T7 promoter (plasmid pCB112). Cultures were grown at 37°C to an OD at 600 nm of 0.5, IPTG was added to 0.5 m*M*, and incubated 130 min with shaking at 37°C. Extracts were prepared by sonication of cells in 0.05*M* KCl buffer A (25 m*M* Hepes, *p*H 7.7, 10% glycerol, 0.5 m*M* EDTA, 10 m*M* 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride, and 0.5 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin A, Sigma), centrifugation, and dialysis of the resulting supernatant against 0.05*M* KCl buffer A. Dialyzed extracts were centrifuged and analyzed in gel shift assays as in (*3*) except that the final concentration of KCl was 10 m*M* and of NACl was 40 m*M*.

36. The final buffer for the DNase I protection assays was 40 m*M* Hepes, *p*H 7.8, 40 m*M* KCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, and sonicated calf thymus DNA (14 μg/ml). The $MgCl_2$ was added with the DNase I. BAS1 was prepared from an overproducing strain of *E. coli* (AR68; *htpR*, λ cI_{857}) that contains a plasmid with the *BAS1* gene controlled by the inducible λ P_L promoter (pCB114). The BAS1 expression plasmid (pCB114) was an inframe fusion of the ATG start codon of the expression vector [pOT5, a derivative of pAS1; M. Rosenberg, Y. Ho, A. Shatzman, *Methods Enzymol.* **101**, 123 (1983)] with the second codon of the *BAS1* coding sequences. Heat induced cells containing the BAS1 protein were sonicated into 0.05*M* KCl buffer A [see (*35*)]. This extract was centrifuged and two volumes of 4*M* ammonium sulfate (*p*H 7.8) was added to the supernatant. After centrifugation, the pellet was resuspended in 0.05*M* KCl buffer A, dialyzed, and loaded onto a heparin-agarose column equilibrated in 0.05*M* KCl buffer A. The column was rinsed with 0.2*M* KCl buffer A; BAS1 was eluted with 0.7*M* KCl buffer A and dialyzed against 0.05*M* KCl buffer A. The BAS1 protein prepared by this method from *E. coli* binds very well to DNA even though it is partially proteolyzed (*20*).

37. Heparin-agarose–purified BAS2 protein [0.10 mg per milliliter of total protein (*36*)] was prepared from BL21(DE3) bacteria [with pLysS plasmid; see (*35*)] that contained a plasmid with the *BAS2* gene downstream of the T7 promoter (plasmid pCB262). Heparin-agarose–purified En protein [0.10 mg per milliliter of total protein, see (*36*)] was prepared from BL21(DE3) bacteria (without pLysS plasmid) that contained a plasmid with the *engrailed* gene downstream of the T7 promoter [plasmid pAR3040; T. Hoey and M. Levine, *Nature* **332**, 858 (1988)]. As determined by PAGE and gel shift analysis, the preparation of En protein was not proteolyzed while the preparation of BAS2 protein, like BAS1 (*20*), contained proteolytic fragments (about 50% of total binding activity, migrating almost twice as fast as full-length BAS2) that bound to the *HIS4* promoter. Expression of BAS2 from the lambda P_L promoter in strain AR68 or from the T7 promoter in other protease deficient strains did not prevent proteolysis. The full-length BAS1 and BAS2 from bacteria, migrated during gel shift analysis to the same position as yeast-derived BAS1 and BAS2. The lane marked by C contains 45 μl of heparin agarose–purified control extract (0.10 μg per milliliter of total protein, prepared identically as for BAS1, BAS2, and En extracts) from control-induced BL21(DE3) bacteria that had the pLysS plasmid and the T7 polymerase expression plasmid with no insert. A similar control (*19*) for the BAS1 extract with an extract prepared from a heat induced control lambda P_L expression strain had results identical to lane C.

38. We thank J. Jaynes and P. O'Farrell for plasmid pAR3040 containing the *engrailed* gene under control of the T7 promoter, M. Goebl for pointing out that BAS2 is a homeodomain protein, and D. Shevell and A. Sutton for comments on the manuscript. G.R.F. is an American Cancer Society Research Professor of Genetics. Supported by NIH grant GM39892 to K.T.A. and GM35010 to G.R.F.

