Physiologic Relevance of the Membrane Attack Complex Inhibitory Protein CD59 in Human Seminal Plasma: CD59 Is Present on Extracellular Organelles (Prostasomes), Binds Cell Membranes, and Inhibits Complement-mediated Lysis

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Summary

We demonstrate here that CD59, an inhibitor of the membrane attack complex (MAC) of the complement system, is present in cell-free seminal plasma (SP) at a concentration of at least 20 μg/ml. Analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and Edman degradation indicated that this protein, SP CD59, was similar, if not identical, to CD59 isolated from erythrocyte (E) membranes (E CD59). Like purified E CD59, SP CD59 also possesses a glycosyl phosphatidyl inositol (GPI) anchor and incorporates into the membranes of heterologous cells where it inhibits lysis by the human MAC. This phenomenon could be demonstrated not only if cells were incubated with purified SP CD59 but also if unfractionated SP were used. Further, CD59 in unfractionated SP bound to washed spermatozoa, increasing their membrane content of the protein. The mechanism by which this protein retains its GPI anchor while apparently present in the fluid phase is of interest and was further investigated. Using the techniques of high-speed centrifugation, fast performance liquid chromatography fractionation, and electron microscopy, we found that all detectable SP CD59 was associated with vesicular extracellular organelles. These organelles, named "prostasomes," were previously known to be present in SP and to interact with spermatozoa, although their function was uncertain. Interaction of heterologous E with prostasomes rendered the cells more resistant to lysis by human MACs. We propose that these organelles represent a pool of CD59 from which protein lost from spermatozoa, perhaps as a result of low level complement attack or of normal membrane turnover, can be replenished.

Exposure of spermatozoa to antisperm antibodies in the male (1, 2) and female (3, 4) reproductive tracts and to functioning complement (C) in the female reproductive tract (4, 5) has been implicated in some cases of infertility. C-mediated immobilization of spermatozoa is dependent on membrane attack complex (MAC)[1] formation (6); control of MAC assembly on these cells may therefore be important for fertility.

The MAC inhibitory protein CD59 is expressed on spermatozoa, and antibody blocking of this protein increases spermatozoal susceptibility to MAC-mediated killing in vitro (6). This, together with the recent identification of a form of CD59 in amniotic fluid that retains the glycosyl phosphatidyl inositol (GPI) anchor and functional activity even in the fluid phase (7), led us to investigate the possible presence and function of CD59 in seminal plasma (SP).

Herein the detection, purification, and characterization of SP CD59 is described. We show that, like erythrocyte CD59 (E CD59) (8) and CD59 isolated from amniotic fluid (7), SP CD59 has the capacity to insert into the membranes of

[1] *Abbreviations used in this paper:* CVF, cobra venom factor; GPE, guinea pig erythrocytes; GPI, glycosyl phosphatidyl inositol; GVB, veronal-buffered saline containing 1% gelatin; MAC, membrane attack complex (of complement); NHS-C8D, normal human serum depleted of C8; NHS-C9D, normal human serum depleted of C9; PIPLC, phosphatidyl inositol–specific phospholipase C; SP, seminal plasma; DAF, decay-accelerating factor; NIMIg, nonimmune mouse immunoglobulin; NIRIg, nonimmune rabbit immunoglobulin; TEM, transmission electron microscopy.

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heterologous cells and to inhibit their lysis by human MACs. This phenomenon was GPI anchor dependent and occurred even when SP CD59 was present in unfractionated SP, suggesting that this protein may incorporate into cell membranes in vivo. We present data indicating that SP CD59 is carried on the membranes of vesicular, extracellular organelles. Our results suggest that, as a result of interaction with these organelles, cell membranes (both of heterologous cells and of washed human spermatozoa) acquire bound CD59 molecules.

Materials and Methods

Reagents for SDS PAGE were obtained from Bio-Rad Laboratories (Richmond, CA). Phosphatidyl inositol–specific phospholipase C (PIPLC; from *Bacillus thuringiensis*) was from two sources: Peninsula Laboratories, Europe (St. Helen's, UK) and ICN Biomedicals Inc. (Costa Mesa, CA). Unless otherwise started, all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Antibodies. Mouse mAb BRIC 229 (anti-CD59) was from Bioproducts Laboratories (Elstree, Herts, UK). Mouse mAb A35 (anti-CD59), rat mAb YTH 53.1 (anti-CD59), and rabbit polyclonal Rb476 (anti-CD59) IgG were the gifts of Dr. A. Davies (Department of Medical Biochemistry, University of Wales College of Medicine). Mouse mAb GB24 (anti–membrane cofactor protein [MCP]) was a gift of Dr. B. L. Hsi (INSERM U210, Faculte de Medecine, Nice, France). Mouse mAb IH6 (anti–decay-accelerating factor [DAF]) was a gift of Dr. T. Kinoshita (Department of Immunoregulation, Research Institute for Microbiological Diseases, Osaka University, Osaka, Japan). Nonimmune mouse IgG (NIMIg) and rabbit IgG (NIRIg) preparations were purchased from Sigma Chemical Co. FITC-conjugated goat anti–mouse IgG (GAM-FITC) was from Sigma Chemical Co.; alkaline phosphatase–conjugated goat anti–mouse IgG (GAM-AP) and goat anti–rabbit IgG (GAR-AP) were from Bio-Rad Laboratories; and goat anti–mouse IgG conjugated with 12-nm gold particles (GAM-gold) was from Jackson Immunoresearch Laboratories Inc. (West Grove, PA).

Cells, Sera, and Complement Components. Guinea pig E (GPE) were from Crane Laboratories (Syracuse, NY). The 3T3 mouse fibroblast cell line was obtained from the American Type Cell Culture Collection (Rockville, MD), and stably transfected 3T3 cells expressing human MCP (3T3-MCP cells) were prepared in our laboratories. C9-depleted normal human (NHS-C9D) and C8-depleted normal human serum (NHS-C8D) were prepared by passage of normal human serum over monoclonal immunoaffinity columns. C8 and C9 were prepared by immunoaffinity chromatography (9, 10) or were purchased from Sigma Chemical Co. Cobra venom factor (CVF) was prepared by the method of Vogel and Muller-Eberhard (11). E CD59 and urine CD59, prepared as described (8), were gifts of Dr. A. Davies. Purified membrane DAF (mDAF) and a purified preparation of a recombinant, anchor-negative, secreted form of DAF (sDAF) were gifts of Dr. I. Caras (Genentech Inc., South San Francisco, CA).

Collection and Storage of SP. Samples of semen (1–3 ml) provided by healthy donors were allowed to liquefy at room temperature for 30 min. Cells and debris were then removed by centrifugation at 10,000 *g* for 10 min. For functional experiments the cell-free SP thus prepared was used immediately. Samples collected for subsequent purification of SP CD59 were stored separately at −20°C.

Preparation of Live Spermatozoa. Live spermatozoa were prepared by a modification of the "swim up" technique of Hellema and Rumke (12) as previously described (6). After washing three times by repeated centrifugation (400 *g* for 10 min) and resuspension, the cells were finally resuspended in MEM containing 1% gelatin (MEM/gelatin) at a density of 10^7 cells/ml.

Measurement of Protein and Lipids. Protein concentrations were measured using a BCA assay kit (Pierce Chemical Co., Rockford, IL). Cholesterol and phospholipid concentrations were measured using enzymatic kits (Wako Chemicals USA, Richmond, VA). The phospholipid assay quantifies the choline-containing glycosphingolipids, including sphingomyelin, but not glycosphingolipids.

SDS-PAGE and Western Blotting. Electrophoresis was performed on 15% gels under reducing or nonreducing conditions according to the method of Laemmli (13). Nitrocellulose membrane (Bio-Rad Laboratories) was used for Western blotting. Unbound protein sites were blocked by incubation with PBS containing 2% BSA (PBS/BSA). Thereafter, anti-CD59-reactive bands were detected by sequential incubations with mAb anti-CD59 (BRIC 229; 1 μg/ml in PBS) and GAM-AP (diluted according to the manufacturer's instructions). Blots were developed using a chromogenic alkaline phosphatase substrate (Bio-Rad Laboratories).

