

A Map of the Guinea Pig Sperm Surface Constructed with Monoclonal Antibodies

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The surface of mammalian sperm is known to exhibit regional heterogeneity. Using monoclonal antibodies, we have analyzed the nature of this regional variation at the molecular level. A map of the surface of guinea pig sperm has been constructed that defines a number of regions in which surface antigens are localized and analyzes the diversity of antigens found in each region. In an initial screening of 117 hybridoma antibodies to a sperm membrane preparation, a remarkable result was obtained: all the antibodies bound to a localized region of the cell. From the initial hybrid lines, we established a collection of 56 stable hybridomas producing antibodies to surface antigens. These antibodies detect antigens localized in five surface regions: anterior head (AH), posterior head (PH), whole head (WH), posterior tail (PT), and whole tail (WT). At least 12 distinct surface antigens are recognized that bind antibodies in one of the localized regions (five AH antigens, three PH, two WH, one PT, and one WT). Some of the recognized antigens have been identified as proteins, comprised of either one or several ^{125}I -labeled polypeptides. The identified AH antigens have labeled polypeptides of molecular weights (M_r) 52,000 (52K); 70K, 62K, 46K, 25K, and 18K; 62K, 52K, and 38K; 16K; and 38K. Identified PH antigens have polypeptides of M_r 60K; 66K, 48K, and 41K; and 58K and 48K. Identified WH antigens have polypeptides of M_r 89K and 45K; and 42K. We conclude that the sperm cell can maintain contiguous membrane domains which have quite different compositions. Its surface is a mosaic consisting of multiple regions and each region can contain several localized antigens.

INTRODUCTION

Mammalian fertilization involves the capacitation of sperm, sperm passage through the cumulus cells, the acrosome reaction, and sperm binding to the zona pellucida and fusion with the egg plasma membrane. In each of these processes, the sperm surface is believed to play a fundamental role (cf. Yanagimachi, 1981; for a recent review). During capacitation, surface molecules may be lost and the plasma membrane undergoes changes resulting in entry of external calcium into the cell. In the acrosome reaction, a specific area of the cell membrane fuses with the outer acrosomal membrane. During the sperm's approach to and ultimate fusion with the egg, the sperm surface interacts with successive layers of the oocyte vestments ending with the egg plasma membrane itself.

A deeper understanding of the sperm's role in these processes will require insight into the molecular anatomy of its surface. Currently, there is a paucity of information about individual surface molecules of mammalian sperm. In the present study, we sought such information about the guinea pig sperm surface. Previous studies of the sperm surface have demonstrated a nonuniform binding of probes that potentially recognize classes of molecules. These probes include lectins (reviewed by Koehler, 1978), filipin, and polymixin B

(Bearer and Friend, 1980; Bearer and Friend, 1982; Elias *et al.*, 1979), ^{125}I -di-iodofluorescein isothiocyanate (Gabel *et al.*, 1979), heterogeneous antibodies (Fellous *et al.*, 1974; Koehler, 1975; Koehler and Perkins, 1974; Koo *et al.*, 1973; Millette and Bellive, 1977; Tung *et al.*, 1979; O'Rand, 1980; O'Rand and Romrell, 1980), and colloidal iron hydroxide (reviewed by Koehler, 1978). These studies have established that the sperm surface has regional heterogeneity. Their limitation is twofold: they do not reveal how many patterns of localization of surface molecules may exist on the sperm and they have not determined the number or identity of the surface molecule(s) that result in nonuniform binding of the probes.

Recent work using monoclonal antibodies (MAbs) has indicated that an individual antigen can be localized in a particular sperm surface region (Myles *et al.*, 1981; Feuchter *et al.*, 1981; Saling and O'Rand, 1981; Schmell *et al.*, 1982). In our previous work, we identified three localized surface proteins of guinea pig sperm, a M_r 52K protein localized on the anterior head, a M_r 60K protein localized on the posterior head, and a M_r 42K protein localized on the whole head (Myles *et al.*, 1981). In the present study, we asked if there are additional patterns of localization or if other surface molecules share the already defined patterns of localization. The approach we have taken is to generate a collection of monoclonal antibodies which can be used to localize the antigens

by immunofluorescence and to identify them by immunoprecipitation. This approach has allowed the construction of an initial map of surface topography for the guinea pig sperm.

MATERIALS AND METHODS

Hybridoma Production

Three C57/B1/6 female mice (Charles River) were immunized with the initial 90,000 g_{av} membrane pellet obtained from extensively washed guinea pig cauda epididymal sperm as previously described (Primakoff *et al.*, 1980). The pellet is composed mainly of membrane vesicles released by sperm in the acrosome reaction and some sperm cells (Myles *et al.*, 1981). Thus, the immunogen is enriched for the anterior head region of the plasma membrane providing a focus on this region combined with a survey of the whole cell. For immunization on Day 1, 150 μ g of membrane protein, in complete Freund's adjuvant, was injected intraperitoneally into each mouse. Two mice received a second intraperitoneal injection of 150 μ g protein, in incomplete Freund's adjuvant, on Day 12. All three mice received intravenous injections of 100 μ g protein in phosphate-buffered saline (PBS) on Day 20 and were sacrificed on Day 23.

The spleen cells were fused as previously described (Myles *et al.*, 1981). Culture supernatants were initially assayed for binding to sperm membrane antigens in a solid-phase radioactive binding assay. An arbitrarily selected subset of the positive supernatants was then tested for binding to fixed sperm by indirect immunofluorescence. Most of the cell lines that were positive by indirect immunofluorescence were frozen and stored in liquid nitrogen. Subsequently they were thawed, grown in small quantities, and supernatants were retested to confirm the indirect immunofluorescence. Some lines were cloned by limiting dilution and grown in large quantity, and the others were grown in quantities of about 5 ml. Results from the cloning by limiting dilution were in each case scored by indirect immunofluorescence on live and fixed cells to establish that all the subclones exhibited the same localization of antibody binding.

Solid-Phase Radioactive Binding Assay

The solid-phase assay for the presence of antibody in hybridoma supernatants was performed as previously described (Myles *et al.*, 1981). To assess the heat lability of particular antigenic determinants, the membrane fraction obtained by sonication and ultracentrifugation (Myles *et al.*, 1981) was placed in a boiling-water bath for 10 min, cooled to room temperature, and allowed to

bind to the microtiter plate. Alternatively, to solubilize the antigen before heating, 1/10 volume of 0.25 *M* *n*-octyl- β -D-glucopyranoside, from Sigma Chemical Company (octylglucoside, OG) was added to a suspension of sperm at 1×10^8 /ml in Mg^{2+} -Hepes medium (Green, 1978) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μ M leupeptin. Incubation in the OG continued for 15 min and the cell remnants were removed by centrifuging at 12,000 g_{av} for 10 min at room temperature. One aliquot of supernatant containing the OG-solubilized surface antigens remained at room temperature, and a second aliquot was boiled for 10 min. Each sample was allowed to bind to the microtiter plate. Radioactivity bound to boiled samples in the solid-phase assay was compared to radioactivity bound to untreated samples. For both boiled and untreated samples, binding of culture supernatant from the parent myeloma line is used as a control for nonspecific binding. The counts per minute (cpm) of ^{125} I-goat anti-mouse second antibody bound in the control is the background of the assay and is subtracted from the cpm bound with each hybridoma antibody.