26 July 1989; accepted 10 October 1989

Vaccination with a Synthetic Zona Pellucida Peptide Produces Long-Term Contraception in Female Mice

SARAH E. MILLAR, STEVEN M. CHAMOW, ANNE W. BAUR, CONSTANCE OLIVER, FRANK ROBEY, JURRIEN DEAN

The zona pellucida surrounding mouse oocytes is an extracellular matrix composed of three sulfated glycoproteins, ZP1, ZP2, and ZP3. It has been demonstrated that a monoclonal antibody to ZP3 injected into female mice inhibits fertilization by binding to the zona pellucida and blocking sperm penetration. A complementary DNA encoding ZP3 was randomly cleaved and 200– to 1000–base pair fragments were cloned into the expression vector λgt11. This epitope library was screened with the aforementioned contraceptive antibody, and the positive clones were used to map the seven–amino acid epitope recognized by the antibody. Female mice were immunized with a synthetic peptide containing this B cell epitope coupled to a carrier protein to provide helper T cell epitopes. The resultant circulating antibodies to ZP3 bound to the zona pellucida of immunized animals and produced long-lasting contraception. The lack of ovarian histopathology or cellular cytotoxicity among the immunized animals may be because of the absence of zona pellucida T cell epitopes in this vaccine.

THERE IS CURRENTLY MUCH INTEREST in the development of a safe and effective contraceptive vaccine for population control. An ideal vaccine should have an effect that is long-lasting and highly specific and should inhibit fertilization as a contraceptive agent rather than disrupt early development as an abortifacient. In addition, the immunogen must induce an immunological response to an endogenous antigen that is effective as a contraceptive without eliciting a cytotoxic response that might result in abnormal reproductive function or other damage.

The mammalian zona pellucida (zona), which surrounds growing oocytes and ovulated eggs, is a potential immunogen for a contraceptive vaccine (*1, 2*). The zona of the mouse is composed of three sulfated glycoproteins (ZP1, ZP2, and ZP3) (*3*). Sperm initially bind to ZP3 via *O*-linked oligosaccharide chains, and continued binding involves ZP2 as a secondary sperm receptor. These two zona proteins form filaments that are crossed linked by ZP1 in the extracellular zona pellucida (*4*). The zona is unique to the ovary, highly antigenic, and accessible to circulating antibody during the intraovarian oocyte growth phase prior to meiotic maturation and ovulation (*1, 2*).

Passive immunization of mice with sera against the zona produces reversible contraception without obvious side effects (*1, 5*). In more recent experiments, rat monoclonal antibodies against ZP2 and ZP3 were injected into female mice. The antibodies bound specifically to the zonae surrounding intraovarian oocytes and produced long-term (more than 8 weeks), reversible contraception by preventing sperm penetration of the zona pellucida (*6, 7*). However, the epitopes recognized on mouse ZP2 and ZP3 by five different rat monoclonal antibodies are not present on other mammalian zonae pellucidae (*6, 7*), limiting their usefulness as contraceptive agents.

The recent cloning of the ZP3 gene and the characterization of its transcript and protein product (*8, 9*) have provided sufficient molecular detail of the zona proteins to suggest an alternative contraceptive strategy based on active immunization with a zona peptide. We have made use of the specificity of a monoclonal antibody to ZP3 known to block fertilization to identify a zona pellucida peptide for testing as a contraceptive vaccine. A 1.0-kb cDNA that contains sequences encoding the epitope recognized by the monoclonal antibody to ZP3 (*8*) was cut into random fragments, which were size selected (200 to 1000 bp) and cloned into the λgt11 expression vector (*10*). This epitope library was screened with a monoclonal antibody to ZP3 (*7*) and the nucleic acid sequence of the cDNA insert from eight positive clones was determined (Fig. 1A).

S. E. Millar, S. M. Chamow, A. W. Baur, J. Dean, Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.
C. Oliver, Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.
F. Robey, Laboratory of Cellular Development and Oncology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.

The 24 nucleotides common to the eight clones code for a seven–amino acid peptide that contains the epitope recognized by the antibody (Fig. 1B). The peptide represents amino acids 336 to 342, which are immediately adjacent to the most hydrophilic portion of ZP3 and partially overlap a region that contains a potential amphipathic α helix (Fig. 1C). The former motif has been associated with B cell epitopes (*11*) and the latter with T cell epitopes (*12*). A 16–amino acid peptide (NH_2-Cys-Ser-Asn-Ser-Ser-Ser-Ser-Gln-Phe-Gln-Ile-His-Gly-Pro-Arg-Gln-COOH) was synthesized and coupled via the NH_2-terminal cysteine to keyhole limpet hemocyanin (KLH) (*13*). The underlined amino acids contain the epitope recognized by the monoclonal antibody.

