

Characterization and isolation of SOB2, a human sperm protein with a potential role in oocyte membrane binding

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G12 monoclonal antibody (mAb), one of a library of constructed mAb directed against human sperm proteins, was found by immunoperoxidase staining to label the post-acrosomal and neck regions of fixed human cauda epididymal and ejaculated spermatozoa. Epithelium and fluid of caput epididymis were strongly labelled while there was no staining on testis and efferent ducts. Western blot analysis revealed that G12 antibody reacted with proteins of 17.5, 18 and 19 kDa in human spermatozoa. This pattern seems to be specific for mature human spermatozoa, as it has not been observed either in other human tissues tested, or in spermatozoa from different animals. SOB2, the corresponding protein, was isolated from NP40-extracted human spermatozoa by using preparative electrophoresis, followed by isoelectrofocusing according to its isoelectric point of 6.4. G12 Fab fragments strongly inhibited binding of human spermatozoa to zona-free hamster oocytes (up to 86% inhibition at 200 µg/ml). Impairment of binding was dependent on the concentration of purified G12 immunoglobulin (Ig)G1, and significant even at 10 µg/ml. There was no inhibitory effect of G12 antibody on sperm motility parameters or triggering of the acrosome reaction and it did not inhibit binding to human zona pellucida. These results indicate that SOB2 is likely to participate in membrane oocyte binding, and may be a potential candidate for the development of a contraceptive vaccine. Key words: contraception/fertilization/oocyte recognition/sperm protein

Introduction

Mammalian fertilization is the result of the successful union of the two gametes. This event involves a cascade of cell–cell and cell–matrix interactions. First, the spermatozoon has to cross the cumulus oophorus and make contact with the zona pellucida (ZP) of the ovum. The attachment of spermatozoa to the ZP requires recognition and interaction between complementary molecules present on both gametes (O’Rand, 1988; Wassarman, 1988). This interaction triggers the acrosome reaction which in turn permits spermatozoa to penetrate the ZP. Then spermatozoa bind to and fuse with the egg plasma membrane.

For most of the mammals studied, including humans, the ZP consists of three glycoproteins: ZP2 and ZP3 form heterodimers which are cross-linked by ZP1. Several sperm molecules have been proposed for primary binding to ZP3. These include FA-1 (Kadam *et al.*, 1995), PH34 (Boue *et al.*, 1994), SP17 (Richardson *et al.*, 1994) and ZRK (Burks *et al.*, 1995) which are expressed in humans. Less is known about secondary binding to ZP2. A glycoprotein, PH20, primarily found in guinea pig but also in humans, has been proposed for this role (Lathrop *et al.*, 1990). Another protein, SOB3, recently purified in our laboratory is suspected to participate in this interaction (C.Martin Ruiz, C.Duquenne, D.Treton *et al.*, unpublished).

The next stage involves sperm contact with, and binding to, the oocyte plasma membrane. Using hybridoma technology, attempts have been made to identify sperm surface molecules involved in oocyte plasma membrane binding in various

species. In humans, a few antigens have been isolated and characterized biochemically and immunologically. Notable among them are: YWKII related to A4 amyloid protein (Chang Yan *et al.*, 1990), HSAg-5 (Lee *et al.*, 1995), Fertilin, originally named PH30, first identified in guinea pig spermatozoa and analogous to snake venom disintegrin (Primakoff *et al.*, 1987; Blobel *et al.*, 1992), CD59, a complementary regulatory protein (Fenichel *et al.*, 1994) and FLB1 (Boue *et al.*, 1995).

Using monoclonal antibodies (mAb) as probes, our laboratory is actively engaged in identifying and characterizing sperm antigens that have a role in human fertilization, (Boue *et al.*, 1992, 1995; C.Martin Ruiz, C.Duquenne, D.Treton *et al.*, unpublished). In a previous communication (Boue *et al.*, 1992), we reported the construction of a library of mAb directed against human sperm proteins as well as the effect of these mAb on sperm function and fertilization. The present study describes the properties of G12, one of these mAbs, in particular its ability to specifically block human sperm binding to and penetration of hamster oocytes. The isolation and the biochemical characteristics of the complementary antigen designated sperm oocyte binding antigen 2 (SOB2) are described.

Materials and methods

Production, purification and collection of Fab fragments

G12 was selected from an mAb library raised against human sperm proteins as described previously by Boue *et al.* (1992). Antibodies

were selected on the basis of two criteria: firstly, their capacity to bind to presumed surface proteins on live washed human spermatozoa, using 0.45 mM Multiscreen-HV filtration plates (Pharmacia LKB, Uppsala, Sweden), as described by Boue *et al.* (1992); and secondly, their ability to impair human sperm binding to zona-free hamster oocytes. G12 was obtained from the supernatant of hybridoma cells cultured in minimum essential medium (MEM) supplemented with 15% de complemented fetal calf serum (FCS) (Gibco Laboratory, Grand Island, NY, USA). It was also produced in fluid ascites from BALB/c mice previously stimulated by pristane (Sigma Chemical Co, St Louis, MO, USA). The ability of each hybridoma supernatant and ascites fluid to bind to human spermatozoa was tested by enzyme-linked immunosorbent assay (ELISA) as described by Boue *et al.* (1992).

G12, which has been shown to be of the immunoglobulin (Ig)G1 subclass, was purified using a Protein A Sepharose 4 Fast Flow column (Pharmacia Biotech, Uppsala, Sweden). The column was initially equilibrated by 0.1 M phosphate buffer pH 8. Either hybridoma supernatant adjusted to pH 8 or ascites fluid 1:2 diluted with 0.1 M phosphate buffer pH 8 were loaded onto the column at a flow rate of 0.4 ml/min. The column was then washed with phosphate buffer. The antibody was eluted with 0.1 M citrate buffer pH 4 and neutralized with Tris-HCl, pH 9. Dialysis against phosphate-buffered saline (PBS) 0.05% sodium azide or 0.1 M phosphate buffer pH 7.4 was performed at 4°C and the solution was finally concentrated to 3 mg/ml and stored at -80°C. Purity was checked by silver staining of 12% acrylamide sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Silver Stain Kit; Biorad, Hercules, CA, USA).

Purified G12 IgG1 was papain digested. For a 3 mg/ml antibody solution in 0.1 M phosphate buffer pH 7.4, the reaction mixture included 1 mg papain/100 mg antibody in 2 mM EDTA (from a 0.1 M solution in 0.2 N NaOH) and 10 mM L-cysteine (from a 0.2 M solution in 0.1 M phosphate pH 7.4). The reaction was run for 2 h at 37°C and stopped by addition of 0.04 M iodoacetamide in 0.1 M phosphate buffer pH 7.4 and incubation for 30 min at 37°C. The solution was dialysed against 0.1 M phosphate buffer pH 8 and loaded at a flow rate of 0.5 ml/min onto a protein A Sepharose column previously equilibrated with 0.1 M phosphate buffer pH 8. Purified G12 Fab fragments were collected directly, dialysed against PBS, concentrated and stored at -80°C.

LB5 and CA6 mAb which bind to sperm antigens were used as controls. They have been shown to specifically inhibit sperm binding to ZP and zona-free oocytes respectively without altering any other sperm or fertilization parameters (Boue *et al.*, 1995; C.Martin Ruiz, C.Duquenne, D.Treton *et al.*, unpublished).

