

# Human Seminal Clusterin (SP-40,40)

## Isolation and Characterization

M. K. O'Bryan,<sup>\*\*</sup> H. W. G. Baker,<sup>\*\*</sup> J. R. Saunders,<sup>\*\*</sup> L. Kirszbaum,<sup>†</sup> I. D. Walker,<sup>‡</sup>  
P. Hudson,<sup>||</sup> D. Y. Liu,<sup>§</sup> M. D. Glew,<sup>‡</sup> A. J. F. d'Apice,<sup>\*</sup> and B. F. Murphy<sup>\*\*</sup>

<sup>\*</sup>St. Vincent's Hospital, <sup>†</sup>University of Melbourne, <sup>§</sup>The Royal Women's Hospital,

<sup>||</sup>Commonwealth Scientific and Industrial Research Organization, Biotechnology Division, Melbourne, Australia

### Abstract

Molecular cloning of the human complement inhibitor SP-40,40, has revealed strong homology to a major rat and ram Sertoli cell product, sulfated glycoprotein-2, known also as clusterin. This study reports the purification and characterization of human seminal clusterin. Two-dimensional gel electrophoresis revealed charge differences between clusterin purified from semen and the serum-derived material. Both preparations demonstrate comparable hemagglutination (clustering) activity and inhibition of C5b-6 initiated hemolysis.

The average clusterin concentration in normal seminal plasma is considerably higher than that found in serum. Mean seminal plasma clusterin concentrations were significantly lower in azoospermia caused by obstruction or seminiferous tubule failure than with oligospermia or normospermia. Only men with vasal agenesis had undetectable seminal clusterin, suggesting that some of the seminal clusterin is produced by the seminal vesicles.

Immunofluorescence of human spermatozoa revealed that clusterin was detected on 10% of spermatozoa, predominantly those that were immature or had abnormal morphology. A pilot study of 25 patients suggests that seminal clusterin concentration, together with sperm motility and morphology, is correlated with the fertilization rate in vitro. The function of seminal clusterin is unknown. Its extensive distribution in the male genital tract and its high concentration in seminal plasma suggests an important role in male fertility. (*J. Clin. Invest.* 1990. 85:1477-1486.) Sertoli cell • semen • complement • infertility • in vitro fertilization

### Introduction

SP-40,40 is a normal human serum protein present in a concentration of 35-105 µg/ml. It was initially identified in human glomerular immune deposits and has been shown to be a component of the fluid phase activated SC5b-9 terminal complement complex (1). SP-40,40 is a glycoprotein of *M<sub>r</sub>* 80 kD comprising two distinct 40-kD polypeptide chains. It shows marked similarity, both physicochemically and in immunohistological distribution, to the single chain, 80 kD, S-protein, an additional component of the SC5b-9 complex.

Address reprint requests to Dr. Murphy, Renal Unit, St. Vincent's Hospital, Fitzroy, Melbourne 3065, Australia.

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Like S-protein, SP-40,40 inhibits C5b-6 initiated complement hemolysis (2) and it is likely that both proteins combine with nascent C5b-7 preventing membrane insertion and cell lysis.

Cloning and sequencing of SP-40,40 cDNA (3) from a human liver library, revealed a strong sequence homology with a major rat Sertoli cell product, sulfated glycoprotein-2, known also as clusterin in the rat and the ram (4). SP-40,40 has been identified in Western immunoblots of human seminal plasma (3) and, for reasons that will be described later, the name clusterin will be used to refer to human serum and seminal SP-40,40 hereafter.

In this study we sought to purify human seminal clusterin and to determine its physicochemical and functional properties. In addition, the identification of a new major human seminal protein raised the possibility that determination of seminal plasma clusterin concentration could be of value in the assessment and diagnosis of male infertility. Normal and abnormal human seminal plasma samples were assayed to determine if any correlation with the cause of infertility or with the success of fertilization in vitro could be identified.

### Methods

**Seminal plasma.** To minimize autolysis, seminal plasma samples used to purify human seminal clusterin were collected as follows. A fresh ejaculate was diluted and mixed well in 10 vol of ice cold 0.1 M phosphate-buffered saline (PBS) containing 1% (wt/vol) aprotinin (Sigma Chemical Co., St. Louis, MO) and 10 mM iodoacetamide (Sigma). The sample was centrifuged at 10,000 g (4°C) for 40 min and the supernatant used immediately for affinity chromatography.

For determination of seminal plasma clusterin concentrations, normal and abnormal semen samples were obtained from fertile semen donors or from men being treated for infertility.

Additional seminal plasma samples were obtained from 25 patients undergoing in vitro fertilization (IVF)<sup>1</sup> treatment; these samples were obtained from the semen remaining after preparation of sperm for IVF. The IVF procedure involved induction of follicular development with sequential clomiphene citrate and human menopausal gonadotrophin. Human chorionic gonadotrophin was then administered and oocyte collection performed by vaginal ultrasound-guided aspiration 34 to 36 h after the chorionic gonadotrophin or the estimated time of commencement of a spontaneous luteinizing hormone surge. Semen was collected by masturbation 2 h before the expected time of insemination and the sperm prepared by the swim up technique. Approximately 100,000 motile spermatozoa were added to each oocyte in 1 ml of culture medium in a plastic multiwell tray. After the mechanical removal of the cumulus if necessary, fertilization was assessed microscopically by the appearance of pronuclei 18-20 h later. Two to four cell stage pre-embryos were transferred to the uterus 40-45 h after insemination. Oocytes that had apparently failed to become fertilized were reexamined up to 60 h after insemination before concluding that fertilization had not occurred.

1. Abbreviations used in this paper: IVF, in vitro fertilization.

Semen samples were analyzed according to the World Health Organization criteria (5) and classified as follows: normal, sperm concentration  $\geq 20 \times 10^6/\text{ml}$ , progressive motility  $\geq 40\%$ , normal morphology  $\geq 30\%$ ; asthenospermia, sperm concentration  $\geq 20 \times 10^6/\text{ml}$ , progressive motility  $< 40\%$ ; teratospermia, sperm concentration  $\geq 20 \times 10^6/\text{ml}$ , progressive motility  $> 40\%$ , normal morphology  $< 30\%$ ; moderate oligospermia, sperm concentration  $1-20 \times 10^6$ ; severe oligospermia, sperm concentration  $< 1 \times 10^6$ ; azoospermia, no sperm seen in the centrifuged deposit. Causes of azoospermia were primary seminiferous tubule failure, diagnosed by high serum FSH levels or abnormal testicular biopsies, secondary seminiferous tubule failure from gonadotrophin deficiency or suppression, or genital tract obstruction including vasal agenesis, vasectomy, and epididymal obstruction. Seminal plasma fructose concentrations were determined as previously described (5). In all cases semen samples had been centrifuged within 4 h of collection and seminal plasma stored at  $-80^\circ\text{C}$ .