Sixteen female random-bred Swiss mice from the National Institutes of Health were immunized intraperitoneally with 100 μg of the mouse ZP3 peptide-KLH conjugate (1 mg/ml), prepared in an equal volume of complete Freund's adjuvant, and then boosted at 10- to 14-day intervals with 100 μg of conjugated peptide in incomplete Freund's adjuvant. Circulating antibodies to the zona pellucida were detected with solubilized whole zonae in an enzyme-linked immunosorbent assay (ELISA) (*14*) and a plateau level of the average response was reached after three immunizations (Fig. 2). Variation in the titers of circulating antibodies to the zona pellucida among the animals was noted, with an almost fivefold difference in the average titers for low (group 1, see below) and high (group 3, see below) responders. Control animals, immunized with KLH in Freund's adjuvant following an identical regimen, had only background levels of antibodies to the zona pellucida (Fig. 2).

The reactivity of sera from immunized animals with individual zona proteins was analyzed in immunoblots of purified zonae separated by SDS–polyacrylamide gel electrophoresis (Fig. 3A) (*15*). Sera from animals immunized with the ZP3 peptide-KLH conjugate reacted with a single zona protein that comigrated with ZP3. No reaction with any of the zona proteins was detected with preimmune or control antisera. Antibodies from experimental mice bound to the zonae surrounding developing oocytes in unfixed frozen sections of mouse ovary (*16*), indicating that the circulating antibodies to the zona can bind native ZP3 protein. There was no detectable fluorescence of sections stained with sera from control mice (Fig. 3B).

To determine if the circulating antibodies to ZP3 bound to the zonae surrounding growing oocytes of the experimental mice, plastic-embedded sections of ovaries were stained with horseradish peroxidase (HRP)–conjugated antibody to mouse immunoglobulin G (IgG) (*17*). Antibodies to zonae pellucidae were observed coating the zonae of the oocytes in sections isolated from four females immunized with ZP3 peptide–KLH conjugate (Fig. 4A). There were no detectable antibodies in ovaries isolated from four control (injected with KLH) mice (Fig. 4B). Ovarian sections of treated and control animals (both immunized with Freund's adjuvant) contained only normal follicles and cell types with no evidence of inflammation or cellular cytotoxicity. Antisera of ZP3-KLH immunized animals did not react with other mouse tissues, including brain, liver, spleen, kidney, heart, lung, intestine, testis, and muscle. These results indicate that immunization with the peptide conjugate elicits a response that is specific for the zona pellucida.

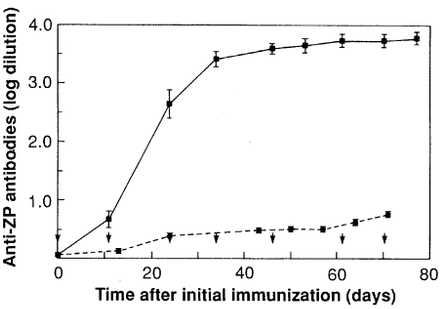


Fig. 2. Time course of production of antibodies to ZP3. Sixteen animals were immunized with 100 μg of ZP3 peptide–KLH conjugate (filled squares) or KLH alone (filled circles). Antibody titers for each animal were measured by ELISA (*14*), and the mean value for the $\log_{10}$ of the dilution for each time point was calculated (bars represent SE). The days on which boosts were administered are indicated by arrows.

The fertility of the remaining 12 experimental and 12 control mice was tested for 9 months by mating them continuously with males proven to be fertile. All of the control (injected with KLH alone) mice gave birth to litters within 3 1/2 weeks of the introduction of males. Three of the 12 experimental mice injected with ZP3 peptide conjugate also gave birth within this period (group 1). These mice were among those that had the lowest titers of antibodies to ZP3 before mating (Fig. 5, inset). In the nine remaining experimental mice, a contraceptive effect was observed that lasted from 16 to 36 weeks (Fig. 5). Three of these animals gave birth to litters after 16 to 24 weeks and initially had intermediate antibody titers (group 2). The last six animals (group 3), which remained infertile for the duration of the study, had the highest initial antibody titers (Fig. 5, inset).

