BIOLOGY OF REPRODUCTION **62,** 318–324 (2000)

Identification of Human Sperm Peptide Sequence Involved in Egg Binding for Immunocontraception[1]

Rajesh K. Naz,[2,3] Xiaolong Zhu,[3] and Arjun L. Kadam[4]

Division of Research,[3] Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo, Ohio 43614
New York Fertility Institute,[4] New York, New York 10028

ABSTRACT

Development of a vaccine based on sperm antigens represents a promising approach to contraception. The sperm-zona pellucida (ZP) interaction constitutes the most important event in the fertilization process, and the molecular sequences involved at this site may provide the most attractive candidates for immunocontraception. In the present study, using the phase peptide display technique, a novel dodecamer sequence, designated as YLP_{12}, was identified that is involved in sperm-ZP recognition/binding. The synthetic 12-mer peptide based on this sequence and its monovalent Fab′ antibodies specifically and significantly ($P < 0.05$) inhibited human sperm-ZP binding. In Western blot and immunoprecipitation procedures, the YLP_{12} peptide recognized the ZP3 component of solubilized human ZP proteins. In the Western blot procedure involving 10 different human tissue extracts, the anti-YLP_{12} Fab′ antibodies recognized a protein band of ~72 ± 2 kDa only in the testis lane. The peptide sequence was localized on the acrosomal region of the human sperm cell. These findings indicate that the novel testis-specific 12-mer YLP_{12} that is present in the acrosomal region and is involved in human sperm-ZP interaction may find applications in contraceptive vaccine development, as well as in diagnosis and treatment of male infertility mediated through sperm dysfunction.

INTRODUCTION

Development of a vaccine based on sperm antigens represents a promising approach to contraception [1,2]. The rationale and feasibility of this approach are provided by experimental immunization studies as well as data from involuntary infertility and vasectomy. Deliberate immunization of male and female animals of various species [3–5] and humans [6,7] with extracts of spermatozoa or mature testes raises an antisperm immune response resulting in infertility. Up to 30% of infertility may be associated with the presence of antisperm antibodies (ASA) in the male and/or female partner of an infertile couple [8], and 70% of vasectomized men form ASA [9,10]. These ASA are not merely associated with but rather are causative factors of infertility [8,11,12]. Thus, the sperm cell has both auto- and iso-antigenic potentials and can generate an immune response in both men and women that is capable of inducing infertility.

[1]Supported in part by NIH grant HD24425 to R.K.N. No funds from this grant were used for human IVF and for studies involving human oocyte zona pellucida.
[2]Correspondence: Rajesh K. Naz, Division of Research, Health Education Building Rm. 211, Medical College of Ohio, 3055 Arlington Avenue, Toledo, OH 43614-5806. FAX: 419 383 4473; e-mail: rnaz@mco.edu

Received: 21 July 1999.
First decision: 2 September 1999.
Accepted: 15 September 1999.

ISSN: 0006-3363. http://www.biolreprod.org

The utility of a sperm antigen in the development of a contraceptive vaccine is contingent on its involvement in the fertilization process and on tissue specificity. During the past decade, several antigens relevant to fertility have been defined (reviewed in [1]). A few of these also affect fertility in actively immunized animals [1,13]. In these studies, purified/semipurified cognate antigens were used for immunization to examine the effect on fertility. However, to obtain Food and Drug Administration approval and to conduct appropriate multicenter fertility trials in a quality-controlled manner, recombinant or synthetic peptide molecules are required. Complementary DNAs encoding for a few sperm antigens have been cloned and sequenced, and the recombinant proteins or their peptides expressed by some of the cloned cDNAs are being examined for their effect on fertility [14–16].

The fertilization process includes a cascade of events that the spermatozoon must undergo before fusing with the oocyte plasma membrane. One of the key steps in the fertilization cascade is the recognition and binding between the complementary molecules present on the spermatozoon and zona pellucida (ZP) of the oocyte [17]. The sperm-ZP interaction constitutes an important event in the fertilization process, and the molecular sequences involved are the most attractive candidates for the development of a contraceptive vaccine.

On the basis of the above findings, the present study was conducted to investigate the sperm peptide sequence(s) involved in recognition and binding to the complementary molecule of the ZP in humans. This was achieved by screening the FliTrx random phage display library (Invitrogen, San Diego, CA) that has expression of 1.77×10^8 different peptide sequences. A solubilized preparation of human oocyte ZP proteins was used as a probe to obtain reactive clones. The long-term objective of this study was to search for sperm peptide sequence(s) that can find potential applications in the development of a contraceptive vaccine and in the diagnosis and treatment of infertility in humans.

MATERIALS AND METHODS

Library Screening by Panning

The FliTrx random phage display library used in the present study was obtained from Invitrogen. The library was screened with solubilized human ZP proteins (HZP) according to the manufacturer's protocol. After the fifth round of panning, the eluted cells were streaked on LB plates containing 100 μg/ml ampicillin to select colonies. Potential positive colonies were further confirmed for reactivity with HZP using the Western blot procedure. The colonies were grown and cells were suspended in 100 μl SDS-PAGE buffer (nonreduced), boiled for 10 min, and centrifuged; the supernatant was run in SDS-PAGE (5–15% gradient gel) [18], and the resolved proteins were trans-

ferred to nitrocellulose paper for Western blot analysis [19]. The blot was reacted with HZP (20 μg/20 ml) and then with rabbit antibodies to HZP (described below; 10 μg/20 ml); the reacted proteins were localized by incubating the antibody-reacted strips first with alkaline phosphatase-conjugated goat anti-rabbit antibodies (heavy- and light-chain specific; Cooper Biomedical Inc., Malvern, PA) and then with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate, as described elsewhere [16,20].

