Biochemical and immunochemical characterization of boar sperm flagellar protein with role in hyperactivation/capacitation process

Margarita Mollova¹, Bojko Atanassov, Ralitza Nedkova, Stanimir Kyurkchiev
Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences, Sofia, Bulgaria

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SUMMARY

Investigations on specific and functionally active sperm antigens could bring about the elucidation of the mechanisms of gamete interaction and help the search for new approaches in prognosis and regulation of fertility. Previously, we reported that the monoclonal antibody (Mab) 3G4 against capacitated boar spermatozoa was capable of inhibiting boar sperm-porcine zona pellucida binding due to its inhibitory effect on sperm hyperactivation and capacitation. The cell and tissue specificity of Mab 3G4 was demonstrated in indirect immunofluorescence (IIF) and ELISA experiments against spermatozoa from different vertebrate species, as well as against extracts of boar reproductive and somatic organs. In the present IIF experiments, it was shown that Mab 3G4 recognized an antigen determinant on the flagellar midpiece region of ejaculated and capacitated boar spermatozoa. It was speculated that the Mab 3G4-
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Corresponding antigen participates in pyruvate/lactate metabolism because of its specific localization in the sperm structure, which is responsible for producing forward motility and its involvement in processes that require the metabolism of pyruvate and lactate. As a possible approach toward investigating the participation of Ag 3G4 in pyruvate/lactate metabolism, Mab 3G4’s effect on lactate dehydrogenase (LDH) was examined. Using an electrophoretic approach we provided evidence that Mab 3G4 stimulates LDH activity in the Triton X-100 and NP40 protein fractions of capacitated boar spermatozoa. In addition, we found that LDH isoenzymes stimulated by Mab 3G4 are of gametic C type. In Western blot, under nonreducing conditions, Mab 3G4 identified a single protein band with a molecular weight of 140 kDa. The biochemical and immunochemical experiments provided evidence supporting the involvement of 3G4 antigen in the sperm pyruvate/lactate metabolism. Reproductive Biology 2006 6 (1):79–94.

Key words: boar spermatozoa, sperm flagellar antigens, monoclonal antibody, LDH

INTRODUCTION

Before fertilization, mammalian spermatozoa acquire the ability to fertilize eggs. This phenomenon is called capacitation. Sperm capacitation involves a series of molecular and cellular changes, including an influx of calcium ions, an increase in cAMP levels, an increase in membrane fluidity due to a cholesterol efflux [12, 37], an increase in protein tyrosine phosphorylation [36] and an increase in actin polymerization [7]. Although the precise role of these changes in capacitation has not yet been clarified, they may facilitate two functions that appear to be essential for fertilization: the acrosome reaction and hyperactivated motility of spermatozoa [37]. Hyperactivated sperm motility is characterized with high-amplitude and asymmetrical flagellar beating that accompanies capacitation in many species and assists spermatozoa in penetrating the oocyte’s zona pellucida [26, 37].

Monoclonal antibodies (Mabs) produced in our laboratory are used as specific probes for detecting and characterizing components specific for
capacitated boar spermatozoa [29, 30, 31]. One of these, Mab 3G4 was found to recognize an antigen on the midpiece structure of boar spermatozoa. In biological experiments, it was shown that Mab 3G4 inhibits boar sperm-porcine zona pellucida (ZP) binding in vitro\(^1\), which can be explained by its inhibitory effect on the hyperactivated/capacitated state of spermatozoa\(^2\). The cell and tissue specificity of Mab 3G4 has been demonstrated in indirect immunofluorescence (IIF) and ELISA experiments against spermatozoa from different vertebrate species, as well as against saline extracts of boar reproductive and somatic organs [33]. The midpiece is a sperm structure that is involved in motility, hyperactivation and capacitation - the processes requiring the pyruvate and lactate metabolism [34, 37]. Due to Mab 3G4’s specific localization, a role for its corresponding antigen in pyruvate/lactate metabolism is suggested. As a possible approach toward investigating the participation of antigen 3G4 in pyruvate/lactate metabolism, the effect of Mab 3G4 on lactate dehydrogenase (LDH) was examined.

