TEMPORAL EXPRESSION OF MEMBRANE ANTIGENS DURING MOUSE SPERMATOGENESIS

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ABSTRACT

The temporal expression of cell surface antigens during mammalian spermatogenesis has been investigated using isolated populations of mouse germ cells. Spermatogenic cells at advanced stages of differentiation, including pachytene primary spermatocytes, round spermatids, and residual bodies of Regaud and mature spermatozoa, contain common antigenic membrane components which are not detected before the pachytene stage of the first meiotic prophase. These surface constituents are not detected on isolated populations of primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene primary spermatocytes, or leptotene and zygotene primary spermatocytes.

These results have been demonstrated by immunofluorescence microscopy, by complement-mediated cytotoxicity, and by quantitative measurements of immunoglobulin (Ig) receptors on the plasma membranes of all cell populations examined. The cell surface antigens detected on germ cells are not found on mouse thymocytes, erythrocytes, or peripheral blood lymphocytes as determined by immunofluorescence and by cytotoxicity assays. Furthermore, the absorption of antisera with kidney and liver tissue does not reduce the reactivity of the antibody preparations with spermatogenic cells, indicating that these antigenic determinants are specific to germ cells.

This represents the first direct evidence for the ordered temporal appearance of plasma membrane antigens specific to particular classes of mouse spermatogenic cells. It appears that at late meiotic prophase, coincident with the production of pachytene primary spermatocytes, a variety of new components are inserted into the surface membranes of developing germ cells. The further identification and biochemical characterization of these constituents should facilitate an understanding of mammalian spermatogenesis at the molecular level.

Spermatogenesis is an excellent model for the study of mammalian cell differentiation. Although many similarities exist between spermatogenesis and systems of somatic cell development, spermatogenesis also provides a number of unique features for investigation. The initiation and regulation of meiosis, for example, may be studied only during germ cell differentiation. In addition, morphological observations reveal that spermatogenesis involves a variety of intracellular structures, including the manchette and the acrosome which are found only during differentiation of spermatogenic cells (9, 10, 11). Finally, intercellular communication during sperm differentiation may also

be unique since round spermatids are connected by extensive cytoplasmic bridges forming a syncytium unlike any present in developing somatic tissues (7, 12). Thus, it is possible that the regulatory mechanisms governing spermatogenesis may contrast with those responsible for other systems of cell differentiation such as lymphopoiesis and erythropoiesis.

Surface molecules of spermatogenic cells may play an important role in the regulation of sperm development and function. The membranes of mature mammalian spermatozoa contain antigenic determinants not detected on most somatic cells (1, 15, 33). Furthermore, glycoproteins of the sperm surface are organized in a nonmobile and polarized manner unlike most somatic cell membrane molecules (28), suggesting differences in the membrane structure of somatic and germ cells. Most studies of specific spermatogenic cell antigens, however, have examined only intracellular components (17, 21, 22, 38), and few investigations have been reported on the temporal appearance of spermatogenic cell surface constituents (14, 27).

Recently, methods for the separation of spermatogenic cell populations from adult mouse seminiferous epithelium have been developed (32). These procedures have been extended to permit the isolation of germ cells from the prepuberal testis (2). Consequently, it is now possible to obtain highly purified populations of mouse germ cells at virtually all stages of spermatogenesis. The isolated spermatogenic cells have been utilized in this study to examine the temporal appearance of antigenic membrane components. Cell membrane antigens specific to spermatogenic cells have been identified. These surface components are not detected on purified populations of primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene primary spermatocytes, or leptotene/zygotene primary spermatocytes, but are present on pachytene primary spermatocytes, round spermatids, residual bodies, and mature spermatozoa.

MATERIALS AND METHODS

Isolation of Spermatogenic Cells

CD-1 mice (Charles River Laboratories, Inc. [Wilmington, Mass.]) were used as testis donors. Homogeneous populations of the respective spermatogenic cells were isolated by sedimentation velocity at unit gravity (32) with some modification (2). Cells were separated on small or medium STA-PUT chambers (SP-120 and SP-

180; Johns Scientific, Toronto, Canada) using maximum loads of 2×10^8 or 5×10^8 cells, respectively. Purified populations of primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene primary spermatocytes, leptotene/zygotene primary spermatocytes, and Sertoli cells were obtained from the seminiferous cords of prepuberal mice of the appropriate age (2). Primitive type A spermatogonia and Sertoli cells were obtained with 6-day-old animals. 8-day-old mice were used for the isolation of type A spermatogonia and type B spermatogonia. Preleptotene primary spermatocytes and leptotene/zygotene primary spermatocytes were obtained from 18-day-old mice. Adult mice, 70–90 days of age, were used for the isolation of pachytene primary spermatocytes, round spermatids, and residual bodies (32). In some experiments, isolated cell mixtures from seminiferous tubules were used without separation. The purity of the isolated spermatogenic cells ranged from 75 to >95% as determined by hemocytometer assay and by ultrastructural examination (2, 32). All cell populations ranged from 95 to 98% viable as determined by the exclusion of trypan blue.

Mature spermatozoa were obtained from the vasa deferentia of adult CD-1 mice. Cells were washed three times in enriched Krebs-Ringer buffer (EKRB) (32) before use.