Indirect Immunofluorescence

Indirect immunofluorescence was performed as previously described (Myles *et al.*, 1981). Cells were fixed prior to staining by addition of 1 vol of buffered formaldehyde made fresh from paraformaldehyde, to 1 vol of cells suspended in Mg^{2+} -Hepes at 2×10^7 /ml.

Iodination and Immunoprecipitation of Cell Surface Proteins

Surface proteins of sperm from the cauda epididymis were labeled with ^{125} I using the Iodo-Gen reagent, from Pierce Chemical Company (Lopo and Vacquier, 1980; Markwell and Fox, 1978). In general, five aliquots each containing 1 ml of washed sperm suspended at 3×10^7 /ml in Mg^{2+} -Hepes were added to five 10-ml Pyrex beakers coated with dried Iodo-Gen and 1 mCi Na- ^{125} I was added to each beaker. The iodination reaction was allowed to proceed for 10 min at room temperature, the sperm were pipetted out of the beakers into a centrifuge tube, diluted to about 15 ml with Mg^{2+} -Hepes containing 10 μ M leupeptin, and washed twice at room temperature. The cells were then resuspended in about 0.1–0.2 ml of residual supernatant (Mg^{2+} -Hepes with 10 μ M leupeptin), cooled to 4°C, and solubilized by addition of 1–1.5 ml of "solubilization buffer" containing 0.14 *M* NaCl, 1% bovine hemoglobin, 10 mM Tris, pH 8.0, 1% Triton, 1 mM PMSF, and 10 μ M leupeptin. Solubilization was allowed to continue for 15 min at 4°C, and subsequently the entire solubilized cell mixture was centrifuged at 100,000 g_{av} for 1 hr at 4°C. Free ^{125} I was

removed from the supernatant by dialysis against two 1-liter aliquots of solubilization buffer for 3 hr each. If the labeled antigen mixture was frozen before use, glycerol was first added to a final concentration of 20%.

Immunoprecipitation, electrophoresis, and autoradiography were done as previously described (Myles *et al.*, 1981). In experiments with PH-20, PH-21, and PH-1022, the immunoprecipitates were alkylated following reduction according to Dwyer and Blobel (1976) and then electrophoresed. Variation in the molecular weight (M_r) determined for iodinated antigens in successive electrophoresis runs was less than or equal to $\pm 5\%$ of the M_r .

Autoradiographic films were allowed sufficient exposure times (noted in the figure legends) to visualize the polypeptide bands clearly. The considerable differences in required times result presumably from different levels of ^{125}I labeling of each antigen, extractability and stability of the antigen in 1% Triton, or affinity of the antibodies for the antigen.

In immunoprecipitation experiments in which the antigen mixture was precleared with AH-30, 400 μl solubilization buffer was added to 4×10^6 cpm of antigen mixture and then incubated 2 hr with 120 μl of AH-30 supernatant at 4°C . Following this incubation, 40 μl of rabbit serum anti-mouse IgG was added and incubation at 4°C continued overnight. The mixture was centrifuged to remove the bulk precipitate, and the supernatant was centrifuged twice more to attempt to remove any residual precipitate; 150- μl aliquots of the final supernatant were then mixed with other MAbs and the second precipitation performed as described (Myles *et al.*, 1981).

Examination of Procedures for Possible Proteolysis

To search for possible proteolysis during our procedures, exogenous, ^{125}I -labeled bovine serum albumin (BSA) was added, carried through the procedures, and then examined for proteolytic fragmentation. The ^{125}I -BSA, a kind gift from Dr. J. F. Dice of Harvard Medical School, was stored frozen at -20°C at a concentration of 0.18 mg/ml and 8.25×10^5 cpm/ μl in PBS. Since the exogenous BSA is soluble and will not centrifuge down with the sperm, it is left behind when the sperm are initially pelleted and it must be readded. Therefore, the control experiment to probe for proteolysis was performed in two steps. In the first step an attempt was made to detect protease activity released during the isolation and initial pelleting of cauda sperm. Twenty microliters of ^{125}I -BSA was added to 20 ml of Mg^{2+} -Hepes, a freshly excised cauda epididymis was added and cut into pieces as usual, and the sperm were pressed out from the cut epididymis with wooden applicator

sticks, and then removed by centrifugation at room temperature. The ^{125}I -BSA was allowed to sit an extra 3 min at room temperature in the supernatant (thus giving it extra exposure time to potential proteases) and then SDS-PAGE sample buffer was added and this sample prepared for electrophoresis. In the second step, another sample of ^{125}I -BSA was exposed to all the conditions that pelleted sperm experience as they are resuspended, iodine labeled, detergent extracted, dialyzed, and incubated with antibodies in our procedures. Sperm were released from the cauda, pelleted, and resuspended in 0.5 ml Mg^{2+} -Hepes containing 40 μl of ^{125}I -BSA. The sperm were then "mock-iodinated" using 50 ng of nonradioactive NaI for 10 min in an Iodo-Gen coated beaker. Subsequently, they were removed from the beaker, leupeptin was added to a final concentration of 6 μM , the cells were pelleted twice at room temperature, and resuspended in their own supernatant so as not to discard the exogenous ^{125}I -BSA. The cell suspension was cooled at 4°C and 0.5 ml of two times concentrated solubilization buffer added. The detergent extract was ultracentrifuged, dialyzed, and frozen. This mock-labeled surface antigen mixture containing ^{125}I -BSA was later thawed and immunoprecipitated in the usual fashion with AH-30 supernatant. The ^{125}I -BSA containing supernatant was saved after each washing centrifugation and after the final wash was mixed with 9 vol of sample buffer and prepared for electrophoresis.

RESULTS

Generation and Screening of the Antibodies

The initial screening indicated that the immunization protocol produced many hybrid lines secreting antibodies to sperm membrane antigens. From three splenomyeloma fusions, 832 culture supernatants were assayed and 318 were found to be positive in the solid-phase assay for binding to a sperm membrane preparation. One hundred and seventy-six of these were arbitrarily selected for scoring of binding to fixed sperm by indirect immunofluorescence. Sixty of the supernatants scored negative and 116 were positive by indirect immunofluorescence. One hundred fifteen of the one hundred sixteen positive supernatants contained antibody that bound to a specific region of the sperm. The one culture which initially produced antibody that bound to the entire sperm was subsequently cloned and split into two hybrid cell lines. One clone produced antibody that bound to the whole head and the other produced an antibody that bound only to the tail. Thus, all the hybridoma antibodies tested from the three fusions bound to a localized region on the sperm.