**MATERIALS AND METHODS**

**Preparation of spermatozoa**

Boar semen was collected at the Camberow Meat Production Farm from Large White boars by artificial vagina. Samples of donor semen were centrifuged in discontinuous multiple step (80%, 70%, 55%, 40%) Percoll gradient (Pharmacia, Fine Chemicals AB, Uppsala, Sweden), at 300×g for 20 min at room temperature (RT). After washing, the 80% gradient layer semen pellet was resuspended in Tris-buffer medium (TBM, pH 7.7; [1]), supplemented with 5 mg/ml bovine serum albumin (BSA, fatty-acid free-Sigma Co., USA) to a final concentration of spermatozoa 1-2×10\(^8\) cells/ml and incubated for 3-4 h at 37ºC (95% air, 5% CO\(_2\)) in order to


achieve capacitation. Sperm capacitation status was determined using the time-exposure photokinesigraphic method, reported earlier [28], which allows registration of hyperactive sperm movement patterns increasing under capacitating condition. Aliquots of capacitated spermatozoa were processed for immunofluorescent and cytochemical investigations. The other part was used for isolation of sperm proteins and their biochemical and immunochemical characterization.

**Indirect Immunofluorescence**

The IIF was performed with live ejaculated, live capacitated and fixed capacitated boar spermatozoa for immunolocalization of Mab 3G4 corresponding antigen. A part of the semen samples with live sperm cells was mixed with an equal volume of Mab 3G4 supernatant (v/v), incubated for 1 h at 37°C and after washing aliquots of sperm suspension were smeared onto glass slides. The remaining part of semen samples after washing was smeared onto glass slides, fixed in acetone for 10 min at RT and incubated with antibody under the same conditions. The smears were then allowed to react (45 min, 37°C) with FITC-conjugated swine anti-mouse immunoglobulins (SEVAC, Prague, Czech Republic, 1:20), washed with phosphate-buffered saline (PBS) 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.

**Cytochemical analysis**

LDH enzyme activity was investigated cytochemically [19]. Semen samples, both capacitated control (untreated) and treated with Mab 3G4, were washed twice in PBS (1000×g, 10 min) and smeared on microslides. The smears were covered with 100-200 µl incubation medium containing 0.2 M Tris/HCl (pH 7.4), 1.0 M sodium lactate, 0.1 M 4-nitroblue tetrazolium chloride (NBT) and 0.2 M nicotinamide adenine dinucleotide (NAD; Serva, Germany) for 30 min at 37°C, washed with distilled water and dried. The color product of the reaction, manifested as formazan granules in the midpiece portion of
spermatozoa was observed under a Zetopan microscope (Reichert, Vienna, Austria). A total amount of 200 cells were analyzed per slide.

**Extraction of sperm proteins**

The electrophoretic pattern of LDH were determined in the proteins of water soluble, Triton X-100 and NP40 extracts of capacitated boar spermatozoa. Capacitated semen samples were washed three times in PBS. A part of sperm pellet was resuspended in distilled water, disintegrated by sonification for $3 \times 10^3$ s (150 W MSE Ultrasonic disintegrator) and frozen (–20°C) for 12 h. After thawing, sperm samples were centrifuged (12 000×g, 30 min, 4°C) and clear supernatant (water soluble protein fraction) was collected and stored at –80°C in the presence of 0.2 mM proteinase inhibitor, phenylmethyl-sulfonylfluoride (PMSF; Sigma, St. Louis, USA) until use. The sperm sediment was subjected to an additional second extraction with 1% solution of Triton X-100 in 0.15 M NaCl for 30 min; then the suspension was centrifuged again and the supernatant containing the additionally extracted and strongly bound proteins (sedimentary protein fraction) was collected and stored at the same conditions [16]. Nonidet P40 (NP40, Sigma, St. Louis, USA) was used for extraction of sperm membrane protein in the following manner: washed spermatozoa were resuspended in PBS containing 1% NP40 detergent and 1 mM PMSF, incubated for 1 h at 4°C and centrifuged at 10 000×g for 10 min to remove insoluble material. NP40 sperm extracts were stored at –80°C until use [21].

