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Reproductive Tract Secretions and Bull Spermatozoa Contain Different Clusterin Isoforms That Cluster Cells and Inhibit Complement-induced Cytolysis

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ABSTRACT: Clusterin from bull rete testis fluid (RTF), cauda epididymal fluid (CEF), and octyl-β-D-glucopyranoside extract of cauda epididymal sperm (CES) was identified and characterized using monoclonal and polyclonal antibodies (Abs) developed against ram clusterin and a β-subunit–specific oligopeptide of porcine clusterin. One-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting showed that bovine RTF clusterin had dimeric and monomeric molecular weights (M_rs) of ≈94 kDa and of 42 and 43 kDa, respectively. Clusterin in CEF and CES had similar dimeric M_rs (74 kDa). Reduced CEF clusterin appeared as three monomers (M_r = 40, 39, and 38 kDa), whereas reduced CES clusterin appeared only at M_r 40 kDa. Enzymatic deglycosylation resulted in similar M_rs of clusterin from RTF, CEF, and CES. The M_r of RTF clusterin decreased from 94 kDa to 51 kDa, indicating a carbohydrate content of 45%. After deglycosylation, the M_r of the CEF clusterin decreased from 74 kDa to two distinct bands at 51 and 50 kDa (with carbohydrate contents of 31 and 32%, respectively), suggesting that two isoforms of the heterodimeric protein are present because of the two isoforms of the α-subunit. Under nonreduced conditions, a β-subunit–specific Ab reacted with M_r of 36–38 kDa, indicating the existence of free clusterin β-subunits in CES. RTF, CEF, and CES extracts all caused mouse fibroblastic L-cell aggregation. CEF cell aggregation was inhibited by Hyb-17 Ab but not by other Abs. Both RTF and CEF caused a dose-dependent inhibition of complement-induced cytolysis, although RTF clusterin was more potent than CEF clusterin. We conclude that several isoforms of clusterin occur in the bull reproductive tract and that the variation in carbohydrate content among these isoforms may affect the biological or functional activity of the protein.

Key words: Epididymal fluid, rete testis fluid, L-cells.
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Clusterin, also known as sulfated glycoprotein-2 (Kissinger et al, 1982; Sylvester et al, 1984), testosterone-repressed prostate message-2 (Leger et al, 1987; Bettuzi et al, 1989; Buttyan et al, 1989), complement lysis inhibitor, serum protein-40,40 (Jenne and Tschopp, 1989; Kirszbaum et al, 1989; Murphy et al, 1989), apolipoprotein-J (de Silva et al, 1990), and sulphated glycoprotein-III (GP-III; Palmer and Christie, 1990) is a heterodimeric, glycosylated acidic protein of 80–85 kDa with a wide range of isoelectric points (pI 3.6 to 6). First described in ram rete testis fluid (RTF), clusterin was found to constitute a major protein (18%) and was shown to cluster various types of mammalian cells (Fritz et al, 1983). In its reduced form, clusterin has an apparent molecular weight (M_r) of 40 kDa and displays heterogeneity (which is removable by neuraminidase treatment; Blaschuk et al, 1983; Fritz et al, 1983). It is thought that clusterin may be involved with a variety of biological functions, such as spermatogenesis (Sylvester et al, 1984; Collard and Griswold, 1987), complement-mediated cell lysis inhibition and sperm protection (Jenne and Tschopp, 1989; Kirszbaum et al, 1989; Murphy et al, 1989; O'Bryan et al, 1990), fertilization (O'Bryan et al, 1990), endocrine cell exocytosis–endocytosis (Patzak and Winkler, 1986), lipid solubilization and redistribution (de Silva et al, 1990), apoptosis and degenerative disease conditions (Leger et al, 1987; Lee and Sensibar, 1987; Bettuzi et al, 1989; Buttyan et al, 1989; Bandyk et al, 1990), cell maturation (Harding et al, 1991; O'Bryan et al, 1993), smooth muscle modulation (Thomas-Salgar and Millis, 1994), and implantation (Brown et al, 1996).

The bovine homologue of clusterin (GP-III) was first identified as a component of the glycoprotein complex of the adrenal chromaffin granule membrane (Geissler et al, 1977; Gavine et al, 1984), with a sugar content of 32% (Fischer-Colbrie et al, 1982). Subsequent studies have de-

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tected GP-III in the soluble content of chromaffin granule (Fischer-Colbrie et al, 1984) and in tissue extracts of the bovine pituitary, hippocampus, parathyroid gland, kidney, liver, testis, and epididymis (Laslop et al, 1993). Clusterin has been shown to occur in two isoforms in the ram (Cheng et al, 1988), rat (Mattmueller and Hinton, 1991; Sylvester et al, 1991), human (O'Bryan et al, 1990), and cow (Laslop et al, 1993), but virtually nothing is known about the isoforms of clusterin in the bull reproductive tract secretions and spermatozoa.

The purpose of this study was as follows: 1) to detect and characterize clusterin in bull reproductive tract secretions and on spermatozoa, and 2) to determine if bull RTF and cauda epididymal fluid containing clusterin would cause cell aggregation and would inhibit complement-induced cytolysis.

Materials and Methods

Collection of RTF, Cauda Epididymal Fluid, and Cauda Epididymal Spermatozoa

Bovine testes from four to eight bulls were collected daily for 5 days and were transported on ice within 4 hours after slaughter (slaughterhouse in South St. Paul, Minnesota). RTF and cauda epididymal fluid (CEF) were collected, essentially as described previously by Crabo (1965).

RTF—Efferent ductules were dissected free at the junction between the testes and the caput epididymides. Since intratesticular fluid in the bull is under positive pressure, puncture of the ductules immediately resulted in escape of RTF, which was trapped in a Pasteur pipette and transferred to ice-chilled Eppendorf tubes for the duration of collection (1 to 2 hours). From batches of 8 to 16 testes per collection, a total of 10 to 300 µl RTF was collected from each testis and pooled together.

Cauda Epididymal Content (CEF and Cauda Epididymal Spermatozoa)—Cauda epididymal fluid containing cauda epididymal spermatozoa (CES) was collected from the cauda epididymides after multiple incisions over region VI and the distal part of region V, as described by Nicander (1958). A total of 100 to 500 µl was collected from each testis and was pooled into ice-chilled Eppendorf tubes. RTF and cauda epididymal content were fractionated by processing twice at 13,000 × *g* in an Eppendorf microcentrifuge (Brinkman, Wesbury, New York) for 6 minutes. RTF and CEF were aspirated and were stored at −20°C. Cauda epididymal sperm pellets were saved for membrane protein extraction.

CES Membrane Protein Extraction—Sperm membrane integral proteins were extracted essentially as described by Samper et al (1995). Cauda sperm pellets were suspended in phosphate-buffered saline (PBS; pH 7.4) and were washed three times by centrifugation (1,500 × *g* for 6 minutes) and then resuspended. The sperm pellets were extracted by suspension in five volumes of 40 mM octyl-β-D-glucopyranoside (OBG) for 1 hour with gentle, periodic shaking. The supernatant was collected after centrifugation and was dialyzed for 24 to 30 hours against 10 mM Tris-HCL (pH 7.2) at 4°C in a 3.5-kDa cutoff dialysis tubing (Spectropore, Cole Palmer Inc., Illinois), followed by centrifugation in Centriprep-10 concentrators (Amicon, Beverly, Massachusetts). All extracted and dialyzed samples were stored at −20°C until analysis.

Protein content of RTF, CEF, and OBG sperm extract was assessed using Bio-Rad-DC protein assay kit II (Bio-Rad, Hercules, California). Absorbance was measured at 750 nm in a Beckman spectrophotometer (Beckman Instruments Inc., Fullerton, California) with bovine serum albumin as a standard. Samples were aliquoted (0.5 to 1 ml) and were stored at −20°C before use in SDS-PAGE, cell clustering, or complement inhibition studies.

