

SOB3, a Human Sperm Protein Involved in Zona Pellucida Binding: Physiological and Biochemical Analysis, Purification

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ABSTRACT LB5 antibody was selected from a monoclonal antibody (mAb) library directed against human sperm proteins. LB5 mAb detected the corresponding protein SOB3 in the neck region and the flagellum of most live ejaculated sperm while it labelled, in addition, the acrosome of about 10–20% of spermatozoa. The percentage of LB5 acrosome-stained sperm was significantly correlated with the percentages of either spontaneous or A23187-induced acrosome-reacted sperm. While SOB3 could not be detected in the testis, it appeared in spermatozoa from the corpus epididymis segment. LB5 mAb impaired neither sperm motion parameters, acrosomal reaction triggering, nor sperm binding to zona-free hamster oocytes. By contrast, LB5 Fab fragments (200 µg/ml) inhibited sperm binding to human zonae pellicidae by 35.7%. If sperm were induced to acrosome react with A23187 prior to LB5 treatment, the inhibitory effect shifted to 59.9%, while no significant effect was observed following A23187 incubation alone. Western blotting of human sperm and cauda epididymis extracts revealed two bands of 18 and 19 kDa. While no cross-reaction was observed with other tested organs, a similar 18-kDa band was revealed in erythrocytes and one of 19 kDa in B-lymphocytes. No cross-reactivity could be evidenced in any animal sperm analyzed. SOB3 was first separated in a 17- to 20-kDa preparative electrophoresis fraction and finally purified by isoelectrofocusing according to its pI of 9.8. These results suggest that SOB3 is localized under the outer acrosomal membrane, that it participates in secondary sperm binding to the zona pellucida, and that it shares homologies with the immune system. *Mol. Reprod. Dev.* 49:286–297, 1998. © 1998 Wiley-Liss, Inc.

Key Words: fertilization; recognition protein; secondary binding

INTRODUCTION

Before reaching the egg plasma membrane, the mammalian spermatozoon must cross the two envelopes surrounding the egg, the cumulus oophorus and the

zona pellucida (ZP). Only capacitated and acrosome-intact sperm seem to be allowed to pass through the cumulus barrier (Cummins and Yanagimachi, 1986). In guinea pigs, the hyaluronidase activity of PH-20, a sperm membrane protein, is required for acrosome-intact sperm to penetrate the cumulus cell layer (Lin et al., 1994; Hunnicut et al., 1996).

Sperm binding to ZP—a species-specific mechanism—has been studied most extensively in the mouse. Schematically, ZP3, one of the three proteins constituting the extracellular coat, initiates binding to the sperm plasma membrane and induces the acrosome reaction (AR) and exocytosis. The release of its proteolytic enzymes allows the spermatozoon to penetrate the egg matrix and fuse with the plasma membrane. If, with respect to the egg, the situation seems relatively simple, that of its sperm counterpart is much more complex. In the rat, several sperm proteins have been identified as potential receptors of ZP3; they have been shown to bind to ZP3 oligosaccharides and/or to elicit intracellular signals. A 95-kDa mouse sperm protein has been identified as a tyrosine kinase-linked receptor by Leyton et al. (1992). Sp56, a galactosyl receptor, has been isolated by means of cross-linking to mouse ZP3 (Cheng et al., 1994). Gene cloning and sequencing have shown Sp56 to belong to a superfamily of receptors that includes the alpha subunit of the complement 4B binding protein (Bookbinder et al., 1995). Furthermore, the aggregation of mouse sperm galactosyltransferase subsequent to binding to ZP3 oligosaccharides has been shown to induce the acrosome reaction through the activation of a heterotrimeric G-protein complex (Gong et al., 1995). Moreover, Sp17, a rabbit sperm protein, is thought to act as a lectin-like molecule that binds to ZP3 carbohydrate (Richardson et al., 1994). In addition, important data from Wassarman's group suggest that another egg protein, ZP2, enables maintenance of attachment of acrosome-reacted sperm to the egg via its

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binding to a sperm trypsin-like protein (Bleil et al., 1988). Several studies performed in boar and rabbit have shown that the enzymatically inactive zymogen proacrosin, which is localized in the acrosomal matrix, exhibits a ZP binding activity (Mori et al., 1995; Richardson and O'Rand, 1996). Furthermore, PH-20, once cleaved and localized on the inner acrosomal membrane after acrosome reaction, expresses its second function, which is participation in this secondary sperm-zona binding (Hunnicut et al., 1996). Taken together, these data suggest a complex cascade of events, each requiring a different sperm protein or proteic domain able to react with a specific ZP component.

The molecular bases of sperm-zona interactions are still far from having been resolved in humans. The gene for the human protein homologue of the murine sperm tyrosine kinase has been cloned recently (Burks et al., 1995). Boué and Sullivan (1996) have shown that certain cases of male infertility are associated with a failure to synthesise P34H, the human equivalent of P26h, a hamster epididymal sperm protein that binds to ZP.

In order to identify human sperm proteins involved in sperm-oocyte interaction, we had previously generated monoclonal antibodies (mAb) directed against sperm proteins. Here we report that one of them, LB5, binds to the acrosomal region of human sperm and inhibits *in vitro* sperm binding to ZP without affecting other sperm parameters and fertilization steps. The biochemical characterization and the purification of the corresponding protein, SOB3 (sperm oocyte binding 3), via preparative electrophoresis and isoelectrofocusing, are described.

MATERIALS AND METHODS

LB5 Antibody

The LB5 antibody was selected from an mAb library raised against human sperm proteins as described by Boué et al. (1995). A preliminary panel of antibodies directed against proteins involved in sperm-ZP recognition was selected, and from this panel, LB5 was chosen for large-scale production by hybridoma culture in Minimum Essential Medium (MEM) supplemented with 15% de complemented fetal calf serum (Gibco Laboratory, Grand Island, NY) and by obtaining fluid ascites from BALB/c mice sensitized with pristane (Aldrich Chemical Co., Milwaukee, WI). The antibody concentration of the hybridoma supernatant used in the different protocols was 8.6 µg/ml. It was shown to belong to the IgG₁ subclass. G12 and CA6, two mouse IgG₁s, were used as negative controls in the form of hybridoma supernatants, purified IgG₁, or Fab fragments in experiments to evaluate the effect of LB5 on sperm fertilizing ability; both have been shown to specifically impair sperm binding to ZP-free hamster eggs, and the most effective concentration of G12 mAb was 200 µg/ml (Boué et al., 1992, 1995; Lefèvre et al., 1997).

Immunochemical Tissue Staining

Human epididymides and testes obtained from a transplant program were removed from three subjects; none of them had received hormonal treatment. Epididymides were dissected in three regions: caput, corpus, and cauda. Tissue fragments were immediately frozen in liquid nitrogen, embedded at -30°C in Tissue Tek II medium (Miles, Elkhart, IN), and stored at -80°C. Then 5-µm-thick sections were cut with a cryotome and placed on slides that were air dried, fixed with methanol at -20°C for 10 min, and stored at -20°C. Antigen localization was performed using an avidin-biotin peroxidase staining procedure (Vectastain ABC Kit, Vector Labs, Burlingame, CA). After incubation for 20 min with 10% goat serum in phosphate-buffered saline (PBS) to eliminate nonspecific binding, sections were treated with either LB5 or a nonsecreting supernatant for 1 hr at room temperature. They were washed three times with PBS, incubated for 30 min with goat biotinylated antimouse IgG (Jackson ImmunoResearch Labs, West Grove, PA), washed again three times with PBS, and then incubated for 30 min with Vectastain ABC reagent. After three washings in PBS, the reaction was developed with 118 mM amino ethyl carbazole (AEC) (Sigma Chemical Co., St. Louis, MO) as peroxidase substrate plus 0.02% H₂O₂. Finally, sections were rinsed, stained by Harris hematoxylin (Orto Diagnostic Systems, Loudwater, UK), and mounted with glycerol solution (Dako, Carpinteria, CA).

