Localization of Sulfatoxygalactosylacylalkylglycerol at the Surface of Rat Testicular Germinal Cells by Immunocytochemical Techniques: pH Dependence of a Nonimmunological Reaction between Immunoglobulin and Germinal Cells

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ABSTRACT The synthesis of sulfatoxygalactosylacylalkylglycerol (SGG) is a marker of germinal cell differentiation during spermatogenesis. Antibodies raised against this lipid have been used to visualize SGG on the surfaces of rat spermatocytes and spermatids. An ionic interaction between SGG and immunoglobulin was shown to occur at physiological pH, resulting in high fluorescence backgrounds for control cells treated with nonimmune sera. Immunofluorescence was therefore performed at alkaline pH such that this interaction was much reduced or eliminated. A method was also developed to detect surface-bound complement fixed in the presence of anti-SGG. SGG was found to be mobile within the plane of the membrane, undergoing ligand-induced "patching" and occasional "capping." However, this phenomenon was independent of temperature.

Male germ cell differentiation offers a unique opportunity to study the biochemistry of differentiation. The strict synchrony of events, dramatic structural reorganization (28) and implicit role of intercellular communication make spermatogenesis an ideal system for the study of cell membrane differentiation. 1-0-hexadecanyl-2-0-hexadecanoyl-3-0-β-[galactopyranosyl(3-0-sulfate)]-glycerol (sulfatoxygalactosylacylalkylglycerol, SGG)[1] is the major glycolipid of mature testes from all mammalian species studied so far (7, 13, 16, 21). It has been shown in SGG both from the rat (12) and from the pig (7) that >90% of the constituent fatty acids were palmitic and >80% of the glyceryl ethers were chimyl. SGG is present in relatively small amounts in brain (17). Testicular SGG appears to be present mainly in germinal cells (12). Sulfation of galactosylacylalkylglycerol (GG) to SGG has been shown to be restricted to a brief phase early in spermatogenesis (11, 14) and SGG is maintained in subsequent germinal cell stages without apparent turnover (11), despite cell division and extensive membrane reorganization. We have recently reported (18) the preparation in rabbits of complement-fixing antibodies against SGG (anti-SGG). In the present study anti-SGG has been used to show the presence of SGG on the surfaces of various rat testicular germinal cells. During the course of the work, it was noted that, at pH 7.4, SGG gave a strong precipitation line by counterimmunoelectrophoresis (CIE) not only with anti-SGG but also with nonimmune immunoglobulin and with other basic proteins; it was therefore not surprising to find that rat testicular germinal cells reacted with both immune and nonimmune sera at pH 7.4. The nonimmunological reaction between SGG and immunoglobulins was believed to be ionic in nature and, indeed, at pH 8.6 there was a marked reduction of both the interaction of nonimmune sera with SGG as detected by CIE and with germinal cells as determined by immunofluorescence. The use of pH 8.6 therefore allowed the demonstration by immunofluorescence techniques of SGG on germinal cell surfaces.

MATERIALS AND METHODS

Immunofluorescence

IgG antibodies that reacted specifically with SGG were raised in female rabbits by repeated i.v. injection as previously described (18). In some experiments anti-SGG was first purified from the crude serum by elution from an affinity

[1] Previously referred to as sulfogalactoglycerolipid.

matrix comprising SGG adsorbed onto cholesterol particles (18). Most experiments were performed using crude or ammonium sulfate fractionated serum. Sera from nonimmunized rabbits or rabbits immunized without specific antigen were used in control incubations. Unless otherwise specified, cells were prepared from the testes of adult (300–350 g) Sprague-Dawley rats by gentle mechanical dissociation (14) in phosphate-buffered saline (PBS). Cell aggregates and seminiferous tubule fragments were allowed to sediment at 1 *g* for 10 min at room temperature. The supernate was centrifuged for 5 min at 1,000 *g*, and the cell pellet was washed three times with PBS. Spermatozoa were prepared by extrusion from the epididymis. Cell surface SGG was detected by indirect immunofluorescence. In initial experiments at pH 7.4, cells were fixed by prior incubation in 2.5% glutaraldehyde for 30 min followed by washing twice with PBS. Fixed cells were incubated with anti-SGG or control serum for 1 h at room temperature, washed twice with PBS, and incubated for a further 30 min with fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.). Cellular fluorescence was then observed under a Leitz Orthoplan fluorescence microscope. Later experiments at pH 8.6 were carried out by washing fixed cells once with TBS (0.05 M Tricine-buffered saline pH 8.6) followed by incubation with control or immune sera that had previously been dialyzed against TBS; after 1 h at room temperature, cells were washed twice with TBS, treated with fluorescein-conjugated IgG at pH 7.4 as described above, and cellular fluorescence was observed. In some experiments, unfixed cells were washed with TBS containing 1% fructose followed by incubation with anti-SGG or control serum (previously dialyzed against fructose containing TBS) for 1 h at room temperature or 4°C. After being washed with the same buffer, cells were treated with fluorescent conjugate for an additional 30 min at the same temperature. Cell fluorescence was then monitored immediately under the fluorescence microscope or cells were transferred to 37°C for 30 min before observation.

In some experiments use was made of the ability of the hyperimmune anti-SGG to fix complement in the presence of SGG. Testicular cell suspensions were fixed with glutaraldehyde as described above. After washing, the cells were first incubated with an equal volume of guinea pig serum (1 h, room temperature) to block any inherent complement binding activity. The cells were then washed twice with PBS (pH 7.4), and aliquots were incubated at room temperature in the presence of anti-SGG or control serum (previously incubated at 56°C for 20 min to inactivate endogenous complement) containing 10% normal human serum. Cells were washed twice and incubated (30 min, room temperature) with goat anti-human C_3 (Miles Laboratories, Inc.). This antiserum showed strict species specificity as judged by Ouchterlony precipitation in agar. Finally, the cells were incubated for 30 min with fluorescein-conjugated rabbit anti-goat immunoglobulin (Miles Laboratories, Inc.), washed, and mounted in phosphate-buffered glycerine, and cell surface fluorescence was monitored under the fluorescence microscope.

Anti-SGG and control sera were routinely used undiluted (100 $\lambda/10^5$ cells). A similar staining pattern was observed using antibody dilutions of up to 1:5 for both methods of fluorescence.

