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In search of the active PZP epitope in white-tailed deer immunocontraception

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Abstract

Native porcine zona pellucida (PZP) has been shown to be highly effective as an immunocontraceptive in white-tailed deer. However, the immunogenicity of PZP extracted from pig ovaries may vary from lot to lot and the extract has the potential of containing either viral or pathogenic material. Determination of the immunocontraceptive epitopes of PZP would allow portions of the molecule to be synthesized or inserted into a recombinant system for production of a consistent and safe vaccine. In this study, epitopes of PZP were selected and tested by in vitro binding, immunogenicity in rabbits, immunogenicity and immunocontraception in deer.

Sera from PZP immunocontracepted deer were tested on ELISA plates containing immobilized peptides from ZP1 and ZP3α. Peptides with which sera from infertile deer reacted (six peptides from ZP1 and six peptides from ZP3α) were selected, synthesized and tested for immunogenicity in rabbits. Deer were then immunized with combinations of peptides from either the ZP1 or ZP3α groups. ZP3α peptides induced high immune titers against native PZP, but did not induce infertility in the deer. Although ZP1 peptides induced lower titers, deer immunized with two ZP1 peptides exhibited multiple estrus events and infertility, typical of that for deer immunized with native PZP vaccine. Competitive inhibition assays using the ZP1 peptides demonstrated that the peptide comprising pins 10–16 was most effective in blocking binding by the serum antibody of native PZP immunized deer. This peptide was used to immunocontracept deer, resulting in a significant reduction in fawning for 1 year. © 2002 Published by Elsevier Science Ltd.

Keywords: PZP; Immunogenicity; Immunocontraceptive epitopes

1. Introduction

A growing interest in non-lethal methods for population control of nuisance or damaging species of wildlife has fostered research to reduce fertility of the pest species.

Immunocontraceptive vaccines prevent conception by stimulating the production of antibodies that bioneutralize proteins or hormones essential for reproduction.

Many immunocontraceptive studies have targeted the interaction of spermatozoa with the zona pellucida (ZP) that occurs during mammalian fertilization. The ZP, a unique extracellular matrix surrounding the mammalian oocyte, is formed during ovarian follicular development. Following ovulation, spermatozoa must bind to and penetrate the zona to fertilize the egg. Dunbar demonstrated that, in addition to the commonly held notion that ZP antibodies block sperm penetration of the ovum, ZP antibodies may also interfere with follicle development and ovulation [1]. Native porcine zona pellucida (PZP) vaccines have been used to produce sterilization in rabbits, dogs, monkeys, horses, burros, baboons, deer and other species [2]. In a PZP contraceptive study of white-tailed deer at Pennsylvania State University, no detrimental health effects were observed in 4 years of the study [3].

Mammalian ZP has a molecular weight of over 250,000 and is typically comprised of three or four glycoproteins with high homology among species [1]. PZP proteins are designated ZP1, ZP2 and ZP3. ZP4 comprises a portion of ZP1. ZP1 has be shown to have sperm receptor activity, mediating sperm contact with the oocyte. ZP2 then acts as a second sperm receptor reinforcing tight interactions. ZP1 cross-links ZP2 and ZP3 forming dimers or oligomers. Gamete binding assays have also implicated PZP3α, but not PZP3β in sperm binding [4].

Sperm recognition and adhesive properties of ZP3α are dependent on both protein and carbohydrate moieties [5]. While the role of carbohydrates has been established in sperm–zona binding, there is considerable variation among species regarding the specific carbohydrates involved [6]. Moreover, the number of O- and N-linked glycosylation sites, the ratios of di-, tri- and tetra-antennary chains, as well

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as the degree of sulphation and sialylation all contribute to the species heterogeneity of the ZP protein [7].