To determine the stability of immunoreactive clusterin in semen, repeated measurements of clusterin concentration were made on two ejaculates maintained at room temperature for 1, 2, 4, and 6 h after ejaculation before centrifugation and freezing of seminal plasma.

A sample of seminal vesicle fluid, obtained from a postmortem specimen within 5 h of death, was also obtained for the measurement of clusterin concentration.

**Affinity purification of clusterin.** Serum derived clusterin was affinity purified from fresh normal human serum as previously described (1). Seminal clusterin was also purified by affinity chromatography using an affinity matrix containing the anti-clusterin MAb G7 prepared as described (1). 20 ml of seminal plasma (diluted 1:10 in cold PBS) was passed over a 10-ml G7 affinity column at  $4^\circ\text{C}$ . The column was then washed with 1 liter of cold PBS and eluted with 10 ml 0.2 M glycine HCl, pH 2.6. The eluate was dialysed against distilled water, lyophilized and stored at  $4^\circ\text{C}$  until needed.

**Two-dimensional polyacrylamide gel electrophoresis.** Two-dimensional electrophoresis was performed as described by O'Farrell (6). Isoelectric focusing was performed in 1 mm polyacrylamide tube gels with ampholines of pI range 3.0 to 6.0 (Pharmacia Fine Chemicals, Piscataway, NJ). The SDS-PAGE second dimension was performed using 10% (wt/vol) acrylamide gels. The samples were analyzed without reduction and protein was stained with Coomassie blue.

**Protein sequencing.** Samples of purified clusterin were reduced, carboxamidomethylated, acetone precipitated, and subjected to automated Edman degradation using a protein sequencer (model 471; Applied Biosystems, Foster City, CA) protein sequencer. PTH amino acids were chromatographically resolved using a Zorbax Bioseries PTH column (Dupont, Australia).

**"Clustering" (hemagglutination) assay.** This method was adapted from that of Fritz et al. (7). Freshly drawn human blood was defibrinated by agitation in the presence of glass beads and the erythrocytes washed three times with PBS. Erythrocytes were then diluted with PBS to a final concentration of  $1 \times 10^5$  cells/ml. For some experiments rabbit, sheep and guinea pig erythrocytes were similarly prepared.

Assays were performed in U-shaped wells of a polyvinylchloride microtitration plate (Flow Laboratories, McLean, VA). The required amount of seminal or serum clusterin in PBS was placed in each well and the volume adjusted to 100  $\mu\text{l}$  with PBS. 25  $\mu\text{l}$  of the erythrocyte suspension was then added to each well and the plates incubated at  $37^\circ\text{C}$  for 20 h, with occasional agitation. Clustering was assessed by inverted phase-contrast microscopy.

**Inhibition of C5b-6-initiated hemolysis.** The effect of seminal clusterin on C5b-6 initiated reactive hemolysis was assessed as previously described (2). Briefly, 0.2 ml of 10% guinea pig erythrocytes were incubated for 10 min with from 1 to 10  $\mu\text{g}$  of clusterin, 100 ng of C5b-6 and excess C7. Lysis was developed by the addition of 10  $\mu\text{l}$  fresh human serum containing 10 mM EDTA and incubation for a further 15 min at room temperature. Lysis was stopped by the addition of cold NaCl, unlysed cells were removed by centrifugation, and the free hemoglobin was measured by determination of the optical density at 541 nm. The median percentage lysis from triplicate determinations was calculated by reference to 100% lysed cells in water.

**Enzyme-linked immunosorbent assay (ELISA) for determination of SP-40,40 levels in seminal plasma.** The dual MAb sandwich ELISA system was used as previously described for the measurement of serum clusterin (1). Seminal plasma samples were thawed, centrifuged (5,000 g, 5 min) to remove aggregates, and then diluted 1:300 in PBS containing 0.1% Tween 20 (Bio-Rad Laboratories, Richmond, CA) before assay. ELISA plates were precoated with the E5 MAb as the capture antibody and alkaline phosphatase (Sigma Chemical Co.) conjugated G7 MAb was used for detection (1). Serial dilutions of purified serum derived clusterin were assayed simultaneously to provide standard curves for each ELISA plate. In the assay system used, the lower limit of detection of clusterin was 25  $\mu\text{g}/\text{ml}$  and the upper limit of quantitation was 20 mg/ml. Repeated measurements yielded an intraassay coefficient of variation of 7% and an interassay coefficient of variation of 11%.

**Immunofluorescence studies of spermatozoa.** These studies were performed to determine if clusterin was present on the surface of spermatozoa. Fresh semen (0.5 ml) was diluted to 10 ml in Tyrode's solution (5) (Commonwealth Serum Laboratories, Melbourne, Australia) containing 0.2% wt/vol bovine serum albumin. The spermatozoa were centrifuged (600 g for 10 min) and resuspended in the same buffer. After further centrifugation the spermatozoa were resuspended in 0.1 ml of the buffer and smeared onto glass slides that were then air dried. Slides were incubated for 30 min with the G7 anti-clusterin MAb (1) (100  $\mu\text{g}/\text{ml}$  in Tyrode's/BSA buffer), washed in distilled water and then incubated for a further 30 min with fluorescein-conjugated rabbit antibody to mouse immunoglobulins (Dako, Copenhagen, Denmark) diluted 1:20. After further washing the smears were covered with cover slips and examined at  $\times 400$  or  $\times 1,000$  using an epifluorescence microscope (Dialux 20; Leitz, Wetzlar, West Germany). Each microscopic field was also examined and photographed using phase-contrast illumination to determine the total number and morphology of the spermatozoa present.

These experiments were repeated using the following alternative antibody labeling technique which preserved sperm viability.  $5 \times 10^6$  washed spermatozoa were incubated in a solution of G7 MAb (100  $\mu\text{g}/\text{ml}$  in Tyrode's/BSA buffer), washed (Tyrode's/BSA buffer) and then incubated in a 1:20 dilution of the fluorescein-conjugated rabbit antibody to mouse immunoglobulins. Sperm were again washed and mounted as a wet preparation in Tyrode's/BSA buffer before microscopic examination.

**Statistical analysis.** Seminal plasma clusterin concentrations were normalized by square root transformation (8) and the significance of group differences determined by analysis of variance.