Human Oocyte ZP Proteins and Their Antibodies

The human oocytes were obtained from female partners (21–35 yr old) of infertile couples who attended the Infertility Service for in vitro fertilization-embryo transfer [21,22]. These infertile couples had infertility attributed to the male partners, and the female partners were without any ovarian abnormality. Appropriate informed consent and Institutional Review Board approval were obtained for study participation and collection of ova. There were an excess number of left-over mature eggs, cryopreserved in 3.5 M propanediol containing 0.27 sucrose and 5% BSA in PBS until used. These eggs were never exposed to sperm and could have otherwise been discarded. They were rapidly thawed, washed in Ham's F-10 medium, and freed of adhering granulosa cells by incubation with 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) in PBS. The eggs were washed (3 times) in PBS to remove adhering cumulus cells, and the zonae were heat solubilized in sodium carbonate buffer (0.001 M, pH 9.0) at 60°C for 1 h [21,22]. The solubilized HZP were dialyzed against PBS for 48 h at 4°C before use.

Rabbit antiserum was prepared against HZP as described elsewhere [23]. Briefly, sexually mature virgin female rabbits (n = 2) were actively immunized systemically with HZP emulsified with Freund's adjuvant; and after three booster injections, the animals developed high titer antibodies (> 1:5012) against HZP, as examined using ELISA. Preimmune serum and the sera from control animals injected similarly with only PBS, emulsified with Freund's adjuvant, were used as positive controls in various assays.

DNA Isolation and Sequencing

DNA was isolated from the uninduced cells/clones that showed positive reaction with HZP in the Western blot procedure after five cycles of panning. The plasmid DNA was isolated using the simple nucleic acid preparation (S.N.A.P.) miniprep kit (cat. #K1900-01; Invitrogen) and sequenced by dsDNA Cycle Sequencing System (Gibco-BRL, Gaithersburg, MD) [16,20]. Sequencing primers were FliTrx forward and reverse sequencing primers. The 36 base pairs corresponding to the dodecamer peptide sequence were inserted at *Rsr*II/*Ava*II (5′ end) and *Ava*II (3′-end) of the vector DNA and could be released using *Ava*II. The search for nucleotide (nt) and amino acid (aa) sequence homology in GenBank, National Biomedical Research Foundation (NBRF), and Swiss sequence banks was performed using Fasta and tFasta search programs [24]. The consensus aa sequence was derived from the various positive peptide sequences by using the Lineup program (Genetics Computer Group, Madison, WI). The peptides were synthesized by solid-phase synthesis using Fmoc chemistry (Biosynthesis Inc., Lewisville, TX). Deprotection was achieved by 20% piperidine in dimethylformamide, and the peptides were cleaved from the resin by 85% trifluoroacetic acid (TFA). The peptides were then precipitated in methyl tert-butyl ether and purified by using reverse-phase HPLC. The fractions eluted with 0.5% TFA in acetonitrile were dried in a speed vacuum, redissolved in water, and lyophilized. All four peptides were water soluble and had > 95% purity level.

Antibodies to Peptide

The peptides were conjugated to tetanus toxoid (TT; Wyeth Laboratories, Radnor, PA) by the method described by Tsong et al. [25], using 1-ethyl-3 (3-dimethylaminopropyl). Antiserum was raised in sexually mature virgin female rabbits of the New Zealand white strain [23], and the antibodies were purified by using a protein A-Sepharose 4B column. The monovalent Fab′s were prepared using pepsin by the method of Nisonoff and colleagues [26] or described elsewhere [27]. The Fab′s were further immunoaffinity purified by using peptide-BSA-Sepharose 4B immunobeads prepared as described below.

Hemizona Assay

The hemizona assay was performed to examine the effects of peptides and their Fab′ antibodies on human sperm-ZP binding [28]. One half of the hemizona pair was experimental (treated with the peptide or Fab′s), and the other half of the same oocyte was used as a control (untreated). Two sets of experiments were performed. In the first set, peptide/Fab′s were incubated (37°C, 1 h) with sperm (100-μl drop), washed, and then incubated (4 h) with the untreated experimental hemizona. Control sperm were incubated with the medium only. In the second set, peptide/Fab′s were incubated (37°C, 1 h) with the experimental hemizona, washed, and then incubated (4 h) with the untreated sperm in a 100-μl drop. Control hemizonae were incubated with the medium only. The number of sperm tightly bound to the outer surface of the hemizona was determined and expressed as the hemizona index: HZI = [Number of sperm bound on experimental hemizona/Number of sperm bound on control hemizona] × 100.

Each sample was tested with 7–12 hemizonae obtained from 3–8 different women in three different experiments performed on different days using sperm from at least three different fertile men. For the immunoadsorption experiments, the Fab′s were incubated (4°C, overnight) with the peptide (1:10, w:w) and centrifuged (10 000 rpm, 5 min), and the supernatant was tested in the hemizona assay.

