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Ram Rete Testis Fluid Contains a Protein (Clusterin) Which Influences Cell-Cell Interactions In Vitro[1]

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ABSTRACT

Ram rete testis fluid is shown to elicit clustering of suspensions of Sertoli cells from testes of immature rats, TM-4 cells derived from mouse testis, and erythrocytes from several species. Details of bioassay procedures and characteristics of the phenomenon are reported. Concanavalin A and wheat germ agglutinin prevent aggregation elicited by rete testis fluid, and this inhibition is specifically prevented by α-methylmannoside and N-acetyl-glucosamine, respectively. Influences of rete testis fluid on cell aggregation are not dependent on exogenous calcium, but clustering is blocked by various metabolic inhibitors such as dinitrophenol. Rete testis fluid addition to mixed suspensions of erythrocytes and TM-4 cells is followed by separate aggregation of each cell type. Using aggregation of TM-4 cells suspended in simple medium at low density in rotation as a bioassay, we have determined which fractions in rete testis fluid retain activity. We have shown that a heat-stable, trypsin-sensitive protein, having an isoelectric point below pH 4.0, retains the capacity to aggregate cells. We discuss the possible functions of this protein, named clusterin, in cell interactions.

INTRODUCTION

During spermatogenesis, a remarkable restructuring occurs in which clones of spermatocytes destined to undergo meiosis and mature into spermatozoa are translocated from the basal compartment of the seminiferous tubule into the adluminal compartment (Fawcett, 1975; Russell, 1977). Mechanisms are unknown by which Sertoli cells recognize the clone of spermatocytes committed to meiosis, and then facilitate its translocation.

Surface proteins are thought to play crucial roles in cellular interactions in morphogenetic assembly during embryogenesis (for reviews, see Moscona and Hausman, 1977; Frazier and Glaser, 1979). In adult mammals, spermatogenesis represents one of the continuing developmental processes which has aspects of cytodifferentiation analogous to those occurring during organogenesis and tissue restructuring in the fetus. Spermatogenesis in most mammals is a steady state process in which type A_o spermatogonia differentiate to form type A spermatogonia, which then become committed to develop into spermatocytes, undergo meiosis, and form spermatozoa (for reviews, see Clermont, 1972; Monesi, 1972; Fritz, 1973, 1978; Setchell, 1978).

In spermatogenesis as in other differentiating systems, cell interactions play an important role during development (Fawcett, 1975; Roosen-Runge, 1977). It is generally accepted that Sertoli cells influence and modulate germinal cell development, first by forming and maintaining the seminiferous tubule barrier (Fawcett, 1975; Setchell and Waites, 1975); second by producing and secreting particular compo-

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nents such as androgen binding protein (for review see Fritz, 1978), plasminogen activator (Lacroix et al., 1977; Làcroix and Fritz, 1982), inositol (Robinson and Fritz, 1979), transferrin (Skinner and Griswold, 1980, 1982), and other less well-defined proteins (Wilson and Griswold, 1979; Wright et al., 1981; Kissinger et al., 1982; De Philip et al., 1982); and third by implementing the restructuring in the tubule which occurs during various stages of spermatogenesis (Russell, 1977). Molecules involved in mediating cell interactions among Sertoli cells during these processes remain to be identified. However, the presence of unique antigenic determinants on the surfaces of the mammalian spermatocytes and spermatids is well documented (Millette and Bellvé, 1977; O'Rand and Romrell, 1977), and these specific plasma membrane proteins have been postulated to be involved in the modulation of germinal cell development (Tung and Fritz, 1978). This modulation would be facilitated by the close apposition of germinal cells to the surfaces of surrounding Sertoli cells in a highly organized arrangement at all stages of spermatogenesis (Fawcett, 1975).

If testicular cells contain specific plasma membrane proteins which influence interactions among germinal cells and Sertoli cells, we thought it possible that the putative compounds or their degradation products might be released into the surrounding tubular fluid in a manner comparable to that described for chick neural cells in culture (Moscona and Hausman, 1977). If this occurs in vivo, plasma membrane components released into tubular fluid would be transported into the rete testis, from which it may readily be collected by cannulation of efferent ducts from the rete testis of rams (Voglmayr et al., 1967), as a potential source of molecules involved in cell aggregation.

In this communication, we report that ram rete testis fluid contains a protein which elicits clustering of suspensions of rat Sertoli cells, mouse testis TM-4 cells, and erythrocytes. We also provide information concerning the nature of cell responses in our assay, together with preliminary data on the properties of the partially purified clustering factor, for which we propose the name "clusterin." We describe elsewhere in greater detail the properties of the protein which we have purified to apparent homogeneity (Blaschuk et al., 1983).

MATERIALS AND METHODS

Assay for Clustering Factor (Clusterin) Activity

The TM-4 cells used in these experiments were a generous gift from J. P. Mather, who derived them from primary cultures of presumptive Sertoli cells prepared from testes of 10-day-old mice (Mather, 1980).

Subcultured cells were routinely grown from 48 to 96 h in 25 cm^2 culture flasks (Falcon, Oxnard, CA) containing 5 ml of a 1:1 (v/v) mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) horse serum and 2.5% (v/v) newborn calf serum (purchased from Gibco, Grand Island, NY).

The TM-4 cells were routinely removed from the substratum by treatment with 2 ml of 0.1% (v/v) lyophilized trypsin (Gibco), dissolved in Hanks' (calcium and magnesium free) buffer (Gibco) and diluted 1:1 (v/v) with the growth medium containing 1 mg/ml trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). In some experiments, cells were removed from the substratum mechanically and dispersed by agitation with a Pasteur pipette. The cell suspension was centrifuged at 360 × *g* for 2 min and the resulting pellet was washed with 1 ml of a 1:1 (v/v) mixture of serum-free Ham's F12 and Dulbecco's modified Eagle's medium (F12-DMEM) containing 1% (w/v) bovine serum albumin (BSA) (Fraction V, Sigma) and 1 mg/ml of a trypsin inhibitor. For cell incubation experiments, the cells were routinely suspended in 1 ml of Dulbecco's phosphate-buffered saline (PBS) and filtered through nylon to remove cell aggregates. For cell culture experiments, F12-DMEM or minimal essential medium (MEM) was substituted for PBS. The number of cells in an aliquot of the filtrate was determined by counting with a hemocytometer, and the cell suspension was then diluted with PBS or culture medium to a final density of 4×10^5 cells/ml.

In cell incubation experiments, the following components were added to each well of a polyvinyl chloride microtitration plate having 96 U-shaped wells (Dynatech, Cat. #1-220-24, Alexandria, VA): 70 μl of PBS, 5 μl of ram rete testis fluid or any other solution to be tested, and 25 μl of the cell suspension (1×10^4 cells) prepared as described above. In cell culture experiments, the same procedures were used with the exception that F12-DMEM or MEM was substituted for PBS. The microtitration plate was mounted in a holder at an angle of 30° and rotated at 1 to 2 rpm in a water-saturated incubator set at 37°C and equilibrated with 5% (v/v) of CO_2 in air. After an incubation period of 1 to 24 h, or a culture period of up to 7 days, cells were examined with the aid of a dissecting microscope (25–50× magnification), and scored according to the criteria described in *Results*. Details concerning other components added are described in the text. Other cells employed included erythrocytes freshly obtained from rats, sheep or humans, and Sertoli cells from testes of immature rats, prepared as described previously (Dorrington et al., 1975; Tung et al., 1975). The final cell density per well was routinely adjusted to 10^4 cells/100 μl, unless indicated otherwise.

Dye-Ligand Chromatography of Ram Rete Testis Fluid

Ram rete testis fluid was collected from conscious rams by the procedures of Voglmayr et al. (1967). The centrifuged, sperm-free supernatant fractions were stored frozen. Ram rete testis fluid (13.5 mg of protein) was thawed and applied to a 0.8 × 8 cm column of Affi-Gel Blue (Bio-Rad Labs., Toronto) equilibrated with 50 mM sodium phosphate, pH 7.4. The column was washed with equilibrating buffer until no more material absorbing at 280 nm was eluted, and then equilibrating buffer containing 1.2 M NaCl was applied. Aliquots of 5 μl were withdrawn from each fraction and assayed for clustering factor activity as described above. The protein content of ram rete testis fluid and other samples were determined by the method of Bradford (1976).