Preparation of Somatic Cells

Thymocytes were obtained from 2- to 4-wk-old CD-1 mice by excision and gentle homogenization of the organ. Spleen cells and erythrocytes were obtained from CD-1 mice 60–90 days old. Liver and kidney tissues were recovered from the same animals, minced, and homogenized. Particulate fractions of these tissues were prepared by filtration on Nitex nylon cloth (80 mesh, Tet/Kressilk, Inc., New York) to remove larger fragments. The filtrate was then centrifuged at 500 g for 10 min at 4°C to obtain the particulate fractions used for immunoglobulin (Ig) absorptions.

Antibody Preparation

Rabbit antisera directed against purified populations of mouse spermatogenic cells were prepared in female New Zealand white rabbits (White Pine Rabbitry, Douglas, Mass.). The spermatogenic cells were suspended in 1–2-ml of EKRB and were injected intravenously at weekly intervals. The average number of cells injected per week was as follows: vas deferens spermatozoa, 30 × 10^6; pachytene primary spermatocytes, 4 × 10^6; round spermatids, 16.5 × 10^6; seminiferous cell mixture, 20 × 10^6. Initial bleedings were taken 7 days after the fifth immunization.

Preparations of IgG were obtained by precipitation with 37% $(NH_4)_2SO_4$ followed by chromatography on diethylaminoethyl (DEAE)-cellulose in 0.0175 M phosphate buffer, pH 6.3 (25). Ig samples were dialyzed against distilled H_2O and lyophilized.

Antibody Absorption Procedures

All IgG preparations were used at a final concentration of 1 mg/ml in EKRB containing 0.5% wt/vol bovine serum albumin (EKRB-BSA). 1 ml of Ig solution was absorbed with 4×10^8 splenocytes. Preparations were also absorbed with 5×10^8 erythrocytes and 5×10^8 thymocytes. Liver and kidney tissues were used in equi-volume amounts for absorption. All absorptions were conducted for 30 min at 4°C.

Immunofluorescence Assays

Indirect immunofluorescence procedures were used in all experiments. Cells were incubated in Ig solutions (1 mg/ml in EKRB-BSA) for 30 min at 4°C. After two washes in EKRB-BSA, the cells were then incubated for 30 min at 4°C in fluorescein-conjugated goat antirabbit Ig (catalog no. 15-645-901, lot 641E, molar F/P ratio 3.4, Behring Diagnostics, American Hoechst Corp., Somerville, N. J.). The fluorescent reagent was diluted 1:10 in EKRB-BSA. Cells were washed twice in the same buffer before observation. Final resuspension was made using 30% glycerin vol/vol in EKRB-BSA.

A Zeiss photomicroscope equipped with transmitted light fluorescence was used. Labeled cells were examined with BG 12 and BG 38 excitation filters and with barrier filters 41 or 44. Cell identification was verified by Nomarski differential interference optics. Photographs were taken on Kodak Tri-X film at ASA 400 using automatic exposure.

Cytotoxicity Assays

Complement-mediated cytotoxicity was performed according to the method of Schachner et al. (33). Ig preparations were diluted serially from an initial concentration of 1 mg/ml. Experimental controls included incubation with (a) antibody alone, (b) complement alone, and (c) antibody plus complement heated to 56°C for 1 h before use. Guinea pig complement (catalog no. 40755, lot J7HAF6, Baltimore Biological Laboratories, Baltimore, Md.) was used at a dilution of 1:5 in EKRB-BSA. The percentage of dead cells was scored immediately.

Quantitation of Ig Receptors

Purified Ig preparations (1 mg/ml) were labeled with [125]I (sp act 17 Ci/mg, NEZ-033, New England Nuclear, Boston, Mass.) using chloramine-T (34). Unconjugated iodine was removed by dialysis against distilled H_2O and EKRB. The specific activity of IgG preparations ranged from 10^6 to 10^7 cpm/mg protein.

For the titration of antibody binding sites on the cell surface, ^{125}I-labeled IgG (1 mg/ml in EKRB-BSA) was incubated with cells in the same buffer for 30 min at 4°C. Increasing aliquots of antibody were used to ensure the saturation of receptor sites. Radiolabeled normal rabbit IgG was used to determine the extent of nonspecific binding. The results were quantitated by liquid scintillation spectrometry (20).

RESULTS

Specificity of Antibody Preparations

The specificity of all Ig preparations was assayed by fluorescence microscopy and by cytotoxicity on somatic cells. Unabsorbed preparations of the antibody IgG fractions directed against purified pachytene primary spermatocytes (AP), against purified round spermatids (ARS), against a mixture of cells obtained from isolated seminiferous tubules (ASC), and against vas deferens spermatozoa (AVDS) were examined. None of these antibodies labeled mouse thymocytes, erythrocytes, or peripheral blood lymphocytes as determined by fluorescence microscopy. However, ~30% of mouse splenic lymphocytes were labeled weakly with all unabsorbed IgG fractions. The percentage of labeled splenocytes was not increased by incubation in higher IgG concentrations or by longer incubation times. Labeled splenocytes were indistinguishable morphologically from unlabeled spleen cells by Nomarski differential interference microscopy or by phase contrast microscopy.