Deglycosylation

Deglycosylation was performed overnight by incubating samples with *N*-glycosidase F (peptide-N^4-[acetyl-β-glucosaminyl] asparagine amidase; Boehringer Mannheim Co., Indianapolis, Indiana) following manufacturer's recommendations. Briefly, 40 µg of RTF, CEF, and OBG sperm extract were denatured at 95°C for 5 minutes in 1% SDS in Tris-HCl–buffered saline (TBS) before deglycosylation, then were divided into two groups (treatment and control). Treatment samples were incubated with 0.4 or 0.6 units of *N*-glycosidase F in a total volume of 20 µl TBS (pH 7.4), 0.1% SDS, 30 mM Tris–HCl–EDTA, and 0.5% nonionic detergent P-40 (NP-40; Sigma Chemical Co., St. Louis, Missouri) for 17 hours at 37°C in a 5% CO_2 humid air chamber; control samples were incubated under the same conditions except for the absence of the enzyme, which was replaced by TBS. SDS-PAGE analysis was performed on the deglycosylated and undeglycosylated samples.

Antibody Preparation

Anti-β-subunit porcine clusterin antibody (β-12) was generated by methods similar to those described by Sanders et al (1993). Briefly, a β-specific oligopeptide of the porcine clusterin corresponding to amino acids 410 through 428 of the cDNA (Diemer et al, 1992) was synthesized. Two mg of the synthetic peptide were conjugated to 2 mg of cationized BSA (cBSA) via imject supercarrier 1-ethyl-3-(3 dimethylaminopropyl) carbamide hydrochloride (EDC) system for peptides (Pierce Rockford, Illinois) and were added to Freund's complete adjuvant. Rabbits were given subcutaneous injections along the spinal column and were subsequently boosted with an equal amount of the peptide in Freund's incomplete adjuvant before sera collection. Antiserum was collected 10 days after the third injection, and the IgG was purified with Protein-A columns (Pierce Rockford). Monoclonal anti-ram clusterin Hyb-17 and Hyb-45 antibodies (mAbs) were generated in mice after immunization with purified clusterin from ram RTF (Blaschuk et al, 1983).

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis and Western Blotting Analysis

Proteins were separated by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (1-D SDS-PAGE) in a modified Laemmli buffer system (Laemmli, 1970). Slab gels with total acrylamide-bis concentrations of 15%, for reduced gels, or 10%, for unreduced gels, were used. All gels were run in duplicates at 150 volts for 1.5 hours with 20 µg protein per lane. After separation, gels were electrophoretically transferred to immobilon polyvinylidine diflouride transfer membranes

(Millipore, Bedford, Massachusetts), as described by Towbin et al (1979), for western blot analysis. Nonspecific binding sites were blocked by 3% BSA/TBS with gentle rocking for 1.5 hours at room temperature. Controls were incubated in 3% BSA/TBS alone, while treatment blots were incubated for 12 to 15 hours at room temperature with Hyb-17, Hyb-45, the mixture of Hyb-17 and Hyb-45 Abs, or β-12 Ab diluted 1:500 or 1:250 in 3% BSA/TBS as primary antibody. Blots were washed thoroughly two times with 0.1% TBS-Tween-20 (TBS-T) and were washed thoroughly three times with TBS over a period of 15 minutes. They then were incubated for 1 hour with the secondary antibody. Peroxidase-conjugated sheep anti-mouse IgG was used as secondary Ab for Hyb-17 and Hyb-45 Abs. Peroxidase-conjugated sheep anti-rabbit IgG was used for the β-12 Ab. Both secondary antibodies were diluted 1:1000 in 3% BSA/TBS. Peroxidase was detected by incubating blots in 0.01 M Tris–HCL buffer (pH 7.6) in 40 ml H_2O containing 30 mg 4-chloro-1-naphthol, 10 ml cold methanol, and 167 μl 30% cold H_2O_2.

Cell-Clustering Activity

Bovine CEF, RTF, and OBG extracts were diluted with PBS to yield a protein concentration of 2.1 mg/ml. Cell-clustering conditions were modified from those described by Fritz et al (1983). Briefly, nearly confluent mouse fibroblastic L-929 cells (ATCC number CCL-1) were removed by trypsinization and were pelleted by centrifugation at 300 × *g* for 3 to 4 minutes. The pellet was washed two times with PBS before it was counted and its number adjusted to 10,000 cells/35 μl PBS. One to 11 μl of CEF, RTF, or OBG extract was added to wells in a 96 U-bottom well polyvinyl chloride microtitration plate (Costar Corp., Cambridge, Massachusetts). The PBS/cell suspension was then added, and the total volume of each well was brought to 100 μl with PBS. Control wells contained PBS and mouse L-cells only. Cell clustering was monitored using an inverted microscope (Nikon Diaphot 300) after 1-, 12-, and 24-hour incubations (with 1 as the minimum score and 3 as the maximum score). Sample volumes and corresponding protein and clusterin amounts that gave a cell-clustering score (CCS) of 1.5 after a 1-hour incubation were recorded for comparison.

Cell-Clustering Inhibition (CCI) Analysis

Pooled CEF from 8 bulls was used as a clustering agent. The minimum amount of the CEF that caused cell clustering was determined in serial dilutions of CEF (0.5 μl containing ≈10 μg protein). Inhibition of cell clustering was determined with varying dilutions (1:200, 1:100, and 1:60) of Hyb-17, Hyb-45, and β-12 antibodies, except for the positive control, which was prepared in a total volume of 100 μl PBS containing 0.5 μl CEF and 10,000 cells per well. Eight duplicate microtiter plate wells were made for the test as follows: 1) a positive control (PC) containing cells and PBS only; 2) a negative control (NC) containing cells and 0.5 μl CEF in PBS; 3) three treatment wells containing 1:200, 1:100, and 1:60 of Hyb-17, Hyb-45, or β-12 Ab dilutions in PBS and 0.5 μl CEF; and 4) three control wells accounting for nonspecific IgG effect, containing 0.5, 1.0, and 1.5 μg porcine IgG and the dilution of the CEF. The microtiter plate was incubated for 7 hours at room temperature on a 35°-tilted rotor set at 2 to 3 rpm, after which the 10,000 cells were added to each well. The plate was incubated at 37°C in 5%CO_2 humid air on a rotor rotating at 2 to 3 rpm. Cell clustering was scored under an inverted microscope after 1, 12, and 24 hours of incubation.

Inhibition of Cytolytic Complement Activity

Bovine RTF was pooled from 36 bulls and was concentrated to 29.5 mg/ml using a speed vac concentrator (Savant Instruments Inc., Farmingdale, New York).CEF samples were pooled from five bulls with protein concentrations ranging from 21–32.4 mg/ml. Clusterin content of the pooled CEF and RTF preparations was estimated to be between 27–28 and 8–9% of the total protein, respectively, by densitometric analysis of Coomassie brilliant blue–stained blots of SDS-PAGE–separated unreduced samples (Bio-Rad imaging densitometer).