At the time of birth of their first litters, the titer of antibodies to ZP3 in group 2 animals (those that reversed) had dropped to below that of group 1 animals, indicating that there is a correlation between antibody concentration and fertility. The litter sizes ranged from 1 to 5 pups (average, 2.8) and were somewhat smaller than those of the control mice (litters of 1 to 9 pups; average, 5.2). However, both groups had fewer than the normal 7 to 14 pups (average, 10). This

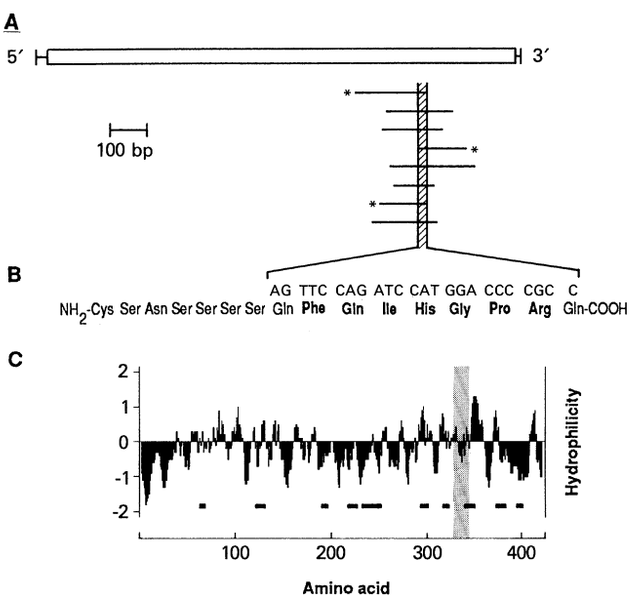


Fig. 1. Definition of a mouse ZP3 epitope. (**A**) Schematic representation of the 1317-nucleotide (nt) ZP3 mRNA. The single 1272-nt open reading frame is indicated by an open bar. The lines below the mRNA represent eight positive cDNA clones isolated from the ZP3 epitope library by the monoclonal antibody to ZP3 (*8*). The clones are aligned on the ZP3 cDNA, and the hatched bar indicates the sequence common to all positive clones. Three clones (*) define the 5′ and 3′ ends of the epitope. (**B**) The DNA sequence of the overlapping region among the eight positive clones and the corresponding amino acid sequence (bold) are shown. The one additional COOH-terminal and eight additional NH_2-terminal amino acids shown flanking the epitope were included in the peptide used for immunization. (**C**) Hydrophilicity of the deduced 424–amino acid ZP3 protein was plotted with a seven-residue moving average (*11*). Horizontal filled-in bars beneath the hydrophilicity plot indicate amphipathic α helical segments predicted by an 11-residue moving average (*12*). The speckled vertical bar represents the 16–amino acid peptide shown in (B) that was used to immunize experimental animals.

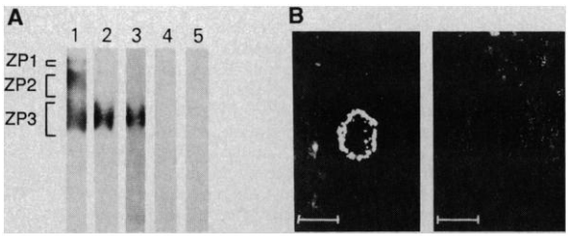


Fig. 3. Characterization of ZP3 peptide–KLH antiserum. (**A**) Immunoblot of purified mouse zonae pellucidae incubated with (lane 1) rabbit polyclonal antibody to mouse zona pellucida; (lane 2) monoclonal antibody to ZP3; (lane 3) serum from animals immunized with ZP3 peptide–KLH conjugate; (lane 4) serum from animals immunized with KLH; and (lane 5) preimmune serum. (**B**) (Left) Frozen mouse ovarian section after incubation with serum from animals immunized with ZP3 peptide–KLH conjugate. Horizontal bar, 50 μm. (Right) Same as left panel, but after incubation with serum from animals immunized with KLH alone. Bound antibody was detected with a fluorescein-conjugated goat IgG antibody to mouse IgG (*16*).