CIE

CIE was carried out in 0.5% agarose (Aldrich Chemical Co., Milwaukee, Wis.) in 50 mM Tris-HCl, pH 7.4, as previously described (18). Serum samples (5 μl) were placed in the anode well and electrophoresed against glycolipid antigens (5 μl, 1 mg/ml 50 mM Tris) in the cathode well for 5 min using an Immunobox electrophoretic chamber (E-C Apparatus Corp., St. Petersburg, Fla.). CIE was repeated under similar conditions except that all buffers were adjusted to pH 8.6.

Electron Microscopy

Testicular cell suspensions were treated with glutaraldehyde and washed with TBS, and aliquots were incubated with anti-SGG or control serum at pH 8.6 at room temperature for 1 h, as described above. The cells were then washed three times with TBS and incubated for an additional 30 min in the presence of ferritin-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc.). Antibody-treated cells were then washed twice with PBS, and the cell pellet was fixed in 0.1 M phosphate buffer containing 1% osmium tetroxide. The cells were embedded in Araldite/Epon and sections (60–90 nm) were observed under a Philips ER 300 electron microscope at 60 kV.

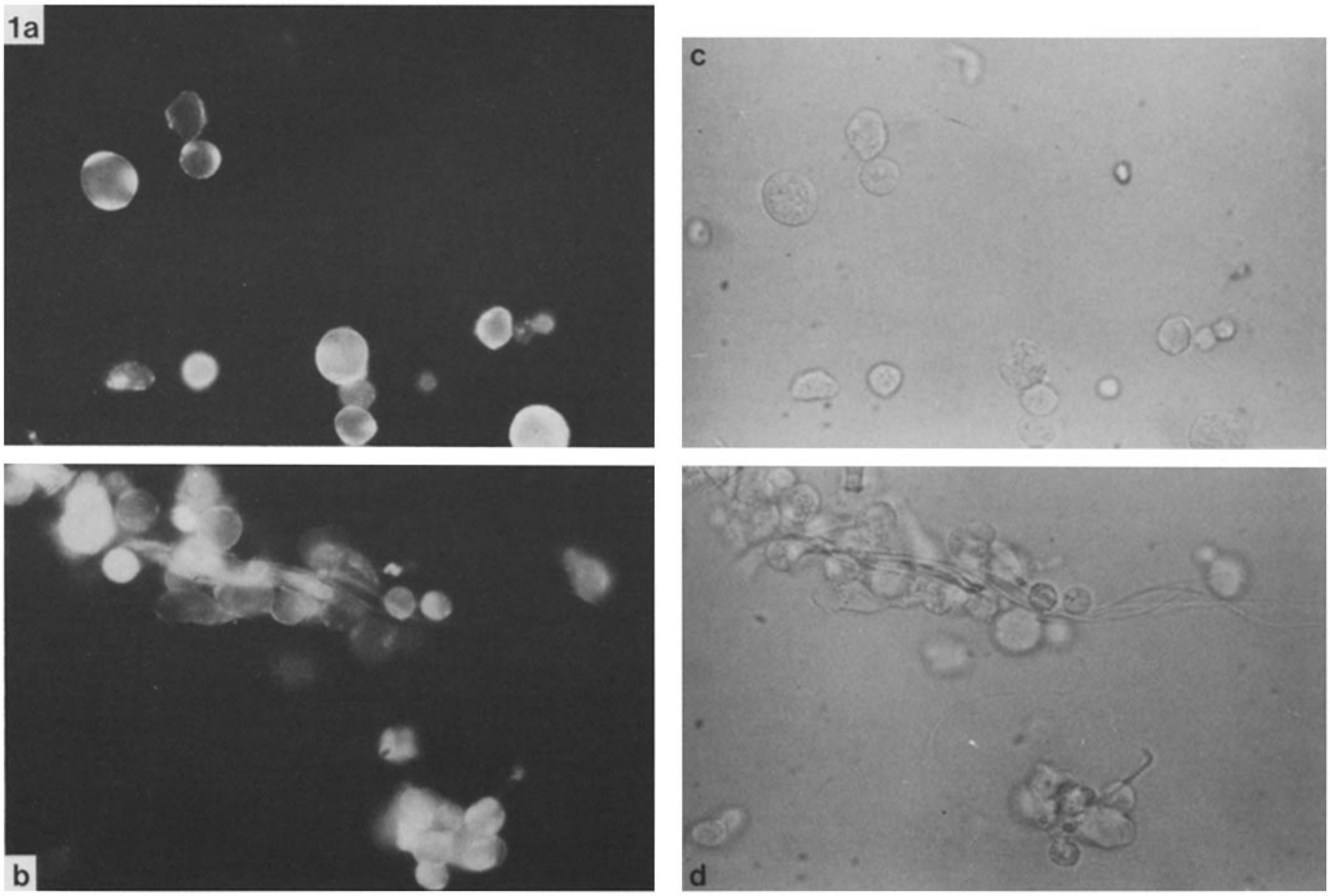


FIGURE 1 Indirect immunofluorescence of testicular cells stained at physiological pH: *a* and *c*, anti-SGG; *b* and *d*, control serum; *a* and *b*, incident UV illumination; *c* and *d*, phase contrast. Bar, 2 μm.

Cell Purification

Pachytene spermatocytes were prepared from the testes of 20 23-d-old rats and round (early) spermatids were prepared from two adult rat testes. Testes were stripped of their tunica and subjected to mechanical dissociation using a razor blade as previously described (14). The mince was suspended in 20 ml of enriched Krebs-Ringer's bicarbonate buffer (EKRB) as described by Bellvé et al. (1) but neither collagenase nor trypsin was used to aid dissociation. Tubules and large clumps were allowed to settle at room temperature for 5 min, the supernate was decanted, and the residue was resuspended in 20 ml of EKRB and again allowed to settle for 5 min. The washing procedure was carried out three times and the combined supernates were loaded on a Beckman elutriator rotor driven by a Beckman J21C centrifuge exactly as described by Grabske et al. (5). Fractions were collected according to the schedule of Meistrich (19) for rat testicular cells. Fractions were centrifuged for 10 min at 800 *g* to sediment cells. A portion of these cells was suspended in buffer and examined with a phase-contrast microscope; cells were also examined by electron microscopy as previously described (14). The pachytene spermatocyte preparation was 82% enriched and the spermatid preparation was 90% enriched according to electron microscopy criteria.

RESULTS

Glutaraldehyde-fixed testicular cells showed membrane staining when treated with anti-SGG at pH 7.4 as described in the Materials and Methods (Fig. 1 *a*). Similarly treated rat peripheral blood cells showed no fluorescence. Surprisingly, however, serum from normal rabbits or from control immunized rabbits showed immunofluorescence with testicular cells similar to anti-SGG (Fig. 1 *b*). Whole serum or 33% ammonium sulfate precipitated immunoglobulin gave similar results.

