Monoclonal Antibodies against Boar Sperm Zona Pellucida-Binding Protein AWN-1. Characterization of a Continuous Antigenic Determinant and Immunolocalization of AWN Epitopes in Inseminated Sows

Juan J. Calvete, Michael Ensslin, Jane Mburu, Antonio Iborra, Paz Martinez, Knut Adermann, Dagmar Waberski, Libia Sanz, Edda Töpfer-Petersen, Karl-Fritz Weitze, Stig Einarsson, and Heriberto Rodriguez-Martinez

Institut für Reproduktionsmedizin, Tierärztliche Hochschule, Hannover, Germany
Swedish University of Agricultural Sciences (SLU), Department of Obstetrics and Gynaecology, Uppsala, Sweden
Institut de Biologia Fonamental “Vicent Villar Palasi,” Unitat d’Immunologia, Universitat Autònoma de Barcelona, Spain
Niedersächsisches Institut für Peptid-Forschung GmbH, Hannover, Germany

ABSTRACT
Boar spermadhesin AWN-1 is a sperm surface-associated 14.7-kDa lectin and a major protein of porcine seminal plasma. AWN-1 binds to β-galactosides and to porcine zona pellucida glycoproteins, suggesting that this protein might play a role in the primary binding of spermatozoa to the egg’s external glycoprotein matrix. We have produced a collection of murine monoclonal antibodies against purified AWN-1. Five monoclonal antibodies recognized sequential antigenic determinants. All these epitopes were located at the C-terminal region of AWN-1 (residues 109–123) by competitive ELISA using overlapping synthetic peptides that cover the complete 133 amino acid sequence of the lectin. In a structural model of spermadhesin AWN-1, the polypeptide stretch 109–123 is fully solvent-exposed, providing a reasonable explanation for its high immunogenicity. In addition to epitope mapping, we have employed anti-AWN monoclonal antibodies for immunolocalization of the protein in the genital tract of inseminated sows. Clusters of AWN epitopes were occasionally found attached to the epithelium of the uterotubal junction and the adjacent lower isthmus. However, neither AWN-1 nor other seminal plasma proteins were found in the isthmic fluid collected 10–26 h after insemination. These results suggest that the whole amount of seminal plasma proteins are absorbed by the epithelium of the female genital tract, supporting the claim that removal of seminal plasma components from spermatozoa might be a major event in both in vitro and in vivo sperm capacitation.

INTRODUCTION
Mammalian spermatozoa leaving the testis are highly differentiated cells but do not have fertilizing capability. They develop this ability while passing through the epididymis [1, 2]. Although spermatozoa do not become fertile simultaneously in this region of the epididymal duct, it is in the midsegment (i.e., proximal corpus region) that most boar spermatozoa attain progressive motility, chromatin stability, and fertilizing ability [3–5].

At ejaculation, spermatozoa mix with the seminal plasma, a complex mixture of secretions originating from the epididymis and the accessory glands. The seminal plasma serves as a vehicle for ejaculated spermatozoa and in addition contains factors that influence the fertilizing ability of the spermatozoa. The effect of seminal plasma on sperm function is complex. Factors that prevent sperm from becoming acrosome-responsive, as well as positive regulators of agonist-induced acrosome reaction, have been reported [6, 7] and references therein.