Sperm Immunostaining

Immunofluorescence was carried out on live human sperm. Sperm samples were washed twice with PBS containing Ca²⁺, Mg²⁺, and 0.4% bovine serum albumin (BSA, i.e., PBS-BSA) and submitted to a discontinuous Percoll gradient, as described by Lassalle and Testart (1994). Live cells were recovered in the pellet, washed twice with PBS-BSA, and incubated with 1% BSA (type I; Sigma Chemical Co) in PBS for 1 hr at room temperature. After two washings, they were incubated for 1 hr at 20°C with either LB5 or a nonsecreting supernatant. They were then washed twice and incubated with a 1:30 dilution of sheep antimouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (Valbiotech, Paris, France). After three washings, the suspensions were diluted 1:2 with Fluoroguard (Bio-Rad, Hercules, CA), smeared on a glass slide, and observed with a Leica TCS 4D confocal laser scanning microscope (Institut Jacques Monod, Paris).

For comparison of percentages of LB5-stained and acrosome-reacted spermatozoa, fresh ejaculated sperm were submitted to a discontinuous Percoll gradient, as described by Lassalle and Testart (1994). Live cells were washed twice with PBS-BSA and divided into two samples. One of them was treated with 25 µM Ca²⁺ ionophore A23187 (Sigma Chemical Co) for 30 min in a 5% CO₂ atmosphere to induce the acrosome reaction. After washing, both samples were stained by LB5 mAb and revealed by immunofluorescence as described above.

An aliquot of each sample was stained for 15 min at room temperature with 100 µg/ml fluoresceinated *Pisum sativum* agglutinin (PSA-FITC; Sigma Chemical Co) according to Cross et al. (1986) in order to evaluate the acrosomal status.

Purification of LB5 Antibody

The antibody was purified using a protein A sepharose 4 fast flow column (Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 0.1 M phosphate buffer (pH 8.0) and charged at a flow rate of 0.5 ml/min with either culture supernatant adjusted to pH 8.0 or ascites fluid diluted 1:2 with phosphate buffer. The column was then washed with phosphate buffer and the antibody eluted with 0.1 M citrate buffer (pH 4), neutralized with Tris HCl (pH 9), dialysed against PBS or phosphate buffer (pH 7.4), concentrated, and stored at -20°C .

LB5 Fab Fragment Production and Purification

The purified antibody was subjected to papain digestion. For a 2.5 mg/ml antibody concentration, the reaction included 5 mM EDTA (from a 0.1 M solution in 0.2 N NaOH), 0.01 M L-cystein (from a 0.2 M solution in 0.1 M phosphate buffer, pH 7.4), and 1 mg papain per 100 mg antibody. The solution was incubated 2 hr at 37°C , and the reaction was stopped by incubation with 0.04 M iodoacetamide (from a 0.4 M solution in 0.1 M phosphate buffer, pH 7.4) for 30 min at 37°C . The Fab fragment purification was carried out on a protein A sepharose column that was equilibrated initially with 1 M phosphate buffer (pH 8) and charged at a flow rate of 0.5 ml/min. LB5 Fab fragments were collected directly and dialysed against PBS, speed-vac concentrated, and stored at -20°C .

Effect of LB5 on Sperm Fertilizing Ability

Sperm motility analysis. Sperm were washed twice with PBS-BSA and incubated for 50 min with either LB5 or G12 supernatant as control. After two washings with PBS-BSA, the samples were diluted in 500 µl of BM1 medium (Ellios Biomedica, Igny, France) and placed in a glass semen analysis slide that was inserted in a computerized sperm analyzer for image analysis (Hamilton Thorn Motility Analyzer 2000, Hamilton-Thorn, Danvers, MA). The mobility parameters of five different sperm samples from five donors exhibiting various percentages of motile sperm were thus analyzed. For each sample, the mean value of nine determinations was calculated, each for a different field. The following parameters were measured: motility (%), progressive motility (%), curvilinear velocity (µm/sec), rectilinear velocity (µm/sec), amplitude of lateral head displacement (µm), and beat cross-frequency (Hz).

Effect on acrosomal reaction triggering. The effect on the acrosome reaction of LB5 supernatant was tested using procedures adapted from Garner et al. (1988) and Cross et al. (1986). A double propidium iodide-FITC-PSA staining was performed in order to estimate basal sperm vitality and the percentage of

acrosome reaction. The acrosome reaction was induced or not by the addition or not of A23187 diluted in BM1, as described above. After three washings in PBS-BSA, induced and control samples were incubated for 1 hr at 37°C in a 5% CO_2 atmosphere with, respectively, either LB5 supernatant or MEM medium and G12 supernatant as controls and washed again. Samples were washed twice, and final pellets were resuspended in 100 µg/ml PSA-FITC and incubated for 15 min at room temperature. At the same time, aliquots of sperm suspensions were incubated for vital staining with 5 µg/ml propidium iodide for 15 min at room temperature, washed twice, and fixed with 95% ethanol for 30 min at -20°C . Two hundred spermatozoa were counted for each condition, and results were expressed with respect to live spermatozoa.

Zona pellucida binding assay. A modified protocol (Liu et al., 1988) was used to estimate the effect of LB5 mAb on ZP binding. Salt-stored human oocytes that had failed to fertilize in an in vitro fertilization (IVF) program were washed twice for 1 hr with PBS-BSA and stored in BM1 medium. Sperm from different normal donors and expressing different motilities were used. Live spermatozoa selected from a Percoll gradient as described above were divided into two samples. The acrosome reaction was induced in one of them by incubating with 25 µM ionophore A23187 for 30 min as indicated above. Washed acrosome-reacted and intact populations were treated for 1 hr at 37°C in a 5% CO_2 atmosphere with either LB5 or G12 mAb (cell culture supernatant or Fab fragments). LB5-treated sperm (incubated or not with A23187) were then washed and stained with 2 mg/ml FITC, while controls (incubated or not with A23187) were stained with 1 mg/ml tetramethyl rhodamine isothiocyanate (TRITC) or the inverse in order to eliminate any artifact related to the dye. A mixture of equal numbers (0.2×10^5 motile spermatozoa in 500 µl BM1 medium) of both stained sperm samples for each condition was incubated with about 20 oocytes at 37°C for 2 hr in a 5% CO_2 atmosphere. The oocytes were washed twice with BM1 medium to eliminate nonadherent sperm, and FITC- and TRITC-labelled sperm bound to the ZP were counted using a light fluorescent microscope with excitation at 450–490 and 546 nm, respectively. Aliquots of each sample were stained with propidium iodide and PSA-FITC in order to evaluate sperm vitality and the proportion of acrosome-reacted spermatozoa.

Heterospecific fertilization test. Sperm fertilizing ability was evaluated using a hetero-IVF procedure. Virgin golden hamsters were induced to superovulate by an intraperitoneal injection of 30 IU PMSG (Intervet, Angers, France) followed by 30 IU human chorionic gonadotropin (Serono, Roma, Italy) 72 hr later. Twenty hours later, animals were killed by cervical dislocation. Cumulus cells were dispersed with 0.05% hyaluronidase (bovine testis, type I; Sigma Chemical Co) in PBS-BSA, and ZP were removed by treatment with 0.05% trypsin in PBS-BSA, as described by Lassalle and Testart (1988). Zona-free oocytes were rinsed in

fresh PBS-BSA and finally placed in BM1 at 37°C in a 5% CO₂ atmosphere.