Effect of G12 mAb on sperm fertilizing ability

Sperm motility analysis

Four different sperm samples from four donors of known fertility, whose initial semen characteristics fell within the World Health Organization criteria for normality were analysed. Spermatozoa were washed twice with PBS containing Ca²⁺ and Mg²⁺, 0.4% bovine serum albumin (BSA) and incubated for 50 min with either G12 supernatant or LB5 supernatant as control. After washing twice with PBS-BSA, the samples were diluted in 500 µl of BM1 medium (Ellios Biomedica, Igny, France) and parameters of sperm motility were measured using a computerized sperm analyser (Hamilton Thorn Motility Analyzer 2000; Hamilton-Thorn, Danvers, MA, USA). For each sample the mean value of nine measurements was calculated, each for a different field. The following parameters were measured: motility (%), progressive motility (%), curvilinear velocity (µ/s), rectilinear velocity (µ/s), amplitude of lateral head displacement (µm) and beat cross frequency (Hz).

Effect on acrosomal reaction triggering

The effect of G12 supernatant on the acrosome reaction was tested through a modified protocol from Garner *et al.* (1986) and Cross *et al.* (1986). Double staining with propidium iodide and *Pisum sativum*-fluorescein isothiocyanate (PSA-FITC) permitted simultaneous estimation of sperm vitality and percentage of acrosome reaction. Spermatozoa were selected from a discontinuous Percoll gradient as described by Lassalle and Testart (1994), diluted in PBS-BSA, divided into two samples and induced or not to acrosome react with 25 µM Ca²⁺ ionophore A23187 for 30 min at 37°C under 5% CO₂. After three washings in PBS-BSA, both samples were incubated for 1 h at 37°C with either G12 supernatant or LB5 supernatant as control and washed again. The four sperm suspensions were washed twice and final pellets were resuspended in 100 µg/ml PSA-FITC, incubated for 15 min at room temperature and observed by fluorescence microscopy. At the same time, in order to evaluate the percentage of live spermatozoa, an aliquot of each sample was incubated with 5 µg/ml propidium iodide for 15 min at room temperature, washed twice and fixed with ethanol 95° for 30 min at -20°C. A total of 200 spermatozoa were counted for each condition and results were expressed with respect to live spermatozoa only.

Zona pellucida binding assay

In order to estimate the effect of G12 mAb on sperm binding to ZP, a modified protocol from Liu *et al.* (1988) was used. Salt-stored human oocytes that had failed to fertilize in an in-vitro fertilization (IVF) programme were washed twice for 1 h with PBS-BSA and stored in BM1 medium. Live human spermatozoa selected from a Percoll gradient as described above were washed three times with PBS-BSA, divided into two samples and incubated for 1 h at 37°C under 5% CO₂ with either G12 supernatant or CA6 supernatant respectively as control. After two washes, the G12-treated sample was stained with 20 mg/ml FITC (Sigma Chemical Co) while the control was stained with 10 mg/ml tetramethyl rhodamine isothiocyanate (TRITC; Sigma Chemical Co). This allocation of stains was reversed in some experiments in order to eliminate any dye-related artefact. In preliminary experiments, both dyes were assayed for their efficiency to label spermatozoa and for absence of toxic effect. The concentration used in this study was the lowest one that allowed efficient labelling without toxic effect. A mixture of equal numbers of motile spermatozoa (0.2 × 10⁵ in 500 µl BM1 medium) from both stained sperm samples were incubated with ~20 oocytes in 500 µl BM1 at 37°C for 2 h under 5% CO₂. The oocytes were then carefully washed twice with BM1 to eliminate non-adherent spermatozoa, and FITC- and TRITC-labelled spermatozoa bound to the ZP were counted by light fluorescence microscopy with excitation at 450–490 and 546 nm respectively.

Hetero-specific fertilization test

Sperm fertilizing ability was evaluated using a hetero IVF procedure modified from Lassalle and Testart (1988). Virgin golden hamsters were stimulated to ovulate by an i.p. injection of 30 IU pregnant mare's serum gonadotrophin (PMSG; Intervet, Angers, France) followed by 30 IU human chorionic gonadotrophin (HCG; Serono, Roma, Italy) 72 h later. They were then killed by cervical dislocation 20 h after the HCG injection. After dispersion of cumulus cells with 0.05% hyaluronidase (bovine testis, type I; Sigma Chemical Co) in M16 medium (Whittingham, 1971), ZP were removed with 0.05% trypsin in M16 medium. Zona-free oocytes were rinsed in BM1 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 with either 200 µg/ml G12 Fab fragments diluted in 0.5 ml of BM1 or 200 µg/ml LB5 Fab fragments as control. For dose-response analysis, spermatozoa were treated with either 10–200 µg/ml G12 purified

IgG1, or 200 µg/ml LB5 IgG1 as control, for 1 h at 37°C under 5% CO₂. After two washes with PBS–BSA, spermatozoa were resuspended in BM1. Finally, 25–30 zona-free oocytes were incubated with 4×10⁴ motile spermatozoa for each condition for 1 h 30 min at 37°C with CO₂. Oocytes were rinsed to remove non-adherent spermatozoa, stained with Syto 15 (Molecular Probes, Eugene, OR, USA) and observed 5 h later. Fluorescent analysis was performed with a Nikon Diaphot inverted microscope interfaced with a Bio-Rad MRC600 confocal laser scanning imaging system (Bio-Rad Laboratories). The number of spermatozoa bound per egg was estimated and ova were considered as penetrated when at least one swollen sperm nucleus or male pronucleus was discernable in the egg ooplasm.

Organ and sperm immunostaining

Human testis and epididymides obtained from a transplant programme were removed from three subjects; none of them had received hormonal treatment. Epididymides were dissected into 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, USA) and stored at –80°C. Sections (5 µm thick) were cut with a cryotome and placed on slides which were air-dried, fixed with methanol at –20°C for 10 min and stored at –20°C. Antigen localization was performed by an avidin–biotin peroxidase staining procedure (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). After incubation for 20 min with 10% goat serum in PBS to eliminate non-specific binding, sections were treated with either G12 or a non-secreting supernatant for 1 h at room temperature. They were washed three times with PBS, incubated for 30 min with a 1:5000 dilution of goat biotinylated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PE, USA), washed again three times with PBS and then incubated for 30 min with Vectastain ABC reagent. After three washes in PBS, the reaction was revealed with 118 mM amino ethyl carbazol (AEC; Sigma Chemical Co) and 0.02% H₂O₂. Finally, sections were rinsed, stained by Harris haematoxylin (Orto Diagnostic Systems, Loudwater, UK) and mounted with glycerol solution (Dako, Carpinteria, CA, USA).

Sperm samples were washed three times with PBS and resuspended to 50×10⁶ spermatozoa/ml. A 20 µl sample of suspension was smeared, air-dried, fixed for 10 min with methanol at –20°C, and stored at –20°C. Slides were further submitted to the avidin–biotin peroxidase staining procedure as described above.

SDS-PAGE and Western blotting

Protein samples were obtained from washed spermatozoa and homogenized tissues by treatment with NP40 extraction buffer (0.1 M Tris–HCl pH 8.0, 0.14 M NaCl, 10% Glycerol and 1% Nonidet P-40). After sonication, samples were submitted to rotatory agitation for 45 min at 4°C, centrifuged at 11 000 g for 15 min at 4°C and their protein concentration evaluated.

