# Expression of a P-selectin Ligand in Zona Pellucida of Porcine Oocytes and P-selectin on Acrosomal Membrane of Porcine Sperm Cells. Potential Implications for Their Involvement in Sperm–Egg Interactions

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***Abstract.*** The selectin family of cell adhesion molecules mediates initial leukocyte adhesion to vascular endothelial cells at sites of inflammation. *O*-glycan structural similarities between oligosaccharides from human leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) and from zona pellucida glycoproteins of porcine oocytes indicate the possible existence of a P-selectin ligand in the zona pellucida. Here, using biochemical as well as morphological approaches, we demonstrate that a P-selectin ligand is expressed in the porcine zona pellucida. In addition, a search for a specific receptor for this ligand leads to the identification of P-selectin on the acrosomal membrane of porcine sperm cells. In vitro binding of porcine acrosome-reacted sperm cells to oocytes was found to be $Ca^{2+}$ dependent and inhibitable with either P-selectin, P-selectin receptor–globulin, or leukocyte adhesion blocking antibodies against P-selectin and PSGL-1. Moreover, porcine sperm cells were found to be capable of binding to human promyeloid cell line HL-60. Taken together, our findings implicate a potential role for the oocyte P-selectin ligand and the sperm P-selectin in porcine sperm–egg interactions.

Cell–cell and cell–matrix interactions play central roles in biological development, such as during fertilization, implantation, placenta formation, embryogenesis, cell differentiation, migration, and organ formation. These types of interactions are also essential for a variety of physiological and pathological processes, such as lymphocyte trafficking, immune defense, hemostasis, wound healing, cancer cell invasion, and metastasis. Cell–cell and cell–matrix interactions are precisely controlled and regulated by cell and matrix adhesion molecules with specificities appropriate for their particular functions (Gumbiner, 1992; Hynes and Lander, 1992; Cross et al., 1994; Wassarman, 1995; Snell and White, 1996).

Recruitment of leukocytes, from the flowing blood stream across the endothelial cells of postcapillary venules into the tissue at sites of inflammation or injury, is a multistep paradigm requiring at least three sequential steps. Three major families of cell adhesion molecules are involved in this process: selectins, integrins, and adhesion molecules of the immunoglobulin superfamily (Butcher, 1991; Lasky, 1992; Springer, 1994). The selectins comprise a subfamily of $Ca^{2+}$-dependent (C-type) animal lectins (Drickamer, 1988, 1993) that are mainly responsible for the initial leukocyte tethering to, and rolling on, the activated endothelial cells. Three members of this family have been described to date (Butcher, 1991; Lasky, 1992; Springer, 1994). L-selectin (CD62L) is a constitutively expressed homing receptor on a majority of leukocytes for lymphatic and vascular endothelial cells. E-selectin (CD62E) is a cytokine-inducible cell surface receptor on vascular endothelial cells, and P-selectin (CD62P) is a rapidly inducible receptor on vascular endothelial cells and platelets. E- and P-selectins function as cell surface receptors for neutrophils, monocytes, T lymphocyte subsets, eosinophils, and basophils.

All three selectins recognize a sialoglycoprotein ligand, P-selectin glycoprotein ligand-1 (PSGL-1)[1] (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995; Spertini et al., 1996; Tu et al., 1996). PSGL-1 is a disulfide-linked dimeric sialomucin expressed on the microvilli of human leukocytes. The recognition of PSGL-1 by the three selectins is $Ca^{2+}$ dependent and sialidase sensitive, characteristics of selectin-mediated leuko-

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1. *Abbreviations used in this paper*: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxylmethyl ester; PGSL-1, P-selectin glycoprotein ligand-1; PUVEC, porcine umbilical vein endothelial cell; Rg, receptor-globulin; TNF-α, tissue necrosis factor-α.

cyte adhesion (Rosen et al., 1985; Yednock and Rosen, 1989; Bevilacqua et al., 1987, 1989; Larsen et al., 1989; Geng et al., 1990).

All three selectins have been reported to bind to the tetrasaccharide structure NeuNAcα2-3Galβ1-4(Fucα1-3) GlcNAc (called sialyl Lewis x or $SLe^x$) and its isomer, NeuNAcα2-3Galβ1-3(Fucα1-4)GlcNAc (called sialyl Lewis a or $SLe^a$); these structures may constitute the minimal recognition motif for the three selectins (Brandley et al., 1990; Varki, 1994). In a previous investigation, we isolated PSGL-1 from [$^3$H]glucosamine-labeled HL-60 cells, a human promyeloid cell line, by P- and E-selectin affinity chromatography. The desialylated *O*-linked oligosaccharides released from this molecule were separated into five well-defined peaks having elution volumes corresponding to glucose oligomers composed of 2.5, 3.5, 6.3, 9.8, and 12.8 glucose units, respectively (Asa et al., 1995). Sequencing of the carbohydrate structures in these peaks resulted in the identification of a set of oligosaccharides (Aeed, P., J.-G. Geng, D. Asa, L. Raycroft, L. Ma, and Å. Elhammer, manuscript submitted for publication), which had considerable similarities to structures previously identified on glycoproteins isolated from the porcine zona pellucida (Hirano et al., 1993).

The involvement of oligosaccharide structures in mammalian gamete interactions is well documented (Florman and Wassarman, 1985). In mouse, for instance, *O*-linked oligosaccharides have been specifically implicated in the sperm–egg binding (Wassarman, 1988; Litscher et al., 1995). To determine whether porcine gametes carry cell adhesion molecules similar to those described for the interaction of leukocytes with vascular endothelial cells, i.e., selectins and sialomucin-type ligand molecules (Butcher, 1991; Lasky, 1992; Springer, 1994), we investigated whether a P-selectin ligand and P-selectin are expressed on porcine oocytes and sperm cells, respectively.

## Materials and Methods

### Materials

Rabbit, mouse, and human IgGs, saponin, L-cysteine, and Hoechst 33258 (H258) were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse IgM was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Calcium ionophore A23187 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tissue necrosis factor-α (TNF-α) was purchased from Genzyme (Boston, MA). 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR). Paraformaldehyde was purchased from Mallinckrodt Inc. (St. Louis, MO). Glass microscope slides were purchased from Baxter Healthcare Corp. (McGaw Park, IL). Frozen porcine ovaries were obtained from PelFreeze Biologicals (Rogers, AR); fresh porcine ovaries were obtained from a local slaughter house; and fresh porcine umbilical cords were collected from a local farm. The membrane extracts of porcine leukocytes were prepared as previously described (Ma et al., 1994; Asa et al., 1995). HL-60 cells (CCL 240) and Ramos cells (CRL 1596) were obtained from American Tissue Culture Collection (Rockville, MD) and cultured as recommended. All other chemicals were purchased from previously described sources (Ma et al., 1994; Asa et al., 1995).

### Proteins and Antibodies

P-selectin was purified from outdated human platelets as published (Ma et al., 1994). E- and P-selectin receptor–globulins (Rg) were prepared as described (Asa et al., 1995).

Monoclonal IgG antibodies against P-selectin, P7 and P23, and monoclonal IgM antibodies against PSGL-1, PL5, and against $SLe^x$, CSLEX, were prepared and characterized as reported (Ma et al., 1994; Asa et al., 1995). Rabbit polyclonal antibodies raised against human platelet P-selectin and E-selectin Rg were prepared as previously described (Toombs et al., 1995).

A polyclonal IgG antibody against PSGL-1 was prepared as follows. A peptide corresponding to residues 41–55 (QATEYEYLDYDFLPEGGC) of the amino acid sequence of PSGL-1 (Sako et al., 1993) was synthesized on a 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). Two glycine residues (underlined) were added before the carboxyl-terminal cysteine to provide a spacer such that flexibility and availability of the peptide would be retained after conjugation. The peptide was coupled to maleimide-activated keyhole limpet hemocyanin via the carboxyl-terminal cysteine (underlined). Two New Zealand white rabbits (C3633 and C3365; Caltag Laboratories, San Francisco, CA) were immunized with the peptide conjugate. Rabbit IgG fractions were isolated from preimmune sera and antisera using mAb Trap G (Pharmacia Biotech, Piscataway, NJ). The IgG fractions were dialyzed against 27.3 mM Tris-phosphoric acid, pH 6.3, and concentrated on a Mono-Q ion-exchange column (Pharmacia Biotech) by elution with the same buffer containing 2 M NaCl. The purified proteins were then dialyzed against PBS, pH 7.4, and stored at 4°C.

For the immunostaining experiments, the protein G–purified IgG fraction (C3633) was further purified by affinity chromatography on the immobilized antigen peptide as follows. The peptide (2.2 mg) was coupled directly onto ~3 ml of SulfoLink Coupling Gel (Pierce Chemical Co., Rockford, IL); nonspecific binding sites on the gel were blocked with 50 mM L-cysteine, according to the manufacturer's protocol. The IgG fraction was incubated with the immobilized peptide beads at 4°C overnight with end-to-end rotation. After washing with 100 ml of PBS, the bound antibody was eluted with 0.1 M glycine-HCl, pH 2.7. The preparation was then concentrated by Mono-Q chromatography as described above. This procedure yielded ~2 mg of the affinity-isolated antibody protein from ~100 mg of the protein G–purified IgG fraction.

### Preparation of Porcine Oocytes and Zona Pellucida

Porcine oocytes and zona pellucida were prepared from frozen or fresh ovaries essentially as described (Dunbar et al., 1980). No obvious contamination by leukocytes could be observed by microscopy. The concentration of total zona pellucida proteins was determined by amino acid compositional analysis.

### Isolation of Porcine Sperm Cells

Fresh porcine sperm were collected at a local farm and kept at 37°C until use. No obvious contamination of blood cells could be found in the white milky suspension. The sperm was kept still for at least 15 min at 37°C in the presence of 5% $CO_2$, and "swim-up" sperm cells were carefully collected on the top layer of the sperm suspension. This was done to avoid the possible contaminating blood cells and to eliminate less viable sperm cells. No obvious contamination by leukocytes could be observed by microscopy.

