BIOLOGY OF REPRODUCTION 33, 177–186 (1985)

Immunolocalization of Clusterin in the Ram Testis, Rete Testis, and Excurrent Ducts[1]

PIERRE S. TUNG and IRVING B. FRITZ[2]

Banting and Best Department of Medical Research
University of Toronto
112 College Street
Toronto, Ontario M5G 1L6
Canada

ABSTRACT

Clusterin, a glycoprotein that elicits cell aggregation, has previously been isolated from ram rete testis fluid, and has been partially characterized. In experiments reported, we have used monoclonal antibodies against clusterin in combination with indirect immunofluorescence microscopy to investigate the distribution of clusterin in the adult ram testis, rete testis, and excurrent ducts. Tissue blocks (5 mm^3) were fixed in periodate/lysine/paraformaldehyde containing 0.1% glutaraldehyde and, after embedding, 5-μM sections were prepared for immunolocalization. In the testis, 2 basic patterns were observed: 1) strong to moderate staining for clusterin in the adluminal region with little staining in the basal region of the seminiferous epithelium and germinal cells; and 2) moderate staining throughout the seminiferous epithelium between germinal cells. In the rete testis, strong clusterin staining was localized intracellularly in the rete epithelial cells, most often associated with the luminal surface. In the epididymis, intracellular clusterin was localized in some principal cells of the caput epididymidis. The luminal surfaces and spermatozoa within the lumen were strongly positive. In the vas deferens, clusterin staining was associated with the luminal surface only. The presence of clusterin was clearly detected in unwashed isolated epididymal spermatozoa, but not in spermatozoa washed with phosphate-buffered saline containing 0.05% Tween 20.

INTRODUCTION

It is generally accepted that cell interactions play an essential role in the development and cytodifferentiation of the seminiferous epithelium (Fawcett, 1975; Roosen-Runge, 1977). Nevertheless, the molecule(s) implicated in germ cell-somatic cell interactions are at present unknown. Rete testis fluid may contain molecules generated by one or more types of testicular cells that could be involved in cell interactions in the seminiferous tubule (Voglmayr et al., 1967; Blaschuk et al., 1983; Fritz et al., 1983). We have recently isolated and characterized a glycoprotein, clusterin, from ram testis fluid that elicits clustering of several types of cells (Blaschuk et al., 1983; Fritz et al., 1983). In this communication, we report the immunohistochemical localization of clusterin in cells of the testis, rete testis, and excurrent ducts.

Accepted Janury 29, 1985.
Received December 3, 1984.
[1] Work reported from this laboratory was supported by a grant from the Canadian Medical Research Council.
[2] Reprint requests.

MATERIALS AND METHODS

Preparation of Tissue Sections

Testes, epididymides, and vasa deferens of young adult rams (Suffolk breed) were obtained from a local abbatoir in February and March and were transported on ice. Within 30 min the organs were dissected on ice, and 5-mm^3 tissue blocks were fixed by immersion in 4°C periodate/lysine/paraformaldehyde (PLP; McClean and Nakane, 1974) containing 0.1% glutaraldehyde. After fixation at 4°C for 24 h, the tissues were thoroughly washed in phosphate-buffered saline (PBS), dehydrated in graded ethanol, and embedded in Paraplast (Sherwood Medical Ind., St. Louis, MO). Sections of 5-μm thickness were prepared for immunostaining.

Isolation and Processing of Spermatozoa

Spermatozoa were isolated from epididymides of adult rams according to procedures previously described (Tung and Fritz, 1978). Briefly, the epididymides were diced into 1–2-mm blocks in PBS and agitated gently using a plastic pipette with an open-tip diameter of 5 mm. Spermatozoa released were separated from the epididymal fragments by filtering through 100-μm mesh stainless steel grids, and by washing (twice) with cold PBS, followed by centrifugation at 1000 × *g* for 10 min. Resuspended cells were adjusted to 1×10^6 cells/ml, and 10-μl samples were smeared on microscope slides. Slides were air-dried for 15 min at room temperature, after which they were fixed either with: 1) PLP containing 0.1% glutaraldehyde for 30 min,

followed by immersion in methanol at −20°C for 7 min; or 2) with PLP-glutaraldehyde alone. Some slides were left unfixed. All slides were then washed in PBS, and the preparations were either used immediately or stored at −20°C for less than 30 days before examination.

For bulk adsorption, isolated spermatozoa were pelleted and fixed with PLP-glutaraldehyde, followed by washing with PBS. Adsorption was carried out as previously described (Tung et al., 1984). Spermatozoa preparations isolated from epididymides were examined for immunostaining before and after being washed further with PBS containing 0.05% Tween 20 (Bio-Rad, Richmond, CA) (PBS-Tween). Unfixed spermatozoa, accommodated in (15-ml) screw-capped centrifuge tubes, were resuspended in PBS-Tween at 4°C. The tubes were agitated at 4°C for 2 h by rotation at 10 revolutions per min. Spermatozoa were washed three times in PBS without Tween 20, resuspended in PBS, smeared, and fixed as described above.

