ELSEVIER

Biochimica et Biophysica Acta 1200 (1994) 48–54

BBA Biochimica et Biophysica Acta

Quantitation of boar spermadhesins in accessory sex gland fluids and on the surface of epididymal, ejaculated and capacitated spermatozoa

Zuzana Dostàlovà [a,b], Juan José Calvete [a,c], Libia Sanz [a], Edda Töpfer-Petersen [a,*]

[a] *Institut für Reproduktionsmedizin, Tierärztliche Hochschule Hannover, Bünteweg 15, 30599, Hannover-Kirchrode, FRG*
[b] *Institute of Molecular Genetics, Academy of Science of the Czech Republic, Czechoslovakia*
[c] *Instituto de Química-Física 'Rocasolano' CSIC, Madrid, Spain*

(Received 17 September 1993)

Abstract

Spermadhesins are multifunctional proteins involved in boar sperm capacitation and gamete recognition. Using anti-AWN antibodies, we have followed the fate of spermadhesin AWN along the maturation and capacitation stages of boar spermatozoa. In addition, the amount of spermadhesins AQN-1, AQN-2, and AQN-3 relative to that of AWN was determined by amino acid analysis after reverse-phase HPLC isolation. Our data show that AWN-1 is the only spermadhesin on the surface of epididymal sperm and that a large amount of AQN-1, AQN-2, AQN-3, AWN-1 and AWN-2 become coated on ejaculated spermatozoa. The number of spermadhesin molecules on ejaculated sperm ($12–60 \times 10^6$/spermatozoa) is sufficient to form a many-molecules-thick coat over the sperm head. However, 50–75% of the AQN-1, AQN-2, and AQN-3 population, and around 90% of coated AWN (1 + 2) are released from ejaculated sperm during capacitation. This indicates that a large subpopulation of each boar spermadhesin is loosely associated to the sperm surface and may function as decapacitation factors. The remaining spermadhesin molecules, which are tightly bound to the sperm head's surface may play a role as either positive capacitation factors and/or in gamete recognition and binding.

Key words: Boar sperm; Spermadhesins; AWN; AQN-1; AQN-2; AQN-3; Sperm capacitation

1. Introduction

Mammalian spermatozoa which are newly synthesized in the testis and released into the lumen of the seminiferous tubules are in a functionally immature state, i.e. unable to fertilize eggs [1–3]. They acquire this biological activity during subsequent passage through the epididymal duct, a process called epididymal maturation [1,4]. In addition, freshly ejaculated sperm of most mammalian species do not respond to acrosome reaction agonists but become capable of undergoing the acrosome reaction after several hours of residence in the female's genital tract, or after incubation in vitro in an appropriate medium [1–3]. The poorly understood events which prepare spermatozoa to undergo the acrosome reaction in response to interaction with its homologous zona pellucida have been collectively denominated 'capacitation' (see Refs. 5 and 6) for recent reviews). It seems plausible that factors attached to the sperm surface upon mixing with seminal plasma at ejaculation prevent premature acrosome reaction and are subsequently released from the sperm surface during capacitation, permitting the expression of endogenous or extrinsic positive regulators of zona pellucida-induced exocytosis [7,8].

One important facet of sperm maturation and capacitation is the continuous remodeling of the sperm's plasma membrane as a consequence of the constantly changing microenvironment [9] found by spermatozoa. In the bovine, heparin-binding proteins (15–17 kDa) from seminal plasma bind peripherally to ejaculated spermatozoa and play a positive regulatory role in capacitation potentiating zonae pellucidae-induced acrosome reactions in epididymal sperm [10]. In the mouse, a 6.4 kDa proteinase inhibitor from seminal vesicle fluid associates to, and inactivates, an epididymal sperm-associated 15 kDa zona pellucida-binding

* Corresponding author. Fax: +49 511 9538504.

SSDI 0304-4165(93)E0139-A

protein at ejaculation [11–14], and is removed during in utero or in vitro incubation [15,16]. In the pig, spermadhesins AQN-1, AQN-2, AQN-3, and AWN (12–14 kDa) present in seminal plasma become associated to the sperm's head surface at ejaculation (17–20; see [21] for a review). They are multifunctional proteins which possess zona pellucida glycoproteins-, serine proteinase inhibitor-, and/or heparin-binding sites [21–23], and may thus play a role in sperm capacitation and gamete recognition. However, whether they represent positive or negative capacitation factors has not been established.