Mab 3G4 supernatant (3 mg/ml total protein) was added to an equal amount of boar semen samples or sperm extracts and incubated for 1 h at 37°C before investigation. In some experiments, control Mab with unrelated specificity (anti-human chorion gonadotropin Mab 1B10) at the same protein concentration was used in parallel control experiments. The total protein content of the Mab 3G4 supernatant was measured by the method of Lowry et al [25].

**Electrophoretic analysis**

The molecular forms of the enzyme were separated by the anode system of Davis [11], on polyacrylamide gel in tubes. Briefly, water soluble, Triton
X-100 and NP40 protein extracts from untreated and treated with Mab 3G4 spermatozoa were run in 7.5% gel tube at 2.5 mA per tube at 4°C. Each sample was loaded into a self-dependent tube with equal protein contents (30 µg/ml). The isoenzyme pattern was demonstrated using the routine buffer medium with sodium lactate as a substrate (0.05 M Tris/HCl buffer containing 0.1 M NBT, 0.5 M NAD, 0.1 M sodium DL lactate, 0.1 M phenazin metasulfate, pH 8.5). The C type of LDH was detected by changing the incubation conditions as follows: a/ warming up the gel at 65°C for 30 min after electrophoresis which resulted in the inactivation of A and B subunits [27]; b/ incubation of the gel in the medium containing 2-oxohexanoate (5.0 mM). This reagent is used as a specific isoenzyme substrate instead of sodium lactate, which gives a selective estimation of LDH-C₄ molecular form [8].

SDS-PAGE and Western blot analysis

The NP40 extract of capacitated boar spermatozoa was mixed with 2×Laemmli sample buffer and loaded onto 12% polyacrylamide gels (Hoefer miniVE, Vertical Electrophoresis System, Amersham, England) following the procedure described by Laemmli [24] under nonreducing conditions. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, S. Louis, USA). After electrophoresis, proteins were electrophoretically transferred [35] to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat dry milk overnight at 4°C. Then, it was washed in PBS with 0.05% Tween 20 and incubated with Mab 3G4 as first antibody or with Mab 1B10 (with unrelated specificity, a negative control) in the form of undiluted supernatants. After extensive washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse antibody (BioRad, Prague, Czech Republic) diluted 1:5000 in blocking solution. The membrane was washed again in Tween-PBS and a color reaction developed with a substrate mixture containing 0.6% 4-chloro-l-naphthol, 0.2% diaminobenzidine and 0.015% H₂O₂. Wide molecular weight electrophoresis calibration kit (Sigma, St. Louis, USA) was run in
the same slab gels in order to determine the relative molecular mass of the analyzed proteins.

RESULTS

Immunolocalization of Mab 3G4 corresponding antigen

The immunofluorescence reactivity of Mab 3G4 with live and fixed ejaculated and capacitated boar spermatozoa was investigated. The antigen recognized by Mab 3G4 was available for the antibody without permeabilization of the sperm membrane, indicating surface localization of this antigen. Mab 3G4 staining was observed in the midpiece portion of boar sperm cells. A strong fluorescent reaction was observed in the

![Figure 1. Indirect immunofluorescence of boar a/live ejaculated, b/live capacitated, and c/ fixed capacitated spermatozoa with Mab 3G4. Bright fluorescence in the midpiece portion of the sperm flagellum is seen (arrows). Control semen samples were negative for specific immunofluorescence staining (d).](image-url)
midpiece of live ejaculated (fig.1a), live capacitated (fig.1b) as well as of fixed capacitated spermatozoa (fig.1c). The spermatozoa from the same samples were negative for specific immunofluorescence with supernatant of myeloma cells or with the FITC-conjugate used as controls (fig.1d).

**Effect of Mab 3G4 on sperm LDH activity**

The effect of Mab 3G4 on LDH was studied using a cytochemical procedure. The color product of the enzyme reaction catalyzed by LDH

Figure 2. Cytochemical localization of LDH enzyme activity in boar a/ capacitated untreated, and b/ treated with Mab 3G4 spermatozoa. Intensified colored reaction is manifested in the midpiece portion of treated with Mab 3G4 spermatozoa (arrows).
makes it possible to determine the difference in the enzyme activity of antibody treated and nontreated semen samples. A more intensive reaction of sperm midpiece was revealed in the presence of the antibody, represented by darker and bigger formazan granules formed (fig. 2b) in comparison to those observed in sperm cells not treated with the antibody (fig. 2a).