Monoclonal Antibodies

Lymphocytes prepared from spleen and inguinal lymph nodes of Balb/c female mice previously immunized against partially purified ram clusterin (Blaschuk et al., 1983) were fused with Sp 2/0-Ag 14 myeloma cells. Hybridomas were grown in hypoxanthine/aminopterin/thymidine medium (Oi and Herzenberg, 1980) for 2 wk, after which they were propagated in hypoxanthine/aminopterin medium. Hybridomas generating immunoglobulin were expaned into 24-well Linbro plates, and assessed using an enzyme-linked immunosorbent assay (ELISA) as previously described (Fritz et al., 1985). Hybridomas that secreted immunoglobulin G_1 (IgG_1) against clusterin were cloned by limiting dilution in 96 well plates. Clones were propagated in RPMI 1640 culture medium containing 10% fetal bovine serum, and were used for the production of ascites fluid in female Balb/c mice (Fritz et al., 1985). Immunoglobulin G_1 was purified from ascites fluid by ammonium sulfate fractionation, and ion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia, Quebec, Canada) equilibrated with 20 mM potassium phosphate (pH 8.0) (Good et al., 1980). In experiments reported in this communication, anticlusterin antibody preparations employed were those secreted by a hybridoma named HCn 17 (Blaschuk et al., 1983; Fritz et al., 1985).

Immunohistochemistry

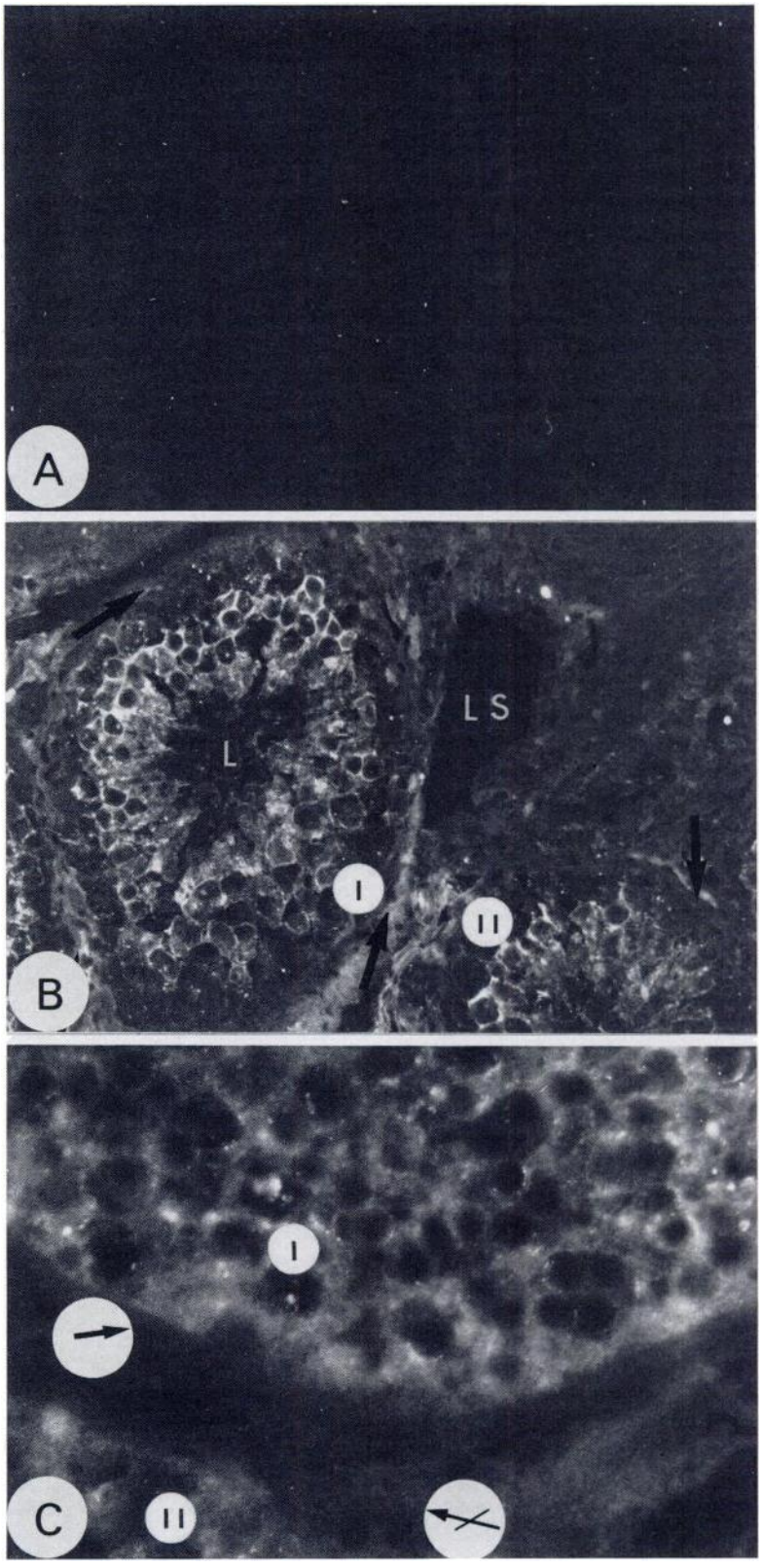
Immediately before immunostaining, tissue sections were deparaffinized and hydrated. Tissue sections or smears were incubated in 1% normal sheep serum in PBS for 30 min at room temperature to block nonspecific staining. The sections were blotted with filter paper, followed by incubation for 30 min with specific monoclonal antibodies at various concentrations in PBS. Sections were washed in PBS-Tween, after which affinity-purified $F(ab')_2$ fragments of sheep antimouse IgG conjugated with fluorescein isothiocyanate (FITC; Cappel Lab., West Chester, PA), diluted 1:10 with PBS, were added. Sections were incubated for another 25 min, followed by additional washing with PBS-Tween. Preparations were mounted in 0.1 M *n*-propyl gallate (Sigma, St. Louis, MO) in glycerol (Giloh and Sedat, 1982), and examined by fluorescent microscopy as previously described (Tung and Fritz, 1978). Controls for the immunofluorescence studies included: 1) monoclonal antibody (IgG_1) against rodent histocompatibility antigen IA^k, which had been demonstrated in pilot experiments to be negative to ram tissues, in place of antibody against clusterin; 2) monoclonal antibody (IgG_1) against clusterin preadsorbed with lyophilized ram rete testis fluid or spermatozoa isolated from ram epididymis (Tung and Fritz, 1984); and 3) PBS alone, followed by FITC-conjugated sheep antimouse IgG. All of these controls showed no specific staining.