A modification of a previously described technique (Mayer, 1961; Tanaka et al, 1987) was used to determine cytolytic complement activity. A total of 7 ml of venous blood was withdrawn from the jugular vein of five clinically healthy cows using a vacutainer system (Becton Dickinson, Rutherford, New Jersey) containing sodium heparin as an anticoagulant. Following centrifugation at 400 × *g* for 10 minutes, all plasma was pooled, divided into aliquots of 2 ml, and frozen at −20°C until used. The 50% hemolytic complement activity (CH_{50}) in bovine plasma was determined in serial 0.3-ml dilutions of plasma in ice-cold 0.1% gelatin veronal buffer supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (GVB^{2+}; Sigma). A total of 0.05 ml antibody-sensitized sheep erythrocytes (Sigma) at a concentration of 1×10^9 cells/ml was added to the plasma dilutions while maintained on ice. Following 60 minutes of incubation in a 37°C waterbath, the reaction was terminated by a 5-minute incubation on ice, followed by centrifugation at 4°C for 10 minutes at 500 × *g*. The absorbance of the supernatant was analyzed at 540 nm in a spectrophotometer (Beckman). Optical density (OD) for 100% hemolysis was determined by incubating 0.05 ml antibody-sensitized sheep erythrocyte suspensions in 0.3 ml distilled water, and the optical density of spontaneous hemolysis was determined by incubating 0.05 ml antibody-sensitized erythrocytes in 0.3 ml GVB^{2+}. Optical density of samples without antibody-sensitized sheep erythrocytes served as serum color controls. Percent hemolysis (*y*) was calculated from the following formula:

$$y = [\text{test sample optical density (OD)} - \text{serum color OD} - \text{spontaneous lysis OD}] \div [100\%\ \text{lysis OD} - \text{spontaneous OD}].$$

CH_{50} for the pooled bovine blood plasma was determined as the reciprocal of the complement dilution at the 50% lysis interception after plotting *y* against the reciprocal of the dilution on a semilogarithmic scale. The CH_{50} for the untreated plasma was compared to cytolytic complement activity for RTF- and CEF-treated samples. RTF ($n = 2$) and CEF ($n = 5$) were assayed for inhibition of CH_{50} activity. Ten μl, 20 μl, 50 μl, or 100 μl of RTF, estimated to contain 250 μg clusterin/100 μl, was added to the cytolytic complement activity assay with a dilution of bovine plasma that gave CH_{50} activity. Similarly, increasing amounts of 10 μl, 25 μl, 50 μl, or 100 μl of CEF, estimated to contain 737 μg clusterin/100 μl, were added to the CH_{50} assay ($n = 5$). Inhibition of complement activity was expressed as percent of CH_{50}.

Table 1. *Reactivity of monoclonal anti-ram clusterin antibodies with unreduced samples of clusterin*

		Molecular weight (M_r; kDa)*							
		Undeglycosylated				Deglycosylated			
Antibody†	Sample source	94	74	38	36	53	52	51	50
Hyb-17	RTF	++	−	−	−	−	−	++	−
Hyb-17	CEF	−	+++	−	−	−	−	++	++
Hyb-17	OBG	−	+	+	+	+	+	+	−
Hyb-45	RTF	+	−	−	−				
Hyb-45	CEF	−	++	−	−				
Hyb-45	OBG	−	+	+	+				
Hyb-17	RTF	++	−	−	−				
+	CEF	−	+++	−	−				
Hyb-45	OBG	−	+	+	+				

The 94-kDa band was not present in all gels in the case of the Hyb-45 antibody, and many gels lacked bands with OBG extract. RTF, rete testis fluid; CEF, cauda epididymal fluid; OBG, octyl-β-D-glucopyranoside extract of cauda epididymal sperm.

* Band presence is indicated by +. Absence is indicated by −. The number of + indicates intensity of reactivity. + = weak; ++ = moderate; +++ = strong.

† Hyb-17 and Hyb-45, monoclonal anti-ram clusterin antibody.

Results

Characterization of Clusterin Isoforms

RTF—Under nonreduced conditions, a band was recognized by western blotting at M_r 94 kDa by both anti-ram mAbs Hyb-17 and anti-ram mAbs Hyb-45 (Fig. 1A; Table 1). However, reactivity with Hyb-45 Ab was often so weak that it was barely visible under nonreduced conditions. This band appeared at $M_r \cong 51$ kDa with Hyb-17 after deglycosylation, indicating a carbohydrate content of approximately 45% (Fig. 1B, C; Table 1).

Under reduced conditions, Hyb-17 detected two bands; a major one at M_r 43 kDa and a minor and very weak one at M_r 42 kDa (Fig. 1D; Table 2). Both Hyb-45 and β-12 antibodies reacted with the M_r 43-kDa band (Fig. 1D, E; Table 2). The M_r 42-kDa band became more discernible when western blots were probed with both Hyb-17 and Hyb-45 antibodies (Fig. 1D). After deglycosylation, all Hyb-17, Hyb-45, and β-12 antibodies detected one band at M_r 27 kDa (Fig. 1 F–H; Table 2). These data suggest that the Hyb-17 antibody binds to both the α- and β-subunits of clusterin and that the Hyb-45 and β-12 antibodies specifically bind to the β-subunit. The carbohydrate content of the α-subunit was estimated from these data to be approximately 37% and that of the β-subunit, 38%.

Cauda Epididymides Fluid—Western blot analysis of nonreduced samples treated with either the Hyb-17 or Hyb-45 antibodies featured a single band at M_r 74 kDa,

Table 2. *Reactivity of monoclonal anti-ram clusterin and anti-β-subunit porcine clusterin antibodies with reduced samples of clusterin*

		Molecular weight (M_r; kDa)*								
		Undeglycosylated						Deglycosylated		
Antibody†	Sample source	43	42	42–38	40	39	38	27	25	22
Hyb-17	RTF	++	+	−	−	−	−	+++	−	−
Hyb-17	CEF	−	−	+++	−	−	−	+++	+++	+++
Hyb-17	OBG	−	−	−	+	−	−	−	+	−
Hyb-45	RTF	++	−	−	−	−	−	+++	−	−
Hyb-45	CEF	−	−	−	++	−	−	+++	−	−
Hyb-45	OBG	−	−	−	+	−	−	−	−	−
Hyb-17	RTF	++	+++	−	−	−	−			
+	CEF	−	−	−	++	++	++			
Hyb-45	OBG	−	−	−	+	−	−			
	RTF	++	−	−	−	−	−	++	−	−
β-12	CEF	−	−	−	++	−	−	++	−	−
	OBG	−	−	−	−	−	−	−	−	−

* Band existence is indicated by (+). Absence is indicated by (−). The number of (+) indicates intensity of reactivity. + = weak; ++ = moderate; +++ = strong.

† Hyb-17 and Hyb-45, monoclonal anti-ram clusterin antibody; β-12, anti-β-subunit clusterin antibody.

RTF, rete testis fluid; CEF, cauda epididymal fluid; OBG, octyl-β-D-glucopyranoside extract of cauda epididymal sperm.