For SDS–PAGE, protein samples were diluted with SDS reducing buffer (10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris–HCl pH 6.8, 2% SDS and 0.005% bromophenol) according to Laemmli (1970), heated at 95°C for 5 min and finally separated on 12% acrylamide gels. Silver staining was performed using the Silver Staining Kit (Sigma Chemical Co) after fixation of the gels for 1 h at room temperature in an aqueous solution containing 10% acetic acid and 30% ethanol.

After migration, proteins were transferred to nitrocellulose membranes according to Towbin *et al.* (1979). Blots were saturated with 10% non-fat-milk in PBS for 2 h at room temperature, washed with PBS, 0.05% Tween (PBS–Tween) and incubated for 2 h with either G12 or a non-secreting hybridoma supernatant. Membranes

were washed twice in PBS–Tween, treated for 2 h with a 1:600 dilution of goat anti-mouse IgG horseradish peroxidase conjugate (Biosys, Compiègne, France) and washed again with PBS–Tween. Immunostaining was revealed by 1.5 mM diaminobenzidine (DAB) in PBS with 0.1% H₂O₂. Alternatively, a chemiluminescence protocol (ECL) was used to enhance sensitivity (Amersham, Buckinghamshire, UK). After mAb treatment, membranes were incubated for 1 h with 1:3000 sheep anti-mouse IgG horseradish peroxidase conjugate (Amersham). Visualization was carried out with Hyperfilm-ECL (Amersham).

SOB2 purification

Preparative SDS electrophoresis

A NP40 sperm extract obtained as described above and containing 10 mg of proteins was diluted 1:2 in 2× SDS reducing buffer, heated for 5 min at 95°C and loaded onto a 4% acrylamide stacking gel and a 12% acrylamide separating gel. Both were polymerized in the 37 mm diameter tube of the preparative gel apparatus Model 491 Prep Cell (Bio-Rad). The electrophoresis was run for 48 h at 4°C with a 40 mA constant current. Proteins were recovered in elution buffer (2 mM Tris, 30 mM NaCl, pH 7.2) as 240×4 ml fractions. 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 SOB2. Positive fractions were pooled, 10× concentrated to ~80 µg/ml and stored at –80°C with 0.2% azide.

Isoelectric focusing (IEF)

Proteins of pooled fractions recovered from preparative electrophoresis were treated with acetone in order to remove SDS before focusing. Samples were diluted 1:5 by dropping on acetone at –60°C and incubated for 2 h at –60°C. After centrifugation at 10 000 g for 20 min at 4°C, pellets were washed in acetone, then with ether. Protein pellets were resuspended with lysis buffer containing 9.5 M urea, 8% CHAPS (Sigma Chemical Co), 5% β-mercaptoethanol and 2% ampholine 3.5–9.5 (Pharmacia) in H₂O, and incubated 1 h at 37°C before being loaded on gels. IEF was run into a vertical 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 and 7.5% ampholine 3.5–9.5. 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 and proteins were loaded at the basic pole. Running conditions were 200 V for 20 h at 4°C (4000 volt hours).

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 h in H₂O, treated for 15 min with 5 mg/l dithiothreitol (DTT) and then with 2.04 g/l AgNO₃ for 1 h. After 2 min washing with H₂O, staining was developed with 34.72 g/l Na₂CO₃, 0.025% formaldehyde and stopped by a 1% acetic acid solution. For Western blotting, proteins were transferred with 0.7% acetic acid buffer and inverted polarity, then treated and immunostained by ECL as described above.

Statistical analysis

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

Results

G12 mAb had been selected previously on account of its binding to live human spermatozoa and its effect on human sperm-binding to zona-free oocytes. It has been shown to

Table I. Effect of G12 Fab fragments on human sperm binding and penetration of zona-free hamster oocytes. Figures in parentheses show numbers of oocytes examined

Sperm	Sperm binding			Fertilization		
	Mean no. spermatozoa/oocyte		Binding inhibition	Penetration rate ^a		Fertilization inhibition
	Control	Treated	%	Control	Treated	%
A	14.6 (38)	4.4 (24)	69.8	30.0	8.3	72.3
A	12.06 (31)	3.4 (19)	71.8	35.2	23.8	32.4
B	5.8 (22)	0.8 (36)	86.2	ND	ND	ND
C	19.4 (38)	11.7 (43)	39.7	36.8	18.3	50.3

Four experiments were performed with four different sperm samples from three donors (A, B and C). Spermatozoa were incubated with either LB5 (control) or G12 Fab fragments (200 µg/ml).

^aPercentages of oocytes inseminated with one or more swollen heads

Mean binding inhibition = 66.8%.

Mean fertilization inhibition = 51.6%.

belong to the IgG1 subclass. These preliminary results led us to purify the mAb and prepare Fab fragments in order to study its physiological impact as well as to characterize and purify the corresponding protein, SOB2.

Effect of G12 mAb on sperm–egg interaction

Hybridoma supernatants were used in studies of sperm motion parameters, induction of acrosomal reaction and sperm binding to ZP while the hamster egg penetration test was performed after incubation with Fab fragments.

Hamster egg penetration test

Preliminary studies had shown that G12 supernatant inhibited human sperm binding to zona-free oocytes. To exclude an artefactual effect via the egg Fcγ receptor, Fab fragments of purified G12 mAb were utilized and their effect was compared with that of LB5 Fab fragments. Table I and Figure 1 show that binding of spermatozoa from frozen ejaculates of three donors to zona-free hamster oocytes was inhibited by incubation with G12 Fab fragments as compared with those incubated with LB5 Fab fragments. Inhibition varied from 39.7–86.2%, depending on the sperm sample analysed. The oocyte penetration rates were also reduced and the percentages of fertilization inhibition observed were consistent with those of sperm attachment inhibition. Furthermore, the effect was dependent on the concentration of purified G12 mAb in the incubation milieu, and significant even at 10 µg/ml G12 mAb (Figure 2).

ZP binding assay

To demonstrate that the sites on spermatozoa which interact with G12 mAb have a specificity of binding restricted to the oocyte plasma membrane, the ability of the mAb to impair binding to ZP was evaluated. The numbers of human spermatozoa bound to human zonae pellucidae after treatment by G12 versus CA6 supernatant as control were compared. Table II shows that, as expected, G12 mAb did not significantly inhibit sperm binding to ZP.

Sperm motility parameter analysis

G12 mAb did not cause any perceptible agglutination or immobilization of spermatozoa. To investigate further whether

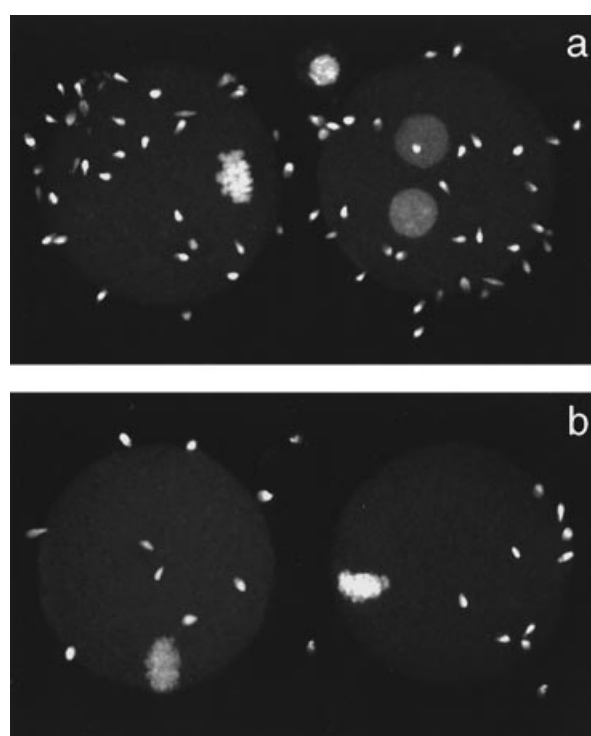


Figure 1. Confocal image of human sperm binding to hamster zona-free oocytes. Spermatozoa were treated with either (a) 100 µg/ml LB5 Fab fragments or (b) 100 µg/ml G12 Fab fragments. (Original magnification $\times 100$).

the effect of G12 mAb on sperm binding is linked with sperm motility, various parameters were studied. LB5 served as control because it was previously shown to have no influence on these parameters (C.Martin Ruiz, C.Duquenne, D.Treton *et al.*, unpublished). Four sperm samples from four donors who exhibited normal semen characteristics, according to the WHO criteria, were analysed. As can be seen in Table III, G12 supernatant had no significant effect on either motility, progressive motility, rectilinear velocity, curvilinear velocity, amplitude of lateral head displacement or beat cross frequency.