Studies with zona peptides have been limited. Temporary infertility has been reported in hamsters immunized with PZP4 [8]. Incubation of gametes with a monoclonal antibody to PZP4 inhibits human sperm binding to ZP in vitro [9]. Hasegawa et al. [10] identified a peptide, CTYVLDPENL comprised of amino acids 50–59 of PZP4 that inhibited porcine oocyte fertilization in vitro. When this 10 amino acid peptide was conjugated to KLH and injected into mice, the resulting antibodies reacted with PZP4 and native PZP. This binding suggests that this epitope is exposed on the outside of the PZP molecule, which may make it more useful as a contraceptive target. Miller et al. [11] tested the contraceptive effects of three rabbits ZP recombinant peptides in white-tailed deer. Although there is a high homology between rabbit ZP and pig ZP, serum ELISA anti-PZP titers from the deer immunized with the rabbit recombinant ZP were quite low (1/1000 or less). There was a small contraceptive effect in two of the three recombinants, however, the recombinants were not as effective as a contraceptive as native PZP.

In practical terms, native PZP has been shown to be an effective immunogen for contraception of a variety of species. FDA has indicated that extracted PZP could be accepted in a final contraceptive vaccine formulation. However, the FDA has expressed concerns about consistency and safety of the final product. Given this background, and the need to understand which and how many epitopes are involved in PZP-induced infertility, we undertook a study to identify the immunocontraceptive epitopes of PZP and evaluate these epitopes in PZP immune deer serum, with the goal of producing the ZP vaccine in the laboratory. Identifying epitopes in a large multi-protein antigen such as PZP is a difficult task. Synthesizing peptides to identify epitopes generally assumes that the epitope is in a linear sequence. However, many epitopes are conformational, that is the epitope is made up of two-folded peptide loops. Such epitopes can disappear when the peptide is straightened out, which happens many times when a peptide is synthesized and may result in errors of interpretation. The peptides used in this study were quite large, not coupled to a carrier, therefore, folding may have occurred with the formation of conformational epitopes. However, until a practical method is developed that can differentiate between the linear and conformational epitopes, it would be difficult and time consuming to distinguish between the two.

2. Materials and methods

2.1. White-tailed deer study

This study was a cooperative effort involving the National Wildlife Research Center (NWRC) and The Pennsylvania State University. It was conducted at the Deer Research Center of The Pennsylvania State University, University Park, PA, as part of a multi-year contraceptive research project involving several contraceptive technologies. The study was approved by the Pennsylvania State University animal care and use committee.

The does were injected with 1 ml of PZP epitopes subcutaneously. The 1 ml prime dose consisting of 0.5 ml of saline containing 500 μg of PZP peptides mixed with 0.5 ml of complete Freund's adjuvant (CFA), was given in September. The booster dose, containing 500 μg PZP peptides in 0.5 ml saline mixed with 0.5 ml of incomplete Freund's adjuvant (IFA), was given in October. The does were exposed to bucks in November.

2.2. Search for active epitopes in PZP immunocontracepted deer

To identify which epitopes were involved in the effective PZP immunocontraception of deer, PZP peptide sequences of PZP1, PZP3α and PZP3β were obtained from the NIH gene-bank and used to prepare immobilized peptide plates by the mimotope system of Chiron Mimotope Systems (San Diego, CA). As recommended by Chiron, the ELISA plate was designed so that each well contained a 14 mer peptide, with an overlap in the sequence of 8 mer between wells. The serum from four deer immunocontracepted with PZP were tested on these plates to determine the potential zona epitopes recognized by the deer immune system. Each deer was tested separately on the plates. An amount of 50 μl of 1:1000 immune deer serum was added to each well and incubated at room temperature for 2 h on a Mimimix shaker (Fisher Scientific Springfield, NJ). The plates were washed with PBS containing 2% non-fat dried milk. Antibodies binding to PZP peptides on the plate were detected with the following linkage: deer anti-PZP binds to peptides on the plate, rabbit anti-deer IgG binds to the deer IgG, goat anti-rabbit-peroxidase (Sigma) binds to the rabbit IgG. The chromogen tetramethylbenzidine was used to develop the color and 2 M H_2SO_4 was used to stop the reaction. The color intensity of the sample was read at 450 nm with a Dynatech MR 5000 ELISA plate reader. The serum from all four deer reacted with the selected epitopes (Fig. 1).

2.3. Synthesis of active epitopes

Selected peptide sequences (six PZP3α and six PZP1) were synthesized by Colorado State University Macromolecular Resources, Fort Collins, CO. The peptides selected ranged from 24 to 44 amino acids and were large enough to be immunogenic without conjugation to a carrier protein.