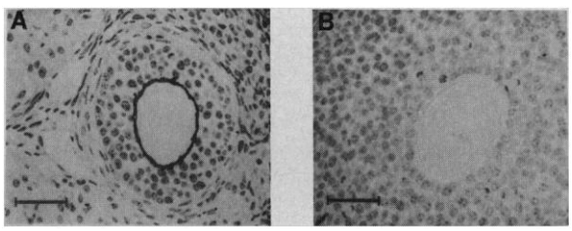


Fig. 4. Binding of circulating antibodies to ZP3 peptide to the ovaries of vaccinated mice. (**A**) Plastic-embedded ovarian section isolated from a ZP3 peptide–KLH immunized mouse and stained with biotinylated antibody to mouse IgG detected with streptavidin-HRP (*17*). The binding of antibody to ZP3 peptide to the zona of immunized mice is indicated by the dark ring of substrate deposition surrounding the sectioned oocytes. (**B**) Same as in (A), except that the ovary was isolated from a control, KLH-immunized mouse. Scale bars, 50 μm.

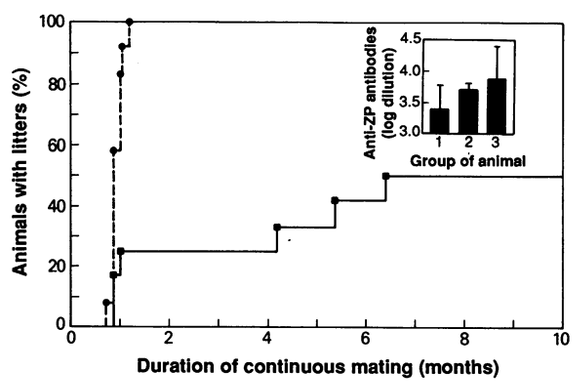


Fig. 5. Contraceptive effects of immunization with ZP3 peptide–KLH conjugate. Two weeks after the last immunization, males, proven to be fertile, were individually and continuously caged with experimental and control mice at a ratio of 1:1. The percentage of females that gave birth to a litter was plotted as a function of the duration of continous mating for animals injected with ZP3 peptide–KLH conjugate (filled squares) and KLH alone (filled circles). (**Inset**) The titers (log_{10} dilution) of antibodies to zona pellucida (anti-ZP) of three groups of ZP3 peptide–KLH immunized mice at the beginning of the mating period: group 1, gave birth within 1 month (three animals); group 2, gave birth between 4 and 7 months (three animals); and group 3, did not give birth to litters within the 9-month study (six animals).

decrease in litter size may be due, in part, to the adverse effects of intraperitoneal administration of Freund's adjuvant on fecundity. Those animals that remained infertile (group 3) also showed a decrease in antibody titer by the end of the study. However, their continued infertility in the latter stages of the study may have been caused by age, since control animals of the same age were no longer producing litters.

Our studies demonstrate that repeated immunization of female mice with a mouse ZP3 peptide–KLH conjugate can result in long-term infertility. The production of antibodies to the zona pellucida occurs despite the fact that the zona peptide is a self antigen. B cells capable of being stimulated by the ZP3 peptide used in our conjugate must have escaped from tolerance of self, perhaps because of low (or absent) levels of zonae pellucidae in the neonate (*4*) during the time tolerance to self is established (*18*).

The reversibility of the contraceptive effect may have been facilitated by the use of a zona peptide–KLH immunogen. The carrier KLH moiety must have contributed most (if not all) of the helper T cell epitopes (*19*), which stimulated the production of antibodies to ZP3. The endogenous ZP3 protein, although containing the same ZP3 peptide as the conjugate, does not contain the helper T cell epitopes of the carrier protein. Thus, the concentration of antibodies to ZP3 declines with time, and as additional oocytes enter into the growth phase they synthesize a zona pellucida in the absence of circulating antibodies to ZP3. When ovulated, these oocytes, uncoated with antibodies, can be fertilized.

Immunization with the ZP3 peptide vaccine did not result in ovarian histopathology, a finding that is consistent with studies in which heterologous rodent zonae preparations were used to immunize rodents (*1, 20*), but in contrast to the widespread ovarian histopathology and dysfunction in rabbits, dogs, and primates after active immunization with zonae pellucidae (*21*). Several studies have suggested that both the amount of zona immunogen and the purity of the preparation contributed to these abnormalities (*1, 22*). Not only does the ZP3-KLH conjugate lack other ovarian antigens, but, perhaps more importantly, by not providing ZP3 T cell epitopes it may avoid provoking a cytotoxic T cell response to the zona. In contrast to the design of vaccines against exogenous infectious agents, the dissociation of zona pellucida B and T cell epitopes may be important in the development of a contraceptive vaccine that elicits an effective, reversible antibody response and avoids ovarian cytotoxicity.