Isoelectric Focusing

Fractions from the Affi-Gel Blue containing biologically active material were dialyzed against PBS, and concentrated to yield 200 μg/ml protein. An aliquot (400 μg of protein) of the partially purified clusterin obtained after dye-ligand chromatography was subjected to preparative isoelectric focusing according to the procedures of Friesen et al. (1971). The carrier ampholyte (LKB Producer AB, Uppsala, Sweden) used had the pH range of 3.5 to 5.0. Initially a potential of 100 volts of current was applied for 16 h, after which time the current had dropped to 1 mA. The potential was then increased to 400 volts for an additional 24 h. Aliquots of 5 μl were withdrawn from each fraction and assayed for activity as described above.

Other Materials

Lectins (concanavalin A, wheat germ agglutinin and peanut agglutinin) and BSA were purchased from Calbiochem, La Jolla, CA, while all sugars (α-methyl-mannoise, N-acetylglucosamine and D-galactose) and other reagents cited (puromycin, iodoacetamide, 2,4-dinitrophenol, N-ethylmaleimide, colcemid, EGTA and mercaptoethanol) were obtained from Sigma. Tissue culture reagents were from Gibco and other chemicals were of highest reagent grade available.

RESULTS

Influences of Ram Rete Testis Fluid on the Clustering of Suspensions of Testicular Cells and Erythrocytes

In suspensions of Sertoli cell aggregates, freshly prepared from testes of 10-day-old rats and maintained in rotation in polyvinyl microtiter plates, addition of ram rete testis fluid to the medium results in the formation of large aggregates (Fig. 1). Suspensions of Sertoli cells prepared from testes of 3-day-old rats show similar responses (data not shown). The clustering of suspensions of TM-4 cells, a cell line from testes of immature mice thought to be derived from Sertoli cells (Mather, 1980), is even more pronounced (Fig. 2). Human erythrocytes (Type AB) also aggregate following the addition of rete testis fluid, but red blood cell packing into a cluster is not as tight or as structured (Fig. 3) as the TM-4 cell packing (Fig. 2B). Suspensions of human erythrocytes of other classes examined (Types A, B, and 0), and of erythrocytes from rats or sheep, each aggregate in response to rete testes fluid addition in a manner similar to that observed for Type AB human erythrocytes (data not shown).

The ease of growing large numbers of TM-4 cells in a relatively simple medium, together with their pronounced responses to rete testis fluid and their known testicular origin, led us to select these cells for a more thorough investigation of the phenomena involved. To evaluate the possibility that rete testis fluid components may be acting only by altering the attachment of cells to the surfaces of polyvinyl microtiter plates, we investigated the responses of cells suspended in hanging drops. TM-4 cells suspended in PBS alone remain dispersed, but they readily form aggregates in the presence of ram rete testis fluid (Fig. 4). Erythrocytes respond in a similar fashion (results not shown). These data indicate that the effects elicited are not restricted to a particular substratum for eliciting aggregation.

The illustrated responses of TM-4 cells to rete testis fluid at varying times after cell plating (Fig. 5) provide a convenient way of describing the qualitative scoring procedures employed in subsequent studies. Within 1 h after plating TM-4 cells in the presence of rete testis fluid, several centers of aggregation appear (Fig. 5A) (scored as "1+"). During the succeeding 3 h, various aggregates progressively cluster more closely together, but remain relatively diffuse (Fig. 5B and C) (scored as "2+"). By 6 h after plating almost no isolated cells or small aggregates are evident. Instead, most of the cells are gathered together in a single cluster in the center of the well (Fig. 5D) (scored as "3+"). At this stage, the core does not have sharply demarcated borders. By 24 h after plating, the cells have formed into a sphere-shaped structure having a relatively dense core with well-demarcated borders (Fig. 2B) (scored as "4+"). By 24 h, the TM-4 cells are sufficiently packed together to resist separation during manipulation with a Pasteur pipette, and the bottom of the sphere-shaped cluster of cells is firmly attached to the surface of the plate.

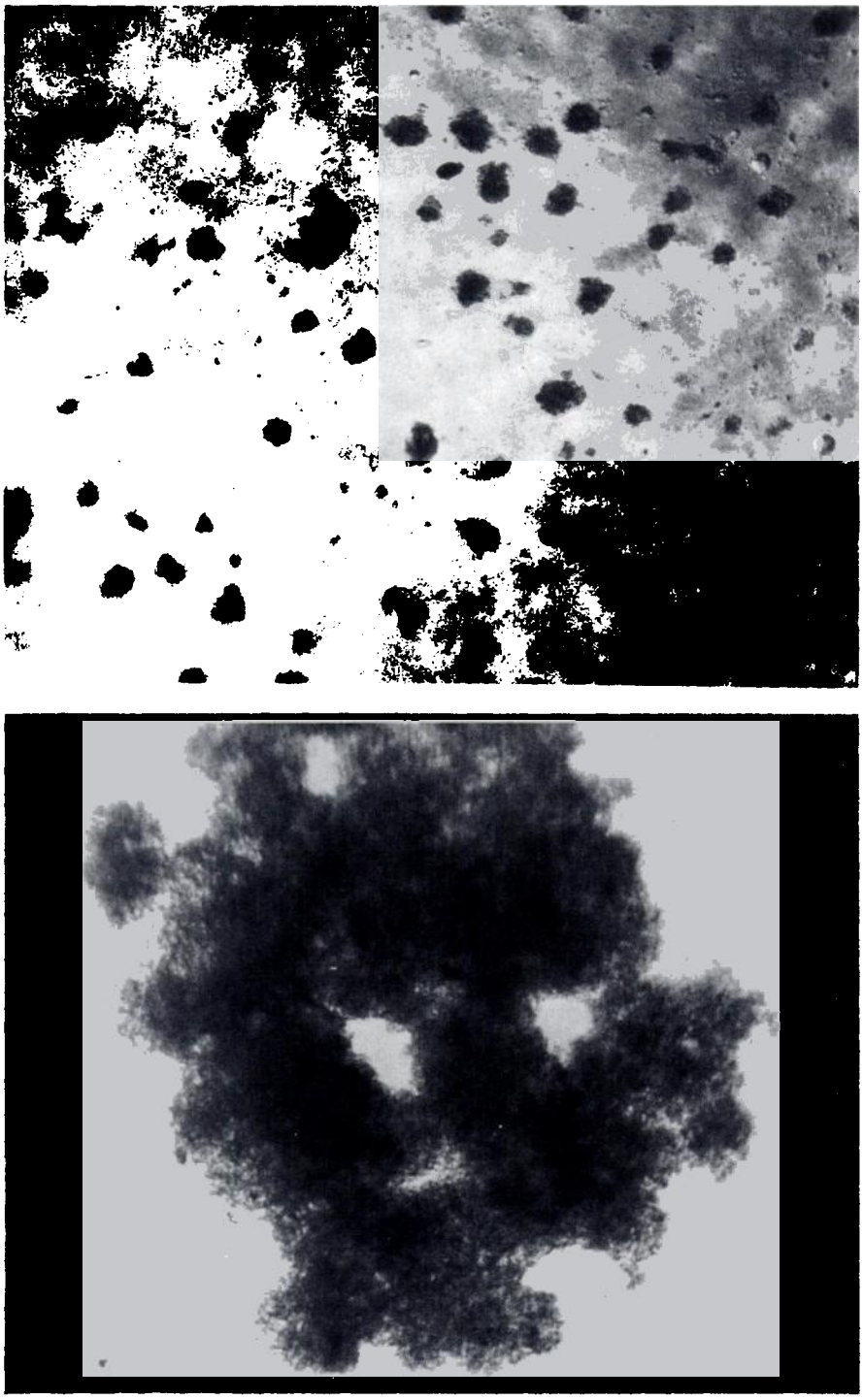


FIG. 1–6. Influences of ram rete testis fluid on the aggregation of cells indicated. All photographs were taken at the same magnification (×50), with a Wild dissecting microscope and camera attachment.