In previous studies we have characterized the major boar seminal plasma proteins that coat the sperm surface at ejaculation (reviewed in [8–10]). A group of homologous proteins, termed spermadhesins, represent over 90% of the total seminal plasma proteins. Although the major biological source of porcine spermadhesins is the secretion of the seminal vesicle epithelium, where the concentration of various spermadhesins (AWN-1, AWN-2, AQN-1, PSP-I, PSP-II, and AQN-3) [11, 12] ranges from 0.6 to 7.2 mg/ml [13], AWN-1 is also synthesized by the tubuli recti and rete testis [14]. It is the only member of its family found on the surface of epididymal spermatozoa [13]. About 6 million AWN-1 molecules have been quantitated on the surface of a single boar epididymal spermatozoon [13]. After ejaculation, 12–60 million molecules of each of AQN-1, PSP-I, AQN-3, AWN-1, and AWN-2 become coated on the apical third of the sperm acrosomal cap. Most of this material, however, is released during in vitro capacitating conditions, and the level of each spermadhesin drops to 5–6 million molecules per spermatozoon [13]. Since boar spermadhesins are multifunctional proteins that display zona pellucida glycoprotein-, serine proteinase inhibitor-, and/or heparin-binding activities [11, 12], these data suggest that different subpopulations of spermadhesins might play diverse roles as either decapacitation or acrosome-stabilizing factors or zona pellucida-binding molecules.

Shortly after sperm deposition in the pig, a population of spermatozoa colonizes the uterotubal junction crypts and the deep furrows formed by the primary folds in the lower adjacent isthmus [15]. These spermatozoa attach to the epithelium [16–18], where they are kept morphologically intact [18, 19], isolated from the rejecting mechanisms of the female tract and fertile for a rather long period (sometimes more than 24 h), during which time they may migrate or be transported toward the ovarian tubal end in relation to ovulation onset [20–22]. Despite the large number of studies so far performed, the nature of the interactions between...
the sperm and the tubal epithelium leading to capacitation remains to be clarified.

To investigate the fate of AWN-1 molecules during the events leading to porcine fertilization, we have produced a collection of murine monoclonal antibodies against the purified protein. Here we report the presence of a high immunogenic sequential epitope at the C-terminal part of AWN-1, as well as the immunolocalization of AWN epitopes in tissue sections of the genital tract of inseminated sows.

MATERIALS AND METHODS

Animals

Male and female mature pigs (purebred boars—Germans and Swedish Yorkshire—and cross-bred multiparous sows—Swedish Landrace × Swedish Yorkshire) were used. All animals were kept indoors in individual pens and given commercial pig feed. All animals had records of high fertility. The use of the animals in the present study was approved by the corresponding Committee for Experimentation with Animals.

Immunization and Hybridoma Production

Spermadhesin AWN-1 was isolated from the heparin-binding fraction of boar seminal plasma by reversed-phase HPLC as described previously [23]. To generate anti-AWN-1 monoclonal antibodies, BALB/c female mice were immunized by i.p. injection of 50 μg AWN-1 per mouse with Freund’s complete adjuvant on Day 0 and with Freund’s incomplete adjuvant on Days 15 and 30. Animals showing the highest antibody titers by ELISA (see below) were selected for the fusion and were immunized without adjuvant 3 days before fusion. On the day of fusion, mice were killed and immune spleen cells were fused with mouse myeloma cells NS-1 (at ratio of 10:1) in HAT (hypoxanthine-aminopterin-thymidine) selective medium using the polyethylene glycol procedure [24, 25]. About 15 days after fusion, when the growing hybridomas showed anti-AWN-1 antibody activity as detected by ELISA, cloning of the positive cells was performed by limiting dilution. The positive clones were recloned. Immunoglobulins obtained from the supernatant were purified by affinity chromatography using protein-A-Sepharose-CL-4B (Pharmacia, Uppsala, Sweden). The antibody peak was eluted with 0.1 M citric acid, pH 4, dialyzed against deionized water, and lyophilized.