### Preparation of Porcine Umbilical Vein Endothelial Cells

Porcine umbilical vein endothelial cells (PUVEC) were prepared from the freshly collected porcine umbilical cords exactly as previously described for human umbilical vein endothelial cells (Geng et al., 1990; Ma et al., 1994). For induction of E-selectin expression, confluent monolayers of PUVEC (third passage) were treated with 300 U/ml of TNF-α at 37°C for 4 h. After washing once with PBS, the cells were harvested by mechanical detachment with a cell scraper (Nunc, Naperville, IL) in the presence of Versine™ (GIBCO BRL, Gaithersburg, MD).

### CHO Cell Line Expressing E-Selectin

A stable CHO-K1 cell line expressing human full-length E-selectin was established by cotransfection of E-selectin cDNA in CDM8 vector (10 μg/ml) with pcDNA1/Neo (1 μg/ml) using a Lipofectin™ (GIBCO BRL) method according to manufacturer's protocol. 3 d later, CHO cells were selected and maintained in DME (high glucose) in the presence of 10% FCS (vol/vol) and 0.4 mg/ml of active Geneticin™ (wt/vol; GIBCO BRL).

## SDS-PAGE and Silver Staining

Aliquots of total zona pellucida proteins (~38 μg per lane) and sperm cells ($5 \times 10^6$ cells per lane; washed three times with ice-cold PBS) were mixed with SDS sample buffer in the presence or absence of 5% β-mercaptoethanol (vol/vol). After boiling for 5 min, samples were subjected to 7% SDS-PAGE. After electrophoresis, proteins were silver stained (Bio Rad Laboratories, Hercules, CA).

## Ligand Blotting

Aliquots of total zona pellucida proteins (~115 μg per lane) and porcine leukocyte membrane extracts (~500 μg per lane) were mixed with SDS sample buffer in the presence or absence of 5% β-mercaptoethanol (vol/vol). Samples were boiled for 5 min and subjected to 7% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). The membranes were probed with P-selectin followed by biotinylated P23 mAb (1 μg/ml). The membranes were subsequently incubated with a streptavidin–peroxidase complex (Vectostain ABC kit; Vector Laboratories, Burlingame, CA). A chemiluminescent detection system (Amersham Corp., Arlington Heights, IL) was used for detection (Ma et al., 1994; Asa et al., 1995). All incubation and washing buffers contained either 1 mM $CaCl_2$ or 2 mM EDTA as indicated.

## Immunoblotting

Aliquots of total zona pellucida proteins (~115 μg per lane), sperm cells ($1 \times 10^6$ cells per lane), and TNF-α–treated PUVECs (confluent monolayer of cells from a 35-mm dish per lane) were subjected to 7% SDS-PAGE. The separated proteins were transferred to the blotting membranes as described above. For the zona pellucida proteins, the blotting membranes were probed with preimmune IgG or PSGL-1 peptide antibody (both at 1 μg/ml). They were then incubated with biotinylated goat antibodies against rabbit IgG (5 μg/ml). For sperm cells and PUVECs, the blotting membranes were probed separately with either biotinylated rabbit P- or E-selectin antibody or biotinylated P7 mAb (all at 1 μg/ml). The membranes were subsequently incubated with a streptavidin–peroxidase complex followed by a chemiluminescent detection system as described above.

## Flow Cytometric Analysis

Porcine sperm cells were used either unwashed or washed three times with HBSS/FCS (1% heat-inactivated FCS in HBSS, vol/vol; 3,000 rpm for 10 min). More than 90% of the washed sperm cells were mechanically capacitated, as determined using Coomassie brilliant blue (Aarons et al., 1991). The sperm cells were resuspended in HBSS/FCS ($2 \times 10^6$ cells per ml) and incubated separately with either an FITC-conjugated rabbit preimmune IgG, an FITC-conjugated rabbit polyclonal antibody against P- or E-selectin, an FITC-conjugated mouse preimmune IgG, or an FITC-conjugated P7 mAb (all at 10 μg/ml) at 22°C for 1 h. For staining of PUVECs, mechanically detached cells (confluent monolayer of cells from a 35-mm dish per aliquot) were resuspended in HBSS/FCS and incubated with an FITC-conjugated rabbit preimmune IgG or an FITC-conjugated rabbit E-selectin antibody, as outlined above. After incubation, the cells were sedimented by centrifugation at 1,500 rpm for 5 min and washed twice with HBSS/FCS. The cells were then resuspended in HBSS/FCS for flow cytometric analysis (FACScan®; Becton Dickinson & Co., Mountain View, CA).

## Immunoelectron Microscopy

Porcine oocytes were washed with HBSS/FCS and incubated with purified platelet P-selectin (10 μg/ml) at 22°C for 1 h. As controls, P-selectin was either omitted or the oocytes were incubated with P-selectin in calcium and magnesium-free HBSS/FCS containing 2 mM EDTA. After washing, the oocytes were incubated with rabbit P-selectin antibody (25 μg/ml) for 30 min. For antibody staining, porcine oocytes were first incubated in 10% (vol/vol) normal goat serum in PBS for 1 h at 22°C. After this step, the oocytes were incubated with 1 μg/ml of PSGL-1 peptide antibody or preimmune IgG for 1 h at 22°C. After washing, samples were incubated with 50 μg/ml of affinity-purified goat anti–rabbit IgG conjugated with HRP (Accurate Chemical Co., Westbury, NY) for 1 h at 22°C. The oocytes were rinsed and fixed with 2.5% glutaraldehyde (vol/vol) and 2% paraformaldehyde (wt/vol) in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.5 mM $CaCl_2$ at 4°C for 30 min. They were then processed for peroxidase cytochemistry and EM as previously described (Raub et al., 1990).

Rabbit P- and E-selectin antibodies were conjugated with gold particles as previously described (Raub and Kuentzel, 1984). Porcine sperm cells and CHO cells expressing E-selectin were rinsed with HBSS/FCS and incubated with a sixfold dilution of the gold-conjugated rabbit P- or E-selectin antibody in HBSS/FCS at 22°C for 1 h. For inhibition experiments as control, sperm cells were incubated with the gold-conjugated P-selectin antibody in the presence of 200 μg/ml of the unconjugated P-selectin antibody. The labeled cells were fixed with 2% paraformaldehyde (wt/vol) in PBS at 4°C for 15 min followed by 0.1 M ammonium chloride for 5 min. The cells were processed for EM as previously described (Raub and Kuentzel, 1984). Semithick (0.25 μm), en face sections through the cells were viewed with a JEM-1200EX electron microscope (JEOL, Inc., Peabody, MA) operated at 100–120 kV.

## Sperm–Egg Binding Assay

A sperm–egg binding assay was set up according to a published procedure (Almeida et al., 1995). Porcine sperm cells and oocytes were gently washed once (1,000 rpm for 10 min) with HBSS/BSA (1% BSA in HBSS; wt/vol) except in controls where calcium and magnesium-free HBSS/BSA containing 2 mM EDTA was used throughout the entire assay. The sperm (0.1-ml aliquots of $2 \times 10^6$ cells per ml) were mixed with oocytes (0.1-ml aliquots of ~500 oocytes per ml), in the presence or absence of 5 μM A23187, at 22°C for 1 h. The cell mixtures were carefully layered on the top of 100% dialyzed and heat-inactivated FCS (1 ml per tube) and spun at 500 rpm on a table-top centrifuge for 2 min (FCS "cushion"). Under these washing conditions, <10% of the free sperm cells were mechanically capacitated as determined using Coomassie brilliant blue (Aarons et al., 1991), and more than 90% of the free sperm cells were viable according to their ability to exclude Hoechst 33258 (Tao et al., 1993). The supernatants were discarded and the cell pellets were fixed with 0.2 ml per tube of 2% paraformaldehyde (freshly prepared in PBS, wt/vol). The preparations were transferred onto glass microscope slides and examined under a microscope (Nikon Phase Contrast-2, EL WD 0.3; Tokyo, Japan), equipped with a screen monitor (VOCON Industries, Inc., New York) and a VC2400 video camera (VICON Industries, Inc., New York). The microscopic images were printed using a color video printer (UP-5200MD; Sony, Park Ridge, NJ).

For the inhibition studies, sperm cells were preincubated with mouse IgG, P23 mAb, or P7 mAb (all at 30 μg/ml) in the presence of 5 μM A23187 at 22°C for 30 min. Oocytes were preincubated with mouse IgM, CSLEX mAb, PL5 mAb, or human IgG, E-selectin Rg, or P-selectin Rg (all at 30 μg/ml) at 22°C for 30 min. The cells were then mixed with sperm cells without washing for the binding assay, as described above.

## Sperm Cell–HL-60 Cell Binding Assay

Freshly collected sperm (0.3 ml) were resuspended into 30 ml of HBSS/BSA and loaded with 2 μM BCECF-AM at 37°C for 30 min (Ma et al., 1994; Asa et al., 1995). The labeled sperm cells as well as HL-60 and Ramos cells were washed three times with PBS (1,000 rpm for 10 min). The cells were resuspended in HBSS/BSA ($2 \times 10^6$ cells per ml), except in controls where calcium and magnesium-free HBSS/BSA containing 2 mM EDTA was used, throughout the assay. Under these washing conditions, ~30–50% of the sperm cells were mechanically capacitated as determined using Coomassie brilliant blue (Aarons et al., 1991), and >80% of the free sperm cells were viable based on their ability to exclude Hoechst 33258 (Tao et al., 1993).