The morphology of the sperm cell and its surface re-

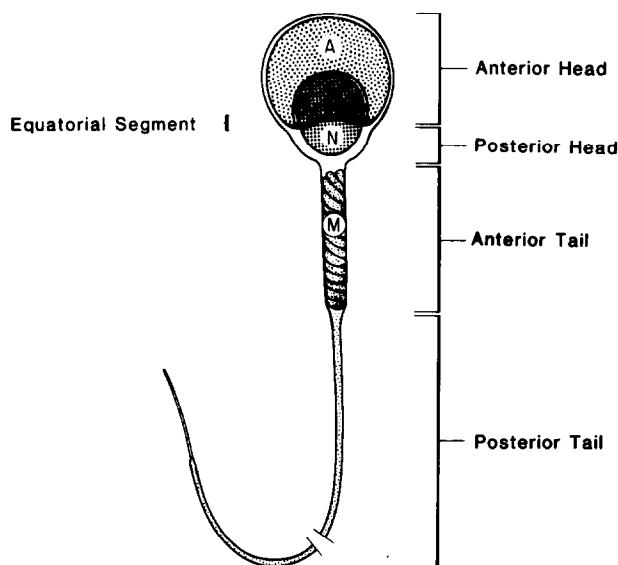


FIG. 1. Diagram of the guinea pig sperm. The asymmetrical sperm cell and the positions of its internal organelles are shown: A, acrosome; N, nucleus; M, mitochondria. The terminology used for sperm surface regions is on the right side of the diagram.

gions are illustrated in the diagram of Fig. 1. The binding patterns to the surface observed with the antibodies included in this study are shown schematically in Fig. 2. From the initial 116 positive hybridomas we now have established 56 stable lines, which, when grown in quantity, continue to secrete antibody to sperm surface antigens (Fig. 2). Letter designations are given to antibodies indicating their localized binding to the anterior head (AH), posterior head (PH), whole head (WH), posterior tail (PT), and the whole tail (WT) (Fig. 2). The anterior head surface is the region that lies over the acrosome and equatorial segment; the posterior head surface overlies the postacrosomal region of the cell; and the posterior tail surface covers the region over the principal piece and endpiece of the tail (Fig. 1). If the antibody is produced by a cloned line it is assigned a number from 1 to 100; if it is produced by an uncloned line it has a number from 1001 to 1100. Thus, for example, PH-30 is an antibody from a cloned line which binds to the posterior head. The results obtained with the individual antibodies are presented for each surface region.

Anterior Head

The binding patterns of antibodies that stain the anterior head and the other distinct regions can be observed on live, swimming cauda epididymal sperm. This is the case for all the antibodies in this study with the exception of the five antibodies in anterior head Group 3a (see below). Since it is possible that the immunofluorescent staining procedure could induce antigen ag-

gregation on live cells, binding to live cells was compared in all experiments with binding to cells fixed in 1.5–4% formaldehyde prior to staining. Alternatively, live sperm at room temperature were compared to live sperm maintained at 4°C throughout the staining procedure. In all cases, the surface domain in which the antibody is observed to bind is the same.

Six anterior head antibodies have been found that precipitate a single ^{125}I -labeled polypeptide, M_r 52K. These antibodies are grouped together under anterior head Group 1 (Table 1), since they apparently recognize the same antigen. A member of this group is AH-2 (Table 1) whose immunofluorescent binding pattern and immunoprecipitation results are illustrated in Figs. 3A,B and Fig. 4 (lane a).

In another group of AH antibodies, anterior head Group 2, each antibody precipitates five polypeptides of M_r 70K, 62K, 46K, 25K, and 18K (Table 1). The immunoprecipitate from Group 2 MAb (AH-40) is shown in Fig. 4 (lane b). AH-40 exhibits a less smooth, more splotchy immunofluorescent pattern on the anterior head (Fig. 3C, D) as compared to the antibodies from AH Group 1 (see AH-2, Fig. 3A, B and AH-1 (Myles *et al.*, 1981).

The major class of antibodies produced in these fusions comprises anterior head Groups 3 and 3a (Table 1). Fifteen of the 56 antibodies studied fall into these two groups. Each antibody in these groups bind specifically to the anterior head (Figs. 3E–H) and precipitates polypeptides of M_r 62K, 52K, and 38K (Fig. 4, lanes c, d). While they precipitate the same ^{125}I -labeled antigen, there are four differences between Group 3 and Group 3a antibodies:

(1) Group 3 antibodies bind to live sperm, whereas Group 3a antibodies do not bind to live sperm. We found that Group 3a antibodies can bind to sperm only if the cells are first treated with saponin, frozen and thawed,

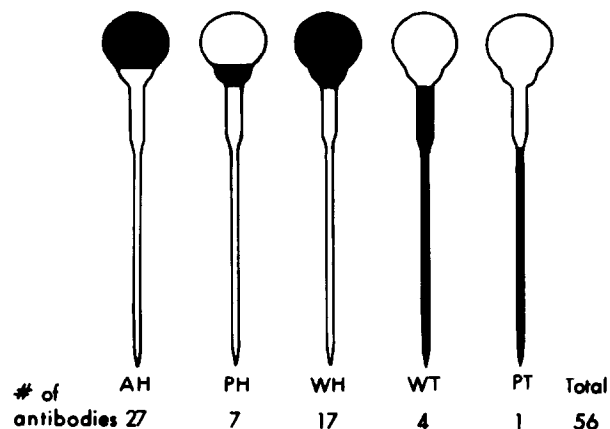


FIG. 2. Summary diagram of the binding patterns observed for the collection of hybridoma antibodies.

TABLE 1
ANTIGENIC SPECIFICITIES OF ANTIBODIES BINDING
TO THE SPERM SURFACE

Localization	M_r of precipitated polypeptides $\times 10^{-3}$	Antibodies
Anterior head		
Group 1	52	AH-1, AH-2, AH-1003, AH-1004, AH-1005, AH-1006
Group 2	70, 62, 46, 25, 18	AH-40, AH-1041, AH-1042
Group 3	62, 52, 38	AH-20, AH-21, AH-1022, AH-1023, AH-1024, AH-1025, AH-1026, AH-1027, AH-1028, AH-1029
Group 3a	62, 52, 38	AH-30, AH-31, AH-1032, AH-1033, AH-1034
Group 4	38, 16	AH-50
Group 5	No bands on gel	AH-1060, AH-1061
Posterior head		
Group 1	60	PH-1
Group 2	66, 48, 41	PH-20, PH-21, PH-1022
Group 3	58, 48	PH-10, PH-1011
Group 4	No bands on gel	PH-30
Whole head		
Group 1	42	WH-1, WH-1002, WH-1003, WH-1040
Group 2	89, 45	WH-30
Group 3	No bands on gel	WH-20, WH-21, WH-1022, WH-1023, WH-1024, WH-1025, WH-1026, WH-1031, WH-1032, WH-1033, WH-1034, WH-1035
Posterior tail		
Group 1	No bands on gel	PT-1
Whole tail		
Group 1	No bands on gel	WT-1, WT-2, WT-1003, WT-1004

or fixed in formaldehyde at concentrations higher than 1% (Figs. 3G, H). Such treatments may result in permeabilizing the cell, thus permitting some access of antibodies to internal components, and under these conditions binding of Group 3a antibodies becomes possible.