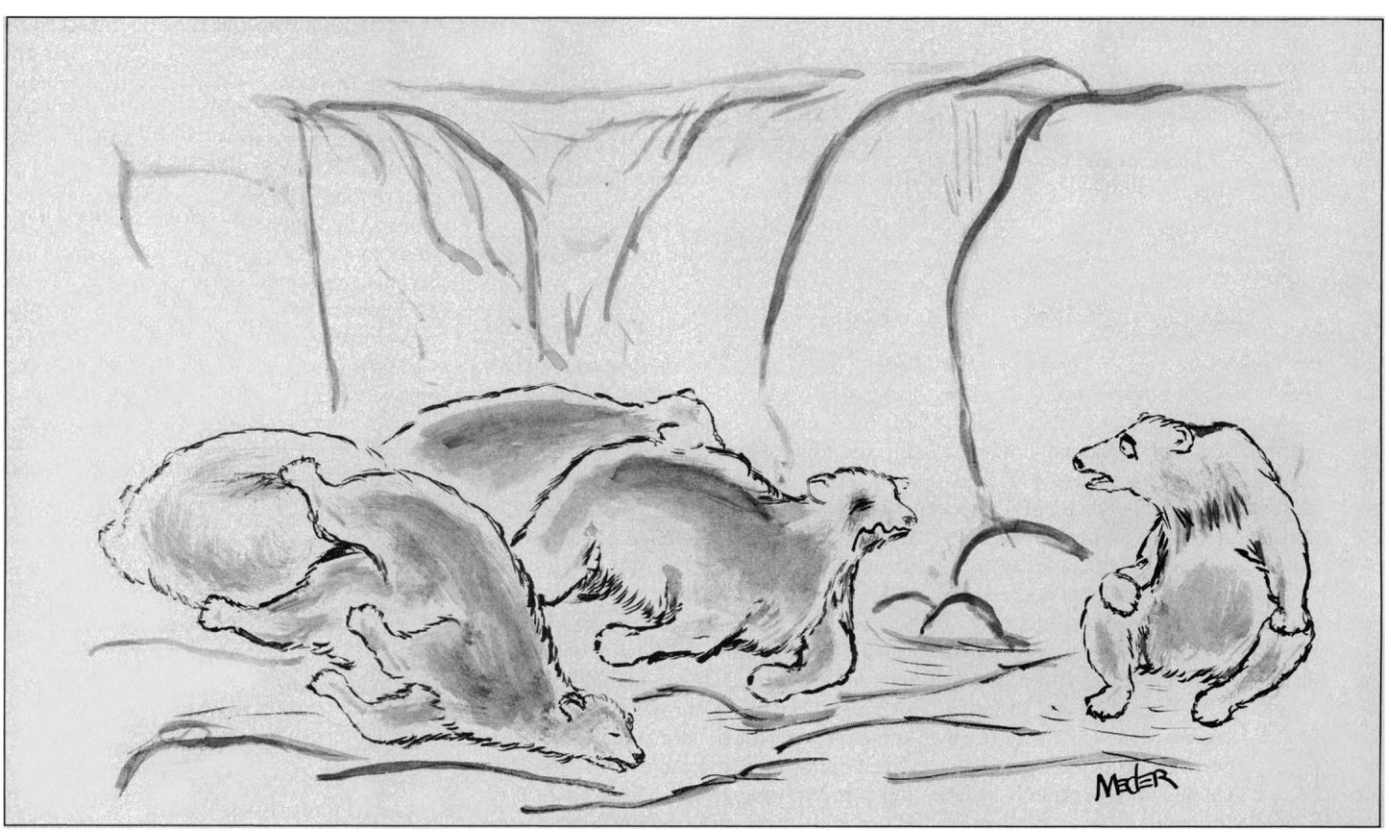
The ZP3 epitope used in this investigation is not detected immunologically in hamster, guinea pig, cat, or dog ovaries (*7*). Thus, the mouse ZP3 peptide would not be expected to act as a contraceptive in other mammalian species. However, our strategy of vaccination with self zona peptides can be applied to other mammals by taking advantage of the conserved nature of the zona genes (*8, 9*). For instance, the recent cloning and sequencing in our laboratory of the human ZP3 gene has shown that it has greater than 70% sequence identity with the mouse ZP3 gene and that the deduced amino acid sequence is also well conserved between mouse and human (*23*). Once the ZP3 gene of a species is cloned, the homologous zona peptide can be deduced from the nucleic acid sequence and tested as a contraceptive vaccine in the particular species.