At the same time, frozen ejaculates from three donors were thawed and washed with PBS-BSA. The pellets were resuspended in 0.5 ml BM1 with or without 200 µg/ml LB5 Fab fragments and incubated for 1 hr at 37°C under 5% CO₂. After two washings with PBS-BSA, sperm were resuspended in BM1. Finally 4×10^4 to 1×10^5 motile spermatozoa per condition were placed in Petri dishes, and 25–30 zona-free oocytes were added to each. After incubation for 1.5 hr at 37°C under 5% CO₂, oocytes were rinsed to remove nonadherent sperm. Five hours later they were examined directly under a phase-contrast microscope or stained with Syto 15 (Molecular Probes, Eugene, OR). Fluorescence analysis was performed with a Nikon Diaphot microscope interfaced with a Bio-Rad MRC600 confocal laser scanning imaging system. In both cases, the number of spermatozoa bound per egg was counted.

Blood Cell Purification

For human erythrocytes, blood samples were washed three times in PBS at 4°C, centrifuged, and the pellets were resuspended 1:30 (v/v) in 5 mM sodium phosphate buffer (pH 8) containing 1 mM EDTA and 5 µl/ml aprotinin. After centrifugation at 17000g for 20 min at 4°C, pellets were resuspended in 300 µl of phosphate buffer supplemented with 1% SDS and directly evaluated for protein concentration.

For lymphocyte purification, the initial whole-blood sample was diluted in 0.9% NaCl, and leukocytes were separated from erythrocytes by centrifugation at 1000g on a continuous Ficoll gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). After washing with 0.9% NaCl, leukocytes were diluted to 20×10^6 cells per milliliter in RPMI 1640 medium (Gibco Life Technologies) supplemented with 5% FCS. They were then mixed with an equal volume of sheep erythrocytes previously treated with 0.14 M amino ethylisothiuronium bromide (AET). After an overnight incubation at 4°C, the aggregated cells were gently resuspended, laid on a continuous Ficoll gradient, and centrifugated at 1700g in order to separate a pellet of rosetted T cells and an annulus containing monocytes and B cells (MB suspension). T-lymphocytes were recovered after sheep erythrocyte lysis obtained by a brief treatment with ACK buffer (0.155 M NH₄Cl, 0.1 mM Na₂EDTA, 0.01 M KHCO₃) at 37°C. B-lymphocytes were selected from the MB suspension by using Dynabeads M-450 Pan-B CD19 (Dyna, Oslo, Norway) and a Dynal magnetic particle concentrator (MPC). The beads were washed with 2% FCS in PBS at 4°C, and the MB cell suspension, adjusted to 30×10^6 cell per milliliter in RPMI 1640 medium (Gibco Laboratory), was added (10 beads per target cell) and incubated for 30 min at 4°C with gentle rotation. The suspension was placed in the MPC at 4°C for 3 min, and the supernatant containing CD19 cells, i.e., monocytes, was collected. After several washings, B-lymphocytes were recovered from the beads by overnight incubation at 37°C in a 5% CO₂ atmosphere in RPMI 1640 and 10%

FCS. Once washed with PBS, the cell suspensions containing T-lymphocytes, monocytes, or B-lymphocytes were subjected to the protein extraction described below.

Electrophoresis, Immunoblotting, and Silver Staining

Protein samples were obtained from washed sperm, cell suspensions (except erythrocytes), and homogenized tissues by treatment with extraction buffer NP-40 (0.1 M Tris HCl, pH 8.0, 0.14 M NaCl, 10% glycerol, and 1% Nonidet P-40). After sonication (average intensity, 1 min, 3 sec pulses at 4°C), the samples were submitted to rotatory agitation for 45 min at 4°C and centrifuged for 15 min at 11,000g and 4°C. Supernatants were then evaluated for protein concentration.

For SDS-PAGE, protein samples were diluted 1:2 with SDS reducing buffer containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 62.5 mM Tris HCl (pH 6.8), and 0.005% bromophenol blue dye, according to Laemmli (1970), heated for 5 min at 95°C, and separated under reducing conditions on 12% acrylamide gels.

After migration, proteins were electroblotted onto nitrocellulose membranes according to Towbin et al. (1979). Membranes were saturated with 10% nonfat milk in PBS for 2 hr at 20°C, then washed with PBS-Tween 0.05%, and incubated for 2 hr with either LB5 supernatant, purified LB5 IgG₁ diluted in PBS, or PBS alone as control. The membrane was again washed twice in PBS, treated with 1% goat serum in PBS for 2 hr, incubated for 2 hr in a 1:600 dilution of peroxidase-conjugated goat antimouse IgG (Biosys, Compiègne, France), and washed with PBS-Tween 0.05%. Results were visualized using 1.5 mM diaminobenzidine (DAB) in PBS with 0.1% H₂O₂.

Alternatively, for a more sensitive staining, the ECL chemiluminescence protocol (Amersham, Buckinghamshire, England) was used. After mAb treatment, the membrane was incubated for 1 hr with a 1:3000 dilution of horseradish peroxidase-linked sheep antimouse IgG (Amersham) in PBS; after each step, the membrane was washed three times with PBS-Tween 0.05%. Visualization was carried out according to the manufacturer's instructions using Hyperfilm-ECL films (Amersham). Silver staining was performed using a silver staining kit (Sigma) after fixation in 10% acetic acid and 30% ethanol.

SOB3 Purification

Preparative electrophoresis. A 12% acrylamide separating gel and a 4% stacking gel were polymerized in the 37-mm-diameter tube of a Model 491 Prep Cell preparative gel apparatus (Bio-Rad). An NP-40 sperm extract obtained as described above and containing 10 mg protein was diluted 1:2 in 2 × SDS reducing buffer, heated for 5 min at 95°C, and loaded on the gel. The electrophoresis was run for 48 hr at 4°C with a 40-mA constant current. Proteins were recovered from the elution chamber with elution buffer (2 mM Tris, 30 mM

NaCl, pH 7.2), and 240 × 4 ml fractions were collected. The first tube containing bromophenol blue marker dye was referred to as fraction number one. Fractions were tested by SDS-PAGE and Western blotting with the ECL procedure in order to detect the presence of SOB3. Positive fractions 32 to 49 were pooled, 10× concentrated to about 80 µg/ml, and stored at -80°C with 0.2% azide.

Isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEPHGE). Pooled fractions recovered from preparative electrophoresis were treated with acetone, as follows, in order to remove SDS before focusing: Fractions were diluted 1:5 by dropping on acetone at -60°C followed by 2 hr of incubation at -60°C. After centrifugation at 10,000g for 20 min at 4°C, pellets were washed in acetone and then in ether. Protein pellets were resuspended with lysis buffer containing 9.5 M urea, 8% CHAPS (Sigma), 5% β-mercaptoethanol, and 2% Ampholine 3.5-9.5 (Pharmacia) in H₂O and incubated for 1 hr at 37°C before being loaded on gels.