Interaction Between the Peptide and Oocyte ZP

The interaction between synthetic peptide and oocyte ZP proteins was examined by Western blot and immunoprecipitation. For Western blot analysis, the HZP was run in two identical SDS gels (5–15% gradient) and transferred to nitrocellulose membranes. BSA was biotinylated and separated from unbiotinylated protein according to the manufacturer's protocol (ECL protein biotinylation module; Amersham, Piscataway, NJ) [20]. The peptide was conjugated to biotinylated BSA by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as described above. The Western blots were incubated with blocking solution for 2 h at room temperature; then one blot was treated with the peptide-biotinylated BSA conjugate in PBS (20 μg/20 ml) and the second identical blot was treated with biotinylated BSA not conjugated to peptide (20 μg/20 ml). The blots were washed, reacted with streptavidin-horseradish peroxidase (1:1500), washed, incubated with substrate, and exposed to

TABLE 1. Sequences of peptides showing binding to human zona pellucida.

Clone	Sequence
1. SNR_{12}	SNRVKLGGDDGK
2. GHR_{12}	GHRARRVEISAS
3. YLP_{12}	YLPVGGLRRIGG
4. $Consensus_{17}$*	GHRGRRVGLGGGGRIGG

* $Consensus_{17}$ was derived from sequences of the above three strongly binding peptides and another six that showed weak binding with zona pellucida.

x-ray film for 1–5 min. BSA was used as a control in these experiments.

For analysis using the immunoprecipitation procedure, the peptide was conjugated to BSA as described above, and the peptide-BSA conjugate or the BSA alone was coupled to cyanogen bromide-activated Sepharose 4B (Sigma) [21,29]. HZP was divided into two aliquots of 20 μg protein/50 μl each. One aliquot was incubated with peptide-BSA-Sepharose 4B beads, and the second was incubated with the BSA-Sepharose 4B beads suspended in radioimmunoprecipitation assay buffer (50 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM PMSF [30]. The reaction mixture was incubated for 2 h at 4°C; unadsorbed proteins were washed off, and the reacted zona protein was eluted from the beads by treating with glycine-HCl (0.1 M, pH 2.8). The eluate was neutralized with solid K_2HPO_4 to pH 7.4, boiled with nonreduced SDS-sample buffer, and subjected to SDS-PAGE; the gel containing the resolved proteins was stained with silver nitrate and visualized.

Tissue-Specific Expression

To examine tissue-specific expression, the solubilized extracts from 10 different human tissues—namely, testis, kidney, liver, spleen, heart, lung, brain, muscle, ovary, and placenta (Human Protein Medley; Clontech, Palo Alto, CA)—were run in SDS-PAGE and transferred to nitrocellulose membrane for Western blot analysis. The membranes were probed with anti-peptide Fab′s/control Fab′s (15 μg/10 ml), and the reacted bands were localized as described above [16,20].

Indirect Immunofluorescence Technique

Indirect immunofluorescence technique was performed on methanol-fixed sperm cells to examine the subcellular site at which the peptide sequence was present [31]. Motile sperm cells were collected from semen of fertile men by the swim-up procedure, washed (3 times) with PBS, air dried at room temperature, fixed in methanol, and air dried again. The slides were then rinsed in PBS and blocked with PBS containing 5% BSA for 45 min; next they were reacted with anti-peptide Fab′s in PBS (10 μg/100 μl) for 1.5 h at room temperature in a moist chamber. After washing in PBS, fluorescein isothiocyanate-labeled goat anti-rabbit IgG (1:40 dilution; Cappel Labs., Malvern, PA) was added and incubated as above for 1.5 h. The slides were washed, mounted in 90% glycerol in PBS containing sodium azide (0.1%) and 1,4-diazabicyclo(2,2,2)octane (10 mg/ml), and examined.

Capacitation was induced by incubating (6–8 h, 37°C in 5% CO_2 and 95% air mixture) the swim-up sperm (10×10^6 sperm/ml) in Ham's F-10 medium containing 5% BSA. The capacitated sperm were washed, methanol fixed, and studied for antibody reactivity as described above.

TABLE 2. Effect of synthetic peptides on human sperm binding in the hemizona assay.

Sample	Concentration (mM)	Number of sperm tightly bound to[a]: treated hemizona	untreated hemizona	HZI
BSA control	75	34 ± 4	36 ± 5	94
Peptide SNR_{12}	25	35 ± 3	40 ± 6	88
	75	33 ± 4	34 ± 3	97
Peptide GHR_{12}	25	32 ± 8	31 ± 7	103
	75	34 ± 7	35 ± 8	97
Peptide YLP_{12}	25	16 ± 4[b]	35 ± 3	46[b]
	75	7 ± 2[b]	33 ± 4	21[b]
Peptide $consensus_{17}$	25	28 ± 8	34 ± 5	82
	75	21 ± 3[c]	32 ± 6	67[c]

[a] Values are mean ± SEM.
[b] Versus respective control, $P < 0.003$.
[c] Versus respective control, $P = 0.019$; all others were nonsignificant ($P > 0.05$).

TABLE 3. Effect of Fab′ fragments on human sperm binding in the hemizona assay.

Sample	Concentration (μg/100μl)	Number of sperm tightly bound to hemizona incubated with: treated sperm[a]	untreated sperm[a]	HZI
Control Fab′[b]	10	36 ± 4	42 ± 6	86
Preimmune Fab′	10	38 ± 7	39 ± 5	97
Antipeptide YLP_{12} Fab′				
Unadsorbed	10	7 ± 2[c]	41 ± 7	17[c]
Immunoadsorbed[d]	10	32 ± 6	38 ± 4	84

[a] Values are mean ± SEM.
[b] From animals injected with tetanus toxoid.
[c] Versus respective control, $P = 0.008$; others were nonsignificant ($P > 0.05$).
[d] Immunoadsorbed with the peptide.