Cytotoxicity assays with unabsorbed antisera revealed that ~30% of mouse splenocytes were lysed by all Ig preparations. The relationship of the splenocyte subpopulation labeled with unabsorbed antisera in immunofluorescence assays to the spleen cell population lysed in cytotoxicity experiments is not clear. No significant numbers of either thymocytes or erythrocytes were lysed by any antiserum.

Although these results indicated that the antibodies did not react strongly with somatic cells, all Ig fractions were absorbed with somatic cells to ensure immunological specificity. Fluorescence microscopy revealed that all reactivity of the antibodies with somatic cells was removed by a single absorption of the antibodies with mouse splenocytes. However, as a precautionary measure, preparations of IgG were also absorbed routinely with erythrocytes, thymocytes, and particulate fractions of both kidney and liver. After absorption with somatic cells, <5% of mouse splenocytes, erythrocytes, or thymocytes were lysed by any antiserum (Table I).

The reactivity of both unabsorbed and absorbed Ig fractions with purified populations of mouse spermatogenic cells was compared by immunofluorescence, by cytotoxicity, and by quantitative measurements using radio-iodinated Ig. No reduction of activity directed against spermatogenic

TABLE I

Cytotoxic Effect of Antibody Preparations on
Somatic Cell Populations

	Dead cells			
	AP	ARS	ASC	AVDS
	%			
Unabsorbed IgG				
Splenocytes	33	29	36	26
Thymocytes	10	8	8	7
Erythrocytes	10	7	11	9
Absorbed IgG				
Splenocytes	4	4	3	4
Thymocytes	3	3	5	4
Erythrocytes	5	3	4	4

Data represent the maximum percent of killed cells at an Ig concentration of 100 μg/ml. All results are the average of three independent determinations.

cells was detectable after absorption. These results, therefore, indicate that all four absorbed antibody preparations (AP, ARS, ASC, and AVDS) exhibit specificity for spermatogenic cell surface antigens which are not shared by a variety of somatic cells.

Temporal Appearance of Spermatogenic Cell Membrane Antigens

To determine the temporal expression of plasma membrane antigens during spermatogenesis, the reaction of antibodies with highly purified populations of spermatogenic cells obtained from both prepuberal and adult mouse testis was examined. Cell populations were assayed by fluorescence microscopy, by cytotoxicity, and by quantitative tests. The cells assayed included primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene primary spermatocytes, leptotene/zygotene primary spermatocytes, pachytene primary spermatocytes, round spermatids, residual bodies, and Sertoli cells.

IMMUNOFLUORESCENT LABELING OF SPERMATOGENIC CELLS: All four antibodies reacted similarly when assayed by immunofluorescence, and the results for AP are shown in Fig. 1. Pachytene spermatocytes, round spermatids, and residual bodies were labeled strongly by all antibodies. All cells in these respective populations were labeled and agglutinated. A decrease in antibody concentration from 10- to 100-fold resulted in the loss of detectable immunofluorescence, but cells continued to agglutinate at the

lower levels of IgG. In contrast to these findings, the antibody preparations did not label primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene spermatocytes, or leptotene/zygotene spermatocytes. These cells were not agglutinated at any Ig concentration assayed (1-100 μg/ml).

The fluorescent labeling of pachytene spermatocytes, round spermatids, or residual bodies was not observed when (a) normal rabbit IgG was substituted for antibody or (b) when fluorescein conjugated goat antirabbit Ig alone was used. These results indicated that the labeling seen by immunofluorescence was not due to simple adsorption of protein to the cell surface. Sertoli cells were labeled weakly by all four antisera, but the percentage of labeled cells varied from <20% to ~50% depending upon the particular antibody and the individual experiment. Preliminary immunofluorescence experiments with an enriched population of Leydig cells indicated that these cells were not labeled by any of the antibody preparations.

The results of the immunofluorescence experiments, then, indicate that cell membrane antigens specific to spermatogenic cells appear at the pachytene stage of the first meiotic prophase. These cell surface constituents are also expressed by spermatogenic cells at subsequent stages of spermatogenesis.