RESULTS

The Testis

By indirect immunofluorescence microscopic procedures, material reactive with monoclonal antibody against clusterin was detected in the seminiferous epithelium (Fig. 1). In contrast, the luminal cavity, the boundary tissue of the seminiferous tubule, the interstitial tissues, the lymphatic spaces, the testicular capsule, and blood vessels were either negative or very weakly immunoreactive to monoclonal antibody staining (Fig. 1). Within the seminiferous tubule, two types of staining patterns were consistently observed: 1) strong to moderate staining in the seminiferous epithelium adjacent to the lumen, with very little staining in the basal region and spherical germinal cells (Fig. 1B); and 2) moderate immunostaining throughout the seminiferous epithelium between the spherical germinal cells (Fig. 1C). Sections

FIG. 1. Indirect immunofluoresence micrographs showing distribution of clusterin in the adult ram testis. *A*): A control section stained with monoclonal IA^k antibody (0.1 mg/ml). *B* and *C*: Sections stained with monoclonal clusterin antibody (0.1 mg/ml). Note moderate to intense immunostaining of the seminiferous epithelium between the spherical germinal cells in both *B* and *C*. *B* shows staining in borders of nearly all cells in the adluminal region, and no staining in most cells in the basal region of the tubule. *C* shows both extracellular and intracellular staining with similar intensity throughout the adluminal and basal regions of the epithelium. The lymphatic space (*LS*) and lumen (*L*) are consistently unstained. Peritubular boundary tissues (*arrows*) and interstitial tissues (*cross-arrow*) are weakly or negatively stained. Different tubules are labeled *I* and *II* for orientation. Magnifications: *A*, *B* ×160; *C* ×630.


A
B
L
LS
I
II
C
I
II

incubated with the monoclonal antibody against IA^k, or with PBS were consistently not stained (Fig. 1A). Adsorption of the monoclonal antibody against clusterin with nonwashed epididymal spermatozoa fixed with PLP-glutaraldehyde, or with ram rete testis fluid, also reduced the staining intensities to negligible levels (data not shown).

The Rete Testis

In the rete testis, distribution of material reactive with antibodies against clusterin was characterized by strong intracellular staining between nuclei of both columnar and cuboidal epithelial cells (Fig. 2B–D). The luminal surface was often stained, but the luminal cavity and the connective tissues (mediastinum) underlying the epithelium were not stained.

The Epididymis

Throughout the entire epididymis, material strongly immunoreactive with monoclonal antibody against clusterin was associated with spermatozoa located within the lumen (Fig. 3A–D). The staining intensity decreased from caput (Fig. 3A,B) to cauda (Fig. 3D) epididymidis but was still detectable in the cauda epididymidis (Fig. 3D). Intracellular staining of the epithelium was observed only in isolated areas of the caput epididymidis where diffuse immunoreactive material was localized in both basal and supranuclear regions of principal cells (Fig. 3A,B). The luminal surfaces of caput, corpus, and cauda epididymidis were strongly immunoreactive to monoclonal clusterin antibody (Fig. 3A–D). The interstitium underlying the epididymal cells was not stained (Fig. 3D).

The Vas Deferens

Immunostaining for clusterin was detected in moderate to weak intensities on the luminal surfaces of the vas deferens, and also in unidentified particles in the lumen. No specific intracellular staining was found within the epithelium, nor in the underlying connective tissues and smooth muscle layers (Fig. 4A).