FIG. 1. Sertoli cells prepared from testis of 10-day-old rats were subjected to rotation for 24 h in wells of polyvinyl microtitration plates containing the following components: 70 μl Eagle's modified minimal essential medium, approximately 5×10^4 cells (0.10 μg DNA) in 25 μl PBS, and either 5 μl BSA (3 mg/ml PBS) *(A)* or 5 μl ram rete testis fluid (0.5 mg/ml) *(B)*.

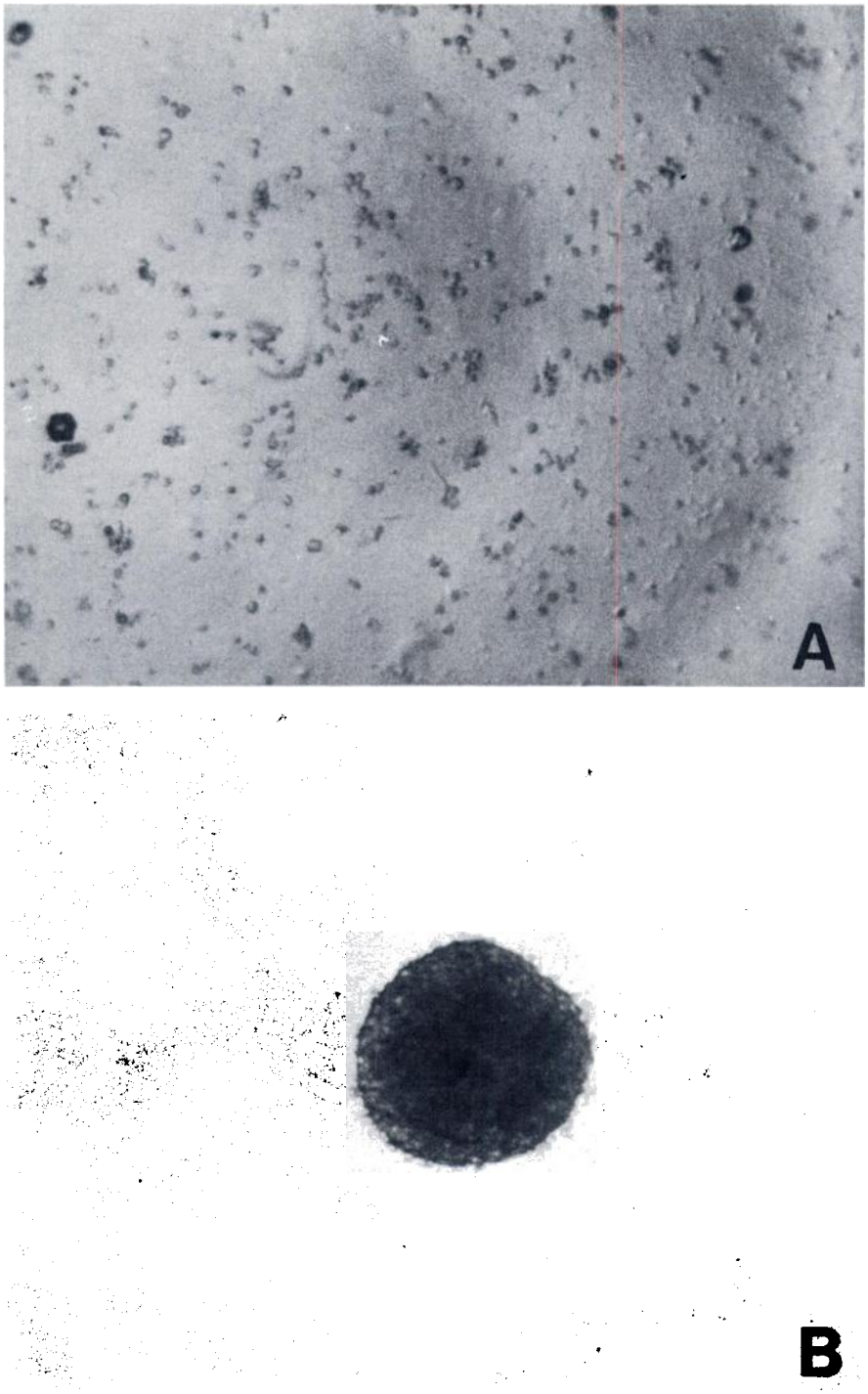


FIG. 2. TM-4 mouse testicular cells were subjected to rotation for 24 h in wells containing the following components: 70 μl PBS, 10^4 cells in 25 μl PBS, and 5 μl BSA (3 mg/ml PBS) *(A)* or 5 μl ram rete testis fluid (0.5 mg/ml) *(B)*.

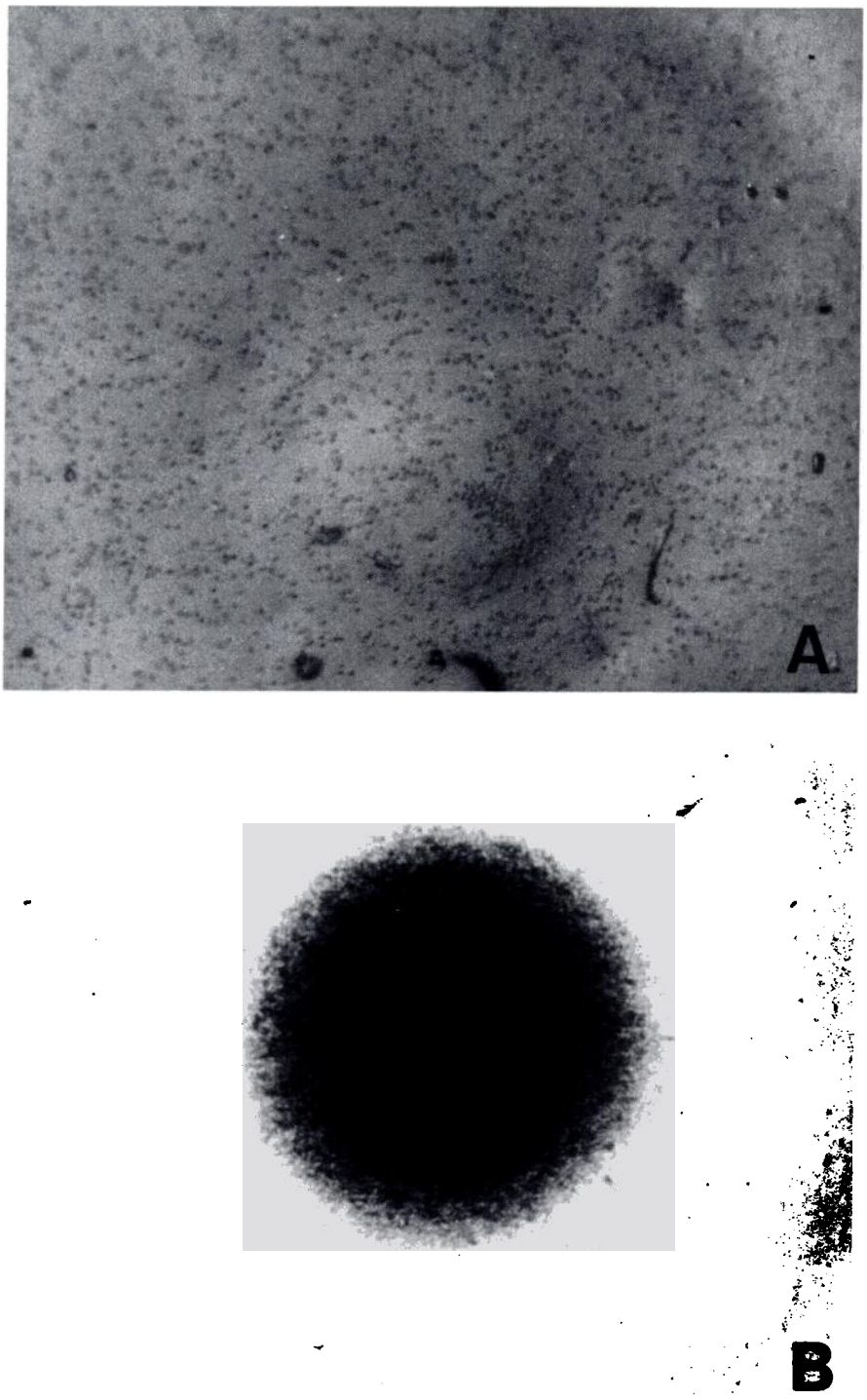


FIG. 3. Red blood cells, human Type AB, were subjected to rotation for 24 h in wells containing the following components: 70 μl PBS, 10^4 cells in 25 μl PBS, and either 5 μl BSA (3 mg/ml PBS) *(A)* or 5 μl ram rete testis fluid (0.5 mg/ml) *(B)*.