CYTOTOXIC EFFECTS OF ANTIBODY PREPARATIONS: To confirm and extend the results obtained by immunofluorescence, complement-mediated cytotoxicity was used to examine the binding of antibodies to mouse spermatogenic cells. Primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene spermatocytes and leptotene/zygotene spermatocytes were not lysed in significant numbers under any conditions tested (Fig. 2). In contrast, significant proportions of pachytene spermatocytes and round spermatids were lysed by all four IgG fractions. All antibody preparations exhibited a relatively weak cytotoxic titer, and little evidence of cytotoxicity at IgG concentrations below 6.25 μg/ml was detected. The maximum cell death occurred at an IgG concentration of 100 μg/ml for all antibodies and all cells. Increased complement concentrations or increased incubation periods did not yield a greater percentage of killed cells. Table II summarizes the cytotoxicity results obtained for mouse spermatogenic cells. Early germ cells, from primitive type A spermatogonia
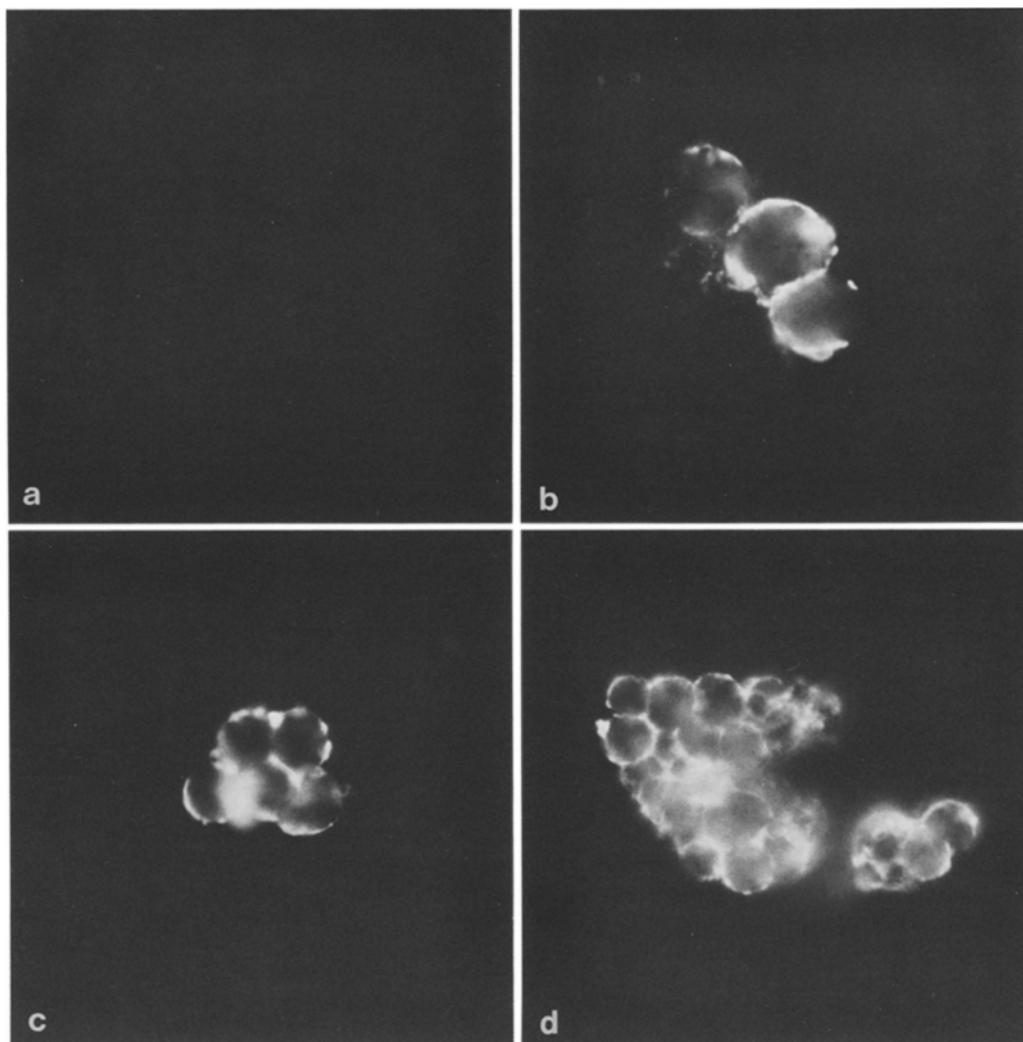
FIGURE 1 Binding of rabbit AP to purified mouse spermatogenic cells: (a) unlabeled type A spermatogonia; (b) pachytene primary spermatocytes; (c) round spermatids; (d) residual bodies. Identical results are obtained with all antibody preparations. × 1,180.

to zygotene spermatocytes, were not readily lysed by any Ig. From 55 to 90% of pachytene spermatocytes and round spermatids were lysed by all antibodies tested. These results are in good agreement with the findings obtained by immunofluorescence. Residual bodies, however, exhibited a reduced capacity for complement-mediated lysis, with a maximum value of 36% lysis obtained in the presence of AVDS.

The cytotoxicity observed in these studies was due to antibody as indicated by control experiments. For instance, normal rabbit IgG (100 μg/ml) in the presence of complement yielded rou-

tinely <5% cell death as did guinea pig complement in the absence of antibody. In addition, antibody (100 μg/ml) in the presence of heat-inactivated complement did not have a significant cytotoxic effect.