Anti-SGG activity present in the hyperimmune serum was originally measured by use of an assay in which complement-fixing anti-SGG/SGG immune complexes were detected by a complement-dependent hemolysin indicator system (18). By this assay nonimmune and control immune sera showed no reactivity towards SGG. However, precipitating activity against SGG could be detected in both control sera and anti-SGG by CIE at pH 7.4 (Fig. 2). Each serum showed a strong precipitation line when electrophoresed against SGG, a reduced reactivity towards GG and cerebroside sulfate but no reaction with

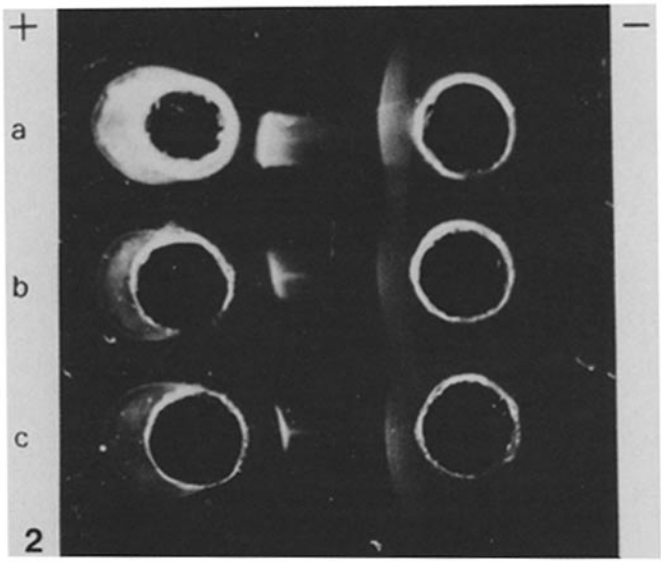


FIGURE 2 Counterimmunoelectrophoresis (CIE) at pH 7.4. Anti-SGG in the anodal well was electrophoresed as described in Materials and Methods against (*a*) SGG, (*b*) cerebroside sulfate, and (*c*) galactosylacylalkylglycerol (GG). After electrophoresis, anti-rabbit Ig was added to the cathode well and allowed to precipitate at 4°C overnight. Similar results were obtained using nonimmune or control serum.

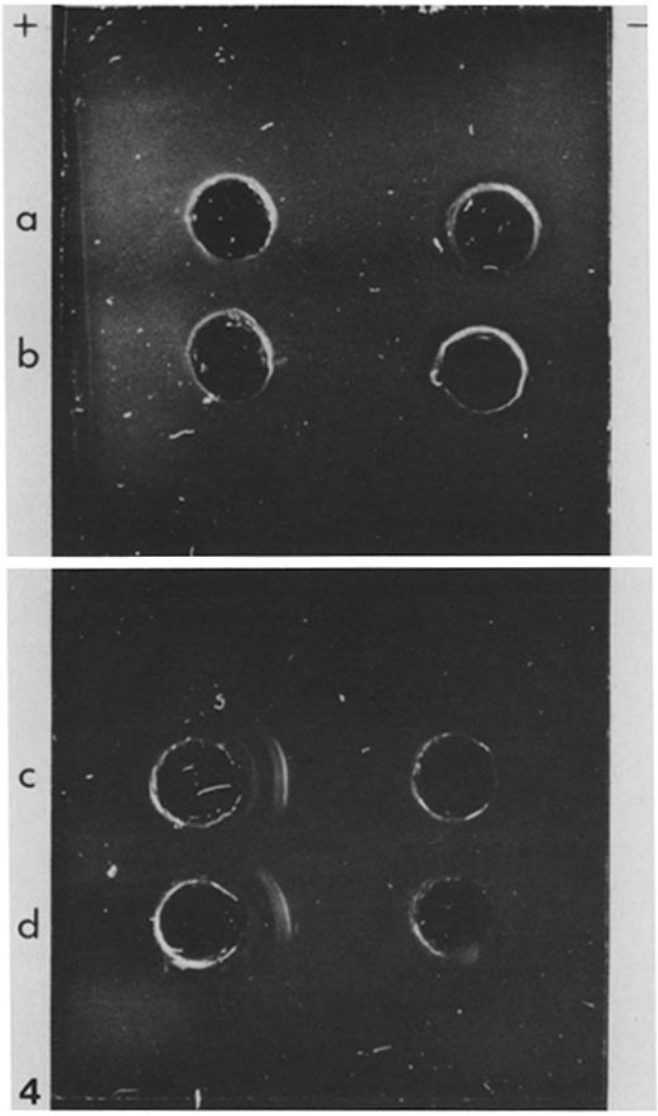


FIGURE 3 Effect of pH on CIE. Antibody samples in the anodal wells were electrophoresed against SGG at pH 8.6 (upper panel) and pH 7.4 (lower panel): (*a* and *c*) anti-SGG; (*b* and *d*) nonimmune serum.

other charged or neutral glycolipids (hematoside, ceramide monohexoside, ceramide dihexoside; data not shown). This precipitation results, we believe, from a nonimmunological electrostatic interaction, because other basic proteins (e.g., myelin basic protein) also precipitate preferentially with SGG when assayed by CIE at pH 7.4 (data not shown); myelin basic protein gave a weak reaction with cerebroside sulfate but no reaction at all with GG or cerebroside. This hypothesis is supported by the finding that precipitation with SGG was found not to occur with either anti-SGG or control sera if CIE was performed at pH 8.6 (Fig. 3) (a faint precipitation line does develop at pH 8.6 for all sera if electrophoresis is continued for 1 h). The lack of a precipitin line between anti-SGG and SGG with CIE at pH 8.6 shows that anti-SGG is a nonprecipitating antibody, because anti-SGG will fix complement in the presence of SGG at pH 8.6 (18); at this pH, the serum behaves as a classic complement-fixing, nonprecipitating antibody. To determine whether the antigen binding activity detected by complement fixation was sufficient for immunofluorescence, we repeated the immunofluorescence experiments at pH 8.6 as

described in Materials and Methods. This procedure effectively eliminated the fluorescent staining observed with nonimmune or control sera at pH 7.4 (Fig. 4*b*). Anti-SGG serum retained its reactivity towards testicular cells at pH 8.6 (Fig. 4*a*).