Acrosome reaction triggering

To rule out the possibility that G12 mAb could influence the onset of the acrosome reaction, the effect of G12 supernatant

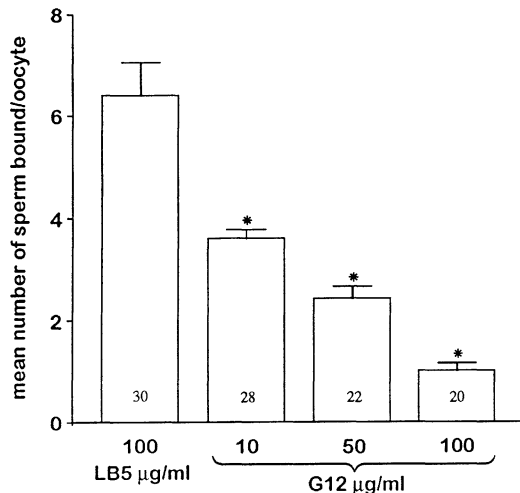


Figure 2. Dose–response effect of G12 purified immunoglobulin (Ig)G1 on human sperm binding to hamster zona-free oocytes. Numbers inside the bars are the number of oocytes used in each test. Values are mean \pm SE of three separate experiments. *Values significantly different from control ($P < 0.05$).

Table II. Effect of G12 mAb on human sperm binding to human zona pellucida. Three experiments were performed with three samples from three donors. Results are given as mean \pm SEM. Figures in parentheses show number of zona pellucida examined

	No. spermatozoa bound/zona pellucida	
	Control	Treated
Experiment 1	44 \pm 5.3	46 \pm 4.9 ^a (26)
Experiment 2	29 \pm 2.9	30 \pm 2.9 ^a (29)
Experiment 3	36 \pm 4.2	34 \pm 4.4 ^a (19)

Sperm samples were incubated with either CA6 supernatant as control or G12 supernatant.

^aNot significantly different from control ($P > 0.05$).

Table III. Effect of G12 mAb on sperm motility parameters

Motion parameters	Control ^a	Treated ^a
Percentage motility	66.8 \pm 13.4	69.7 \pm 6.4 ^b
Percentage progressive motility	50.8 \pm 7.3	54.9 \pm 8.9 ^b
Rectilinear velocity μ /s	51.7 \pm 4.8	47.8 \pm 6.0 ^b
Curvilinear velocity μ /s	76.1 \pm 6.8	70.6 \pm 7.1 ^b
Amplitude of lateral head displacement μ /s	3.8 \pm 0.2	3.9 \pm 0.2 ^b
Beat cross frequency Hz	16.5 \pm 0.4	18.0 \pm 0.2 ^b

Sperm samples were treated with either LB5 supernatant as control or G12 supernatant.

^aMean measure \pm SEM of four sperm samples from four donors; percentage motility varied from 50.1 to 89.1 and 59.7 to 88.5 for control and treated spermatozoa respectively.

^bNot significantly different from controls ($P > 0.02$).

was assessed. LB5 supernatant had previously been shown to have no effect on either the spontaneous- or A23187-induced acrosome reaction of fresh human spermatozoa (C.Martin Ruiz, C.Duquenne, D.Treton *et al.*, unpublished). As can be seen in Table IV, G12 supernatant had no significant effect on the percentages of either spontaneous or induced acrosome-reacted spermatozoa when compared with LB5 supernatant.

Table IV. Effect of G12 mAb on spontaneous and induced acrosome reaction. Three experiments were performed with three sperm samples from three donors. Sperm samples were preincubated without (–) or with (+) A23187 and treated with either LB5 supernatant as control or G12 supernatant. Results are given as mean \pm SEM

	Percentage of reacted spermatozoa			
	Control		Treated	
A23187	–	+	–	+
Sperm sample 1	7 \pm 0.8	25 \pm 3.2	6 \pm 0.5 ^a	23 \pm 2.7 ^a
Sperm sample 2	6 \pm 0.6	13 \pm 1.5	8 \pm 0.9 ^a	14 \pm 1.8 ^a
Sperm sample 3	3 \pm 0.5	9 \pm 1.1	3 \pm 0.2 ^a	8 \pm 0.8 ^a

^aNot significantly different from controls ($P > 0.05$).

Organ and sperm localization of SOB2

We tried to determine at what stage of sperm development the antigenic determinant of G12 appeared. As can be seen in Figure 3a and b, testis and efferent ducts displayed no staining. In contrast, the mAb strongly labelled the epithelium and spermatozoa of the caput and corpus epididymis (Figure 3e) while no staining was observed in control slides (Figure 3c). Spermatozoa in the lumen of the caput epididymis showed a diffuse labelling (Figure 3d) which was progressively restricted to the post-acrosomal and neck regions in the corpus epididymis (Figure 3e). G12 mAb also stained the postacrosomal and neck regions of $>90\%$ of ejaculated spermatozoa (Figure 3f = control; Figure 3g = stained with G12).

SOB2 biochemical characterization

Western blotting of human sperm extracts separated by electrophoresis under reducing conditions and incubated with G12 supernatant displayed three immunoreactive bands of apparent molecular weights 17.5, 18 and 19 kDa (Figure 4I). The same pattern was observed under non-reducing conditions, indicating that these are not components linked by disulphide bonds (data not shown). No staining was observed in testis extracts of up to 200 μ g proteins. G12 blotting revealed two major bands of 37 and 47 kDa in caput epididymis and in deferent duct while no staining was observed in the 17.5–20 kDa area even for loads of up to 400 μ g/well for epididymal proteins (Figure 4).

One band of 53 kDa and one band of 33 kDa were observed in ovary and placenta extracts respectively (Figure 4II). By contrast, blots of both male and female liver, spleen and skin displayed no immunoreactivity.

As can be seen in Figure 5, in samples of rabbit, ram, hamster, mouse and rat spermatozoa, G12 mAb did not react with protein bands of molecular size similar to those with which it reacted in humans. Bands of 26, 28 and 50 kDa were detected in rat, hamster and rabbit extracts respectively.