Here, we have quantitated the amount of spermadhesins AWN (isoforms 1 and 2), AQN-1, AQN-2, and AQN-3 in epididymal fluid, epididymal sperm, seminal vesicle fluid, seminal plasma, freshly ejaculated sperm, swim-up spermatozoa, and 3 h and 24 h capacitated sperm. Our results show that only AWN-1 is present on epididymal sperm ($5.9–7.5 \cdot 10^6$ molecules/sperm), though it is absent from epididymal fluid. On the contrary, high amounts of all spermadhesins (ranging from 0.6 to 7.2 mg/ml) are present in seminal vesicle fluid and seminal plasma. Following ejaculation, $12–60 \cdot 10^6$ molecules of each AQN-1, AQN-2, AQN-3, and extra $50 \cdot 10^6$ AWN molecules (isoforms 1 and 2) become coated to the surface of spermatozoa. However, around 60% of adsorbed spermadhesins AQN-1, AQN-2, and AQN-3 are released after 3 h in vitro capacitation, the amount of AWN-1 decreases to the level found on epididymal sperm, and the whole AWN-2 population is lost. This figure changes only slightly upon 24 h capacitation. Our data indicate that different subpopulations of spermadhesins may play diverse roles as either decapacitation factors, positive capacitation elements, and/or receptors for zona pellucida.

2. Materials and methods

Sample preparation

Epididymal spermatozoa and seminal vesicle fluid were released from the epididymal duct and seminal vesicles, respectively, of recently (0.5–1 h) sacrificed boars at the local slaughter by cutting the tubules at different sites. Sperm was separated from epididymal fluid by centrifugation at room temperature at $160 \times g$ for 5 min. Spermatozoa were washed twice with 20 mM phosphate, 135 mM NaCl, pH 7.4, (PBS buffer) by centrifugation, and resuspended in this buffer. Sperm count was determined using a Neubauer chamber.

Freshly ejaculated boar sperm was collected as described [24], and separated from seminal plasma by centrifugation as above. Spermatozoa were washed with PBS buffer, and resuspended in this buffer. Alternatively, washed sperm was extracted overnight at 4°C with five (pellet) volumes of 2.5% (v/v) acetic acid, 10% glycerol, with gentle stirring (acid extraction) [25]. After centrifugation at $14000 \times g$ for 15 min at room temperature, the clear supernatant was dialyzed against water, and lyophilized.

Swim-up and in vitro capacitation

To obtain the swim-up fraction, freshly ejaculated boar spermatozoa were washed three times with Hepes buffer (20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 95 mM NaCl, 1 mM K_2HPO_4, 5 mM glucose, 60 mM saccharose, 5 mM sodium pyruvat, 0.5% bovine serum albumin, pH 7.4), resuspended in this buffer, and 1 ml deposited at the bottom of a tube containing 5 ml Hepes buffer. The tube was placed at an inclination of 45° for 1 h at 37°C in an incubator under a 5% CO_2 atmosphere [26]. The swim-up spermatozoa in the upper 1 ml fraction were recovered by centrifugation ($160 \times g$, 5 min), resuspended in Hepes buffer, and its concentration determined as above. For capacitation, the pelleted, washed Swim-up fraction was resuspended with two volumes of capacitation buffer (25 mM sodium bicarbonate, 29 mM sodium lactate, 10 mM Hepes, 2,3% (w/v) bovine serum albumin, 10 g/l TCM medium (Seromed-Biochrom, KG Berlin) (pH 7.8) (adjusted just before use), preconditioned overnight at 37° C under a 5% CO_2 atmosphere [1]. Spermatozoa were allowed to capacitate in a high humidity, 5% CO_2 incubator. Aliquots were taken at 3 h and 24 h, the spermatozoa recovered by centrifugation, washed three times with PBS buffer, and resuspended in this buffer at the desired sperm count.

Solid-phase anti-AWN binding assay

Polyclonal monospecific anti-AWN antibodies (recognizing both isoforms of AWN) were raised in chicken, the IgG fraction was purified from the egg's yolk as described [27], dialyzed against 20 mM phosphate, 135 mM NaCl, 1 mM NaN_3, pH 7.4, and stored at −20°C until used. The suitable working dilution was determined using an ELISA procedure. To this end, microtiter plates were coated with 1 μg purified AWN-1 [20] in 100 μl 50 mM sodium carbonate buffer (pH 9.6) overnight at 4°C, blocked with 200 μl 20 mM Tris/HCl, 150 mM NaCl, 0.5% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100, pH 7.4 (washing buffer) for 1 h at 37°C, and incubated with 100 μl/well of different anti-AWN dilutions for 1 h at 37°C. The plate was then extensively washed with washing buffer, incubated for 1 h at 37°C with 100 μl/well of a 1:2000 (v/v) dilution of goat biotinylated anti-chicken IgG (Vector Laboratories, Burlingame, California), washed, and incubated with 100 μl/well of a 1:4000 (v/v) dilution of streptavidin-peroxidase conjugate (Sigma) for 1 h at 37°C. After washing, the plate was developed with 100 μl/well of 2 mg/ml O-phenylendiamine in 20 mM citric acid, 50 mM phosphate, pH 5.0, containing