2.4. Rabbit anti-ZP-peptide antibody study

To determine if the synthesized peptides were immunogenic, they were first tested in rabbits (Fig. 2). Each peptide was prepared as a vaccine by mixing it with Freund's adjuvant and injected into two rabbits. The prime vaccine

36 IGVNQ LVNTAFPGIV TCHENRMVVE
61 FPRILGTKIQ YTSVVDPI - ***GL EMMNCTYVLD PENLTLKAPY EA***CTKRVRGH HQMTIRLIDD
121 **NAALRQEALM** YHISCPVMGA EGPDQHSGST ICMKDFMSFT -***FNFFPGMADE NVKR***EDSKQR
181 **MGWSLVVGDG *EKARTLTFQE AMTQGY***- NFLI GNQKMNIQVS FHATGVTRYS QGNSHLYMVP
241 LKLKHVSHGQ SLILASQLIC VADPVTCNAT HVTLAIPEFP GKLKSVNLGS GNIAVSQLHK
301 HGIEMETTNG LRLHFNQT **LL KTNVSEKCLP HQLYLSSLKL TFHSQLEAVS MVIYPECL** CE
361 STVSLVSEGL CTQDGFMDVK VHSHQTKPAL NLDTLRVG- ***DS SCQPTFKAPA QGLVQFRIPL***
421 ***NGCGTRHKFK* NDK VIYENEI HALWAD- *PPSA VSRDSEFRMT VR** CSYSSSNM LINTNVESLP
481 SPEASV- ***KPGPLTLTLQTYPD NAYLQPYGDK EYPVVKYLRQ* PIYLEVRILN RTDPNIKL VL**
541 DDCWATSTED PASLPQWNVV MDGCEYNLDN HRTTFHPVGS SVTYPNHHQR FDVKTFAFVS
601 GAQGVSQLVY FHCSVFICNQ LSPTFSLCSV TCHGPSRSRR ATGTTEEEKM IVSLPGPILL
661 LSDGSSLRDA VNSKGSRTNG YVAFKTMVAM VASAGIVATL GLISYLHKKR IMMLNH

	Selected Amino Sequences	
	Mimotope Pin Number	Amino Acid Number
	10-16	79-130
	20-25	161-206
	40-44	319-358
	50-54	399-432
	54-58	434-462
(A)	61-67	487-538

1 MWLRPSIWLC FPLCLALPGQ SQPKAADDLG GLYCGPSSFH FSINLLSQDT ATPPALVVWD
61 RRGRLHKLQN DSGCGTWVHK GPGSSMGVEA SYRGCYVTEW DSHYLMPIGL EEADAGGHRT
121 VTETKLFK ***-CP VDFLALDVPT IGLCDAVPVW DRLPCAPPPI TQGECKQLGC CYNSEE*** -VPSC
181 YYGNTVTSRC TQDGHFSIAV SRNVTS- ***PPLL WDSVHLAFRN DSECKPVMET*** - IITFVLF -***RFPF***
241 ***SSCGTAKRVT GNQAVYENEL VAARDVRTWS HGSITRDS- -IF RLRVSCIYSV SSSALPVNIQ***
301 ***VFTLPPPLPE THPGPLTLEL QIAKDERYGS YY*** -NASDYP- ***VV KLLREPIYVE VSIRHR*** -TDPS
361 LGLHLHQCWA TPGM- ***SPLLQP QWPMLVNGCP YT*** -GDNYQTKL IPVQKASNLL FPSHYQRFSV
421 STFSFVDSVA KQALKGPVYL HCTASVCKPA GAPICVTTCP AARRRRSSDI HFQNGTASIS
481 SKGPMILLQA TRDSSERLIK YSRPPVDSHA LWVAGLLGSL IIGALLVSYL VFRKWR

	Selected Amino Acid Sequences	
	Mimotope Pin Number	Amino Acid Number
	21-28	129-176
	34-39	207-230
	39-45	237-278
	46-54	279-332
	56-59	341-356
(B)	62-64	375-392