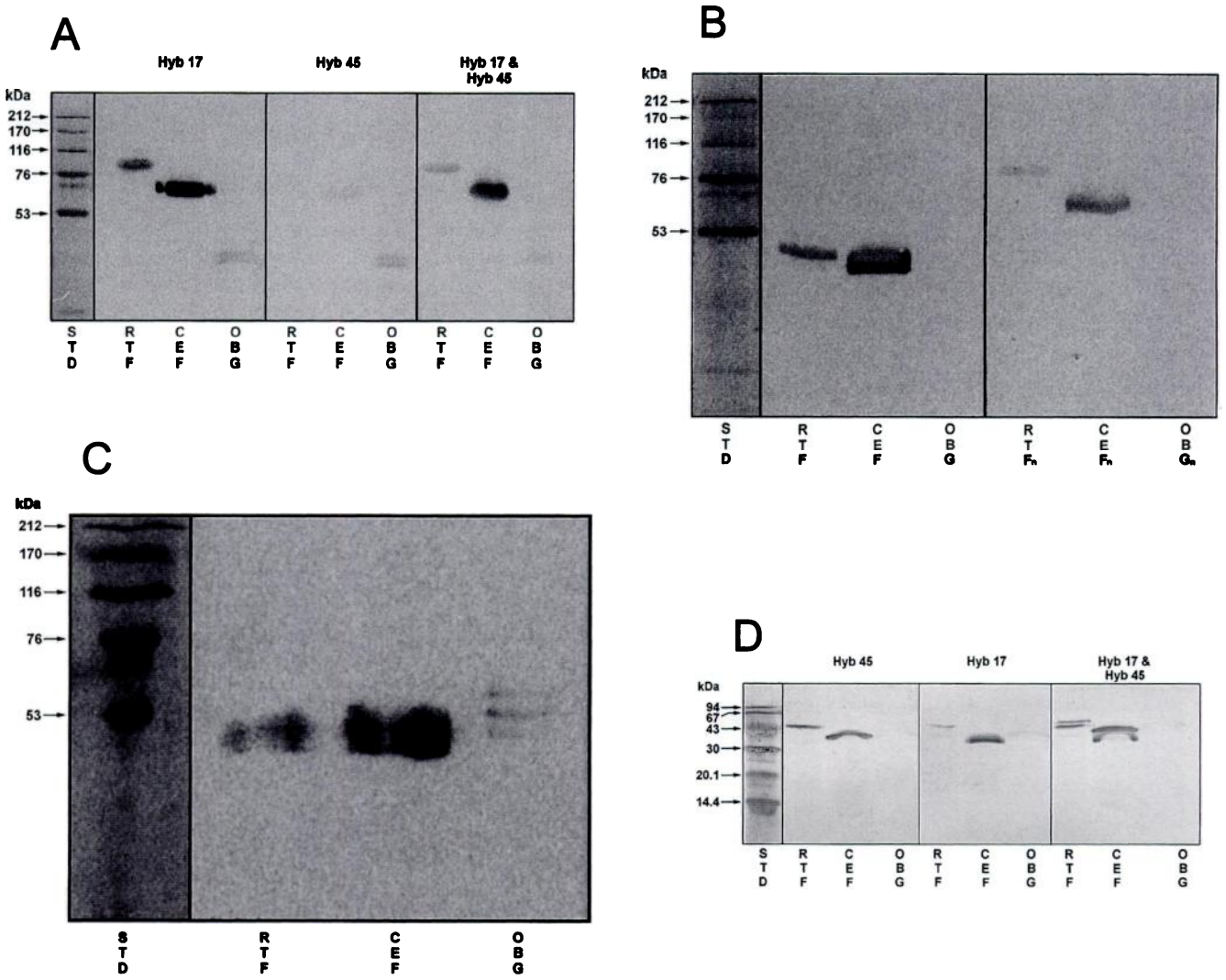

FIG. 1. (A) Immunoreactivity of unreduced bovine RTF, CEF, and OBG sperm extracts with mAbs Hyb-17 and Hyb-45 separately or together. Immobilon blots of the separated proteins were treated with anti-ram clusterin, Hyb-17 antibody (left panel), Hyb-45 antibody (middle panel), and the mixture of both antibodies (right panel) in 3% BSA/TBS. Reactivity was detected by immunoperoxidase system. STD = molecular weight standards (kDa). **(B)** Immunoreactivity of deglycosylated (left panel) or undeglycosylated (right panel) RTF, CEF, and OBG sperm extract with mAb Hyb-17. Reactivity was detected by immunoperoxidase system. STD = molecular weight standards (kDa).**(C)** Immunoreactivity of deglycosylated RTF, CEF, and OBG sperm extract with mAb Hyb-17. Reactivity was detected by immunoperoxidase system. STD = molecular weight standards (kDa). **(D)** Immunoreactivity of reduced RTF, CEF, and OBG sperm extract with mAbs Hyb-45 and Hyb-17, separately or together. Immobilon blots of the separated proteins were treated with anti-ram clusterin Hyb-45 antibody (left panel), Hyb-17 antibody (middle panel), and the mixture of Hyb-17 and Hyb-45 antibody (right panel) in 3% BSA/TBS. Reactivity was detected by immunoperoxidase system. STD = molecular weight standards (kDa). **(E)** Immunoreactivity of reduced RTF, CEF, and OBG sperm extract with polyclonal anti-β-subunit porcine clusterin Ab. Immobilon blots of the separated proteins were treated with anti-β-subunit porcine clusterin Ab in 3% BSA/TBS (right panel). A control blot (left panel) was treated with preimmune rabbit serum in 3% BSA/TBS. Reactivity was detected by the immunoperoxidase system. STD = molecular weight standards (kDa). **(F)** Immunoreactivity of reduced deglycosylated (right panel) or undeglycosylated (left panel) RTF, CEF, and sperm OBG extract with mAb Hyb-17. Control samples (undeglycosylated; RTFn, CEFn, and OBGn) were incubated for the same period of time under the same chemical conditions except for the absence of the enzyme. Reactivity was detected by the immunoperoxidase system. STD = molecular weight standards (kDa). **(G)** Immunoreactivity of deglycosylated (right panel) or undeglycosylated (left panel) bovine RTF, CEF, and OBG sperm extract with mAb Hyb-45. Reactivity was detected by the immunoperoxidase system. STD = molecular weight standards (kDa).**(H)** Immunoreactivity of deglycosylated (right panel) or undeglycosylated (left panel) bovine RTF, CEF, and OBG sperm extract with anti-β-subunit porcine clusterin Ab. Reactivity was detected by immunoperoxidase system. STD = molecular weight standards (kDa).

although the band resulting from treatment with the Hyb-45 antibody was weaker (Fig. 1A; Table 1). After deglycosylation, the Hyb-17 antibody reacted with two bands at M_rs 51 kDa and 50 kDa (Fig. 1B, C; Table 1).

In reduced samples, probing with mAb Hyb-17 caused the appearance of a broad band at $M_r \approx 38–42$ kDa (Fig. 1D; Table 2). The reactivity to the Hyb-45 and β-12 antibodies defined a narrow band at $M_r \approx 40$ kDa (Fig. 1D,