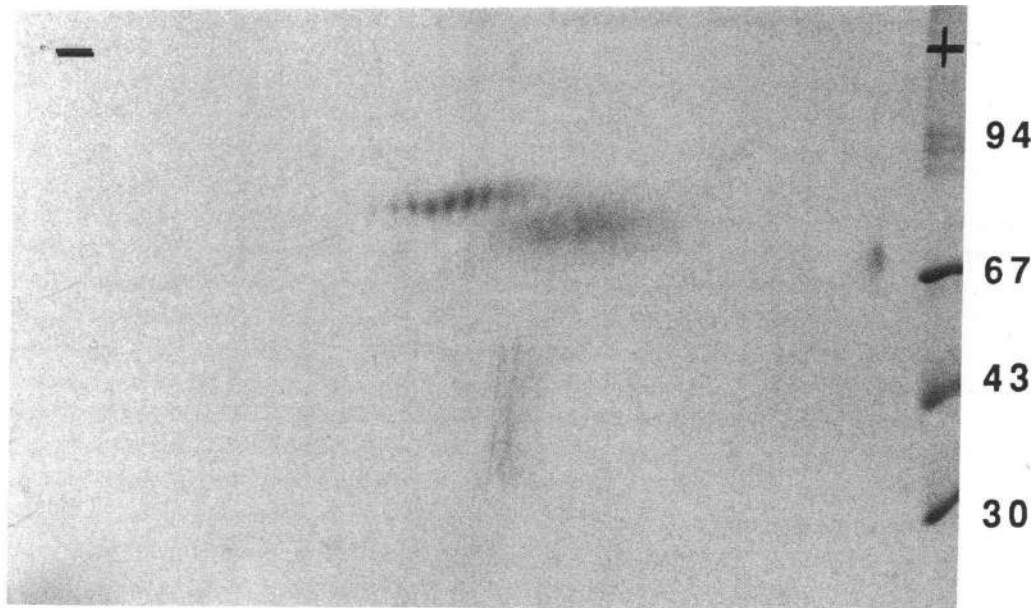
Maximum likelihood logistic regression analysis was used, as previously described (9), to determine which semen factors were related to the probability of fertilization of oocytes in vitro. Covariate adjustment was used for two missing sperm morphology measurements.

## Results

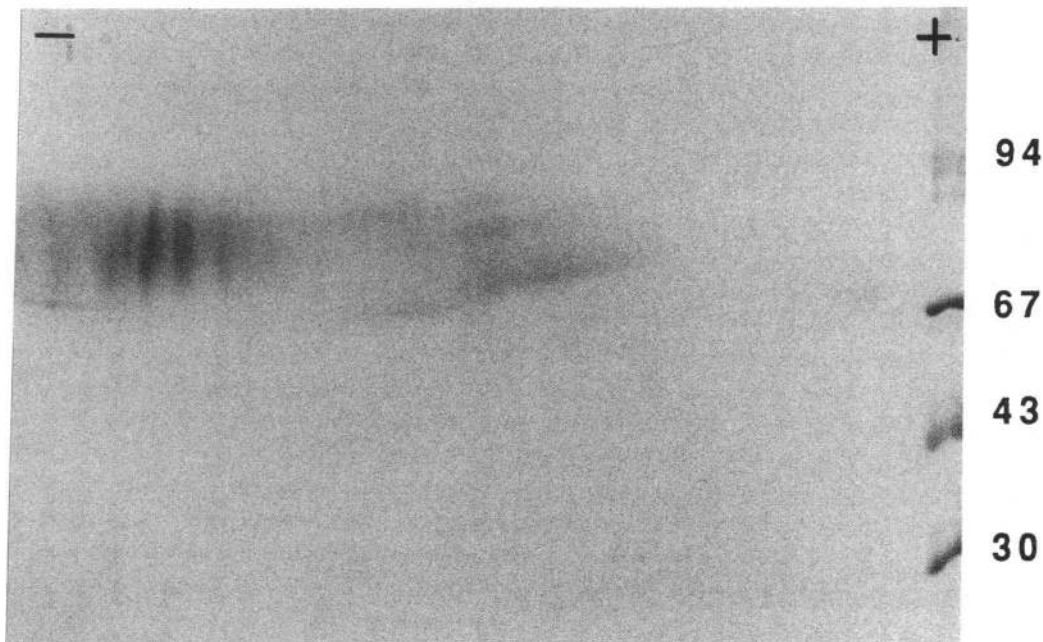
**Biochemical comparison of serum and semen-derived clusterin.** Two-dimensional PAGE revealed a complex pattern of stained protein species with both preparations of clusterin (Fig. 1). The seminal and serum clusterin patterns were clearly different. The seminal clusterin pattern extended over a broader pI range and was also consistently more diffuse despite attempts to minimize proteolysis after the collection of semen.

To investigate whether proteolysis contributed to the tissue-specific electrophoretic differences in clusterin, purified semen-derived clusterin was subjected to amino terminal sequence analysis. The  $\text{NH}_2$ -terminal sequence of seminal clusterin produced a high background in addition to the predicted amino terminal sequences of the  $\alpha$  and  $\beta$  chains (3). Analysis of the background signals revealed an additional sequence corresponding to residues 175-181 within the cDNA sequence and probably represents a major site of proteolytic cleavage.

Additional sequencing was performed with seminal clus-



**Serum Clusterin**



**Seminal Clusterin**

*Figure 1. Two-dimensional PAGE analysis of ~ 50 µg of serum-derived clusterin and 100 µg seminal clusterin. The molecular weight marker proteins are on the right of each gel with their migration (kD). Gels were stained with Coomassie blue.*

