chosen and 2-fold dilutions were tested. The highest reactive serum dilutions were found to be 1:20 with horse oocytes and 1:800 with pig oocytes. The comparatively lower endpoint titre obtained with horse oocytes can be expected when cross-reacting antibodies are involved. The results confirm the cross-reactivity between pig and horse antigenic zona determinants as published earlier.

In-vivo block of fertilization

The 8-month period of exposure of feral mares to fertile stallions consisted of the last 2 months of the current physiological mating season, an intermediate 4-month anoestrous period and the first 2 months of the following year's mating season. Examination *per rectum* of the 9 feral mares that could be recaptured revealed one pregnancy of 21–23 days and no evidence of pregnancy in the remaining 8 mares. The pregnancy was not confirmed by ultrasonography because of the difficulty of applying this method to feral mares. Attempts to recapture the 10th mare failed, but observation from approximately 20 feet did not indicate any evidence of advanced pregnancy, and 8 months later it was seen without a foal by its side. When analysed for anti-zona antibodies, the pregnant mare's serum was negative. Among the 8 remaining non-pregnant mares, 7 demonstrated substantial antibody levels (mean absorbance ratio = 0·82) and one was found to be negative (Fig. 1).

Of the 4 fertile immunized domestic mares, 2 (AP, SH) did not conceive for an 8·5-month period after the 4th inoculation (Fig. 2). Of this 8·5-month period, 4·5 months consisted of active physiological mating period and the remaining 4 months of anoestrous and transitional phase periods. Conception occurred when antibody concentrations had decreased from peak absorbance ratios of 1·16 to 0·64 (AP) and from 1·14 to 0·91 (SH). At 25 days, the pregnancies were diagnosed by ultrasonography (embryo and heartbeat) and terminated. After subsequent booster injections, antibody concentrations regained high levels (absorbance ratios 1·11 and 1·12) and remained high for 27 months during which time continued insemination of Mare AP did not result in pregnancy. Because of persistent ovarian inactivity, possibly resulting from its blindness, Mare SH was not mated for the remaining part of the study.

How long the block of fertilization after the first immunization series lasted for the fertile domestic Mares ST and GO cannot be stated with certainty as the mares were in anoestrus with low
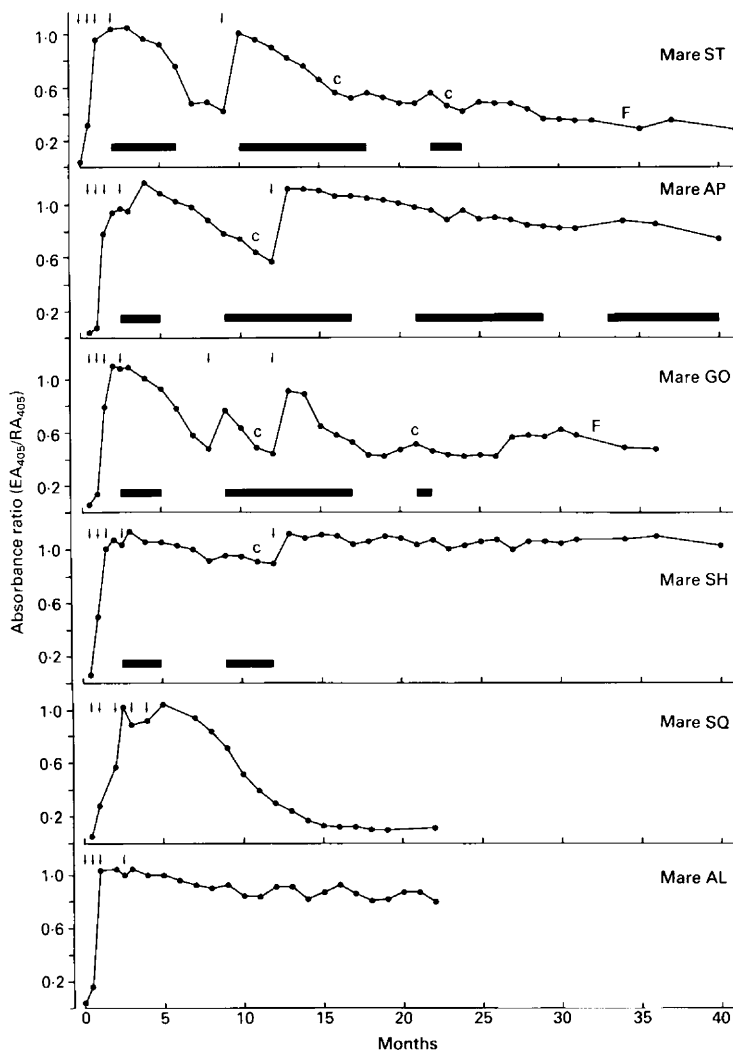
Fig. 2. Immune response to pig zona vaccinations shown individually in 6 domestic horses as measured by ELISA using pig zona antigen. Mares ST, AP, GO and SH were fertile, Mare SQ was infertile (63XO) and Horse AL was a gelding. Results are expressed as means of duplicate absorbance ratios: 57% of the standard errors (not shown) were <0·010, the mean of the remaining errors was 0·021 (range 0·010–0·052). ■, Period when inseminated; ↓, vaccination; C, conception; F, foaling.