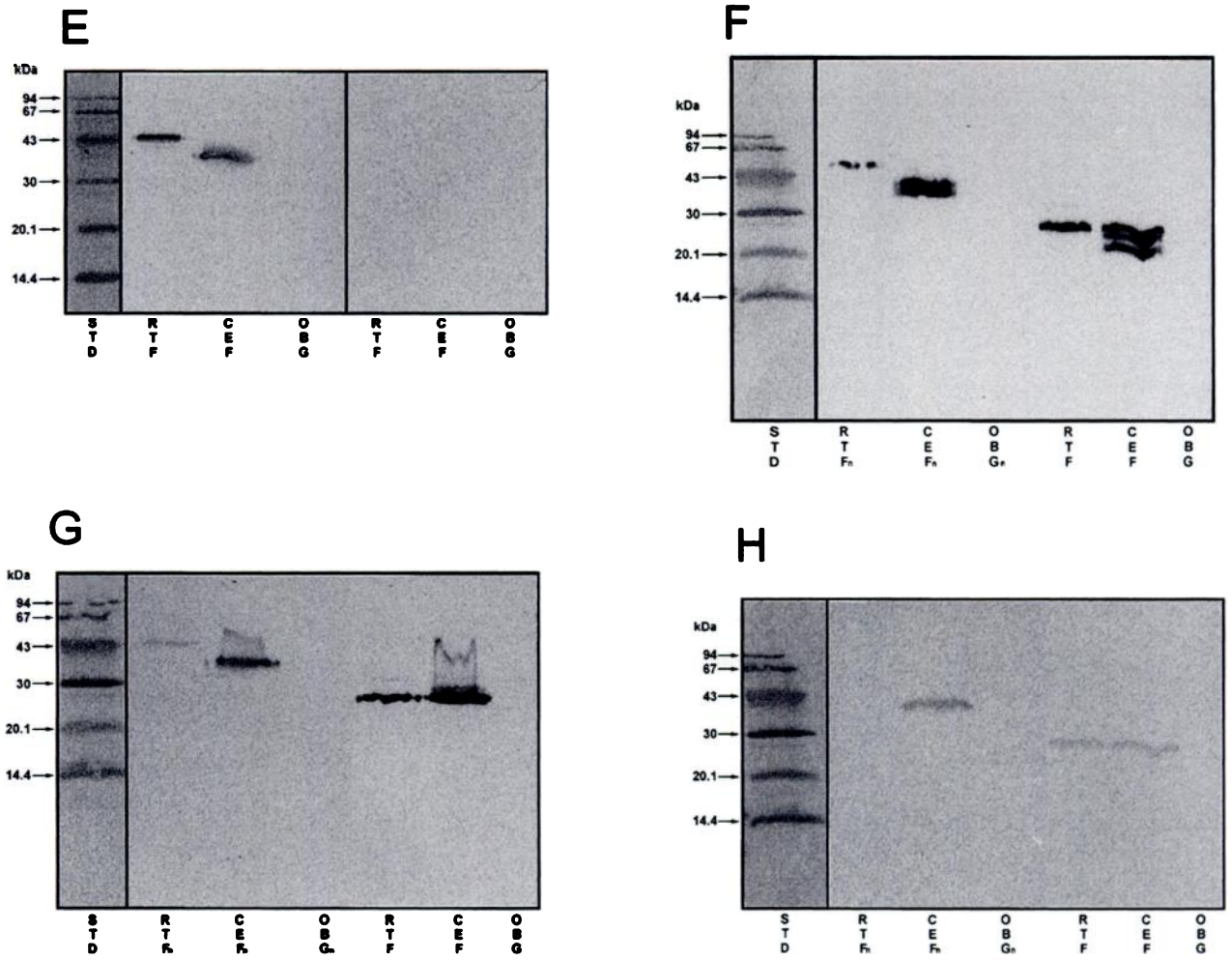

FIG. 1. *Continued*

E; Table 2). When mAbs Hyb-17 and Hyb-45 were used simultaneously, two lighter bands appeared at M_r ≈38 kDa and 39 kDa in addition to the M_r 40-kDa band (Fig. 1D; Table 2). After deglycosylation, mAb Hyb-17 detected bands at M_rs 27 kDa, 25 kDa, and 22 kDa (Fig. 1F; Table 2). Only the M_r 27-kDa band was detected with the Hyb-45 and the β-12 antibodies (Fig. 1G, H), suggesting that this peptide represented the β-subunit and that the two smaller peptides detected by Hyb-17 Ab represented variants of the clusterin α-subunit. The carbohydrate content of cauda fluid dimeric clusterin and the α- and β-subunits was similar, at approximately 32% and 37–38%, respectively.

CES—Western blot analysis of nonreduced samples of OBG sperm extracts of washed cauda epididymal sperm featured minor bands at M_rs 74 kDa, 38 kDa, and 36 kDa using mAbs Hyb-17 and Hyb-45. These bands did not show up on all blots. The M_r 74 kDa band was very weak and was difficult to reproduce photographically (Fig. 1A; Table 1). This suggests the existence of free clusterin subunits in the sperm membrane. After deglycosylation, three bands migrating at M_rs 51 kDa, 52 kDa, and 53 kDa were detected with mAb Hyb-17; these apparently resulted from deglycosylation of the M_r 74 kDa glycoprotein (Fig. 1C; Table 1).

Under reduced conditions, both mAbs Hyb-17 and Hyb-45 detected, very weakly, a band at M_r 40 kDa (Fig. 1D; Table 2), but there was no reactivity with the β-12 antibody, possibly because of low concentration of the subunits in the OBG sperm extract. After deglycosylation, there was a weak reaction at M_r 25 kDa with the Hyb-17 mAb (Fig. 1F; Table 2), but there was no visible reaction with the other antibodies. Thus, the sperm membrane dimeric clusterin appears to have approximately 30% carbohydrate content as determined under nonreduced conditions and 38% carbohydrate content on the basis of data obtained using reduced conditions. The free clusterin subunits detected in the sperm membrane may consist of two

Table 3. *Comparison of cell clustering activity*

Samples*	CCS† (1 hour after incubation)	Volume added (μl)	Protein content (μg)	Clusterin content‡ (%)	Clusterin content (μg)
CEF	1.5	1	2.1	27–28	0.6
RTF	1.5	4	8.4	8–9	0.6
OBG	1.5	8	16.8	4–5	0.8
PBS	0.0	X§	0.0	0.0	0.0

* Samples were standardized to a protein concentration of 2.1 mg/ml before addition. CEF, cauda epididymal fluid; RTF, rete testis fluid; OBG, octyl-β-D-glucopyranoside extract of cauda epididymal sperm; PBS, phosphate-buffered saline (denotes control wells, in which cells were incubated in PBS only).

† Cell-clustering score.

‡ Clusterin content was determined by densitometric analysis of Coomassie brilliant blue–stained SDS-PAGE blots.

§ PBS was added to make total test volume = 100 μl.

β-subunit isoforms that are similar to the α-subunits detected in cauda fluid (under reduced conditions) but not in RTF.

Cell-Clustering Activity and Inhibition

After a 1-hour incubation with the diluted samples, a similar degree of clustering of L-cells (score 1.5) was produced by RTF, CEF, and OBG sperm extract in the presence of approximate clusterin amounts of 0.6, 0.6, and 0.8 μg, respectively (Table 3).

Of all anticlusterin antibodies tested, only Hyb-17 inhibited clustering after 1 to 12 hours of incubation. Partial inhibition of clustering occurred with 0.5 μl antibody solution in the well (clustering score decreased from 3 to 0.5; Fig. 2A1, Table 4), while maximum inhibition was seen with 1 and 1.5 μl of the antibody solution (clustering score decreased from 3 to 0.1; Fig. 2A2, A3; Table 4). After 24 hours of incubation, cell clustering in Hyb-17-treated wells increased slightly but was always less than in the control (porcine IgG-containing) wells (Fig. 2B1–B3).

Inhibition of Cytolytic Complement Activity

Both RTF and CEF inhibited complement-induced cytolysis in a dose-dependent fashion (Fig. 3). At 50% inhibition, RTF showed a twofold higher inhibitory effect on complement activity than did cauda epididymal fluid, on a clusterin-equivalent basis. Approximately 200 μg cauda epididymal fluid clusterin equivalent was needed to achieve 50% inhibition of complement-induced hemolysis, as compared to 100 μg RTF clusterin equivalent.

Discussion

We have shown that bovine clusterin from RTF, CEF, and the sperm membrane differ in size, glycosylation, and biological potency. The M_r for dimeric RTF clusterin was found to be 94 kDa; however, the more accurate estimate by reduced conditions suggested that sum of the subunit M_rs was 85 kDa. This is only slightly larger than the CEF and sperm membrane clusterin M_rs of 74–80 kDa. However, the grossly different M_rs of the unreduced forms suggest a major conformational difference between the RTF and the CEF clusterin. Sperm membrane and CEF clusterin migrated with similar M_rs (74 kDa), which excludes the presence of extractable testicular clusterin in the sperm membrane. Additionally, using unreduced conditions, the sperm membrane extract contained a lower molecular weight doublet that was not detectable in CEF or RTF and that may consist of free clusterin subunits. Further evidence was obtained for two forms of CEF clusterin with apparently similar M_rs. The confinement of the larger clusterin isoform to RTF and of the 74 kDa isoform to CEF, together with the absence of intermediate forms in the CEF, suggests tissue-specific synthesis of the protein in the bull reproductive tract, as was suggested for the rat (Mattmueller and Hinton, 1991; Sylvester et al, 1991). Thus, in the male bovine reproductive tract, clusterin is produced in two distinct parts, the testis and the epididymis, each of which is unique for its posttranslational modifications during synthesis of the protein.

The M_r of the bovine adrenal gland clusterin (GP-III) precursor peptide was calculated to be 51.1 kDa (Palmer and Christie, 1990), which is virtually identical to the immunoblotting results presented here for the mature, deglycosylated, unreduced RTF clusterin and for two CEF clusterin fractions migrating closely together at M_r 50 and 51 kDa. The lower molecular weight protein, which is unique for CEF, may be a result of an additional tissue-specific posttranslational modification. This is the first report of 51- and 50-kDa clusterin isoforms in a native reproductive tract secretion, but the presence of the M_r 51- and 50-kDa peptides was also observed as an *in vitro* translation product of an immunopurified mRNA from cultured rat Sertoli cells (Collard and Griswold, 1987).