SOB2 purification

NP40 extracts of human spermatozoa were submitted to preparative electrophoresis on a 12% acrylamide gel. Fractions 50 to 67 with higher SOB2 content were selected, pooled and concentrated to ~ 80 μ g/ml. This pool was thereafter referred to as Fraction D. Figure 6 shows the comparison between silver stained SDS–PAGE of total spermatozoa and Fraction

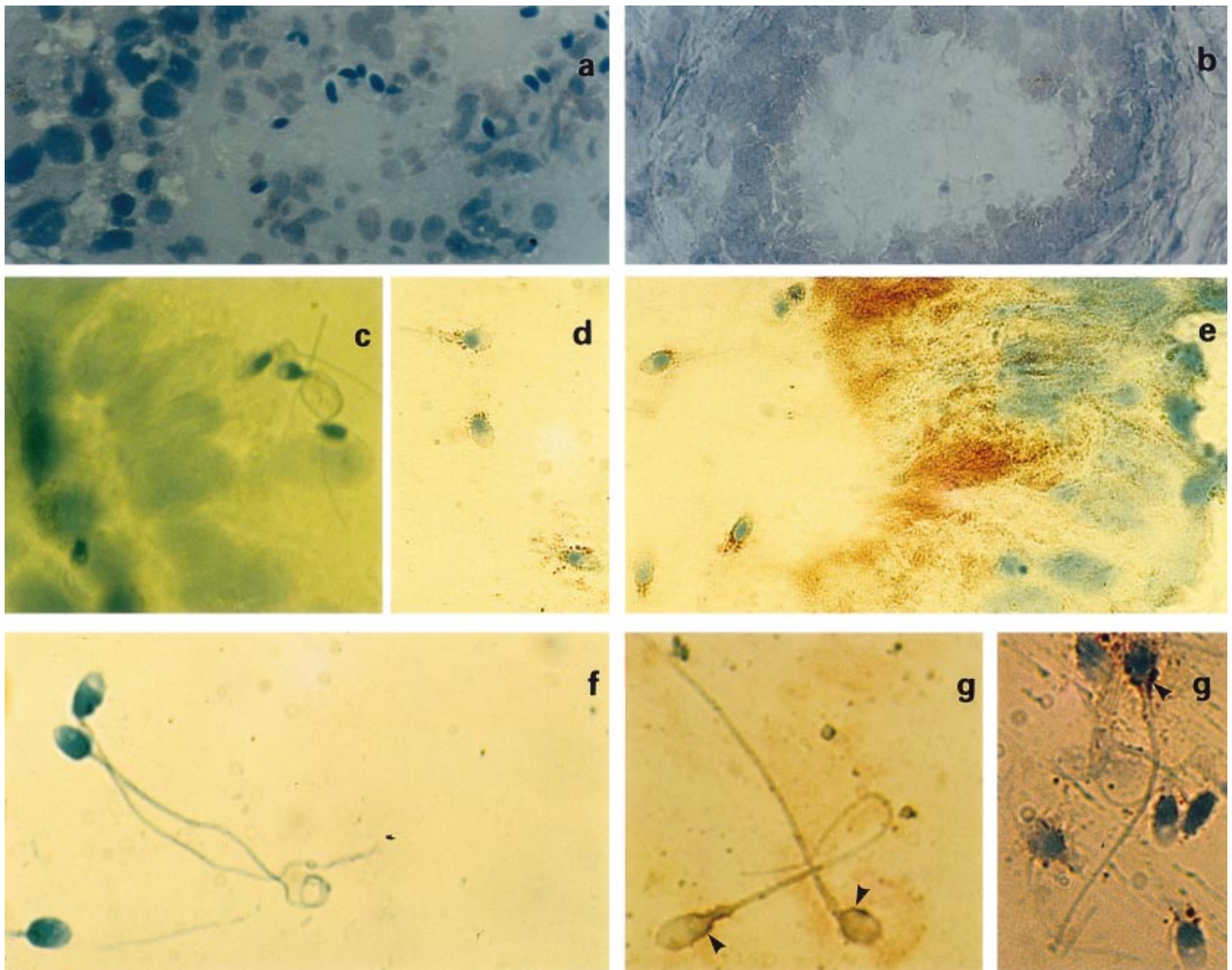


Figure 3. Immunoperoxidase staining localization of the antigen defined by G12 mAb of methanol-fixed human testis, epididymis and spermatozoa. Controls were performed with a negative hybridoma supernatant. (a) G12-treated testis tubules (original magnification $\times 300$); (b) G12-treated vasa efferentia (original magnification $\times 300$); (c) control corpus epididymis (original magnification $\times 1200$); (d) G12-treated spermatozoa in caput epididymis (original magnification $\times 750$); (e) G12-treated corpus epididymis (original magnification $\times 750$); (f) control (original magnification $\times 1200$) and (g) G12-treated ejaculated spermatozoa (original magnification $\times 1200$). Arrowheads indicate G12 labelling of post-acrosomal and neck regions.

D protein extracts with G12 Western blotting and illustrates the relative purification of SOB2. Fraction D submitted to IEF and Western blotting showed that SOB2 immobilized at its isoelectric point (pI) of 6.4 in an area where no other protein was detected by silver staining (Figure 7).

Discussion

The G12 mAb had previously been selected from a collection of mAbs to human sperm antigens for its capacity to bind to presumed surface proteins on live spermatozoa, as assessed by ELISA using filtration plates, as well as for its potent inhibition of sperm-oocyte binding (Boue *et al.*, 1992). G12 mAb, which is of the IgG1 subclass, bound to the post-acrosomal region and the neck of epididymal and ejaculated spermatozoa. The role of the post-acrosome region in sperm binding and fusion is implicit in the fact that PH 30 antigen, shown to play a role in both binding to and fusion with the egg

plasma membrane oocyte via its β and α subunit respectively, is located in this region (Primakoff *et al.*, 1987; Blobel *et al.*, 1992).

As fertilizing capacity develops during the transit of spermatozoa through the epididymis, we established at what stage of sperm development SOB2 first appears. While G12 mAb did not bind testis or efferent ducts, it strongly labelled the epithelium and spermatozoa in caput and corpus epididymis. Sperm labelling which was diffuse in the caput appeared clearly restricted to the post-acrosome and the neck in corpus epididymis.

G12 mAb revealed three bands of 17.5, 18 and 19 kDa on reducing or non-reducing SDS-PAGE of sperm extracts. Rather than multiple gene products, this heterogeneity of the peptides identified by Western blotting may reflect post-translational modifications of the same protein, such as glycosylations, or proteolytic processing as well as alternative splicing as shown for SP10, an intra-acrosomal antigen (Herr *et al.*, 1992;

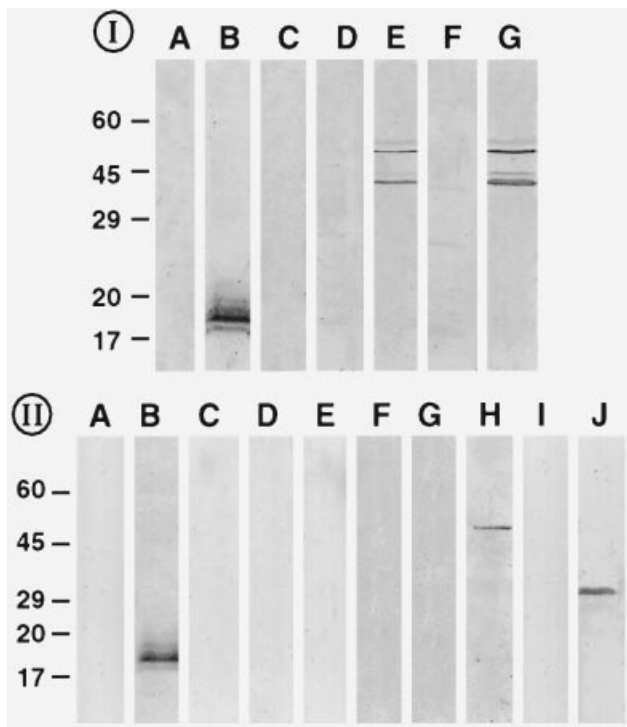


Figure 4. Western blotting of (I) human spermatozoa and male genital tract organs, (II) tissue from other organs. Protein extracts were submitted to SDS-PAGE and incubated with either G12 supernatant or a negative hybridoma supernatant as control. The amount of protein applied per lane was 20 μ g. (IA, B) control and G12-treated spermatozoa; (IC) G12-treated testis; (ID, E) control and G12-treated epididymis; (IF, G) control and G12-treated deferent duct; (IIA, B) control and G12-treated spermatozoa; (IIC, D) G12-treated male and female liver; (II E) G12-treated spleen; (II F) G12-treated skin; (IIG, H) control and G12-treated ovary; (IJI, J) control and G12-treated placenta. The molecular weights of standards are indicated on the left.