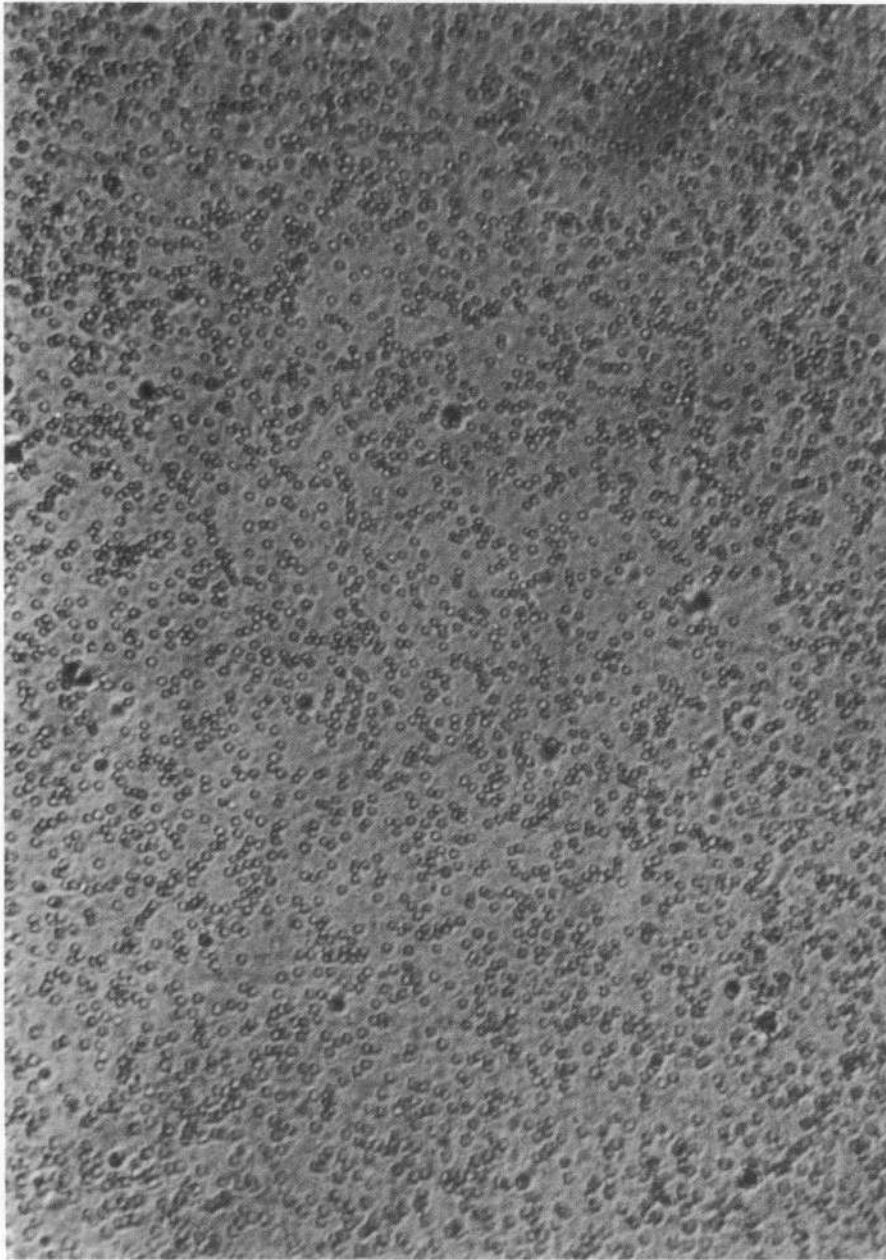
terin purified from seminal plasma which was collected after semen had been kept for 4 h at room temperature. The background was indistinguishable from that obtained with freshly processed clusterin protected by protease inhibitors.

*Seminal and serum clusterin produce erythroagglutination (clustering).* Seminal clusterin produced erythrocyte clustering as described with ram clusterin (7) (Fig. 2). Clustering was produced at a minimum clusterin concentration of 5 µg/ml and was not human ABO blood group or red cell species specific; rabbit, sheep, and guinea pig erythrocytes exhibited comparable clustering.

Human serum derived clusterin was also tested in this assay and produced clustering at a minimum concentration comparable to the seminal form (10 µg/ml).

*Seminal clusterin inhibits C5b-6 initiated hemolysis.* Fig. 3 shows the effect on hemolysis of the addition of clusterin of both seminal and serum origin to C5b-6 before addition of C7. Serum clusterin inhibits hemolysis, in a dose-dependent manner, as previously described (2). Seminal clusterin also inhibits hemolysis and appears to be comparably potent to the serum form.

*Clusterin concentration in normal seminal plasma.* Seminal clusterin concentration in samples from 16 normal subjects showed a wide range from 2 to 15 mg/ml (Fig. 4). Repeated measurements of two seminal plasma samples frozen 1, 2, 4, and 6 h postejaculation showed no significant fall in seminal plasma clusterin concentration, indicating that, at least immunoreactive seminal clusterin is stable.



*Figure 2.* Erythroagglutination (clustering) produced by seminal clusterin. A photomicrograph of a control well is shown on the left with no aggregation and on the right the effect of 20 µg affinity purified seminal clusterin is shown with dense clustering of the human erythrocytes. Similar results were seen with serum derived clusterin (not shown).

*Clusterin concentration in abnormal seminal plasma samples.* Fig. 4 also shows the results of determination of clusterin concentration in abnormal seminal plasma samples obtained from 160 patients with various causes of infertility.

Azoospermic individuals had significantly lower mean seminal plasma clusterin concentrations than normals although there was considerable overlap between the groups. All patients with congenital absence of the vasa had undetectable seminal clusterin. This contrasts with the vasectomized patients who were similar to the other azoospermics. There was a significant correlation in azoospermic individuals between seminal plasma fructose concentration and clusterin concentration (Spearman nonparametric correlation,  $r = 0.51$ ,  $P < 0.009$ ).

Seminal plasma clusterin concentrations of oligospermic men were not significantly different from those of normal men (Fig. 4) and there was no correlation in nonazoospermic men

between seminal plasma clusterin and fructose concentrations ( $r = 0.14$ ,  $P < 0.26$ ).

Three additional individual samples were of considerable interest. A sample of fluid taken directly from a seminal vesicle at autopsy from a normal subject had a clusterin concentration of 9.9 mg/ml. A seminal plasma sample from a normospermic man with seminal vesicle obstruction or malfunction (low semen volume and fructose, data not shown) showed a clusterin concentration within the normal range whereas a sample from a patient with ejaculatory duct obstruction did not contain detectable clusterin by ELISA.

*Clusterin concentrations in semen used for in vitro fertilization.* In the 25 semen samples used for in vitro fertilization, logistic regression analysis was used to determine whether seminal clusterin concentration, when considered together with other parameters of semen analysis by multiple regression, was predictive of successful fertilization. Table I shows the results