Statistical Analysis

Significance of differences between treated and control groups in Tables 2 and 3 was analyzed by using unpaired and paired Student's *t*-test. A P value of < 0.05 was considered significant.

RESULTS

After biopanning ~1.2×10^6 phage clones five times, 21 clones were selected that showed varied degrees of affinity for HZP. Clones were cultured in RM medium (Invitrogen) to obtain a larger quantity of protein for analysis in the Western blot procedure. Of these clones, 18 showed positive reactivity with HZP in the Western blot procedure, as seen by the presence of the expected ~53-kDa protein band. With loading of the same amount of total protein from each clone, 6 of the 18 clones showed a weaker band, and the remaining 12 showed a band of 3- to 5-fold stronger intensity. DNA from all 18 clones was isolated, purified, and sequenced using the FliTrx forward and reverse primers to obtain the sequence coding for the dodecamer. The clones were sequenced several times to confirm the sequence obtained. Twelve strongly reacting clones exhibited three aa sequences designated as SNR_{12}, GHR_{12}, and YLP_{12} (Table 1). SNR_{12} had a frequency of 16.7% (2 of 12), GHR_{12} of 33.4% (4 of 12), and YLP_{12} of 50% (6 of 12) among these clones. The six weakly reacting clones exhibited six different sequences. Consensus sequence of 17 aa,

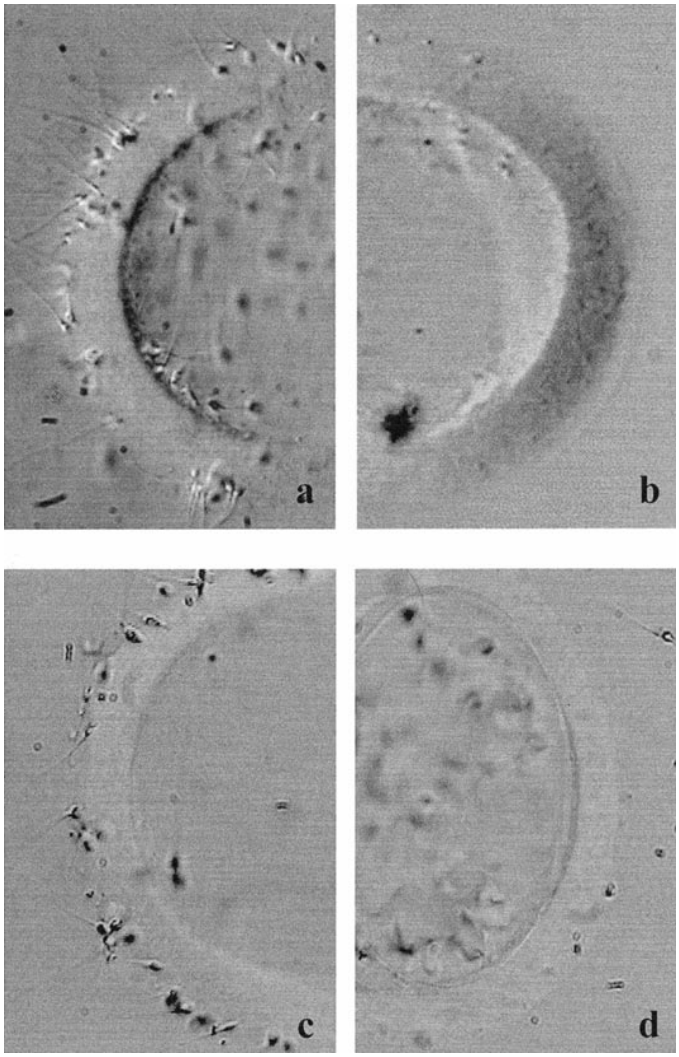

FIG. 1. Effect of YLP_{12} peptide and its Fab′ antibodies on sperm-zona binding in the hemizona assay. Treatment of hemizona with YLP_{12} peptide prior to coincubation with sperm caused a significant inhibition of sperm binding to ZP (**b**). The control hemizona from the same oocyte, not treated with the peptide, demonstrated binding to sperm (**a**). Treatment of sperm with anti-YLP_{12} Fab′ antibodies also significantly inhibited sperm binding to ZP (**d**), while the control hemizona from the same oocyte, incubated with sperm not treated with the antibodies, demonstrated binding to sperm (**c**). **a–d,** ×820 (published at 62%).