Fig. 1. PZP1 (PZ4 1–133 = PZ1 36–168) (A) and PZP3α (B) amino acid sequences, obtained from the NIH gene-bank, were synthesized and immobilized on 96-well plates. Sera from native PZP-immunized/contracepted deer were diluted 1:1000 and added to the plates. The bold underlined sequences represent potential zona epitopes recognized by the deer sera using a standard ELISA procedure. These epitope areas were synthesized for further testing.

was made by mixing 500 μg of the peptide with Freund's complete adjuvant and the boost dose was made by mixing 500 μg of peptide with incomplete Freund's adjuvant. Both prime and boost were injected in 0.5 ml total volume. After a prime and a boost, the serum from each rabbit was tested for antibody response to native PZP by ELISA. Most of the peptides induced an antibody response in rabbits to the native PZP protein.

2.5. White-tailed deer study with pooled PZP1 and PZP3α epitopes

Because the cost of testing each of the 12 selected peptides separately in deer was prohibitive, the peptides of ZP1 (see Fig. 1) were pooled together to form a single vaccine. The same was done with the ZP3α peptides. Each combination of peptides was injected into two deer. A third vaccine was also prepared by combining the ZP1and ZP3 peptides, injected into two deer. A fourth vaccine, tested in two deer, was prepared from the ZP3α pins 46–54, which had an exceptional immune response in rabbits (Figs. 3 and 4).

2.6. White-tailed deer study with pooled PZP1

In our long-term contraceptive study using PZP, most treated deer exhibited multiple estrus with an extended breeding period. The two deer in the ZP1 group that