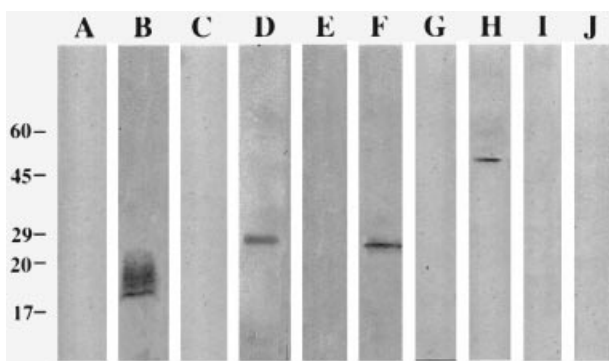


Figure 5. Comparison of SDS-PAGE Western blotting of human and animal spermatozoa. The amount of protein applied per lane was 20 μ g. The blots were incubated with either G12 supernatant or a negative hybridoma supernatant as control. (A, B) control and G12-treated human spermatozoa; (C, D) control and treated hamster spermatozoa; (E, F) control and G12-treated rat spermatozoa; (G, H) G12-control and treated rabbit spermatozoa; (I, J) G12-treated ram and mouse spermatozoa. The molecular weights of standards are indicated on the left.

Freemerman *et al.*, 1995). The mAb did not reveal any protein in this molecular weight area in the human tissues tested. The absence of signal in testis and epididymis as well raises the

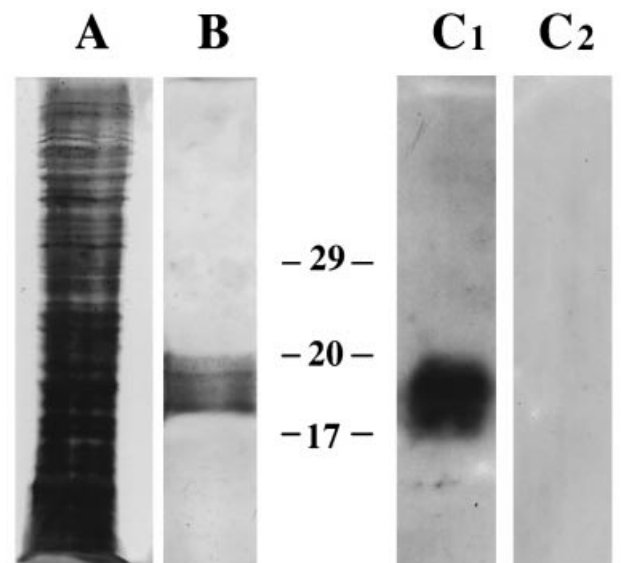


Figure 6. SDS-PAGE of preparative electrophoresis purified SOB2: comparison of NP40 sperm extracts and Fraction D protein patterns after silver staining and G12 immunoblotting. Silver staining of (A) NP40 sperm extracts and (B) Fraction D. (C1) G12 Western blotting of Fraction D; (C2) control (incubated with a negative hybridoma supernatant). The amounts of protein applied were 20 μ g and 2.5 μ g/lane for NP40 extracts and Fraction D respectively. The molecular masses of standards are indicated in the middle.

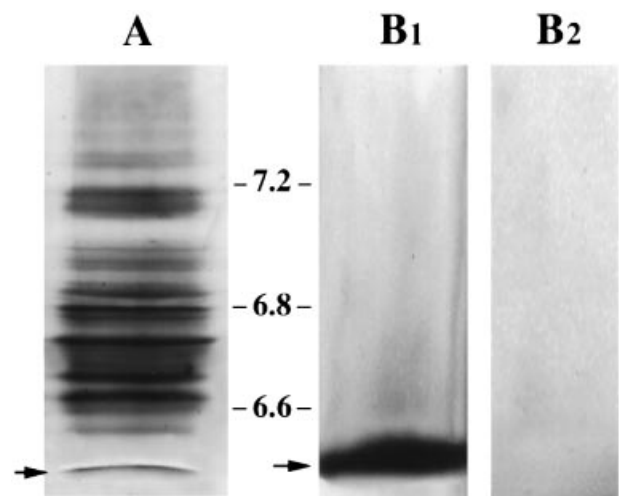


Figure 7. Isoelectric focusing of Fraction D. The amount of protein applied was 2.5 μ g/lane. (A) silver staining. (B1) G12 Western blotting; (B2) control (incubated with a negative hybridoma supernatant). The isoelectric points of standards are indicated on the left.

question of the origin of SOB2. Whether SOB2 derives from the epididymal fluid and binds to spermatozoa in the course of its epididymal transit or whether the antigen pre-exists in testicular germ cells, in a conformation that does not allow G12 mAb recognition, will be resolved with microsequencing of the purified SOB2, and subsequent cloning of its gene. Interestingly, PH30 was first detected on spermatozoa in the proximal cauda epididymis on the basis of immunostaining analysis (Primakoff *et al.*, 1987), while it was later cloned after PCR amplification of guinea pig testis cDNA (Blobel

et al., 1992), suggesting a belated appearance of its antigenicity. In fact, as in the caput epididymis spermatozoa were already labelled by G12, the absence of signal in the epididymis extracts may reflect the paucity of epididymal fluid in sperm cells prior to protein extraction. The presence of two major staining bands of 37 and 47 kDa in caput epididymis and deferent ducts is intriguing. Even though one can postulate that SOB2 is secreted in the epididymis in an immature form, and integrates with the spermatozoa after proteolysis, it would be difficult to understand why the same protein would also be secreted by deferent ducts, while spermatozoa are already coated with SOB2 in corpus epididymis. Therefore, it is likely that these two bands represent unrelated proteins, sharing a common epitope with SOB2, rather than immature forms of SOB2. The 53 and 33 kDa bands observed respectively in ovary and placenta are also likely to be unrelated proteins. G12 mAb failed to detect the three bands of 17.5, 18 and 19 kDa in any other mammalian spermatozoa tested, including rodents, attesting to the specificity of the epitope revealed in humans. However, based solely on molecular weight analysis, it cannot be concluded that the bands stained by G12 in extracts from rabbit, hamster and rat are unrelated to the three protein species comprising human sperm SOB2. Only protein sequence analysis will reliably establish whether or not these proteins are related.

