STUDIES ON THE DISTRIBUTION OF ANTIGENIC SITES ON THE SURFACE OF RABBIT SPERMATOZOA

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ABSTRACT

Antibodies to rabbit spermatozoa isolated from immunized virgin female rabbits were conjugated to hemocyanin and have been used to determine the pattern of antigenic sites over the surface of rabbit spermatozoa under a variety of conditions. When the spermatozoa are incubated with antibody at 0°C, labeling is almost entirely restricted to the postacrosomal sheath. Incubation at room temperature (22°C) or at 37°C results in progressively increased acrosomal labeling as well as enhanced postacrosomal sheath labeling. Initial incubation at low temperature followed by washing and high temperature incubation yields cells which indicate little tendency for postacrosomal sheath sites to migrate toward the acrosomal region. Absorption of the antibody preparation with rat lymphocytes has no effect on labeling density, whereas absorption with rabbit lymphocytes diminishes the intensity of labeling and yields cells with markers primarily over the postacrosomal sheath. It is concluded that the postacrosomal sheath plasma membrane has specific antigenic properties distinct from other regions of the head.

The antigenicity of spermatozoa was established by Landsteiner at the turn of the century and numerous studies have been carried out exploring the properties and possible function of these antigenic substances (see reviews by Piko [21] and Metz [19]). Only recently have ultrastructural techniques been employed specifically to tag antigenic sites on the sperm surface with the aid of morphological markers such as ferritin (13), tobacco mosaic virus (16), and hemocyanin (15). In the former work, specific histocompatibility antigens were being investigated, whereas in the latter study antisperm antibodies were utilized. It has also been shown that sperm-specific lactate dehydrogenase-x can be localized on mouse sperm surfaces by use of immunoferritin technique (6).

Concomitantly, a number of studies, restricted primarily to established cell lines, have shown that the detailed pattern of antibody labeling on such cells is a function of the temperature of incubation and the concentration of antibody used (4, 9). Such temperature effects in the labeling pattern are apparently caused by membrane mobility phenomena which are activated at temperatures above 0°C. Karnovsky et al. (12) have shown convincingly that the capping and patching phenomena associated with lymphocyte-antibody reactions are related to the movement of membrane moieties culminating in the interiorization of such surface sites by endocytosis. Incubation of the preparations at low temperatures (0°–5°C), on the other hand, prevents such membrane mobility and thus serves as a “control” for subsequent higher temperature membrane transformations. Furthermore, different classes of membrane moieties, IgG and H-2 receptors for example, appear to have quite different degrees of freedom of movement—suggesting variations in the possible mechanisms
of binding of these components within or on the surface of the plasmalemma (12).

In view of the importance of incubation temperature on the final site distribution obtained, together with the observation that many sperm antigens appear to have a differential location on the sperm (head vs. tail, acrosomal region vs. postacrosomal sheath [17, 18]), a study of this parameter has been carried out. Such information may be of importance in determining the stability of site loci and membrane conformations during the various physiological and morphological changes which mammalian spermatozoa undergo during capacitation and the acrosome reaction before making contact with the ovum.

MATERIALS AND METHODS
Preparation and Purification of Antiserum

Freshly collected and washed (Hank’s salt solution) cauda epididymal sperm from New Zealand White rabbits were injected intradermally into two virgin females of the same strain. The inocula consisted of a mixture of 2.7 x 10^7 cells and 2.7 x 10^7 cells of Freund’s complete adjuvant. Five such injections were delivered at 10-day intervals, and the final injection contained 6.6 x 10^7 cells. The animals were exsanguinated by cardiac puncture 2 wk after the last injection, and the antiserum was separated from the whole blood and decomplemented at 56°C for 30 min. The purification of the IgG fraction was accomplished by salting out with ammonium sulfate and DEAE-cellulose chromatography in the same manner as described in a prior report (15). Immunoelectrophoresis of the isolated fraction against goat-antirabbit globulins confirmed that only IgG activity was present in the preparation.

An approximate titer of the preparation was obtained by incubating dilutions of the IgG with rabbit epididymal spermatozoa for 0.5 h at 37°C in the presence of complement. Macroscopic agglutination, as well as microscopic changes, was determined. Concentrations ranged from 300 µg/10^7 cells to 1 µg/10^7 cells. Sperm incubated with the higher concentrations were macroscopically agglutinated and appeared clumped and immotile microscopically. At a dilution of 7 µg/10^7 cells a shift occurred toward less agglutination and higher motility, whereas higher dilutions showed no agglutination and normal motility.

Preparation of Hemocyanin and Coupling to IgG

The procedure of Karnovsky et al. (12), derived from the work of Smith and Revel (23), was followed to obtain and purify hemocyanin from commercially available Busycan canaliculatum (Woods Hole Biological Supply, Woods Hole, Mass.). This method is also summarized in a prior report (15) and will not be repeated here.