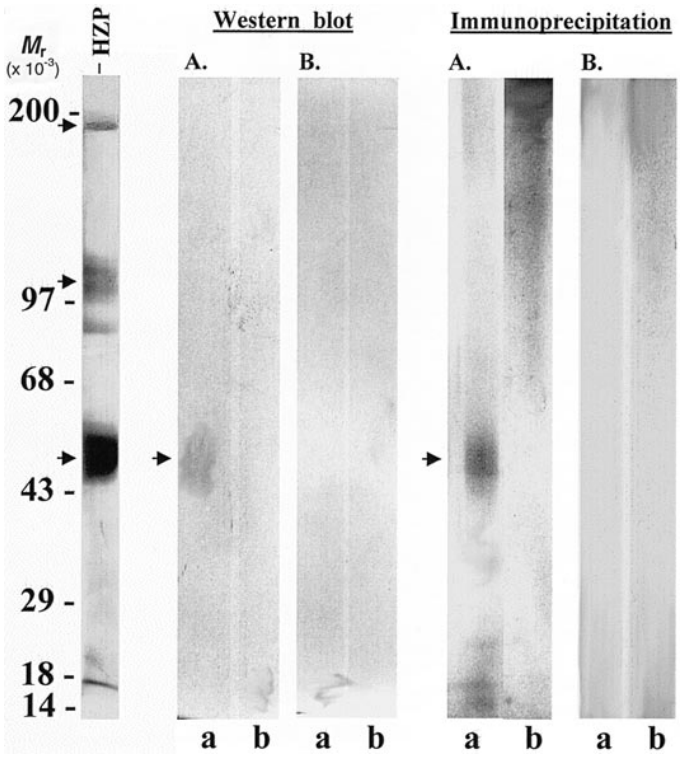

FIG. 2. Interaction between the YLP_{12} peptide and ZP3 of human oocyte ZP. The solubilized ZP preparation from human oocytes (HZP) was separated in SDS-PAGE and then transferred to nitrocellulose membrane for the Western blot procedure. HZP predominantly showed three major protein bands with median molecular masses of 50, 110, and 195 kDa and two minor bands of 95 kDa and 17 kDa (far left lane). BSA was biotinylated, separated from the unbiotinylated protein according to the manufacturer's protocol, and divided into two aliquots. One aliquot was conjugated to YLP_{12} peptide and the other was used per se. **Western blot**) Composed of HZP (a lanes) or BSA (b lanes), and incubated with YLP_{12} peptide-biotinylated BSA conjugate (A) or with biotinylated BSA (B), washed and allowed to react with streptavidin-horseradish peroxidase (1:1500), washed again, incubated with substrate, and exposed to x-ray film for 1–5 min. The YLP_{12} peptide-biotinylated BSA conjugate specifically reacted with the ZP3 component of ~50 kDa of HZP and not with BSA (Aa and Ab, respectively). Biotinylated BSA not conjugated to peptide did not react with any component of HZP or BSA (Ba and Bb, respectively). **Immunoprecipitation**) YLP_{12} peptide conjugated to BSA and coupled to Sepharose 4B beads (YLP_{12} beads) specifically precipitated a ZP3 component of ~50 kDa from HZP and no protein from the BSA solution (Aa and Ab, respectively). BSA alone coupled to Sepharose 4B beads (BSA beads) did not precipitate any protein from HZP or BSA solution (Ba and Bb, respectively).

designated as $Consensus_{17}$, was obtained using the Lineup program including all nine (three strongly and six weakly reacting) sequences. An extensive computer search in GenBank, NBRF, and the Swiss sequence banks did not identify any known nt/aa sequence having a complete homology with these four sequences.

The peptides were synthesized based on these four sequences, and the purified peptides were examined for their effects on binding of human sperm with human ZP in the hemizona assay. Of these peptides, two, namely YLP_{12} and $Consensus_{17}$, caused a significant ($P < 0.05$) inhibition of sperm binding with ZP (Table 2, Fig. 1b). The effects were concentration dependent, with a stronger inhibition at higher doses. The effect was much stronger with the YLP_{12} as compared to $Consensus_{17}$ peptide at 75 mM concentration. The inhibition was apparent only when the hemizonae were treated with the peptides before coincubation with sperm, not vice versa. The other two peptides did not affect binding, and the results were similar to those with BSA control (Table 2). Further studies focused on the YLP_{12} peptide. Immunization with YLP_{12} peptide conjugated to TT raised high-titer (> 1:5120, ELISA titer) antibodies against the peptide. The immunoaffinity-purified Fab′s against the YLP_{12} peptide significantly inhibited sperm-zona binding (Table 3, Fig. 1d). Control Fab′s from animals immunized with TT alone and with preimmune serum did not affect sperm binding. Immunoadsorption of the anti-YLP_{12} Fab′s with purified peptide completely abolished inhibitory activity. The inhibition was apparent only when sperm were treated with the anti-YLP_{12} Fab′s before coincubation with hemizona, not vice versa.

To examine the component of the human ZP that is recognized by the YLP_{12} peptide, Western blot and immunoprecipitation procedures were performed. In Western blotting, the peptide YLP_{12} conjugated to biotinylated BSA specifically reacted with the ZP3 component of human ZP and not with BSA (Fig. 2, Western blot, lanes Aa and Ab,