(2) Group 3 antibodies show a relatively smooth fluorescent pattern (Figs. 3E, F). Group 3a antibodies have a clearly different, uneven fluorescent pattern that often exhibits holes (Figs. 3G, H).

(3) Group 3 antibodies precipitate lower amounts of the three ^{125}I -labeled polypeptides (Fig. 4 lanes c and d; exposure time lane c, Group 3, 47 days; exposure time lane d, Group 3a, 8 days).

(4) Group 3 antibodies recognize heat-sensitive antigenic determinants whereas Group 3a antibodies recognize heat-stable determinants. In the experiment that revealed this difference, antibody binding to a sperm membrane preparation or to the antigen solubilized in octylglucoside (OG) was measured in the solid-phase assay. The antigenic material was either untreated or

boiled. All 10 Group 3 antibodies recognize heat-sensitive antigenic determinants, whereas all five Group 3a antibodies recognize heat-stable determinants (Table 2).

Anterior head Groups 4 and 5 include the other AH antibodies obtained (Table 1). AH-50, the single antibody in Group 4, has a bright fluorescent pattern (Figs. 3I, J) and precipitates labeled polypeptides of M_r 38K and 16K (Fig. 4, lane e). Group 5 antibodies exhibit a smooth and bright fluorescence (Figs. 3K, L), but show no labeled bands in their immunoprecipitates (Table 1). These two Group 5 antibodies (AH-1060, 1061) may represent unique antigenic specificities. Alternatively, if further characterized they could turn out to recognize, perhaps with low affinity following detergent solubilization, one of the Group 1–Group 4 antigen types.

Relationships may exist between the antigens recognized by antibodies in AH Groups 1–4. Figure 4 shows that AH Groups 3 and 3a precipitate three polypeptide bands with apparent M_r 62K, 52K, and 38K. A band that consistently comigrates with the 62K band is precipitated by Group 2 antibodies (Fig. 4, lanes b–d); a band that consistently co-migrates with the 38K band, is precipitated by Group 4 antibodies (Fig. 4, lanes c–e); and a band that consistently comigrates with the 52K band is precipitated by Group 1 antibodies (Fig. 4 lanes a–d). A "preclearing" experiment was performed to ask (1) if the 62K band recognized by Group 2 AH-40 (70K, 62K, 46K, 25K, 18K) is the same as the 62K band recognized by the Group 3, 3a (62K, 52K, 38K) MAbs, and (2) if the 38K band recognized by Group 4 AH-50 (38K, 16K) is the same as the 38K band recognized by Group 3, 3a (62K, 52K, 38K) MAbs. The ^{125}I -labeled Triton extract of sperm was first precleared by immunoprecipitating with AH-30 (62K, 52K, 38K), and subsequently precipitated either with AH-40 (70K, 62K, 46K, 25K, 18K) or AH-50 (38K, 16K). The pattern obtained with AH-40 following preclearing was unchanged from the control AH-40 pattern indicating that the AH-40 62K polypeptide is not recognized by AH-30 (62K, 52K, 38K). However, after AH-30 (62K, 52K, 38K) preclearing, AH-50 (38K, 16K) gives an immunoprecipitation pattern in which the 38K band is significantly reduced, whereas the 16K band is relatively unchanged (Fig. 4, lane f). This experiment shows that AH-30 (62K, 52K, 38K) recognizes an antigenic determinant on the 38K polypeptide precipitated by AH-50 and these two 38K polypeptides may well be identical. The experiment, in addition, indicates that the MAb AH-50 recognizes two distinct antigens with a common determinant, M_r 38K and M_r 16K, as these two polypeptides are not coprecipitated in the preclearing, and thus apparently are not covalently or noncovalently associated.

The electrophoretic result (Fig. 4, lane f) also illustrates the limitations of this type of preclearing ex-