Table 1
Spermadhesin content in fluids of the boar sex accessory glands and in spermatozoa at different stages of maturation (1) expressed in $\mu g/\mu l$; (2) Spermadhesin molecules/spermatozoa (in millions); (3) determined using competitive ELISA; (4) determined

	Seminal Fluid [1]	Seminal Plasma [1]	Epididymal Sperm [2]	Ejaculated Sperm [2]	Swim-up [2]	Capacitated Sperm [2]	
						3 h	24 h
AWN[3]	2.1 (0.2)	1.8 (0.2)	6.7 (0.8)	50.4 (24.2)	37.6 (11.4)	9.5 (1.5)	7.3 (1.2)
AQN-1[4]	1.2 (0.1)	1.1 (0.2)	–	21.9 (10.5)	16.4 (4.8)	9.3 (1.1)	7.1 (1.3)
AQN-2[4]	7.2 (0.5)	6.4 (0.7)	–	61.5 (29.3)	45.9 (13.4)	20.3 (8.2)	15.4 (2.8)
AQN-3[4]	0.6 (0.1)	0.5 (0.1)	–	12.5 (6.2)	9.4 (2.8)	9.7 (3.6)	7.4 (2.4)

70 μl H_2O_2 30%/ 100 ml. The color developed was measured at 492 nm using an automated microELISA reader (ICN). The working dilution of anti-AWN, defined as that causing 95% of maximal antibody binding, was 1:3000 (v/v).

Quantitation of AWN by enzyme immunoassay

The total AWN content (isoforms 1 and 2) in epididymal fluid, epididymal sperm, seminal vesicle fluid, seminal plasma, ejaculated spermatozoa, swim-up sperm, and capacitated sperm samples, was determined by competitive ELISA. To this end, 50 μl of different dilutions of each sample (in PBS buffer) was mixed with an equal volume of a 1:3000 (v/v) anti-AWN antibody dilution, and incubated for 1 h at 37°C. The samples were then centrifuged (14000 × g, 15 min) and the free anti-AWN antibodies in the clear supernatant were titrated by ELISA as described above. A standard inhibition curve obtained by incubating different amounts of purified AWN-1 with anti-AWN was run in parallel in each experiment. For each sample the IC_{50} was defined as the amount of sample (μl fluid, number of spermatozoa, or μg of purified AWN) which neutralizes 50% of the initial anti-AWN antibodies. The amount of AWN in a given sample was determined using the following equation:

$$\text{number of AWN molecules} = \left[\frac{(IC_{50}\text{AWN-1})/(IC_{50}\text{Sample})}{M_w\text{AWN-1}}\right]\text{xN}$$

where M_w AWN-1 is 14471.2 [17], and N is Avogadro's number ($6.023 \cdot 10^{23}$). The IC_{50} of AWN is given in μg and the units of the IC_{50} of the sample are either number of spermatozoa or μl of fluid.

To rule out the possibility that IgG molecules bind unspecifically to the sperm surface, a control experiment was carried out which consisted in pre-incubating the boar spermatozoa preparations with different amounts of non-immune IgG before adding the anti-AWN antibody solution.