The isoenzyme pattern of LDH and the effect of Mab 3G4 on the LDH molecular forms identified in sperm protein extracts incubated in sodium lactate as oxidative substrate is demonstrated in Figure 3. The isoenzyme forms of the water-soluble fraction (fig. 3a) were not affected by the antibody (fig. 3a'). Mab 3G4 was found to stimulate the activity of two isoenzymes in Triton X-100 extract (fig. 3 b-b'1) and one isoenzyme of NP40 extract (fig. 3 c-c'1) without changing LDH migration rates

Figure 3. Disc electrophoretic pattern of lactate dehydrogenase (LDH) in sperm protein fractions incubated in sodium lactate medium. a, b, c: control untreated samples; a', b', c': spermatozoa treated with Mab 3G4; arrows show isoenzyme activity affected by the antibody.

Figure 4. Disc electrophoretic pattern of lactate dehydrogenase (LDH) in sperm protein fractions heating for 30 min (65°C) after electrophoresis and incubated in normal medium with sodium lactate. a, b, c: control untreated samples; a', b', c': spermatozoa treated with Mab 3G4; arrows show isoenzyme activity affected by the antibody.
during electrophoresis. When gels were heated at 65°C for 30 min after electrophoresis and incubated in normal medium with sodium lactate, the stimulated isoenzymes of Triton X-100 and NP40 extracts were found to be identified in sperm extracts both untreated (fig. 4b, c) and treated with Mab 3G4 (fig. 4b\(_1\), c\(_1\)).

Similar result was observed when the extracts were incubated in a medium containing 2-oxohexonate (fig. 5). No enzyme activity was detected in water soluble protein fraction (fig. 5a, a\(_1\)). The same two isoenzymes of Triton X-100 extract (fig. 5b) and one isoenzyme of NP40 extract (fig. 5c) was visible. These bands were intensified after treating spermatozoa with the antibody (fig. 5b\(_1\), c\(_1\)).

**Figure 5.** Disc electrophoretic pattern of lactate dehydrogenase (LDH) in sperm protein fractions incubated in 2-oxohexonate medium. a, b, c: control untreated samples; a\(_1\), b\(_1\), c\(_1\): spermatozoa treated with Mab 3G4; arrows show isoenzyme activity affected by the antibody.
Immunochemical characterization of Mab 3G4 corresponding antigen

The reactivity of Mab 3G4 against capacitated boar spermatozoa was analyzed by Western blotting which identified specific proteins recognized by the monoclonal antibody. After SDS-PAGE of NP40 extracts from capacitated boar spermatozoa under non-reducing conditions, a number of protein bands in the range from Mr 205 to 36 kDa were observed after staining with Comassie Brilliant Blue (CBB; fig. 6, lane a). After Western blotting, Mab 3G4 identified a single specific protein band with a molecular weight of 140 kDa (fig. 6, lane d). No staining with a control antibody (of different antigenic specificity; fig. 6, lane e) was observed.

*Figure 6. SDS-PAGE and Western blot of boar sperm NP-40 protein fraction using Mab 3G4. Lines: a/ SDS-PAGE of NP-40 extract from capacitated spermatozoa, b and c/ molecular markers, d/ Western blot of NP-40 extract from capacitated spermatozoa with Mab 3G4, e/ Western blot of NP-40 extract from capacitated spermatozoa with Mab 1B10. In Western blotting, Mab 3G4 identified a single specific protein band with molecular weight of 140 kDa.*
DISCUSSION

Mab 3G4 was selected from a panel of monoclonal antibodies produced in our laboratory against capacitated boar spermatozoa [29, 30, 31]. In the present IIF experiments, it was shown that Mab 3G4 reacted with a flagellar structure that is located in the midpiece region of the live and fixed ejaculated and capacitated spermatozoa. The sperm flagellum is responsible for producing forward motility through the coordinated activities of specific organelles such as the mitochondria in the midpiece, microtubules of the axoneme and other cytoskeletal components operating in conjunction with physiological factors such as adenylate cyclase and cAMP-depended kinases [37]. Our previous experiments showed that Mab 3G4 significantly inhibited boar sperm-porcine ZP binding due to its inhibitory effect on sperm hyperactivation.