To accede to the status of potential candidate for binding to the oocyte membrane, a protein has to fulfil specific conditions, and before reaching the plasma membrane, the spermatozoon expressing that protein must succeed in several trials, i.e. it has to cross the cumulus oophorus, bind to a first receptor on the ZP, undergo the acrosome reaction, bind to a second receptor and only then can it approach the plasma membrane. The successful molecular candidate for this last connection is likely to remain uninvolved in any of the previous steps of the fertilization process listed above. Therefore, any proposed protein should be examined according to this hypothesis. This was our procedure with SOB2. Before claiming that SOB2 is specifically involved in oocyte plasma membrane binding, we evaluated the ability of its corresponding mAb to modify the parameters of sperm motility, to block zona pellucida recognition, or to trigger the acrosome reaction. Neither the percentage of motile spermatozoa, nor the different parameters that account for sperm movement, were affected by the presence of G12 mAb. G12 supernatant did not modify the occurrence of either spontaneous or A23187-induced acrosome reaction. The percentage of spermatozoa bound to human ZP was not perturbed by the presence of G12 mAb in the medium. By contrast, G12 Fab fragments drastically reduced the number of human spermatozoa bound to zona-free hamster oocytes (by up to 86% for a concentration of 200 µg/ml) and, subsequently, inhibited fertilization, whereas LB5 Fab, which has been shown to inhibit binding to human ZP (Martin Ruiz, C.Duquenne, D.Treton *et al.*, unpublished), had no effect. Furthermore, the inhibition of sperm binding was dose dependent. That these effects were provoked by Fab fragments, which excludes sperm binding through egg Fcγ receptors, argues for the specificity of the effect of G12 mAb, and therefore, the

direct involvement of SOB2 in sperm-egg plasma membrane binding.

With respect to its potential to inhibit fertilization, SOB2 mAb is not alone. The Vic-1 mAb was effective in inhibiting human sperm penetration of zona-free hamster oocytes, but it also inhibited the acrosome reaction and several parameters accounting for sperm hyperactivation (Naz *et al.*, 1993). Therefore, the corresponding FA-2 antigen is likely to be involved in some step that is vital for human sperm capacitation and/or the acrosome reaction rather than in oocyte plasma membrane binding.

On the other hand, the protein DE, which has been described in the mouse, has been shown to almost completely abolish sperm penetration at a concentration of 200 µg/ml, without affecting the number of bound spermatozoa per egg (Rochwerger *et al.*, 1992). The authors showed that transfer of oocytes with bound spermatozoa to DE-free medium restored a quasi normal fertilization rate. On the other hand, they observed that when sperm-egg binding assays were conducted in the absence of Ca²⁺, a condition in which binding but not fusion occurs, the number of bound spermatozoa per egg did not differ from that obtained in the presence of Ca²⁺. The authors conclude that DE participates in an event subsequent to binding, probably membrane fusion. In the same way, results obtained in guinea pig with fertilin, previously named PH30, were in favour of fertilization being a multiphasic event. Fertilin has been cloned in guinea pig, and the deduced amino acid sequence showed its organization into two subunits, α and β (Blobel *et al.*, 1992). The β subunit contains an integrin-binding disintegrin domain, with sequence homology with the snake venom disintegrin, and would probably mediate sperm-egg binding (Blobel *et al.*, 1992; Myles *et al.*, 1994). The α subunit contains a region that fulfils all the criteria for a fusion peptide (Blobel *et al.*, 1992; Muga *et al.*, 1994). The role of the α subunit has been lately called into question in the human, as Jury *et al.* (1997) have demonstrated the absence of a functional human fertilin α, as well as the absence of a fertilin α-β complex in macaque. On the other hand, human fertilin β subunit has been recently cloned (Gupta *et al.*, 1996). Its sequence is 59% identical to guinea pig fertilin β protein and shows a disintegrin domain. As a matter of fact, the involvement of integrins in adhesive mechanisms in the fertilization process is further supported by several recent findings. Bronson and Furst (1990) demonstrated that the RGD (Arg-Gly-Asp) sequence, which is a ligand recognition motif for integrins, was involved in the binding of human spermatozoa to zona-free hamster oocytes and in their subsequent penetration. Furthermore, RGD binding receptors were detected on the surface of zona-free eggs from human and several other mammals (Fusi *et al.*, 1992). Additionally, integrin subunits α₂, α₅ and α₆ were shown to be expressed by human and hamster oocytes (Fusi *et al.*, 1993) while human testicular germ cells expressed α₃, α₅ and α₆ chains of β₁ integrin as well as fibronectin which is known to contain an RGD sequence (Schaller *et al.*, 1993). The positive correlation observed by Klentzeris *et al.* (1995) between expression of β₁ integrin and fertilizing ability of human spermatozoa *in vitro*, together with the fact that ejaculated spermatozoa contain mRNA transcripts

of β_1 integrins (Rohwedder *et al.*, 1996) strengthen the hypothesis of the role played by integrins in the gamete recognition process.

Taken together, these results are in favour of a multistep phenomenon occurring in sperm-egg fusion which implies that several proteins may be implicated prior to egg activation, SOB2 appears to be a serious candidate for a role in the first step corresponding to a site-to-site recognition between the gametes.

Another protein, CD59, a complement regulatory protein associated with other adhesion molecules in the rosette phenomenon, pinpoints a homology between sperm-egg fusion and adhesion processes in the immune system (Fenichel *et al.*, 1994). Like G12 mAb, CD59 mAb reduces the number of spermatozoa bound per egg. It is unlikely that CD59 is related to SOB2, even though their molecular weights are similar: its distribution on spermatozoa differs, its testicular origin is attested, and it is expressed on peripheral blood cells whereas SOB2 is not. Exploiting this analogy with the immune system, one might hypothesize that more than one protein is necessary to permit binding and fusion of spermatozoa with the egg, and that all interactions occur in a precise sequence. Therefore, when all the different molecules involved in fertilization have been elucidated, including integrins, fertilin β , CD59, SOB2 and others, much work will remain to reconstitute the sequences which relate the various elements of the puzzle. Understanding this puzzle will permit improved management of human infertility and also the design of the cocktail which will probably be necessary for a 100% effective contraceptive vaccine. To date, fertilin β is the only specific sperm protein involved in membrane oocyte binding that has been used with some success in a contraceptive trial in non-human primates (Ramarao *et al.*, 1996).

In conclusion, our data indicate that SOB2 (in its 17.5, 18 and 19 kDa forms) is a specific human sperm protein, as its complementary G12 mAb failed to reveal any epitope in the same molecular weight area in several human tissues as well as in the other mammalian spermatozoa tested. SOB2 antigen can be isolated from NP40 human sperm extracts using preparative electrophoresis followed by isoelectrofocusing according to its pI of 6.4. The G12 mAb inhibits sperm binding to and penetration of zona-free hamster oocytes, suggesting that SOB2 antigen may be directly involved in binding of spermatozoa to the oocyte plasma membrane. It is, thus, a potential candidate for the development of a contraceptive vaccine. One logical and necessary next experimental step would be to sequence SOB2 from the gels.

