Role of a Capacitation-Related Protein on Some Sperm Functional Parameters

(boar sperm-ZP interaction / monoclonal antibody / sperm antigens)

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Abstract. In previous studies a series of Mabs against boar capacitated sperm have been produced. One of these Mabs – 4B12 – was found to recognize a surface membrane-associated protein located in the acrosome portion of the spermatozoa that became accessible to antibody after capacitation. In biological experiments it was shown that Mab 4B12 significantly inhibited boar sperm-porcine ZP binding. In attempts to investigate the mechanisms by which Mab 4B12 affected sperm-ZP binding, the role of the cognate protein on some functional parameters such as sperm motility and ability of the capacitated spermatozoa to undergo AR was studied. Experimental models of premature AR and AR physiologically induced with ZP were applied to study the effect of Mab 4B12 on boar sperm AR using PSA staining to estimate the acrosome-reacted state of spermatozoa. Sperm motility characteristics were determined by the time-exposure photokinesigraphic method. The results obtained in the present study, together with previously established inhibition of sperm-ZP binding by Mab 4B12, documented the participation of the 4B12 protein in primary sperm-ZP binding. The protein is not connected with sperm motility and secondary sperm-ZP binding.

The principal mechanisms of the phenomenon of fertilization are connected with expression of specific determinants on the sperm surface, their interaction with complementary sites on the surface of oocytes and activation of cellular effector systems which lead to such changes of gametes that are needed for fertililization (Yanagimachi, 1994). Investigations on specific and functionally active sperm antigens would bring about elucidation of the mechanisms of gamete recognition and would help in the search for new approaches to prognosis and regulation of fertility.

In boar, sperm zona-binding proteins belonging to the sperm adhesin protein family (Jonakova et al., 1991, Veselsky et al., 1999) and molecules that are either a part of the plasma membrane (Peterson et al., 1991, Capkova et al., 1997, Mollova et al., 1999) or of the acrosomal contents (Jones, 1991, Peknicova et al., 2001) have been considered to mediate the binding of acrosome-intact and acrosome-reacted sperm to the zona pellucida (ZP).

In our previous experiments, a series of monoclonal antibodies (Mabs) have been produced against boar capacitated sperm (Mollova et al., 1995, 1996, 1999). One of these Mabs – 4B12 – was found to recognize a surface membrane-associated protein (~300 kDa) located in the acrosome portion of the spermatozoa. This antibody recognized the protein specific for capacitated but not for freshly ejaculated spermatozoa. The protein corresponding to Mab 4B12 seems to be a conservative component common for different animal species. In biological experiments it was shown that Mab 4B12 inhibited boar sperm-porcine ZP binding (Mollova et al., 1996). In attempts to clarify the mechanisms by which Mab 4B12 inhibited sperm-ZP binding, the role of the 4B12 protein on some functional sperm parameters as well as on the earliest steps of gamete recognition was investigated. Since the effect of sperm parameters such as motility and premature acrosome reaction (AR) might result in impairment of the sperm ability to bind ZP (Lin et al., 1989), we studied the ability of its corresponding Mab to modify these sperm parameters. In order to further elucidate the role of protein 4B12 in the mechanisms of gamete interaction, the effect of Mab 4B12 on the ability of capacitated spermatozoa to undergo the AR triggered with ZP was also studied.

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Abbreviations: Ah – average lateral sperm head displacement, AR – acrosome reaction, BSA – bovine serum albumin, DMSO – dimethylsulphoxide, Mab – monoclonal antibody, PBS – phosphate-buffered saline, PSA – fluorescein-labelled *Pisum sativum* agglutinin, TBM – Tris-buffered medium, Vp – linear velocity of progression, ZP – zona pellucida.