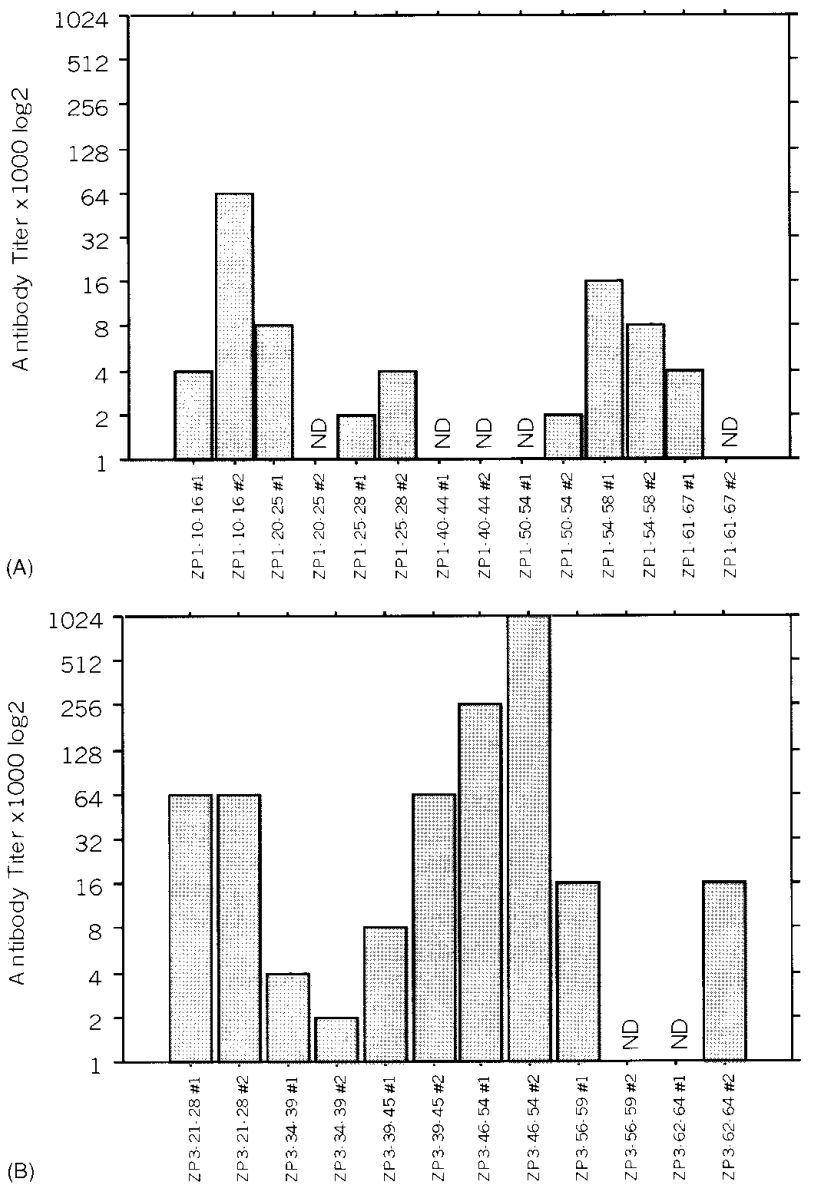

Fig. 2. PZP1 (A) and PZP3α (B) peptide sequences selected by PZP immunized deer were synthesized and each peptide was injected into two rabbits. Resulting rabbit antibody against native PZP was determined by an ELISA procedure.

were contracepted exhibited the same multiple estrous and extended breeding period as observed in the PZP treated deer. Therefore, a follow-up study was performed as described in the following sections to test more deer with the ZP1 group of peptides.

The six PZP1 peptides were pooled and injected into seven deer. Each deer received a 500 μg prime and a 500 μg boost given 30 days apart. This resulted in two of the seven deer showing multiple estrus events similar to that seen with PZP immunized deer. Serum from the two PZP1 immunocontracepted deer that recycled multiple times and still did not conceive, was used for an epitope competition study to determine which peptide or peptides may be responsible for the contraceptive activity.

2.7. ELISA competitive inhibition studies with PZP1 peptides

The ELISA plate was coated with native PZP and blocked as in the earlier study. Serum from deer with a high titer to native PZP was diluted 1:4000 in a PBS 2% milk solution. The binding of this diluted serum was considered 0% inhibition. A 12–1536 ng (in eight concentration steps) of each ZP1 peptide and the combined six peptides were incubated at 37 °C for 30 min with the diluted serum before adding the serum to the ELISA plate.

The ability of a peptide to reduce the binding to native PZP was used as an indication that peptides bound to anti-PZP antibodies present in the serum.

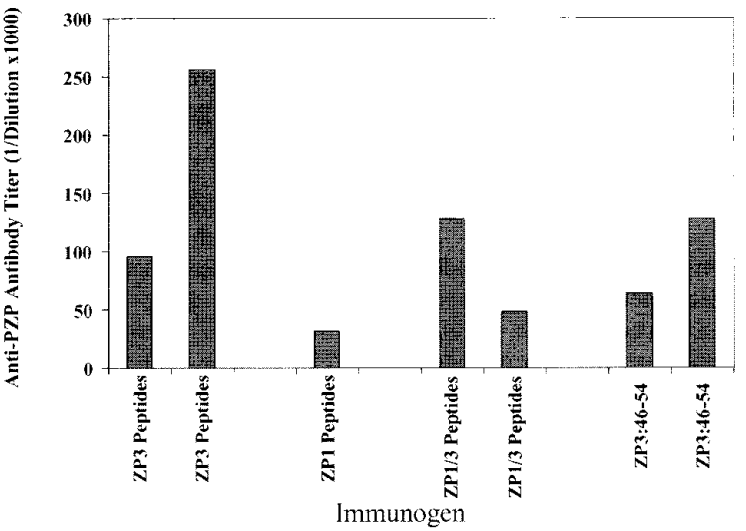

Fig. 3. Three groups of pooled peptides and one promising ZP3α peptide (ZP3α, ZP1, ZP1/ZP3α and ZP3α 46–54) were each injected into two deer. Resulting antibody titer against native PZP was determined by an ELISA procedure. The mean fawning rate of all treated groups was 1.0 fawn/doe and one ZP1 doe had three cycles and did not fawn.

2.8. White-tailed deer study with PZP1 pin 10–16 (amino acids 79–130)

Seven deer were injected with PZP1 10–16 (1000 μg prime and a 500 μg boost after 30 days). The peptide, which is found within the ZP4 region (ZP1 31–168) of PZP (Fig. 1), was dissolved in water and mixed with an equal volume of complete Freund's adjuvant in the prime and incomplete Freund's adjuvant in the boost dose: 79-GLEMMNCTYVLDPENLTLKAPYEACTKRVRGHH-QMTIRLIDDNAALRQEALM-130.