ELISA for the Selection of Monoclonal Antibodies

The ELISA procedure employed was essentially as described previously [26]. Briefly, 10 μg of purified AWN-1 spermadhesin in 100 μl of 50 mM Na2CO3/NaHCO3, pH 9.6, was coated to flat-bottom polystyrene plates overnight at 4°C. After blocking with 1% (w/v) BSA in 20 mM phosphate, 135 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4 (PBS/Tween), for 1 h at room temperature, 100 μl of the hybridoma supernatants was added, and incubation was performed for 2 h at room temperature. The plates were washed three times with PBS/Tween, incubated with goat anti-mouse IgG conjugated with horseradish peroxidase for 2 h at 37°C, washed, and developed with 0.8 mM 3-methyl-2-benzo-thiazolinone hydrazide hydrochloride/40 mM 3-dimethylaminobenzoic acid, 5 mM H2O2, for 20 min. The reaction was stopped with 2 M sulfuric acid, and the optical density at 620 nm was measured with an Anthos 2001 ELISA plate reader (Labtec International, Uckfield, East Sussex, UK).

Immunoblotting Analysis

Boar seminal plasma proteins were separated by SDS-PAGE [27] using 15% polyacrylamide gels and then electroblotted onto polyvinylidene fluoride membranes [28] for 3 h at 1 mA/cm². Immunoblotting analysis was performed with the mouse anti-AWN-1 monoclonal antibodies using a concentration of 0.05–0.25 μg/ml. The second antibody was a 1:1000 (v:v) dilution of a rabbit anti-mouse IgG conjugated with peroxidase (Boehringer-Mannheim, Mannheim, Germany), and color was developed with 1 mg/ml 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) in 20% H2O2/100 ml Tris-HCl, 500 mM NaCl, pH 7.4, containing 20% (v:v) methanol and 15 μl H2O2/100 ml.

Peptide Synthesis

Overlapping peptides, which cover the complete amino acid sequence of porcine spermadhesin AWN-1 (133 residues) [29], were synthesized through use of a standard Fmoc protocol [30], employing preloaded ρ-alkoxybenzyl alcohol resins or a Rink amide resin (peptide P10), on an SMPS 350 automated multiple peptide synthesizer (Zinsser, Frankfurt, Germany) equipped with a 48-syringe synthesis block (Multisynthet, Bochum, Germany). The amino acid sequences and relative position within the AWN-1 sequence of peptides P1-P12 and P12a-P12f are displayed in Figure 1. Peptides were characterized by amino acid analysis using an Alpha Plus (Pharmacia) amino acid analyzer after sample hydrolysis with 6 M HCl for 24 h at 110°C in evacuated and sealed ampules, and by matrix-assisted laser-desorption mass spectrometry (using a Shimadzu MALDI-I instrument [Shimadzu-Europa, Duisburg, Germany] and α-cyano-4-hydroxycinnamic acid saturated in acetone as matrix). The four cysteine residues of AWN-1 (positions 9, 30, 53, and 74), which form two disulfide bridges between nearest neighbor residues [29], were replaced by alanine residues.

Competitive Assay

The ability of synthetic peptides P1-P12 and P12a-P12f to inhibit the binding of monoclonal antibodies to purified AWN-1 was assessed using an ELISA. To this end, 1 μg of AWN (purified as described previously [29]) in 100 μl 50 mM Na2CO3/NaHCO3 buffer, pH 9.5, was coated overnight at 4°C onto ELISA plate wells. After blocking with 10 mM Tris, 150 mM NaCl, pH 7.4 (TBS buffer), containing 5% (w:v) BSA for 2 h at 37°C, the immobilized proteins were incubated with 100 μl of solutions containing 1–5 μg of purified monoclonal IgG preincubated for 1 h at 37°C with increasing concentrations (10 ng–10 μg) of a given synthetic peptide in TBS. After washing three times with TBS containing 0.5% (w:v) BSA (washing buffer), samples were incubated with 100 μl streptavidin-peroxidase (1:4000, w:v; Calbiochem, La Jolla, CA) in washing buffer, washed three times, and developed with 100 μl/well of 2 mg/ml α-phenylenediamine in 20 mM citric acid, 50 mM NaH2PO4 buffer, pH 5, containing 70 μl H2O2 (30%)/100 ml. The color developed was measured at 492 nm with an automated micro-ELISA reader (ICN, Costa Mesa, CA).