The labeled sperm cells ($1 \times 10^6$ cells in a 0.5-ml aliquot) were mixed with either Ramos cells or HL-60 cells ($2 \times 10^5$ cells in a 0.1-ml aliquot) in the presence or absence of 5 μM A23187 at 22°C for 1 h, with end-to-end rotating. The unbound sperm cells were removed on a FCS cushion (0.5 ml of FCS per tube, centrifuged at 700 rpm on a table-top centrifuge for 2 min). The cell pellets were fixed with 2% paraformaldehyde (wt/vol; 0.5 ml per tube) for flow cytometric analysis (FACScan®). The binding of the fluorescence-labeled sperm cells to the Ramos or HL-60 cells was measured as the mean fluorescence intensity from >100,000 cells in the gated windows for Ramos or HL-60 cells.

For the inhibition studies, the labeled sperm cells were preincubated with mouse IgG, P23 mAb, or P7 mAb (all at 30 μg/ml) in the presence of 5 μM A23187 at 22°C for 30 min. HL-60 cells were preincubated with either mouse IgM, CSLEX mAb, PL5 mAb, or human IgG, E-selectin Rg, or P-selectin Rg (all at 30 μg/ml) at 22°C for 30 min. The cells were subsequently mixed with oocytes without washing for the binding assay.

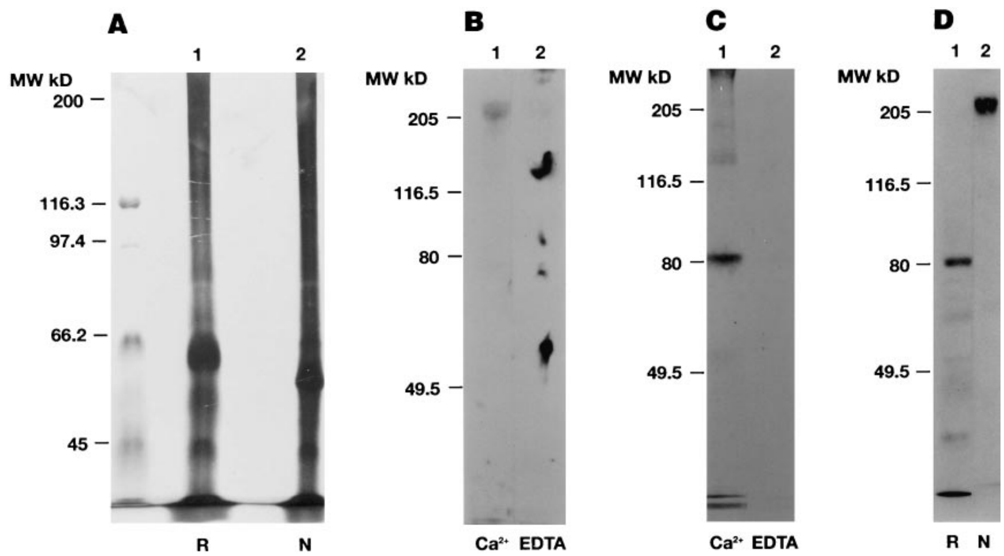


*Figure 1.* $Ca^{2+}$-dependent binding of P-selectin to a dimeric molecule from porcine zona pellucida. (*A*) Silver staining of total zona pellucida proteins (~38 μg per lane) separated on 7% SDS-PAGE in the presence (*R*) or absence (*N*) of 5% β-mercaptoethanol (vol/vol). (*B*) P-selectin ligand blotting of zona pellucida proteins (~115 μg per lane), fractionated on 7% SDS-PAGE under nonreducing conditions. After electrophoresis, the proteins were transferred onto a blotting membrane and probed with human platelet P-selectin followed by a biotinylated P23 mAb in the presence of 1 mM $CaCl_2$ or 2 mM EDTA, as indicated. The blots were visualized with a chemiluminescence detection system (Amersham Corp.). (*C*) P-selectin ligand blotting of the zona pellucida proteins under reducing conditions. (*D*) P-selectin ligand blotting of the membrane extracts of porcine leukocytes (~0.5 mg per lane) under reducing (*R*) and nonreducing (*N*) conditions. Blotting procedures were exactly as described for *B*.

## Results

### *Expression of a P-selectin Ligand in Zona Pellucida of Porcine Oocytes*

To investigate whether the zona pellucida of porcine oocytes contains a specific ligand for P-selectin, we took advantage of the cross-reactivity of human platelet P-selectin with porcine neutrophils, i.e., the fact that human P-selectin supports adhesion of porcine neutrophils (Geng, J.-G., unpublished observations). As shown in Fig. 1 *A*, porcine zona pellucida contains many proteins as visualized by silver staining. Based on densitometric measurements, >50%

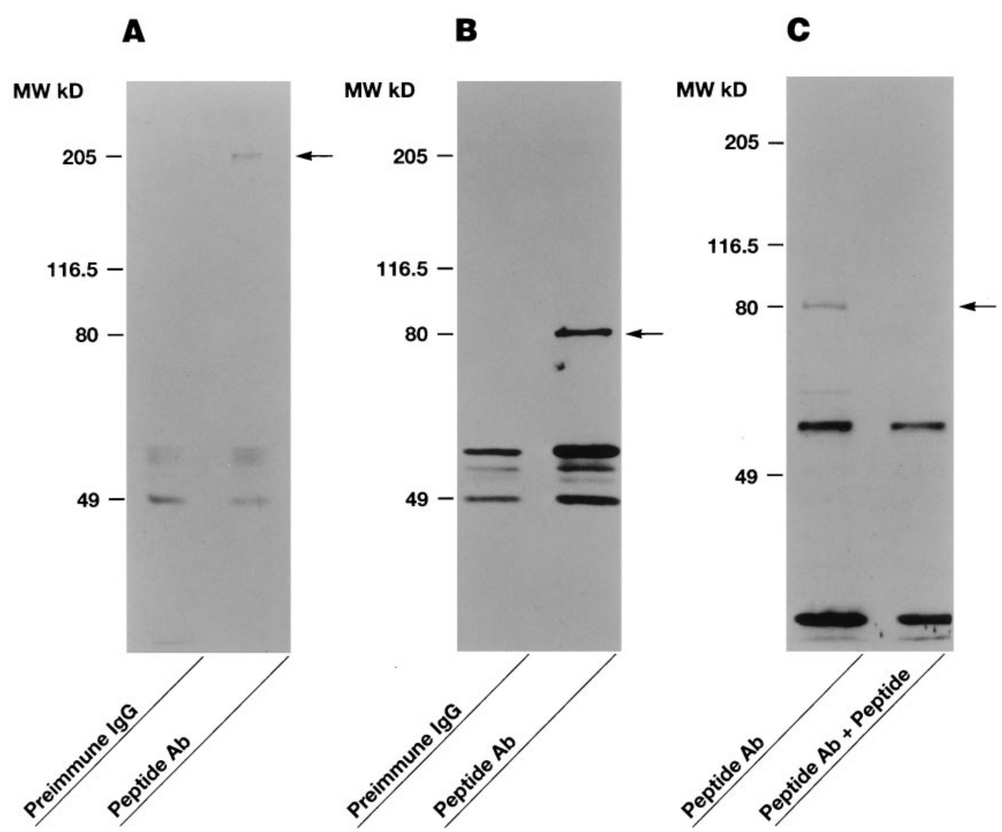


*Figure 2.* Immunoblotting of zona pellucida proteins with PSGL-1 peptide antibody. Porcine zona pellucida proteins, fractionated by 7% SDS-PAGE under nonreducing (*A*) and reducing (*B* and *C*) conditions and transferred to blotting membranes, were probed with rabbit preimmune IgG or PSGL-1 peptide antibody followed by biotinylated antibody to rabbit IgG. Immunoreactive proteins were visualized as outlined in the legend to Fig. 1.

of the proteins were recovered in the 40–70-kD ranges, under reducing conditions. This is consistent with published data (Dunbar et al., 1980).

In a parallel experiment, the zona pellucida proteins, separated by 7% SDS-PAGE and transferred to blotting membranes, were probed with human platelet P-selectin. Surprisingly, P-selectin specifically bound to a single protein with a molecular mass of ~210 kD under nonreducing conditions (Fig. 1 *B*, lane *1*) and ~80 kD under reducing conditions (Fig. 1 *C*, lane *1*). The binding was $Ca^{2+}$ dependent as it was completely abolished by performing the P-selectin incubation in the presence of 2 mM EDTA (Fig. 1, *B* and *C*, lanes *2*). P-selectin also specifically recognized a dimeric molecule from porcine leukocyte membrane extracts, with molecular masses identical to those of the zona pellucida protein under both nonreducing and reducing conditions (Fig. 1 *D*).

Three additional weaker bands (at ~210, ~180, and ~140 kD under reducing conditions) were also recognized by P-selectin (Fig. 1 *C*, lane *1*). The presence of the ~210-kD band may be due to incomplete reduction of the ~210-kD dimer, a phenomenon frequently observed for human leukocyte PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994). The nature of ~180- and ~140-kD bands is unclear; they were not recognized by P-selectin (Fig. 1 *B*, lane *1*) and PSGL-1 peptide antibody (see Fig. 3 *A*) when proteins were separated under nonreducing conditions. Plus, they were not recognized by PSGL-1 peptide antibody (Fig. 3, *B* and *C*), under reducing conditions. In addition, there was some background staining spots on the blots, which were not similar to the protein bands (Fig. 1 *B*, lane *2*).

To corroborate the above findings, the porcine zona pellucida proteins were also probed with an antibody against a synthetic peptide encoding residues 41–55 of the amino acid sequence of PSGL-1. Fig. 2 shows that this antibody, but not preimmune IgG, bound to the ~210-kD protein under nonreducing conditions (*A*, *arrow*) and the ~80-kD protein under reducing conditions (*B*, *arrow*). Preincubation of the antibody with the synthetic peptide abrogated this binding (*C*, *arrow*). The protein bands at ~50–60 kD were most likely due to nonspecific binding, since (*a*) they existed in the blots probed with both preimmune IgG and PSGL-1 peptide antibody (*A* and *B*), and (*b*) they were not inhibited with the respective peptide antigen (*C*).