The relatively diffuse reactivity of CEF clusterin with Hyb-17 Ab (Fig. 1D) may be explained by the existence of three distinct peptides in deglycosylated CEF under reduced conditions (Fig. 1F), although differences in the carbohydrate moieties cannot be excluded. The three peptides consisted of one β-subunit and two α-subunits of the protein (M_rs of 27 kDa and of 25 and 22 kDa, respectively). It is possible that the 25- and 22-kDa α-subunit peptides correspond to two clusterin isoforms detected in the unreduced, deglycosylated CEF clusterin (Fig. 1B, C). Thus, two types of clusterin appear to exist in the bovine CEF, but the degree of glycosylation makes them migrate at approximately the same molecular weight (74 kDa; Fig. 1A). Since only one gene for clusterin has been detected (Palmer and Christie, 1990), it is possible

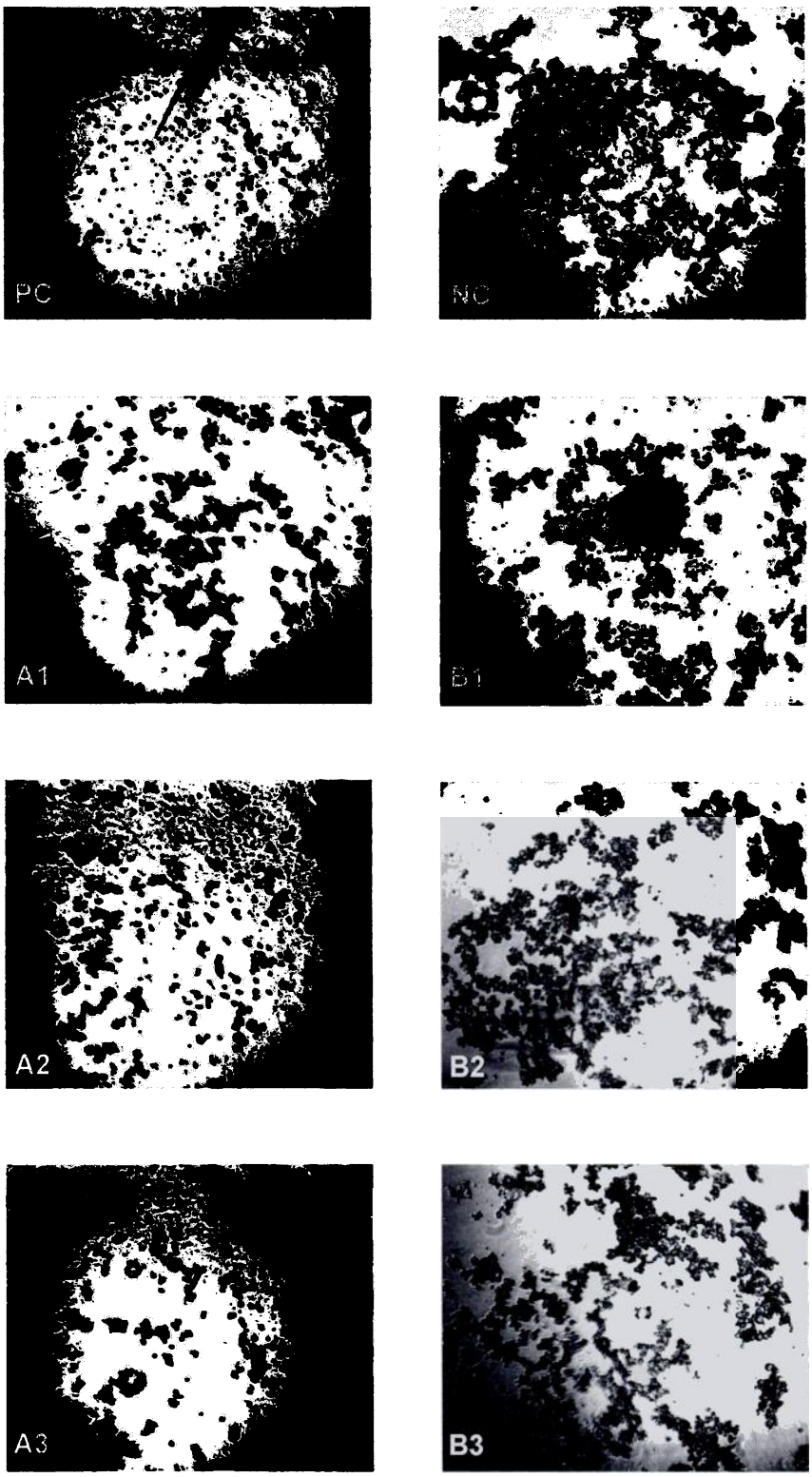

FIG. 2. Effect of Hyb-17 Ab on cell-clustering activity of CEF. Mouse fibroblast L-cells were incubated for 12 hours, in TBS only (positive control; PC); in TBS plus 0.5 μl CEF (negative control; NC); or in TBS plus 0.5 μl CEF and 0.5, 1, or 1.5 μl Hyb-17 Ab **(A1, A2, and A3)**, or 0.5, 1, and 1.5 μl pig IgG **(B1, B2, and B3)**. Partial cell-clustering inhibition (CCI) was seen at 0.5 μl Hyb-17 **(A1)** treatment. Cells appeared partially separated from each other as compared to the positive control, where cells were completely separated from each other except for spontaneous clustering. Maximum CCI was seen at 1 μl and 1.5 μl Hyb-17 **(A2 and A3)** treatment. Pig IgG **(B1, B2, and B3)** did not inhibit cell clustering.

Table 4. *Effect of Hyb-17 anti-ram clusterin antibody on cell-clustering activity of caudal epididymal fluid sample*

Antibody (μl)	Cell-clustering score (CCS) after incubating					
	1-hour incubation		12 hour incubation		24 hour incubation	
	Hyb-17	Pig IgG	Hyb-17	Pig IgG	Hyb-17	Pig IgG
0.0*	0.0	0.0	0.0	0.0	0.0	0.0
0.0†	3.0	3.0	3.0	3.0	3.0	3.0
0.5	0.5	2.0	0.5	1.5	1.5	2.5
1.0	0.1	2.5	0.1	2.0	1.5	2.0
1.5	0.1	2.0	0.1	2.0	1.5	2.0

* Positive control; cells were incubated in Tris-HCl–buffered saline (TBS) alone.

† Negative control; cells were incubated in TBS with 0.5 μl caudal epididymal fluid.

that epididymis-localized endopeptidase(s) may have cleaved the original α_1-subunit (mature M_r 39 kDa) at a specific site and generated the M_r 38 kDa, α_2-subunit.

The monoclonal Hyb-45 antibody showed greater affinity for β-subunits of the reduced bovine reproductive tract clusterin than for unreduced (native) clusterin, in which the conformation of the protein may have sometimes completely concealed the reactive sites (Fig. 1A, D, G). Conversely, Hyb-17 Ab showed less reactivity to β-subunits but reacted equally strongly to α-subunits and to native clusterin (Fig. 1A–D, F). The similarity in the binding pattern between Hyb-45 Ab and β-12 Ab provides strong evidence that the Hyb-45 Ab is specific for the β-subunit of bovine reproductive tract clusterin (Fig. 1 E, G, and H). Similarly, Murphy et al (1988) and O'Bryan et al (1994), using monoclonal anti-human clusterin antibodies, showed that some clusterin epitopes are shared by both subunits, some are subunit specific, and others are conformationally dependent on the three-dimensional structure of the dimeric form.

The detection of a doublet migrating at M_r 38 kDa and 36 kDa by both Hyb-17 and Hyb-45 Ab in the unreduced OBG extract of sperm (Fig. 1A) suggests the existence of free clusterin β-subunits on the sperm. Since an α-specific antibody was not available, the methodology used could not exclude the presence of free α-subunits. The fact that this subunit-size protein was consistently detected in sperm extracts but not in RTF or CEF, taken together with the absence of any intermediate forms, suggests that this molecule is original to the sperm, rather than a proteolytic product.