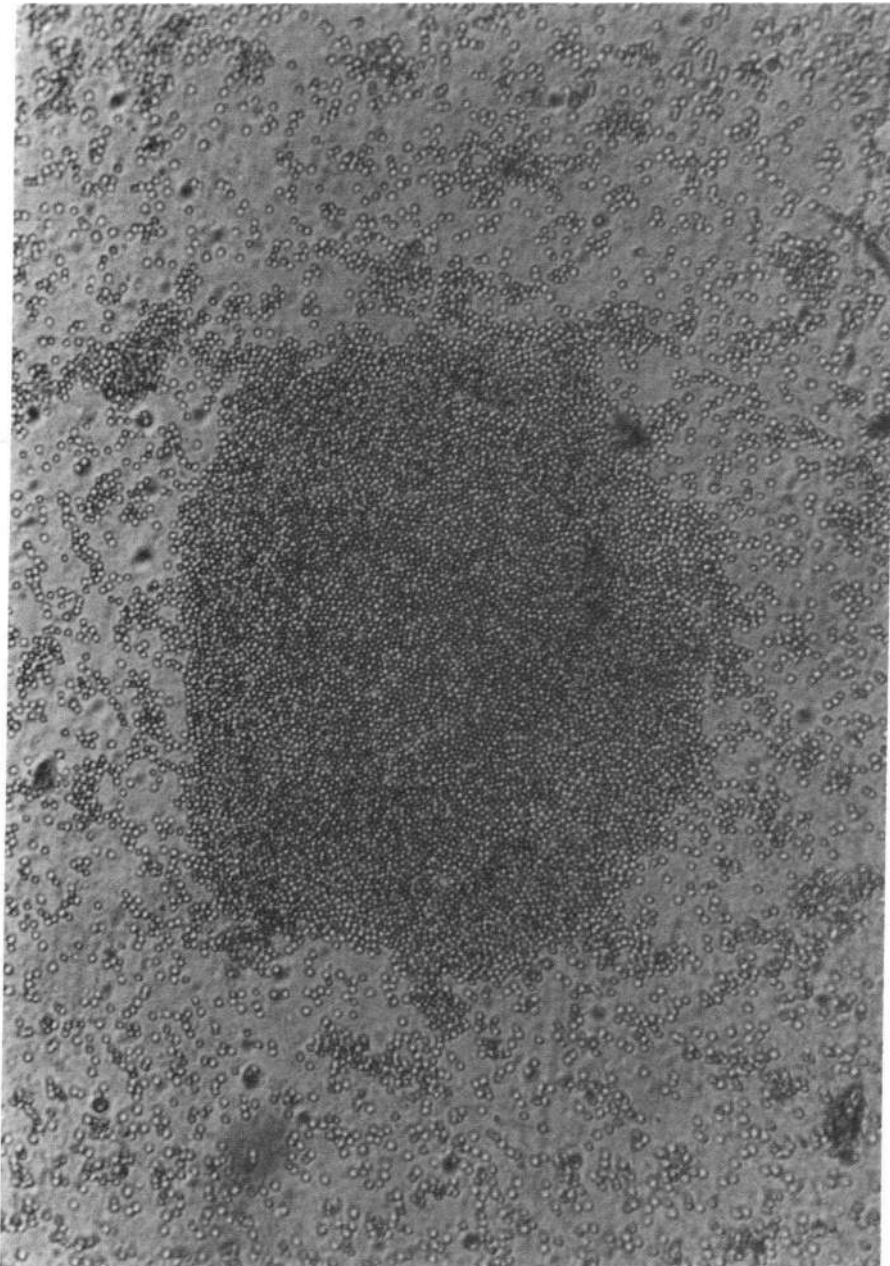


Figure 2 (Continued)

of semen analysis and measurement of clusterin in seminal plasma, together with the actual number of oocytes fertilized by IVF and the predicted number of fertilized oocytes using the logistic regression model. The semen factors that significantly predicted successful fertilization are shown in Table II. The results were similar if the data for subjects 17 and 18 without sperm morphology results were omitted. Seminal clusterin was significantly predictive of successful fertilization together with motility index, proportion of sperm with normal morphology and number of oocytes inseminated.

*Immunofluorescence of spermatozoa.* Four separate experiments, with either of the methods of antibody labeling, gave similar results. Fluorescence was found in the midpiece region and occasionally the tail of ~10% of human spermatozoa (range 5–18%). Immature forms, particularly those with cytoplasmic droplets, and abnormal forms were labeled with the anti-clusterin MAb more frequently than morphologically normal spermatozoa, which were only rarely labeled (Fig. 5).

## Discussion

An unexpected finding from the molecular cloning of the recently identified human serum complement inhibitor, SP-40,40 was its 77% identity to a rat Sertoli cell product, sulfated glycoprotein-2 (3).

Sulfated glycoprotein-2 (SGP-2) is the major secretory glycoprotein of rat Sertoli cells in culture (10–13). It is a heterodimeric protein but unlike SP-40,40 has constituent polypeptide chains of different sizes, 47 and 34 kD. SGP-2 has a wide tissue distribution on Northern blot analysis, has been identified in serum and is produced by epididymal and testicular organ cultures. Sertoli cells, however, are the only testicular cells to produce significant amounts of SGP-2. The function of SGP-2 is not known, but it binds to rat spermatozoa in their final stages of development and is present on all mature spermatozoa. Recently, the cloning and sequencing of cDNA corresponding to a previously identified androgen repressed



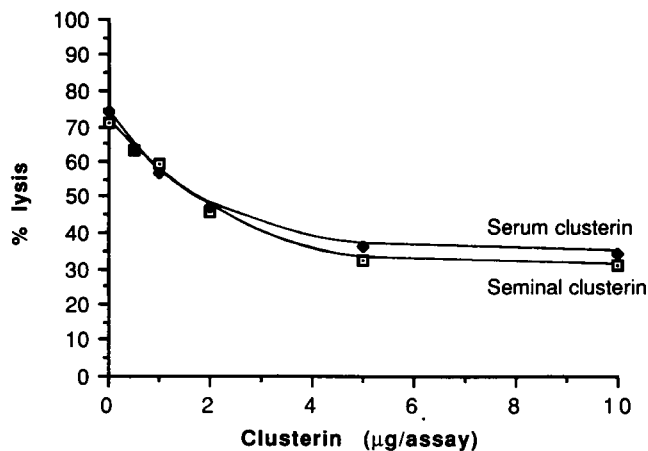


Figure 3. Inhibition of C5b-6 initiated hemolysis by seminal clusterin and serum-derived clusterin. A dose-dependent inhibition of hemolysis is seen with both preparations.

mRNA from rat ventral prostate has revealed this also as coding for SGP-2 (14). This message has been identified in a variety of rat organs (14) and is induced to high levels, coordinate with the onset of cell death, in rodent models of inducible tissue damage (15).

Concurrent with the identification of SGP-2, a protein with potent cell agglutination properties was isolated from ram rete testis fluid. This protein was called clusterin because of its ability to induce aggregation of a variety of cell types including a testicular cell line and erythrocytes from a variety of species (7). Clusterin also has a heterodimeric structure, is produced by ram Sertoli and rete testis cells in culture and is present on mature spermatozoa (16, 17). A serum form of clusterin, which lacks the cell aggregating activity of the seminal form has also been identified (18). Using the same clustering bioassay, a rat form of clusterin has recently been isolated from Sertoli cell cultures (4). Amino terminal sequence analysis and cDNA cloning revealed that rat clusterin and SGP-2 are the same protein (4).