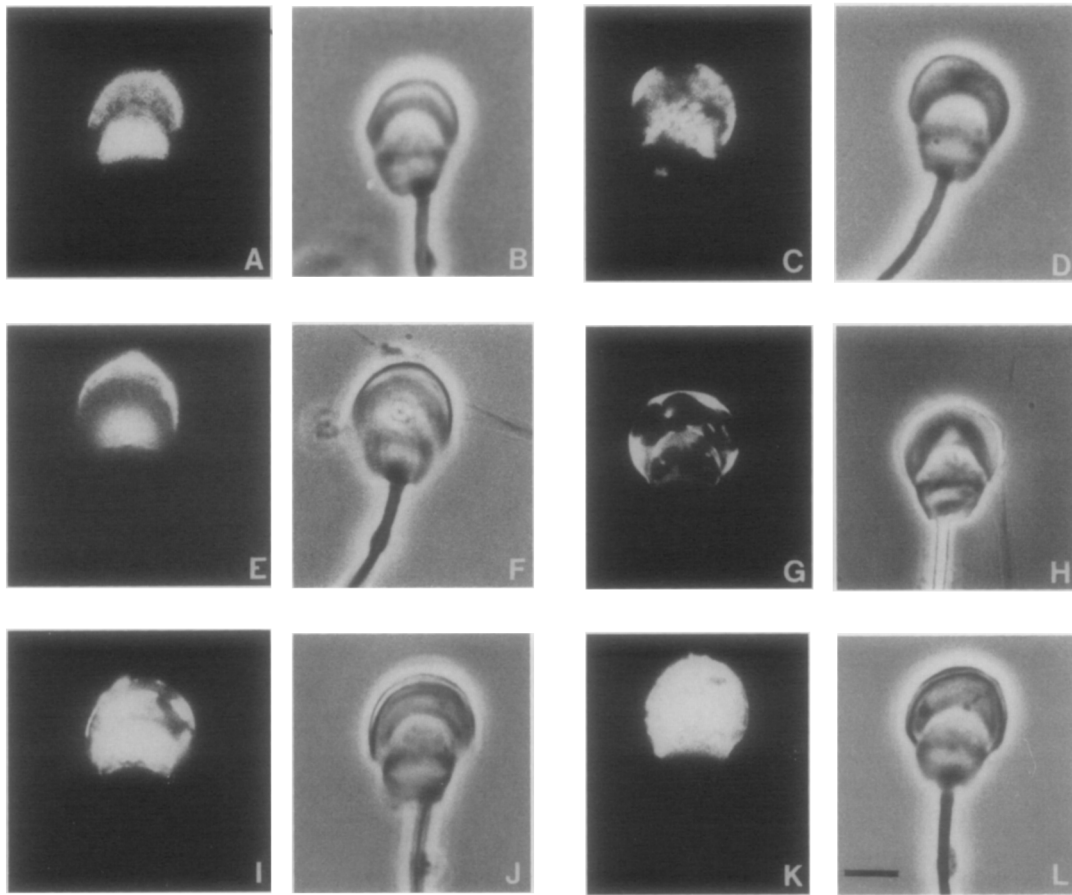


FIG. 3. Binding patterns of six AH antibodies demonstrated by indirect immunofluorescence. Left micrograph in pair shows fluorescence; right micrograph is the corresponding phase contrast image of the same cell (A,B) AH-2, live staining; (C,D) AH-40, live staining (E,F); AH-20, staining after fixation in 1.5% formaldehyde; (G,H) AH-30, staining after fixation in 1.5% formaldehyde. (I,J) AH-50, live staining; (K,L) AH-1060, staining after fixation in 4% formaldehyde. Bar, 5 μ m.

periment: though we varied the conditions of precipitation, it was not possible to quantitatively remove all of the 38K band by preclearing with AH-30 (62K, 52K, 38K). Thus, a low level of the 38K band remains to be precipitated by AH-50 (38K, 16K). Furthermore, a small amount of the 52K band, the strongest band in the AH-30 (62K, 52K, 38K) pattern, appears with the second (AH-50) immunoprecipitate (Fig. 4, lane f). This autoradiographic detection of 52K material probably occurs because less than 100% of the initial AH-30 antigen-MAb complex is removed in the preclearing. This residual complex is then precipitated by the second precipitation step. This appearance of a residual 52K labeled band in the second precipitate after preclearing with AH-30 (62K, 52K, 38K) made it impossible to determine whether AH-30 (Group 3a) could preclear the 52K band recognized by Group 1 antibodies (Fig. 4, lane a, Table 1).

Posterior Head

The seven antibodies that bind to the posterior head can be classified into four groups, whose fluorescent pat-

terns are presented in Fig. 5. Group 1 (Figs. 5A, B) contains a single MAb, PH-1, that precipitates an ^{125} I-labeled polypeptide of M_r 60 K (Myles *et al.*, 1981). Group 2, represented by PH-20, has a fluorescence pattern on the posterior head which differs from the PH-1 pattern, since PH-20 fluorescence is not as bright nor as uniformly distributed in the PH region (Figs. 5A, B, E, F). PH-20 precipitates three bands on a reducing gel with M_r 66K, 48K, and 41K (Fig. 6, lane a). If the immunoprecipitate is run on a nonreducing gel, one band is obtained with M_r 70K (Fig. 6, lane b).

PH-10 (Figs. 5C, D) represents the third group of PH antibodies and has a fluorescent pattern that is similar to that of PH-1 (Figs. 5A, B). It precipitates two labeled polypeptides with M_r 58K and 48K (Fig. 6, lane c). These two polypeptides appear as only weak bands on the film after a 27-day exposure. A third weak band of M_r 65K also appears (Fig. 6, lane c). This third band was apparently a contaminant, since it was present in all the lanes on this particular gel, including control lanes of immunoprecipitates from MAbs that do not bind to the sperm surface (Fig. 6, lane d).

A fourth type of MAb that binds to the posterior head has a fluorescent pattern shown in Figs. 5G, H. This MAb, PH-30, shows no labeled bands in its immunoprecipitate and thus may recognize a unique antigen or have low affinity, following detergent solubilization, for one of the identified posterior head antigens.

Whole Head

Although it appears that the sperm cell has several antigens segregated into either the anterior head or the posterior head surface, other antigens localized on the head are present in both of these domains. These are detected by whole head antibodies and three distinct groups of WH antibodies have been found.

Group 1 contains four antibodies: WH-1, WH-1002, WH-1003, and WH-1040. These antibodies give a bright, smooth fluorescent pattern on the whole head (Figs. 7A, B and WH-1 fluorescence, Myles *et al.*, 1981). WH-1 (Myles *et al.*, 1981), WH-1002, and WH-1003 precipitate a single ^{125}I -labeled polypeptide with M_r 42K (Fig. 8, lane a). WH-1040 precipitates a single band that consistently migrates slightly faster than the 42K WH-1,

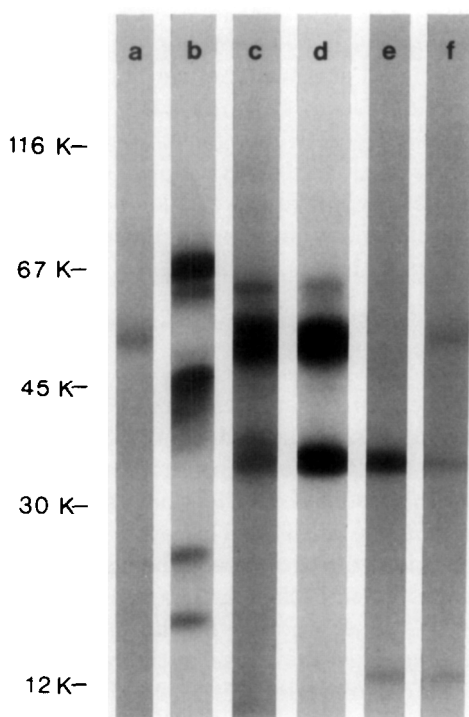


FIG. 4. Analysis of immunoprecipitates of AH antibodies by SDS-PAGE and autoradiography. Lane a, AH-2, 47 days exposure; lane b, AH-40, 27 days exposure; lane c, AH-20, 47 days exposure; lane d, AH-30, 8 days exposure; lane e, AH-50, 28 days exposure; lane f, AH-50 following preclearing with AH-30, 28 days exposure. Molecular weight markers are β -galactosidase, 116K; bovine serum albumin, 67K; ovalbumin, 45K; carbonic anhydrase, 30K; and cytochrome c, 12.4K.

TABLE 2
HEAT LABILITY OF ANTIGENIC DETERMINANTS RECOGNIZED BY
ANTERIOR HEAD ANTIBODIES IN GROUPS 3 AND 3a

	Counts per minute bound to membranes boiled/untreated	Counts per minute bound to OG extract boiled/untreated
Group 3		
AH-20	0/1127	0/229
AH-21	0/1376	0/1533
AH-1022	233/1468	337/2346
AH-1023	102/1710	352/2794
AH-1024	31/1348	0/1303
AH-1025	96/1562	0/2329
AH-1026	0/1492	0/2055
AH-1027	3/902	0/1772
AH-1028	64/950	65/1792
AH-1029	6/350	0/1397
Group 3a		
AH-30	2132/2434	1808 ^a /1643
AH-31	2667/3536	1803/3089
AH-1032	2462/2558	1940 ^a /1718
AH-1033	1789/2052	1842 ^a /891
AH-1034	680/818	1398 ^a /896

^a This increase in antibody binding after heating is unexplained. It may reflect somewhat increased attachment of the heated, solubilized antigen to the solid phase (microtiter plate).