antibody levels (0·42 and 0·49) at 5·5 and 7 months after inoculation and received booster injections before the start of the new mating season. In addition, the booster injection and subsequent immunological response of Mare GO was considered invalid because of the development of an abscess at the injection site. The injection was repeated 4 months later when a 25-day pregnancy was diagnosed (absorbance ratio 0·44) and terminated. Following the booster injection it required 7 months for Mare ST and at least 5 or at most 9 months (considering the anoestrous period) for Mare GO to regain fertility. At the time of conception the antibody levels were expressed as absorbance ratios of 0·46 and 0·51, respectively. The second pregnancies of Mares ST and GO were not interrupted and both delivered healthy foals after normal pregnancies.

Antibody-dependent in-vitro block of sperm binding

Repeated efforts to develop a binding assay for horse spermatozoa and oocytes failed because of inconsistent controls. This is consistent with the lack of reports of in-vitro fertilization or sperm binding techniques in the horse. The only alternative was to study the blocking abilities of the horse anti-zona sera using pig oocytes and boar spermatozoa. Sera collected at different periods from the same mare (AP) were selected on the basis of their different antibody levels and were tested simultaneously. The oocytes displayed normal sperm binding (95% heavy + 5% intermediate) after preincubation with pre-immune serum and similarly strong binding was found after treatment with a random non-immune serum and PBS (Fig. 3). Preincubation of the oocytes with the various antisera, however, reduced sperm binding in apparent relationship to the antibody levels of the sera. The weakest of the antisera used in this experiment (absorbance ratio = 0·57), collected in Month 12 of the study (Fig. 2), blocked sperm binding in 25% of the treated oocytes. Blocking of sperm binding gradually increased with higher antibody levels. The strongest antiserum (absorbance ratio = 1·16, Month 4) prevented sperm binding in 99% of the oocytes.
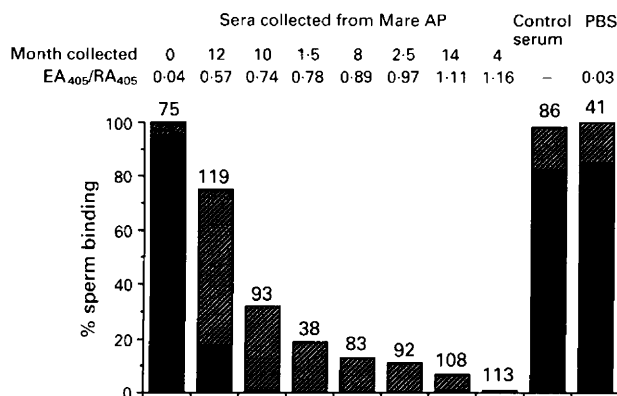

Fig. 3. In-vitro block of boar sperm binding (■, heavy; ▨, intermediate) to pig oocytes by horse anti-pig zona antibodies. Sera collected from Mare AP before (pre-immune) and at different times after immunization were selected for sperm binding experiments on the basis of their antibody level diversity. The results were placed in order of the antibody levels of the sera. The control serum originated from a non-immunized random mare. The total number of oocytes counted per test appears above each column.

Histological examination of the ovaries

The ovaries of 3 of the domestic mares appeared normal. Most of them showed severe hyalinization of the arteries in the capsules, a common finding in many ovaries of untreated mares. Graafian follicles, developing and atretic follicles, functional corpora lutea and recent ovulation sites were present in all the ovaries examined. No active inflammation was noted in the arteries. Both ovaries from Mare SH were inactive: no follicles or corpora lutea were noted in either ovary.