Dot Blotting. Dot blotting was used for rapid detection of CD59 in fractions eluted from columns and for semiquantitative measurements. Aliquots (2 μl) of each fraction were spotted onto nitrocellulose membrane. After drying the membrane was blocked and stained as described above for Western blots.

ELISA for CD59. Microtiter plates were coated with mAb MEM 43.5 (anti-CD59; 1 μg/ml) overnight at 4°C and unbound protein sites were blocked by incubation with PBS/BSA/0.1% Tween 20 for 1 h at 37°C. After washing twice in PBS/0.1% Tween 20, standards and samples (diluted in PBS/0.1% NP-40) were applied in triplicate and incubated for 1 h at 37°C. Wells were washed twice, incubated with biotin-labeled mAb YTH 53.1 (anti-CD59; 1 μg/ml) overnight at 4°C, washed twice, and incubated with Streptavidin peroxidase (Amersham, Cardiff, UK; diluted 1:1,000 in PBS) for 1 h at 37°C, and washed two more times before development using a chromogenic horseradish peroxidase (HRP) substrate (Sigma Chemical Co.).

PIPLC Treatment. To cleave the GPI anchor of SP CD59, purified protein (50 μg/ml in PBS/0.2% NP-P40) or fresh, unfractionated, undiluted SP was incubated with PIPLC (0.1 U/ml) for 2 h at 37°C.

Removal of GPI-anchored proteins from washed spermatozoa was achieved by incubating the cells (10^7/ml in MEM/gelatin) with PIPLC (0.1 U/ml) for 1 h at 37°C. The cells were then washed by centrifugation at 400 *g* for 10 min and used immediately.

Phase Separation of SP and CD59 in Solution of Triton X-114. To determine whether SP CD59 was amphiphilic or hydrophilic, experiments were conducted according to the method of Bordier (14) with minor modifications. Briefly, SP (100 μl) or purified SP CD59 (10 μg in 100 μl PBS) was diluted in 100 μl of Tris/NaCl (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.5% Triton X-114 at 0°C. The solution was incubated at 30°C for 3 min after which condensation of the detergent occurred (as evidenced by clouding). The detergent and aqueous phases were separated by centrifugation at 2,500 *g* for 3 min at room temperature; the detergent phase formed an oily droplet at the bottom of the tube. The aqueous phase was collected and extracted twice more with 0.5% Triton X-114. Finally, the three detergent phases were pooled and the aqueous phase was rinsed with 2% Triton X-114 (the detergent phase from this rinse was discarded). Aqueous and detergent phases were dialyzed separately against PBS and aliquots (20 μl) of these and of the input sample were analyzed by SDS-PAGE and Western blotting. SP and CD59 treated with PIPLC were subjected to the same analysis.

Purification of CD59 from SP. Samples of frozen, cell-free SP

were thawed and pooled (60 ml total volume). Diaminobenzidine (1 mM), EDTA (5 mM), and PMSF (2 mM) were added and the pool was centrifuged at 10,000 *g* for 30 min to remove any residual debris. NP-40 was added to a final concentration of 2%. The pool was agitated at 4°C for 1 h, diluted 10× in PBS containing the protease inhibitors listed above, and filtered through a glass sintered funnel. The diluted, filtered plasma was applied to a column of mAb anti-CD59 (BRIC 229) coupled to Sepharose 4B, which had been equilibrated in PBS/0.2% NP-40 containing the additives listed above. After washing the column with five column volumes of the same buffer, bound protein was eluted with 50 mM diethylamine/0.2% NP-40 (pH 11.0). pH was normalized by collection of fractions into 1 M Tris/HCl (pH 7.4) containing 0.2% NP-40. CD59-containing fractions were identified by dot blotting, pooled, dialyzed against PBS/0.2% NP-40, and concentrated to 500 μg/ml in an ultracentrifugation cell using a PM10 membrane (Amicon Corp., Beverly, MA). SP CD59 was further purified from this preparation by a second chromatography with the same column. The final sample was concentrated to 200 μg/ml.

Radiolabeling of SP CD59 and Incorporation into GPE. SP CD59 (50 μg in 0.4 ml PBS/NP-40) was labeled with ^{125}I using derivatized chloramine T (Iodobeads; Pierce and Warriner, Chester, UK) according to the manufacturer's instructions. Free iodine was removed by extensive dialysis. The specific activity of the labeled protein was 0.5–1 μCi/μg. The radiolabeled protein was incubated at various concentrations with GPE (5×10^6/ml, 200 μl total volume) for 15 min at 37°C. The cells were washed three times in cold veronal-buffered saline containing 1% gelatin (GVB) to remove unbound protein, and bound radioactivity was then measured in a gamma counter.

Protein Sequence Analysis of SP CD59. Amino-terminal sequence analysis using 24-nmol samples of reduced and alkylated protein was performed by D. McCourt (Howard Hughes Medical Institute's Protein Chemistry Facility, Washington University School of Medicine, St. Louis, MO), using a gas phase sequencer (470A; Applied Biosystems, Inc., Foster City, CA).

High-Speed Centrifugation of SP. Aliquots (500 μl) of fresh, cell-free SP were centrifuged at 200,000 *g* for 1 h at 4°C in a benchtop centrifuge (T100; Beckman Instruments, Inc., Fullerton, CA). Supernatant was decanted into a separate tube and the pellet resuspended in a volume of PBS equal to the original volume of SP.

Fast Performance Liquid Chromatography (FPLC) Analysis of SP. SP was fractionated using an FPLC system consisting of a P-500 pump and an LCC-500 liquid chromatography controller (both from Pharmacia Fine Chemicals, Piscataway, NJ) by the following technique. Two Superose 6 HR 10/30 columns (Pharmacia Fine Chemicals), each with a bed volume of 25 ml, were connected in series. SP (1.5 ml) was diluted 1:1 with column eluant (saline/1 mM EDTA/0.02% NaN_3) and applied to the columns. Fractions were eluted at 0.35 ml/min and 90 × 0.5-ml fractions were collected. The concentrations of protein, phospholipid, and cholesterol in each fraction were measured and CD59-containing fractions were identified by dot blotting (2 μl) and Western blotting (15 μl) of each fraction.

Cell Binding Assay. This assay was used to determine whether proteins present in SP or in purified or partially purified preparations had the ability to bind to cell membranes. 3T3 or 3T3-MCP cells were suspended in PBS/2% BSA at a density of 10^7/ml. Aliquots (100 μl = 10^6 cells) were incubated with the samples to be assayed (100 μl) for 30 min at 37°C (unless otherwise stated). Cells were then washed three times with 1 ml cold PBS/BSA to remove unbound protein, incubated with first antibody (1 μg/ml of mAb BRIC 229 anti-CD59, 2 μg/ml of mAb anti-DAF IA6, or 2 μg/ml of mAb anti-MCP GB24) for 30 min at 4°C, washed three times, and incubated with second antibody (GAM-FITC, diluted according to instructions) for 30 min at 4°C, before final washing and analysis by flow cytometry on a FACS 440® (Becton Dickinson & Co., Mountain View, CA). Cells incubated with assay sample and then with NIMIg instead of first Ab and cells incubated with PBS instead of assay sample served as controls.