SP-40,40, SGP-2, and clusterin, therefore are the species homologues of the same protein existing in both a serum and a seminal form (3, 4). While SGP-2 and clusterin were initially identified and are well characterized in the reproductive tract, human SP-40,40 was identified and characterized as a serum complement-related protein. Rather than continuing the confusing use of different names for species homologues, the name clusterin has been used in this paper to refer to the human seminal and serum protein. It is proposed that in future this protein should be referred to as clusterin in all species.

This study reports the purification and characterization of human seminal clusterin. Affinity chromatography of human seminal plasma produced a high yield of the protein. Two-dimensional PAGE analysis revealed significant differences between semen and serum-derived clusterin. Sequence analysis revealed evidence of proteolytic cleavage of seminal clusterin which did not appear to be affected by the conditions of sample collection before affinity purification. This suggests that some of the proteolysis may occur in vivo in the male reproductive tract. Proteolysis may account for some of the differences in two-dimensional PAGE between seminal and serum clusterin. Glycosylation differences, which are seen between the serum and seminal forms of other proteins, for example transferrin (19) may also be contributing to the electrophoretic differences between seminal and serum clusterin.

In functional studies, however, both seminal and serum clusterin display the same properties. Human seminal clusterin exhibits erythroagglutinating activity as reported for ram and rat clusterin. The serum form of clusterin also exhibited erythroagglutinating activity with a similar potency to the seminal form. This contrasts with previous studies which failed to show this activity with ram serum clusterin (18).

Purified human seminal clusterin is capable of inhibiting C5b-6 initiated hemolysis, a property recently identified in serum clusterin (2). Seminal plasma has previously been shown to have hemolytic complement-inhibiting activity (20, 21); it is possible that clusterin is responsible for at least some of this activity.

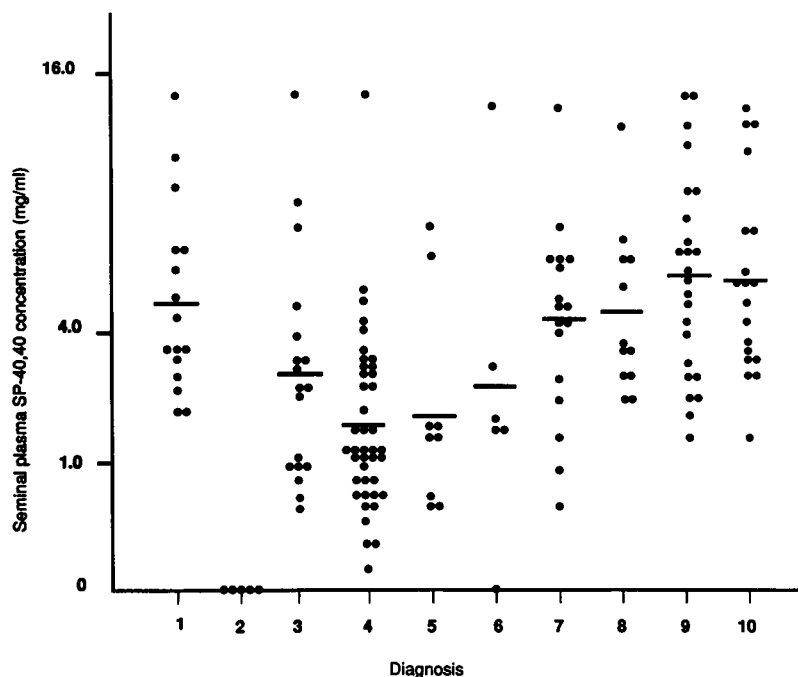


Figure 4. Seminal plasma clusterin (SP-40,40) concentration determined by ELISA in normal and infertile males. Values have been normalized by square root transformation and, for presentation, are back transformed on the vertical axis. Column 1, Normal semen ( $n = 16$ ); column 2, Vasal agenesis ( $n = 5$ ); column 3, Epididymal obstruction ( $n = 18$ ); column 4, Vasectomy ( $n = 41$ ); column 5, Azoospermia with primary seminiferous tubule failure ( $n = 9$ ); column 6, Azoospermia with secondary seminiferous tubule failure ( $n = 6$ ); column 7, Moderate oligospermia,  $1 \times 10^6/\text{ml}$ . to  $20 \times 10^6/\text{ml}$ . ( $n = 17$ ); column 8, Severe oligospermia,  $< 1 \times 10^6/\text{ml}$ . ( $n = 12$ ); column 9, Either asthenospermia or teratospermia ( $n = 24$ ); column 10, Both asthenospermia and teratospermia ( $n = 19$ ). The mean clusterin concentrations in all azoospermic groups (columns 2-6) were significantly below normal.

Table I. Seminal Clusterin, Semen Analysis, and Results of IVF in 25 Couples

Patient	Seminal clusterin mg/ml	Sperm concentration 10 <sup>6</sup> /ml	Sperm motility %	Motility index	Normal morphology %	Oocytes		
						Inseminated	Fertilized	Predicted*
						n	n	n
1	6.0	109	63	78	27	5	0	2
2	12.6	25	62	108	15	13	12	11
3	7.5	20	64	118	6	4	0	1
4	10.5	120	57	106	3.5	4	3	3
5	13.5	90	76	134	24	4	3	3
6	13.5	43	50	87	19	3	1	2
7	3.0	118	44	79	45	12	8	8
8	10.0	243	70	93	59	2	2	2
9	6.0	11	39	60	40	6	2	3
10	15.0	64	68	77	30	4	3	3
11	2.1	25	60	106	43	14	12	11
12	15.0	150	76	133	30	3	3	2
13	8.1	75	53	90	17	7	6	3
14	9.0	111	56	97	30	2	2	1
15	10.5	59	63	106	18	1	1	0
16	15.0	29	48	80	9	2	0	1
17	7.5	111	60	106	—	1	1	1
18	11.7	40	62	102	—	7	6	6
19	6.0	55	53	99	3	7	2	2
20	7.5	170	59	99	42	9	6	7
21	15.0	80	68	118	32	3	3	2
22	7.5	116	41	59	34	8	6	4
23	6.0	176	45	80	38	7	4	4
24	6.3	83	74	131	38	9	6	7
25	13.5	14	35	44	29	10	5	7
Lower limit of normal	2.5	20	40	100	30			

\* Number of fertilized oocytes predicted from the logistic regression equation.