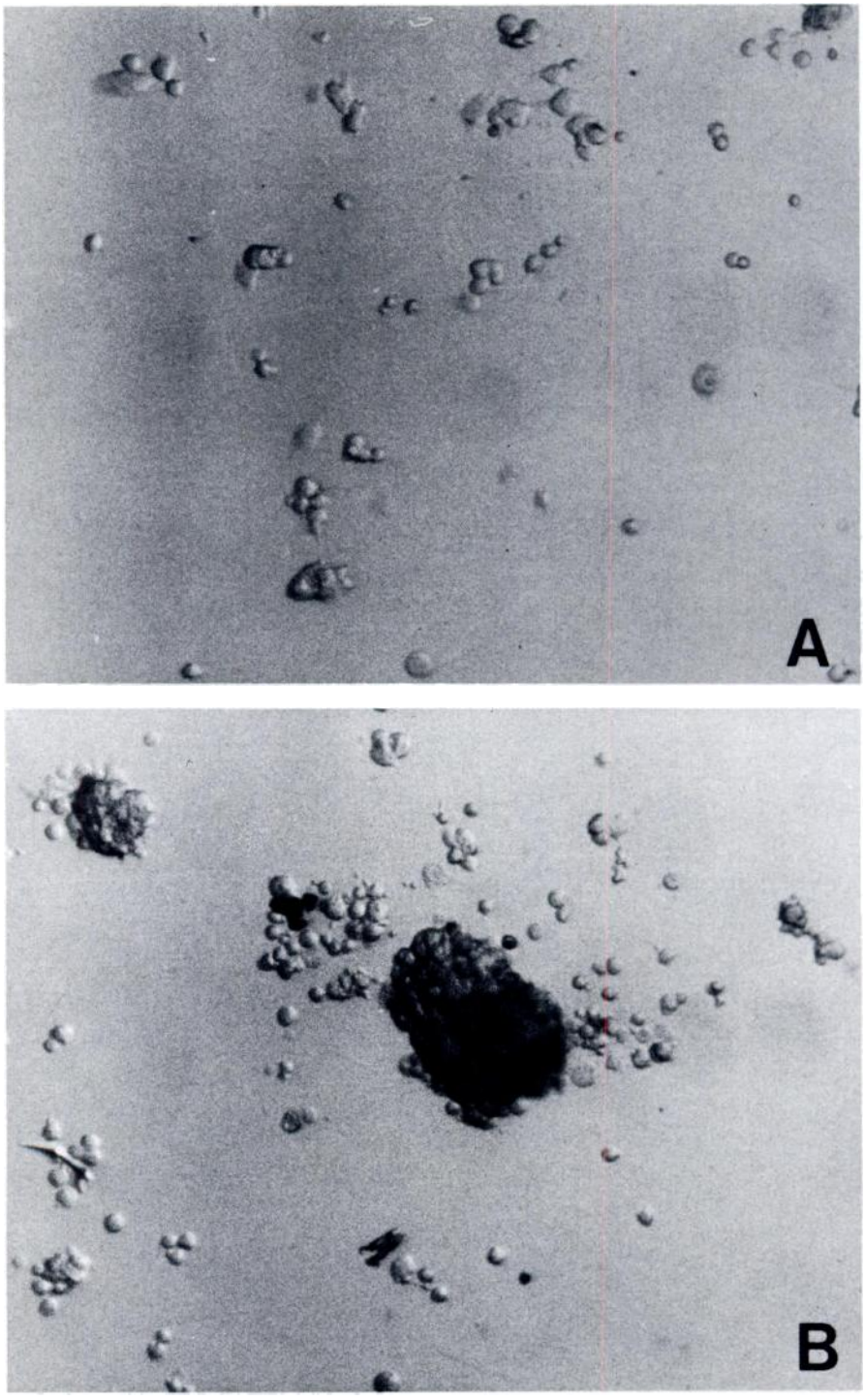


FIG. 4. TM-4 mouse testicular cells were dispersed into a hanging-drop mounted on a glass slide, and incubated at room temperature for 6 h. The 25-μl droplets of PBS contained 2.5×10^3 cells and either 15 μg BSA *(A)* or 1.2 μg protein from ram rete testis fluid *(B)*.