Using EM, the distribution of the P-selectin ligand in the zona pellucida of porcine oocytes was examined after labeling either with P-selectin followed by P-selectin antibody or with PSGL-1 peptide antibody. The experiments demonstrated that the P-selectin ligand was associated with membrane fragments and vesicles embedded throughout

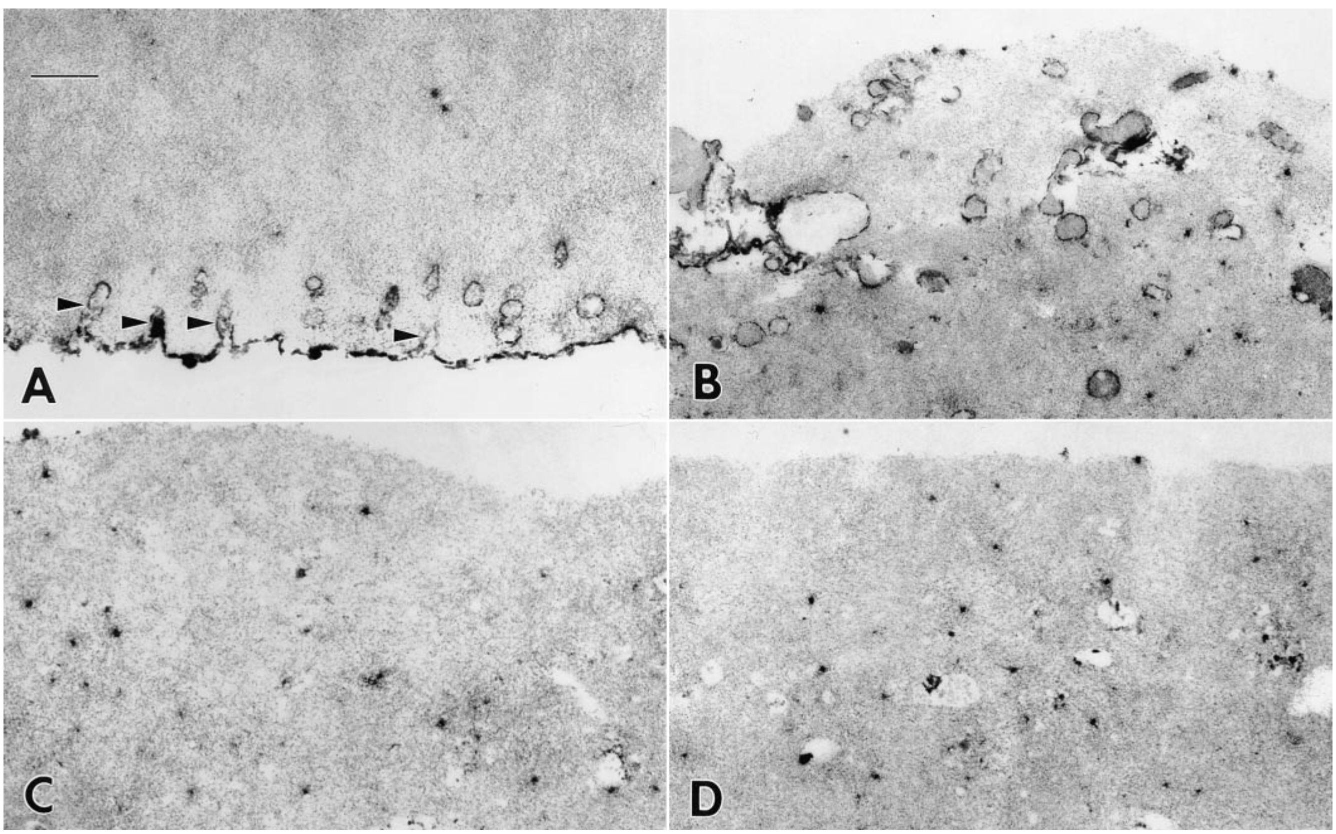


*Figure 3.* Distribution of a P-selectin ligand in porcine zona pellucida. Porcine oocytes were washed and incubated sequentially with platelet P-selectin, rabbit P-selectin antibody, and an HRP-conjugated secondary antibody. P-selectin binding was visualized by the electron-dense reaction product. P-selectin is bound to the membrane vesicles and fragments embedded within the zona pellucida at or near the (*A*) inner surface (*arrows*) of the zona pellucida, which is adjacent to the oocyte removed during the staining procedures, and (*B*) outer surface. (*C*) Zona pellucida stained in the absence of P-selectin. (*D*) Zona pellucida stained with P-selectin in the presence of EDTA. Bar, 500 nm.

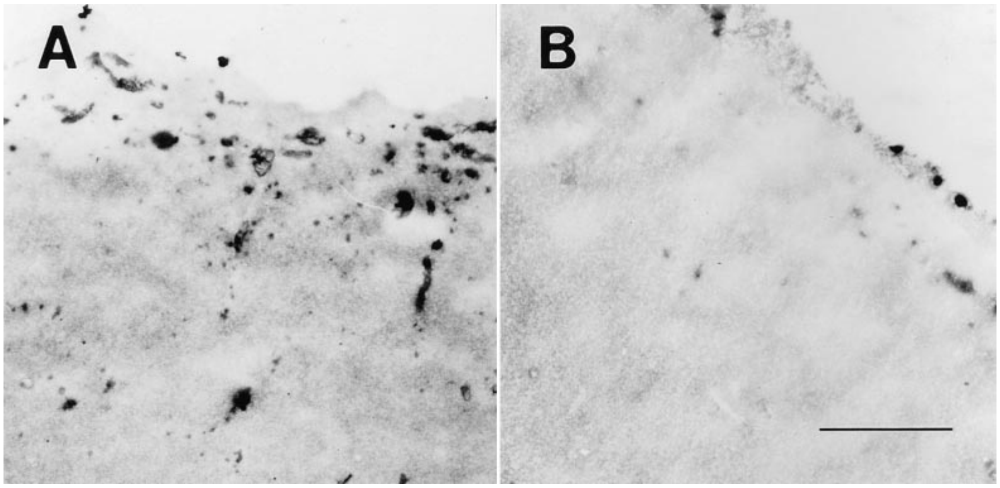


*Figure 4.* Staining of porcine zona pellucida with PSGL-1 peptide antibody. Zona pellucida of porcine oocytes were stained with either (*A*) PSGL-1 peptide antibody or (*B*) preimmune IgG followed by indirect immunoperoxidase EM as described above. The outer surfaces of the zona are shown. Bar, 2 μm.

the matrix of the zona pellucida (Figs. 3 and 4). The specificities of these approaches were confirmed by the absence, or marked reduction, of peroxidase reaction product in control oocytes, where either P-selectin (Fig. 3 *C*) or P-selectin antibody (data not shown) was omitted, where P-selectin was incubated in the presence of 2 mM EDTA (Fig. 3 *D*), or where preimmune IgG was used (Fig. 4 *B*).

### *P-selectin Expression on Acrosomal Membrane of Porcine Sperm Cells*

The expression of P-selectin on porcine sperm cells was first established by FACS® analysis, using two different P-selectin antibodies, an FITC-conjugated rabbit P-selectin antibody and an FITC-conjugated P7 mAb. Both antibodies were raised against human platelet P-selectin and both reacted with porcine platelet P-selectin (see Fig. 7 *C*). As shown in Fig. 5, rabbit P-selectin antibody (*A*) and P7 mAb (*B*) bound to repeatedly washed sperm cells whose plasma membranes were no longer intact (see Fig. 8). By contrast, FITC-conjugated rabbit E-selectin antibody did not bind to the sperm cells (Fig. 5 *C*), although it clearly reacted with the TNF-α–treated PUVEC (*D*). Interestingly, P-selectin polyclonal antibody did not bind to the unwashed sperm cells (Fig. 6 *A*) unless they were treated with A23187 (a calcium ionophore known to induce the acrosomal reaction; Fig. 6 *B*), repeated washing (causing disruption of the plasma membranes, as demonstrated in Fig. 8; Fig. 6 *C*), or saponin (a detergent that selectively permeabilizes the plasma membrane; Fig. 6 *D*). Together, these results suggest that porcine sperm cells express P-selectin, but not E-selectin, and that P-selectin is expressed on the acrosomal membrane of the sperm cells, but not on the plasma membrane.

To corroborate the finding of P-selectin expression on porcine sperm cells, an immunoblotting experiment was carried out. Intact sperm cells were washed with ice-cold PBS and lysed in SDS sample buffer. The total sperm cell proteins were separated by SDS-PAGE and stained by silver staining. This resulted in numerous protein bands with various molecular masses, under reducing and nonreducing conditions (Fig. 7 *A*). The separated proteins were also transferred to blotting membranes and probed with either rabbit P- or E-selectin antibody or with P7 mAb. Both P-selectin antibodies bound to ~120-kD proteins under nonreducing conditions (Fig. 7 *B*, *arrow*). The observed molecular mass is identical to the molecular mass for human platelet P-selectin (Ma et al., 1994) and porcine platelet P-selectin (Fig. 7 *C*) (Toombs et al., 1995). The identities of the additional protein bands, with lower molecular masses, observed on the blots are not known. They may represent proteolytic fragments of P-selectin generated

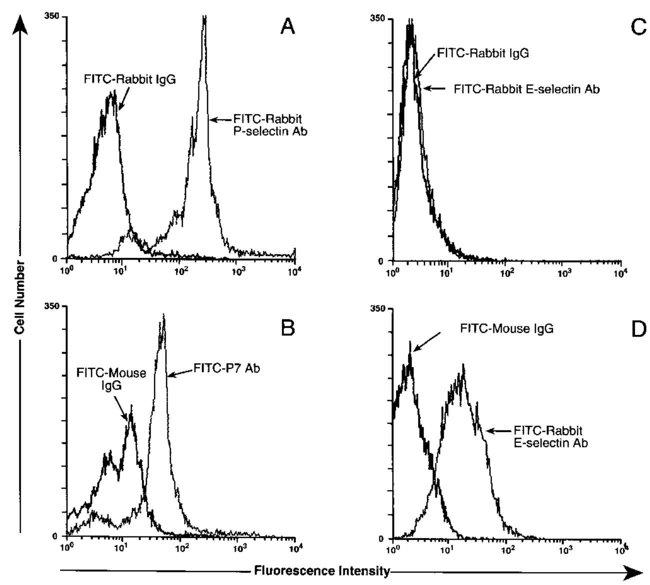


*Figure 5.* P-selectin antibody binding to sperm cells. Repeatedly washed porcine sperm cells (2 × $10^6$ cells per ml) were incubated separately with FITC-labeled rabbit IgG, an FITC-labeled rabbit P-selectin antibody (*A*), FITC-labeled mouse IgG, an FITC-labeled P7 mAb against P-selectin (*B*), or an FITC-labeled rabbit E-selectin antibody (*C*), respectively, at 22°C for 1 h (all at 10 μg/ml). Similarly, TNF-α–treated PUVECs were incubated separately with FITC-labeled rabbit IgG or an FITC-labeled rabbit E-selectin antibody (*D*). After washing, the sperm and endothelial cells were analyzed by flow cytometry (FACScan®). Results were presented as histograms of log fluorescence intensities over cell numbers from 10,000 cells.