MAC Inhibition Assay. The following assay was used to measure the MAC inhibitory activity of CD59 present in SP or in purified or partially purified preparations. GPE (5% suspension in GVB) were incubated with NHS-C9D (final dilution, 1:10) and CVF (1 μg/ml) for 30 min at 37°C to form C5b-8 sites on the cells (EC5b-8). EC5b-8 were washed three times and resuspended in GVB at a density of 5% before use. Aliquots (100 μl) of the cells were incubated with 100 μl of the sample to be tested (or 100 μl GVB for control cells) for 30 min at 37°C unless otherwise stated. After washing three times with 1 ml of cold GVB to remove unbound protein, cells were incubated with purified C9 (2 μg/ml in 200 μl GVB) for a further 30 min. After centrifugation, percent hemolysis was estimated by measuring the OD of the supernatant at 412 nm in a plate reader (MR700; Dynatech Laboratories Inc., Chantilly, VA). In some experiments, EC5b-7, rather than EC5b-8, were generated by incubation of GPE with NHS-C8D (final concentration, 1:20) and with CVF (1 μg/ml). After incubation with samples to be tested and washing to remove unbound protein, MACs were completed on the cells by addition of C8 and C9 (each 5 μg/ml).

The effect of depletion of CD59 from SP on this fluid's MAC inhibitory activity was investigated. SP (100 μl) was incubated with mAb anti-CD59 (BRIC 229) coupled to Sepharose 4B (solid phase anti-CD59; 100 μl) for 1 h at 4°C. Solid phase anti-CD59 was removed by centrifugation and depletion of CD59 from the SP was confirmed by dot blotting. SP incubated with Sepharose 4B coupled to NIMIg (solid phase NIMIg) served as a control.

The extent to which CD59 that was bound to EC5b-7 or EC5b-8 could be blocked by anti-CD59 Ab was determined. To do this, the cells were first incubated with CD59-containing material (pure CD59, SP, or fractions of SP), then washed and incubated with either $F(ab)_2$ mAb anti-CD59 (YTH 53.1; 50 μg/ml) or with polyclonal anti-CD59 (Rb476; 100 μg/ml) for 15 min. Cells were then washed and C8 and C9 were added before analysis as described above.

Electron Microscopy. Pellets produced by high-speed centrifugation, either of unfractionated SP or of pooled CD59-containing fractions from FPLC of SP, were fixed in modified Karnovsky's fixative (2% glutaraldehyde, 1% paraformaldehyde, 2 mM $CaCl_2$ in 0.08 M sodium cacodylate buffer) for 2 h, postfixed in osmium tetroxide (1%), dehydrated in graded ethanols, and embedded in Spurr's embedding resin. Sections were mounted on 200-mesh nickel grids, counterstained with uranyl acetate and lead citrate, and viewed on a transmission electron microscope (TEM).

Immunoelectron Microscopy of Vesicles. Immunoelectron microscopy of vesicles present in the CD59-containing fractions from FPLC of SP was conducted by a modification of the method of Watts et al. (15). Briefly, droplets (10 μl) of pooled, vesicle-containing fractions were placed on formvar-coated nickel grids and left undisturbed at room temperature for 1 min. Excess liquid was blotted off with filter paper. The grids were then incubated with 50% rabbit serum in PBS (50 μl) for 30 min and washed by sequential incubation in 3 × 50-μl aliquots of PBS for 1 min each. Excess liquid was again blotted off and the grids were incubated either with first Ab (BRIC 229; 20 μg/ml in PBS), with NIMIg (20 μg/ml), or with irrelevant primary antibody (UPC-10, 20 μg/ml; Sigma Chemical Co.) for 30 min at room temperature. After washing three times

in PBS the grids were incubated with GAM-gold (diluted according to manufacturer's instructions) for 1 h at room temperature, washed a further three times, and then fixed and counterstained by incubation with uranyl acetate in 50% ethanol for 1 min before viewing in the microscope. In some experiments, vesicles were fixed with 2% paraformaldehyde or permeabilized with 30% methanol before the blocking step.

Results

Identification of CD59 in Samples of SP. Samples of SP, subjected to SDS-PAGE under nonreducing conditions followed by Western blotting, contained an anti-CD59-reactive band of 20 kD that comigrated with E CD59 (Fig. 1). This band was detected in all SP samples tested, including 15 samples from men who were presumed fertile and four samples from vasectomized men whose semen was azoospermic (not shown). The concentration of CD59 in samples of SP from four fertile donors was found by ELISA to be 19.7, 24.5, 17.6, and 15.9 μg/ml (mean, 19.4 μg/ml; SD, 3.7).

Ability of CD59 in SP to Bind Cell Membranes. The uptake of SP CD59 by heterologous, CD59-negative cells was demonstrated using two murine fibroblast cell lines (Table 1). The first was the 3T3 line, cells of which are negative for the human GPI-linked proteins CD59 and DAF, and for the human transmembrane protein MCP (CD46). The second was the 3T3-MCP line, cells of which are negative for CD59 and DAF but express high levels of MCP. After incubation with purified CD59 (GPI anchor positive), washing and analysis by immunofluorescent staining, and flow cytometry, cells of both lines were CD59 positive. Cells incubated with mDAF (anchor positive) were rendered DAF positive whereas those incubated with sDAF (anchor negative) remained DAF negative. These data established that binding of these proteins to cells requires a GPI anchor.

SP retained CD59 with the ability to bind to cells even after storage at 4°C for 1 wk. Although we detected DAF in SP by Western blotting (not shown), cells incubated with SP remained DAF negative. The characterization of DAF in SP is under further investigation.

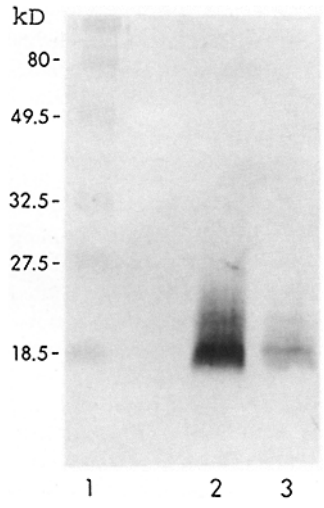

Figure 1. Detection of CD59 in SP. Shown is a Western blot of a 15% SDS-PAGE gel, stained for CD59. Lane *1*, molecular mass markers; lane *2*, purified E CD59 (1 μg); lane *3*, unprocessed SP (10 μl).

Table 1. *Binding of Purified Proteins and SP Proteins to Murine Fibroblasts*

Test sample	First Ab	Mean fluorescence 3T3	Mean fluorescence 3T3-MCP
PBS	–	5	4
PBS	NIMIg	5	4
PBS	Anti-CD59	5	4
PBS	Anti-DAF	6	5
PBS	Anti-MCP	7	250
mCD59	NIMIg	6	5
mCD59	Anti-CD59	42	44
mDAF	NIMIg	4	4
mDAF	Anti-DAF	29	34
sDAF	NIMIg	4	3
sDAF	Anti-DAF	7	6
SP	NIMIg	6	5
SP	Anti-CD59	40	70
SP	Anti-DAF	7	10
SP	Anti-MCP	8	280

3T3 or 3T3-MCP cells (10^6 cells in 200 μl) were incubated with PBS, mCD59 (6 μg/ml in PBS), mDAF (10 μg/ml in PBS), sDAF (10 μg/ml in PBS), or SP (50% in PBS) for 30 min at 37°C, washed, incubated with primary Ab or with NIMIg as stated, washed, and incubated with FITC-labeled second Ab before analysis by flow cytometry. Unstained cells were used to set the gate. 10,000 events were counted in each case. Mean fl, mean fluorescence.

MAC Inhibitory Activity of CD59 in SP. Incubation of EC5b-8 with SP inhibited lysis of these cells on subsequent addition of C9 (Fig. 2 *A*). Cells were washed before exposure to C9, indicating that the effect depended on binding of protein to the cell membrane. Percent inhibition of lysis increased with the concentration of SP. Maximal inhibition (>90%) was observed when 2.5% SP was used but significant inhibition occurred at much lower doses. Preincubation of SP with solid phase anti-CD59 reduced the MAC inhibitory activity of this fluid by >50%. Incubation of SP with solid phase NIMIg had no effect on MAC inhibitory activity (not shown).