REFERENCES AND NOTES

1. C. J. Henderson, M. J. Hulme, R. J. Aitken, *J. Reprod. Fertil.* **83**, 325 (1988).
2. B. S. Dunbar, in *Mechanisms and Control of Animal Fertilization*, J. F. Hartmann, Ed. (Academic Press, New York, 1983), pp. 140–175; A. T. Sacco, *Am. J. Reprod. Immunol. Microbiol.* **15**, 122 (1987).
3. J. D. Bleil and P. M. Wassarman, *Dev. Biol.* **76**, 185 (1980); S. Shimizu, M. Tsuji, J. Dean, *J. Biol. Chem.* **258**, 5858 (1983).
4. R. Yanagimachi, *Physiology of Reproduction*, E. Knobil and J. Neil, Eds. (Raven, New York, 1988), vol. 1, pp. 135–185; P. M. Wassarman, *Annu. Rev. Biochem.* **57**, 415 (1988); J. D. Bleil and P. M. Wassarman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6778 (1988); C. C. Moller and P. M. Wassarman, *Dev. Biol.* **132**, 103 (1989).
5. Y. Tsunoda and T. Sugie, *Jap. J. Zootech. Sci.* **48**, 784 (1977); A. G. Sacco, *J. Reprod. Fertil.* **56**,