Material and Methods

Preparation of spermatozoa

Samples of boar semen were collected from Large White boars by artificial vagina at the Camberow Meat Production Farm. Ten semen specimens were obtained from five donors and after washing in phosphate-buffered saline (PBS) were subjected to a swim-up separation of the sperm motile fractions. The fractions with highly motile spermatozoa were washed by centrifugation and sperm pellets were resuspended in Tris-buffered medium (TBM), pH 7.7, supplemented with 5 mg/ml bovine serum albumin – fraction V (BSA, Sigma Co., St. Louis, MO) to a final concentration of spermatozoa 1×10^8 cells/ml and incubated for 3 to 4 h at 37°C, 5% CO_2 to achieve capacitation. Mab 4B12 supernatant was added to spermatozoa (1 : 1 – vol/vol) 1 h before the end of capacitation. For appropriate controls, spermatozoa incubated in medium only or with the supernatant of myeloma cells (1 : 1 – vol/vol) were used. Aliquots of capacitated treated and untreated spermatozoa were processed for assessment of their motility parameters and acrosome state.

Indirect immunofluorescence (IIF)

The IIF was performed with live sperm suspension of capacitated boar spermatozoa. The samples were incubated for 1 h with hybridoma supernatant at 37°C and aliquots of sperm suspension were smeared onto glass slides. The smears were reacted with FITC-conjugated swine anti-mouse immunoglobulins (SEVAC, Prague, Czech Republic), diluted 1 : 20 in PBS for 45 min at 37°C, washed with PBS and water, and mounted in 50% glycerol in PBS, pH 9.0. For appropriate controls, smears were incubated with the supernatant of myeloma cells, and with the FITC-conjugate only.

Sperm motility analysis

Sperm motility characteristics were determined by time-exposure photokinesigraphy under dark-field microscopy, obtained by combining a single wreath-like phase diaphragm (corresponding to a 100x objective) with a 10x plan achromatic objective and a 8x applanate ocular fitted on a Zetopan-Reichert (Reichert, Vienna, Austria) microscope (Tchacarof and Nachev, 1964). Photoregistration of boar sperm motility was made in conditions described by Mollova et al. (1992). Each motile cell was visualized on the photokinesigrams as a trajectory which was used to measure the percentage of progressive spermatozoa, the linear velocity of progression (Vp), the average lateral sperm head displacement (Ah) and the nature of movement. At least ten photokinesigrams with more than 100 motile cells in each were analysed per sample to obtain statistically significant results.

ZP-induced acrosome reaction

Porcine oocytes were recovered from fresh ovaries (2 to 3 h after slaughter) by puncturing and aspirating 3 to 5 mm follicles, collected in 1% BSA-PBS medium, washed twice in 0.5% BSA-TBM and stored in 2 M dimethylsulphoxide (DMSO) in 0.5% BSA-PBS at –20°C until use. After thawing and washing in 0.5% BSA-TBM the oocytes were incubated within 1–2 h at 37°C, 5% CO_2, and only oocytes with intact ZPs were used for co-incubation (30 min at 37°C, 5% CO_2) with either controls or pretreated with Mab 4B12-capacitated boar spermatozoa ($1–2 \times 10^6$ cells/ml). At the end of co-incubation time, oocytes were processed to dissolve zonae for dispersion of tightly bound spermatozoa according to the method of Moohan and Lindsay (1995). Briefly, the ZPs were washed by vigorous pipetting to remove any loosely attached spermatozoa. The zonae were then placed individually in microwells and dissolved by exposure to acidified (pH < 2.0) medium to form a fluid monolayer and the number of acrosome-reacted spermatozoa in the monolayer was counted.

Assessment of acrosome status

The acrosome status of sperm was determined with fluorescein-labelled *Pisum sativum* agglutinin (PSA, Sigma) according to a modification (Mendoza et al., 1992) of the original method of Cross et al. (1986). Sperm smears were fixed in methanol for 30 s and then stained in 50 µg/ml PSA in PBS for 30 min at room temperature. The slides were then washed, allowed to dry and 200 sperm per sample were counted with a fluorescence microscope (Leitz, Vienna, Austria). The sperm that were stained throughout the whole acrosome region were scored as acrosome-intact. Sperm with a bright band of labelling at the equatorial segment or without fluorescence were considered as acrosome-reacted.