Focusing was run in a Mini Protean II Dual Slab Cell (Bio-Rad) with 4% acrylamide gels containing 9.5 M urea, 2% CHAPS, 12.5% Readysol IEF (Pharmacia), 10% glycerol, 7.5% Ampholine 3.5-9.5, 0.03% ammonium persulfate, and 0.3% TEMED. IEF was carried out with 0.02 M NaOH as cathode solution in the upper chamber and 0.01 M H₃PO₄ as anode solution in the lower chamber. In order to confirm IEF results and to obtain a better resolution in the basic pH range, NEPHGE also was carried out with inversion of both buffers and of polarity. Thus protein samples were charged at the basic pole for IEF and at the acidic pole for NEPHGE. In both cases, running conditions were 200 V for 20 hr at 4°C (4000 volthours).

Proteins were transferred with 0.7% acetic acid buffer and inversed polarity and then immunostained by ECL as described above. Gel silver staining was performed according to a modified protocol from Morrissey (1981), with initial fixation in a 20% trichloroacetic acid solution. Gels were washed for 1 hr in H₂O and treated for 15 min with 5 mg/liter dithio-tritrol (DTT) oxidizing solution and then with 2.04 g/liter AgNO₃ for 1 hr. After a 2-min wash with H₂O, staining was developed with 34.72 g/liter Na₂CO₃ and 0.025% formaldehyde and stopped with a 1% acetic acid solution.

Statistics

Means are expressed ± SEM. Significance of differences was calculated using Student's *t*-test for unpaired samples.

RESULTS

Organ and Sperm Staining

Immunohistochemical staining of paraffin-embedded human testis exposed to LB5 mAb supernatant did not reveal any binding to spermatogenic cells within the seminiferous tubules, and no staining was observed in vasa efferentia and caput epididymis sections (Fig. 1).

By contrast, LB5 mAb labelled the fluid and the acrosomal region of spermatozoa in the lumen of corpus epididymis fragments, while no staining was observed in control slides. The same pattern was observed in cauda epididymis (not shown).

Two staining patterns were observed on live ejaculated spermatozoa. As can be seen in Fig. 2, LB5 mAb stained only the neck region and part of the flagellum of most spermatozoa (B), while about 10-20% exhibited, in addition, a clear labelling of the acrosome (D).

In order to determine the acrosomal status corresponding to each pattern, we compared the percentages of sperm whose acrosome was stained by LB5 mAb with the percentages of either spontaneous acrosome-reacted spermatozoa or sperm induced to acrosome-react by A23187 treatment (Fig. 3). A positive correlation was observed between the proportion of LB5-stained acrosomes and the proportion of sperm that were not labelled by PSA and thus acrosome-reacted according to the criteria of Cross et al. (1986) ($R^2 = 0.94$, $P < 0.0001$).

Effect of LB5 on Sperm Maturation and Sperm-Egg Interaction

Sperm motion parameters. Mean measures ± SEM of motion parameters evaluated on five sperm samples from five donors are presented in Table 1. Sperm samples with from 7.8-80.6% motile spermatozoa were used. Statistical analysis revealed no significant effect of LB5 mAb on any of the measured parameters.

Acrosomal reaction triggering. As can be seen in Table 2, LB5 mAb supernatant had no significant effect on the percentages of spontaneous and induced acrosome reaction of human sperm as compared with control mAb supernatants CA6 and G12.

ZP binding assay. The effect on sperm binding to human ZP of LB5 mAb alone was first evaluated using sperm samples from different donors in order to eliminate possible side effects due to individual parameters. In a preliminary series of experiments, LB5 supernatant was shown to reduce the number of spermatozoa bound to eggs by between 22.5 and 69.4%.

In a second series of experiments, we used only sperm samples having 10-20% spontaneous reacted acrosomes. Since there was some variation in binding ability between sperm samples, each experimental result was expressed as the percentage of sperm bound under control conditions. As can be seen in Fig. 4B, binding of spermatozoa preincubated in either LB5 supernatant or Fab fragments (200 µg/ml) was decreased by 27.9 and 35.7%, respectively, as compared to binding of control spermatozoa treated with an irrelevant mAb (G12 supernatant or G12 Fab fragments).

At the same time, in the light of the immunostaining results, spermatozoa were induced to acrosome react prior to LB5 mAb incubation. As can be seen in Fig. 4A, a preliminary experiment had shown that the treatment by A23187 alone had no significant effect on sperm binding to ZP ($P > 0.05$). The same treatment

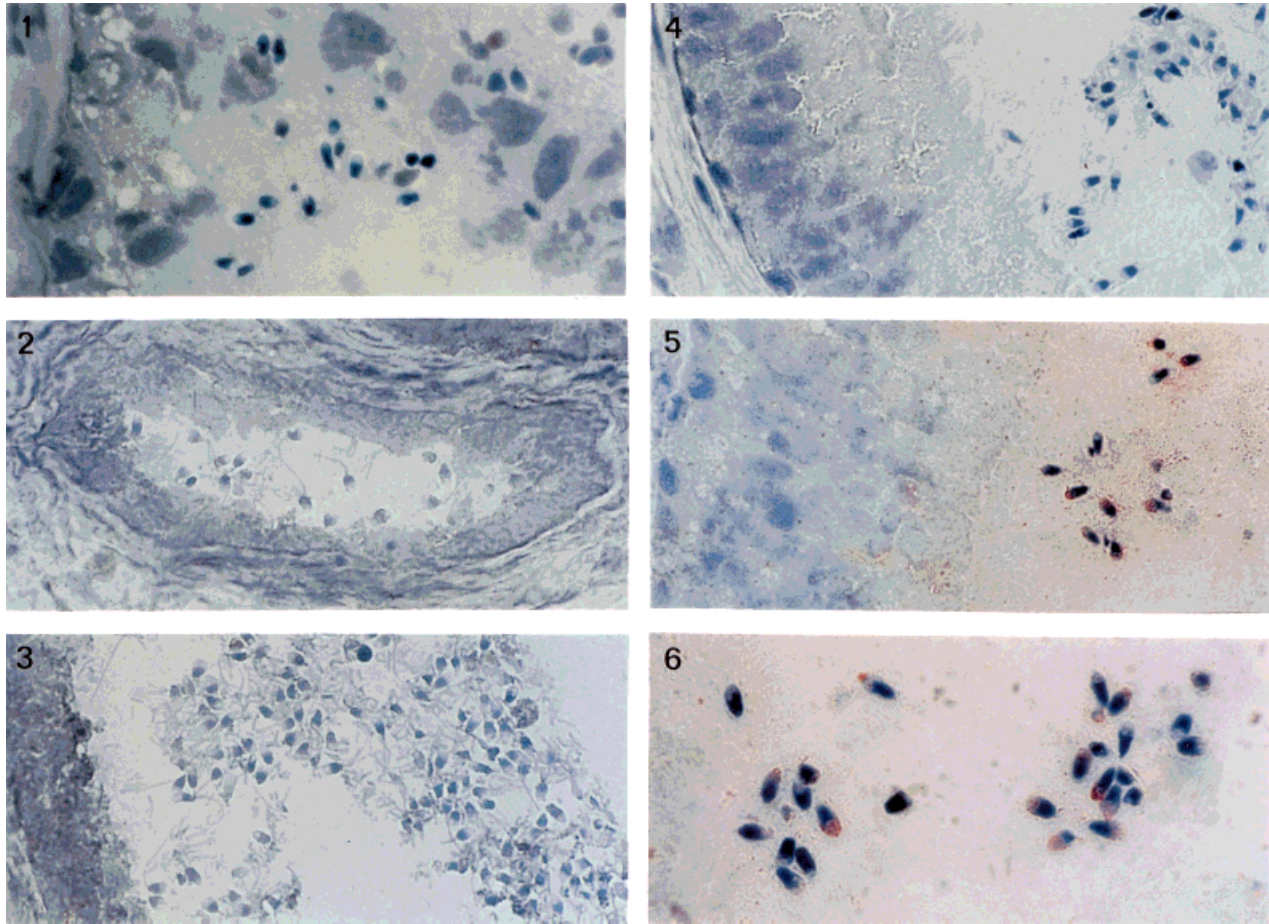


Fig. 1. Immunostaining of methanol-fixed human testis and epididymis sections. (1) LB5-treated testis tubule. (2) LB5-treated vasa efferentia. (3) LB5-treated caput epididymis. (4) control corpus epididymis. (5,6) LB5-treated corpus epididymis showing a positive reaction in the lumen and on sperm acrosome. Original magnification: $\times 500$ (1 to 5), $\times 750$ (6).