533 (1979); R. J. Aitken *et al.*, *ibid.* **62**, 597 (1981); Y. Tsunoda, T. Sugie, J. Mori, S. Isojima, K. Koyama, *J. Exp. Zool.* **217**, 103 (1981); Y. Tsunoda and D. G. Whittingham, *J. Reprod. Fertil.* **66**, 585 (1982).
6. I. J. East and J. Dean, *J. Cell Biol.* **98**, 795 (1984); I. J. East, D. R. Mattison, J. Dean, *Dev. Biol.* **104**, 49 (1984); I. J. East, A. M. Keenan, S. M. Larson, J. Dean, *Science* **225**, 938 (1984).
7. I. J. East, B. J. Gulyas, J. Dean, *Dev. Biol.* **109**, 268 (1985).
8. M. J. Ringuette, D. A. Sobieski, S. M. Chamow, J. Dean, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4341 (1986).
9. C. C. Philpott, M. J. Ringuette, J. Dean, *Dev. Biol.* **121**, 568 (1987); M. J. Ringuette, M. E. Chamberlin, A. W. Baur, D. A. Sobieski, J. Dean, *ibid.* **127**, 287 (1988); R. A. Kinloch, R. J. Roller, C. M. Fimiani, D. A. Wassarman, P. M. Wassarman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6409 (1988); M. E. Chamberlin and J. Dean, *Dev. Biol.* **131**, 207 (1989).
10. The cDNA insert of pZP3.1 (*8*) was digested with deoxyribonuclease in the presence of 1.5 m*M* $MnCl_2$ and 200- to 1000-bp size-selected fragments [V. Mehra, D. Sweetser, R. A. Young, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7013 (1986)] were ligated into Lambda ZAP (Stratagene). *Escherichia coli* BB4 cells were infected with the unamplified epitope library and screened (*8*) with a monoclonal antibody to ZP3 (7). Positive clones were plaque-purified, and the sequence of the insert DNA was determined from isolated plasmid DNA [F. Sanger, S. Nicklen, A. R. Coulson, *ibid.* **74**, 5463 (1977)].
11. T. P. Hopp and K. R. Woods, *ibid.* **78**, 3824 (1981); J. A. Berzofsky, *Science* **229**, 932 (1985); E. D. Getzoff, J. A. Tainer, R. A. Lerner, H. M. Geysen, *Adv. Immunol.* **43**, 1 (1988).
12. H. Margalit *et al.*, *J. Immunol.* **138**, 2213 (1987); J. B. Rothbard and W. R. Taylor, *EMBO J.* **7**, 93 (1988).
13. The 16–amino acid peptide was synthesized [R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963)] on a Model 430A Applied Biosystems Solid Phase Synthesizer, deprotected, and released from the phenylacetamidomethyl resin with anhydrous hydrogen fluoride containing 10% anisole and 10% thiophenol at 0°C for 2 hours. The crude peptide was purified by high-performance liquid chromatography on a Vydac C4 column and conjugated to KLH, coupling the NH_2-terminal Cys to KLH through a maleimido linkage [R. A. Lerner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3403 (1981)].
14. ELISA plates coated with acid-solubilized zonae (100 ng/well) and blocked with 1% bovine serum albumin (BSA) in tris-HCl, *p*H 7.5, and 0.15*M* NaCl [tris-buffered saline (TBS)] were incubated with sera diluted in TBS with 1% BSA and 0.1% Tween-20. Plates were washed (TBS with 0.1% Tween-20), incubated with HRP-conjugated goat antibody to mouse IgG, and developed with an HRP Kit (Bio-Rad). Absorbance was measured at 414 nm. Sera were diluted to fall within the linear range of the assay and the $\log_{10}$ dilution that gave an A_{414} of 0.5 was calculated for each bleed.
15. Individual lanes of immunoblots [W. N. Burnette, *Anal. Biochem.* **112**, 195 (1981)] were probed with sera diluted in TBS with 3% BSA. Filters were washed (TBS with 0.1% Tween), incubated with HRP-labeled second antibody of the appropriate specificity (Jackson ImmunoResearch) diluted 1:1000 in TBS with 3% BSA, and developed with 4-chloro-1-naphthol.
16. Frozen ovarian sections (5 μm) mounted on gelatin-coated slides were incubated for 1 hour with sera (undiluted) and stained for 30 min with fluorescein isothiocyanate–conjugated goat antiserum to mouse IgG (Jackson ImunoResearch Laboratories). Sections were mounted in Fluormount-S (FisherBiotech) and photographed with Ektachrome 200 film.
17. Dissected ovaries were fixed for 1 hour in 1% glutaraldehyde, rinsed in PBS, and embedded in JB4 plastic. Engodenous antibody was detected in 4-μm sections with a streptavidin-HRP kit against mouse IgG (Zymed).
18. A. M. Pullen, J. W. Kappler, P. Marrack, *Immunol. Rev.* **107**, 125 (1989); G. J. Nossal, *J. Immunol.* **141** (suppl. 7) S21 (1988); C. G. Goodnow *et al.*, *Nature* **334**, 676 (1988).
19. J.-G. Guillet *et al.*, *Science* **235**, 865 (1987); M. F. Good *et al.*, *ibid.*, p. 1059; D. R. Milich, A. McLachlan, G. B. Thornton, J. L. Huges, *Nature* **329**, 547 (1987); M. J. Francis *et al.*, *ibid.* **330**, 168 (1987).
20. R. B. L. Gwatkin, D. T. Williams, D. J. Carlo, *Fertil. Steril.* **28**, 871 (1977).
21. R. B. L. Gwatkin, O. F. Andersen, D. T. Williams, *Gamete Res.* **1**, 19 (1980); D. M. Wood, C. Liu, B. S. Dunbar, *Biol. Reprod.* **25**, 439 (1981); C. A. Mahi-Brown, T. T. F. Huang, Jr., R. Yanagimachi, *J. Exp. Zool.* **222**, 89 (1982); B. J. Gulyas, R. B. L. Gwatkin, L. C. Yuan, *Gamete Res.* **7**, 299 (1983); A. G. Sacco, M. G. Subramanian, E. C. Yurewicz, R. J. DeMayo, W. R. Dukelow, *Fertil. Steril.* **39**, 350 (1983); S. M. Skinner, T. Mills, H. J. Kirchick, B. S. Dunbar, *Endocrinology* **115**, 2418 (1984); A. K. Bamezai, A. Suman, C. Das, G. P. Talwar, *J. Rep. Immunol.* **9** suppl., 85 (1986); A. G. Sacco, D. L. Pierce, M. G. Subramanian, E. C. Yurewicz, W. R. Dukelow, *Biol. Reprod.* **36**, 481 (1987).
22. S. M. Skinner and B. S. Dunbar, *Biol. Reprod.* **30** (suppl. 1), 73 (abstr. 85) (1984); C. A. Mahi-Brown, R. Yanagimachi, J. C. Hoffman, T. T. F. Huang, Jr., *ibid.* **32**, 761 (1985).
23. M. E. Chamberlin, thesis, The Johns Hopkins University (1989).
24. We thank J. A. Berzofsky and R. T. Simpson for critical reading of the manuscript and P. Bowie for technical assistance.

26 June 1989; accepted 5 October 1989



"I can't sleep."