2.9. Hormonal, ELISA and fawning data

In each study, blood was drawn 3 weeks after the boost dose for progesterone assays, and anti-PZP titers. Ultrasound, to detect pregnancy was performed in February, and fawning was recorded in the spring.

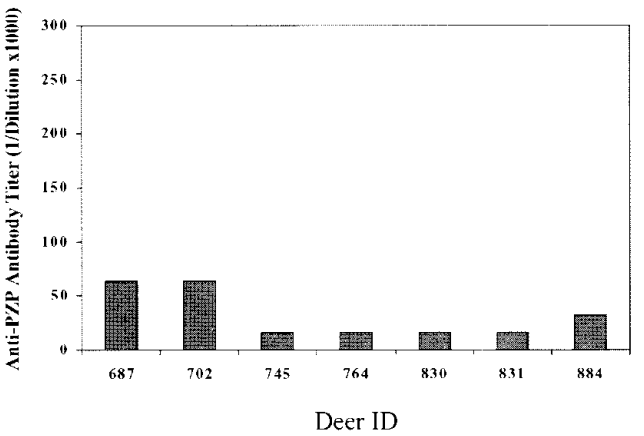

Fig. 4. Pooled ZP1 peptides were injected into seven deer. Resulting antibody against native PZP was determined by an ELISA procedure. The fawning rate by the seven injected deer was reduced to 1.28 fawns/doe compared to 1.8 fawns/doe for the control deer.

3. Results

3.1. Peptide selection

It is well known that epitopes (the smallest peptide unit that binds to an antibody) are approximately 3–8 amino acids in size. However, the peptide sequences selected by the mimotope technology were substantially larger than predicted for a single epitope. Furthermore, the recognition patterns for the four immunized deer did not completely overlap, therefore, we included the entire peptide recognition area represented by the four deer. The resulting peptides included up to 50 amino acids of 7000–8000 MW (Fig. 1). The peptides were injected without any coupling to carrier protein, to allow as much natural folding to occur as possible.

3.2. Rabbit peptide study

Rabbits immunized with PZP3α peptides produced high titers (up to 1 million) to native PZP compared to those immunized with PZP1. The highest titer was produced in response to PZP3α pin 46–54. This area is reported to contain two *O*-glycosylation sites that are necessary for sperm binding [9] (Fig. 2A). Fewer PZP1 peptides induced antibody production and the titers produced were lower than those found in the PZP3α group. PZP1 pins 10–16 stimulated a 64,000 titer to native PZP in one rabbit (Fig. 2B). These high titers against native PZP on the ELISA plates were unexpected since the recombinant peptides developed in Dr. Dunbar's laboratory and tested in both rabbits and deer only had titers of approximently1000 against native PZP [10].

3.3. Deer peptide studies

PZP is a highly immunogenic contraceptive antigen which, during active immunocontraception in white-tailed deer, induced antibody titers of over 1 million. In one study, fawning rate in immunized does was reduced to 0.23 fawns/doe as compared to 1.8 fawns/doe in the control population [2].

Immuno-reactions of four serum samples (two bleedings from each of two PZP-immunized deer) demonstrated some pin variations in reactive regions between bleeds, therefore, the peptide area selected for the determination of specific reactive epitopes was broader than if one bleeding had been used. Peptide areas of 16–52 amino acids were selected (Fig. 1A and B). Because the mimotope for ZP3β did not demonstrate definite areas of antibody recognition, no ZP3β peptides were selected for further analysis. Mimotope results using pre-immune sera showed a clean plate with no strong areas of reactivity.

The deer immunized with the pooled peptides selected from ZP1, ZP3, ZP3 pin 46–54 and ZP1/ZP3 produced high antibody titers against native PZP. The overall fawning rate was reduced from 1.8 to 1.1 fawn/doe. One deer in the ZP1 group cycled three times and had no fawns, which is characteristic of native PZP-immunized deer [2]. The mean overall fawning rate of the ZP1 group was reduced to 1.0 fawn/doe compared to 1.8 fawn/doe in the controls.