The concentration of each monoclonal antibody used was the concentration that in the absence of inhibiting peptide gave a total absorbance of 2.0–2.5 at 492 nm. The concentration of peptides was determined by amino acid analysis using an Alpha Plus (Pharmacia) amino acid analyzer after sample hydrolysis with 6 M HCl for 24 h at 110°C in evacuated ampules.
Immunofluorescence of Boar Spermatozoa

Localization of AWN-1 epitopes on boar spermatozoa recovered from a swim-up fraction [13] was studied by indirect immunofluorescence. Ten microliters of the sperm suspension (10^6/ml) was spread on slides, air dried, and fixed for 15 min in methanol. The preparations were incubated with 20 mM sodium phosphate, 135 mM NaCl, pH 7.4 (PBS buffer), containing 50 mg/ml BSA (blocking buffer); they were then incubated with either blocking buffer (control) or with a 1–5 μg/ml monoclonal antibody solution (AWN-1; control) or with a 1-5 μg/ml monoclonal antibody solution (AWN-1). After washing with blocking buffer, both samples were incubated for 2 h at 37°C in a humid chamber. After washing with blocking buffer, both samples were incubated for 2 h at 37°C with 20 μg/ml of fluorescein isothiocyanate-labeled goat anti-mouse IgG polyclonal antibody (Boehringer-Mannheim). The preparations were exhaustively washed with blocking buffer and were observed under a fluorescence microscope (Carl Zeiss, Thornwood, NY; ×100 objective, ×10 ocular).

Recovery of Oviductal Isthmic Fluid

Twelve cross-bred multiparous sows (Swedish Landrace × Swedish Yorkshire) were used for recovery of oviductal isthmic fluid. The animals were kept indoors in the Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Uppsala, Sweden, and fed with commercial pig feed. Estrus detection was performed twice daily in the presence of a teasing boar, and transrectal ultrasonography was used to assess ovulation [31].

During a second normal spontaneous estrus, the sows were submitted to midventral laparotomy under general narcosis at 8 h preovulation (expected, n = 4), during ovulation (n = 4), and 8 h postovulation (n = 4). The 8-h preovulation time point was calculated from the previously occurring ovulation, and the other two time points were determined by the occurrence of ovulation at ultrasonography, performed presurgery [31]. The ovarian morphology was assessed, and an oviduct and the tip of the uterine horn were selected at random and exposed. The thin wall of the lower ampulla (~3 cm away from the ampullary-isthmic junction) was perforated with a blunt instrument, avoiding bleeding; and a silastic catheter (0.30 i.d., 0.65 in o.d.; Dow Corning, Midland, MI) that had lateral notches in one end was threaded through the ampulla toward the ampullary-isthmic junction and forced a few millimeters into the isthmic segment where it was manually held in place. A similar opening was made in the tip of the uterus (~2 cm from the uterotubal junction, UTJ); a button-ended 19-gauge needle was inserted into the isthmus (~2 cm from the UTJ), and a syringe containing warm saline solution was fixed to it. The isthmus lumen was gently flushed with 1 ml of saline solution, and fluid was collected into an Eppendorf tube from the silastic catheter inserted in the distal isthmus. After collection, the fluid was filtered through a 0.6-μm Millipore (Bedford, MA) filter and stored at −20°C until analyzed. The filter was fixed by flushing with a 2.5% solution of glutaraldehyde in 0.01 M cacodylate buffer, pH 7.2, 500 mOsm. The fixed Millipore filters were processed for scanning and transmission electron microscopy and examined in a Cambridge (Watertown, MA) 150 SEM electron microscope.

After sampling of isthmic intraluminal fluid, a surgical resection of the UTJ and the adjacent unflushed lower isthmus was performed. The sampled tissue segments were cleaved longitudinally; one half were cryopreserved in liquid nitrogen, and the other half were fixed by immersion in a 1% solution of paraformaldehyde in PBS (pH 7.2) and stored in the fixative at 4°C until further processing for histology and immunocytochemistry.