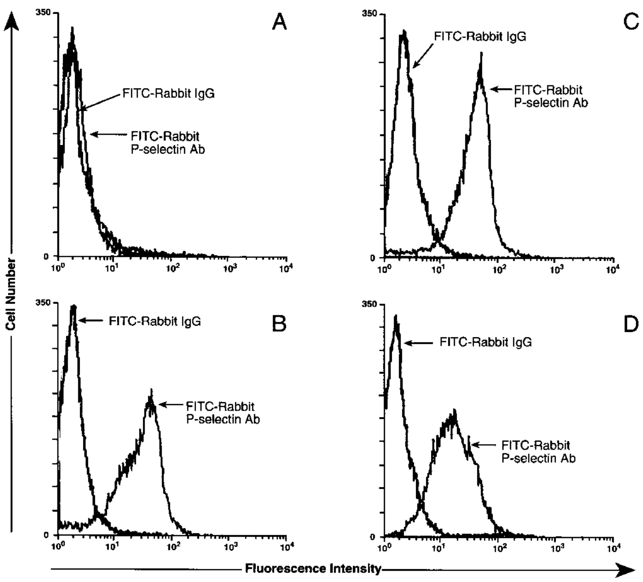


*Figure 6.* P-selectin antibody binding to sperm cells after acrosomal reaction or permeablization. Porcine sperm cells ($2 \times 10^6$ cells per ml) without prior washing (*A*), without prior washing in the presence of 5 μM A23187 (*B*), with prior repeated washing (*C*), and without prior washing in the presence of 0.01% saponin (*D*) were incubated with either FITC-labeled rabbit IgG or FITC-labeled rabbit P-selectin antibody at 22°C for 1 h (all at 10 μg/ml). After washing, the cells were analyzed by flow cytometry as above.

during the lysis of the sperm cells, since (*a*) sperm cells were known to contain a variety of proteases (Eddy, 1988), and (*b*) the antibodies used in this experiment specifically bound to P-selectin among all the proteins present in the platelet lysates (Fig. 7 *C*). The relatively sharper ~120-kD bands from sperm cells (Fig. 7 *B*, *arrow*), as compared with the relatively broad bands from platelets (Fig. 7 *C*), could also result from the proteolytic cleavage of some forms of the sperm P-selectin. Differential protease accessibility of heterogeneous glycosylated platelet P-selectin has been demonstrated previously (Johnston et al., 1989). E-selectin antibody did not recognize any protein in the porcine sperm cells, although it bound avidly to a ~90-kD protein from TNF-α–treated PUVEC (Fig. 7 *D*).

Further studies, using immunoelectron microscopy and a gold-conjugated rabbit P-selectin antibody, revealed that the localization of P-selectin on the porcine sperm cells was confined to the region of the sperm head, containing the dense nucleus, covered only by the exposed acrosome (Fig. 8 *A*, *1*), including the acrosomal cap (Fig. 8 *A*, *arrow*). The gold-conjugated P-selectin antibody did not label the lower one-third of the sperm head (Fig. 8 *A*, *2*), the neck (Fig. 8 *A*, *3*), or the tail (Fig. 8 *A*, *4*). Consistent with the results from the flow cytometric studies (Fig. 6), label was not observed on sperm cells with the plasma membrane still intact (Fig. 8 *B*). Incubation of sperm cells with the gold-conjugated P-selectin antibody in the presence of the unconjugated P-selectin antibody abolished the binding (Fig. 8 *C*). Again, gold-conjugated rabbit E-selectin antibody did not label the sperm cells (Fig. 8 *D*). Neither the broken plasma membrane (Fig. 8 *D*, *arrow*) nor the acrosome was labeled, although it labeled the surface of a CHO cell line expressing human E-selectin (Fig. 8 *E*). Thus, these results confirm the expression of P-selectin, but not E-selectin, on the acrosomal membranes, but not on the plasma membrane, of porcine sperm cells. Furthermore, the detection of P-selectin after acrosomal reaction suggests the possible localization of the molecule on the inner acrosomal membrane.

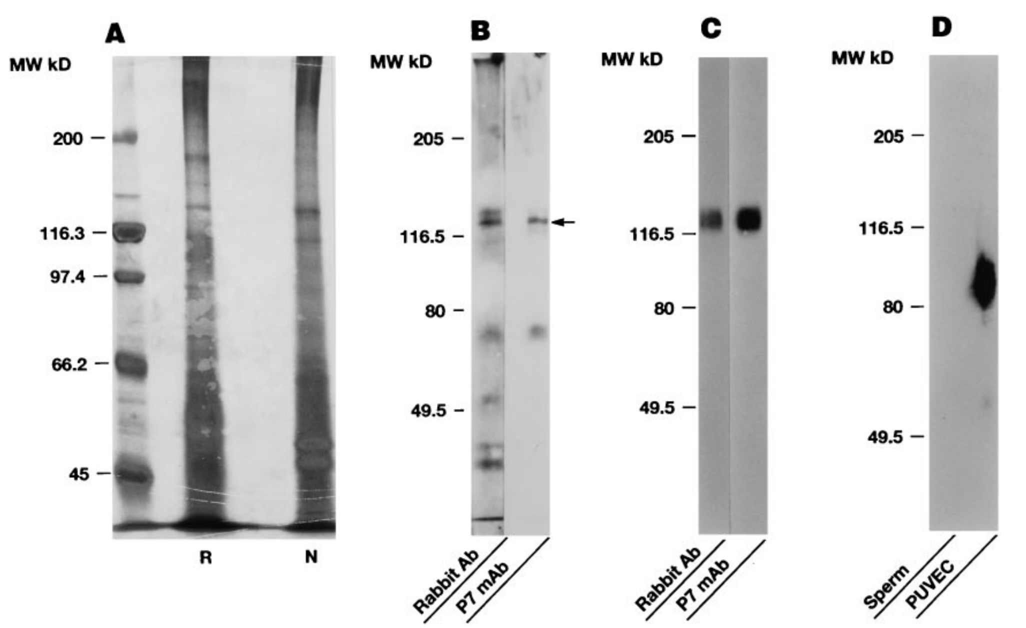


*Figure 7.* Immunoblotting of porcine sperm cells. Porcine sperm cells ($5 \times 10^5$ cells per lane for silver staining and $1 \times 10^6$ cells per lane for immunoblotting), porcine platelets ($1 \times 10^5$ cells per lane), and PUVEC (confluent monolayer of cells from a 35-mm dish per lane) were mixed with SDS sample buffer and boiled for 5 min. After electrophoresis under reducing (*A*, *R*) and nonreducing conditions (*A*, *N*; *B–D*), proteins were either silver stained (*A*) or transferred to blotting membranes and probed with 1 μg/ml of biotinylated rabbit P-selectin antibody, biotinylated P7 mAb against P-selectin (*B* for sperm cells and *C* for platelets), or biotinylated rabbit E-selectin antibody (*D*). Immunoreactive proteins were visualized as outlined in the legend to Fig. 1.