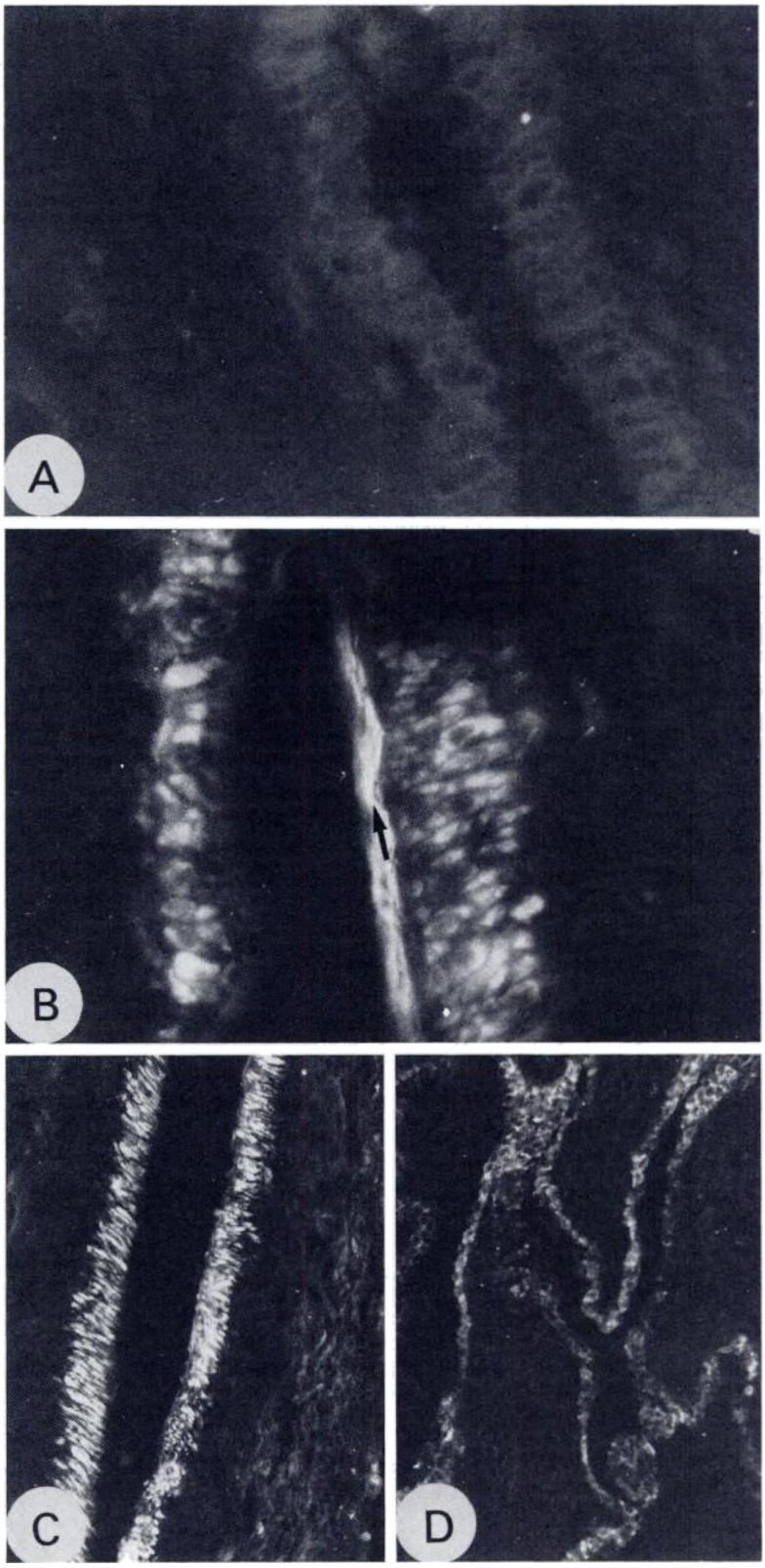
The Spermatozoa

Spermatozoa isolated from ram epididymis specifically stained with the monoclonal antibody against clusterin. Fixation by PLP-glutaraldehyde, with or without permeabilization by methanol, exhibited identical patterns of immunostaining, suggesting that clusterin was associated exclusively with the surface of the spermatozoan. The immunostaining pattern was characterized by a strong to moderate staining intensity on the sperm head, with a punctate appearance. In contrast, the tail was uniformly stained (Fig. 4B). Washing of sperm with PBS-Tween prior to fixation totally removed immunostaining (Fig. 4C), suggesting that clusterin may be nonspecifically bound to spermatozoa.

DISCUSSION

We have reported the immunohistochemical distribution of clusterin in the testis, rete testis, epididymis, vas deferens, and isolated spermatozoa of the ram. Data presented indicate that immunoreactive clusterin can be localized in situ in the seminiferous tubule, with a distribution characteristic of that associated with Sertoli cells. These data are consonant with our previous observations that clusterin can be synthesized by Sertoli cell-enriched preparations in culture (Blaschuk et al., 1983; Blaschuk and Fritz, 1984). In addition, the present paper also demonstrates the apparent intracellular localization of clusterin in rete epithelial cells and in some principal cells of the caput epididymidis. This localization is of particular interest in light of previous findings that rete epithelial cells lack ultrastructural features characteristic of secretory cells (Dym, 1976). Furthermore, we have demonstrated in this paper that clusterin is loosely associated with spermatozoa found in the epididymis and vas deferens, and with the luminal surfaces throughout the epididymis and vas deferens. This is consistent with previous observations that clusterin is present in significant concentrations in epididymal fluids (Fritz et al., 1985). Clusterin was readily removed

FIG. 2. Indirect immunofluorescence micrographs showing distribution of clusterin in the rete testis of an adult ram. *A*: A control section stained with monoclonal IA^k antibody (0.05 mg/ml). *B,C,* and *D*): Sections stained with 0.05 mg/ml monoclonal antibody against clusterin. Note intracellular distribution of immunostaining in columnar cells (*B, C*), and cuboidal cells (*D*) in the rete epithelium. Portions of the luminal surface (*arrow*) are also stained (*B*), whereas the mediastinum surrounding the lumen is weakly or negatively stained. Magnifications: *A,B* ×630; *C,D* ×160.