Progesterone profiles

Progesterone profiles obtained from the 4 domestic mares at the end of the study are shown in Fig. 4. Mare SH revealed no evidence of luteal function and remained acyclic, whereas Mares ST, GO and AP demonstrated evidence of ovulation, luteal function and normal cyclicity. Progesterone

concentrations of the 3 cycling mares peaked with a mean of 8·9 ng/ml, ranging from 5·6 to 13·1 ng/ml. Ovulation to ovulation (1 complete oestrous cycle) required a mean of 22·3 days, ranging from 21 to 25 days.
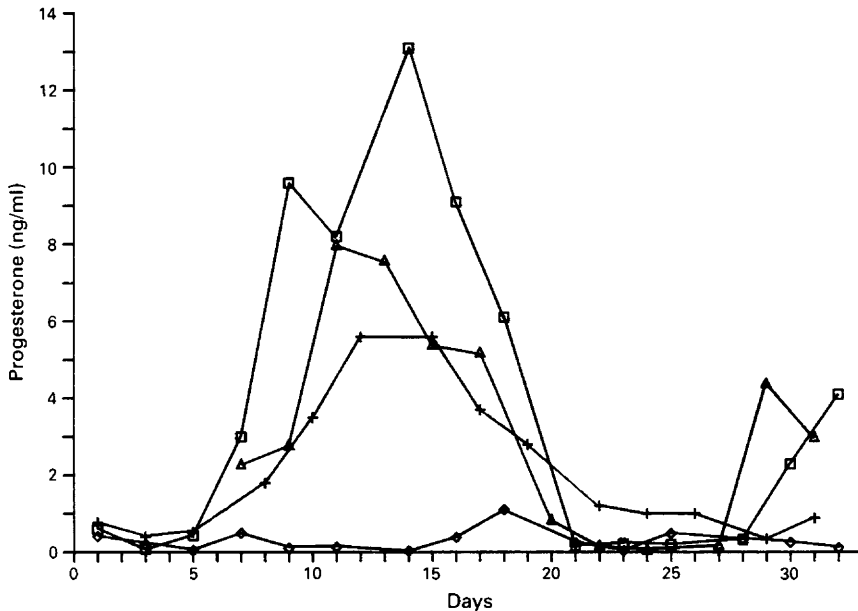

Fig. 4. Progesterone profiles of the 4 fertile domestic mares ($\triangle$ = ST, + = AP, $\square$ = GO, $\diamond$ = SH) through one complete oestrous cycle (ovulation to ovulation, with the initial one occurring on Day 5) were obtained before ovariectomy.

Discussion

An immune response was provoked in horses by active heteroimmunization with heat-solubilized pig zonae. Other investigations confirm the effects of heteroimmunization when compared with alloimmunization (Gwatkin *et al.*, 1977; Wood *et al.*, 1981; Mahi-Brown *et al.*, 1982), whereby heteroimmunization appears to be the more immunogenic and therefore preferable method of eliciting anti-zona antibodies.

In this preliminary study, the contraceptive effect correlated with high anti-pig zona antibody concentrations, persisted for at least 8 months in most of the animals studied and the effect diminished as antibody levels declined. As in other investigations in which large quantities of zonae from the immunized species are unavailable, the horse anti-pig zona antibodies were studied by using pig and not horse antigen. Such data permit insight into the kind of immune response elicited, although correlation of the direct antibody titres with the more important cross-reactive antibody titres exists only to a certain extent within the same individual (Sacco *et al.*, 1983) and may vary between individuals, especially when the immune response is directed against different antigenic determinants of the zona. In an indirect immunofluorescent assay it was demonstrated that horse anti-pig zona antibodies cross-reacted with horse zona components at a considerably lower titre than with pig zona components. Such cross-reactivity (Shivers & Liu, 1982), as well as cross-reactivity among zona components of several mammalian species, has been reported elsewhere (Gwatkin *et al.*, 1978; Sacco *et al.*, 1981). While ELISA absorbance ratios of 0·64 or less appeared to indicate the level at which most mares became susceptible to pregnancy, one of the fertile

domestic mares conceived at an absorbance ratio of 0·91 and one feral mare had not conceived in the absence of antibody against pig zonae. It was not determined why conception occurred in Mare SH at such a high anti-pig zona antibody titre, but it is possible that the associated cross-reactivity with horse zona antigens may have had a lower titre than in the other mares. The failure of the feral mare to conceive could have been due to one or more of several reasons (e.g. uterine infections following copulation, persistence of a corpus luteum, or early embryonic loss at 40 days and over, resulting in persistence of PMSG secretion) unconnected with the immunization programme.