QUANTITATION OF CELL SURFACE LABELING: Quantitative measurements of IgG binding to spermatogenic cells were made using radio-iodinated antibodies. The number of specific antibody binding sites detected on purified spermatogenic cells is shown in Table III. Fewer than 1,000 antibody receptors per cell were detected on primitive type A spermatogonia, type A sper-
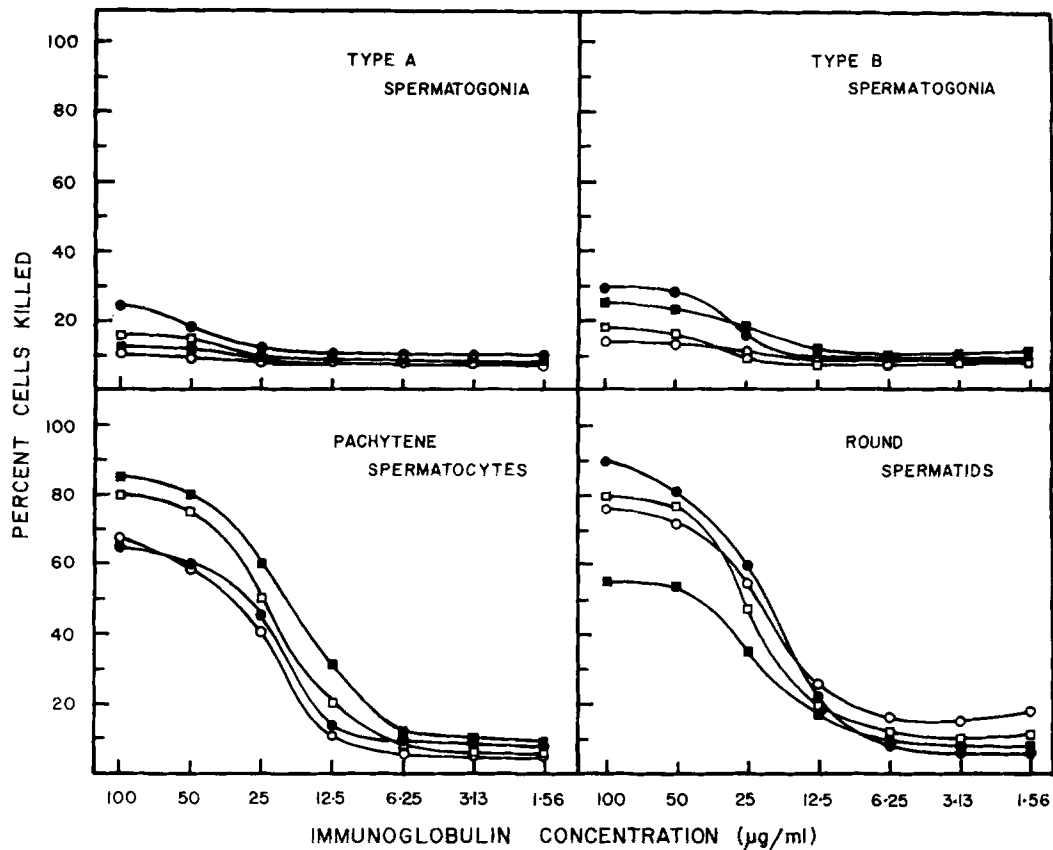
FIGURE 2 Cytotoxic effects of antibodies on purified mouse spermatogenic cells. Only pachytene primary spermatocytes and round spermatids are lysed in significant numbers. Occasional Sertoli cell contaminants were excluded from these data. Sertoli cells may be easily identified by Nomarski differential interference microscopy. ●, ARS; ■, AP; ○, ASC; □, AVDS.

TABLE II

Cytotoxic Effect of Antibody Preparations of Spermatogenic Cell Populations

Cell type	Dead cells			
	AP	ARS	ASC	AVDS
			%	
Primitive type A spermatogonium	<5	6	<5	<5
Type A spermatogonium	<5	14	<5	7
Type B spermatogonium	14	19	5	7
Preleptotene spermatocyte	<5	<5	<5	<5
Leptotene-zygotene spermatocyte	<5	<5	<5	<5
Pachytene spermatocyte	85	65	66	80
Round spermatid	55	90	76	80
Residual body	25	22	22	36

Data represent the maximum percent of killed cells at an Ig concentration of 100 μg/ml. All results are the average of three independent determinations.

TABLE III

Quantitation of Ig Receptors on Spermatogenic Cell Populations

Cell type	Sites per Cell ($\times 10^3$)			
	AP	ARS	ASC	AVDS
Primitive type A spermatogonium	<1	<1	<1	<1
Type A spermatogonium	<1	<1	<1	<1
Type B spermatogonium	<1	<1	<1	<1
Preleptotene spermatocyte	<1	<1	<1	<1
Leptotene-zygotene spermatocyte	<1	<1	<1	<1
Pachytene spermatocyte	274	180	241	183
Round spermatid	98	75	63	89
Residual body	103	86	99	106
Spermatozoon	62	61	90	147

Data represent the average of triplicate determinations. Background labeling, as defined by the binding of ^{125}I-labeled normal rabbit IgG, has been subtracted from the data. Nonspecific labeling was always <5% of the total binding.

matogonia, type B spermatogonia, preleptotene spermatocytes, or leptotene/zygotene spermatocytes. Pachytene spermatocytes, however, have ~2 × 10⁵ membrane receptors per cell. Round spermatids, residual bodies, and mature spermatozoa also exhibit from 6 × 10⁴ to 1.5 × 10⁵ receptor sites per cell, depending upon the particular cell type and IgG fraction being assayed (Table III). Control experiments using unlabeled antibody as a competitive inhibitor of binding indicated that virtually all cell surface labeling was specific and not the result of simple adsorption of protein onto the plasma membrane. Radiolabeled normal rabbit IgG did not bind to the membranes of any mouse spermatogenic cells.

The quantitative results with all IgG preparations are in excellent agreement with the data obtained by immunofluorescence and by complement-mediated cytotoxicity.