A
B
C
D

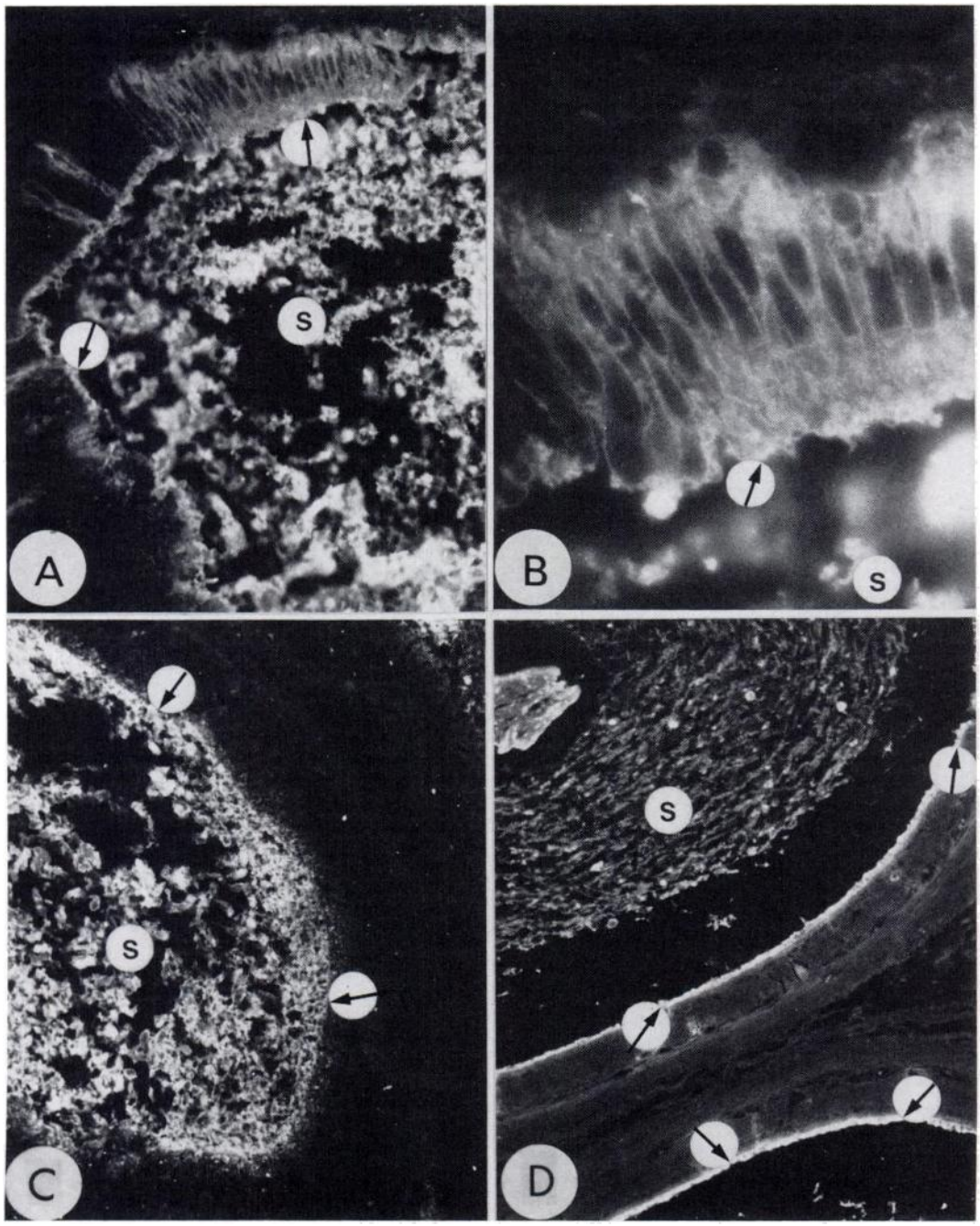


FIG. 3. Indirect immunofluorescence micrographs showing distribution of clusterin in the caput (*A* and *B*), corpus (*C*), and cauda epididymidis (*D*). Note occasional intracellular immunostaining principal cells of the caput epididymidis (*A* and *B*), but negative intracellular staining in epithelial cells of the corpus and cauda epididymidis (*C* and *D*). The spermatozoa (*s*) in the lumen and the luminal surfaces (*arrows*) are consistently positive throughout the epididymis, but the connective tissues underlying the epithelium are unstained. Magnifications: *A*,*C*, and *D* ×160; *B* ×630.

from unfixed sperm by washing (Fig. 4), but remained bound to sperm in fixed tissue sections (Fig. 3). We interpret these data to indicate that clusterin is nonspecifically bound to spermatozoa, but that it can be cross-linked to sperm surfaces by treatment with glutaraldehyde.