Immunofluorescent staining showed that 3T3 cells incubated with SP acquired bound CD59 molecules. The quantity bound increased with the dose of SP up to 50% (Fig. 2 *B*). Binding of CD59 to 3T3 cells incubated with SP occurred in a time-dependent manner during the first 30 min of incubation. Thereafter, only a small additional increase in binding was observed (Fig. 2 *C*). Pretreatment of SP with PIPLC prevented binding of CD59 to 3T3 cells (Fig. 2 *D*), indicating that binding depended on a GPI anchor. No decrease was observed in the amount of SP CD59 that bound to 3T3 cells when 10 mM EDTA was added to SP (Fig. 2 *E*).

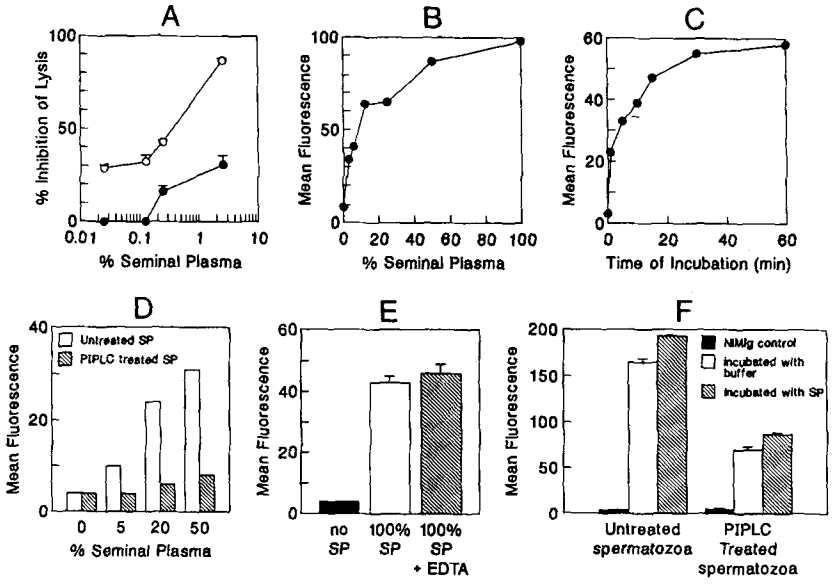

Figure 2. Ability of CD59 in SP to bind cells. Each panel is representative of at least three experiments. (*A*) Inhibitory effect of SP on C9-mediated hemolysis of EC5b-8: dose dependence and effect of immunoaffinity depletion of CD59 from SP. EC5b-8 (5% suspension in GVB) were incubated with SP at the stated doses for 30 min at 37°C, washed, and exposed to C9 (2 μg/ml) for 30 min at 37°C for 30 min before centrifugation and estimation of percent hemolysis by measurement of the OD of the supernatant at 414 nm. (○) SP preincubated with solid-phase NIMIg, and (●) SP incubated with solid-phase anti-CD59. Points are the means of triplicates and error bars represent SD of the triplicates. (*B–E*) Binding of CD59 in SP to 3T3 cells. Aliquots of 3T3 cells (10^6 cells in 100 μl PBS/BSA) were incubated with dilutions of SP in PBS/BSA or with PBS/BSA alone (100 μl) under the conditions described below, washed, stained for CD59, and analyzed by flow cytometry. Unstained cells were used to set the gate and 10,000 events were counted in each case. (*B*) Effect of varying doses of SP on mean fluorescence of 3T3 cells stained for CD59. Cells were incubated with SP at the concentrations stated for 30 min at 37°C before washing and staining. (*C*) Time course of binding of CD59 in SP to 3T3 cells. Cells were incubated with 50% SP at 37°C for the times stated before washing and staining. (*D*) Effect of pretreatment of SP with PIPLC on binding of SP CD59 to 3T3 cells. 50% SP (in PBS/BSA) was incubated with or without PIPLC (1 U/ml at 37°C for 30 min). Cells were then incubated with the PIPLC-treated or untreated SP for 30 min at 37°C before washing and staining. (*E*) Effect of inclusion of 10 mM EDTA on the ability of SP CD59 to bind 3T3 cells. Cells were incubated with 100% SP with or without 10 mM EDTA or with PBS/BSA alone for 30 min at 37°C before washing and staining. Values are the means of triplicates and error bars represent SD of the triplicates. (*F*) Binding of CD59 in SP to spermatozoa. Washed spermatozoa and washed, PIPLC-pretreated spermatozoa (both suspended at a density of 10^7/ml) were incubated with SP (50% in MEM/gelatin) or with MEM/gelatin alone for 4 h at 37°C before washing and staining for CD59 as described for the 3T3 cells. NIMIg control represents cells incubated with NIMIg in place of first Ab during the immunofluorescent staining procedure. Values are the means of triplicates and error bars represent SD of the triplicates.

CD59 present in SP was also able to bind washed human spermatozoa (Fig. 2 *F*). Mean fluorescence of spermatozoa incubated with MEM/gelatin in the absence of SP for 4 h and then stained for CD59 was 165 ± 4 U, whereas cells incubated with SP for the same period had mean fluorescence 193 ± 1 U. PIPLC treatment reduced mean fluorescence of spermatozoa stained for CD59 by >50%. PIPLC-treated cells incubated with MEM/gelatin and stained for CD59 had mean fluorescence 70 ± 4 U, whereas those incubated with SP had mean fluorescence 87 ± 2 U. Spermatozoa were at least 95% motile at the end of these experiments.

Purification and Characterization of CD59 from Pooled SP. CD59 was purified from pooled SP by affinity chromatography. After one filtration through the immunoaffinity column the preparation contained a major protein band of 20 kD plus contaminating low molecular mass material. After a second pass over the same column the preparation contained a single 20-kD band (Fig. 3 *A*). This 20-kD band reacted strongly with mAb anti-CD59 in the region of 17–25 kD (Fig. 3 *B*) on Western blotting. Final yield of SP CD59 was 17 μg/ml SP, consistent with the results obtained by ELISA of whole SP. Edman degradation of this protein revealed an amino-terminal sequence from residues 1 to 38 that was identical to that reported by Davies et al. (8) for E CD59. No residue could be identified at position 18, suggesting that, as for the E protein, this position is occupied by an N-glycosylated Asn residue.

Radiolabeled SP CD59 incorporated into GPE in a dose-

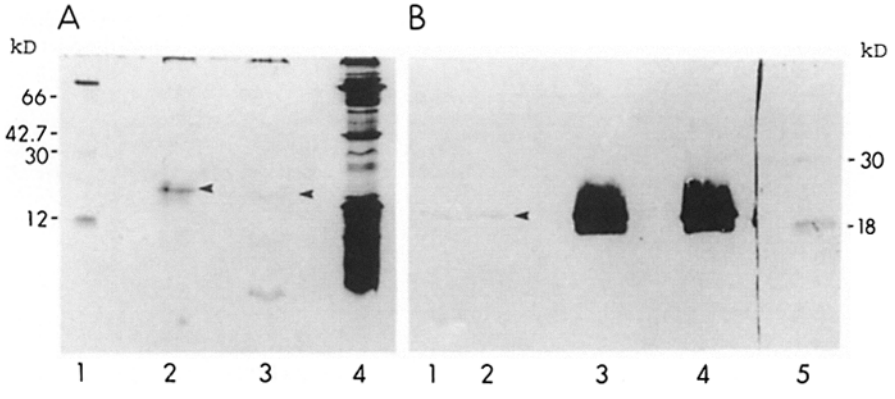

Figure 3. Purification of SP CD59. (*A*) 15% SDS-PAGE gel (silver stained). Lane *1*, molecular mass markers; lanes *2* and *3*, SP CD59 after the second and first immunoaffinity chromatographies, respectively (3 μg protein was loaded in each case); lane *4*, unfractionated SP (10 μl). Arrowheads mark SP CD59. (*B*) Western blot of a 15% gel stained for CD59. Lanes *1* and *2*, two samples of SP (10 μl each); lanes *3* and *4*, SP CD59 after one and two passes over the column (1 μg of protein in each case); lane *5*, molecular mass markers. Arrowheads mark SP CD59.