To further verify these results, we developed an alternate method to circumvent the positive staining of cells by control or nonimmune sera. The method was devised to visualize complement specifically bound at the site of antigen/antibody complex formation on the testicular cell surface. Anti-SGG serum will fix complement specifically in the presence of SGG. Control or nonimmune sera show no such activity. In the case of plasma membrane SGG, anti-SGG will fix complement at the cell surface antigen site. To prevent complement-mediated lysis, cells were first prefixed with glutaraldehyde as described above. Fixed testicular cells were then pretreated with guinea pig serum to block any endogenous complement-binding activity and treated at pH 7.4 with heat-inactivated anti-SGG or nonimmune serum in the presence of 10% fresh human serum as complement source. Cell-bound human complement was then detected by a specific indirect immunofluorescence technique as described in Materials and Methods. Only cells treated with anti-SGG showed positive fluorescence for membrane-bound complement (Fig. 5). Nonimmune serum showed no complement-fixing activity in the presence of SGG (18) and also showed no complement binding at the cell surface.

The above three methods for staining fixed cells with anti-SGG gave similar cell surface fluorescent patterns (cf. Figs. 1, 4*a*, and 5*a*). Similar results were also obtained by indirect immunofluorescence at pH 8.6 with affinity-purified anti-SGG (data not shown). The fluorescence seemed to fall into two patterns. The first type is a continuous membrane staining characterized by fluorescent rings at the cell periphery; a cell preparation enriched in spermatids (Fig. 6*a*) showed primarily this type of fluorescence. The second type shows localized and discontinuous areas of intense fluorescence implying an asymmetric distribution of SGG on the cell surface; this pattern was observed most frequently in cell populations enriched in pachytene spermatocytes (Fig. 6*b*). Cell samples prepared from the testes of rats 11 d after birth, when Sertoli cell precursors and spermatogonia are the predominant cell types (28), show no fluorescence after treatment with anti-SGG at pH 8.6. More surprisingly, spermatozoa from the epididymis were also negative for SGG staining (data not shown).

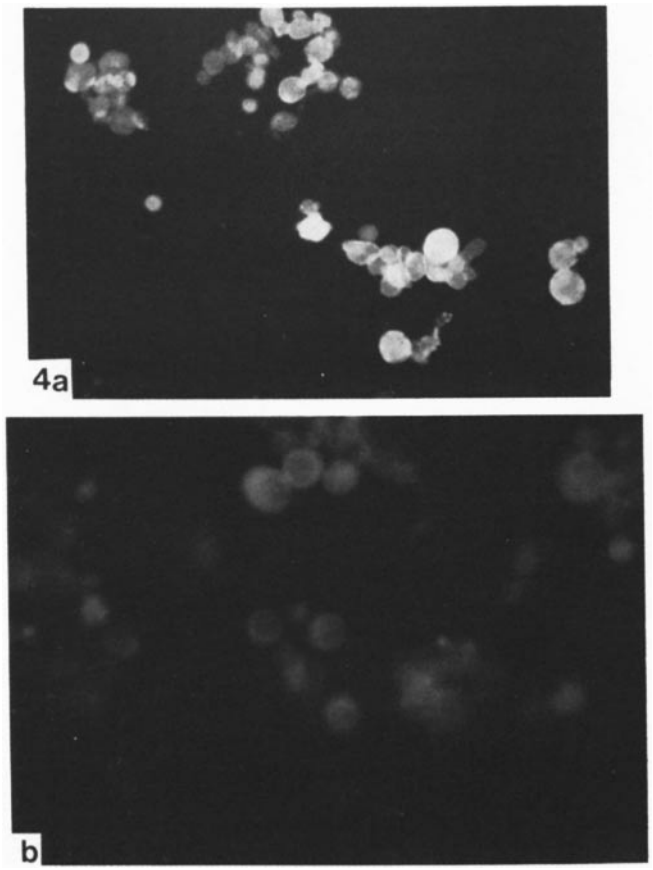


FIGURE 4 Indirect immunofluorescence of fixed testicular cells stained at pH 8.6. Serum samples were dialysed against saline containing 0.05 M Tricine/NaOH buffer, pH 8.6 (TBS). Cells were washed once with TBS before antibody treatment. (*a*) Anti-SGG-treated cells; (*b*) control serum-treated cells.

Preliminary electron microscope immunocytochemistry has been carried out with anti-SGG. Ferritin-labeled anti-rabbit IgG showed a discontinuous or "micropatched" distribution along the surface membrane of spermatids and spermatocytes previously treated with anti-SGG at pH 8.6 (Fig. 7). No surface ferritin was observed with a control serum lacking anti-SGG activity. More detailed immunoelectron microscopy experiments will be reported elsewhere.

Preliminary experiments were also performed with unfixed testicular cell suspensions to investigate the ability of cell surface SGG to cap in the manner described for other cell surface components, both proteins (2, 4, 26) and lipids (24, 29). Cells were treated with antibody at 4°C. Labeled cells were then incubated at 37°C for various times and observed under the fluorescence microscope. Unlike results reported for other systems, this did not induce cap formation in the majority of testicular cells. Most cells showed a patched fluorescence (a typical example is shown in Fig. 8*a*) and a small percentage of cells showed "caplike" localization of fluorescence (Fig. 8*b*). However, the relative frequency of these cells did not increase on incubation of cells at 37°C (Fig. 8*e*).

DISCUSSION

SGG is the major glycolipid of the mature testes of every mammal so far tested. With the exception of the brain, in which it is a minor fraction, SGG is found in no other organ of the rat.[2] The discrete synthesis and metabolic conservation of SGG during spermatogenesis strongly imply a specific germinal cell function for this glycolipid. Previous studies have shown that the synthesis of SGG during the first wave of spermatogenesis occurs, in the rat, between 15 and 20 d after birth, corresponding to the appearance of early primary spermatocytes (12). However, purified preparations of pachytene spermatocytes do not show an enhanced rate of SGG synthesis when compared with the total cell complement (14), which implies that SGG is synthesized before the pachytene spermatocyte stage. The present experiments have shown by both immunofluorescence (Fig. 6) and immunoelectron microscopy (Fig. 7) that this glycolipid is present on the surface of primary spermatocytes and round spermatids. The immunofluorescence results suggest that the surface expression of SGG is reduced for spermatids. As expected, no cell surface fluorescence was detected for cells prepared from the testes of 11-d-old rats when

[2] Lingwood et al. Tissue distribution of sulfolipids in the rat—restricted location of sulfatoxygalactosylacylalkylglycerol. *Can. J. Biochem.* In press.