increased by 62.9% the number of acrosome-reacted spermatozoa. Simultaneous treatment with A23187 and either LB5 supernatant or LB5 Fab fragments resulted in a significant synergistic inhibition of sperm binding as compared with untreated sperm ($P < 0.02$) (Fig. 4B). When associated with A23187, the LB5 supernatant and LB5 Fab fragments were nearly as potent and induced a 58 and 59.9% inhibitory effect, respectively. The proportion of live acrosome-reacted spermatozoa determined by PSA binding for the same time period was 57–74% higher after exposure to A23187, depending on the sperm sample. A positive correlation was observed between this stimulation range and the inhibitory effect on sperm binding (results not shown).

Hamster egg penetration test. Preliminary studies had shown that LB5 supernatant had no effect on sperm binding to zona-free hamster oocytes as compared with an irrelevant mAb (Boué et al., 1992). We compared the number of sperm bound per oocyte with and without preincubation with LB5 Fab fragments. As can be seen in Table 3, LB5 Fab fragments did not

impair sperm binding even at 200 $\mu\text{g/ml}$ dilution. Similar results were observed with higher sperm numbers using an optical microscope. In a representative experiment, 27.7 ± 3.6 and 29.7 ± 4.8 sperm bound per egg were counted for control and LB5 experimental conditions, respectively.

SOB3 Biochemical Characterization

Western blots of human sperm and cauda epididymis extracts performed under reducing conditions and incubated with LB5 supernatant showed two immunoreactive protein bands of 18 and 19 kDa, whereas controls without LB5 supernatant incubation were negative (Fig. 5). Blots of human protein extracts from caput epididymis, testis, ovary, and both male and female liver displayed no immunoreactivity. However, LB5 mAb revealed similar bands of 18 and 19 kDa in human spleen (Fig. 6). It precisely cross-reacted with an 18-kDa protein in erythrocytes and with a 19-kDa protein

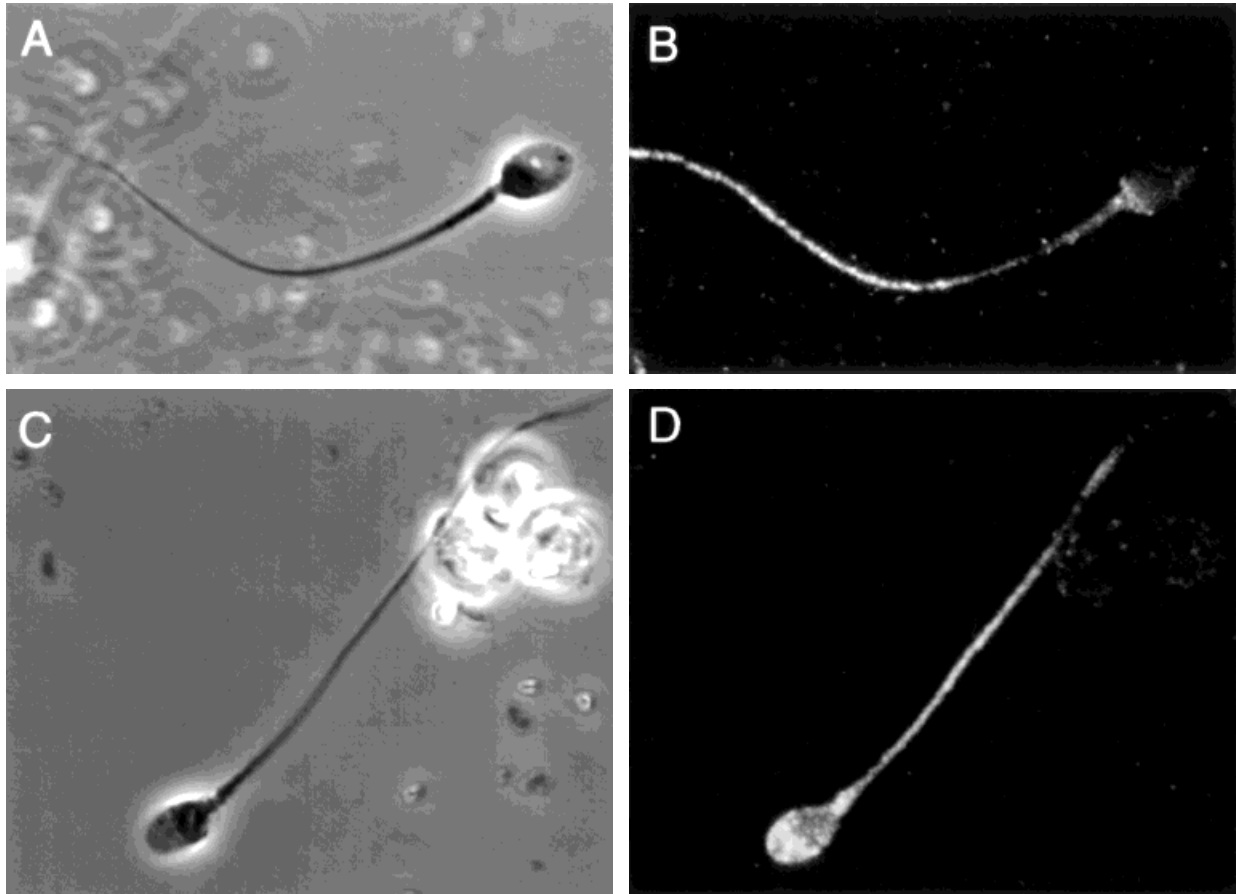


Fig. 2. Immunofluorescence staining of live human ejaculated sperm. (B) Staining most commonly observed in the sperm population. (D) Specific staining of the acrosome observed in 10–20% sperm. (A) and (C) light micrographs corresponding to (B) and (D), respectively.

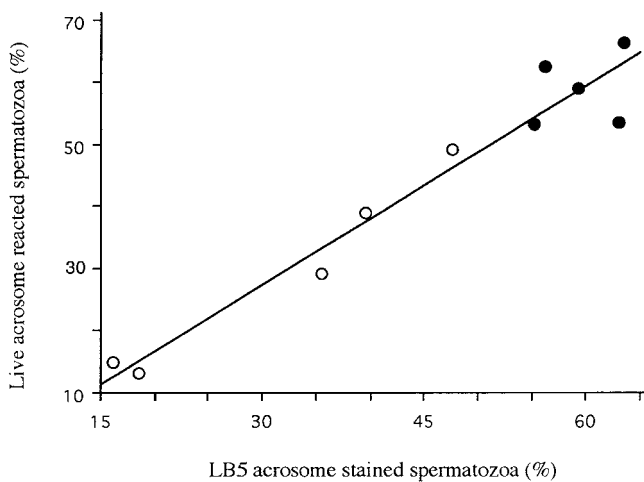


Fig. 3. Comparison of proportions of LB5-stained acrosomes with proportions of acrosome-reacted sperm as assessed by the absence of PSA staining. Five ejaculates from different normal donors were used. Sperm were either not treated (○) or treated with 25 μM A23187 (●). The equation of the straight-line regression and the correlation coefficient were calculated ($R^2 = 0.94$, $P < 0.0001$), and linearity was tested using the F test.