Postoperative recovery was uneventful, and at the next spontaneous estrus the same animals were either naturally mated or artificially inseminated with the neat semen of two proven-fertile boars, 18 h before expected ovulation. The same procedures (ultrasonography, surgery, collection of isthmic fluid and UTJ-isthmic tissue samples) were repeated in the contralateral, intact oviduct.

Immunocytochemistry of Oviductal Tissues

The immunolocalization of AWN-1 in the UTJ and isthmic tissues sampled was studied at the light microscopic level using an ABC-Elite staining procedure (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). In brief, 5-μm-thick frozen sections from both the cryopreserved and the paraformaldehyde-fixed samples were cut in a cryostat (Leica CM 1800; Leica, Stockholm, Sweden) and mounted on poly-L-lysine (Sigma)-coated glass slides. The sections were left at room temperature (20–22°C) for 1 h and then stored at −70°C. Before incubation, the sections
were fixed in pure acetone for 10 min and air dried for 10 min. The sections were thereafter briefly washed in buffer (0.5 M Tris-HCl, 135 mM NaCl, pH 7.6) and then incubated with 0.3% H₂O₂ for 20–30 min in order to block endogenous peroxidase activity. Pretreatment with 2% BSA in the buffer was carried out to minimize nonspecific binding to immunoglobulins before the sections were incubated with anti-AWN-1 monoclonal antibodies. The sections were incubated with the primary antibodies, diluted in Tris-HCl buffer, for 30–60 min; they were then rinsed in buffer and incubated with biotinylated goat anti-mouse IgG antibody (1:200) prior to application of the ABC-Elite complex and further incubation in ethylcarbazole solution for 8 min to visualize the antibody-antigen complex. Sections were mounted with glycerin-gelatin either uncounterstained or after nuclear counterstaining with Mayer’s hematoxylin. Selected sections were photographed with a Nikon (Garden City, NJ) microphot-FXA photomicroscope with and without Nomarski interference contrast optics. Controls were run by omission of the primary antibody and by replacement of the primary antibody with an irrelevant antibody raised in the rabbit species (rabbit anti-rat, Z 494; Dakopatts, Glastrop, Denmark) and preimmune serum (mouse immunoglobulins as negative control, ×931; Dakopatts), respectively. As positive control, samples of the freshly ejaculated boar semen were subjected to the same protocol as above. Monoclonal antibodies D11 and B12 gave the best immunopositive reaction (staining AWN epitopes in both seminal plasma and spermatozoa) and were therefore selected for subsequent immunocytochemical studies.

Sections adjacent to those submitted to immunocytochemistry were stained with hematoxylin and eosin for morphology assessment.

**Two-Dimensional Electrophoresis of Isthmic Intraluminal Fluid and Seminal Plasma**

Two-dimensional electrophoresis was carried out essentially as previously described [32] using 11-cm-long Immobiline Dry-Strips (pH 3–11; Pharmacia) for isoelectric...
focusing (overnight at 15°C and 22.650 Vh). Sample buffer was 9 M urea, 2% (v:v) Nonidet P-40, and 2% (w:v) dithiothreitol. The second dimension was performed using SDS-(15%) polyacrylamide gels run as described previously [24]. Gels were either silver-stained [33] or blotted (1 h, 1 mA/cm²) onto polyvinylidiene fluoride membranes [30], and the blotted proteins were visualized using Coomassie blue R-250. Protein spots were excised, and the N-terminal amino acid sequence was determined using an Applied Biosystems (Foster City, CA) 477A/120A sequencer. The amino acid sequence similarity search was done via the Internet World Wide Web facilities using the program BLAST [34] and the SwissProt database (National Center for Biotechnology Information, NIH, Bethesda, MD).