WH-1002, WH-1003 polypeptide with a mobility corresponding to M_r 40K. This polypeptide could be a modified form of the 42K antigen or a distinct antigen, and cloning of the WH-1040 line and further characterization will be needed to find out which is the case.

The second defined type of WH antibody gives a fluorescent pattern (Figs. 7C, D) that generally resembles the smooth, uniform fluorescence of WH Group 1. The single representative of this WH Group 2, WH-30, precipitates two polypeptides, M_r 89K and 45K, in very low amounts that can be visualized by autoradiography after a long exposure (47 days, Fig. 8, lane b).

A large class, 12 whole head antibodies, was obtained which gives no labeled immunoprecipitate (WH Group 3, Table 1). Staining obtained with one (WH-20) of these antibodies is shown in Figs. 7E, F. The binding pattern is less uniform than that observed with WH Group 1, with relatively sparser staining in some subregions. For example, often the posterior head near the neck or the apical portion of the anterior head stains less intensely (Figs. 7E, F).

Posterior Tail

Only one antibody that bind exclusively to the PT region has been isolated. This MAb, PT-1, binds from the tip of the tail up to the junction of the PT with the anterior tail (Myles *et al.*, 1981) and no labeled immunoprecipitate has been obtained.

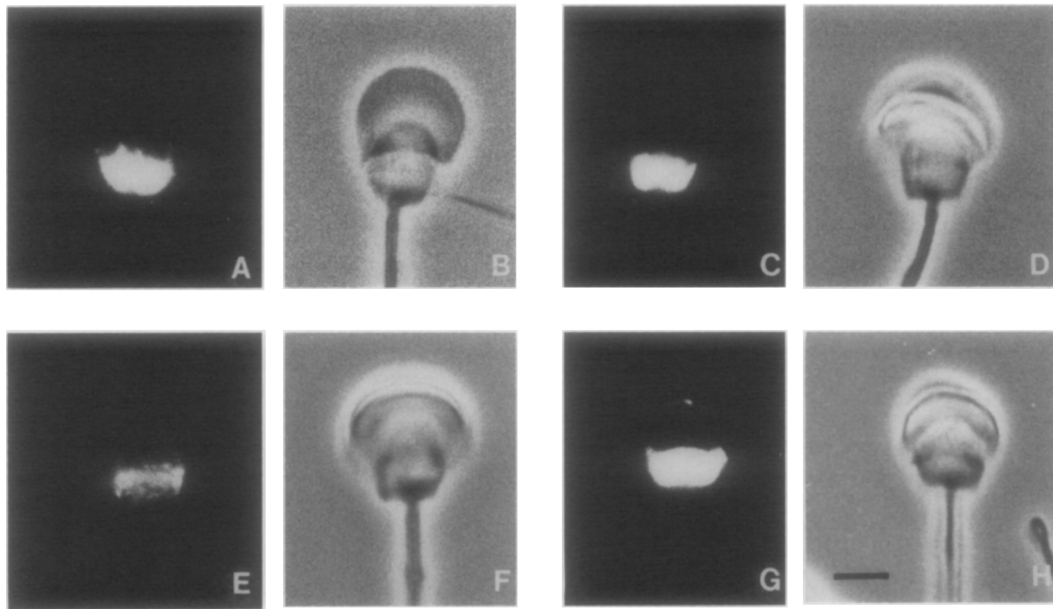


FIG. 5. Binding patterns of four PH antibodies demonstrated by indirect immunofluorescence. Left micrograph in pair shows fluorescence; right micrograph is the corresponding phase contrast image of the same cell. (A,B) PH-1, live staining; (C,D) PH-10, live staining; (E,F) PH-20, live staining; (G,H) PH-30, live staining. Bar, 5 μ m.

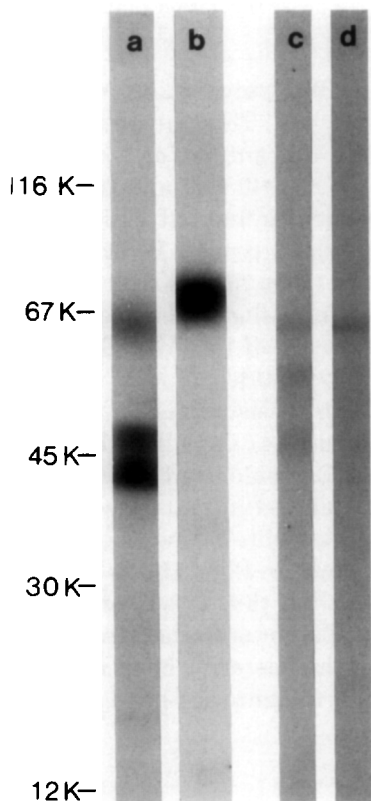


FIG. 6. Analysis of immunoprecipitates of PH antibodies by SDS-PAGE and autoradiography. Lane a, PH-20, 47 days exposure; lane b, PH-20, nonreducing gel, 51 days exposure; lane c, PH-10, 27 days exposure; lane d, control for PH-10; IX C6 α C3, a MAb isolated in these fusions that does not bind to the sperm surface (unpublished results), 27 days exposure. This IX C6 α C3 immunoprecipitate was

Whole tail

We did find another localization of antibody binding on the sperm tail surface, the whole tail region (Table 1). Immunofluorescent staining with WT-2 shows fluorescence along the entire length of the tail and no binding to the head (Figs. 9A, B). None of the four whole tail antibodies precipitates a labeled polypeptide (Table 1), so we do not know how many distinct antigens may be recognized with this localization.

Examination of Experimental Procedures for Possible Proteolysis

Several of the MAbs studied here precipitate multiple ^{125} I-labeled bands, visualized on the SDS-gels. In one case (PH-20, PH-21) it appears that the multiple bands are in part the result of disulfide bonds linking subunits into a multimeric antigen. However, this one antigen is the exception; all the other labeled immunoprecipitates were tested on both reducing and nonreducing gels and show the same number of bands under both conditions, indicating that these antigens do not contain disulfide-linked subunits.

The possibility was considered that the multiple bands arise from proteolysis during our procedures. To minimize any released protease activity, two protease in-

obtained in the same experiment as the PH-10 immunoprecipitate and run in another lane on the same gel. All lanes on this gel showed the 65K band. Molecular weight markers are the same as those in Fig. 4.