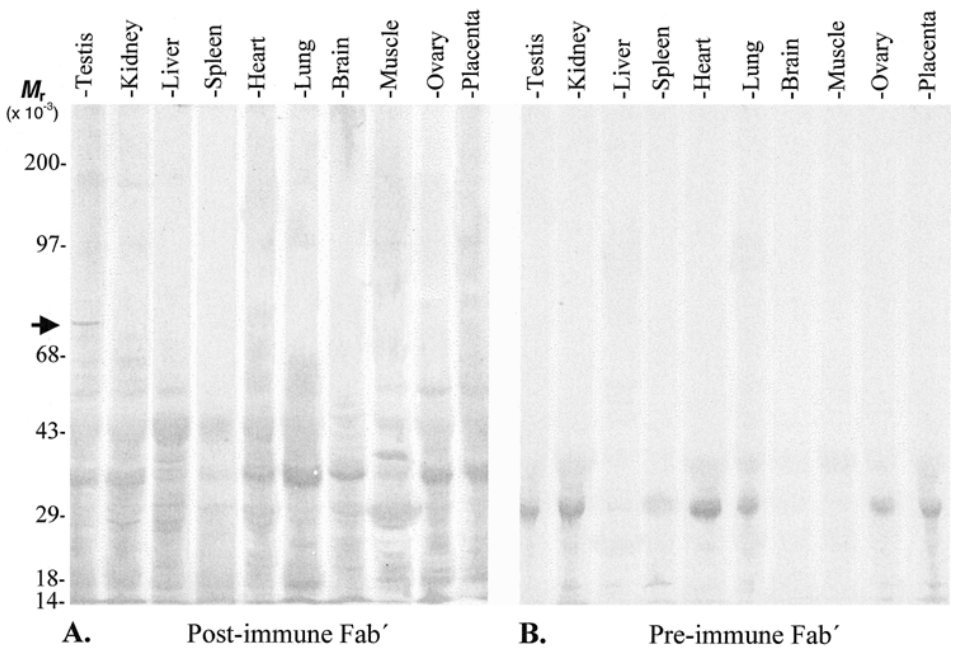

FIG. 3. Western blot indicating the testis-specific expression of YLP_{12} peptide sequence. Solubilized extracts from 10 different human tissues were run in SDS-PAGE and transferred to nitrocellulose membrane for Western blot analysis. Anti-YLP_{12} Fab′s (**A**) from postimmune serum, and not the Fab′s from control or preimmune serum (**B**), recognized a specific band of ~72 ± 2 kDa (arrow) only in the testis. There were some nonspecific protein bands reactive in each tissue extract that were also recognized by the control Fab′s. The Western blot procedure was repeated seven times on different days using various concentrations (100–300 μg protein per lane) of tissue extracts, and each time an identical pattern was obtained. Silver-stained SDS-PAGE of these tissue extracts showed multiple sharp and well-resolved protein bands without any smears.

respectively). The biotinylated BSA not conjugated to peptide did not react with HZP or BSA (Fig. 2, Western blot, lanes Ba and Bb, respectively). In the immunoprecipitation procedure, similar results were obtained. The peptide conjugated to BSA coupled to Sepharose 4B beads pulled out the ZP3 component from HZP and nothing from the BSA solution (Fig. 2, Immunoprecipitation, Aa and Ab, respectively). The BSA-Sepharose 4B beads not conjugated to peptide did not react with HZP or any protein in the HZP preparation or BSA solution (Fig. 2, Immunoprecipitation, Ba and Bb, respectively).

Anti-YLP_{12} Fab′s specifically recognized a protein band of ~72 ± 2 kDa only in the testis lane in the Western blot procedure involving solubilized extracts of 10 different human tissues (Fig. 3).

Anti-YLP_{12} Fab′s reacted with the acrosomal region of the methanol-fixed human sperm cell, whether noncapacitated (Fig. 4b) or capacitated (Fig. 4d). Fab′s from control or preimmune immunoglobulins did not bind to any region of the sperm cell (Fig. 4f).

DISCUSSION

In the present study, we used phage display technology, a unique tool for identifying novel sequences. Previous studies have employed predominantly hybridoma technology to delineate fertilization-related antigens [1,2,13–16,20], which is more laborious and time consuming. Using the peptide display library containing 1.77×10^8 unique sequences, we identified at least three 12-mer aa sequences that showed stronger binding with human ZP. In addition, a 17-mer consensus sequence was delineated based upon these three strongly binding and six relatively weakly binding sequences. All four sequences were novel, since an extensive computer search in the GenBank, NBRF, and Swiss sequence banks did not show any known nt/aa sequence having a complete identity with them. All four synthetic peptides based on these sequences were water soluble.

When tested in the hemizona assay, only two, YLP_{12} and $Consensus_{17}$, significantly inhibited sperm binding, with YLP_{12} having a stronger effect. The other two, SNR_{12} and GHR_{12}, did not inhibit sperm binding with hemizona, although these peptide sequences showed relatively stronger binding with HZP in the five cycles of biopanning and Western blotting. In both these screening procedures using solubilized HZP, the complementary linear sequences rather than conformational epitopes of the interacting molecules are involved in recognition and binding. In contrast to the situation with solubilized HZP, in the intact HZP the complementary sequences may be present as conformational epitopes and/or may not be exposed on the surface, thus not recognizable by the linear sequences of these peptides in the hemizona assay. Even if the complementary epitopes present on the intact ZP are accessible to these peptides, they may not be relevant to sperm binding. Some specific sequences present in ZP are involved in sperm binding, and others are not [21,32–34]. Various studies have shown that the ZP of the human oocyte is composed of at least three components, namely, ZP1, ZP2, and ZP3, and the ZP3 molecule acts as a primary receptor for sperm binding in various species of animals [21,32–34]. It is possible that SNR_{12} and GHR_{12} sequences react with ZP1 and/or ZP2 instead of ZP3 and thus are unable to inhibit sperm binding in the hemizona assay.

The inhibitory effects of YLP_{12} and $Consensus_{17}$ peptides were specific, since 1) the second halves of the zonae (hemizonae) from the same oocyte that were used as controls showed sperm binding; 2) the effects of peptides were apparent only when they were preincubated with hemizonae instead of sperm before insemination; and 3) two other same-sized (12-mer) peptides (SNR_{12} and GHR_{12}) and BSA did not affect binding at the same concentration. Also, the inhibitory effect of YLP_{12} was further confirmed with use of its monovalent Fab′ antibodies, which are devoid of nonspecific binding Fc portion. Immunoadsorption of the anti-YLP_{12} Fab′s with the peptide abolished the inhibitory effect, and the control Fab′s from preimmune serum and control animals immunized with the TT alone did not affect sperm binding, indicating specificity of the inhibitory effects. None of the peptides or the Fab′s caused agglutination of sperm or any apparent deleterious effect on motility (percent or progressive) of the spermatozoa.