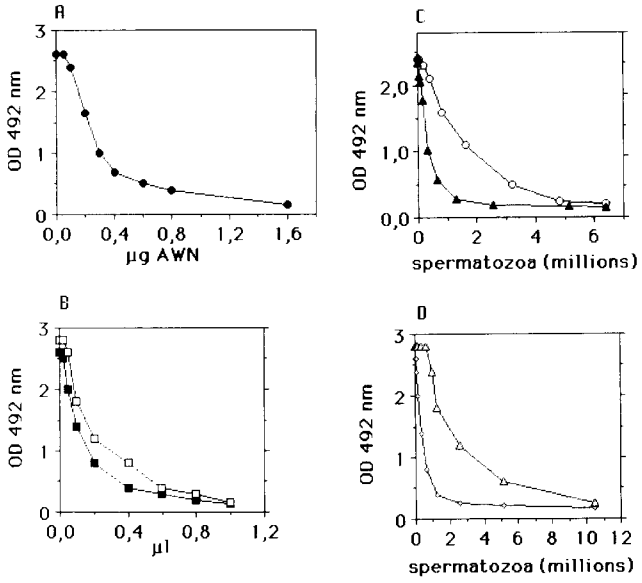

Fig. 1. *Quantitation of AWN using competitive enzyme immunoassay.* A) Standard curve of inhibition of anti-AWN antibodies (1:3000) binding to 1 μg AWN-1 immobilized on microtiter plate wells by soluble AWN-1 (IC_{50} = 0.3 μg). B) Inhibition curves obtained with seminal vesicle fluid (■ ——— ■) (IC_{50} = 0.14 μl) and seminal plasma (□ ——— □) (IC_{50} = 0.17 μl). C) Quantification of AWN in epididymal sperm (○ ——— ○) ($IC_{50} = 1.86 \cdot 10^6$ spermatozoa) and ejaculated sperm (▲ ——— ▲) ($IC_{50} = 0.24 \cdot 10^6$ spermatozoa). D) The same as in C) but using Swim-up sperm (■ ——— ■) (IC_{50} = $0.35 \cdot 10_6$ spermatozoa) and 24 h capacitated sperm (△ ——— △) ($IC_{50} = 1.71 \cdot 10^6$ spermatozoa).

Reverse-phase HPLC analysis and quantitation of AQN-1, AQN-2, and AQN-3

For determination of the concentration of spermadhesins AQN-1, AQN-2, and AQN-3, the proteins from samples whose AWN content had been previously established by competitive ELISA (see above) were isolated by reverse-phase chromatography using either a Lichrospher RP-100 (Merck) C18 column (25 · 0.4 cm, 5 μm particle size) eluting at 1 ml/min with a gradient of 0.1% trifluoroacetic acid in (A) water and (B) acetonitrile as described [20]. The whole AQN-1, AQN-2, AQN-3, and AWN (1 + 2) [20] fractions were dried using a Speed-Vac (Savant), and the protein content determined by quantitative amino acid analysis (using an Alpha Plus automated amino acid analyzer, Pharmacia-LKB) after hydrolysis with 6 N HCl for 24 h. The concentration of protein was calculated based on the established amino acid sequence of each protein [17–19], taken as reference the values obtained for alanine, valine, and lysine. The results were corrected