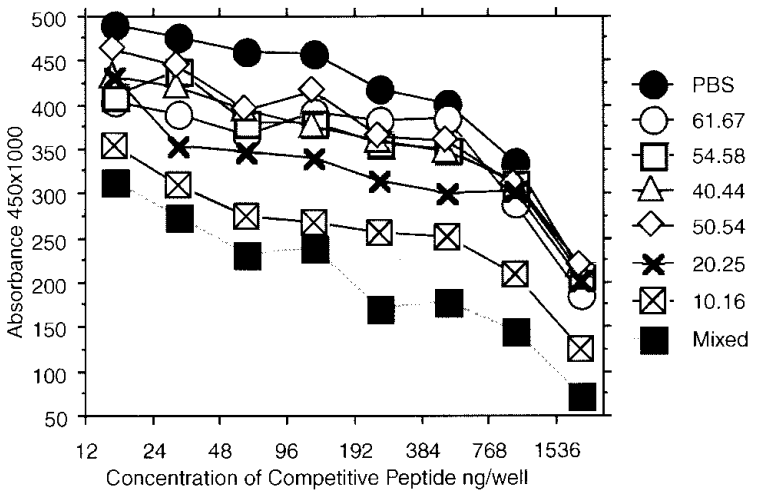

Fig. 5. To determine which of the 6 ZP1 peptides contained dominant antigenic determinants, a peptide inhibition assay was performed. Peptides in eight serial dilutions were pre-incubated with a 1:4000 dilution of the serum of ZP1 injected deer number 702. Peptide from pins 10–16 demonstrated the greatest absorption of ZP1 antibody; close to that absorbed by the combined peptides.

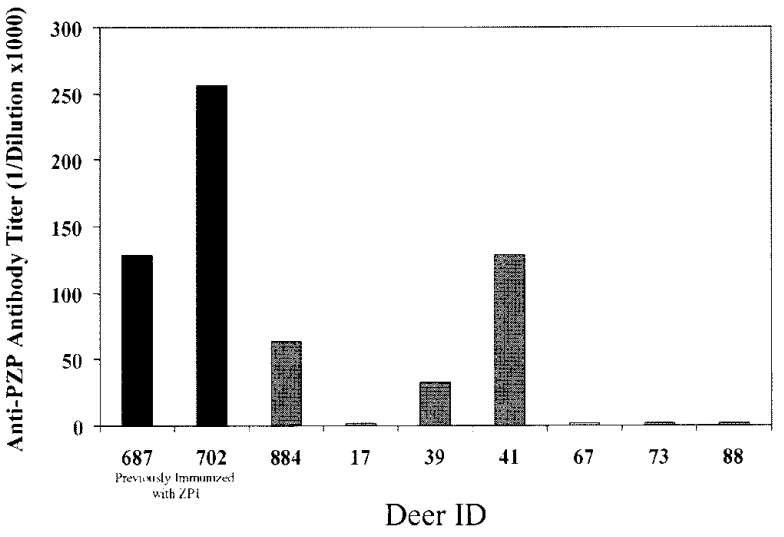

Fig. 6. Peptide from pins 10–16 was used to immunize nine deer. The immune response to the 52 amino acid peptide was variable. One deer in the group demonstrated multiple cycling and the fawning rate in this group was 0.89 fawns/doe.

3.4. PZ1 peptide deer study

Because does immunized with peptides from ZP1 showed evidence of contraception in the preliminary study, we injected seven additional deer with the combination of the six ZP1 peptides. The fawning rate was reduced to 1.28 fawns/doe compared to 1.8 fawns/doe in the control deer. Two of the seven deer were observed having multiple estrous cycles. One deer cycled five times and did not fawn. The second deer cycled three times and had one late fawn that died at birth. This suggested that within the six peptides there are one or more contraceptive active epitopes.

3.5. ELISA peptide inhibition assay

To determine which epitopes might be contributing to infertility, a peptide inhibition assay was performed. ZP1 pins 10–16 had the greatest inhibition of the six ZP1 peptides, indicating that the major portion of the PZP binding antibody in the ZP1-treated deer was directed to the ZP1 10–16 peptide. The level of inhibition by this peptide approached that of the combined peptides (Fig. 5).