Table 2. *Incorporation of Radiolabeled SP CD59 into GPE*

Input of SP CD59		Counts bound		Molecules bound	
cpm × 10^3	*μg*	*cpm* × 10^3	%	× 10^3	
50	0.25	4.6	9.2	6.9	(1.5)
20	0.1	3.1	15.5	4.7	(0.9)
5	0.025	1.9	37.2	2.8	(1.4)
2	0.01	1.0	49.0	1.5	(0.9)

GPE (10^7 cells in 0.2 ml GVB) were incubated with varying doses of ^{125}I-labeled SP CD59, washed, and the cell-bound radioactivity was measured. Molecules bound per cell were calculated from the known specific activity of the labeled protein. Radiolabeled BSA was used as a control for nonspecific binding of iodinated protein, and the values observed for control cells were substracted from the values documented in this table. Results are the means of data obtained in three experiments and the numbers in parentheses are the SD of the values from the three experiments.

dependent manner (Table 2). Although the amount of protein incorporated increased with the dose of SP CD59, incorporation was more efficient at lower doses. At the lowest dose of SP CD59 used (0.01 μg/ml), 49% of the radiolabeled protein bound to the cells.

SP CD59 inhibited MAC-mediated lysis of EC5b-7 in a dose-dependent manner (Fig. 4 *A*). When SP, E, and urine CD59 were directly compared, the MAC inhibitory activity of SP CD59 was between 59 and 75% of E CD59 activity. In contrast, urine CD59 (which lacks a GPI anchor) produced slight inhibition only at the highest concentrations used. PIPLC pretreatment reduced the ability of SP CD59 to inhibit MAC-mediated lysis of EC5b-8 cells by >70% (Fig. 4 *B*). Consistent with its known resistance to cleavage by PIPLC (16), treatment of E CD59 with PIPLC caused only a modest (20–30%) decrease in MAC inhibitory activity (data not included). Incubation of EC5b-7 with $F(ab)_2$ fragment of mAb anti-CD59 (YTH 53.1) after incorporation of SP CD59 and before addition of C9 markedly reduced the inhibition of MAC-mediated lysis attributable to SP CD59 (Fig. 4 *C*).

CD59 Is Present in SP as a GPI-anchored, High Molecular Mass Species. When SP and purified SP CD59 were subjected to partitioning in solution of Triton X-114, anti-CD59-reactive material was detected only in the detergent phase. In both cases, PIPLC pretreatment of the input sample greatly reduced the amount of CD59 partitioning into the detergent phase (Fig. 5 *A*).

10 samples of fresh, cell-free SP were fractionated by ultracentrifugation. The precipitated pellets consistently contained ~10% of the SP protein but >90% of the CD59 (Fig. 5 *B*). In other experiments, silver-stained gels of SP, supernatant, and pellet revealed multiple protein bands in each. Pretreatment of SP with PIPLC prevented precipitation of the CD59. Purified preparations of E CD59 and SP CD59 were not precipitated by ultracentrifugation.

SP and the high-speed pellet and supernatant were compared with respect to their content of functionally active CD59

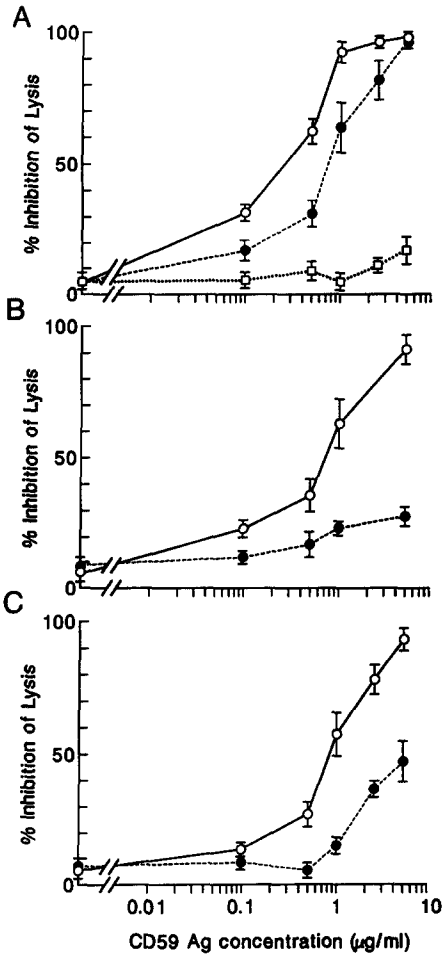

Figure 4. Functional activity of purified SP CD59. (*A*) Comparison of the inhibitory effect of purified CD59 from various sources on C8 + C9–mediated hemolysis of EC5b-7. EC5b-7 (5% suspension in GVB) were incubated with purified SP CD59, E CD59, or urine CD59 at the concentrations stated (in GVB) for 30 min at 37°C. After washing the cells were incubated with C8 and C9 (each 5 μg/ml in GVB) for 30 min at 37°C before centrifugation and estimation of percent hemolysis by measurement of the OD of the supernatant at 412 nm. (●) SP CD59; (○) E CD59; (□) urine CD59. (*B*) Effect of PIPLC treatment on the MAC inhibitory activity of purified SP CD59. EC5b-7 were incubated with PIPLC-treated or untreated SP CD59 at the concentrations stated (in GVB) for 30 min at 37°C before washing, exposure to C8 and C9, and estimation of percent hemolysis as described in Fig. 3 *A*. (○) Untreated SP CD59; (●) PIPLC-pretreated SP CD59. (*C*) Effect of $F(ab)_2$ fragment of mAb anti-CD59 YTH 53.1 on the MAC inhibitory activity of SP CD59. EC5b-7 were incubated with purified SP CD59 at the concentrations stated for 30 min at 37°C, washed, and incubated with $F(ab)_2$ fragment of mAb anti-CD59 (YTH 53.1; 50 μg/ml in GVB) or with GVB alone for 15 min at 37°C before washing, exposure to C8 and C9, and estimation of percent hemolysis as described in Fig. 3 *A*. (●) SP CD59–treated EC5b-7 cells that were incubated with $F(ab)_2$ anti-CD59; (○) those that were incubated with GVB alone. In each case, points are the means of triplicates, error bars represent SD of the triplicates, and data are representative of at least three experiments.