In the mature testis, two basic (adluminal and panepithelial) patterns of distribution of clusterin were observed within the seminiferous tubule. The variations in the distribution of clusterin might be dependent on the stage of the cycle of the seminiferous epithelium, a phenomenon known to occur in the localization of androgen-binding protein (Ritzen et al., 1980; Attramadal et al., 1981) and in the distribution of plasminogen activator (Lacroix et al., 1981; Fritz and Karmally, 1983). In the mature testis, the tight junctional complexes among Sertoli cells limit the transport of fluids and macromolecules between the basal and adluminal compartments (Fawcett, 1975; Setchell and Waites, 1975). Clusterin is probably secreted by Sertoli cells primarily into the adluminal portion of the seminiferous epithelium, released into the tubular lumen, and then transported into the rete testis. In some sections, clusterin was also detected in the basal region of the epithelium. Relatively small quantities could have been released into the lymphatics, accounting for the clusterin present in testicular lymph (Fritz et al., 1985). Androgen-binding protein release into the basal compartment is also known to occur (Bardin et al., 1981). The blood-testis barrier has been shown to be structurally (Dym, 1976) and functionally (Tung et al., 1971) incomplete in the rete testis. Nevertheless, most protein molecules do not seem to escape from the plasma across the rete epithelium into the lumen (Setchell and Waites, 1975; Dym, 1976). It remains possible that small quantities of clusterin could have been released from the basal surfaces of rete epithelial cells through the poorly vascularized mediastinum (Kormano and Suoranta, 1971; Dhingra, 1977) into the testicular lymph, especially if the rete testis epithelial cells synthesize clusterin.

The intracellular localization of clusterin in rete epithelial cells indicates a cell-specific distribution within the tissue. The data indicate that rete cells may be capable of clusterin biosynthesis, or that they absorb clusterin from the rete testis fluid. Absorption by rete testis epithelial cells seems to be minimal, as judged by the observations that Thorotrast injected into the lumen of hamster and rat rete testes is rarely detected within rete epithelial cells (Dym, 1976). Although lacking the ultrastructure suggestive of active secretory functions (Dym, 1976), rete epithelial cells have been demonstrated to possess secretory activities (Renzali, 1957; Goyal and Dhingra, 1973, Setchell, 1974). Using cultures of enriched preparations of ram rete testis cells for immunocytochemical localization and immunoassay, we have recently observed that rete testis cells synthesize clusterin to an extent comparable to that detected in Sertoli cell-enriched preparations in culture (Tung and Fritz, in preparation). We have previously reported that the concentrations of clusterin in epididymal fluid (30–120 μg/ml) are only moderately lower than those in rete testis fluid (80–200 μg/ml) (Fritz et al., 1985). By contrast, concentrations of clusterin in testicular lymph (4–16 μg/ml) and systemic plasma (0.8–3.2 μg/ml) are very low in comparison to those in rete testis or epididymal fluids (Fritz et al., 1985). These observations suggest that clusterin is poorly resorbed by epithelial cells throughout the rete and the excurrent ducts. We interpret available data to indicate that immunoreactive clusterin localized intracellularly in rete epithelial cells represents active in situ biosynthesis rather than resorption of this molecule. The data also suggest that clusterin in rete testis fluid is derived from rete epithelial cells as well as from Sertoli cells. The precise amounts contributed by each class of cells in vivo remain to be determined.

Clusterin induces marked aggregation of several cell types in vitro (Blaschuk et al., 1983; Fritz et al., 1983). Its possible role in cell-cell interactions in the testis has previously been postulated (Fritz et al., 1983; Blaschuk and Fritz, 1984), but it has not been ruled out that high levels of clusterin within the rete and the excurrent ducts may have functions other than those involved in cell cohesion. For example, clusterin could be a candidate among molecules uniquely present in high concentrations in testicular and epididymal fluids that play a role in providing immunologic privilege to germinal cells. It is generally accepted that the seminiferous tubule barrier (often erroneously called the blood-testis barrier) protects germinal cells in the adluminal compartment from the immunologic surveillance system (Dym and Fawcett, 1970; Johnson, 1973; Gilula et al., 1976; Neaves, 1977). However, other factors may be involved in shielding the germinal cells