Statistical analysis was performed using unpaired Student's *t*-test.

Results

The protein recognized by Mab 4B12 was found to be located in the acrosome portion of the spermatozoa. It was shown that Mab 4B12 stained the acrosome of live capacitated spermatozoa quite intensively and regularly (Fig. 1). The spermatozoa from the same samples were negative after staining with supernatant of myeloma cells or with the FITC-conjugate only. Subsequently, the acrosome status of capacitated spermatozoa was followed in each experiment using FITC-conjugated PSA for staining of the acrosome. The quantitative representation of patterns visualized by this method (Fig. 2) showed that the majority of live capacitated spermatozoa (about 70–75%) were with intact acrosome.

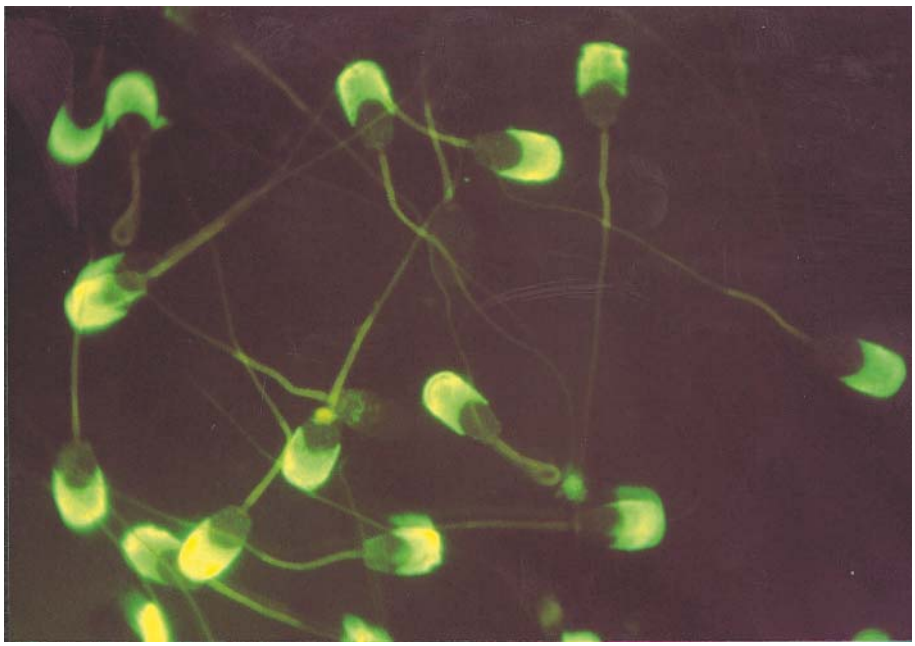

Fig. 1. Immunofluorescence staining of capacitated boar spermatozoa with Mab 4B12