Differential Specificity of Antibody Preparations

Immunofluorescence studies conducted with spermatogenic cells indicated similar patterns of reactivity for all four Ig fractions (Fig. 1). The results of cytotoxicity experiments (Table II) and of quantitative assays (Table III), however, revealed significant differences in the binding of the antibodies to individual cell types. For example, AP lysed 85% of a population of purified pachytene spermatocytes, but killed only 55% of a population of round spermatids. Likewise, ARS lysed only 65% of pachytene spermatocytes, but killed 90% of the round spermatids. These data suggested that the four IgG fractions used in these

studies had different immunological specificities.

Further investigations were made, therefore, to test the reactivity of the antisera. In particular, the binding of spermatogenic specific IgG fractions to spermatozoa obtained from the vas deferens was examined. By immunofluorescence, it was observed that all four antisera reacted with mature spermatozoa, but that in each instance the topographical localization of labeling on the sperm surface differed (Fig. 3).

AVDS labeled the entire cell surface, except for the lateral postacrosomal regions of the sperm head. In contrast, ASC labeled only the sperm principal piece. AP labeled only the sperm membrane overlying the acrosome and the midpiece. The postacrosomal region of the head and the principal piece were unlabeled. Finally, ARS revealed a binding pattern similar to that detected using AVDS, although binding to the principal piece was weaker than binding to the midpiece. Control experiments using normal rabbit IgG indicated that the labeling patterns seen on mature spermatozoa were specific to each antibody.

The immunofluorescence studies of antibody binding to mature spermatozoa demonstrate that the four Ig preparations tested in these experiments are not identical, but that each has a distinctive immunological specificity. This suggests that the cell surface antigens detected on mouse spermatogenic cells may be heterogeneous, representing multiple molecular species.

DISCUSSION

Cell surface antigens specific to spermatogenic cells have been identified. These antigens appear
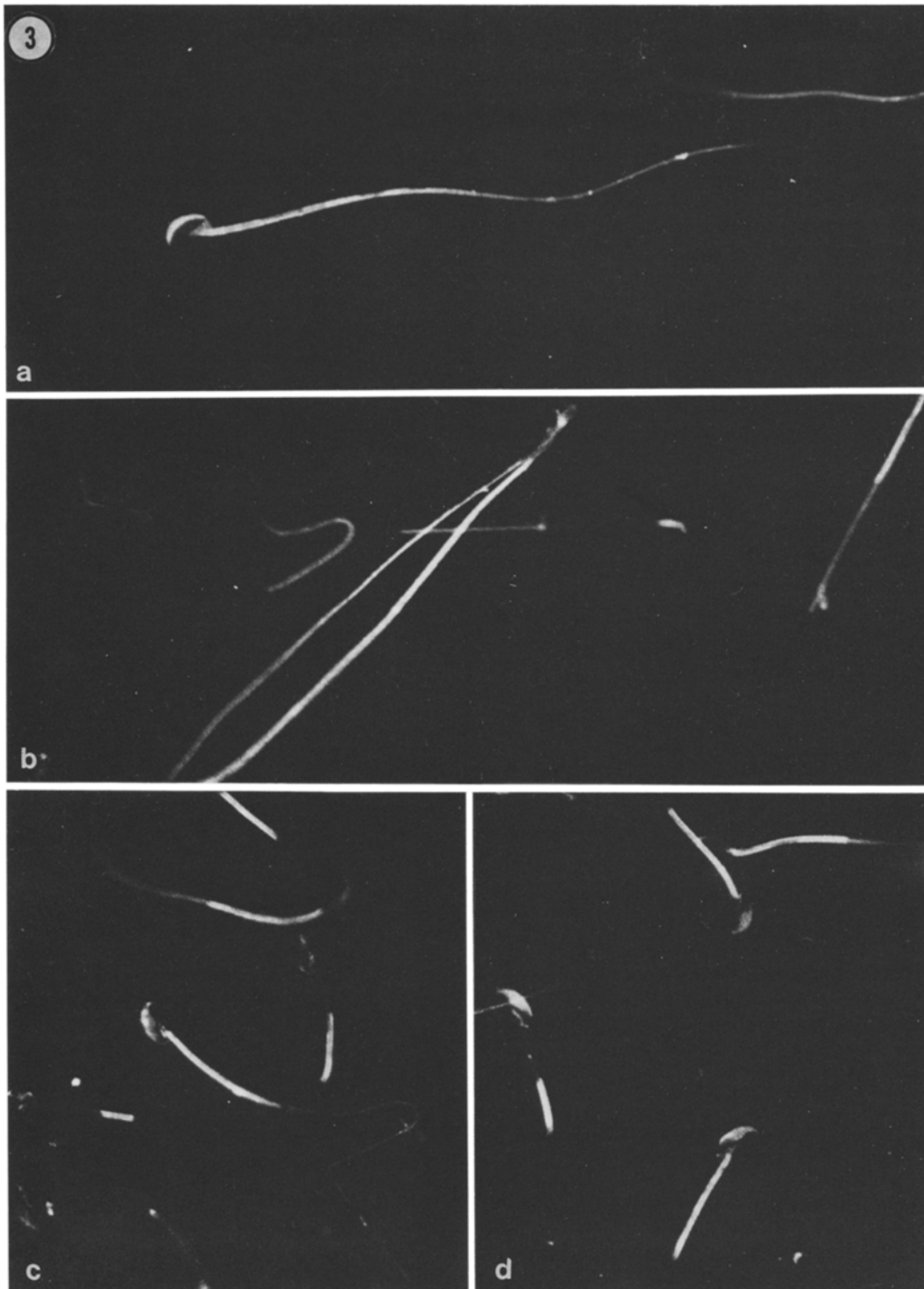
FIGURE 3   Differential labeling of mouse vas deferens spermatozoa by antibodies. (a) AVDS: All of the sperm plasma membrane is labeled except the postacrosomal lateral surface of the head. (b) ASC: Only the plasma membrane of the sperm tail is labeled. The labeling is uniform along the length of the tail, and the midpiece cannot be readily distinguished from the principal piece. No sperm heads are labeled. (c) AP: The sperm membrane overlying the acrosome and the middle piece is labeled. (d) ARS: The sperm surfaces overlying the acrosome and the middle piece are labeled strongly, but weak binding is also detectable on the principal piece. × 1,180.