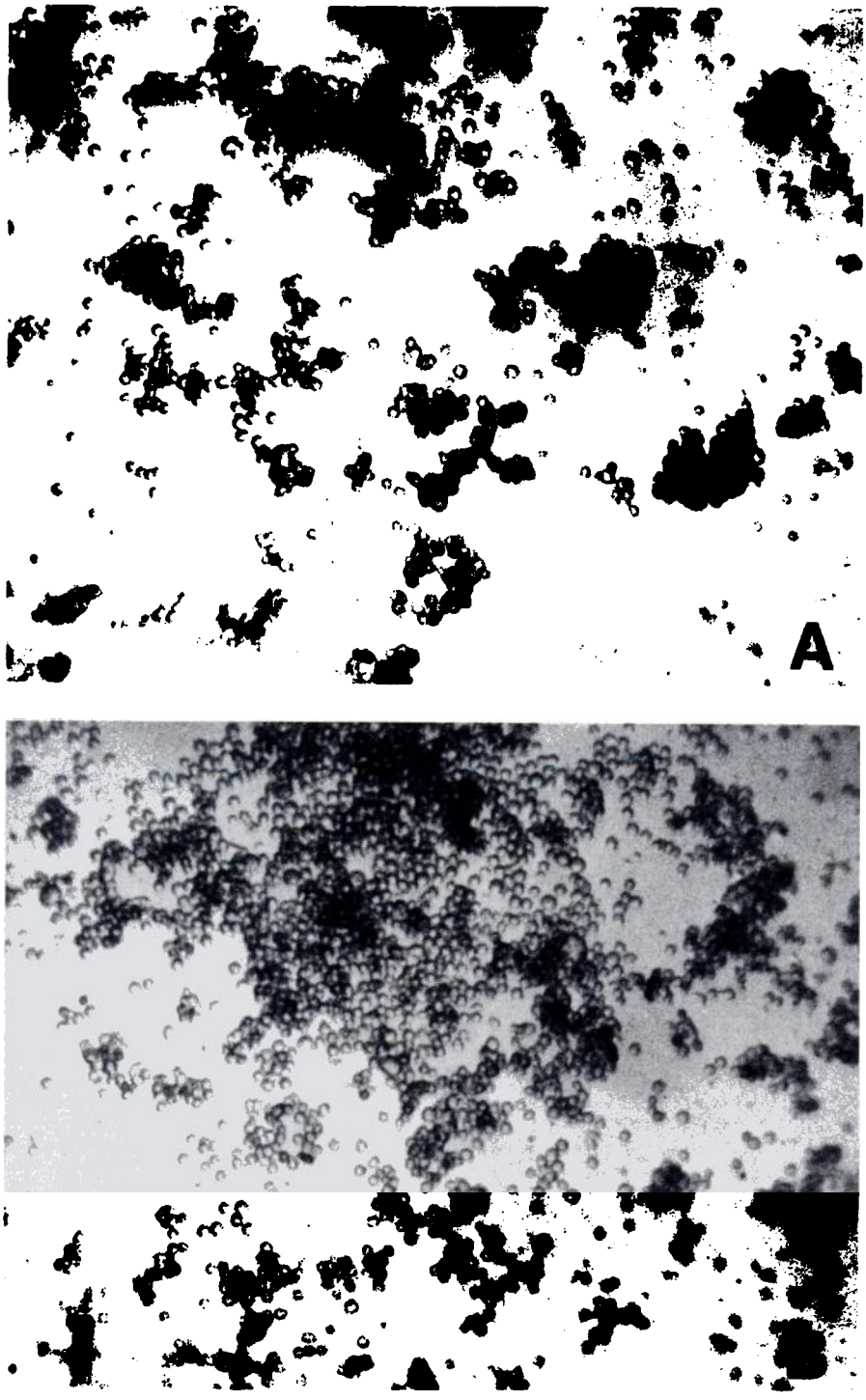
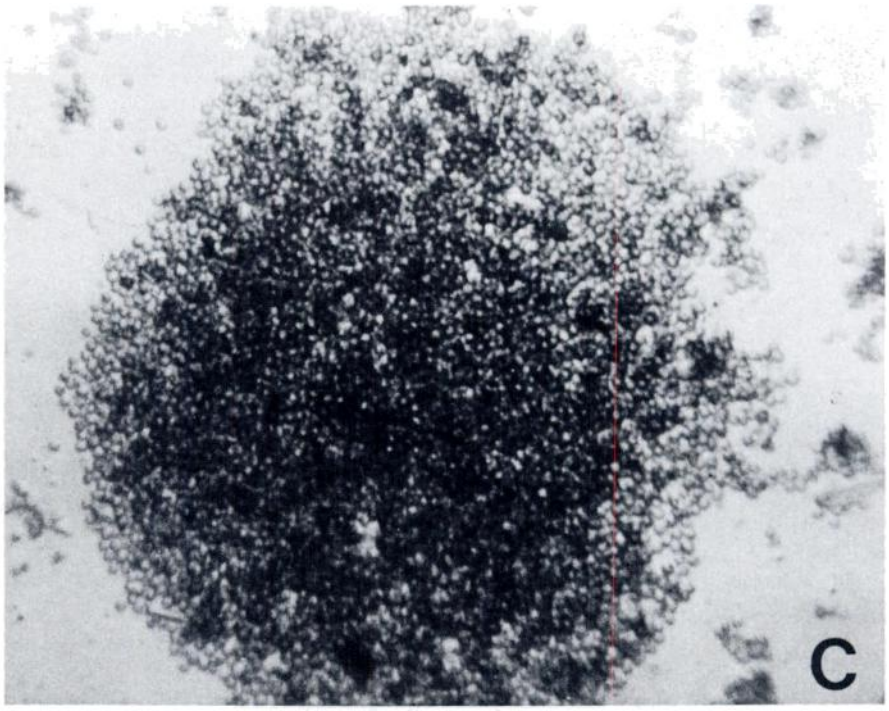
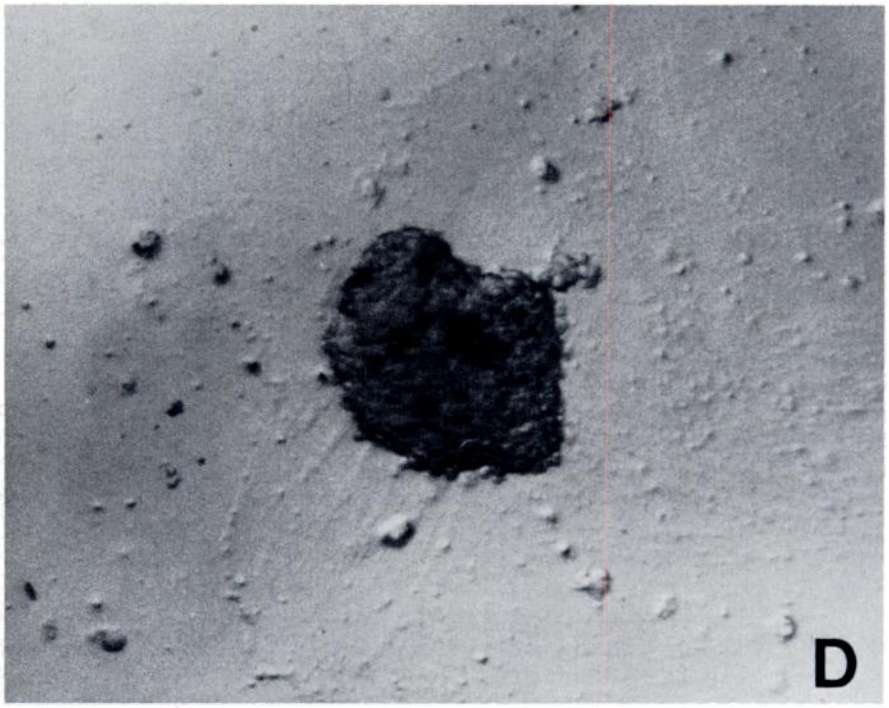


FIG. 5. TM-4 mouse testicular cells were subjected to rotation for times indicated in wells containing the following components: 70 μl PBS, 10^4 cells in 25 μl PBS, and 5 μl BSA (3 mg/ml) *(E)* or 5 μl ram rete testis fluid (0.5 mg/ml) *(A–D)*. Photographs were taken of cells incubated for 1 h *(A)*; 3 h *(B)*; 4 h *(C)*; and 6 h *(D* and *E)*.





Comparable suspensions of TM-4 cells maintained in rotation in the absence of rete testis fluid remain well dispersed. In some preparations, limited numbers of aggregates form containing a few cells per aggregates (Fig. 2A and 5E). This same pattern occurs in control TM-4 cells maintained for 48 h. We routinely terminated incubation experiments at 24 h after plating the cells in PBS. For cells cultured in F12-DMEM or MEM, we extended the period of observation up to 7 days. We obtained the same qualitative results in all cases, provided we plated the density of cells indicated in the polyvinyl chloride wells. Results reported were observed both with TM-4 cells which had been removed from the substratum by trypsin treatment, followed by neutralization of the trypsin, and with TM-4 cells removed mechanically and dispersed with a Pasteur pipette.

In wells in which erythrocytes and TM-4 cells are added together in the absence of rete testis fluid, both cell types remain dispersed, as when either cell type is plated alone (Fig. 2A and 3A). In the presence of rete testis fluid, the red cells gather loosely together at the base of the well, and primarily TM-4 cells cluster more tightly together in a spherical configuration (Fig. 6). It appears that clustering factor in rete testis fluid facilitates interactions among similar cells, and does not elicit co-clustering of TM-4 cells and erythrocytes. In the case of erythrocyte clustering elicited by rete testis fluid (Fig. 3), relatively diffuse aggregates form at the bottom of the well, and these cells can readily be dispersed with a Pasteur pipette.