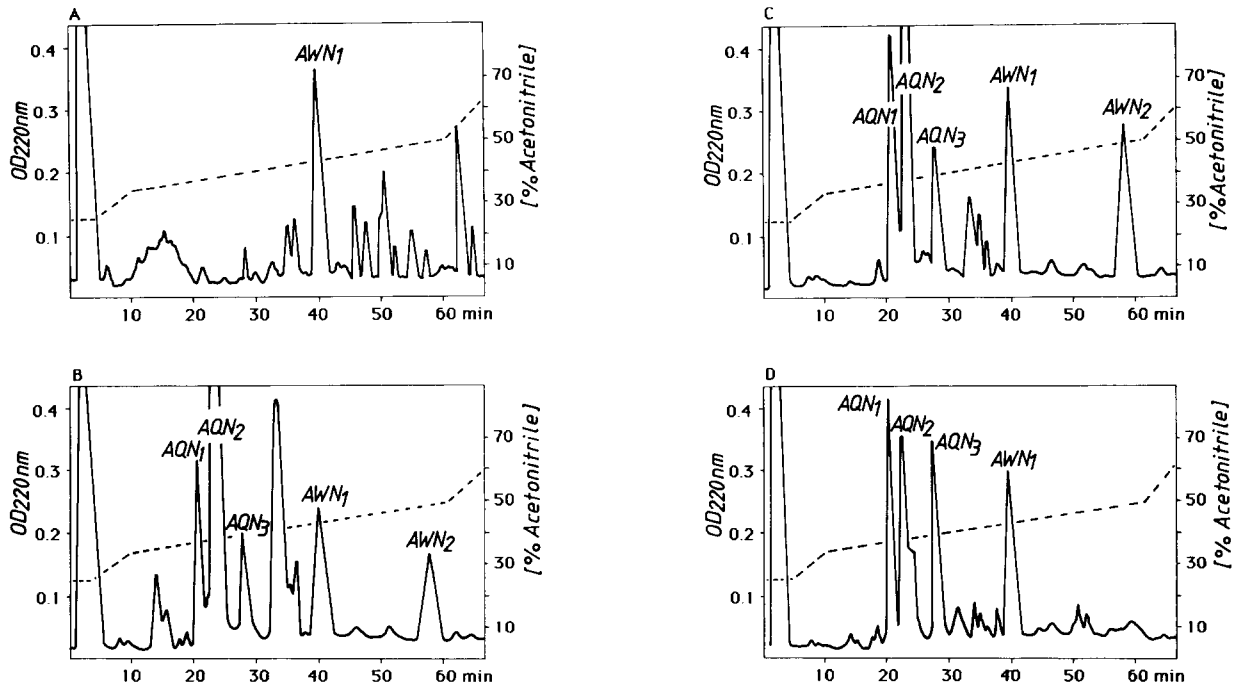

Fig. 2. *Isolation of boar spermadhesins.* Purification of the different boar spermadhesins (labeled AQN-1, AQN-2, AQN-3, AWN-1, and AWN-2) from samples of A) acid extract of epididymal sperm; B) Seminal vesicle fluid; C) acid extract of ejaculated spermatozoa; and D) acid extract of 24 h capacitated sperm, was achieved by reverse-phase chromatography.

for handling loses using as a coefficient the ratio between the amount of AWN determined in the same sample by ELISA and by amino acid analysis (AAA) after HPLC isolation:

μg AQN-x = μg AQN-x by AAA x

(μg AWN by ELISA/μg AWN by AAA)

This correction coefficient was consistently 4–8.

3. Results

Quantitation of AWN by competitive ELISA

The amount of spermadhesin AWN (isoforms 1 and 2) in secretions of the male sex accessory glands and in different sperm samples was determined using competitive ELISA by comparing the amount of purified AWN-1 which neutralizes 50% of anti-AWN antibodies binding to immobilized AWN-1 with the amount of sample (μl or number of spermatozoa) causing the same effect (Fig. 1). The concentration of AWN found in seminal plasma (Table 1) indicates that this protein represents around 7–8% of the total boar seminal plasma proteins (which typically contains 28–30 mg/ml). The AWN content in seminal vesicle fluid was always slightly, but consistently, greater than in the seminal plasma obtained after centrifugation of fresh ejaculates (Table 1). This may be due to dilution of seminal fluid with secretions of the other male sex accessory glands (prostate, Cowper's gland and Littre's gland) or to the large amount of AWN molecules which are coated to the sperm surface upon mixing of epididymal sperm with seminal fluid at ejaculation. Indeed, the population of AWN molecules increases

Table 2
Relative distribution of individual spermadhesin populations along the maturation process of boar spermatozoa. (1) The mean values in Table 1 were used for calculation of percentages.

	Percentage of the total spermadhesin population [1]					
	Epididimal Sperm	Seminal Fluid	Ejaculated Sperm	Swim-up	Capacitated	Sperm
					3 h	24 h
Spermadhesin						
AWN	100	18.9	34.4	34.4	19.4	19.6
AQN-1	–	10.8	14.9	15.1	19.1	19.1
AQN-2	–	64.9	42.1	41.9	41.6	41.4
AQN-3	–	5.4	8.5	8.6	19.9	19.9

almost 10 times on the surface of ejaculated sperm in comparison with epididymal sperm (Fig. 1C, Table 1). On the other hand, the amount of AWN molecules coated to ejaculated spermatozoa decreased a 25% in swim-up sperm (Table 1) and and this decoating effect was more dramatic in capacitated spermatozoa, where the number of AWN molecules per spermatozoa was of the same order of magnitude than in epididymal sperm (Fig. 1D, Table 1).

Quantitation of spermadhesins AQN-1, AQN-2, and AQN-3

Due to the lack of specific antibodies against proteins of the AQN family, we have quantitated the amount of each spermadhesin by amino acid analysis after reverse-phase HPLC separation (Fig. 2). In parallel, the total amount of AWN in each sample was established as described above. Table 1 shows the results obtained with different samples. Interestingly, the distribution of each individual spermadhesins on spermatozoa at different stages of maturation does not change in parallel (Fig. 2, Table 2). Thus, the only spermadhesin found in epididymal spermatozoa is AWN-1 (Fig. 2A). Spermadhesin AWN-2 is first found on seminal vesicle fluid (Fig. 2B) and is subsequently coated to ejaculated sperm (Fig. 2C). Moreover, the relative concentration of both AQN-1 and AQN-3 increases during sperm maturation, whereas the population of AWN-1 constantly decreases, AWN-2 is completely lost during capacitation, and the amount of AQN-2 remains stable from ejaculation to 24 h capacitation. The decrease in spermadhesin concentration on capacitated spermatozoa was not due to membrane exocytosis since under the conditions used less than 15% of spermatozoa underwent the acrosome reaction.