TABLE 1. Effect of LB5 Supernatant on Sperm Motility Parameters^a

| Motion parameters | Control ^b | Treated ^b |
|--|----------------------|----------------------------|
| Percent motility | 35.6 ± 12.96 | 38.54 ± 13.31 ^c |
| Percent progressive motility | 17.8 ± 7.80 | 21.28 ± 11.37 ^c |
| Rectilinear velocity, μm/s | 45.30 ± 5.61 | 49.14 ± 8.4 ^c |
| Curvilinear velocity, μm/s | 72.60 ± 5.25 | 73.02 ± 6.43 ^c |
| Amplitude of lateral head displacement, μm/s | 4.44 ± 0.14 | 4.34 ± 0.29 ^c |
| Beat cross-frequency, Hz | 17.40 ± 0.70 | 17.30 ± 0.46 ^c |

^aSperm were treated with either LB5 supernatant or G12 supernatant as control.

^bMean measure ± SEM of five sperm samples from five donors; percent motility varied from 7.8 to 80.6 and 7.5 to 81.6 for control and treated sperm, respectively.

^cNot significantly different, $P > 0.05$.

in B-lymphocytes, while no immunoreactivity was observed in either monocyte or T-lymphocyte extracts.

Studies of species specificity showed no cross-reactivity with sperm of any of the species analyzed, including rabbit, ram, hamster, mouse, and rat (Fig. 7). Moreover, a similar protein could not be detected in either rat caput or cauda epididymis (sperm or organ extract).

TABLE 2. Effect of LB5 Supernatant on Spontaneous and Induced Acrosome Reaction of Live Sperm^a

| A23187 ^b | Percent of reacted sperm | | | | | |
|---------------------|--------------------------|------------|------------|------------|-------------------------|-------------------------|
| | Sperm treatment | | | | | |
| | MEM | | CA6 | | LB5 | |
| | - | + | - | + | - | + |
| Experiment 1 | 14 | 21 | 8 | 22 | 13 | 18 |
| Experiment 2 | 12 | 30 | 13 | 25 | 10 | 28 |
| Experiment 3 | 9 | 16 | — | — | 11 | 16 |
| Experiment 4 | — | — | 11 | 22 | 9 | 18 |
| Means ± SEM | 11.6 ± 1.4 | 22.3 ± 4.0 | 10.6 ± 1.4 | 23.0 ± 1.0 | 10.7 ± 0.8 ^c | 20.0 ± 2.7 ^c |

^aSperm samples of four donors were treated with either LB5 supernatant or MEM medium alone and CA6 supernatant as controls.

^bSperm samples were preincubated without (-) or with (+) 25 μM A23187.

^cNot significantly different from controls, $P > 0.05$.

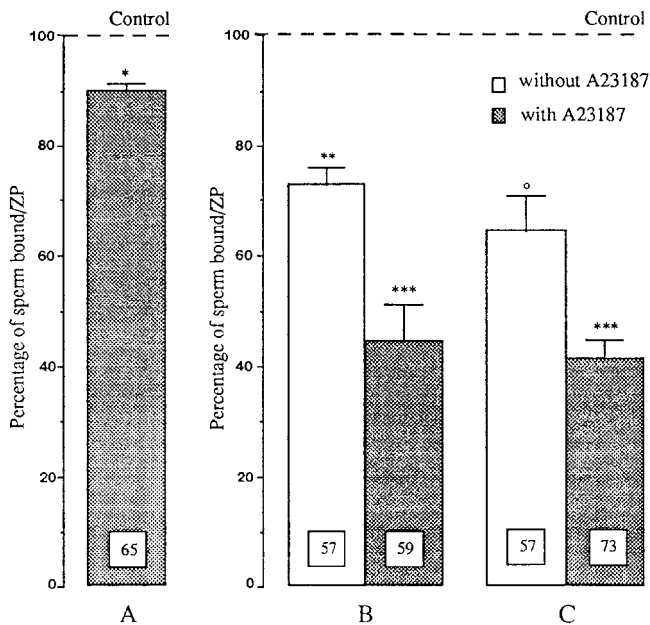


Fig. 4. Effect of LB5 mAb and A23187 on sperm binding to human ZP. In Experiment A, the number of sperm treated with 25 μM A23187 and bound to ZP was expressed as the percentage of untreated bound sperm. In Experiments B and C, the number of bound sperm treated or not with A23187 and exposed to either LB5 supernatant (B) or LB5 Fab fragments (C) were expressed as percentages of bound sperm exposed to either G12 supernatant or G12 Fab fragments. The mAb supernatants were used undiluted and the Fab fragments at 200 μg/ml. Three experiments were performed for each condition with sperm from different normal donors, and values are means ± SEM. For controls, the average numbers of sperm bound per ZP were 37.51 ± 10.49 for Experiment A (MEM medium), 17.75 ± 3.12 for Experiment B (G12 supernatant), and 16.93 ± 2.28 for Experiment C (G12 Fab fragments). The numbers of ZP examined for each condition are indicated in the columns. * $P > 0.05$, $\circ P = 0.06$, ** $P < 0.05$, *** $P < 0.02$.

SOB3 Purification

SOB3 was detected in preparative electrophoresis fractions 27–50. Fractions 32–44, having higher SOB3 content, were pooled and concentrated to about 80 μg/ml protein. This pool was thereafter referred to as fraction C. The comparison of silver-stained sperm and SDS-PAGE fraction C patterns with fraction C submit-

TABLE 3. Effect of LB5 Supernatant on Human Sperm Binding to Hamster Oocytes^a

| | No. of eggs examined | No. of sperm bound per egg |
|--------------------|----------------------|----------------------------|
| Control | 97 | 5.1 ± 0.2 |
| LB5 Fab, 200 μg/ml | 76 | 6.2 ± 1.3^b |

^aThree experiments were performed with three donors. Values are means ± SEM. Sperm were incubated with or without LB5 Fab fragments.

^bNot significantly different, $P > 0.05$.

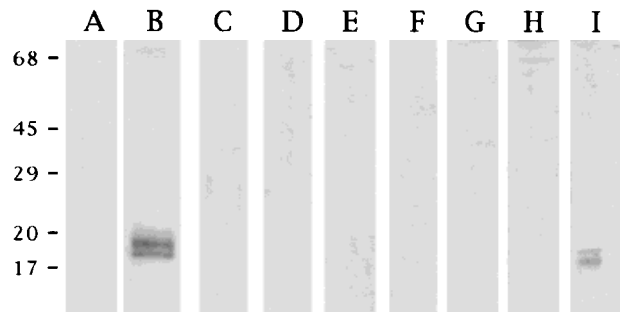


Fig. 5. Immunodetection of SOB3 in various extracts of human organs. Proteins were separated by SDS-PAGE and incubated with LB5 supernatant. (A,B) Control and treated sperm. Treated testis (C), ovary (D), male liver (E), female liver (F), caput epididymis (G). (H,I) Control and treated cauda epididymis. The amount of protein applied was 10 μg per lane. The molecular masses of standards are indicated on the left.

ted to LB5 blotting illustrates the separation of SOB3 (Fig. 8). As can be seen in Fig. 9, fraction C submitted to IEF and LB5 Western blotting showed that SOB3 focused to the basic pole; its estimated pI was 9.8. When submitted to NEPHGE in order to confirm this result and eliminate artifacts due to a possible low solubility of SOB3, before probing by LB5, the same sample yielded the same band of pI 9.8 at the bottom of the gel. In this highly basic area, only one protein is detected by silver staining after both IEF and NEPHGE. Western blotting patterns suggest that it is SOB3.