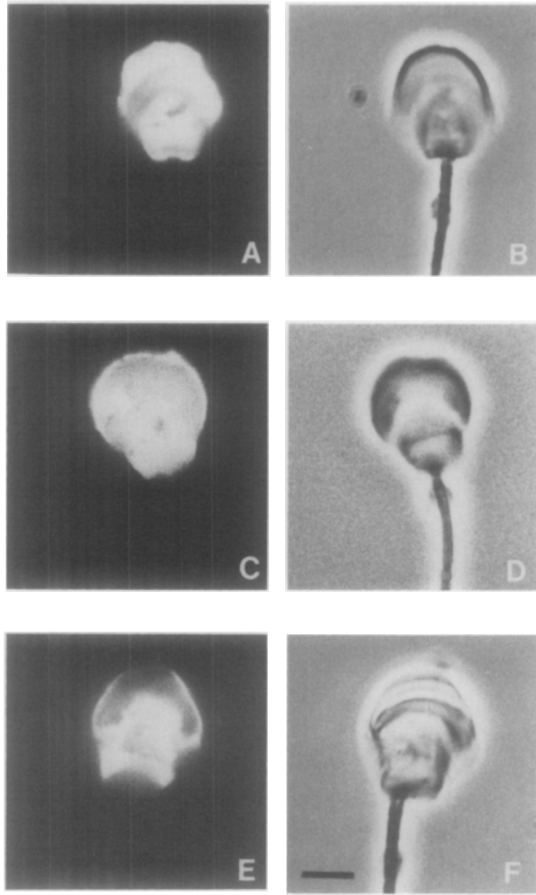


FIG. 7. Binding patterns of three WH antibodies demonstrated by indirect immunofluorescence. (A,B) WH-1002, staining after fixation in 1.5% formaldehyde; (C,D) WH-30, live staining; (E,F) WH-20, live staining. Bar, 5 μ m.

hibitors (leupeptin and PMSF) were added during the cell washing, extraction, and immunoprecipitation. In addition, in a two-step control experiment, exogenous ^{125} I-labeled bovine serum albumin was used to probe for proteolysis at any point during the procedures. After being carried through the procedures, the ^{125} I-BSA was examined by gel electrophoresis to see if any proteolytic fragmentation could be detected. A first aliquot of ^{125} I-BSA was added in the medium with the cauda epididymis as soon as the epididymis was excised and allowed to incubate with the epididymal fragments while sperm were pressed out and then pelleted. A second aliquot of ^{125} I-BSA was added to pelleted sperm and carried through all the steps of labeling, Triton extraction, dialysis, and immunoprecipitation. The gel pattern of these two treated ^{125} I-BSA samples is shown in Fig. 10, lanes a and c, compared to ^{125} I-BSA that remained in the freezer (Fig. 10, lanes b and d). No proteolytic degradation of the treated samples is seen and this suggests that proteolysis of sperm surface proteins is not occurring during our various procedures.

DISCUSSION

Topographical Organization of the Sperm Surface

Our primary conclusion is that the sperm cell can maintain surface domains which have a number of distinct components not present in neighboring domains. An initial understanding of the location of these domains and their molecular composition has been developed. Using the current collection of antibodies, five localizations of surface antigens have thus far been defined: anterior head (AH), posterior head (PH), whole head (WH), posterior tail (PT), and whole tail (WT). These regions of localization are large, each representing a sizeable fraction of the total surface area. At least 12 distinct surface antigens are recognized that can bind antibodies in one of the localized regions (five AH antigens, three PH, two WH, one PT, one WT, cf. Table 1). Twelve distinct antigens is a minimum estimate for two reasons. First, we do not know how many different antigens are recognized by the 20 antibodies (Table 1)

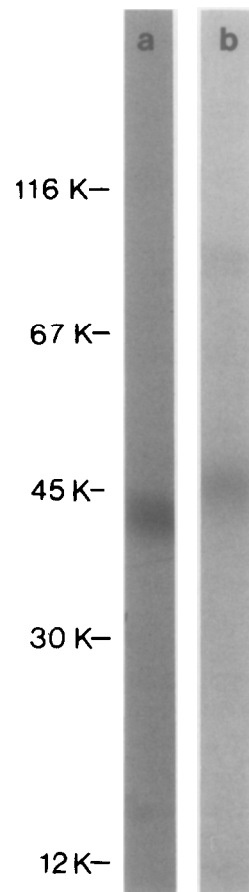


FIG. 8. Analysis of immunoprecipitates of WH antibodies by SDS-PAGE and autoradiography. Lane a, WH-1002, 26 days exposure; lane b, WH-30, 47 days exposure. Molecular weight markers are the same as those in Fig. 4.

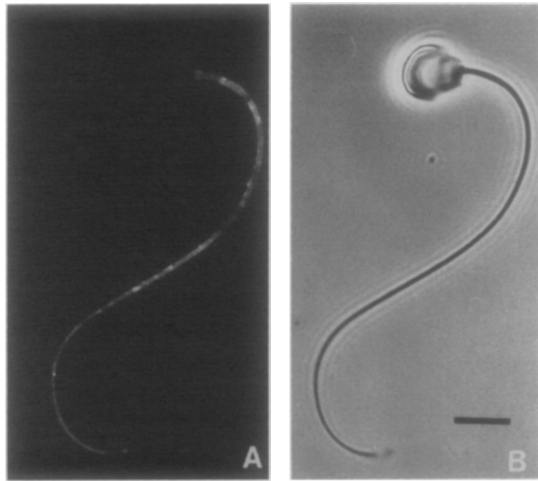


FIG. 9. Binding pattern of a WT antibody demonstrated by indirect immunofluorescence. (A,B) WT-1, staining after fixation in 4% formaldehyde. Bar, 10 μ m.

that fail to give a labeled immunoprecipitate. Second, some individual antibodies (like AH-50, antigens of M_r 38 K and M_r 16K) may be precipitating two or more antigens which have a common determinant. Thus, our results suggest that the sperm surface is a complex mosaic in which distinct regions may have several distinctive molecules, and yet may share other localized molecules with adjacent regions (e.g., WH antigens occupy both the AH and PH domains).

A remarkable finding concerning the sperm surface mosaic is that among over 100 hybridoma antibodies initially examined with indirect immunofluorescence, none was found that bound to the whole cell. This may mean that evenly distributed sperm surface molecules are present at much lower concentration or are much weaker antigens in the mouse hybridoma system than are the localized surface molecules. Alternatively, it may be that there are no evenly distributed antigens on the surface of guinea pig sperm. To proceed in asking whether evenly distributed antigens exist, the current MAb collection could be used to remove immunodominant antigens to yield an immunogen for "second-generation" hybridoma antibodies, following the cascade approach used by Springer (1981).