4. Discussion

Many studies have indicated that the spectrum of proteins that bind to the sperm surface varies during the maturation process and may be responsible for inducing changes which facilitate the acquisition of fertilizing ability by the spermatozoa. Thus, though epididymal spermatozoa from many mammalian species are fertile to some extent, interaction of spermatozoa with constituents of epididymal fluid during epididymal maturation, seminal plasma at the time of ejaculation, and during capacitation in the female reproductive tract, may modulate their fertilizing ability and may ensure that only spermatozoa which reach the vicinity of the egg react in a proper manner with acrosome reaction agonists, i.e. the zona pellucida network. This effect may depend on both the absolute and the relative concentration of the constituents of the different fluids that are coated to, or released from, the spermatozoa surface along the different stages of its maturation and capacitation process.

Here, we have investigated the time-course of appearance of proteins of the spermadhesin family [21] on the surface of boar spermatozoa and their fate during the different stages of the sperm's live, i.e. epididymal maturation, mixing with seminal plasma at ejaculation, and in vitro capacitation. Surprisingly, only one member of the spermadhesin family, AWN-1, was present on epididymal sperm (Table 1, Fig. 1A). Since AWN-1 is a zona pellucida binding protein, we hypothesize that it may be one of the factors which contribute to the fertilizing activity of epididymal spermatozoa. In addition, the fact that AWN-1 is not present in epididymal fluid indicates that either it is quantitatively adsorbed on sperm or, more probably, that it is synthesized and coated to the spermatozoa surface before sperm enter the epididymal duct, i.e. in the testis or rete testis. Indeed, immunohystochemical studies using an anti-AWN antibody show positive material in the rete testis (F. Sinowatz, W. Amselgruber, personal communication).

Following ejaculation, high amounts of all spermadhesins are coated to the sperm surface (Table 1, Fig. 1C). The relative amounts of each spermadhesin on the surface of ejaculated sperm does not reflect their relative contribution to seminal fluid (or plasma) composition (Table 2), however. Thus, spermadhesins AWN, AQN-1, and AQN-3 become 2-, 1.5-, and 1.5-fold enriched on sperm whereas the concentration of AQN-2 decreases 1.5 times. The reason for this uneven coating of different spermadhesins on the sperm surface is not clear. Whether this implies that individual spermadhesins are preferentially adsorbed to different sperm surface domains needs a detailed investigation. Nevertheless, assuming that a spermadhesin molecule may have molecular dimensions similar to an immunoglobulin domain [28], which are typically $30 \times 35 \times 25$ Å, one can calculate that a single molecule will cover around 800 Å^2 of sperm surface. Since the sperm head's surface is about 150 μm^2, it follows that the AWN-1 population on epididymal sperm is sufficient to cover one-third of the entire sperm head surface, i.e. the whole acrosomal cap where it has been localized by indirect immunofluorescence [17], with a one-molecule-thick coat. On the other hand, adsorption of further 150 million seminal plasma spermadhesin molecules on ejaculated spermatozoa (Table 1) may involve the formation of a ~8-molecule-thick coat over the sperm head. Since spermadhesins show a pronounced tendency to self-aggregation at pH above 5.0 (unpublished results), we speculate that this might be the driving force for adsorption. On the other hand, whether the first spermadhesin shell is attached to the sperm surface by specific membrane receptors or directly to lipid moieties, needs further investigation.

The first possibility is used in the binding of the major seminal plasma protein to bull ejaculated spermatozoa [29], while the latter case would resemble the binding of the sea urchin sperm-egg adhesion molecule bindin [30,31].

Interestingly, 75% of the total spermadhesin population on ejaculated sperm is subsequently released from the surface of capacitated spermatozoa (Table 1). Our working hypothesis is that a large amount of spermadhesin molecules, loosely attached to the sperm surface (spermadhesin-spermadhesin aggregation), may act as decapacitation, or acrosome stabilizing, factors, while the remaining spermadhesin pool may function as capacitation factors, i.e. by their heparin-binding activity [23] or, together with other sperm membrane-associated moieties, as primary receptors for zona pellucida glycoproteins [21].