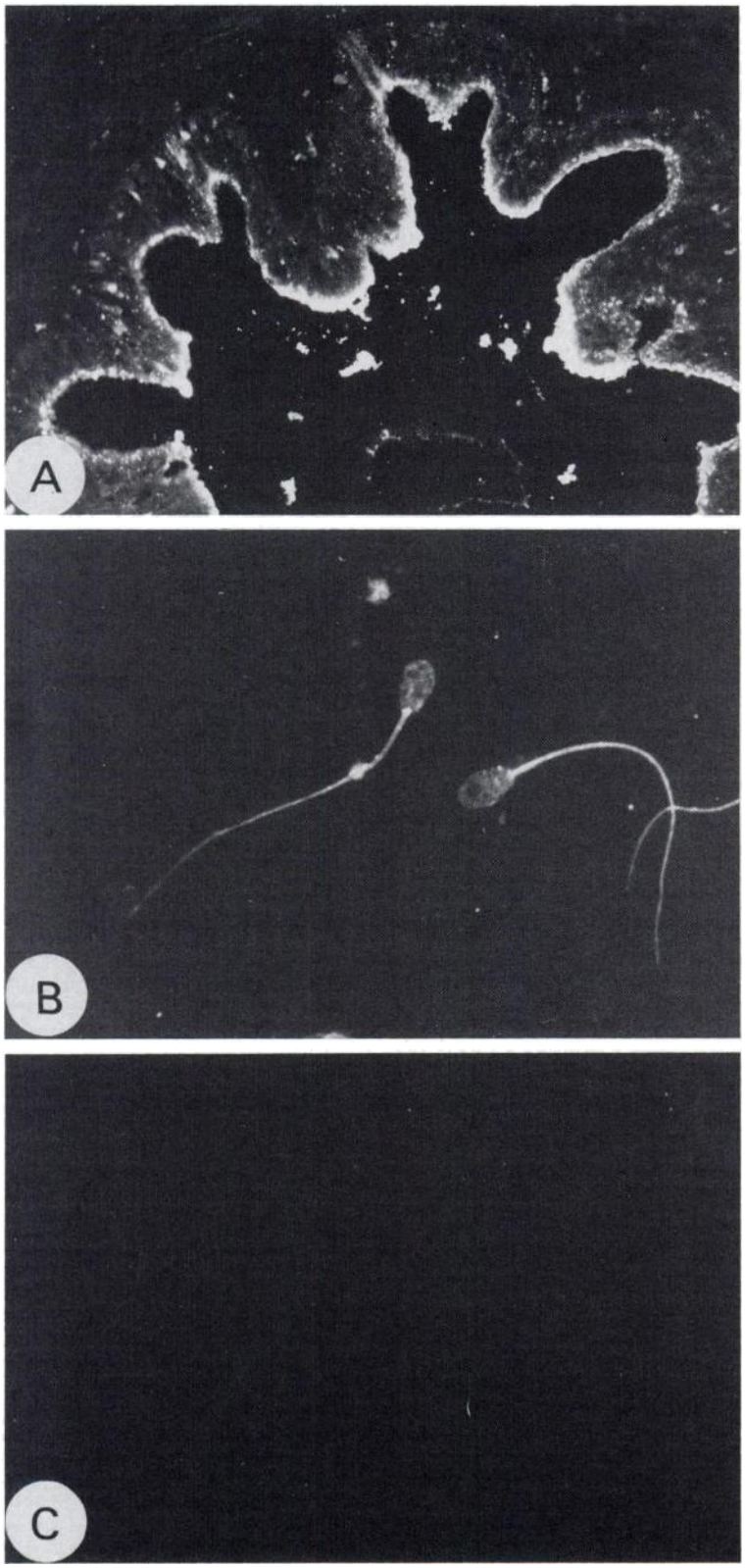

A
B
C

FIG. 4. Localization of clusterin by indirect immunofluorescence in the ram vas deferens (*A*); spermatozoa isolated from the epididymis and washed with PBS (*B*); and epididymal spermatozoa washed with PBS-Tween (*C*). Note strong immunostaining associated with the luminal surface and residues in the vas lumen (*A*) and with epididymal spermatozoa washed only with PBS (*B*), and the total removal of specific staining from spermatozoa washed with PBS-Tween (*C*). Magnifications: *A* X 160; *B* and *C* X640.

from potentially deleterious immunoglobulins and self-reactive lymphocytes (Tung and Fritz, 1984), especially in the rete testis, the epididymis, and the vas deferens duct, in which the barrier is functionally incomplete in secluding spermatozoa from immunoreactants (Kormano, 1967; Koskimies et al., 1971; Tung et al., 1971; Johnson, 1972; Koskimies and Kormano, 1973; Suzuki and Nagano, 1978). Lymphocytes are present among the resident epithelial cells of the rete testis and epididymis (Dym and Romrell, 1975), suggesting that the potentially immunogenic sperm cells in the lumen are indeed under immunosurveillance. It therefore seems plausible to postulate the presence of components in these portions of the male reproductive tract that act as local immunosuppressants. Experiments are in progress to determine if clusterin may have local immunosuppressing activity, perhaps by blocking recognition by lymphocytes of germinal cells as "foreign."

ACKNOWLEDGMENTS

We thank Dr. O. Blaschuk for stimulating discussion, Krystyna Burdzy and Lyn Dean for excellent technical assistance, and Fern Teodoro and Donna McCabe for typing the manuscript. We are grateful to Dr. Terry L. Delovitch for providing ascites fluid containing mouse monoclonal antibody against the rodent histocompatibility antigent IA^k.

REFERENCES

Attramadal, A., Bardin, C. W., Gunsalus, G. L., Musto, N. A. and Hasson, V. (1981). Immunochemical localization of androgen binding protein in rat Sertoli and epididymal cells. Biol. Reprod. 25:983–988.

Bardin, C. W., Muato, N. A., Gunsalus, G. L., Kotite, N., Cheng, S.-L., Larrea, F. and Becker, R. (1981). Extracellular androgen binding proteins. Annu. Rev. Physiol. 43:189–198.

Blaschuk, O. and Fritz, I. B. (1984). Isoelectric forms of clusterin isolated from ram rete testis fluid and from secretions of primary cultures of ram and rat Sertoli cell-enriched preparations. Can. J. Biochem. Cell Biol. 62:456–461.