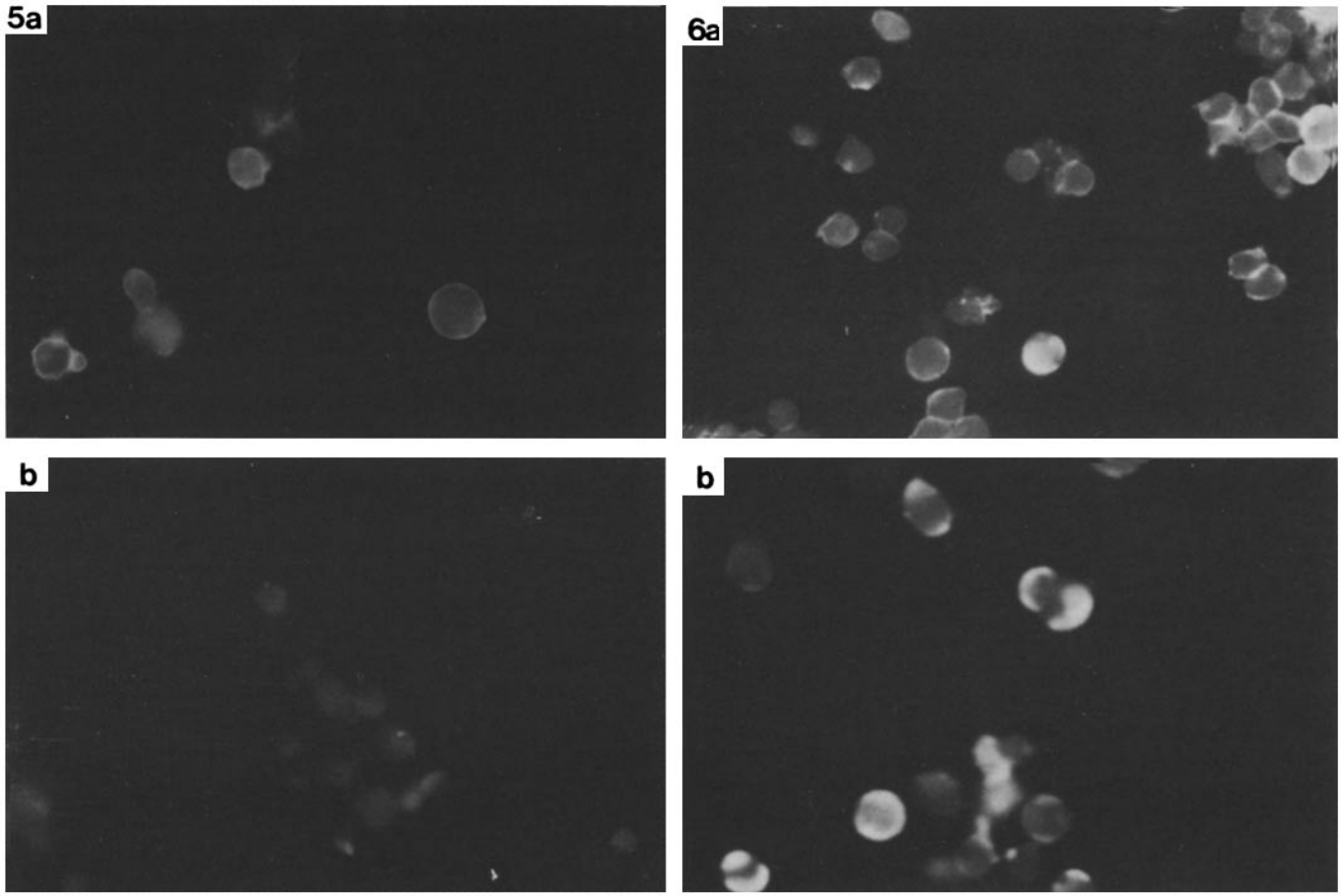


FIGURE 5 Cell surface SGG detected by membrane-bound complement. Fixed testicular cells were treated with antibody in the presence of complement as described in Materials and Methods. Cells were then treated with goat anticomplement followed by fluorescein-conjugated rabbit anti-goat immunoglobulin. (*a*) Anti-SGG-treated cells; (*b*) control serum-treated cells.

FIGURE 6 Immunofluorescent staining of purified testicular cell populations with anti-SGG at pH 8.6. (*a*) Spermatids; (*b*) pachytene spermatocytes.

the synthesis of SGG had not yet begun. More surprising was the finding that mature spermatozoa from the epididymis also showed no fluorescence, since SGG can be isolated from these cells. It is possible that this observation is an optical effect. If the surface antigen density were the same as that seen for spermatids, then the small size of the spermatozoan cell might not allow sufficient "compounding" of the fluorescence at the cell periphery to permit detection. Alternatively, the SGG has in some way become cryptic or masked during the last stages of differentiation. Studies have shown that the spermatozoan plasma membrane undergoes further modification, by adsorption of extracellular components, after release from the seminiferous tubule (6, 8). Such exogenous modification may obscure SGG. Immunological techniques have been used to demonstrate the presence of cell surface antigens unique to primary spermatocytes and the appearance of sperm-specific differentiation antigens during spermiogenesis (9, 20, 22, 23, 25, 30, 31). SGG, however, is present at a constant level in all germinal cell types subsequent to the early primary spermatocyte, and it is SGG reorganization that must account for the differential anti-SGG-dependent surface immunofluorescence observed for cells at different stages in spermatogenesis (Fig. 6). Other studies carried out in this laboratory have shown that SGG is greatly enriched in a plasma membrane fraction prepared from mature rat testis homogenates (27). Attempts to cleave SGG on the spermatozoan cell surface with arylsulfatase A were unsuccessful although SGG proved an effective substrate in vitro.[3] This result complements the present findings and lends support to the concept that germinal cell surface membrane SGG undergoes modulation during differentiation.

The finding that fixed testicular cells showed positive fluorescence when treated with control or nonimmune serum at physiological pH was initially confusing, because these sera showed no activity against SGG when assayed by complement fixation (18). Activity against SGG was detected in these sera when measured by CIE at pH 7.6 (Fig. 2), and the presence of naturally occurring antibodies to testicular cells in the sera of nonimmune animals has been reported (9, 10). It was therefore at first assumed that CIE was detecting a non-complement-fixing, naturally occurring antibody against SGG. However, the findings that myelin basic protein also showed a preferential reactivity towards SGG when measured by CIE and that the reaction between SGG and immunoglobulin was not detected at pH 8.6 (Fig. 3) led to the conclusion that in this case CIE was indeed detecting a nonimmunological, ionic interaction between SGG and basic immunoglobulin. This is not a completely satisfactory explanation, because the serum samples also reacted to some extent with the desulfated lipid GG (Fig.