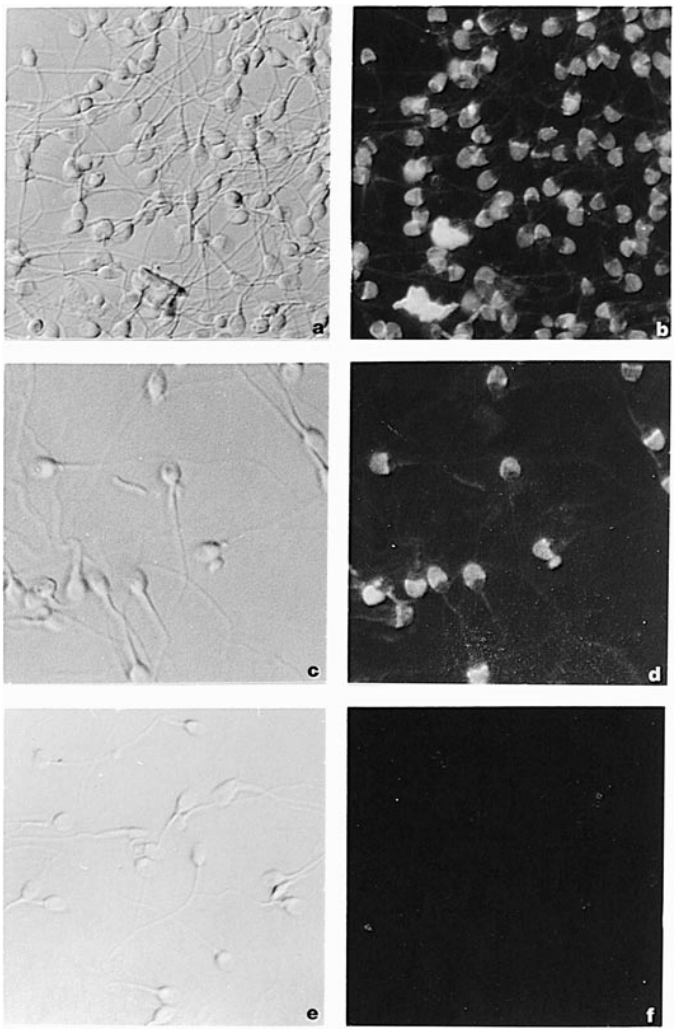

FIG. 4. Epifluorescent photomicrographs indicating the indirect immunofluorescent reaction pattern of the anti-YLP_{12} Fab′s with methanol-fixed human sperm. Anti-YLP_{12} Fab′s reacted with the acrosomal region of sperm, whether noncapacitated (**b**) or capacitated (**d**). Fab′s from control or preimmune immunoglobulins did not bind to any region of sperm cell (**f**). Panels on the left are the corresponding phase-contrast pictures, respectively, of panels on the right. **a–f**, ×720 (published at 67%).

The YLP_{12} peptide specifically recognized the ZP3 component of the ZP of the oocyte in Western blot and immunoprecipitation procedures. The binding with ZP3 and inhibition of sperm-zona binding by the peptide indicate that the YLP_{12} sequence may be a complementary receptor sequence on the sperm surface that is involved in recognition/binding to the ZP of the oocyte. The interaction of ZP3 with the peptide also indicates that the recognition/binding may involve protein-protein interaction, recognizing the linear sequences of the complementary molecules of two interacting cells (sperm/oocyte), as is becoming apparent from recent studies using recombinant ZP and sperm proteins [35].

The specific binding of the Fab′s with the ~72 ± 2-kDa protein band only in the testis extract indicates the testis-specific expression of the protein having the YLP_{12} aa sequence. In addition to involvement in the fertilization process, tissue specificity is an important criterion for the selection of an antigen as a candidate for contraceptive vaccine development. The binding of the Fab′s with the acrosomal region of the noncapacitated as well as the capacitated sperm cell in the indirect immunofluorescence technique indicates that the ~72 ± 2-kDa protein with YLP_{12} sequence is present in the acrosomal subcellular region of the sperm cell and is not lost during capacitation.

In conclusion, by using the phage display technique we have identified a testis-specific novel peptide sequence, present in the acrosomal region of the human sperm cell, that is involved in recognition/binding to the ZP3 component of the ZP of human oocytes. Besides helping to provide further insight into molecular mechanisms underlying sperm-zona interaction, the sequence may find applications in contraceptive vaccine development and in diagnosis and treatment of male infertility mediated through sperm dysfunction.

ACKNOWLEDGMENTS

We are thankful to Monica H. Leslie, Nadia Ashraf, and Linda Wagner for technical and typing assistance and Professor Michael Boyle for helpful suggestions. The clinical help and resources provided by Majid Fateh, M.D., are gratefully acknowledged.