RESULTS

Immunofluorescence of Boar Spermatozoa and Localization of a Continuous Epitope in the C-Terminal Region of AWN-1

Fourteen hybridoma clones (termed A11, A12, A13, B11, B12, B13, D11, D12, D13, B21, B22, B23, D21, and D23) producing monoclonal antibodies against purified porcine seminal plasma spermadhesin AWN-1 were generated. Immunoglobulins isolated from their supernatants were purified by affinity chromatography. All of them recognized seminal plasma AWN-1 on swim-up boar spermatozoa (Fig. 2, A and B).

It is noteworthy that the 14 anti-AWN-1 antibodies recognized the antigen after separation of seminal plasma proteins by SDS-PAGE and electroblotting onto nitrocellulose sheet. Prior reduction of disulfide bonds reduced, but did not abolish, the binding of all monoclonal antibodies except for B11, whose binding to reduced AWN-1 was enhanced. These results suggest either that the antigenic determinants for this panel of monoclonal antibodies are continuous in the primary structure of AWN-1 or that the SDS-denatured and reduced spermadhesin at least partially recovered its native structure in the blotting membrane. In addition, B11 may recognize an epitope that is more accessible in the denatured state. This is not surprising, since the monoclonal antibodies were produced against HPLC-isolated AWN-1.

To distinguish between continuous and conformational epitopes, the complete amino acid sequence of AWN-1 was synthesized in overlapping peptides (Fig. 1), and the ability of the synthetic peptides to inhibit the binding of the monoclonal antibodies to the parent protein was tested. The only peptide displaying inhibitory activity was P12, which blocked, in a concentration-dependent manner (Fig. 3), the interaction of monoclonal antibodies B21, D12, D13, D21, and D23 with AWN-1. In each case, 50% inhibition (IC₅₀) of the binding of monoclonal antibody to 1 μg of immobilized AWN-1 (molecular mass: 14,770 Daltons; 67 pmol) was achieved with 0.25–0.5 μg peptide (molecular mass: 1762 Daltons; 141–283 pmol). This indicates that synthetic peptide P12 (AWN-1 109–123) mimics quite faithfully the epitope recognized by the monoclonal antibodies in native AWN-1. The polypeptide stretch 109–123 is localized in the C-terminal region of the spermadhesin molecule (Fig. 1), which, in a proposed molecular model for spermadhesins [35, 36], protrudes from the protein core and is fully solvent-exposed (Fig. 4).

The fact that the IgGs from five hybridoma clones recognized the same 15-mer polypeptide indicated that the antibodies may bind to the same regions or to overlapping distinct regions. To assess this point, we investigated the ability of overlapping synthetic peptides P12a-P12f (Fig. 1) to inhibit the binding of monoclonal antibodies B21, D12, D13, D21, and D23 to AWN-1. None of the peptides (up to 10 μg) blocked the binding of monoclonal antibodies D21 and D13 to AWN-1. Peptide P12a (10 μg) inhibited 40% and 35% of maximal D12 and D23 binding, respectively, and 10 μg of peptides P12a-d blocked 54% of maximal binding of B21 to immobilized AWN-1. These results indicate that the complete structure of peptide P12 is necessary for effective recognition by the monoclonal antibodies. Comparison of the inhibitory activities of peptides P12 and P12a–f shows that the N-terminal dipeptide (109AD110) either plays an essential role in maintenance of the appropriate conformation of the epitopes expressed by P12, or it forms an integral part of these epitopes. This is particularly evident for monoclonal antibodies D21 and D13. The partial inhibitory effect of peptides P12a and P12a–d on the binding of monoclonal antibodies D12/D23 and B21, respectively, to the parent spermadhesin molecule indicates...
the presence of at least three different, although overlapping, epitopes in P12.