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References

- Blobel, C.P., Wolfsberg, T.G., Turk, C.W. *et al.* (1992) A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. *Nature*, **356**, 248–252.
- Boue, F., Lassalle, B., Duquenne, C. *et al.* (1992) Human sperm proteins from testicular and epididymal origin that participate in fertilization: modulation of sperm binding to zona-free hamster oocytes, using monoclonal antibodies. *Mol. Reprod. Dev.*, **33**, 470–480.
- Boue, F., Berube, B., de Lamirande, E. *et al.* (1994) Human sperm-zona pellucida interaction is inhibited by an antisera against a hamster sperm protein. *Biol. Reprod.*, **51**, 577–587.
- Boue, F., Duquenne, C., Lassalle, B. *et al.* (1995) FLB-1, a human sperm protein of epididymal origin that is involved in the sperm-oocyte recognition process. *Biol. Reprod.*, **52**, 267–278.
- Bronson, R.A. and Fusi, F.M. (1990) Sperm-oolesmal interaction: role of the Arg-Gly-Asp (RGD) adhesion peptide. *Fertil. Steril.*, **54**, 527–529.
- Burks, D.J., Carballada, R., Moore, H.D.M. and Saling, P.M. (1995) Interaction of a tyrosine kinase from human sperm with zona pellucida at fertilization. *Science*, **269**, 83–86.
- Chang Yan, Y., Bai, Y., Wang, L. *et al.* (1990) Characterization of a cDNA encoding a human sperm membrane protein related to A4 amyloid protein. *Proc. Natl. Acad. Sci. USA*, **87**, 2405–2408.
- Cross, N.L., Morales, P., Overstreet, J.W. and Hanson, F.W. (1986) Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res.*, **15**, 213–226.
- Fenichel, P., Cervoni, F., Hofmann, P. *et al.* (1994) Expression of the complement regulatory protein CD59 on human spermatozoa: characterization and role in gametic interaction. *Mol. Reprod. Dev.*, **38**, 338–346.
- Freemerman, A.J., Flickinger, C.J. and Herr, J.C. (1995) Characterization of alternatively spliced human SP-10 mRNAs. *Mol. Reprod. Dev.*, **41**, 100–108.
- Fusi, F.M., Vignali, M., Busacca, M. and Bronson, R.A. (1992) Evidence for the presence of an integrin cell adhesion receptor on the oolemma of unfertilized human oocytes. *Mol. Reprod. Dev.*, **31**, 215–222.
- Fusi, F.M., Vignali, M., Gailit, J. and Bronson, R.A. (1993) Mammalian oocytes exhibit specific recognition of the RGD (Arg-Gly-Asp) tripeptide and express oolemmal integrins. *Mol. Reprod. Dev.*, **36**, 212–219.
- Garner, D.L., Pinkel, D., Johnson, L.A. and Pace, M.M. (1986) Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. *Biol. Reprod.*, **34**, 127–138.
- Gupta, S.K., Alves, K., O'Neil Palladino, G.E.M. and Hollis, G.F. (1996) Molecular cloning of the human fertilin β subunit. *Biochem. Biophys. Res. Commun.*, **224**, 318–326.
- Herr, J.C., Klotz, K., Siannon, J. *et al.* (1992) Purification and microsequencing of the intraacrosomal protein SP10. Evidence that SP10 heterogeneity results from endoproteolytic process. *Biol. Reprod.*, **47**, 11–20.
- Jury, J.A., Frayne, J. and Hall, L. (1997) The human fertilin α gene is non-functional: implications for its proposed role in fertilization. *Biochem. J.*, **321**, 577–581.
- Kadam, A.L., Fateh, M. and Naz, R.K. (1995) Fertilization antigen (FA-1) completely blocks sperm binding to human zona pellucida: FA-1 antigen may be a sperm receptor for zona pellucida in humans. *J. Reprod. Immunol.*, **29**, 19–30.
- Klentzeris, L.D., Fishel, S., McDermott, H. *et al.* (1995) A positive correlation between expression of β_1 -integrin cell adhesion molecules and fertilizing ability of human spermatozoa *in vitro*. *Mol. Hum. Reprod.*, **1**, see *Hum. Reprod.*, **10**, 728–733.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **15**, 680–685.
- Lassalle, B. and Testart, J. (1988) Effects of glutathione (GSH and GSSG) and glutathione reductase on zona-free hamster oocyte to decondense human sperm. *Gamete Res.*, **24**, 21–30.
- Lassalle, B. and Testart, J. (1994) Human zona pellucida recognition associated with removal of sialic acid from human sperm surface. *J. Reprod. Fertil.*, **101**, 703–711.

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- Lathrop, W.F., Carmichael, E.P., Myles, D.G. and Primakoff, P. (1990) cDNA cloning reveals the molecular structure of a sperm surface protein, PH-20, involved in sperm-egg adhesion and the wide distribution of its gene among mammals. *J. Cell. Biol.*, **111**, 2939–2949.
- Lee, C.Y.G., Yoshiki, T., Chui, L. *et al.* (1995) Epitope analysis of a sperm acrosomal antigen defined by HSA-5 monoclonal antibody. *J. Reprod. Immunol.*, **29**, 223–238.
- Liu, D.Y., Mahan, M., Lathrop, W.F. *et al.* (1988) A human sperm zona pellucida binding test using oocytes that failed to fertilize *in vitro*. *Fertil. Steril.*, **50**, 782–788.
- Morrissey, J.H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.*, **117**, 307–310.
- Muga, A., Neugebauer, W., Hiram, T. and Surewicz, W.K. (1994) Membrane interaction and conformational properties of the putative fusion peptide of PH-30, a protein active in sperm-egg fusion. *Biochemistry*, **33**, 4444–4448.
- Myles, D.G., Kimmel, L.H., Blobel, C. *et al.* (1994) Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion. *Proc. Natl. Acad. Sci. USA*, **91**, 4195–4198.
- Naz, R.K., Morte, C., Garcia Framis, V. *et al.* (1993) Characterization of a sperm specific monoclonal antibody and isolation of 95 kilodalton fertilization antigen-2 from human sperm. *Biol. Reprod.*, **49**, 1236–1244.
- O’Rand, M.G. (1988) Sperm-egg recognition and barriers to interspecies fertilization. *Gamete Res.*, **19**, 315–328.
- Primakoff, P., Hyatt, H. and Tredick-kline, J. (1987) Identification and purification of a sperm surface protein with a potential role in sperm-egg membrane fusion. *J. Cell. Biol.*, **104**, 141–149.
- Ramarao, C.S., Myles, D.G., White, J.M. and Primakoff, P. (1996) Initial evaluation of fertilin as an immun contraceptive antigen and molecular cloning of the *Cynomolgus* monkey fertilin β subunit. *Mol. Reprod. Dev.*, **43**, 70–75.
- Richardson, R.T., Yamasaki, N. and O’Rand, M. (1994) Sequence of a rabbit sperm zona pellucida binding protein and localization during the acrosome reaction. *Dev. Biol.*, **165**, 688–701.
- Rochwerger, L., Cohen, D.J. and Cuanescu, P.S. (1992). Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion. *Dev. Biol.*, **153**, 83–90.
- Rohwedder, A., Liedigk, O., Schaller, J. *et al.* (1996) Detection of mRNA transcripts of β_1 integrins in ejaculated human spermatozoa by nested reverse transcription-polymerase chain reaction. *Mol. Hum. Reprod.*, **2**, 499–505.
- Schaller, J., Glander, H.J. and Dethloff, J. (1993) Evidence of β_1 integrins and fibronectin on spermatogenic cells in human testis. *Hum. Reprod.*, **8**, 1873–1878.
- Towbin, H., Stehelin, T. and Gordin, H. (1979) Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and applications. *Proc. Natl. Acad. Sci. USA*, **75**, 4350–4354.
- Wasserman, P.M. (1988) Zona pellucida glycoproteins. *Ann. Rev. Biochem.*, **57**, 415–442.
- Whittingham, D. (1971) Culture of mouse oocytes. *J. Reprod. Fertil.*, **14** (Suppl.), 7–21.

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