Optimal Conditions for the Bioassay of Clustering Factor Activity

We determined that 10^4 TM-4 cells, suspended in 100 μl PBS, provide the simplest conditions to permit easily reproducible scoring of clustering factor activity present in crude rete testis fluid or in various partially purified fractions. We employ polyvinyl microtiter plates having 96 U-shaped wells, and rotate these plates (1 to 2 rpm) at an angle of approximately 30° in an incubator maintained at 37°C, having a water-saturated atmosphere containing 95% air and 5% CO_2. We have obtained qualitatively similar results with TM-4 cells suspended in wells in stationary plates, but central packing either did not occur, or was not as pronounced. We observed a maximal degree of clustering

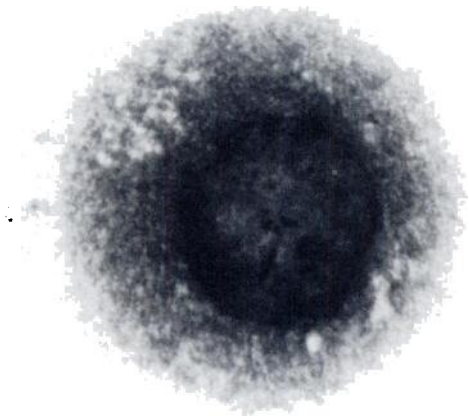


FIG. 6. TM-4 mouse testicular cells and human erythrocytes (Type AB) were mixed together for 24 h in wells containing 10^4 TM-4 cells in 25 μl PBS, 10^4 RBC in 25 μl PBS; 5 μl ram rete testis fluid (0.5 mg/ml); and 45 μl PBS. Either cell alone, plus or minus rete testis fluid, is depicted in Figs. 2 and 3. In the absence of rete testis fluid, rotated cells remained dispersed, as in Figs. 2A and 3A.

corresponding to "3+" for TM-4 cells or erythrocytes following the addition of rete testis fluid to suspensions of stationary cells. Freshly prepared, well-dispersed cells (25 μl, 4 × 10^5/ml) are routinely added last to medium containing components listed and varying amounts of fractions from ram rete testis fluid (routinely 5 μl, having 0.5 mg protein/ml) or BSA (routinely 5 μl, 3.0 mg/ml). In an examination of the possible effects of purified albumin on cells maintained in PBS, we noted the absence of clustering factor activity at levels of albumin up to 25 μg/100 μl. In contrast, the quantity of rete testis fluid protein required to elicit clustering of TM-4 cells at a "4+" score ranged between 0.5 to 5.0 μg/100 μl in samples collected from different rams.

While searching for optimal assay conditions in preliminary experiments, we have observed that the degree of clustering obtained in response to rete testis fluid is influenced by cell type, the cell density, the medium employed, the incubation temperature, conditions of incubation, and the nature of the plates. When TM-4 cells are added to medium in flat-bottomed wells of Immulon II or Linbro microtiter plates, the presence of rete testis fluid elicits the formation of multiple aggregates, corresponding to a score of approximately "2+" by criteria described above (Figs. 5B and C). In flat-bottomed wells, suspensions of TM-4 cells incubated in the presence of rete testis fluid form multiple aggregates, each having well-defined boundaries, but the aggregates never gather together to form a single cluster. To facilitate scoring, we have chosen to utilize Dynatech plates with U-shaped wells. Using a dissecting microscope at 25 or 50× magnification, we routinely score responses of TM-4 cells to various fractions prepared from ram rete testis fluid, employing 10^4 TM-4 cells suspended in 100 μl PBS in U-shaped wells in polyvinyl chloride plates rotated at 1–2 rpm for 24 h, as described above. Under these conditions, we are able to utilize our qualitative scoring procedure (Figs. 2 and 5) to determine which rete testis fluid fractions retain clustering factor activity.

Preliminary Characterization of the Chemical Properties of Clustering Factor(s) in Rete Testis Fluid

There is no loss of clustering factor activity in samples of rete testis fluid immersed for 10 min in a boiling water bath. Full activity is retained after lyophilization. Similarly, complete activity remains after extensive dialysis against PBS, or after adsorption with charcoal (2%)-dextran (0.2%).

In preliminary experiments, we have established that clustering factor appears to be a large molecular weight protein. We first showed that it is retained by an ultrafilter having a 10,000 dalton cut-off. It is destroyed by treatment with trypsin, with total loss of clustering factor activity in 1.0 ml ram rete testis fluid incubated for 30 min at 37°C with 0.5 ml trypsin (0.1% in PBS), followed by the addition of 0.5 ml soybean trypsin inhibitor (0.1% in PBS). The presence of this inactive trypsin-treated rete testis fluid does not inhibit the usual responses of TM-4 cells to untreated rete testis fluid, such as those illustrated in Figs. 2 and 5.

Protein(s) having clustering factor activity in ram rete testis fluid are readily adsorbed to Affi-Gel Blue (Fig. 7). In material eluted from an Affi-Gel Blue column with 1.2 M NaCl,

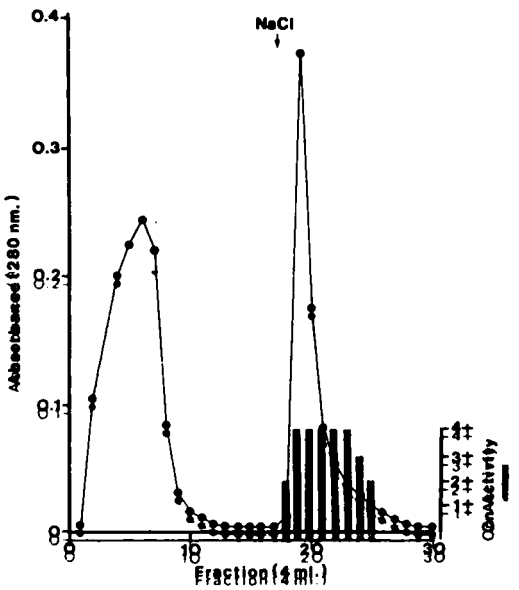


FIG. 7. Partial purification of clusterin by chromatography of ram rete testis fluid on Affi-Gel blue. Ram rete testis fluid (13.5 mg protein) was applied to a 0.8 cm × 8 cm column of Affi-Gel blue equilibrated with 50 mM sodium phosphate, buffered at pH 7.4. At the point indicated by the *arrow*, the solution was changed to 1.2 M NaCl in sodium phosphate buffer, and aliquots of proteins eluted were assayed for clustering factor activity, using TM-4 cells as described in Figs. 2 and 5. Clusterin *(Cn)* activity is shown by the *shaded bars*.

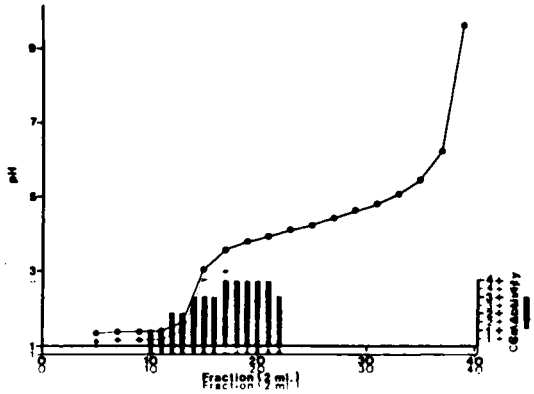


FIG. 8. Partial purification of clusterin from ram rete testis fluid by preparative isoelectric focusing. An aliquot (400 μg protein) of clusterin-enriched fraction eluted by Affi-Gel blue (Fig. 7) was subjected to preparative isoelectric focusing, using an ampholyte pH range of 3.5 to 5.0 (Friesen et al., 1971). The presence of clusterin in fractions assayed was monitored as described in Figs. 2 and 5, and clusterin *(Cn)* activity is shown by the *shaded bars*.

dialyzed, concentrated, and then subjected to preparative isoelectric focusing, all activity appears in fractions more acidic than pH 4.0 (Fig. 8). A more precise determination of the low isoelectric point, and a more detailed chemical characterization of the purified protein clusterin, are described elsewhere (Blaschuk et al., 1983).

Factors Influencing the Aggregation of Cells in Response to Components in Rete Testis Fluid

Influences of Ca^{2+} and effects of various inhibitors. Aggregation of suspensions of erythrocytes or TM-4 cells occurs in response to rete testis fluid addition when cells are suspended in Ca^{2+}-Mg^{2+}-free Hanks' solution, and similar responses ("2±" to "3±") take place among cells suspended on 0.15 M NaCl. Addition of EDTA or EGTA (up to 5 mM) does not prevent these responses to rete testis fluid, suggesting that cell aggregation elicited does not require exogenous Ca^{2+}. However, the subsequent formation of a very tight aggregate among TM-4 cells to form a structure corresponding to a score "4±" (Fig. 2) does not occur in the absence of added Ca^{2+} (data not shown). The clustering response of TM-4 cells to rete testis fluid is inhibited by dinitrophenol, puromycin, or N-ethylmaleimide, with a partial block at concentrations of 0.1 or 0.5 mM, and total inhibition at 1.0 mM (Table 1). Partial inhibition is obtained in the presence of iodoacetamide (1 mM), but there is essentially no inhibition in the presence of NaF (1.0 mM) or mercaptoethanol (1.0 mM) (Table 1). Indeed, mercaptoethanol at concentrations up to 10 mM does not cause inhibition. Although the presence of 100 mM mercaptoethanol in the medium does block TM-4 cell clustering in response to rete testis fluid, the effect is reversible. In samples of rete testis fluid treated with 100 mM mercaptoethanol, and subsequently dialyzed, full clustering factor activity ("4±") is restored after dialysis (data not shown).