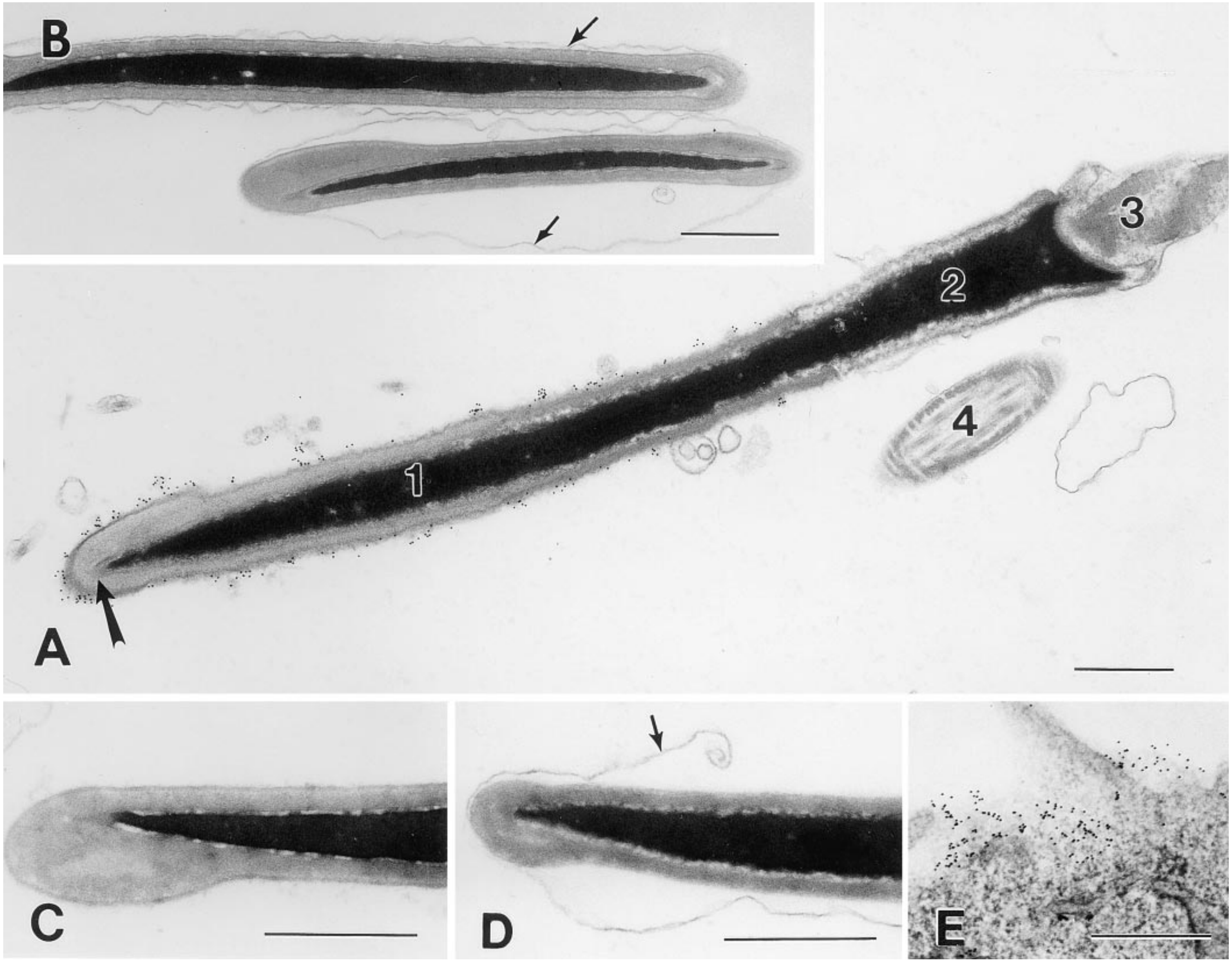


*Figure 8.* Localization of P-selectin on sperm cells. Washed sperm cells were incubated with a gold-conjugated P- or E-selectin antibody and examined by EM. (*A*) The staining of P-selectin antibody was confined to the region of the sperm head (*1*), which contained the dense nucleus, covered only by the acrosome including the acrosomal cap (*arrow*). P-selectin was not expressed on the lower third of the head region (*2*), the neck (*3*), or the tail (*4*). (*B*) The gold-conjugated P-selectin antibody did not label sperm cells with the plasma membrane (*arrow*) still intact. (*C*) Control sperm cell was incubated with the gold-conjugated P-selectin antibody in the presence of 200 μg/ml of the unconjugated P-selectin antibody. (*D*) The gold-conjugated E-selectin antibody did not label the sperm cell. Neither the broken plasma membrane (*arrow*) nor the acrosome were labeled. (*E*) The gold-conjugated E-selectin antibody labeled the cell surface of a CHO cell expressing human E-selectin. Bar, 0.5 μm.

### *Function of the Oocyte P-selectin Ligand and the Sperm Cell P-selectin*

To evaluate the function of the P-selectin ligand in the zona pellucida of porcine oocytes and P-selectin on the acrosomal membrane of porcine sperm cells, an in vitro sperm–oocyte binding assay was carried out, essentially according to the published procedure (Almeida et al., 1995). In this assay, acrosome-reacted sperm cells bound to oocytes in numbers usually exceeding 15 sperm cells per oocyte (referring to the number of bound sperm cells viewed in the single optical plane used; the total number of sperm cells bound to the entire oocyte was considerably larger; Table I). The binding was $Ca^{2+}$ and $Mg^{2+}$ dependent, since it could be dramatically reduced (to less than three sperm cells per oocyte) by treatment with EDTA. The requirement for divalent cations for sperm–egg binding is consistent with previous observations (Yanagimachi, 1988).

Sperm–egg binding was also significantly reduced (to less than five sperm cells per oocyte) by preincubation of the sperm cells with P7 (a leukocyte adhesion blocking mAb against P-selectin), or by preincubation of the oocytes with PL5 (a leukocyte adhesion blocking mAb against PSGL-1), purified platelet P-selectin, or recombinant P-selectin Rg (Table I). By contrast, the sperm–egg binding was not affected by preincubation of the oocytes with mouse IgM, CSLEX (an mAb against $SLe^x$), mouse IgG, P23 (a leukocyte adhesion nonblocking mAb against P-selectin), or E-selectin Rg (Table I).

These results strongly suggest that both the P-selectin ligand in the zona pellucida of porcine oocytes and P-selectin on the acrosome-reacted sperm cells are biologically func-

*Table I. Adhesion of Sperm Cells to Oocytes*

| Condition | Sperm cells per oocyte* |
|---|---|
| − | 18 ± 3 |
| EDTA | 1 ± 1 |
| Mouse IgG | 20 ± 4 |
| P23 mAb | 19 ± 5 |
| P7 mAb | 4 ± 3 |
| Mouse IgM | 19 ± 6 |
| CSLEX mAb | 17 ± 2 |
| PL5 mAb | 5 ± 4 |
| Human IgG | 17 ± 2 |
| P-selectin Rg | 2 ± 2 |
| E-selectin Rg | 16 ± 5 |
| P-selectin | 3 ± 1 |

Porcine sperm cells ($2 \times 10^6$ cells per ml) and oocytes (~500 oocytes per ml) were resuspended in HBSS/BSA (−), or calcium and magnesium–free HBSS containing 2 mM EDTA (*EDTA*). The sperm cells and oocytes were mixed in the presence of A23187 unless specifically indicated. Aliquots of oocytes were preincubated separately with mouse IgM, CSLEX (an mAb against $SLe^x$), PL5 (an mAb against PSGL-1; all at 30 μg/ml), or platelet P-selectin (10 μg/ml), or human IgG, P-, and E-selectin Rgs (all at 30 μg/ml), respectively. Aliquots of sperm cells were preincubated with mouse IgG, P7 (a leukocyte adhesion blocking mAb against P-selectin), or P23 (a leukocyte adhesion nonblocking mAb against P-selectin; all at 30 μg/ml) at 22°C for 30 min. All results were expressed as the mean ± SD number of adherent sperm cells per oocyte in the optical plane studied in five separate experiments; more than 50 oocytes were observed for each condition.

*Refers to the number of bound sperm cells viewed in the single optical plane used.

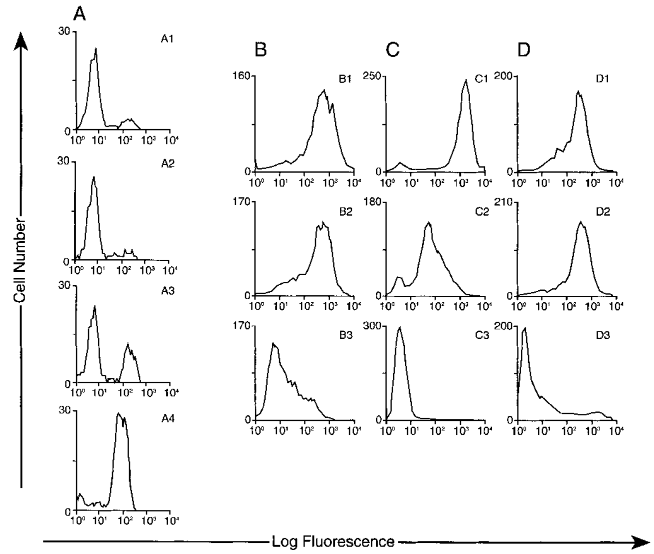


*Figure 9.* Binding of porcine sperm cells to human myeloid cell line HL-60. Freshly collected sperm cells were loaded with a fluorescent dye, BCECF-AM. The labeled sperm cells ($1 \times 10^6$ cells in 0.5-ml aliquots) were mixed with either Ramos cells or HL-60 cells ($2 \times 10^5$ cells in 0.1-ml aliquots) at 22°C for 1 h in the presence of 5 μM A23187, 1.26 mM $CaCl_2$, and 0.81 mM $MgCl_2$, unless otherwise indicated. After removal of unbound sperm cells, the cell pellets were fixed with 2% paraformaldehyde. Binding of the fluorescence-labeled sperm cells to Ramos or to HL-60 cells was measured by flow cytometric analysis (FACScan®). (*A*) Binding of labeled sperm cells to Ramos cells in the presence of EDTA (*A1*) or A23187 (*A2*). Binding of labeled sperm cells to HL-60 cells in the absence (*A3*) or presence (*A4*) of A23187. (*B*) Binding of labeled sperm cells to HL-60 cells in the presence of mouse IgG (*B1*), P23 mAb (a leukocyte adhesion nonblocking mAb against P-selectin; *B2*), or P7 mAb (a leukocyte adhesion blocking mAb against P-selectin; *B3*). (*C*) Binding of labeled sperm cells to HL-60 cells in the presence of mouse IgM (*C1*), CSLEX (an mAb against $SLe^x$; *C2*), or PL5 (an mAb against PSGL-1; *C3*). (*D*) Binding of labeled sperm cells to HL-60 cells in the presence of human IgG (*D1*), E-selectin Rg (*D2*), or P-selectin Rg (*D3*).

tional. To corroborate this finding, we investigated whether porcine sperm cells could bind to HL-60 cells, a human promyeloid cell line that expresses the functional PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995; Spertini et al., 1996; Tu et al., 1996). Fig. 9 shows that, in the presence of $Ca^{2+}$, $Mg^{2+}$, and A23187, fluorescently labeled sperm cells avidly bound to HL-60 cells (A4), but not to Ramos cells (A1 and A2), a human lymphoblast cell line that does not express the functional PSGL-1 (Vachino et al., 1995). This binding was reduced when the experiment was carried out in the absence of A23187 (A3), a calcium ionophore that induces the sperm acrosomal reaction (Aarons et al., 1991; Tao et al., 1993). The partial binding observed in the absence of A23187 (A3) was likely attributed to the broken cytoplasmic membrane on some sperm cells, caused by the repeated washing procedures used in their preparation (Fig. 8). The requirement for A23187 for full binding activity is consistent with the expression of P-selectin on the acrosomal membrane of sperm cells (Figs. 6 and 8).