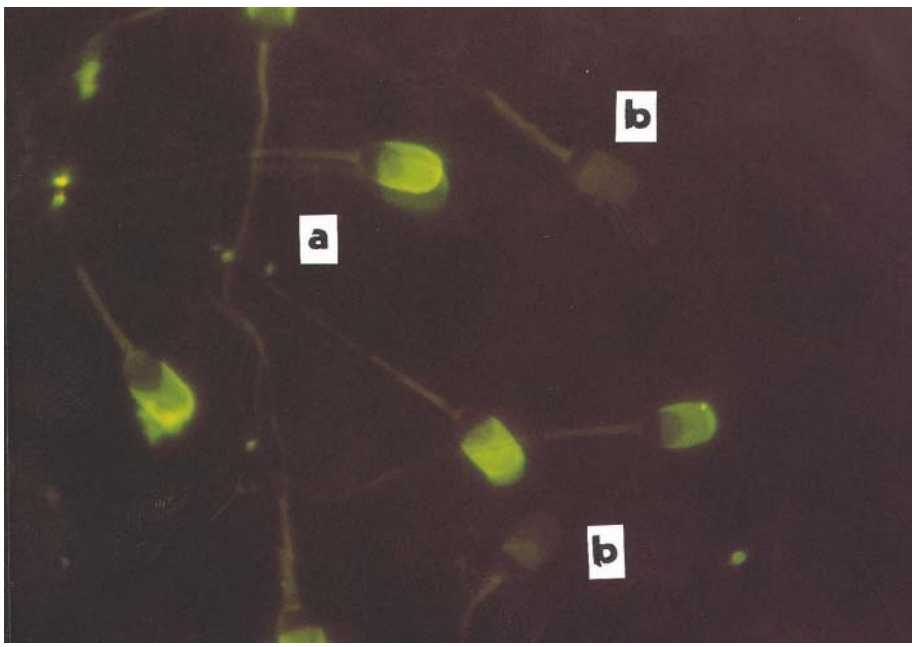

Fig. 2. Fluorescence patterns of capacitated boar spermatozoa visualized by PSA staining: acrosome-intact (a) and acrosome-reacted (b) spermatozoa

Table. 1. Sperm motility analysis after capacitation in vitro in the presence of monoclonal antibody 4B12

Parameter (%)	**Capacitated medium/treatment**		
	MoAb[a]	**Supernatant of myeloma cells**	**None**
Total motility	78.0 ± 0.63	73.34 ± 2.13*	76.49 ± 1.79*
Progressive helicoidal motility	15.86 ± 2.42	14.65 ± 2.91*	15.18 ± 2.10*
Progressive non-helicoidal motility	7.14 ± 2.11	8.06 ± 1.16*	6.65 ± 1.40*
Hyperactivated "whiplash-like" motility	44.16 ± 2.56	42.80 ± 0.40*	46.10 ± 2.77*
Mean velocity of progression (Vp) (mm/sec)	53.47 ± 0.76	51.11 ± 1.17*	51.24 ± 0.65*

[a]Differences between the values for Mab 4B12-treated samples and those for controls determined using Student's t-test, * $P > 0.05$, $N = 10$

The effect of Mab 4B12 on sperm motility parameters was investigated. It was found that in Mab-free capacitation medium, 76.49% of the spermatozoa manifested progressive movement (Table 1). Among them, 15.18% moved with helicoidal trajectories, 6.65% with non-helicoidal and 46.10% with trajectories typical for hyperactivated sperm. Mab 4B12 did not significantly modify the percentages of either total sperm motility (78%) or progressive motility (15.86% helicoidal, 7.14% non-helicoidal and 44.16% hyperactivated

Table. 2. Effect of monoclonal antibody 4B12 on spontaneous and ZP-induced AR

Capacitated medium/treatment	AR (%)	
	Spontaneous	ZP-induced
Mab 4B12[a]	22.53 ± 0.36	48.95 ± 1.81
Supernatant of myeloma cells	20.88 ± 0.78*	52.78 ± 3.26*
Without treatment	21.58 ± 0.26*	50.19 ± 3.15*

[a]Differences between the values for Mab 4B12-treated samples and those for controls determined using Student's t-test, * $P > 0.05$, $N = 10$

sperm populations, $P > 0.05$). Similar statistically non-significant changes in motility parameters studied were recorded when supernatant of myeloma cells was used as a control ($P > 0.05$).

In order to investigate the ability of Mab 4B12 to trigger premature acrosome reaction, boar spermatozoa were incubated for capacitation in the presence of antibody and were stained with PSA to examine the proportion of acrosome-reacted cells. As it is demonstrated in Table 2, the presence of Mab 4B12 did not alter the percentage of spontaneous acrosome-reacted sperm (22.53% for Mab-treated versus 21.58% and 20.88% for the untreated spermatozoa and spermatozoa treated with supernatant of myeloma cells, respectively, $P > 0.05$).