**Fate of AWN-1 Epitopes in the Reproductive Tract of the Sow**

The freshly ejaculated boar sperm showed, following immunocytochemistry, the same qualitative immunolabeling pattern as described above; i.e., the monoclonal antibody B12 was distributed over the whole acrosome surface while D11 bound to a narrow region covering the apical rim of the acrosome. Immunocytochemistry using the same monoclonal antibodies, D11 and B12, was used to study samples of tissue from the UTJ and its adjacent unflushed lower isthmus; none of the immunocytochemical controls or the tissue samples from noninseminated oviducts (each animal acted as its own control) showed immunolabeling. Immunolabeling was, however, localized sporadically in the UTJ and adjacent isthmus from the oviducts collected 10 hours after insemination. The antigen formed part of large aggregates bound to the epithelium of the UTJ and isthmus (Fig. 5). No obvious immunostaining was present in the specimens collected from animals inseminated 18 or 26 h prior to the operation. The intensity of the immunolabeling was stronger with frozen samples as compared to the paraformaldehyde-fixed counterparts. Normal histological views were present in hematoxylin/eosin-stained sections from the noninseminated versus the inseminated, unflushed oviducts from each one of the sows.

The oviductal isthmic fluid from the same specimens was subjected to two-dimensional PAGE to study the possible presence of AWN-1 (Fig. 6, A–C). Samples were analyzed with and without addition of seminal plasma to aid in the identification of seminal plasma proteins (Fig. 6, A–C). Two bands migrating with apparent molecular masses (12–16 kDa)
and isoelectric points (pI 8–9) similar to those of AWN-1 (and other spermadhesin molecules) were identified as the α and β chains of hemoglobin by N-terminal sequence analysis of electrophoretic gels (Fig. 6A). In addition, immunoblotting analysis could not detect AWN-1 epitopes.

Examination of the Millipore filters used for filtration of the isthmic flushings showed epithelial cell debris, mainly cilia, and confirmed the presence of boar spermatozoa only in the inseminated specimens.

DISCUSSION

Characterization of Monoclonal Antibody Epitopes

The binding sites for all of the purified anti-AWN-1 IgGs on sperm were located on the acrosomal cap membrane. However, their immunofluorescence labeling patterns were not identical. Thus, A11, A13, B11–13, D12, and D23 were distributed over the whole acrosomal surface (Fig. 2A), whereas A12, D11, D13, B22, and D21 bound to a narrow region covering the apical rim of the acrosome (Fig. 2B). These patterns, which were obtained with semen of different (n = 3) fertile boars, show that AWN-1 epitopes are unevenly distributed on the spermatozoal surface, indicating that the coating of sperm by seminal plasma AWN-1 molecules at ejaculation may not be homogeneous. In seminal plasma, AWN-1 molecules together with other proteins form large aggregates that are heterogeneous in size and composition. These aggregates may display different surface coating characteristics, and the various different aggregates may be removed at different stages or by different mechanisms during sperm washing and/or immunolabeling.

Using synthetic peptides, we have shown that monoclonal antibodies B21, D12, D13, D21, and D23 are directed against at least three different, although overlapping, conformational epitopes localized within the C-terminal region of spermadhesin AWN-1 (residues 109–123). In a proposed molecular model for spermadhesins [35, 36], the polypeptide stretch 109–123 protrudes from the protein core and is fully solvent-exposed (Fig. 4). Looking at the proposed structure of AWN-1, made up of a β barrel with short loops connecting the β strands, it seems reasonable to hypothesize that only the N- and C-terminal tails (upstream from strand A and downstream from strand G, respectively) will generate sequential epitopes. On the other hand, since the combining region of immunoglobulins typically binds to 800 ± 175 Å2 on the antigen surface [37, 38], antigenic determinants located in the β barrel or including connecting loop segments will have, most probably, a conformational nature. The polypeptide stretch 109–123 of AWN-1 is a proline-rich region for which a proline-based helix or a multiple turn conformation is predicted. Such a structure would potentially explain the "conformational nature" of the "linear epitopes" defined by the anti-AWN-1 monoclonal antibodies. Indeed, comparison of the inhibitory activities of peptides P12 and P12α–f indicated that the dipeptide (109AD110) appears either to play an essential role in maintenance of the appropriate conformation of the epitopes or to form an integral part of these epitopes.