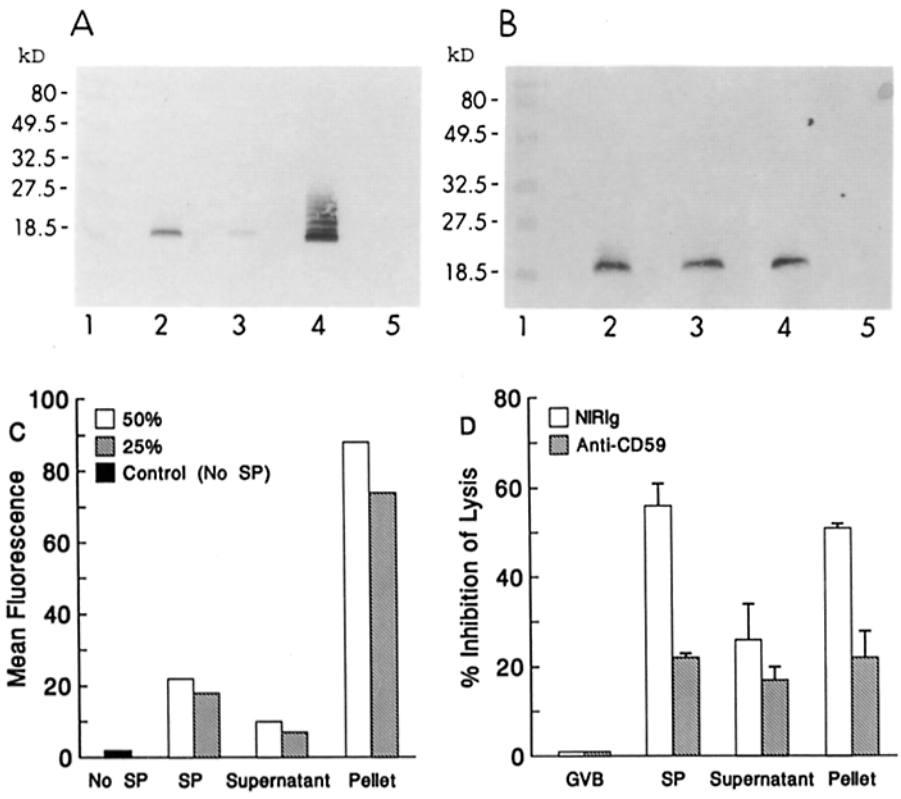

Figure 5. Further characterization of CD59 in SP: detergent phase partitioning and precipitation by ultracentrifugation of SP. (*A*) Partitioning of SP CD59 in solution of Triton X-114. Unprocessed SP and purified SP CD59 were incubated with or without PIPLC (0.1 U/ml) for 2 h at 37°C and then subjected to analysis by phase separation in solution of Triton X-114 as described in Materials and Methods. Shown is a Western blot of a 15% SDS-PAGE gel, stained for CD59, on which the detergent phases collected from the Triton X-114 experiment were loaded. Lane *1*, molecular mass markers; lane *2*, untreated SP (detergent phase); lane *3*; PIPLC-treated SP (detergent phase), anti-CD59-reactive material was much reduced; lane *4*, purified SP CD59 (detergent phase); lane *5*, PIPLC-treated SP CD59 (detergent phase), anti-CD59-reactive material was no longer detected. (*B*) Ultracentrifugation of SP. SP (1 ml) was subjected to ultracentrifugation at 200,000 *g* for 1 h. Shown is a Western blot of a 15% SDS-PAGE gel, stained for CD59. Lane *1*, molecular mass markers; lane *2*, purified SP CD59 (3 μg); lane *3*, unfractionated SP (15 μl); lane *4*, ultracentrifugation pellet (15 μl of a pellet resuspended in a volume of PBS equal to the original volume of SP), anti-CD59-reactive material was present; lane *5*, ultracentrifugation supernatant (15 μl), only very slight anti-CD59 reactivity was detected. (*C*) Binding of SP CD59 to 3T3 cells incubated with SP and the ultracentrifugation pellet and supernatant. 3T3 cells were incubated with SP or with the ultracentrifugation pellet or with the supernatant for 30 min at 37°C, washed, stained for CD59, and analyzed by flow cytometry. 10,000 events were counted in each case. The figure is representative of three experiments. (*D*) MAC inhibitory activity of SP and the ultracentrifugation pellet and supernatant. EC5b-8 were incubated with samples to be tested (100 μg/ml of protein) or with GVB (control cells) for 30 min at 37°C, washed, and incubated with polyclonal anti-CD59 IgG (100 μg/ml) or NIRIg (100 μg/ml) for 15 min at 37°C before exposure to C9 and estimation of percent hemolysis as described in Fig. 1 *A*. Values are the means of triplicates and error bars represent SD of the triplicates. The data are representative of three experiments.

by cell binding and MAC inhibition assays. 3T3 cells incubated with the resuspended pellet before staining with anti-CD59 and FITC-labeled second Ab had a mean fluorescence greater than fourfold that of cells incubated with unfractionated SP; the increased ability of CD59 present in the resuspended pellet to bind cells may be due to the absence of inhibition by lipoproteins present in SP (see Discussion). In contrast, the fluorescence of cells incubated with supernatant was only slightly increased relative to control cells (Fig. 5 *C*).

Lysis of EC5b-8 cells on exposure to C9 was inhibited by 56 ± 5% when the cells were incubated with SP. Incubation of the cells with anti-CD59 after incubation with SP and before exposure to C9 abrogated this inhibitory activity by >50%. The resuspended pellet gave a degree of inhibition comparable to that achieved with SP (51 ± 1%), which was also abrogated by >50% when anti-CD59 was included in the system. In contrast, incubation of EC5b-8 with supernatant inhibited their subsequent lysis by 26 ± 8%, and this inhibitory activity was not significantly reduced by inclusion of anti-CD59 in the assay (Fig. 5 *D*).

The above data indicate that SP CD59 possesses a GPI anchor and is present in SP in a high molecular mass form.

FPLC Fractionation of SP. Samples of SP from four donors were analyzed by FPLC on Superose 6. Protein, phospholipid, and cholesterol content of fractions from a representative experiment are shown in Fig. 6. Protein eluted in two bands: an early, minor band present in the void volume of the column (fractions 4–14) and a later, broad band containing the bulk of the protein (fractions 20–85). Phospholipid also eluted in two bands: an early, minor band that coeluted with the early protein band, while the bulk of the phospholipid was detected in a later band (fractions 50–80). Cholesterol coeluted with the early protein and phospholipid bands and was not detected in later fractions. Anti-CD59-reactive material of 20 kD (detected by Western blot) was present in fractions of the early protein-, phospholipid-, and cholesterol-containing band, and was not detected in later fractions.

CD59-containing fractions were pooled. Total protein content of the pool was 1.1 ± 0.1 mg (mean of four experiments). Cholesterol was measured in three experiments; values were 0.8, 0.83, and 0.73 μg cholesterol/mg protein. Phospholipid was measured in two experiments. Phospholipid/cholesterol ratios (μg/μg) were 1:1.5 and 1:1.4.

Comparable data were obtained when pools of CD59-

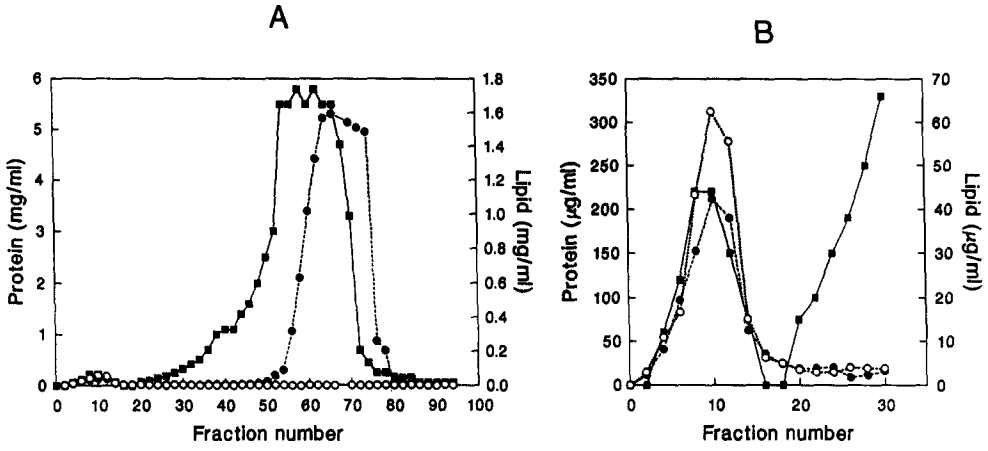

Figure 6. FPLC fractionation of SP. (*A*) Results of biochemical analysis of fractions (94 × 0.5 ml) from FPLC analysis of SP (1.5 ml) on Superose 6. (○) Cholesterol; (●) phospholipid; (■) protein. (*B*) Biochemical analysis of fractions 1–30 from FPLC analysis of SP. Note that in this case the scale for protein and lipid is in μg/ml. Symbols are as in *A*.

containing fractions from three donors were analyzed by SDS-PAGE and Western blotting. Unfractionated SP contained multiple protein bands. Pooled CD59-containing fractions also displayed multiple bands, although fewer bands than were present in SP. These included a major band that comigrated with SP CD59 (Fig. 7 *A*) and reacted with anti-CD59 (Fig. 7 *B*). Densitometry of the silver-stained gels indicated that the 20-kD band comprised 13.2, 12.0, and 16.8% of the total protein in the respective pools. The mean CD59 content of pooled vesicles from three separate preparations was 12.6% of total protein (129 μg/mg of total protein; SD, 29). These data were in agreement with those obtained by both semi-quantitative dot blotting and Western blotting.