DISCUSSION

The antibody LB5 was selected from a library of mAb directed against human ejaculated sperm for its ability

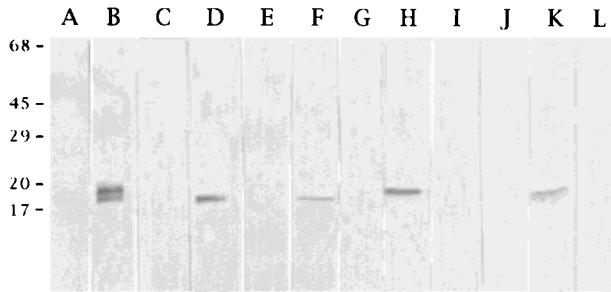


Fig. 6. Comparison of human sperm and blood cell immunoblots incubated with LB5 supernatant. (A,B) Control and treated sperm. (C,D) Control and treated spleen. (E,F) Control and treated erythrocytes. (G,H) Control and treated mix of monocytes and B-lymphocytes. (I) T-lymphocytes. (J,K) Control and treated B-lymphocytes. (L) Monocytes. The amount of protein applied was 10 μ g per lane. The molecular masses of standards are indicated on the left.

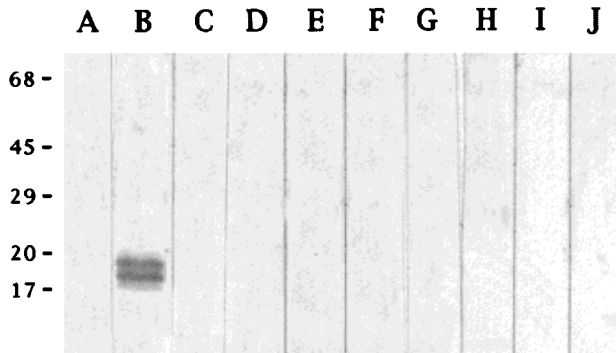


Fig. 7. Comparison of sperm immunoblots from humans and animals incubated with LB5 supernatant. (A,B) Control and treated human sperm. Treated sperm from rabbit (C), ram (D), hamster (E), and mouse (F) cauda epididymis. (G,H) Control and treated rat caput epididymis. (I,J) Control and treated rat sperm from cauda epididymis. The amount of protein applied was 10 μ g per lane. The molecular masses of standards are indicated on the left.

to inhibit sperm binding to human ZP. It specifically recognized an antigen on human sperm and yielded two distinct staining patterns in live spermatozoa: Either the neck region and part of the flagellum were slightly stained, or this pattern was conserved and the acrosome was clearly labelled in addition.

In order to determine whether these patterns were correlated with the acrosomal status, we compared the percentage of LB5 acrosome-labelled spermatozoa with the percentage of acrosome-reacted sperm as assessed by the absence of PSA staining (Cross et al., 1986). Since PSA labels the acrosome region of acrosome-intact sperm only, the strong correlation that we observed suggests that the antigen SOB3 becomes accessible only on completion of the acrosome reaction.

Immunohistochemistry and Western blotting failed to detect SOB3 in testis, vasa efferentia, and caput epididymis. The earliest demonstrable presence of SOB3 was in association with the acrosome of methanol-fixed spermatozoa in the lumen of corpus epididymal segments. Many modifications of human sperm associated

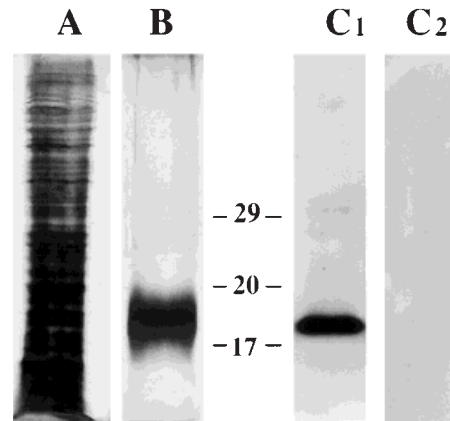


Fig. 8. Comparison of sperm and fraction C protein patterns after silver staining and LB5 immunoblotting. Silver staining of (A) total sperm NP40 extract and (B) fraction C. (C1) Western blot of fraction C. (C2) Control. The amounts of protein applied were 10 and 2.5 μ g per lane for total sperm extract and fraction C, respectively. The molecular masses of standards are indicated in the middle.

with epididymal transit and acquisition of the ability to fertilize have been described (Hinrichsen and Blaquier, 1980; Moore et al., 1983), and some investigators have reported modifications in the immunoreactivity of the intraacrosomal material (Anakwe et al., 1991; Toshimori et al., 1995). Immunoreactivity appearing in the epididymis may result from exposure of a preexisting cryptic epitope, acquisition of a new antigenic determinant following posttranslational modification, or attachment of a new protein to the sperm surface. To the extent that SOB3 is likely to be part of the acrosomal content, the first two hypotheses are to be preferred. Whether SOB3 is an intrinsic component of spermatogenic cells or originates from either testis fluid or epididymal epithelium remains to be elucidated.

LB5 mAb had no effect on any of the sperm parameters analyzed, i.e., motility, progressive motility, rectilinear velocity, amplitude of lateral head displacement, or beat cross frequency, suggesting that it does not affect capacitation. Neither did it impair triggering of the acrosome reaction or human sperm binding to ZP-free hamster oocytes.

By contrast, LB5 supernatant was shown to inhibit sperm binding to human ZP. As could be expected, considering that SOB3 is likely to be localized under the external acrosome membrane, this effect was relatively weak (27.9%). Its specificity is supported by the fact that the same range of inhibition (35.7%) was observed with 200 μ g/ml LB5 Fab fragments. Furthermore, ionophore A23187 increased to 59.9% the inhibitory effect on sperm binding observed with LB5 Fab fragments. This inhibition is synergistic; the inhibition due to simultaneous A23187 and LB5 mAb treatment was 58% (supernatant) or 59.9% (Fab fragments), against that of A23187 alone (9.8%) and 27.3% or 35.7%, respectively, for LB5 supernatant or LB5 Fab fragments.