As expected, the binding activity was blocked by preincubation of the sperm cells with P7 (a leukocyte adhesion blocking mAb against P-selectin; B3), but not with mouse IgG (B1) or P23 (a leukocyte adhesion nonblocking mAb against P-selectin; B2). Preincubation of HL-60 cells with PL5 (a leukocyte adhesion blocking mAb against PSGL-1; C3) or P-selectin Rg (D3) also neutralized the binding, but mouse IgM (C1), human IgG (D1), or E-selectin Rg (D2) did not. Taken together, the results provide independent and convergent evidence for the biological function of the zona pellucida P-selectin ligand and the sperm P-selectin.

It should be mentioned that the anti-$SLe^x$ mAb, CSLEX, partially inhibited the interaction in this assay. This partial inhibition, by CSLEX mAb, of the binding of sperm cells to HL-60 cells (Fig. 9; *C2*), but not on the binding of sperm cells to the oocytes (Table I), may be due to subtle differences in assay formats. Alternatively, the difference in cell type (different oligosaccharide structures on porcine oocytes vs human HL-60 cells) and/or the particulars of the CSLEX mAb specificity (recognizes other carbohydrate epitopes besides $SLe^x$; Stroud et al., 1996*a*,*b*) may contribute to this partial inhibition. In addition, the lack of inhibition by E-selectin Rg, particularly in sperm cell binding to HL-60 cells (Fig. 9; *D2*), raises several possibilities, such as (*a*) there is an insufficient amount of E-selectin Rg in the assay; (*b*) the binding determinant(s) on PSGL-1 for P- and E-selectin is quite distinct and separated; (*c*) there is sufficient other ligand(s) for E-selectin; or (*d*) PSGL-1 is not a functional ligand for E-selectin. Obviously, further experiments are required to clarify these issues.

## Discussion

Attachment of the sperm cell to the oocyte is the first step in mammalian fertilization. This process involves a cascade of cell–cell and cell–matrix interactions with at least six consecutive steps (Wassarman, 1995; Snell and White, 1996). Sperm cells first bind to the zona pellucida, a large extracellular matrix surrounding the oocyte. This binding triggers the acrosomal reaction to facilitate the penetration of the sperm cell through the zona pellucida. After penetration, the sperm cell binds to and fuses with the plasma membrane of the oocyte. The fertilized egg finally undergoes implantation, placenta formation, and embryonic development.

Several adhesion molecules have been implicated to play important roles in gamete binding. The sperm binding to the zona pellucida in mouse is reportedly mediated by ZP3 glycoprotein (Bleil and Wassarman, 1980), a reaction that induces the acrosomal reaction on the mouse sperm cells (Bleil and Wassarman, 1983). Interestingly, this interaction appears to be mediated by *O*-linked carbohydrates on ZP3 molecule (Florman and Wassarman, 1985; Litscher et al., 1995). On the other hand, the binding of sperm cells to the plasma membrane of the oocyte is mediated by fertilin, a mouse sperm surface protein, and its counterligand, $\alpha 6\beta 1$ integrin, on the oocyte surface (Blobel et al., 1992; Almeida et al., 1995).

The specific distribution pattern of the P-selectin ligand in the zona pellucida matrix argues against the possibility that the detection of the ligand in this tissue represents an artifact. An erroneous signal caused by contaminating leukocytes is unlikely for the following reasons. First, as discussed above, our microscopic studies show that both P-selectin and PSGL-1 peptide antibody specifically bind to membranous structures within the zona pellucida. Second, large amounts of leukocytes (~0.5 mg proteins of membrane extract from ~100 million leukocytes per lane) are typically required for positive detection with the P-selectin blotting method used in this report (Fig. 1 *D*; Ma et al., 1994). Hence, if the molecule detected in the zona pellucida represents the leukocyte ligand, truly major leukocyte contaminations of the zona pellucida preparations would be required to produce the signal observed on the blots. However, light and EM examination of these preparations failed to show such leukocyte presence. Third, nonspecific binding of P-selectin and PSGL-1 peptide antibody to a contaminating or otherwise unrelated polysaccharide and/or protein structure(s) on the surface of the membranous structures in the zona pellucida is unlikely since our blotting experiments clearly show that both P-selectin and PSGL-1 peptide antibody primarily recognize one protein among the considerable numbers of proteins present in the zona preparation; in addition, this protein is one of the minor constituents of the zona pellucida (Fig. 1, compare *A*, *B*, and *C*). Finally, the expression of a P-selectin ligand in the zona pellucida is consistent with the detection of PSGL-1 mRNA in the mouse ovary, as demonstrated by Northern analysis (Yang et al., 1996).

The P-selectin ligand from porcine zona pellucida and leukocytes described in this report shares many characteristics with human leukocyte PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995). These include biochemical properties (disulfide-linked dimeric protein), functional properties (recognition by P-selectin in a $Ca^{2+}$-dependent manner), and polypeptide properties (recognition by PSGL-1 peptide antibody). However, there is a difference in molecular masses between the human leukocyte PSGL-1 (~240 kD under nonreducing conditions and ~100 kD under reducing conditions) (Ma et al., 1994) and the porcine oocyte and leukocyte P-selectin ligands (~210 kD under nonreducing conditions and ~80 kD under reducing conditions) (Figs. 1 and 2). Therefore, since amino acid sequencing information is not available for the porcine oocyte P-selectin ligand, the question as to whether this molecule is identical to PSGL-1 remains to be answered.

In this study we failed to detect any expression of E-selectin on sperm cells by flow cytometry, immunoblotting, or immunogold EM. The apparent absence of the expression of E-selectin on porcine sperm cells argues against a biological role for this lectin in gamete interactions. However, an immunohistologic study has demonstrated the expression of both E- and P-selectin on vascular endothelial cells in the decidua basalis, but not on decidua parietalis (Burrows et al., 1994). Therefore, it is conceivable that E-selectin, along with P-selectin, on vascular endothelial cells in the decidua basalis may interact with the zona pellucida ligand during trophoblast implantation.

The expression of P-selectin on the acrosomal membrane of porcine sperm cells is supported by several experimental findings. First, blotting of sperm cell extracts separated on SDS-PAGE with two well-characterized P-selectin antibodies (Ma et al., 1994; Asa et al., 1995; Toombs et al., 1995) resulted, for both reagents, in the detection of a protein band with an apparent molecular mass identical to those of human platelet P-selectin (Ma et al., 1994) and porcine platelet P-selectin (Fig. 7). Since purified sperm cells (see Materials and Methods) were used for this experiment, it is unlikely that platelet contamination is responsible for the signal on the blot; as argued above, a considerable amount of platelets are required for a positive signal in this type of experiment. Second, immunoelectron microscopies of the porcine sperm cells quite unequivocally demonstrate the abundant presence of a molecule recognized by a P-selectin antibody, on what appears to be the acrosomal membrane of the sperm cells (Fig. 8). This distribution to a specific membranous compartment on the sperm cells is in itself an argument against nonspecific labeling, caused by unspecific binding of the antibody as well as by unspecific adsorption of (soluble) antigen to the sperm cell surface. The fact that only acrosome-reacted sperm cells are capable of binding the antibodies also argues against nonspecific results. Finally, the capacity of acrosome-reacted porcine sperm cells to attach to oocytes and HL-60 cells in a manner that is inhibitable by P-selectin antibodies strongly suggests a P-selectin function on the acrosome-reacted sperm cells (Table I; Fig. 9).

The ultrastructural distribution of the zona pellucida P-selectin ligand indicates that it is less likely a component of the zona pellucida matrix. Instead, the molecule appears to be located exclusively in membranes embedded within the matrix of the zona pellucida. The membrane structures in the zona pellucida may originate from the long oocyte microvilli and/or from the follicle cell projections that transverse the zona pellucida and make contact (gap junctions) with the oocyte plasma membrane (Austin, 1968). The morphological localization of P-selectin ligand–containing membranes in the zona pellucida implicates a potentially functional role for a P-selectin ligand during the penetration by the acrosome-reacted sperm cells through the thick, gel-like matrix of the zona pellucida. In this regard, the expression of functional P-selectin on the acrosomal membrane of sperm cells supports this hypothesis.

The findings in this report implicate a potentially biological role for the zona pellucida P-selectin ligand and the sperm P-selectin in porcine gamete interactions. However, since homozygous mice deficient in P-, E-, or L-selectin, by homologous recombination, have no apparent deficiencies in breeding (Myadas et al., 1993; Arbonès et al., 1994; Labow et al., 1994), this interaction may be only one of several molecular mechanisms involved in fertilization in vivo (Wassarman, 1995; Snell and White, 1996). A similar, apparently redundant pathway has been described for leukocyte-endothelial cell interactions. The phenotypes of P-, E-, and L-selectin knockout mice appear normal until the animals are challenged by inflammatory mediators (Myadas et al., 1993; Arbonès et al., 1994; Labow et al., 1994).

Taken together, our in vitro studies suggest that a mechanism, similar to that involved in leukocyte recruitment, may be involved in sperm–egg binding. However, the specific role(s) of these molecules in the different steps of sperm–egg interaction, especially in vivo, clearly require(s) further investigation.