Although physiological functions of hyperactive motility have been established, little is known about its initiating mechanism. It is proposed that physiological factors, such as Ca$^{2+}$, cAMP, bicarbonate and metabolic substrates, are essential for the initiation and maintenance of hyperactivated motility in vitro [18]. It was demonstrated that hyperactivation is tightly correlated with capacitation [36] and these processes require pyruvate and lactate [34, 37]. The data on midpiece localization of Mab 3G4 cognate antigen together with its role in sperm hyperactivation/capacitation suggest a role for this antigen in pyruvate/lactate metabolism. This assumption was verified by the electrophoretic analysis of lactate dehydrogenase activity in protein extracts from Mab 3G4-treated and untreated spermatozoa.

Lactate dehydrogenase (E.C.1.1.1.27) is an oxydoreductase which plays a key role in cellular metabolism by regulating the pyruvate: lactate and NAD:NADH ratio. In somatic tissues and organs, five isoenzyme forms of LDH were found [17], each representing a tetramer formed by A and B

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types of a polypeptide chain. Goldberg [14] and Blanko and Zinkham [4] demonstrated that in the testis of human and other mammalian as well as avian species there is a new additional isoenzyme of LDH, termed LDH-C₄. Homotetrameric LDH-C₄ units are synthesized under the control of distinct genetic locus [5] and function in lactate metabolism and glycolysis of developing and mature spermatozoa [15, 23]. Investigations on boar spermatozoa [10, 22] indicated the participation of LDH-C₄ in a shuttle system utilizing the redox couple lactate/pyruvate to transfer H⁺ from cytosol to mitochondria, a process known to occur in mouse [9], rat and rabbit spermatozoa as well [13].

The activity and the electrophoretic pattern of LDH in this study were determined in the proteins of water soluble, Triton X-100 and NP40 extracts of capacitated boar spermatozoa, representing respectively soluble, sedimentary and membrane forms of the enzyme. Electrophoretic analysis of the soluble, sedimentary and membrane forms of LDH demonstrated different electrophoretic patterns and showed that only sedimentary and membrane forms of the enzyme are affected by Mab 3G4. It was also demonstrated that the affected sedimentary and membrane isoforms of the LDH did not change their configuration and activity after warming and incubation in specific for LDH-C₄ medium. Owing to this, it is assumed that the isoenzymes affected by the antibody are of gametic C type. In Western blot analysis under nonreducing conditions, Mab 3G4 clearly identified a single protein band with a molecular weight of 140 kDa. Since the molecular mass of the tetramer form of LDH is 140 kDa [6, 32], the data from immunoblotting appears to confirm this assumption.

In our experiments, Mab 3G4 appears to recognize LDH-C₄ proteins in 1% Triton X-100 fraction that represents strongly bound proteins (mainly from the mitochondria), and in NP40 fraction that represents the plasma membrane proteins of spermatozoa. Burgos et al [9] demonstrated dual localization of LDH-C₄ in the cytosol and in the matrix of sperm-type mitochondria in the middle piece of spermatozoa. Our results on the existence of a membrane form of the enzyme are in accordance with the results of Beyler and Goldberg [2] who identified LDH-C₄ on the surface of human and murine sperm, using a solid phase radioimmunoassay with

In conclusion, the data reported here show that Mab 3G4 enhances the catalytic activity of LDH-C4 isoenzymes in Triton X-100 and NP40 protein extracts of boar capacitated spermatozoa. In Western blot under nonreducing conditions Mab 3G4 identified a single protein band with a molecular weight of 140 kDa. The biochemical and immunochemical experiments provided evidence supporting the involvement of 3G4 antigen in the sperm pyruvate/lactate metabolism. Further experiments are in progress on studying the mechanism of this antigen action in sperm capacitation and fertilization process.

REFERENCES