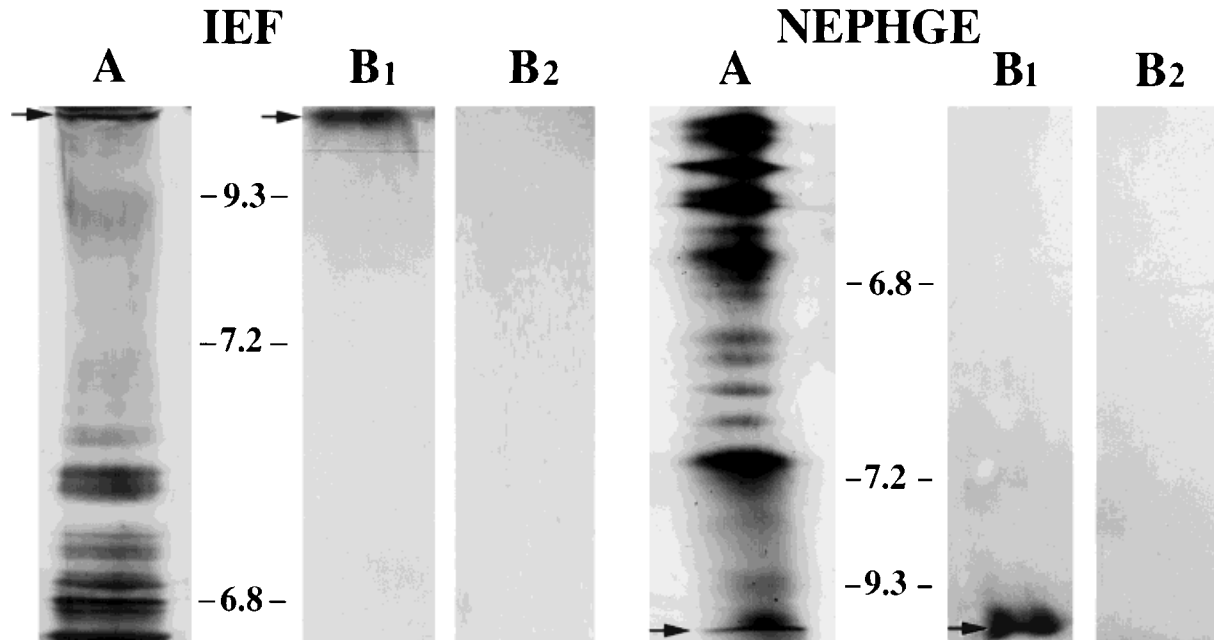


Fig. 9. IEF and NEPHGE of fraction C. (A) Silver staining. (B1) LB5 Western blotting. (B2) Control. The amount of protein applied was 2.5 µg per lane. pIs of standards are indicated for each configuration.

It is clear that in the interpretation of these results, the impact of A23187 on the acrosome reaction and, in turn, the impact of this reaction on sperm-zona binding are important. While Morales et al. (1989) and Van de Voort et al. (1992) report that in humans and macaques, acrosome-intact and acrosome-reacted sperm bind with equal efficiency to the ZP, other workers report a substantial inhibitory effect of the A23187-induced acrosome reaction on sperm-ZP binding in mice and humans (Bleil et al., 1988; Liu and Baker, 1990). These apparently conflicting observations can be reconciled by noting that while the other authors mentioned above did not control the percentages of reacted sperm in the samples, Liu and Baker (1990) demonstrated that sperm-ZP binding decreases with increasing proportions of acrosome-reacted spermatozoa, and this from about 12% reacted cells. We therefore worked only with sperm exhibiting low rates of spontaneous and induced acrosome reaction (on average 10 and 20%, respectively), which enabled us to study the effect of the LB5 mAb itself and reveal its synergy with A23187.

The potentializing effect of A23187 on LB5 mAb-induced inhibition of sperm binding suggests that SOB3 does not act at the ZP3 binding step. In the absence of A23187, LB5 recognizes SOB3 in the small sperm population that has undergone a spontaneous acrosome reaction and it weakly inhibits secondary binding. In the presence of A23187, more spermatozoa undergo the AR with consequent enhancement of access of the mAb to SOB3 and thus of the inhibitory effect. LB5 binds to SOB3 and therefore exerts its inhibitory effect following completion of the AR. Furthermore, we evaluated the number of bound sperm 5 hr after the

beginning of the coincubation of spermatozoa and oocytes, at a time when sperm-ZP3 interaction is probably disrupted as a consequence of the AR and when ZP2, the secondary sperm receptor, replaces ZP3 to maintain sperm binding (Bleil et al., 1988). Taken together, our results suggest that SOB3 is involved in secondary binding to ZP. This leads to the testable hypothesis that induced-acrosome-reacted sperm are able to bind to ZP2.

Up to the present, very few sperm proteins have been proposed as candidates for secondary binding to ZP. In guinea pigs, anti-PH-20 mAb has been shown to preferentially inhibit the binding of reacted spermatozoa to ZP (Myles et al., 1987) while having no effect on the hyaluronidase activity of PH-20 (Hunnicuttt et al., 1996). In both studies, the authors conclude that PH-20 is a candidate for secondary ZP binding. The PH-20 sequence has been determined for several species including guinea pig (Lathrop et al., 1990), monkey, and humans (Lin et al., 1993), and a rat homologue has been characterized recently (Hou et al., 1996). The mammalian sperm proacrosin-acrosin couple has long been believed to be essential to ZP binding and proteolysis, and indeed, amino acid sequences of porcine Sp38 analogous to the proacrosin sequence are required for binding to the 90-kDa glycoprotein family of porcine ZP, which corresponds to human and mouse ZP2 (Mori et al., 1995). However, the danger of hasty conclusions is shown by the fact that male mice homozygous for a targeted mutation in the acrosin gene and lacking acrosin protease activity were nonetheless fertile (Baba et al., 1994). The authors conclude that, in the mouse, acrosin would appear not to be essential for sperm

penetration of the ZP. Recent data indicate that SP-10 antibodies reduce sperm zona secondary binding in a bovine *in vitro* fertilization model (Coonrod et al., 1996). However, the same antibodies also have been shown to affect the motility of capacitated sperm, while an mAb directed against a human intraacrosomal protein similar to SP-10 has been shown to act at the zona-free oocyte binding level (Jimenez et al., 1994).

Concerning the distribution of SOB3, immunoblots of human sperm extracts revealed two protein bands of apparent molecular weights 18 and 19 kDa; a similar protein could not be detected in either rabbit, ram, hamster, mouse, or rat sperm. Moreover, the fact that LB5 mAb revealed no protein in the other human organs tested argues for the relative organ specificity of SOB3.

However, LB5 mAb did recognize similar epitopes in blood cells, where it reacted with 18- and 19-kDa proteins in erythrocytes and B-lymphocytes, respectively. Whether these two proteins are similar to those evidenced in sperm remains to be elucidated. There was no cross-reaction with T-lymphocytes and monocytes. It is perhaps relevant that several human sperm proteins have been shown to be related to lymphocyte differentiation antigens. CDw52, synthesized by the epididymis, is associated with sperm maturation (Kirchhoff et al., 1993), while CD46, CD55, and CD59 may take part in the immune protection of sperm during their transit through the female genital tract (Cervoni et al., 1992, 1993; Fénelich et al., 1994). Moreover, a potential role for CD46 and CD59 in gamete adhesion during fertilization has been proposed (Fénelich et al., 1994; Seya et al., 1993). These observations and ours reveal substantial and intriguing links between the immune and reproductive systems and recall that both processes necessitate fusion molecules.

In order to further describe SOB3 and to define its physiologic role, it was purified from ejaculated sperm via preparative electrophoresis and isoelectrofocusing. The first step allowed us to select proteins between 17 and 20 kDa. The complete purification of SOB3 was achieved by isoelectrofocusing according to its pI of 9.8. In the basic area between pH 7.2 and 10, only one protein is detected after silver staining; comparison with the corresponding LB5 Western blot suggests that it is SOB3. These results were confirmed by submitting the same samples to NEPHGE.

In conclusion, immunologic studies have established that SOB3 is detectable in corpus epididymis and that the corresponding mAb specifically impairs sperm binding to human ZP. The question as to whether the role of SOB3 is that of direct adhesion to the ZP or whether, in association with other membrane receptors, it is involved in activation during human gamete interaction requires further investigations. In particular, protein binding studies and gene cloning will be required to establish whether or not this protein fulfills a role as partner for the secondary binding to ZP.

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