CD59 activity in the pools was investigated by MAC inhibition assay. Incubation of EC5b-8 cells with the pooled material at a protein concentration of 100 μg/ml inhibited their lysis on subsequent exposure to C9 by 47 ± 4%.

Transmission Electron Microscopy. On examination by TEM, the pellet obtained by ultracentrifugation of SP was found to consist of a dense population of bilamellar vesicles (10–200 nm in diameter), together with an amorphous substance that we have not identified (Fig. 8 *a*). The ultracentrifugation pellet of pooled CD59-containing fractions from FPLC of SP consisted almost entirely of vesicles without contaminating material (Fig. 8 *b*).

Unfixed vesicles placed on formvar-coated grids and probed with anti-CD59 and gold-labeled secondary Ab stained positively for CD59 (Fig. 8, *c* and *d*). When the vesicles were fixed in 2% paraformaldehyde or permeabilized with 50% methanol before staining, the number of beads present per vesicle was unaffected. Of 100 vesicles selected at random 67 had gold particles, and the mean number of gold beads per vesicle was 1.0. The mean number of gold beads per vesicle when only stained vesicles were counted was 1.5 (range, 1–4). No gold beads were present on the matrix between vesicles and no gold particles were associated with control vesicles incubated with NIMIg or with irrelevant primate antibody in place of first antibody.

Discussion

We here report the presence in SP of the MAC inhibitory protein CD59 at a concentration of at least 20 μg/ml. This protein, SP CD59, was apparently identical with E CD59 in terms of molecular mass, antigenicity, and amino-terminal amino acid sequence. Moreover, it had the capacity to bind to the membranes of GPE, murine fibroblasts, and human spermatozoa even when present in unfractionated SP, indicating that this phenomenon may occur in vivo. Incubation of heterologous E with SP rendered the cells more resistant to lysis by human MACs. The MAC inhibitory capacity of SP could be partly removed by immunoaffinity depletion of CD59 from this fluid and partly abrogated by incubation of SP-treated EC5b-8 cells with anti-CD59 Ab before exposure

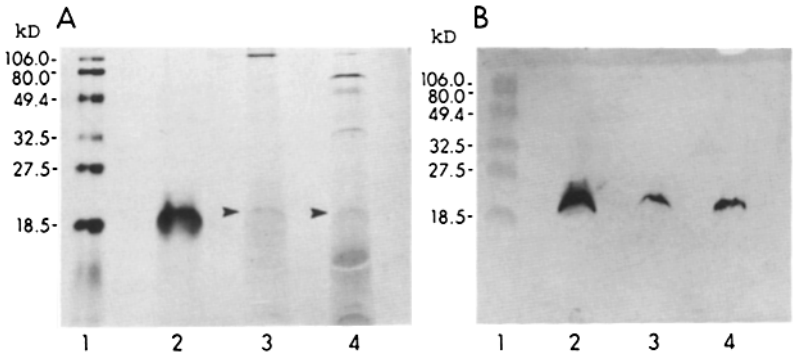

Figure 7. Analysis of vesicles purified from SP by FPLC analysis. (*A*) Silver-stained, 15% SDS-PAGE gel. Lane *1*, molecular mass markers; lane *2*, purified SP CD59 (3 μg); lane *3*, vesicles purified by FPLC (3 μg): multiple protein bands are present, including a 20-kD band; lane *4*, unfractionated SP, a greater number of bands are present than in lane *3*, including a 20-kD band (arrowheads). (*B*) Shown is a Western blot of a 15% gel stained for CD59. Lane *1*, molecular mass markers; lane *2*, purified SP CD59 (1 μg); lane *3*, FPLC-purified vesicles (2 μg); lane *4*, unfractionated SP (20 μg).

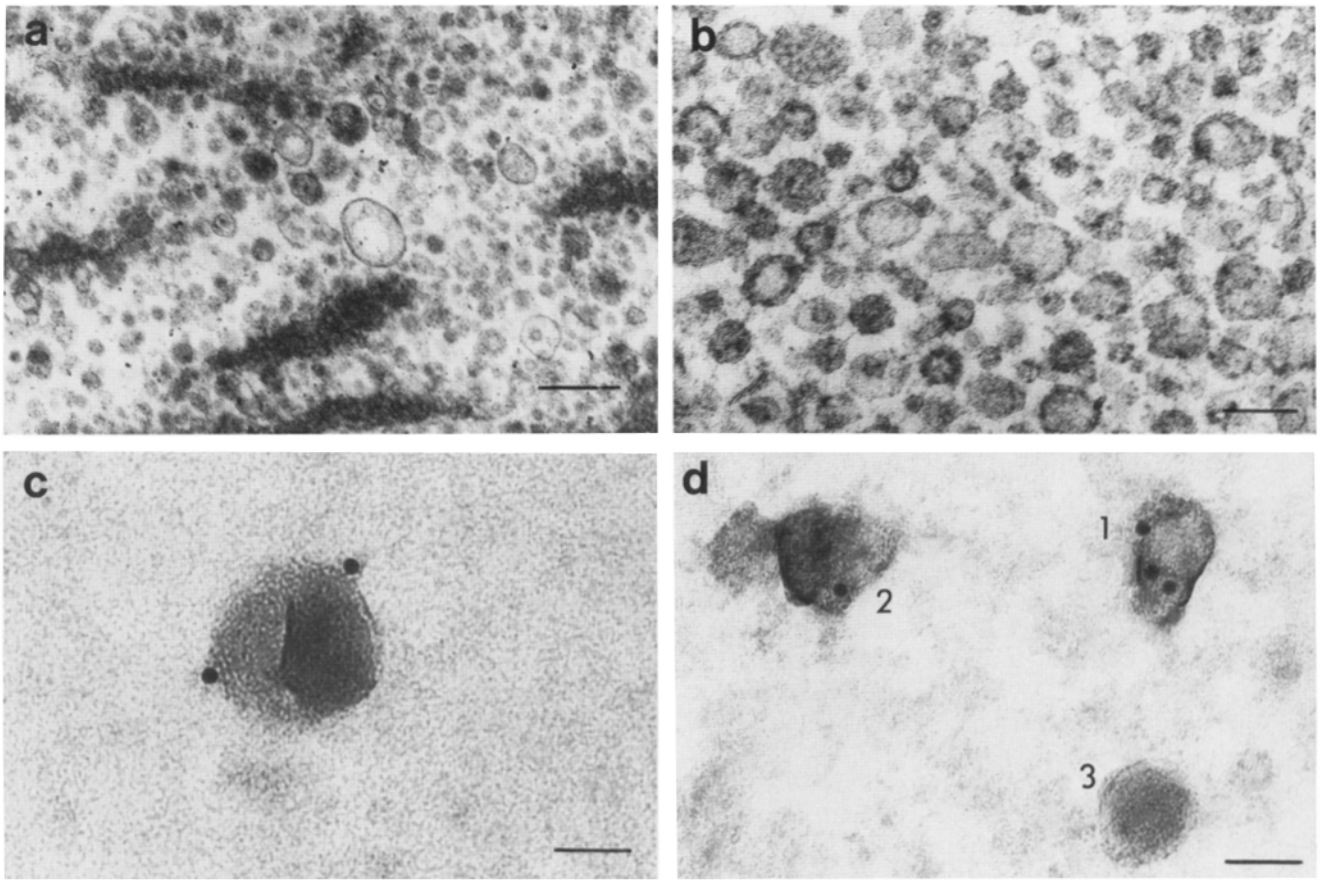

Figure 8. Electron microscopy. (*a*) Shown is TEM of a pellet obtained by ultracentrifugation of SP. The pellet was fixed, embedded in resin, and sectioned as described in Materials and Methods. Bilamellar vesicles are present, together with an amorphous material (×19,250). The bar represents 200 nm. (*b*) TEM of a pellet obtained by ultracentrifugation of FPLC purified vesicles. The pellet was fixed, embedded, and sectioned as described. Vesicles are almost free of contaminating material (×38,500). The bar represents 100 nm. (*c*) FPLC-purified vesicles that were stained unfixed with anti-CD59 and gold-labeled secondary antibody and viewed without embedding or sectioning. The field shows one vesicle, with two gold particles at the periphery. The surrounding matrix is free of gold particles (×77,000). The bar represents 50 nm. (*d*) FPLC-purified vesicles stained as in *c* after fixation in paraformaldehyde (2%). The field shows three vesicles, one with three gold particles (*1*), one with one gold particle (*2*), and one without gold particles (*3*). The surrounding matrix is free of gold particles (×77,000). The bar represents 50 nm.