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. 258:7714–7720.

Dhingra, L. D. (1977). Mediastinum testis. In: The Testis: Advances in Physiology, Biochemistry and Function, Vol. IV (A. D. Johnson and W. R. Gomes, eds.). Academic Press, New York, pp. 451–460.

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

Dym, M. and Fawcett, D. W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol. Reprod. 3:308–326.

Dym, M. and Romrell, L. J. (1975). Intraepithelial lymphocytes in the male reproductive tract of rats and rhesus monkeys. J. Reprod. Fertil. 42:1–7.

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.

Fritz, I. B., and Karmally, K. (1983). Hormonal influences on formation of plasminogen activator by cultured testis tubule segments of defined stages of the cycle of the seminiferous epithelium. Can. J. Biochem. Cell Biol. 61:553–560.

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

Fritz, I. B., Blaschuk, O. W. and Burdzy, K. (1985). Properties of clusterin, a glycoprotein which elicits cell aggregation, and immunochemical determination of levels in ovine tissues. In: Symposium on Gonadal Peptides (M. R. Sairam and L. E. Atkinson, eds.). World Scientific Publishing Co., Philadelphia.

Giloh, H. and Sedat, J. W. (1982). Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Science 217:1252–1255.

Gilula, N. B., Fawcett, D. W. and Aoki, A. (1976). The Sertoli cell occluding junctions and gap junctions in mature and developing mammalian testes. Dev. Biol. 50:142–168.

Good, H. A., Wofsy, L., Kimura, J. and Henry, C. (1980). In: Selected Methods in Cellular Immunology (B. B. Mishell and S. M. Shiigi, eds.). W. H. Freeman and Co., San Francisco, pp. 278–281.

Goyal, H. O. and Dhingra, L. D. (1973). Postnatal study on the histology and histochemistry of mediastinum testis in buffalo (Bubalus bubalis) from birth to one year. Acta. Anat. 86:65–70.

Johnson, M. H. (1972). The distribution of immunoglobulin and spermatozoal autoantigen in the genital tract of the male guinea pig: its relationship to autoallergic orchitis. Fertil. Steril. 23: 383–392.

Johnson, M. H. (1973). Physiological mechanisms for the immunological isolation of spermatozoa. Adv. Reprod. Physiol. 6:279–324.

Koskimies, A. I. and Kormano, M. (1973). The proteins in fluids from the seminiferous tubules and rete testis of the rats. J. Reprod. Fertil. 34: 433–444.

Koskimies, A. I., Kormano, M. and Lahti, A. (1971). A difference in the immunoglobulin content of seminiferous tubule fluid and rete testis fluid of the rat. J. Reprod. Fertil. 27:463–465.

Kormano, M. (1967). Dye permeability and alkaline phosphatase activity of testicular capillaries in the postnatal rat. Histochemie 9:327–338.

Kormano, M. and Suoranta, H. (1971). Microvascular organization of the adult human testis. Anat. Rec. 170:31–39.

Lacroix, M., Parvinen, M. and Fritz, I. B. (1981). Localization of testicular plasminogen activator in discrete portions (stages VII and VIII) of the seminiferous tubule. Biol. Reprod. 25:143–146.

McClean, I. W. and Nakane, P. K. (1974). Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectronmicroscopy. J. Histochem. 32:1077–1087.

Neaves, W. B. (1977). The blood-testis barrier. In: The Testis, Vol. 4 (A. D. Johnson and W. R. Gomes, eds.). Academic Press, New York, pp. 125–161.

Oi, V. T. and Herzenberg, L. A. (1980). In: Selected Methods in Cellular Immunology (B. B. Mishell and S. M. Shiigi, eds.). W. H. Freeman and Co., San Francisco. pp. 289–301.

Renzali, A. (1957). Secretory epithelium of the rete testis in bull. Atti. Soc. Ital. Sci. Vet. 10:502.

Ritzen, E. M., Parvinen, M., Hansson, V. and French, F. S. (1980). Role of the Sertoli cell in spermatogenesis. VIth International Congress Endocrinology, Melbourne Program, Abstr. S-25.

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

Setchell, B. P. (1974). Secretion of the testis and epididymis. J. Reprod. Fertil. 37:165–177.

Setchell, B. P. and Waites, G.M.H. (1975). The blood-testis barrier. In: Handbook of Physiology, Vol. V, Sect. 7, Male Reproduction (R. O. Greep and F. W. Hamilton, eds). American Physiological Society, Bethesda, MD, pp. 143–172.

Suzuki, F. and Nagano, T. (1978). Development of tight junctions in the caput epididymal epithelium of the mouse. Dev. Biol. 63:321–334.

Tung, K.S.K., Unanue, E. and Dixon, F. (1971). Pathogenesis of experimental allergy orchitis. II. The role of antibody. J. Immunol. 106:1463–1472.

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. and Fritz, I. B. (1984). Autoimmune orchitis can be induced by thymic lymphocytes autosensitized against syngeneic Sertoli cells *in vitro*. Biol. Reprod. 30:979–990.

Tung, P. S., Skinner, M. K. and Fritz, I. B. (1984). Fibronectin synthesis is a marker for peritubular cell contaminants in Sertoli cell-enriched cultures. Biol. Reprod. 30:199–211.

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.