A significant fraction (20 of 56) of the antibodies studied here fail to yield an ^{125} I-labeled immunoprecipitate. It is possible that some of these 20 antibodies recognize localized glycolipids. The regions of localization that we find for identified protein antigens correspond well with the regions in which particular lipids appear to be relatively concentrated, as defined in the work of Elias, Goerke, Bearer, and Friend (Bearer and Friend, 1980; Bearer and Friend, 1982; Elias *et al.*, 1979; Friend, 1982). Using freeze fracture to visualize membrane protuber-

ances caused by certain probes, Bearer and Friend found that the anterior head plasma membrane is the one region that shows extensive polymyxin B binding (indicating high anionic phospholipid) (Bearer and Friend, 1980, 1982). The anterior head and posterior tail show high filipin binding (indicating high 3β -hydroxysterol content), whereas the posterior head and anterior tail show sparse filipin binding (Elias *et al.*, 1979; Friend, 1982). The data on lipid probes and the results in this paper indicate that the individual regions have several unique protein antigens and differences in their lipid complement. In intestinal epithelial cells, the other mammalian cell type that has been well studied in regard to membrane domains, there are two distinct large plasma membrane regions that contain unique proteins and different lipid compositions (Brasitus and Schacter, 1980; Fujita *et al.*, 1973; Fujita *et al.*, 1972; Kawai *et al.*, 1974). Thus it is possible that the male gamete is an example of a general rule that a polarized cell's large membrane domains will differ in both their protein and their lipid components.

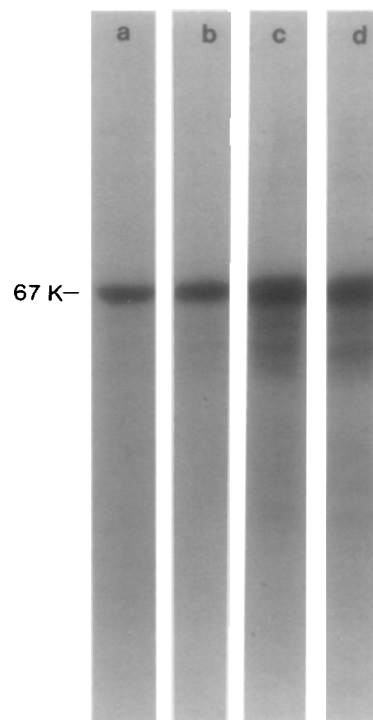


FIG. 10. Comparison of treated and untreated ^{125} I-BSA by SDS-PAGE and autoradiography. Lane a, 10 μ l of ^{125} I-BSA sample that was added to the excised epididymis, exposure 6 days; lane b, 10 μ l of ^{125} I-BSA sample that remained in freezer, exposure 6 days; lane c, 40 μ l of ^{125} I-BSA sample that was carried through labeling, extraction, dialysis, and immunoprecipitation steps, exposure 6 days; lane d, 40 μ l of ^{125} I-BSA sample that remained in freezer, exposure 6 days. Molecular weight markers used were the same as those in Fig. 4. The position of the marker BSA band (67K) is shown.

Nature of the Identified Antigens

Three guinea pig sperm surface antigens were identified with MAbs in our previous report (Myles *et al.*, 1981) and autoantigenic polypeptides of the guinea pig sperm surface have been identified on SDS-PAGE by Teuscher *et al.* (1982). The MAbs characterized in this study have allowed us to identify several additional sperm surface antigens. In some cases, further structural information about the antigens was suggested by the experiments.

The MAb AH-50 precipitates two ^{125}I -labeled polypeptides with M_r 38K and M_r 16K. The 38K band can be selectively removed by pre-clearing with AH-30 (62K, 52K, 38K). This indicates that the 38K and 16K polypeptides are not associated, but rather are two distinct antigens that have a common determinant, recognized by AH-50.

PH-20 recognizes an antigen that exhibits three bands on a reducing gel, M_r 66K, 48K, and 41K. When this immunoprecipitate is run on a nonreducing gel, one band is observed with M_r 70K. It is not apparent how to explain the presence of just the one band with M_r 70K under the nonreducing condition. A possible explanation would be that the 66K band observed on the reducing gel is the result of an artifactual reformation of disulfide bonds (during electrophoresis) between the two lower-molecular-weight bands. This possibility appears unlikely, since the immunoprecipitate was first reduced and then alkylated to prevent reformation of disulfides. An alternative possibility is that both the 66K band, as well as a disulfide-bonded dimer of the 48K and 41K polypeptides, migrate under nonreducing conditions with a mobility corresponding to M_r 70K. Further characterization of the native size and subunit structure of this antigen will be necessary to clarify this result.

The anterior head antigen 62K, 52K, and 38K is precipitated by two sets of antibodies, AH Groups 3 and 3a. However, while the Group 3 antibodies bind to live sperm, the Group 3a antibodies do not bind to live cells and apparently recognize a determinant which is inaccessible in the intact cell. This inaccessibility is not a consequence of the size of the antibody molecules because all the Group 3a (as well as most of the Group 3) antibodies are of the IgG class as shown by analysis on Ouchterlony plates. The Group 3a recognized determinants become accessible to antibodies following cell freezing and thawing, saponin treatment, or fixation in formaldehyde at concentrations of 1% and greater. Formaldehyde fixation of other cell types has been reported (e.g., Satake *et al.*, 1981) to open the cytoplasmic surface of the membrane and internal structures to antibody staining. Guinea pig sperm appear to have altered accessibility to antibodies after 1% (or higher) formaldehyde treatment.

There are several possible explanations for the properties of the two antibody groups. One simple explanation is that in treated cells, Group 3a antibodies are binding to the surface antigen recognized on live cells by Group 3 antibodies. This suggests that the 62K, 52K, 38K antigen may be a transmembrane protein and that Group 3a antibodies bind to a cytoplasmic portion of this protein. Alternatively, they could bind to a cytoplasmic structure (cytoskeletal?) to which this transmembrane protein is itself tightly bound. Although we expected that the external-binding Group 3 antibodies might in some cases recognize heat-stable determinants (carbohydrate), this expectation proved incorrect. The external, Group 3 determinants are heat labile, and for some unknown reason, the cytoplasmic, Group 3a determinants are heat stable.

Some Questions Raised by the Results on Surface Topography

The present data suggest certain new ideas and questions about sperm surface topography. Regional heterogeneity of the sperm surface has frequently been postulated to have a relationship to regional functions of the surface during fertilization. Our finding of five distinct localization patterns of surface antigens, some of them overlapping, suggests that complex models will be necessary to understand the functional significance of molecular localization. A related provocative question concerns the functional role of any group of antigens that are all localized to the same region. Are they part of one multistep process that occurs specifically in that region? Are several of them there to maintain one key antigen in its localized position? Or do they function independently in several distinct, region-specific surface activities? In addition, the mechanisms for the maintenance and development of sperm surface antigen localization remain unknown. One can now ask if different protein antigens in the same region of the sperm surface are maintained there by the same mechanism and if they arrive there during differentiation via the same developmental pathway.

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