at the pachytene stage of the first meiotic prophase and are expressed by all germ cells at subsequent stages of differentiation, including mature spermatozoa. These membrane components are not detected on germ cells at any stage of development preceding the pachytene stage of meiotic prophase. The appearance of these antigenic components on the surface of pachytene primary spermatocytes implies that they are synthesized during this particular stage of spermatogenesis. Preleptotene primary spermatocytes, as illustrated in Fig. 4, are small germ cells with an average diameter of 7.8 μm (2). During meiotic prophase, however, cell volume increases progressively as differentiation continues through the leptotene and zygotene stages of meiotic prophase I. Pachytene spermatocytes are large cells with an average diameter of 16 μm (32) and an estimated cell surface area of 800 μm^2 (28), four times that of preleptotene spermatocytes. These data suggest that large amounts of plasma membrane must be synthesized throughout meiotic prophase but that significant and sudden qualitative changes occur during the final stages of meiotic prophase. Further studies are required, however, to examine the biosynthesis and function of plasma membrane components of spermatogenic cells before pachynema.

A number of studies demonstrate that pachytene spermatocytes synthesize both RNA and protein. Monesi (29, 30), Kierszenbaum and Tres (23, 24), and Tres (35), for example, have shown by autoradiographic techniques a high level of RNA synthesis in pachytene spermatocytes of the mouse. Monesi (29, 30) has also presented evidence demonstrating the incorporation of labeled amino acids into protein by pachytene spermatocytes. Recently, Meistrich et al. (27) have shown that LDH-X, a sperm-specific isozyme of lactate dehydrogenase, is first synthesized during the late pachytene stage of spermatogenesis. Pachytene primary spermatocytes are, therefore, capable of extensive gene transcription and translation. Although *de novo* synthesis of new plasma membrane components during the first meiotic pro-
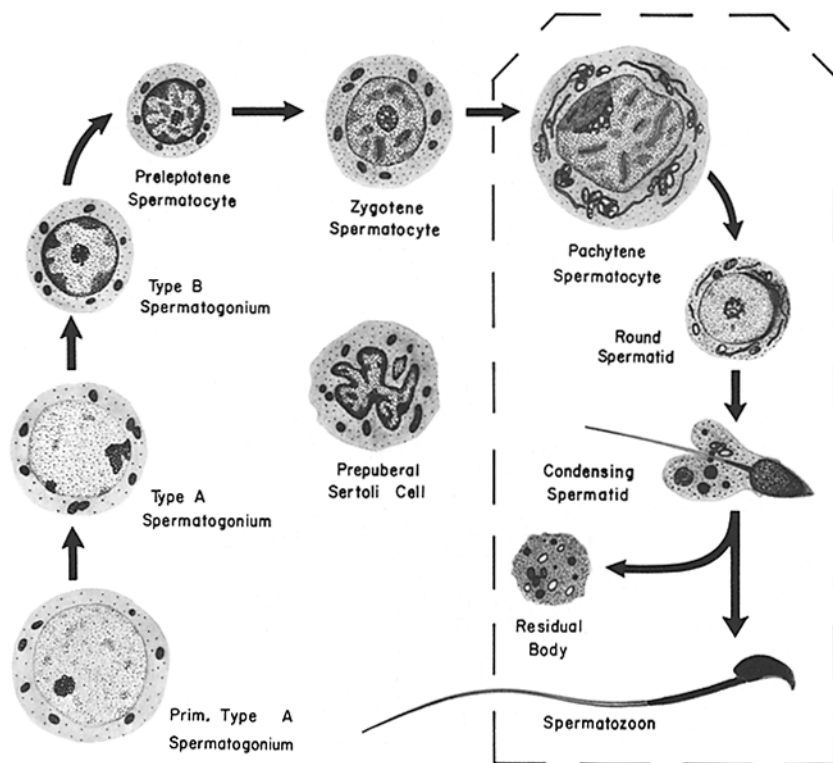

FIGURE 4 A diagrammatic representation of spermatogenesis in the mouse. All cell types illustrated may be isolated by sedimentation at unit gravity. The dashed box encloses those stages of spermatogenesis where specific cell surface antigens have been detected.

phase remains to be proven, this seems to be the most likely explanation for the current observations. The temporal expression of cell surface constituents during mammalian spermatogenesis is also important in view of results indicating the haploid expression of particular plasma membrane components on mature mouse spermatozoa (39, 40).