Although CEF and sperm membrane clusterin migrated similarly at M_r 74, they may not necessarily be identical. Western blot analysis showed that there was consistently a much weaker immunoreactivity with sperm OBG extracts and sometimes no reactivity. This was likely because of conformational differences between sperm membrane clusterin and extracellular clusterin from RTF or CEF, since increasing the amount of protein in the OBG lane did not improve reactivity. This suggests low accessibility of these antibodies to the sperm–clusterin binding sites, or, alternatively, that the amount of sperm membrane clusterin was low and varied among samples.

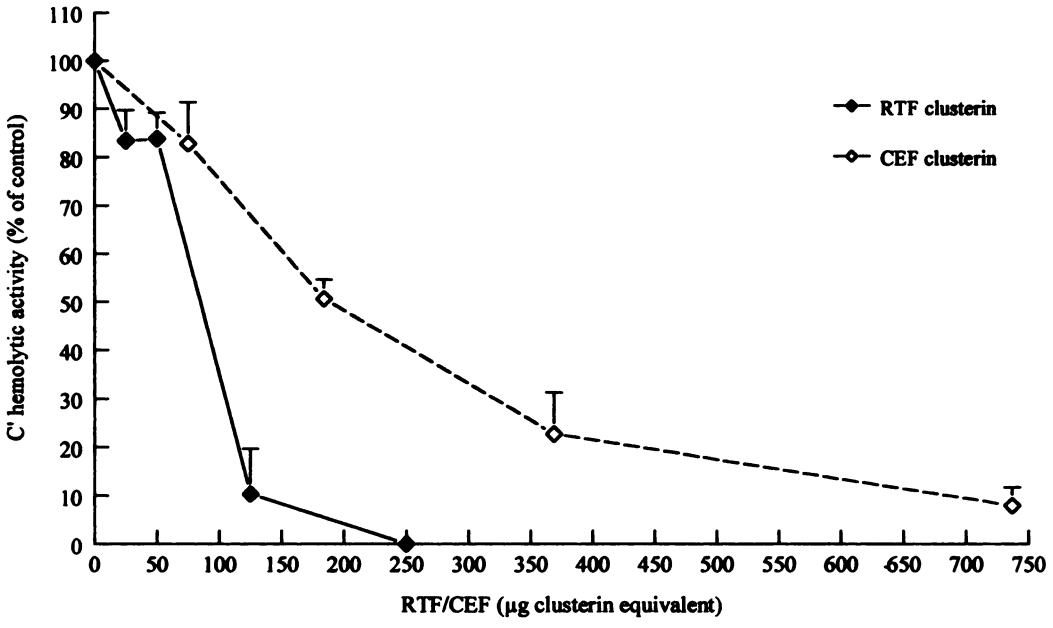

FIG. 3. The inhibitory effect of bovine RTF (RTF) and cauda epididymal fluid (CEF) on the cytolytic complement activity. CH_{50} for the pooled bovine blood plasma was determined as described in Materials and Methods. Increasing amounts of RTF (from 23.6–26.6 μg to 236–265.5 μg clusterin equivalents) and of CEF (56.7–90.7 to 567.0–907.2 μg clusterin equivalents) were added to the CH_{50} assay. Complement lytic activity was expressed as percent of CH_{50}.

A function for clusterin in the male reproductive tract has not been established with certainty. Clusterin has been implicated in the following: spermatogenesis (Sylvester et al, 1984; Collard and Griswold, 1987), protecting spermatozoa against complement attack from epididymal and uterine secretions (Jenne and Tschopp, 1989; Murphy et al, 1989); agglutination of abnormal spermatozoa (O'Bryan et al, 1994); and fertilization (O'Bryan et al, 1990). These functions may vary with the three-dimensional structure and posttranslational modification of the protein associated with the origin and/or location of the molecule. Clusterin originating from the bovine chromaffin granule membrane and from ram serum, for example, was unable to cause cell aggregation of mammalian red blood cells (Cheng et al, 1988; Palmer and Christie, 1990), whereas the bovine reproductive tract clusterin described in this study had strong cell-clustering activity. The Hyb-17 antibody caused a dose-dependent inhibition of cell clustering, which strongly suggests that CEF clusterin was responsible for this effect (Fig. 2, Table 4). Under current experimental conditions, neither anti-β-subunit porcine clusterin Ab nor Hyb-45 Ab was able to inhibit cell clustering. This suggests that more than one epitope is required for the biological activity of clusterin (Murphy et al, 1988). If clusterin mediates its function(s) via carbohydrate moieties alone or in combination with peptide chain domains, an antibody or a combination of antibodies that could simultaneously block these sites is necessary for biological studies. The slight decrease in Ab inhibition of cell clustering observed 24 hours after incubation (Table 4) could be due to Ab dissociation from the effective sites or be due to spontaneous clustering occurring after relatively long incubation times (Fritz et al, 1983).

Human seminal plasma has been shown to inhibit complement-induced cytolysis (Tarter and Alexander, 1984). Clusterin is a major constituent of human seminal plasma (O'Bryan et al, 1990), and clusterin concentrations in bovine RTF and CEF were estimated to be considerable (up to 7.5 mg/ml in CEF). We attribute the cytolytic complement inhibitory effect of these secretions mainly to clusterin, although other, undescribed factors cannot be ruled out. Monoclonal Hyb-17 antibody, which inhibited cell clustering, was unable to neutralize the complement cytolysis inhibition effect of CEF and RTF. However, this does not rule out this role of clusterin in these fluids because it appeared in the present study that Hyb-17 antibody is directed against peptide epitopes, on the basis of the findings that the reactivity was present and even much stronger after deglycosylation. In agreement with reports by Murphy et al (1989) and Jenne and Tschopp (1989), our results suggest that reproductive-tract clusterin isoforms may actively prevent complement-mediated sperm destruction. Human blood and seminal clusterin have been shown to be comparably efficient in inhibiting assembly of the membrane attack complex proteins during cytolytic complement activation (Jenne and Tschopp, 1989; Kirszbaum et al, 1989; Murphy et al, 1989; O'Bryan et al, 1990). Inhibition of the cytolytic activity of purified nascent C5b–7 complexes occurred at 1.5 μg/ml of purified blood plasma clusterin (Jenne and Tschopp, 1989). In the present study, 50% complement-induced cytolysis inhibition by CEF and RTF was noticed at predicted clusterin values of 200 and 100 μg per treatment, respectively. Interestingly, RTF clusterin equivalent was twofold more potent than CEF clusterin equivalent in this effect. This difference in activity is likely due to the observed difference in carbohydrate content or folding of the protein, as evidenced by much higher relative mobility of RTF clusterin than CEF clusterin under nonreduced conditions. The higher concentration of clusterin in CEF versus RTF may be a natural compensation for the difference in activity between the two isoforms, ensuring that inhibition of complement-induced cytolysis is maintained in the lower part of the male reproductive tract. The high concentration of clusterin in both RTF and CEF may be necessary because the blood testis barrier is structurally and functionally incomplete in the rete testis (Tung et al, 1971; Dym, 1976).

References

Bandyk MG, Sawczuk IS, Olsson CA, Katz AE, Buttyan R. Characterization of the products of a gene expressed during androgen-programmed cell death and their potential use as a marker of urogenital injury. *J Urol* 1990;143:407–413.

Bettuzi S, Hiipakka RA, Gilna P, Liao S. Identification of an androgen-repressed mRNA in rat ventral prostate as coding for sulphated glycoprotein 2 by cDNA cloning and sequence analysis. *Biochem J* 1989; 257:293–296.

Blaschuk O, Burdzy K, Fritz IB. Purification and characterization of a cell-aggregating factor (Clusterin), the major glycoprotein in ram rete testis fluid. *J Biol Chem* 1983;25:7714–7720.