Binding assays with boar spermatozoa and pig oocytes pretreated with horse anti-pig zona sera demonstrated antibody-dependent interference with sperm binding. The sperm blocking ability of the horse anti-pig zona antibodies, in conjunction with the evidence of their cross-reactivity with horse zona, suggests that stallion sperm binding sites at the zona surface may be blocked by steric hindrance from cross-linked antibodies as described by Aitken *et al.* (1982). According to Ahuja & Tzartos (1981), the antibodies are interacting with structures in close proximity to the binding sites rather than directly with the less immunogenic sperm receptors. Thus, sperm attachment and subsequent penetration are prevented as long as sufficient levels of cross-reacting antibodies are present.

Unfortunately, the contraceptive effect of zona antibody injections was not evaluated during one continuous physiological mating season (March–October). Instead, the study was initiated in June and the effects on fertility were studied during the second half of one mating season and the first half of the following mating season. Because of the apparent block of in-vivo fertilization well into the second mating season and the persistence of high antibody levels, it is reasonable to assume that the contraceptive effect would have lasted through one continuous physiological mating season. Possible causes, other than immunological, for the temporary inhibition of fertility in the 4 fertile domestic mares after zona injections were investigated. Clinically, no abnormalities were found regarding follicular development, cyclicity, ovulation and duration of oestrus. After 2 mares had regained fertility they conceived normally and produced healthy foals following normal pregnancies. Endocrinological profiles and histological sections of the ovaries ∼3 years after the last immunization revealed no ovarian malfunction and/or abnormal effects on the oestrous cycle with the exception of Mare SH. It has not been determined whether the development of inactivity of the ovaries of Mare SH was due to the effects of zona injections or to adverse effects of bilateral blindness because the photoperiod is critical to the initiation of cyclicity in mares. Ovarian malfunction and follicular changes have been reported as resulting from approximate dose equivalents of 4000 pig zonae/kg in rabbits (Skinner *et al.*, 1984) and 4–6 monthly doses of 118 and 59 zonae/kg in dogs (Mahi-Brown *et al.*, 1985). In contrast, the horses of this study received only 4 doses of 4 or 11 zonae/kg in 3 months and a single dose of 44 zonae/kg 6–10 months later. The possible absence of ovarian side effects in this study may be attributed to the proportionately lower dose of zona antigen administered.

Preliminary data on the use of an aluminium hydroxide gel as immunization adjuvant suggested satisfactory immune responses in horses without apparent side effects. However, more information is needed before any conclusions can be drawn.

Control mares receiving sham injections were not used in this study. Instead, the conception rates of untreated feral mares at the sanctuary and untreated domestic mares at the Equine Research Laboratory's breeding facility were considered alternative controls. The normal breeding behaviour of a healthy mare in the presence of fertile stallions is to produce one foal per breeding season and year. Annual conception rates of the untreated feral mares maintained at the sanctuary are estimated at 60–70%. This is consistent with the findings reported in other feral herds throughout the northern hemisphere (Kirkpatrick & Turner, 1986). Untreated fertile domestic mares maintained at the same facility with the 4 fertile zona-immunized mares conceived at an estimated annual rate of 90% when mated to the same fertile stallions.

In the past, stallions in wild horse populations in the Northern hemisphere have been the target for temporary sterilization as a means of controlling overpopulation. These attempts have met with minimal success. Provided a mare is fertile and inhibition of fertilization can be accomplished for

one physiological mating season, a period of 2 years will yield the birth of only one foal. This represents approximately one half the normal number of foals produced annually for each mare immunized. This preliminary study suggests that the use of preparations of pig zonae pellucidae may serve as an attractive alternative for the control of wild horse populations for which management levels are desired.

Further studies are needed, however, to purify the antigen used for contraceptive purposes, minimize the number of doses required, and improve the method of its administration, before any realistic management procedures can be adopted for use in feral horse populations. Immunization of pregnant mares and its effect upon the fetus and the mare's long-term fertility also should be investigated.

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