The expression of testis-specific antigens is facilitated by the blood testis barrier (6, 13) which is composed of a basal lamina and an extensive system of specialized intercellular Sertoli junctions near the periphery of the seminiferous tubule (16, 31). The network of occluding tight junctions prevents the passage of macromolecules from the testicular blood supply into the adluminal compartment of the tubule. Thus, it is possible to maintain antigens specific to spermatogenic cells in the adluminal compartment without autoimmune reactions. Spermatogonia and preleptotene primary spermatocytes lie on the basal side of the Sertoli cell junctions (5), whereas leptotene, zygotene, and pachytene spermatocytes and round spermatids are located in the adluminal compartment of the seminiferous epithelium. This topographical arrangement may provide a physiological basis for the antigenic differences detected between spermatogonia and other germ cells in the present studies.

The identification of particular cell surface antigens on purified populations of mammalian germ cells is hampered by the necessary exposure of these cells to collagenase and trypsin. Sufficiently highly purified mammalian germ cell populations cannot be obtained in good yield or purity without enzymatic treatment. Romrell et al. (32) demonstrated that the sequential treatment of adult mouse testis with collagenase and trypsin permits the isolation of purified mononucleated cells. Unfortunately, nonenzymatic methods of testicular cell separation result in an increased proportion of multinucleated cells and greatly reduce the total yield and purity of viable cells.

Although some fibroblastic cell surface proteins are susceptible to degradation by exogenous protease (4, 41), the effects of enzymes on the surface molecules of spermatogenic cells are not yet clear. There is substantial evidence, however, suggesting that major alterations of membrane antigens do not occur. First, by fluorescence microscopy no differences have been detected in the binding of antibodies, prepared against isolated germ cells after enzyme treatment, to spermatogenic cells at

all stages of differentiation dissociated from the testis by purely mechanical means. This result indicates that the antigenic determinants of spermatogenic cell surfaces are not removed by collagenase and trypsin. Second, NS-4, a cell surface determinant present on cerebellar tissue and on mouse spermatozoa, is not removed by trypsin (33). Third, H-2 and Ia antigens on the membranes of mammalian spermatogenic cells are not susceptible to collagenase (14). Fourth, a variety of lectins have been shown to bind specifically to the membranes of mouse germ cells after isolation by enzymatic procedures (28). This latter result indicates that many cell surface glycoproteins are not removed from spermatogenic cells by exposure to enzymes. Unfortunately, it is not yet possible to culture isolated populations of spermatogenic cells in order to allow the resynthesis of any degraded membrane components (26). The development of suitable in vitro culture conditions for mouse spermatogenic cells is being pursued at present.

The biochemical identification of the membrane antigens detected in the current experiments will require the isolation and characterization of individual cell surface constituents by means of techniques of membrane extraction and immunocoprecipitation (18, 19). It seems apparent, however, that the components recognized by the Ig fractions used here represent multiple molecular species. Immunofluorescence observations indicate that these antisera have different specificities as demonstrated by the variety of labeling patterns seen with mature spermatozoa. In addition, both the cytotoxicity results and the quantitative data reveal significant variations in the reactivities of the antibodies with different germ cell classes. This suggests that the antigens on spermatogenic cells may differ both qualitatively and quantitatively.

The plasma membrane constituents described here do not correspond to any well-characterized molecular components of mammalian testicular cells. The F9 teratocarcinoma antigen (1, 3) is present on all germ cells, including spermatogonia (15). Its presence on spermatogenic stem cells indicates that F9 is different from any membrane constituents detected in this study. Similarly, preliminary results indicate that NS-4 is not expressed in high quantity on the surfaces of pachytene spermatocytes or round spermatids. Also, initial experiments suggest that absorption of IgG fractions with cerebellar tissue does not reduce antibody

binding to spermatogenic cells. LDH-X is expressed temporally in a manner like that seen in the current experiments (27), but this enzyme is predominantly intracellular. Although some LDH-X may be present on the postacrosomal surfaces of mature spermatozoa (8), this enzyme cannot be responsible for the antigenic activities described in this study. The Ig fractions which were examined do not label the postacrosomal region of mature mouse spermatozoa (Fig. 3).

Many investigators have studied intracellular antigens of mammalian testicular cells and in a few instances have reported detailed biochemical studies of these components. The isolation of specific testicular antigens has been stimulated by investigations of experimental allergic orchitis (36, 37). Many of these aspermatogenic antigens are glycoproteins (17, 21, 22, 38), but the expression of these constituents on the plasma membranes of particular spermatogenic cell types remains to be demonstrated.

The results presented in this investigation provide an alternative approach towards understanding the regulation of spermatogenesis at the molecular level. The isolation of highly purified populations of mouse spermatogenic cells at virtually all stages of differentiation allows the analysis of cell surface determinants in nonpathological conditions. A detailed biochemical knowledge of the surface constituents of individual spermatogenic cells may shed light on the role of the plasma membrane in many aspects of sperm differentiation including the induction of meiosis, the interaction between germ cells and Sertoli cells and the process of spermiogenesis. In addition, the development of specific antisera directed against known surface determinants of mammalian germ cells may provide an immunological method for contraception in the male.

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