[3] M. Levine and R. K. Murray. Personal communication.

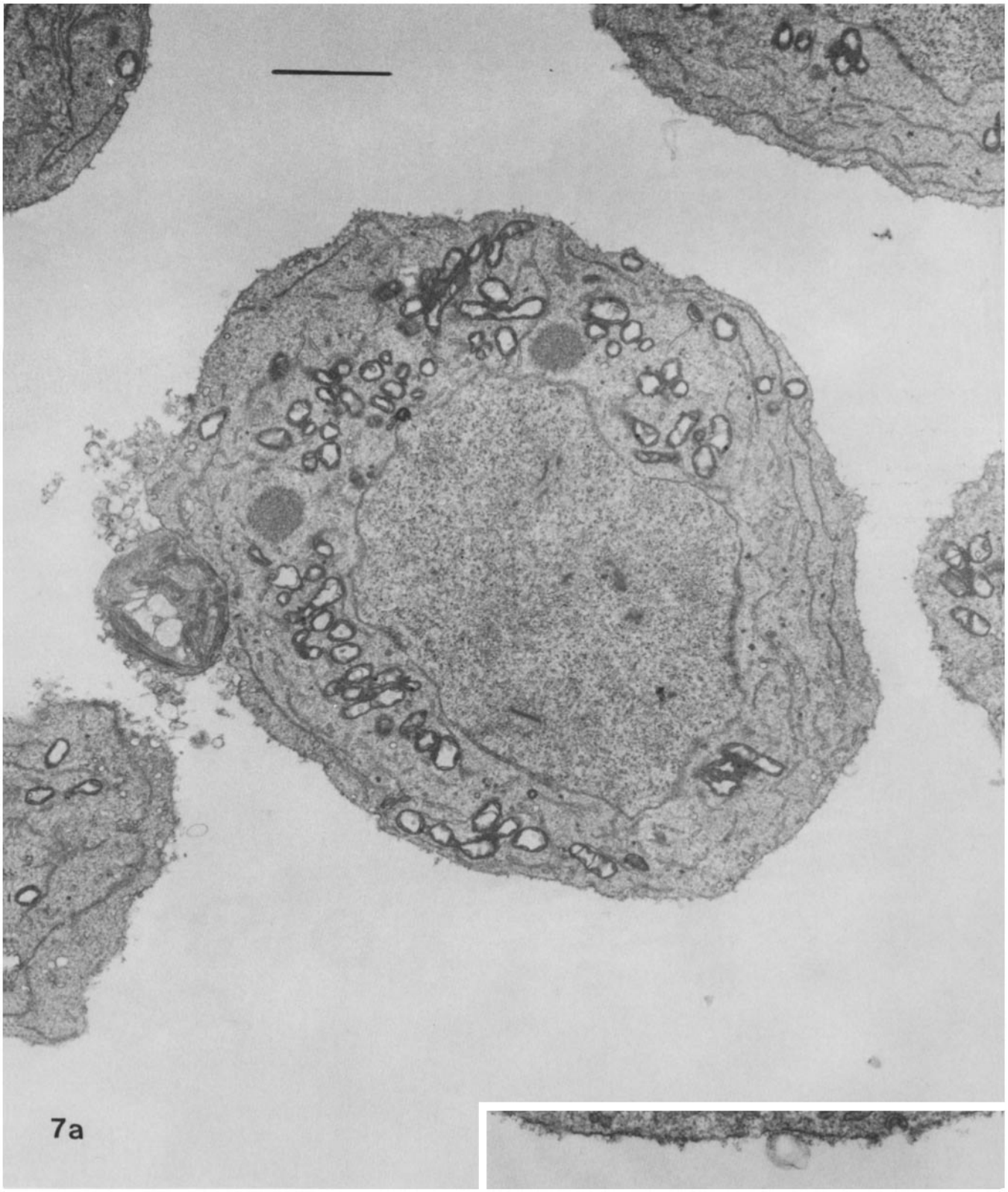


FIGURE 7 Immunoelectron micrographs. Cell surface localization of ferritin conjugate after treatment with anti-SGG at pH 8.6. (*a*) Primary spermatocyte. Bar, 200 nm. × 11,900; *inset*, × 32,600. (*b*) Spermatid. Bar, 200 nm. × 9,900; *inset*, × 36,000.

2); this may be attributable to the presence of a low titer of antibody against GG in control and nonimmune sera (18).

When immunofluorescence experiments were repeated with sera dialyzed against buffer at pH 8.6, control sera showed little reactivity with testicular cells, whereas the hyperimmune anti-SGG sera retained the fluorescent staining capacity originally observed at physiological pH (Figs. 4 and 6). This implies that the reactivity of the control sera towards testicular cells was a result of an ionic interaction between immunoglobulin and cell surface SGG and that at pH 8.6 the immunofluores-