of the cells to C9, strongly suggesting that SP CD59 was at least partly responsible for the MAC inhibition. Binding of SP CD59 to 3T3 cells occurred in a time- and dose-dependent manner. Failure of 100% of the CD59 present in SP to bind to cells may be due to the influence of lipoproteins in SP: lipoproteins present in serum are known to inhibit binding of purified GPI-linked proteins to cell membranes (17, 18).

Pretreatment of SP or of purified SP CD59 with PIPLC markedly reduced the capacity of SP CD59 to bind cells and to inhibit their lysis by human MACs, and greatly decreased the amount of SP CD59 that partitioned into the detergent phase of Triton X-114 (Fig. 4 *A*). These data provide compelling evidence that SP CD59 possesses a GPI anchor. The mechanism by which the anchor is retained is of interest. We present here evidence that SP CD59 is carried on the surface of vesicular organelles and that cells may acquire new CD59 molecules as a result of interaction with these organelles. Analysis of fractions by Western blotting and semiquantitative dot blot indicated that >90% of SP CD59 was precipitated by ultracentrifugation of SP. These data were supported by cell binding and MAC inhibition assays. PIPLC treatment of SP prevented precipitation of SP CD59. We conclude that CD59 was present in SP in particulate form and that this particularity was GPI anchor dependent. The residual MAC inhibitory activity in the high-speed supernatant, together with the failure of anti-CD59 to abrogate MAC inhibitory activity on supernatant-treated cells or to abrogate completely the MAC inhibitory activity of unfractionated SP, may imply the function of other cell-binding, MAC inhibitory proteins in SP.

On direct examination by TEM the high-speed pellet was found to consist of a population of bilamellar vesicles (10–200 nm in diameter) and an amorphous substance that we did not attempt to identify. Our observations concur with those of Ronquist et al. (19), who named the vesicles "prostasomes" (20) and proposed that the amorphous material consists of protein deriving from the seminal vesicles (21). We further

purified the vesicles by FPLC fractionation of SP on Superose 6. TEM of the pellet generated by ultracentrifugation of phospholipid-, protein-, and cholesterol-containing fractions that eluted in the void volume of the columns revealed a clean population of vesicles with minimal contaminating material. Biochemical analysis of the vesicles established that their composition was broadly comparable with that reported for prostasomes (22). We found the cholesterol content of the vesicles to range between 0.73 and 0.83 μg/mg protein, while the values reported by Arvidson et al. (22) range from 0.46 to 0.67 μg/mg.

All detectable CD59 was found by Western blotting to be associated with the vesicles, and this was confirmed by immunoelectron microscopy and MAC inhibition assay. However, our ELISA measurements of CD59 concentration in the purified vesicles are not compatible with those that we obtained for unfractionated SP. For example, the measured concentration of CD59 in SP was $\sim$20 μg/ml and, since 1.5 ml of SP was applied to the FPLC system, a total of not more than 30 μg recovered protein was expected. However, $\sim$100 μg of CD59 was recovered (all in vesicle-containing fractions). Semiquantitative Western blotting and dot blotting of SP, the ultracentrifugation pellet, and the FPLC-purified vesicles, together with data from densitometry analysis of the purified vesicles, indicate that the concentration of CD59 in SP is $\sim$100 μg/ml and that this protein comprises >10% of the total protein content of the vesicles. Together, these data suggest that the direct ELISA measurements of CD59 in unprocessed SP underestimate the amount of CD59 that is present in this fluid. Comparison of data from several different quantitative assays will be required before a more correct estimation can be made. We therefore state here that the concentration of CD59 in human SP is at least 20 μg/ml, with the reservation that the true concentration may be greater.

Prostasomes are extracellular, vesicular, bilamellar organelles, of 150-nm average diameter (20). Although these particles possess a number of enzymic activities (21), promote spermatozoal motility (21), and inhibit mitogen-induced proliferation of lymphocytes (23), our data provide the first direct evidence of a physiologically important role for a prostasome membrane protein.

Data from EM suggest that prostasomes are present within "storage vesicles" in cells of the prostatic acinar epithelium. Two mechanisms have been proposed to operate in their release: exocytosis and "diacytosis," the latter being a process by which intact storage vesicles are expelled from the cell (21). Although we found all detectable SP CD59 to be associated with membrane vesicles, semiquantitative analysis indicated that the concentration of this protein was greater in the SP of fertile men than in that of men postvasectomy (our unpublished results). SP CD59 may therefore arise from sites on both sides of transection of the ductus deferens. Vesicles present in the SP of other species originate from epididymis and seminal vesicles (24–26), and it is possible that prostasomes derive from more than one organ. We are examining their cellular origin and biosynthesis using cells in culture.

Bilamellar vesicles released by other cell types in vitro are enriched in GPI-anchored proteins, relative to the cell plasma membrane (27–29). On cell membranes, GPI-anchored proteins are preferentially expressed in glycosphingolipid-rich domains (caveolae) (30, 31). Others have speculated that these domains are precursors of endocytic vesicles, although vesiculation of caveolae has not been demonstrated (30, 31). We suggest that prostasomes derive from caveolae. This mechanism, if correct, suggests a biologic advantage for the GPI anchor and a role for vesicles in body fluids.

Compelling evidence indicates that the interaction of prostasomes with spermatozoa involves hydrophobic bond formation (32). Our data could be explained by a continuing association of intact prostasomes with cell membranes. Alternatively, CD59 and other membrane proteins might be transferred from prostasome membranes to the membranes of the interacting cell type. Experiments are in progress to elucidate this question.

Inhibition of C activation on spermatozoa has been the focus of much scientific attention in recent years. Another C regulatory protein, MCP, has been localized on spermatozoa (33, 34). However, this protein is not exposed, and is therefore unlikely to protect spermatozoa, until after the acrosome reaction has occurred ($\sim$7 h after entry to the female reproductive tract). The relative abundance of the fluid phase MAC inhibitor, SP 40,40, in semen has prompted suggestions that this may be the most important regulator of C activation on spermatozoa (35, 36). However, the demonstration that neutralization of CD59 on spermatozoa renders the cells highly susceptible to MAC-mediated damage (6) implies an important role for this protein in vivo. Prostasomes may represent a pool of CD59 from which protein lost from spermatozoa, possibly as a result of normal membrane turnover or of low level C attack, may be replenished, thus ensuring that the cells reach higher levels of the female reproductive tract with a full complement of the protein on their membranes.

Binding of SP CD59 to infective organisms may contribute to the persistence of infection within the male and female reproductive tracts. We are therefore investigating the capacity of this protein to bind to bacterial cell walls.

Finally, the findings presented here may facilitate the development of preparations of membrane-bound GPI-linked proteins, including CD59, suitable for use in the therapy of C-related and other diseases.

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