Molecules with similar sperm coating properties as boar spermadhesins have been identified in a number of species. An acrosome stabilizing factor (ASF), a large glycoprotein (360 kDa) made up of two subunits (M_w 92000 and 38000), has been described in rabbit seminal plasma on the basis of its ability to reversibly alter sperm capacitation and block the in vitro induced acrosome reaction [32]. In the rat, the amount of the epididymal acidic glycoprotein DE (37 kDa, pI 4.9–5.1) [33,34] bound to spermatozoa increases progressively from the caput to the cauda epididymal spermatozoa [35]. Incubation of cauda epididymal spermatozoa in utero or in a capacitating medium for 6 h reduced the amount of bound protein to the level observed in caput sperm, suggesting that it may prevent the occurrence of premature acrosome reaction in the male genital tract. Protein DE bind to the plasma membrane in the acrosomal and postacrosomal region of cauda spermatozoa, and antibodies against it block fertilization [36]. Thus it appears that the epididymal protein DE might be a component of a sperm structure involved in the process of fertilization [37]. In man, a basic 140 kDa seminal vesicle protein (SBP) whose synthesis is androgen-dependent binds to spermatozoa at ejaculation [38,39]. SBP 140 kDa is immunologically related to rat seminal vesicle secretory (SVS) protein IV (17 kDa) [38,39], which also binds to spermatozoa. In the bovine, major acidic seminal plasma proteins BSP-A1/2 and BSP-A3 (PDC-109) (15–17 kDa) bind to spermatozoa at ejaculation and have been involved in sperm capacitation by heparin-like glycosaminoglycans [10]. Noteworthy, the amino acid sequences of rabbit ASF [40], rat DE protein [41], rat SVS-IV [42], and the bovine heparin-binding proteins BSP-A1/2 [43] and PDC-109 [44] are neither homologous between them nor show similarity to boar spermadhesins [21]. Thus, the exiting question of whether capacitation (i.e. acrosome stabilizing) factors are species-specific molecules, deserves a detailed investigation.

Acknowledgements

This work was financed by grants Tö 114/1–2 from the Deutsche Forschungsgemeinsam (E.T-P.) and PB92–0096 from the Dirección General de Investigación Científica y Técnica (J.J.C.). The excellent technical assistance of Ms. Diana Riedel and Vike Petersen-Mahrt is gratefully acknowledged.

References

[1] Yanagimachi, R. (1988) The physiology of reproduction (Knobil, E., Neill, J., Ewing, L.L., Greenwald, G.S., Markert, C.L. and Pfaff, D.W., eds.) Raven Press, New York, pp. 135–185
[2] Chang, M.C. (1951) Nature (London) 168, 697–698
[3] Austin, C.R. (1951) Austral. J. Sci. Res. B 4, 581–592.
[4] Shivaji, S., Scheit, K-H. and Bhargava, P.M. (1990) Proteins of Seminal Plasma, John Wiley and Sons, New York.
[5] Zaneveld, L.J.D., DeJonge, C.J., Anderson, R.A. and Mack, S.R. (1991) Human Reproduction 6, 1265–1274.
[6] Florman, H.M. and Babcock, D.F. (1991) Elements of Mammalian Fertilization (Wassarman, P.M., ed.) CRC Press, Boca Raton, Florida, pp. 105–132.
[7] Cross, N.L. (1993) Molec. Reprod. Develop. 35, 316–323.
[8] Florman, H.M. and First, N.L. (1988) Dev. Biol. 128, 464–473.
[9] Jones, R. (1989) Oxford Reviews of Reproductive Biology vol. 11 (Milligan, S.R., ed.), Oxford University Press, Oxford, pp. 285–337.
[10] Miller, D.J., Winner, M.A. and Ax, R.L. (1990) Biol. Reprod. 42, 899–915.
[11] Poirier, G.R., Robinson, R., Richardson, R., Hinds, K. and Clayton, D. (1986) Gamete Res. 14, 235–243.
[12] Benau, D.A. and Storey, B.T. (1987) Biol. Reprod. 36, 282–292.
[13] Irwin, M., Nicholson, N., Haywood, J.T. and Poirier, G.R. (1983) Biol. Reprod. 28, 1201–1206.
[14] Aarons, D., Speake, J.L. and Poirier, G.R. (1984) Biol. Reprod. 31, 811–817.
[15] Robinson, R., Richardson, R., Hinds, K., Clayton, D. and Poirier, G.R. (1987) Gamete Res. 16, 217–228.
[16] Boettger, H., Richardson, R., Free, D., Rushing, S. and Poirier, G.R. (1989) J. Exp. Zool. 249, 90–98.
[17] Sanz, L., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R., Amselgruber, W., Sinowatz, F., Ehrhard, M. and Töpfer-Petersen,E. (1992) FEBS Lett. 300: 213–218.
[18] Sanz, L., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R. and Töpfer-Petersen, E. (1991) FEBS Lett. 291, 33–36.
[19] Sanz, L., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R. and Töpfer-Petersen, E. (1992) Eur. J. Biochem. 205, 645–652.
[20] Sanz, L., Calvete, J.J., Schäfer, W., Mann, K. and Töpfer-Petersen, E. (1992) Biochim. Biophys. Acta 1119, 127–132.
[21] Calvete, J.J., Sanz, L. and Töpfer-Petersen, E. (1992) Spermatogenesis, fertilization, contraception. Molecular, cellular and endocrine events in male reproduction (Nieschlag, E. and Habernicht, U-F., eds.), pp 395–417, Springer Verlag, Berlin.
[22] Sanz, L., Calvete, J.J., Jonàkovà, V. and Töpfer-Petersen, E. (1992) FEBS Lett. 300, 63–66.
[23] Sanz, L., Calvete, J.J., Mann, K., Gabius, H-J. and Töpfer-Petersen, E. (1993) Molec. Reprod. Develop. 35, 37–43.
[24] Céchovà, D., Töpfer-Petersen, E. and Henschen, A. (1988) FEBS Lett. 241, 136–140.
[25] Jonàkovà, V., Sanz, L., Calvete, J.J., Henschen, A., Cechovà, D. and Töpfer-Petersen, E. (1991) FEBS Lett. 280, 183–186.
[26] Peterson, R.N., Russell, L.D., Bundman, D., Conway, M. and Freund, M. (1981) Dev. Biol. 84, 144–156.