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### *References*

Aarons, D., H. Boettger-Tong, G. Holt, and G.R. Poier. 1991. Acrosome reaction induced by immunoaggregation of a proteinase inhibitor bound to the murine sperm head. *Mol. Reprod. Dev.* 30:258–264.

Almeida, E.A.C., A.-P.J. Huovila, A.E. Sutherland, L.E. Stephens, P.G. Calarco, L.M. Shaw, A.M. Mercurio, A. Sonnenberg, P. Primakoff, D.G. Myles, et al. 1995. Mouse egg integrin α6β1 functions as a sperm receptor. *Cell.* 81: 1095–1104.

Arbonès, M.L., D.C. Ord, K. Ley, H. Ratech, C. Maynardcurry, G. Otten, D.J. Capon, and T.F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity.* 1:247–260.

Asa, D., L. Raycroft, L. Ma, P.A. Aeed, P.S. Kaytes, Å.P. Elhammer, and J.-G. Geng. 1995. The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. *J. Biol. Chem.* 270:11662–11670.

Austin, C.R. 1968. Ultrastructure of Fertilization. Holt Press, New York. 196 pp.

Bevilacqua, M.P., J.S. Pober, D.L. Mendrick, R.S. Cotran, and M.A. Gimbrone, Jr. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 84:9238–9242.

Bevilacqua, M.P., S. Stengelin, M.A. Gimbrone, Jr., and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science (Wash. DC).* 243:1160–1165.

Bleil, J.D., and P.M. Wassarman. 1980. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell.* 20:873–882.

Bleil, J.D., and P.M. Wassarman. 1983. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosomal reaction by a zona pellucida glycoprotein. *Dev. Biol.* 95:317–324.

Blobel, C.P., T.G. Wolfsberg, C.W. Turck, D.G. Myles, P. Primakoff, and J.M. White. 1992. A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. *Nature (Lond.).* 356:248–252.

Brandley, B.K., S.J. Swiedler, and P.W. Robbins. 1990. Carbohydrate ligands of the LEC cell adhesion molecules. *Cell.* 63:861–863.

Burrows, T.D., A. King, and Y.W. Loke. 1994. Expression of adhesion molecules by endovascular trophoblast and decidual endothelial cells: implications for vascular invasion during implantation. *Placenta.* 15:21–33.

Butcher, E.C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell.* 67:1033–1036.

Cross, J.C., Z. Werb, and S.L. Fisher. 1994. Implantation and placenta: Key pieces of the development puzzle. *Science (Wash. DC).* 266:1508–1518.

Drickamer, K. 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* 263:9557–9560.

Drickamer, K. 1993. Biology of animal lectins. *Annu. Rev. Cell Biol.* 9:237–264.

Dunbar, B.S., N.J. Wardrip, and J.L. Hedrick. 1980. Isolation, physicochemical properties, and macromolecular composition of *zona pellucida* from porcine oocytes. *Biochemistry.* 19:356–365.

Eddy, E.M. 1988. The spermatozoon. *In* The Physiology of Reproduction. E. Knobil and J.D. Neil, editors. Raven Press, New York. 27–68.

Florman, H.M., and P.M. Wassarman. 1985. *O*-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell.* 41:313–324.

Geng, J.-G., M.P. Bevilacqua, K.L. Moore, T.M. McIntyre, S.M. Prescott, J.M. Kim, G.A. Bliss, G.A. Zimmerman, and R.P. McEver. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature (Lond.).* 343:757–760.

Gumbiner, B.M. 1992. Epithelial morphogenesis. *Cell.* 69:385–387.

Hirano, T., S. Takasaki, J.L. Hedrick, J.J. Wardrip, J. Amano, and A. Kobata. 1993. *O*-linked neutral sugar chains of porcine *zona pellucida* glycoproteins. *Eur. J. Biochem.* 214:763–769.

Hynes, R.O., and A.D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell.* 68:303–332.

Johnston, G.J., A. Kurosky, and R.P. McEver. 1989. Structural and biosynthetic studies of the granule membrane protein, GMP-140, from human platelets and endothelial cells. *J. Biol. Chem.* 264:1816–1823.

Labow, M.A., C.R. Norton, J.M. Rumberger, K.M. Lombard-Gillooly, D.J. Shuster, J. Hubbard, R. Bertko, P.A. Knaack, R.W. Terry, M.L. Harbison et al. 1994. Characterization of E-selectin-deficient mice: demonstration of overlapping function of the endothelial selectins. *Immunity.* 1:709–720.

Larsen, E., A. Celi, G.E. Gilbert, B.C. Furie, J.K. Erban, R. Bonfanti, D.D. Wagner, and B. Furie. 1989. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell.* 59: 305–312.

Lasky, L.A. 1992. Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science (Wash. DC).* 258:964–969.

Lenter, M., A. Levinovitz, S. Isenmann, and D. Vestweber. 1994. Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. *J. Cell Biol.* 125:471–481.

Litscher, E.S., K. Juntunen, A. Seppo, L. Penttilä, R. Niemelä, O. Renkonen, and P.M. Wassarman. 1995. Oligosaccharide constructs with defined structures that inhibit binding of mouse sperm to unfertilized eggs *in vitro*. *Biochemistry.* 34:4662–4669.

Ma, L., L. Raycroft, D. Asa, D.C. Anderson, and J.-G. Geng. 1994. A sialoglycoprotein from human leukocytes functions as a ligand for P-selectin. *J. Biol. Chem.* 269:27739–27746.

Moore, K.L., N.L. Stults, S. Diaz, D.F. Smith, R.D. Cummings, A. Varki, and R.P. McEver. 1992. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J. Cell Biol.* 118:445–456.

Myadas, T.N., R.C. Johnson, H. Rayburn, R.O. Hynes, and D.D. Wagner. 1993. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell.* 74:541–554.

Raub, T.J., and S.L. Kuentzel. 1984. Kinetic and morphological evidence for endocytosis of mammalial cell integrin receptors by using an anti-fibronectin receptor β subunit monoclonal antibody. *Exp. Cell Res.* 184:407–426.

Raub, T.J., M.J. Koroly, and R.M. Roberts. 1990. Endocytosis of wheat germ agglutinin binding sites from the cell surface into a tubular endosomal network. *J. Cell. Physiol.* 143:1–12.

Rosen, S.D., M.S. Singer, T.A. Yednock, and L.M. Stoolman. 1985. Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. *Science (Wash. DC).* 228:1005–1007.

Sako, D., X.-J. Chang, K.M. Barone, G. Vachino, H.M. White, G. Shaw, G.M. Veldman, K.M. Bean, T.J. Ahern, B. Furie et al. 1993. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell.* 75:1179–1186.

Snell, W.J., and J.M. White. 1996. The molecules of mammalian fertilization. *Cell.* 85:629–637.

Spertini, O., A.-S. Cordey, N. Monai, L. Giuffrè, and M. Schapira. 1996. P-selectin glycoprotein ligand 1 is a ligand for L-selectin on neutrophils, monocytes, and $CD34^+$ hematopoietic progenitor cells. *J. Cell Biol.* 135:523–531.

Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell.* 76:301–314.

Stroud, M.R., K. Handa, M.E.K. Salyan, K. Ito, S.B. Levery, S. Hakomori, B.B. Reinhold, and V.N. Reinhold. 1996*a*. Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 1. Separation of E-selectin binding from nonbinding gangliosides, and absence of sialosyl-$Le^x$ having tetraosyl to octaosyl core. *Biochemistry.* 35:758–769.

Stroud, M.R., K. Handa, M.E.K. Salyan, K. Ito, S.B. Levery, S. Hakomori, B.B. Reinhold, and V.N. Reinhold. 1996*b*. Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 2. Characterization of E-selectin binding fractions, and structural requirements for physiological binding to E-selectin. *Biochemistry.* 35:770–778.

Toombs, C.F., G.L. Degraaf, J.P. Martin, J.-G. Geng, D.C. Anderson, and R.J. Shebuski. 1995. Pretreatment with a blocking monoclonal antibody to P-selectin accelerates pharmacological thrombosis in a primate model of arterial thrombosis. *J. Pharmacol. Exp. Ther.* 275:941–949.

Tao, J., E.S. Critser, and J.K. Critser. 1993. Evaluation of mouse sperm acrosomal status and viability by flow cytometry. *Mol. Reprod. Dev.* 36:183–194.

Tu, L., A. Chen, M.D. Delahunty, K.L. Moore, S.R. Watson, R.P. McEver, and T.F. Tedder. 1996. L-selectin binds to P-selectin glycoprotein ligand-1 on leukocytes. *J. Immunol.* 157:3995–4004.

Vachino, G., X.-J. Chang, G.M. Veldman, R. Kumar, D. Sako, L.A. Fouser, M.C. Berndt, and D.A. Cumming. 1995. P-selectin glycoprotein ligand-1 is the major counter-receptor for P-selectin on stimulated T cells and is widely distributed in non-functional form on many lymphocytic cells. *J. Biol. Chem.* 270:21966–21974.

Varki, A. 1994. Selectin ligands. *Proc. Natl. Acad. Sci. USA.* 91:7390–7397.

Wassarman, P.M. 1988. Zona pellucida glycoproteins. *Annu. Rev. Biochem.* 57: 415–442.

Wassarman, P.M. 1995. Towards a molecular mechanism for gamete adhesion and fusion during mammalian fertilization. *Curr. Opin. Cell Biol.* 7:658–664.

Yanagimachi, R. 1988. Mammalian fertilization. *In* The Physiology of Reproduction. E. Knobil and J.D. Neil, editors. Raven Press, New York. 135–185.

Yang, J., J. Galipeau, C.A. Kozak, B.C. Furie, and B. Furie. 1996. Mouse P-selectin glycoprotein ligand-1: molecular cloning, chromosomal localization, and expression of a functional P-selectin receptor. *Blood.* 87:4176–4186.

Yednock, T.A., and S.D. Rosen. 1989. Lymphocyte homing. *Adv. Immunol.* 44: 313–378.