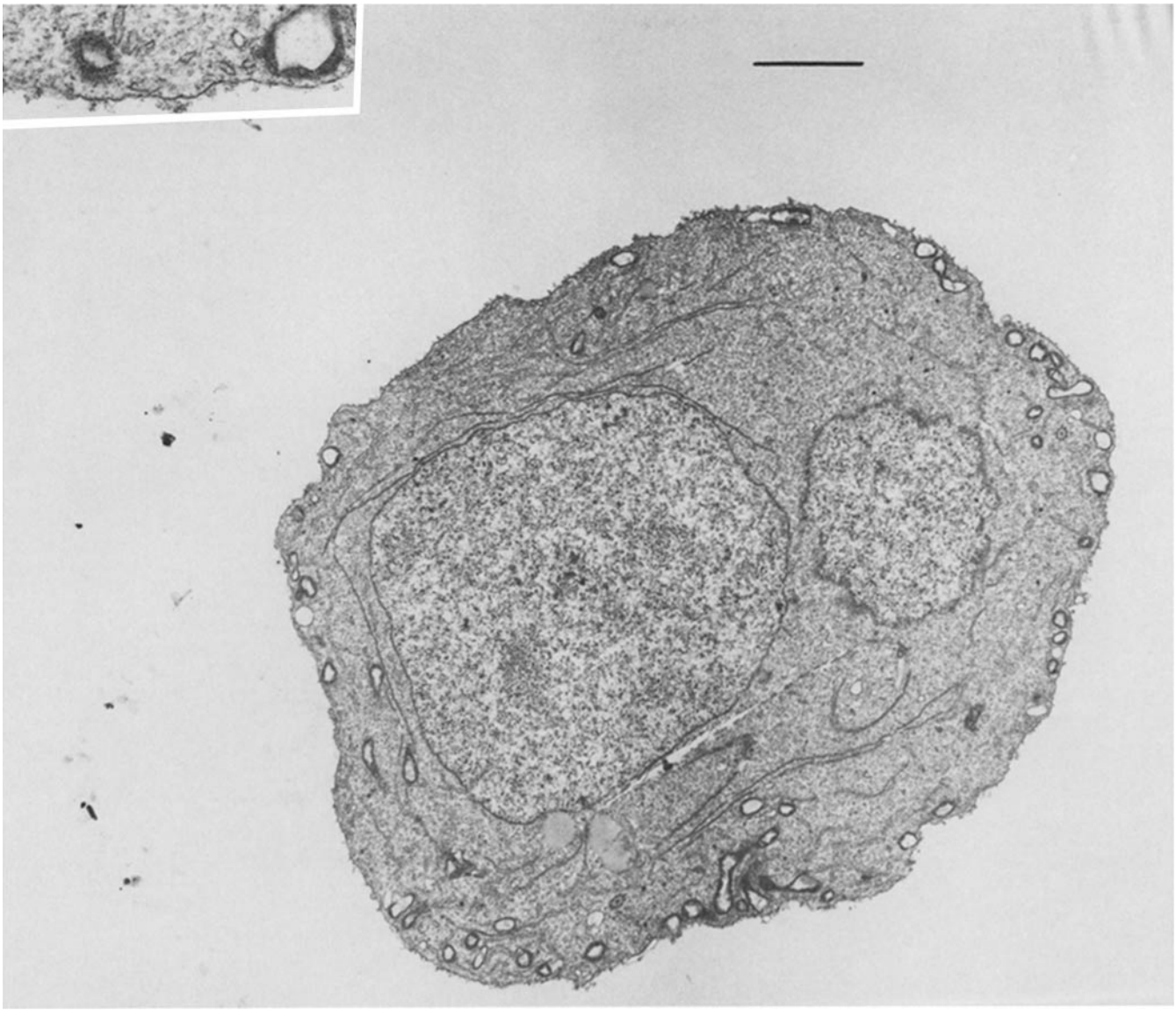
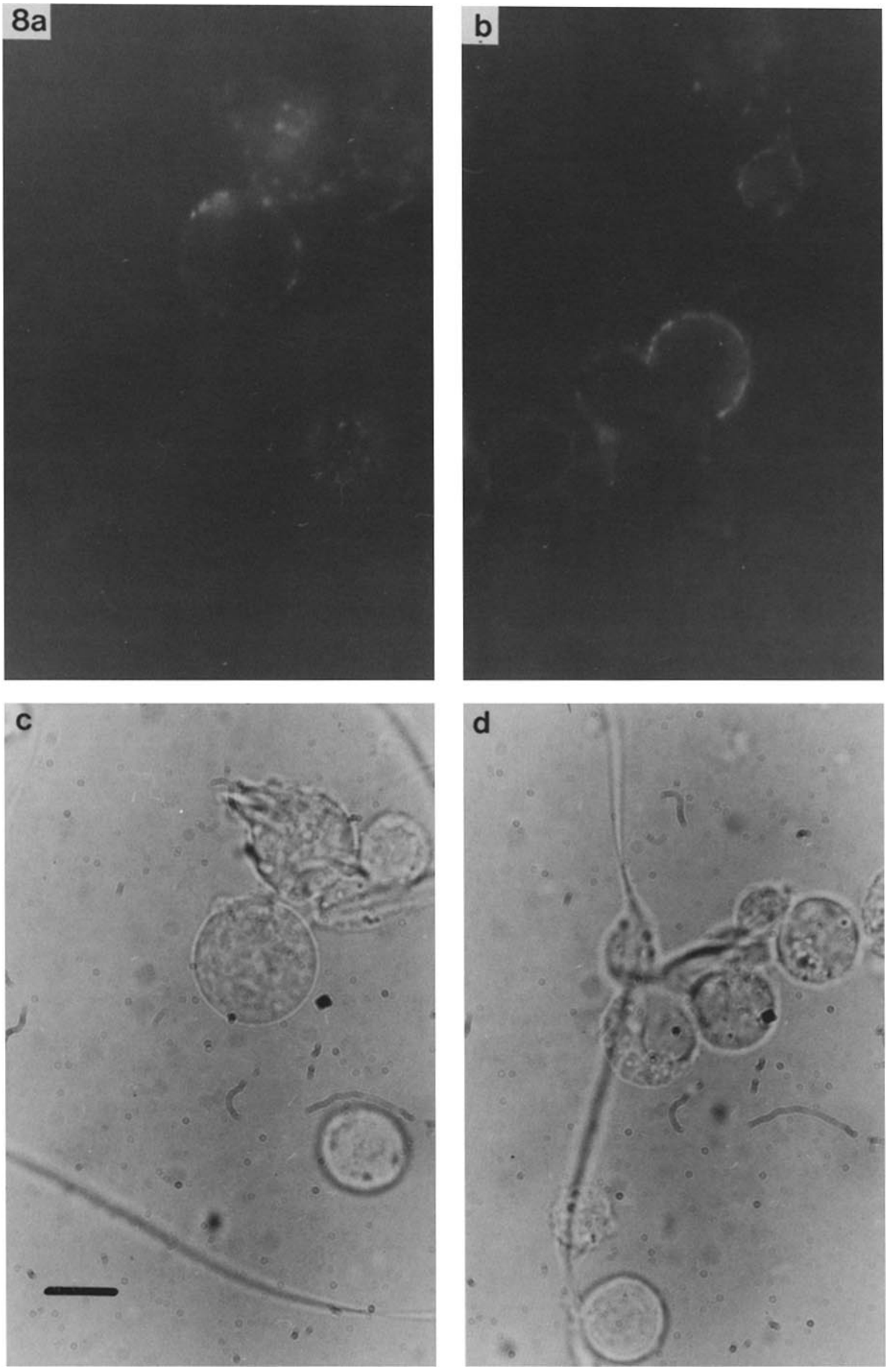


FIG. 7 *b*

cence reflects the complement fixing activity of the serum. This conclusion was further verified by the detection by immunofluorescence of complement bound to the cell surface in the presence of anti-SGG (Fig. 5); control sera showed no such activity. Thus the hyperimmune anti-SGG serum can be used to detect cell surface SGG. The results obtained using indirect immunofluorescence were verified by staining fixed, anti-SGG-treated cells with goat anti-rabbit Ig-ferritin conjugate and observing membrane localization of ferritin under the electron microscope (Fig. 7). The ferritin particles appear to be distributed as clusters along the plasma membrane.