Brown TL, Moulton BC, Witte DP, Swertfeger DK, Harmony JAK. Apolipoprotein J/clusterin expression defines distinct stages of blastocyst implantation in the mouse uterus. *Biol Reprod* 1996;55:740–744.

Buttyan R, Olsson CA, Pintar J, Chang C, Bandyk M, Ng P-Y, Sawczuk IS. Induction of the TRPM-2 gene in cells undergoing programmed cell death. *Mol Cell Biol* 1989;9:3473–3481.

Cheng CY, Mathur PP, Grima J. Structural analysis of clusterin and its subunits in ram rete testis fluid. *Biochemistry* 1988;27:4079–4088.

Collard MW, Griswold MD. Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. *Biochemistry* 1987; 26:3297–3303.

Crabo B. Studies on the composition of epididymal content in bulls and boars. *Acta Vet Scand (Suppl)* 1965;5:1–94.

de Silva HV, Harmony JAK, Stuart WD, Gil CM, Robbins J. Apolipoprotein J: structure and tissue distribution. *Biochemistry* 1990;29: 5380–5389.

Diemer V, Hoyle M, Baglioni C, Millis AJT. Expression of porcine complement cytolysis inhibitor mRNA in cultured aortic smooth muscle cells changes during differentiation in vitro. *J Biol Chem* 1992;267: 5257–5264.

Dym, M. The mammalian rete testis—a morphological examination. *Anat Rec/* 1976;186:493–524.

Fischer-Colbrie R, Schachinger M, Zangerle R, Winkler H. Dopamine β-hydroxylase and other glycoproteins from the soluble content and the membranes of adrenal chromaffin granules: isolation and carbohydrate analysis. *J Neurochem* 1982;38:725–732.

Fischer-Colbrie R, Zangerle R, Frischenschlager I, Weber A, Winkler H. Isolation and immunological characterization of a glycoprotein from adrenal chromaffin granules. *J Neurochem* 1984;42:1008–1016.

Fritz IB, Burdzy K, Setchell B, Blaschuk O. Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interaction in vitro. *Biol Reprod* 1983;28:1173–1188.

Gavine FS, Pryde JG, Deanne DL, Abbs DK. Glycoproteins of the chromaffin granule membrane: separation by two-dimensional electrophoresis and identification by lectin binding. *J Neurochem* 1984;43:1243–1252.

Geissler D, Martinek A, Margolis RU, Margolis RK, Skrivanek JA, Ledeen R, König P, Winkler H. Composition and biogenesis of complex carbohydrates of ox adrenal chromaffin granules. *Neuroscience* 1977; 2:685–693.

Harding MA, Chadwick LJ, Gattone VH, Calvet JP. The SGP-2 gene is developmentally regulated in the mouse kidney and abnormally expressed in collecting duct cysts in polycystic kidney disease. *Dev Biol* 1991;146:483–490.

Jenne DE, Tschopp J. Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc Natl Acad Sci USA* 1989;86:7123–7127.

Kirszbaum L, Sharpe JA, Murphy B, d'Apice, AJF, Classon B, Hudson P, Walker ID. Molecular cloning and characterization of the novel human complement-associated protein, SP-40,40: a link between the complement and reproductive system. *EMBO J* 1989;8:711–718.

Kissinger C, Skinner MK, Griswold MD. Analysis of Sertoli-cell secreted proteins by two dimensional electrophoresis. *Biol Reprod* 1982;27: 233–240.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

Laslop A, Steiner H-J, Egger C, Wolkersdorfer M, Capelari S, Hogue-Angeletti R, Erickson JD, Fischer-Colbrie R, Winkler H. Glycoprotein III (clusterin, sulfated glycoprotein 2) in endocrine, nervous, and other tissues: immunochemical characterization, subcellular localization and regulation of biosynthesis. *J Neurochem* 1993;61:1498–1505.

Lee C, Sensibar JA. Proteins of the rat prostate. II. Synthesis of new proteins in the ventral lobe during castration-induced regression. *J Urol* 1987;138:903–908.

Leger JG, Montpetit ML, Tenniswood MP. Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. *Biochem Biophys Res Commun* 1987;147:196–203.

Mattmueller DO, Hinton BT. In vivo secretion and association of clusterin (SGP-2) in luminal fluid with spermatozoa in the rat testis and epididymis. *Mol Reprod Dev* 1991;30:62–69.

Mayer MM. Complement and complement fixation. In: Kabat EA, Mayer MM, eds. *Immunochemical Immunochemistry.* 2nd ed. Springfield Illinois: Charles C. Thomas publisher; 1961:133–240.

Murphy BF, Kirszbaum L, Walker ID, d'Apice AJF. SP-40,40, a newly identified normal human serum protein found in the SC5b–9 complex of complement and in the immune deposits in glomerulonephritis. *J Clin Investig* 1988;81:1858–1864.

Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID, d'Apice AJF. SP-40,40 is an inhibitor of C5b–6 initiated hemolysis. *Int Immunol* 1989;1:551–554.

Nicander L. Studies on the regional histology and cytochemistry of the ductus epididymis in stallions, rams and bulls. *Acta Morph Neerl-Scand* 1958;I:337–362.

O'Bryan MK, Baker HWG, Saunders JR, Kirszbaum L, Walker ID, Hudson P, Liu DY, Glew MD, d'Apice AJF, Murphy BF. Human seminal clusterin (SP-40,40) isolation and Characterization. *J Clin Investig* 1990;85:1477–1486.

O'Bryan MK, Cheema SS, Bartlett PF, Murphy BF, Pearse MJ. Clusterin levels increase during neuronal development. *J Neurobiol* 1993;24: 421–432.

O'Bryan MK, Murphy BF, Liu DY, Clarke GN, Baker HWG. The use of anticlusterin monoclonal antibodies for the combined assessment of human sperm morphology and acrosome integrity. *Hum Reprod* 1994; 8:1490–1496.

Palmer DJ, Christie DL. The primary structure of glycoprotein III from bovine adrenal medullary chromaffin granules. Sequence similarity with human serum protein 40,40 and rat Sertoli cell glycoprotein 2. *J Biol Chem* 1990;265:6617–6623.

Patzak A, Winkler H. Exocytotic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling. *J Cell Biol* 1986;102:510–115.

Samper JC, Hamilton DW, Pryor JL, Loseth KJ, Troedsson MHT, Crabo BG. Mechanism of Sephadex trapping of capacitated stallion spermatozoa. *Biol Reprod Mono* 1995;1:729–737.

Sanders ML, Farris JA, Strauch AR, Foster DN, Crabo BG. α-Specific anti-clusterin antibody: development and characterization. *Mol Biol Cell (Suppl)* 1993;4:109a.

Sylvester SR, Morales C, Oko R, Griswold MD. Localization of sulfated glycoprotein-2 (Clusterin) on spermatozoa and in the reproductive tract of the male rat. *Biol Reprod* 1991;45:195–207.

Sylvester SR, Skinner MK, Griswold MD. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biol Reprod* 1984;31:1087–1101.

Tanaka S, Kitamura F, Suzuki T. Studies on the hemolytic activity of the classical and alternative pathway of complement in various animal species. *Complement* 1987;4:33–41.

Tarter TH, Alexander NJ. Complement-inhibiting activity of seminal plasma. *Am J Reprod Immunol* 1984;6:28–32.

Thomas-Salgar S, Millis AJT. Clusterin expression in differentiating smooth muscle cells. *J Biol Chem* 1994;269:17879–17885.

Towbin H, Staehelen T, Gordon J. Electrophoretic transfer of proteins of polyacrylamide gels to nitrocellulose sheets. *Proc Natl Acad Sci USA* 1979;76:4350–4354.

Tung KSK, Unanue E, Dixon F. Pathogenesis of experimental allergy orchitis. II the role of antibody. *J Immunol* 1971;106:1463–1472.