Functionally, therefore, both serum and seminal clusterin appear to have the same properties. The analogy with serum S-protein/vitronectin, also a component of the SC5b-9 complement complex, is of interest. Both proteins inhibit C5b-9 lytic activity and both have cell adhesion properties that could be of importance in the tissue localization and stability of the SC5b-9 complex. S-protein/vitronectin contains the Arg-Gly-Asp (RGD) adhesion sequence and its receptor, one of the integrin family, is well characterized (22). The molecular basis of clusterin induced cell aggregation is currently unknown.

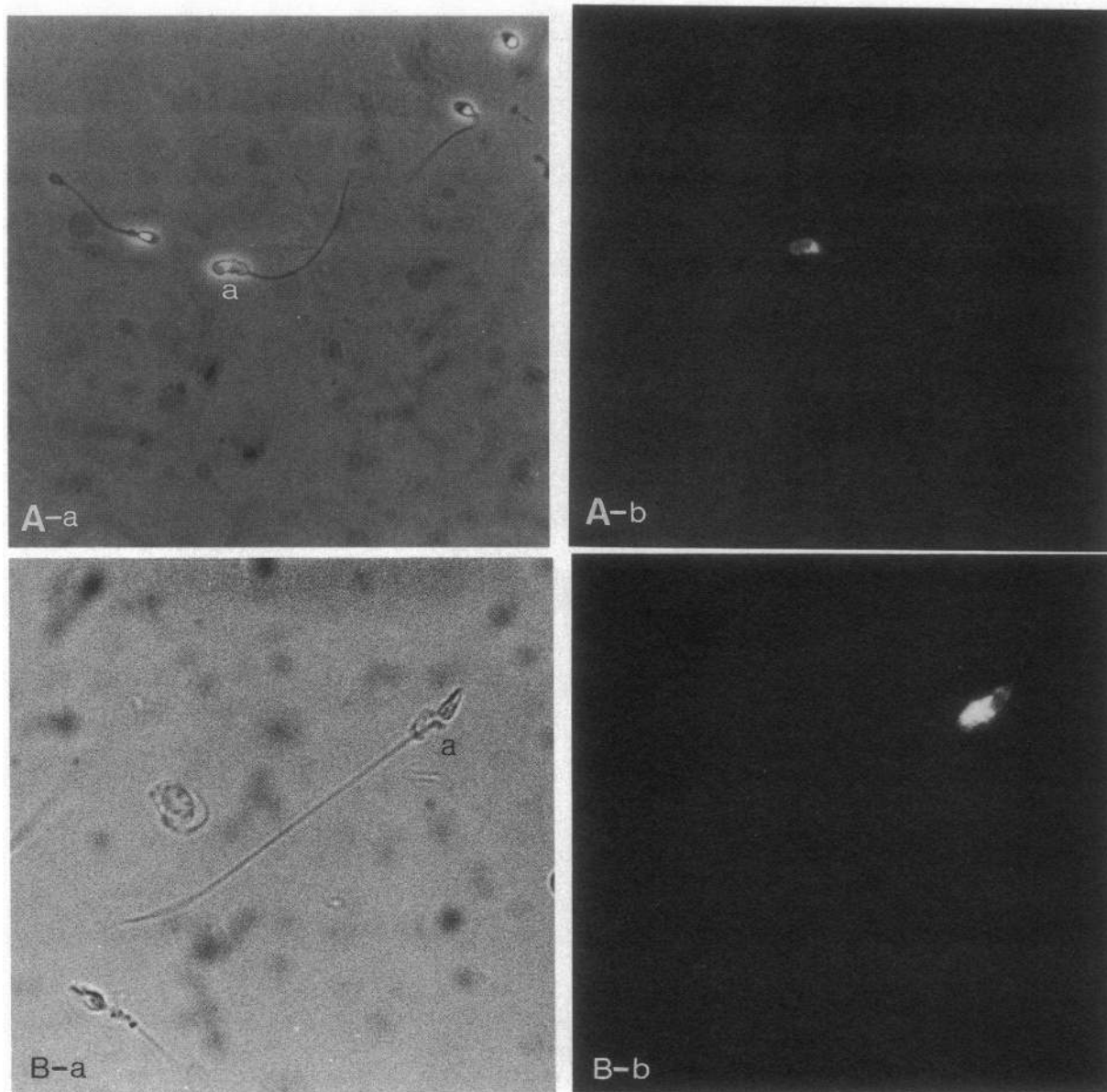
The concentration of clusterin in normal human seminal plasma is considerably higher than that found in serum. Concentrations up to 15 mg/ml were found suggesting that, like

the rat and ram homologues, clusterin is a major glycoprotein of human reproductive tract.

Analysis of the clusterin concentrations of abnormal seminal plasma samples yielded surprising results. By analogy with rat and ram data, we had anticipated that, as product of testicular and epididymal cells, clusterin concentration would be low in men with seminiferous tubule failure and undetectable in obstructive azoospermia and after vasectomy. While azoospermic seminal plasma samples did have significantly lower mean clusterin concentrations, there was a wide spread and a number of samples, including postvasectomy samples, had levels in the normal range. These data suggest that clusterin is also produced in accessory sex glands. The consistently undetectable seminal clusterin in patients with congenital vasal agenesis implicates the seminal vesicles as a site of extratesticular production. Absence of the seminal vesicles accompanies congenital vasal agenesis (23) and these patients therefore do not have the seminal vesicle contribution to the ejaculate, which is present with epididymal and vasal obstruction. Seminal vesicle production of clusterin is also indicated by the significant correlation between seminal clusterin and seminal fructose concentrations in azoospermic men in whom testicular and epididymal contribution to seminal clusterin would be expected to be absent or low. Fructose is produced almost exclusively in the seminal vesicles (23). Seminal vesicle fluid

Table II. Logistic Regression Analysis of Fertilization Rate In Vitro

Semen factor	Partial regression coefficient	Standard error	P value
Clusterin concentration	0.161	0.058	0.006
Motility index	0.023	0.008	0.007
Normal morphology	0.047	0.017	0.008
Number of oocytes inseminated	0.147	0.061	0.016



**Figure 5.** Indirect immunofluorescence labeling of human spermatozoa with anti-clusterin MAb. The left hand panels show light micrographs under phase contrast illumination. The right panels are fluoromicrographs of the same fields under epifluorescence illumination. (A-a) Sperm *a* with an amorphous head and a cytoplasmic droplet ( $\times 400$ ). (A-b) The MAb labels the head and, particularly, the cytoplasmic droplet of the sperm. (B-a) Sperm *a* has a big cytoplasmic droplet and a normal head ( $\times 400$ ). (B-b) The MAb labels the cytoplasmic droplet. (C-a) Sperm *a* has an abnormal head and a big cytoplasmic droplet ( $\times 1,000$ ). (C-b) The MAb labels the head, cytoplasmic droplet and tail of the sperm. (D-a) Sperm *a* has an abnormal head and a big mid-piece ( $\times 400$ ). (D-b) The MAb labels the mid-piece.

obtained directly at autopsy was shown to have a high concentration of clusterin. This sample would be unlikely to be contaminated with epididymal fluid and provides further confirmation of clusterin production by the seminal vesicles. The presence of clusterin in the seminal plasma of a normospermic patient with nonfunctional seminal vesicles is in accord with production of clusterin by both the testis and the seminal vesicles. The absence of clusterin in the seminal plasma of a patient with a blocked ejaculatory duct confirms the results with congenital absence of the vasa and suggests that the other accessory sex glands (prostate, Cowper's, and urethral glands) do not produce the protein.