[27] Lösch, U., Schranner, I., Wanke, R. and Jürgens, L. (1986) J. Vet. Med B 33, 609–619.
[28] Bork, P. and Beckmann, G. (1993) J. Mol. Biol. 231, 539–545.
[29] Aumüller, G., Vesper, M., Seitz, J., Kemme, M. and Scheit, K.H. (1988) Cell Tissue Res. 252, 377–384.
[30] Glabe, C.G. (1985) J. Cell Biol. 100, 794–799.
[31] Lopez, A., Miraglia, S.J. and Glabe, C.G. (1993) Dev. Biol. 156, 24–33.
[32] Oliphant, G., Reynolds, A.B. and Thomas, T.S. (1985) Am. J. Anat. 174, 269–283.
[33] Brooks, D.E. and Tiver, K.K. (1983) J. Reprod. Fertil. 69, 651–657.
[34] Brown, C.R., von Glos, K.I. and Jones, R. (1983) J. Cell Biol. 96, 256–264.
[35] Kohane, A.C., Echevarria, F.M.G., Pineiro, L. and Blaquier, J.A. (1980) Biol. Reprod. 23, 737–742.
[36] Cameo, M.S., Echeverria, F.M.G., Blaquier, J.A and Burgos, M.H. (1986) Gamete Res. 15, 247–257.
[37] Cuasnicu, P.S., Echeverria, F.M.G., Piazza, A.D., Cameo, M.S. and Blaquier, J.A. (1984) J. Reprod. Fertil. 72, 467–471.
[38] Abrescia, P., Lombardi, G., DeRosa, M., Quagliozzi, L., Guardiola, J. and Metafora, S. (1985) J. Reprod. Fertil. 73, 71–77.
[39] Metafora, S., Lombardi, S., DeRosa, M., Quagliozzi, L., Ravagnan, G., Peluso, G. and Abrescia, P. (1987) Gamete Res. 16, 229–241.
[40] Wilson, W.L. and Oliphant, G. (1987) Biol. Reprod. 37, 159–169.
[41] Brooks, D.E., Means, A.R., Wright, E.J., Singh, S.P. and Tiver, K.K. (1986) Eur. J. Biochem. 161, 13–18.
[42] Harris, S.E., Mansson, P-E., Tully, D.B. and Burkhart, B. (1983) Proc. Natl. Acad. Sci. USA 80, 6460–6464.
[43] Seidah, N.G., Manjunath, P., Rochemont, J., Sairam, M.R. and Chretien, M. (1987) Biochem. J. 243, 195–203.
[44] Esch, F.S., Ling, N.C., Bohlen, P., Ying, S.Y. and Guillemin, R. (1983) Biochem. Biophys. Res. Commun. 113, 861–867.