Colcemid (at concentrations up to 2×10^{-6} M) does not influence the response of TM-4 cells to rete testis fluid. Clustering also occurs in TM-4 cells subjected to cytochalasin B (at concentrations up to 5×10^{-6} M), although the response to rete testis fluid is incomplete ("2±" to "3±").

Specific inhibition by lectins of effects of

TABLE 1. Effects of metabolic inhibitors on clustering of TM-4 cells in response to ram rete testis fluid.[a]

Compound added	Concentrations of inhibitor 10 μM	50 μM	100 μM	500 μM	1000 μM
Dinitrophenol	4+	N[b]	1+	N	0
Iodoacetamide	4+	N	3+	N	2+
N-Ethylmaleimide	N	2+	1+	N	0
Puromycin	N	3+	N	2+	0
Sodium fluoride	N	N	N	N	3+
Mercaptoethanol	4+	N	4+	N	4+

[a] 10^4 TM-4 cells were added last to 100 μl PBS containing concentrations of inhibitors indicated, in the presence of 5 μl ram rete testis fluid. The degree of clustering was scored as indicated in the text (Figs. 2 and 5), with "0" representing no clustering and "4+" representing maximal.

[b] *N*=not determined.

rete testis fluid. Concanavalin A addition inhibits the clustering of TM-4 cells in response to testis fluid. Partial inhibition is observed at 0.2 to 0.5 μg/ml, with complete inhibition at 1.0 μg/ml or higher. The presence of α-methylmannoside, but not N-acetylglucosamine or D-galactose, completely prevents this inhibition (Table 2). The inhibition of clustering by concanavalin A in response to rete testis fluid is also observed in suspensions of rat Sertoli cells or human erythrocytes, and this inhibition is also prevented by the presence of 15 mM α-methylmannoside (data not shown). Wheat germ agglutinin (10 μg/ml), but not peanut agglutinin (10 μg/ml), inhibits the clustering of TM-4 cells in response to rete testis fluid. This inhibition is prevented by the presence of N-acetylglucosamine but not α-methylmannoside or D-galactose (Table 2). The clustering of TM-TM-4 cells elicited by rete testis fluid is not altered by the presence of α-methylmannoside, N-acetylglucosamine or D-galactose (15 mM). The lectins alone, at concentrations indicated, do not cause cell aggregation (Table 2).

DISCUSSION

We have shown that ram rete testis fluid elicits clustering of suspensions of rat Sertoli cells, TM-4 mouse testicular cells, and erythro-

TABLE 2. Effects of lectins on clustering of TM-4 cells in response to rete testis fluid (RTF).[a]

Additions to PBS	Lectins added: None Control	None RTF	Concanavalin A (1 μg/ml) Control	Concanavalin A (1 μg/ml) RTF	Wheat germ agglutinin (10 μg/ml) Control	Wheat germ agglutinin (10 μg/ml) RTF	Peanut agglutinin (10 μg/ml) Control	Peanut agglutinin (10 μg/ml) RTF
None	0	4+	0	0	0	0	0	4+
α-Methylmannoside (15 mM)	0	4+	0	4+	0	0	0	4+
N-Acetylglucosamine (15 mM)	0	4+	0	0	0	4+	0	4+
D-Galactose (15 mM)	0	4+	0	0	0	0	0	4+

[a] TM-4 cells (10^4/100 μl PBS) were incubated in rotation culture in the presence and absence of 5 μl rete testis fluid (RTF), and the degree of clustering was scored as described in Figs. 2 and 5 at 24 h after plating the cells.

cytes maintained in rotation in wells of microtiter plates, or in hanging drops. Using primarily the responses of TM-4 cells, we have developed a simple bioassay with which we have been able to monitor clustering factor activity in fractions prepared from ram rete testis fluid. On the basis of data presented in this paper, we judge the biologically active material to be a heat-stable protein having an acidic isoelectric point. We propose the name "clusterin" for this protein. Since lectins (concanavalin A and wheat germ agglutinin) at relatively low concentrations specifically block the clustering of TM-4 cells in response to rete testis fluid (Table 2), it seems likely that clusterin and/or its cellular binding site is a glycoprotein. In experiments to be reported elsewhere, we have determined that purified clusterin contains 20 moles glucosamine per 80,000 molecular weight unit (Blaschuk et al., 1983).

In experiments on the influences of various metabolic inhibitors (Table 1), we observed that 2,4-dinitrophenol and N-ethylmaleimide at lower concentrations (0.1 mM) inhibited considerably the response of TM-4 cells to clustering factor activity in rete testis fluid, while higher concentrations (1.0 mM) totally abolished the response. Although each of these inhibitors acts at more than one site, it is known that dinitrophenol inhibits oxidative phosphorylation (Loomis and Lipmann, 1948), resulting in lowered ATP levels, and that N-ethylmaleimide reacts readily with thiol groups of a variety of enzymes and other proteins, resulting in an inhibition of respiration (Webb, 1966). If these are the primary sites affected by dinitrophenol and N-ethylmaleimide in the system described, it may tentatively be concluded that a continuing energy source is required to permit TM-4 cells to aggregate in the presence of rete testis fluid. The lesser degree of inhibition by iodoacetamide (1.0 mM) observed (Table 1) is consistent with the inhibition of anaerobic glycolysis but not oxidative phosphorylation by iodoacetamide (Webb, 1966), which therefore would not lower ATP levels as much. Other interpretations are possible, but these data suggest that the aggregation phenomenon is energy-dependent. It may also be dependent on continuing protein synthesis since puromycin, an analogue of tRNA which blocks protein synthesis, also inhibited cell aggregation in response to rete testis fluid (Table 1). Additional data are needed to establish if this is the case, since high concentrations of puromycin are known to inhibit other processes (Volkin et al., 1980).

The possible functions of clusterin in rete testis fluid are unknown. We are unable to evaluate whether demonstrated effects on cell interactions in vitro are relevant to cell interactions in vivo. Effects on cell aggregation reported have provided a useful bioassay for the purification of clusterin (Blaschuk et al., 1983). It remains to be determined whether clusterin is a normal secretory product of testicular cells, or whether it is a proteolytic product of a component on the plasma membrane which has been shed. Several precedents exist in which plasma membrane components appear in the medium during cell culture (for review see Moscona and Hausman, 1977). For example, neural cell adhesion molecule is released by embryonal chick retinal cells in culture (Rutishauser et al., 1976; Thiery et al., 1977). In almost all cases, proteins thought to be implicated in avian or mammalian cell-cell adhesion have been isolated from embryonic tissues, but interesting exceptions have been observed in hepatocytes from newborn rats and from certain cell lines in culture, such as 3T3 fibroblasts (for review see Frazier and Glaser, 1979). Clusterin represents a protein present in a developing system in an adult mammal which potentially is involved in cell-cell interactions.

Bordy et al. (1979) reported that addition of follicle-stimulating hormone stimulated the aggregation of Sertoli cells in culture. Glycoproteins appeared to be implicated in this phenomenon, since certain lectins, such as ricin, inhibited aggregation. These authors reported that wheat germ agglutinin (10 μg/ml) and concanavalin A (10 μg/ml) did not inhibit the aggregation phenomenon investigated. Since the latter lectins completely abolished the influences of rete testis fluid on cell clustering (Table 2), it appears unlikely that clusterin was involved in mediating the effects described by Bordy et al. (1979).