The experiments carried out with unfractionated testicular cell suspensions and anti-SGG (Figs. 1, 4, and 5) resulted in surface fluorescence of ~80–90% of the cells. These suspensions are rich in germinal cells (15), and it is therefore likely that it is the germinal cells that are expressing SGG. Previous studies have shown that the synthesis of SGG during the first wave of spermatogenesis occurs between 15 and 20 d after birth, corresponding to the appearance of early primary spermatocytes (12). At earlier times SGG is not detectable by chemical means. The present results from the testes of 11-d-old rats show no fluorescence with anti-SGG. The data obtained by use of highly enriched cell populations (Fig. 6) and immunoelectron microscopy (Fig. 7) offer conclusive evidence that anti-SGG reacts with antigen on the cell surface of pachytene spermatocytes and round spermatids. Subcellular localization of SGG in tissue sections is difficult because of removal of SGG during the required fixing. However, frozen sections have been obtained under very mild conditions of fixation for study by immunoelectron microscopy. These studies will be reported elsewhere.

The majority of the experiments reported here were performed on cells that had been prefixed with glutaraldehyde. When this fixation procedure is omitted, the pattern of anti-SGG cell surface fluorescence is somewhat different. Instead of general membrane staining with localized areas of greater intensity, the pattern of fluorescence is granular (Fig. 8 *a*) or patched. Occasional cap fluorescence can be observed (Fig. 8 *b*). Thus, SGG is mobile within the cell membrane and does undergo ligand-induced redistribution. However, capped cells can be observed at 4°C, at room temperature and at 37°C (~1% of cells in each case). This suggests that the control of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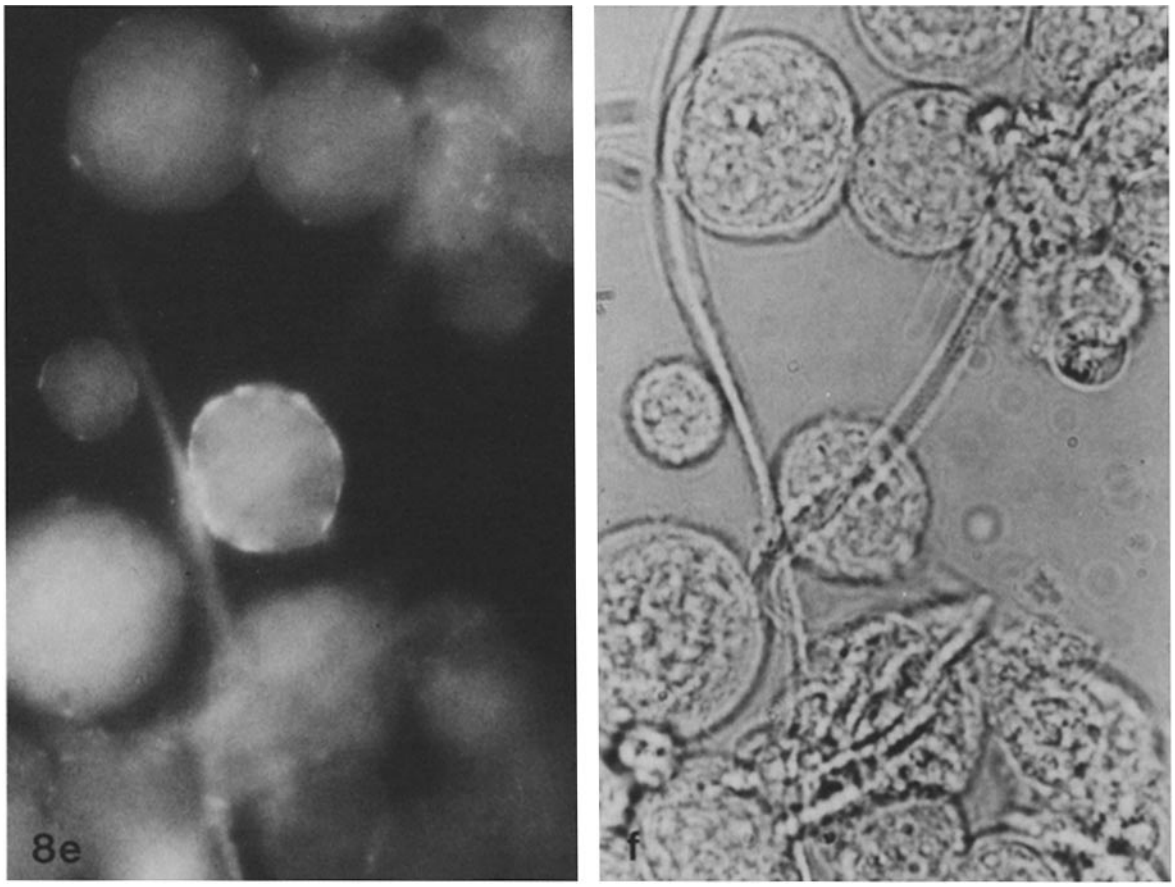

FIGURE 8 Indirect immunofluorescence of unfixed testicular cells after treatment with anti-SGG at 4°C at pH 8.6. (*a* and *c*) "Patching"; (*b* and *d*) "capped" fluorescence; (*e* and *f*) cells incubated at 37°C for 30 min before observation. (*a*, *b*, and *e*) UV illumination; (*c*, *d*, and *f*) phase contrast. Bar, 1 μm.

SGG capping may be distinct from that observed for membrane components of lymphocytes and fibroblasts (2–4, 24, 29) or for other differentiation antigens of spermatogenesis (26).

The finding of a pH-dependent interaction between immunoglobulin and testicular cells raises questions regarding reports of natural antibody to testicular cells (9, 10) and perhaps regarding antibodies in general when detected by immunofluorescence. Most cells have a negatively charged surface but the lack of fluorescence observed for erythrocytes treated with normal serum suggests that this is not a problem for all cells but may be restricted to testicular cells. There are examples of true autoantibodies to testicular cells measured by cytotoxicity (30). Misinterpretation may, however, result when immunofluorescence is used for the study of testicular cell surface antigens.

In conclusion, we have shown that SGG is expressed on the surface of rat germinal cells. Spermatogonia do not contain this glycolipid, whereas primary spermatocytes show strong fluorescence. Fluorescence is decreased on the surface of spermatids and is apparently absent on spermatozoa (see Figs. 1 and 8) although the latter contain SGG, implying a reorganization of SGG during spermatogenesis.

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