The effect of Mab 4B12 on ZP-induced AR was investigated by co-incubation of antibody-pretreated boar spermatozoa with intact porcine oocytes. It was observed (Table 2) that 48.95% from the population of tightly bound sperm treated with Mab 4B12 have undergone acrosomal exocytosis on the surface of ZP. Similar, not significantly different values of spermatozoa-induced AR on the surface of ZP were observed for controls – untreated sperm samples and sperm samples treated with supernatant of myeloma cells (50.19% and 52.78%, respectively, $P > 0.05$).

Discussion

Before asserting that protein 4B12 is specifically involved in ZP binding we studied the ability of its corresponding Mab to impair sperm motility and induction of premature AR. Mab 4B12 did not cause any perceptible agglutination and immobilization of spermatozoa (Mollova et al., 1996). To investigate further whether the effect of antibody on sperm-ZP binding is linked to sperm motility, various kinetic parameters of sperm were studied by the photokinesigraphic method, some of which correspond to the hyperactivated state of sperm. Hyperactivated sperm manifested low forward progressive movement with marked lateral head displacement and "whiplash" trajectory (Yanagimachi, 1994). Using time-exposure photokinesigraphy, it was possible to registrate hyperactivated boar sperm movement patterns observed to increase under capacitating conditions and described elsewhere (Mollova et al., 1992). The results from photokinesigraphic analysis of sperm motility in the presence of Mab 4B12 showed no statistically significant changes in the percentage of progressively motile helicoidal, non-helicoidal and hyperactivated sperm as well as in the linear velocity of progression in comparison to control sperm samples used. The data give evidence that Mab 4B12 did not modulate sperm parameters that account for sperm movement and for the hyperactivation phenomenon.

Another factor such as premature AR may affect the sperm ability to bind ZP. AR is a stimulus-secretion coupled exocytotic event where the outer acrosomal membrane fuses with the plasma membrane over the entire apical region of the sperm head (Brucker and Lipford, 1995). During *in vitro* capacitation, the presence of stimulating agents in the medium could provoke spontaneous acrosome reaction. Unlike ZP-induced AR, the spontaneous acrosomal exocytosis in capacitation conditions is a non-physiological event (Kopf and Gerton, 1991), and it might influence negatively on zona binding and on the penetrating capability of sperm due to premature loss of membrane structures and acrosomal content. Our results demonstrated that Mab 4B12 did not modify the occurence of spontaneous AR in boar spermatozoa.

A body of evidence has been accumulated for the inhibition of AR by antibodies against sperm antigens (Brucker et al., 1992). The possible mechanisms by which this inhibitory effect occurs included indirect interaction with molecules crucial for the AR or blocking of the early steps of acrosomal exocytosis. As ZP should be used as the physiological inducer to study the mechanisms of AR (Liu et al., 2002), the effect of Mab 4B12 on sperm-porcine ZP-triggered AR was investigated. Our results demonstrating that the occurrence of ZP-induced AR was not affected by the presence of antibody suggest that protein 4B12 does not have a functional role in sperm acrosome exocytosis.

The data obtained in the present study did not give ground for suggesting that the 4B12 protein might be involved in the mechanisms regulating motility and acrosome exocytosis in boar spermatozoa. Although a possible inhibition of sperm-ZP binding due to steric hindrance by the antibody cannot be excluded, it is tempting to suggest that boar sperm-zona binding inhibition by Mab 4B12 may be due primarily to the blocking of a fertilization-relevant sperm molecule rather

than to a direct influence on sperm functional parameters.

In conclusion, the results reported here show that Mab 4B12 did not modify the motility parameters and the occurrence of spontaneous AR in capacitated boar sperm. Also, the antibody did not affect the ability of capacitated sperm to undergo an AR induced by ZP. We suppose that protein 4B12, which is exposed during capacitation, participates in the primary binding of sperm to ZP. We have documented that this protein is not connected with motility and secondary sperm-ZP binding as well as with the AR of spermatozoa. The role of the 4B12 protein in the early steps of fertilization will be further investigated.

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