The data indicate that, in man, seminal clusterin is pro-

duced in the testis and epididymis, by analogy with the rat and the ram, and also in the seminal vesicles. This suggests that the function of the protein in the reproductive system is more complex than the previously suggested role in spermatogenesis (10).

A pilot study of couples undergoing IVF treatment for infertility showed a significant relationship between the clusterin concentration in the semen sample used for preparation of the sperm for IVF and the fertilization rate. The relationship between semen factors, such as the proportion with normal morphology, and the fertilization rate has been noted before but motility is usually not significant when other factors are taken into consideration in the regression model (9). A large scale



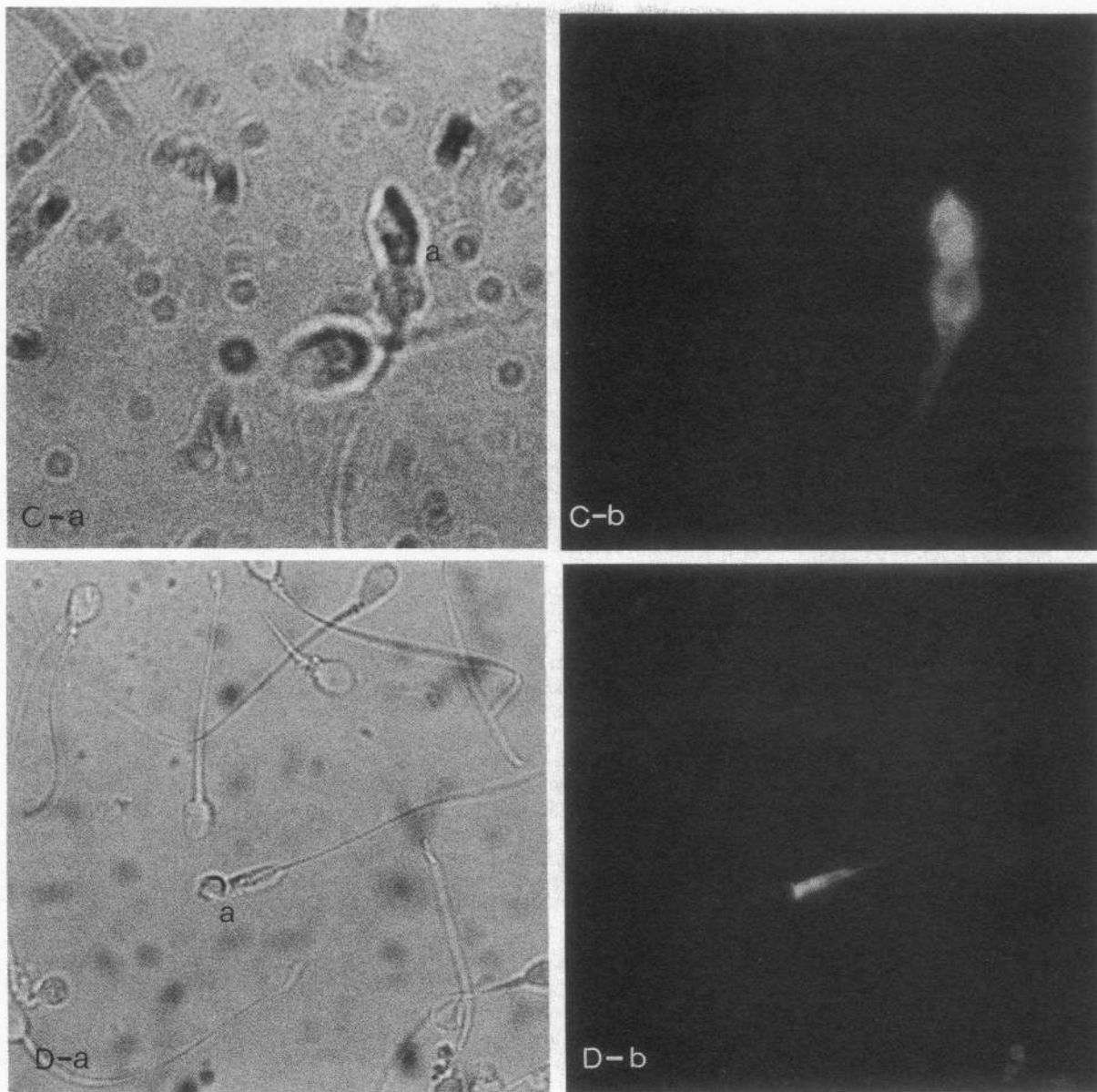


Figure 5 (Continued)

study is necessary to confirm the current observations and to determine whether measurement of clusterin levels are of predictive value in assessing the success of IVF.

The immunofluorescence studies of spermatozoa showed clusterin detectable on only 10% of human sperm. In particular, there was the preferential localization of clusterin to abnormal or immature spermatozoa. This contrasts with the rat and the ram where clusterin/SGP-2 binds to all or most spermatozoa (10, 17). The methodology employed in the ram immunofluorescence studies was similar to ours, but in the rat studies, Sylvester et al. (10) used a more sensitive technique (biotinylated second antibody followed by avidin-FITC). Sylvester et al. also used a polyclonal, rather than monoclonal, anti-SGP-2 antibody and their sperm preparations were all alcohol fixed. These methodological differences preclude direct comparison between the current data and those reported for rat spermatozoa. If the selective binding of clusterin to abnormal human spermatozoa is reproducibly found, how-

ever, this phenomenon could be of future diagnostic value and could provide further insight into the function of the protein in semen.

Clusterin is clearly a major glycoprotein of human semen. It is produced at more than one site along the reproductive tract and may have an important role in fertility.

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