3.6. ZP1 pin (10–16) peptide study

The following year, nine deer were immunized with the ZP1 peptide 10–16. There was a large variation in the immune response (antibody titer) to PZP in this group of deer. The deer with the highest titer to PZP exhibited three estrous cycles and had no fawns. Although other deer in the group (with lower antibody titers) did not exhibit multiple estrous cycles, the fawning rate was reduced to 0.89 fawns/doe. All the does that fawned in this group had only one fawn, which is typical of a partial contraceptive effect (Fig. 6).

4. Discussion

Based on reports in the literature, we began this study assuming that the immunocontraceptive activity of PZP would be found in the ZP3 regions. Indeed, our rabbit immunogenicity studies found that ZP3 peptides induced higher antibody titers than did ZP1 peptides, with ZP3 peptide pin 46–54 (ZP3 279–332) producing the highest anti-native PZP titer of all the peptides. It has been demonstrated that this ZP3α region contains O-linked oligosaccarides and has sperm receptor activity [7]. Therefore, it was not surprising that this region induced a high antibody titer against PZP. However, an unexpected result was that ZP1 peptide immunization reduced fertility and produced physiological effects such as multiple estrous cycles. The multiple estrous cycles were similar to those induced by native PZP immunization, indicating that the mechanism of infertility is preventing sperm penetration of the zona without affecting estrus. The data from this study clearly suggest that epitopes within PZP, that can result in contraception of deer, lie within the ZP1 pins 10–16 peptide region.

The practical challenge of using PZP peptides in place of native PZP is that they are typically much less immunogenic than the native molecule, which results in 95–100% of the deer responding with high titers and contraception. As the peptide size is reduced, the percentage of deer responding with a high titer is reduced.

One possible reason for a less consistent response to PZP peptides compared to whole molecules is that the smaller peptides may not contain sufficient T cell epitopes to ensure sufficient binding by MHC and presentation to T cells in any given deer. Unlike antibodies, T cells recognize antigen as a combination with MHC on the surface of cells. Each allelic form of MHC binds epitopes with a certain configuration. When many epitopes are present, such as in whole PZP, the chance that the MHC expressed in a given deer will be able to bind a sufficient number of those epitopes is high, but when less T cell epitopes are present, the probability of sufficient MHC binding of T cell epitopes is lower. Unless T cells are stimulated by antigen (presented with MHC), antibody production is stifled. To overcome this limitation, peptides will need to be carefully designed to contain sufficient T cell epitopes, or be coupled to larger carrier proteins (e.g. ovalbumin, albumin, keyhole limpet hemocyanin) that can provide the necessary T cell stimulation.

In its native form, PZP is a large three dimensional glycosolated structure which when used as an immunogen presents epitopes that stimulate an immunocontraceptive response. There may be multiple epitopes involved in PZP immunocontraceptive activity. When epitopes which are only small portions of the native PZP molecule are synthesized, the three-dimensional conformation and the glycosolation, which are both important to the immunocontraceptive activity of the native molecule, are limited. However, since many of the these peptides induced high levels of antibody that reacted with native PZP on an ELISA plate, these peptides may be part of the immunological response observed in the PZP vaccine.

Hasegawa et al. [12] demonstrated that monoclonal antibody produced to PZP4 inhibited the binding of human spermatozoa to human zona pellucida in vitro. Active immunization with PZP4 induced temporary infertility in hamsters leading to the conclusion that PZP4 contains an antigen epitope that may be useful for developing a contraceptive vaccine. The PZP4 peptide selected was 85-CTYVLDPENLTLKAPYEA-102. Interestingly this peptide is found within the PZP1 pin 10–16 peptides 79–130 that we found was effective in causing multi-estrous cycles in deer.

It is noteworthy that an overlap exists between the peptide that Hasegawa found to have contraceptive properties and the hydrophilic portion (as determined by a Wellington

antigenicity scale profile) of our large peptide selected from our 3 years of contraceptive deer studies [13].

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