REFERENCES

1. Naz RK, Sacco A, Singh O, Pal R, Talwar GP. Development of contraceptive vaccines for humans using antigens derived from gametes (spermatozoa and zona pellucida) and hormones (human chorionic gonadotropin): current status. Hum Reprod 1995; 1:1–18.
2. Naz RK, Vaneck CM. Testis-specific proteins and their role in contraceptive vaccine development. Front Biosci 1998; 3:39–48.
3. Edwards RG. Immunological control of fertility of female mice. Nature 1964; 203:50–53.
4. Menge AC. Immune reactions and infertility. J Reprod Fertil Suppl 1970; 10:171–185.
5. Allardyce RA. Effect of ingested sperm on fecundity in the rat. J Exp Med 1984; 159:1548–1553.
6. Baskin MJ. Temporary sterilization by injection of human spermatozoa: a preliminary report. Am J Obstet Gynecol 1932; 24:892–897.
7. Mancini RE, Andrada JA, Saraceni D, Bachman AE, Lavier JD, Nemirovsky M. Immunological and testicular response in men sensitized with human testicular homogenate. J Clin Endocrinol Metab 1965; 25:859–875.
8. Naz R, Menge AC. Antisperm antibodies: origin, regulation, and sperm reactivity in human infertility. Fertil Steril 1994; 61:1001–1013.
9. Liskin L, Pile JM, Quillan WF. Vasectomy-safe and simple. Popul Rep 1983; 4:61–100.
10. Naz RK, Deutsch J, Phillips TM, Menge AC, Fisch H. Sperm antibodies in vasectomized men and their effects on fertilization. Biol Reprod 1989; 40:163–173.
11. Ohl DA, Naz RK. Infertility due to antisperm antibodies. Urology 1995; 46:591–602.
12. Schulman S. Sperm antibodies and autoantibodies: effects on fertility. Am J Reprod Immunol Microbiol 1986; 10:86–89.
13. Primakoff P, Lathrop W, Wollman L, Cowan A, Myles D. Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. Nature 1988; 335:543–547.
14. Sirinivasan S, Tinge R, Wright JC, Herr J, Curtiss III R. Oral immunization with attenuated Salmonella expressing human sperm antigen induces antibodies in serum and the reproductive tract. Biol Reprod 1995; 53:462–471.
15. Hardy CM, Clarke HG, Nixon B, Grigg JA, Hinds LA, Holland MK. Examination of the immunocontraceptive potential of recombinant rabbit fertilin subunits in rabbit. Biol Reprod 1997; 57:879–886.
16. Naz RK, Zhu X. Recombinant fertilization antigen-1 causes a contraceptive effect in actively immunized mice. Biol Reprod 1998; 59: 1095–1100.
17. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 189–318.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
19. Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76:4350–4354.

20. Zhu X, Naz RK. Fertilization antigen-1: cDNA cloning, testis-specific expression, and immunocontraceptive effects. Proc Natl Acad Sci USA 1997; 94:4704–4709.
21. Naz RK, Ahmad K. Molecular identities of human sperm proteins that bind human zona pellucida: nature of sperm–zona interaction, tyrosine kinase activity, and involvement of FA-1. Mol Reprod Dev 1994; 39: 397–408.
22. Naz RK, Zhu X, Menge AC. Expression of tumor necrosis factor-α and its receptors type I and type II in human oocytes. Mol Reprod Dev 1997; 47:127–133.
23. Naz RK. Effects of antisperm antibodies on early cleavage of fertilized ova. Biol Reprod 1992; 46:130–139.
24. Pearson WP, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 1988; 85:2444–2448.
25. Tsong YY, Chang CC, Nash HA. Formulation of a potential antipregnancy vaccine based on the β-subunit of human chorionic gonadotropin (β-hCG). J Reprod Immunol 1985; 7:139–149.
26. Nisonoff A, Wissler FC, Lipman LN, Woernley DL. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. Arch Biochem Biophys 1960; 89:230–244.
27. Naz RK, Ahmad K, Menge AC. Antiidiotypic antibodies to sperm in sera of fertile women that neutralize antisperm antibodies. J Clin Invest 1993; 92:2331–2338.
28. Kadam AL, Fateh M, Naz RK. Fertilization antigen (FA-1) completely blocks human sperm binding to human sperm zona pellucida: FA-1 antigen may be a sperm receptor for zona pellucida in humans. J Reprod Immunol 1995; 29:19–30.
29. Naz RK, Phillips TM, Rosenblum BB. Characterization of the fertilization antigen 1 for the development of a contraceptive vaccine. Proc Natl Acad Sci USA 1986; 83:5713–5717.
30. Naz RK, Morte C, Ahmad K, Martinez P. Hexokinase present in human sperm is not tyrosine phosphorylated but its antibodies affect fertilizing capacity. J Androl 1996; 17:143–150.
31. Naz RK, Ahmad K, Kaplan P. Expression and function of *ras* protooncogene proteins in human sperm cells. J Cell Sci 1992; 102:487–494.
32. Chamberlin ME, Dean J. Human homolog of the mouse sperm receptor. Proc Natl Acad Sci USA 1990; 87:6014–6018.
33. Millar SE, Dean J. Targeting the zona pellucida for immunocontraception. In: Naz RK (ed.), Immunology of Reproduction. Boca Raton: CRC Press; 1993: 37–60.
34. Shabanowitz RB. Mouse antibodies to human zona pellucida: evidence that human ZP3 is strongly immunogenic and contains two district isomer chains. Biol Reprod 1990; 43:260–270.
35. Zara J, Naz RK. The role of carbohydrates in mammalian sperm-egg interactions: how important are carbohydrate epitopes? Front Biosci 1998; 3:1028–1038.