AWN-1 Epitopes in the Reproductive Tract of the Sow

Only a small number of spermatozoa enter the oviduct in the pig (reviewed in [39]), with the UTJ and the adjacent lower isthmus acting as barriers/reservoirs of the potentially fertile spermatozoa [15, 40]. Whether the seminal plasma accompanying the spermatozoa enters the oviduct has been controversial. While radio-opaque fluid could not be seen entering the tubes [41], radiolabeled molecules of various sizes, added to seminal plasma, could be traced into the oviducts of gilts inseminated 24 h after estrus [42]. A facilitating role of seminal plasma in the passage of spermatozoa into the pig oviduct has been shown [43], but which seminal plasma component is the responsible one is yet to be determined.

The results of the present study led us to conclude that seminal plasma proteins may have been absorbed by the epithelium of the swine genital tract between the site of semen deposition in the uterus and the UTJ, as well as by the immediate (1 cm) adjacent isthmus region, during the time that elapsed between insemination and sample collection (10–26 h). Although translation of observations made in a few animals to the whole species is always a concern, our conclusion makes sense because if seminal plasma AWN-1 reached the place of fertilization, the spermadhesin would bind to the oocyte zona pellucida and block the binding of spermatozoa [29]. Furthermore, our conclusion ties in with the current view that in species like the pig, in which semen is deposited in the uterus, spermatozoa seem to complete all or most parts of capacitation in the lower segment of the isthmus where fertilizing spermatozoa are stored (reviewed in [1]). The relative rate of absorption of the seminal plasma proteins along the porcine female genital tract remains, however, to be determined. This will be studied in future investigations into the distribution of seminal plasma proteins (i.e., AWN-1) in the genital tract of inseminated sows as a function of the time period that has elapsed from insemination and in relation to the stage of estrus at mating.

Major events of in vivo capacitation are believed to be the removal of a stabilizer or protective coat from the sperm plasma membrane and the interaction of spermatozoa with the oviducal epithelium ([1, 44] and references cited). In the hamster, this interaction appears to be mediated by sperm carbohydrate-binding proteins [45]. On the other hand, when viewed under the scanning electron microscope, pig and bull spermatozoa in the isthmus and UTJ are in close contact with the epithelium [16–18, 46]. Circumstantial evidence has suggested that spermatozoa in close contact with the epithelium of the furrows of mucosal folds and crypts of the isthmus and UTJ represent the subpopulation that eventually fertilize the egg (reviewed in [1, 47, 48]). The mechanism by which isthmus-bound spermatozoa are released is not clear but seems to be related both to changes in the sperm plasma membrane associated with capacitation and to hyperactivated motility. Our results are in line with these hypotheses. Thus, we argue that the bulk of the seminal plasma protein coating material, which in the boar consists essentially of a loosely associated multilayered layer of spermadhesin lectins [13], will be released from the sperm surface either passively or through interaction with the epithelium of the genital tract, or in both ways. The aggregates containing AWN-1 epitopes detected in the uterotubal and lower isthmus regions could, in this model, represent the last sperm-associated seminal plasma protein remnant whose release allows capacitated spermatozoa to leave the isthmic reservoir as they prepare for fertilization.

In conclusion, using a panel of monoclonal antibodies we have characterized an antigenic region within a continuous region at the C-terminus of spermadhesin AWN-1 and have followed the fate of AWN-1 epitopes in the genital tract of inseminated sows. Our results support a role for
AWN-1, and possibly other seminal plasma proteins, in sperm capacitation. A major challenge is now to assess the actual role of AWM-1 in sperm-egg interaction, i.e., by investigating the presence or absence of spermadhesin molecules in the subpopulation of spermatozoa that fertilize the egg.

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