In this paper, we have reported that addition of rete testis fluid to dilute suspensions of cells results in a relatively slow aggregation, with the type of aggregation varying among cells investigated. Interestingly, when suspensions of erythrocytes and TM-4 cells are present in the same wells, addition of rete testis fluid does not elicit random co-aggregation (Fig. 6), indicating that a specific form of cell "sorting out" takes place.

It appears unlikely that clustering factors in

rete testis fluid function as chemotatic molecules, because cell adhesion elicited is not dependent upon motility of cells. We assume that under conditions prevailing in our assay, the cells in suspension are tending to contact each other randomly, under the influence of rotation or gravity. It appears possible that the presence of clustering factors facilitates the formation of stable cohesion forces among similar types of cells in a manner independent of exogenous Ca^{2+}. From a broader viewpoint, the presence of clustering factors investigated may act to facilitate the expression of general surface forces involved in cell cohesion, while expression of specificity of cell interactions is dependent upon other recognition factors essential for homotypic cohesion and subsequent steps involved in structure formation (Frazier and Glaser, 1979). The sequence of interactions among TM-4 cells at varying times after plating in the presence of rete testis fluid (Figs. 2 and 5) indicates that a series of events occurs prior to the formation of the firmly packed, spherical-shaped, cluster of cells with well-demarcated borders.

Experiments are in progress to determine the processes involved in this model system, and the mechanisms by which clusterin in rete testis fluid may influence the behavior of testis cells. The specificity of these phenomena and their physiological significance remain to be determined.

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REFERENCES

Blaschuk, O., Burdzy, K. and Fritz, I. B. (1983). Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. J. Biol. Chem. (in press).

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72:248–254.

Bordy, M. J., Berger, S., Desjardins, C. and Davis, J. C. (1979). Active cell aggregation by immature rat Sertoli cells in primary culture: a role for cell surface glycoproteins. J. Cell Physiol. 99:175–182.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. Physiol. Rev. 52:198–236.

De Philip, R. M., Feldman, M., Spruill, W. A., French, F. S. and Kierzenbaum, A. L. (1982). The secretion of androgen binding protein and other proteins by rat Sertoli cells in culture: a structural and electrophoretic study. Ann. N.Y. Acad. Sci. 383:360–371.

Dorrington, J. H., Roller, N. F. and Fritz, I. B. (1975). Effects of follicle stimulating hormone on cultures of Sertoli cell preparations. Mol. Cell. Endocrinol. 3:57–70.

Fawcett, D. S. (1975). The ultrastructure and functions of the Sertoli cell. In: Handbook of Physiology. vol. V, sec. 7, Male Reproduction (R. O. Greep and F. W. Hamilton, eds.). American Physiological Society, Bethesda, MD, pp. 21–55.

Frazier, W. and Glaser, L. (1979). Surface components and cell recognition. Ann. Rev. Biochem. 48: 491–523.

Friesen, A. D., Jamieson, J. C. and Ashton, F. E. (1971). Effect of nonionic detergent on fractionation of proteins by isoelectric focusing. Anal. Biochem. 41:149–157.

Fritz, I. B. (1973). Selected topics on the biochemistry of spermatogenesis. In: Curr. Top. Cell. Regul. 7:129–174.

Fritz, I. B. (1978). Sites of action of androgens and follicle stimulating hormone on cells of the seminiferous tubule. In: Biochemical Actions of Hormones, vol. 5 (G. Litwack, ed.). Academic Press, New York, pp. 249–281.

Kissinger, C., Skinner, M. K. and Griswold, M. D. (1982). Analysis of Sertoli cell-secreted proteins by two-dimensional gel electrophoresis. Biol. Reprod. 27:233–240.

Lacroix, M. and Fritz, I. B. (1982). The control of the synthesis and secretion of plasminogen activator by rat Sertoli cells in culture. Mol. Cell. Endocrinol. 26:247–258.

Lacroix, M., Smith, F. E. and Fritz, I. B. (1977). Secretion of plasminogen activator by Sertoli cell enriched cultures. Mol. Cell. Endocrinol. 9:227–236.

Loomis, W. F. and Lipmann, F. (1948). Reversible inhibition of the coupling between phosphorylation and oxidation. J. Biol. Chem. 173:807–808.

Mather, J. P. (1980). Establishment and characterization of two distinct mouse testicular epithelial cell lines. Biol. Reprod. 23:243–252.

Millette, C. F. and Bellvé, A. R. (1977). Temporal expression of membrane antigens during mouse spermatogenesis. J. Cell Biol. 74:86–97.

Monesi, V. (1972). Spermatogenesis and the spermatozoa. In: Reproduction in Mammals: Germ cells and fertilization, vol. I (C. R. Austin and R. V. Short, eds.). Cambridge University Press, Cambridge, pp. 46–84.

Moscona, A. A. and Hausman, R. E. (1977). Biological and biochemical studies on embryonic cell-cell recognition. In: Cell and Tissue Interactions (J.

W. Lash and M. M. Burger, eds.). Raven Press, New York.

O'Rand, M. G. and Romrell, L. J. (1977). Appearance of cell surface auto and isoantigens during spermatogenesis in the rabbit. Dev. Biol. 55: 347–358.

Robinson, R. and Fritz, I. B. (1979). Myoinositol biosynthesis by Sertoli cells, and levels of myoinositol biosynthetic enzymes in testis and epididymis. Can. J. Biochem. 57:962–967.

Roosen-Runge, E. C. (1977). The process of spermatogenesis in animals. In: Developmental and Cell Biology Series, vol. 5 (M. Abercrombie, D. R. Neuth and J. G. Torrey, eds.). Cambridge University Press, Cambridge, pp. 1–200.

Russell, L. (1977). Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am. J. Anat. 148:313–328.

Rutishauser, U., Thiery, J. P., Brackenbrug, R., Sela, B. A. and Edelman, G. M. (1976). Mechanisms of adhesion among cells from neural tissues of the chick embryo. Proc. Natl. Acad. Sci. USA 73: 577–581.

Setchell, B. P. (1978). The Mammalian Testis. ELEK Books Ltd., London, pp. 181–248.

Setchell, B. P. and Waites, G.M.H. (1975). The blood testis barrier. In: Handbook of Physiology, vol. 5, Male Reproduction (R. O. Greep and E. W. Hamilton, eds.). American Physiological Society, Bethesda, MD, pp. 143–172.

Skinner, M. K. and Griswold, M. D. (1980). Sertoli cells synthesize and secrete transferrin-like protein. J. Biol. Chem. 255:9523–9525.

Skinner, M. K. and Griswold, M. D. (1982). Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. Biol. Reprod. 27:211–221.

Thiery, J. P., Brackenbury, R., Rutishauser, U. and Edelman, G. M. (1977). Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. J. Biol. Chem. 252:6841–6845.

Tung, P. S. and Fritz, I. B. (1978). Specific surface antigens on rat pachytene spermatocytes and successive classes of germinal cells. Dev. Biol. 64: 297–315.

Tung, P. S., Dorrington, J. H. and Fritz, I. B. (1975). Structural changes induced by follicle stimuating hormone or dibutyryl cyclic AMP on presumptive Sertoli cells in culture. Proc. Natl. Acad. Sci. USA 72:1838–1842.

Voglmayr, J. K., Scott, T. W., Setchell, B. P. and Waites, G.M.H. (1967). Metabolism of testicular spermatozoa and characteristics of testicular fluid collected from conscious rams. J. Reprod. Fertil. 14:87–99.

Volkin, E., Boling, M. E., Jones, M. H., Lee, W. H. and Pike, L. M. (1980). Suppression of the biosynthesis of guanosine triphosphate by protein synthesis inhibitors. J. Biol. Chem. 255:9105–9109.

Webb, J. L. (1966). Enzyme and metabolic inhibitors, vol. 3. Academic Press, New York, pp. 60–87 and 337–365.

Wilson, R. M. and Griswold, M. D. (1979). Secreted proteins from rat Sertoli cells. Exp. Cell Res. 123:127–135.

Wright, W. W., Musto, N. A., Mather, J. P. and Bardin, C. W. (1981). Sertoli cells secrete both testis-specific and serum proteins. Proc. Natl. Acad. Sci. USA 78:7565–7569.