35 cm³ of 0.1 M phosphate buffer at pH 6.8, 10 cm³ of hemocyanin solution containing 400 mg protein, and 6.0 cm³ of rabbit-antirabbit sperm globulin (RARSG) containing 40.2 mg protein were combined in a beaker. Conjugation was effected by the dropwise addition of 1 cm³ of 5% glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.), followed by 2 h of slow magnetic stirring. Dialysis of the conjugate against phosphate-buffered salt solution (PBS, pH 6.9) was followed by concentration in an Amicon (Lexington, Mass.) unit fitted with an XM-300 filter. Since this filter will allow passage of immunoglobulin but not hemocyanin, it was possible, by measuring the residual protein concentration in the effluent, to estimate that about 20% of the globulins remained uncoupled. One could then use this fact to arrive at a corrected value for the concentration of conjugated immunoglobulin. The final preparation was sterilized by passage through a Millipore syringe (Millipore Corp., Bedford, Mass.) and stored in small vials at 4°C. Normal (nonimmunized) rabbit IgG (Cappel Laboratories, Downingtown, Pa.) was coupled to hemocyanin in the same manner described above and was used as a control.

Labeling of Spermatozoa

Rabbit spermatozoa collected from the cauda epididymis or ejaculated via artificial vagina were washed in Hanks’ balanced salt solution and counted in a hemocytometer. Seven male rabbits were utilized in the study. Small droplets containing about 10⁷ cells were incubated with concentrations of labeled RARSG ranging from 20 to 100 µg at various temperatures (0°C–37°C). Similar incubations were made containing control media such as hemocyanin-labeled normal rabbit IgG, normal rabbit serum (NRS), or Hanks’ balanced salt solution. The preparations were washed twice in Hanks’ solution after incubation and fixed for 1 h in a cacodylate-buffered glutaraldehyde solution suggested by Jones

![Figure 1](image_url)
(11) to be effective in minimizing membrane shrinkage artifacts. The preparations were washed, stored in distilled water at 4°C, and carbon-platinum replicas were prepared as described previously (15). Freeze-etching (deep-etching) was carried out on cells as prepared above after pretreatment with 5% glycerol for several hours to improve intracellular preservation after quick freezing. This low concentration of glycerol does not significantly interfere with the visualization of external surfaces after deep etching. Replicas were examined in a Philips 201 electron microscope. All figures are printed directly from the negatives resulting in white shadows, and have been mounted so that shadow direction is from the bottom toward the top. Several figures (Figs. 8, 11, 12, and 15) have been rotated in order to maintain specimen orientation.

RESULTS

Freeze-etching can be utilized to view the surface of the plasma membrane when the ice table surrounding the fractured specimen is lowered via deep etching. The hemocyanin label on the surface of antibody-treated rabbit spermatozoa can be viewed in this way (Fig. 1). The postacrosomal sheath-acrosome boundary is ornamented with a zigzag pattern and terminates caudally in the posterior ring. Cords of intramembranous particulate material appear to reinforce the basal region of the sperm head. These structures have been fully described in earlier work (8, 14). It may be worth noting that the particle size distribution seen on the A-face membrane of these sperm heads appears not to be uniform. The membrane overlying the acrosome displays a majority of large particles in the 150-175-Å size class, whereas the particles overlying the postnuclear sheath are, on the average, considerably smaller (100-125 Å). Examination of Fig. 1 will confirm this asymmetry. This observation may be of relevance when considered together with the distribution of labeled antibody over the sperm surface. The determination of the general pattern of antibody distribution over the sperm head can be much more easily assessed by the analysis of standard surface replicas of sperm populations (Fig. 2) and forms the basis of the experimental work which follows.

In addition to the agglutination studies described earlier, a number of control and absorption experiments were performed in order to determine the specificity of the conjugated antibody preparation. Epididymal hamster spermatozoa did not label when incubated under standard conditions (room temperature for 0.5 h) in either the fixed (glutaraldehyde) or the unfixed condition. No hemocyanin markers were found on replicas of rabbit spermatozoa incubated with high concentrations of purified hemocyanin (uncoupled) after two brief washings with Hanks' salt solution, indicating no significant "stickiness" of the hemocyanin molecules to the plasma membrane. Incubation of sperm with labeled, normal rabbit IgG at levels comparable to the immune treated sperm (100 g/10^7 cells) resulted in largely negative cells. Occasionally, a few sparse markers could be detected, particularly at the periphery of the acrosome (Fig. 3). Absorption of the labeled RARSG with rat lymphocytes and subsequent treatment of rabbit sperm with absorbed supernate resulted in undiminished labeling densities as compared to controls (Figs. 4, 5). A reduction in labeling density resulted when the RARSG was first absorbed with rabbit lymphocytes. In these experiments an evaluation was made on cells treated with RARSG first absorbed either once or twice serially with lymphocytes from both male and female rabbits. No significant difference was detectable in the degree of label reduction between the male and female absorbed specimens (Figs. 6-8). Most of the reduction in label seemed to be at the expense of the acrosomal region rather than the postacrosomal sheath.

Preliminary experiments indicated that incubation of sperm with antibody at room temperature resulted in more total labeling of the sperm head, whereas treatment at low temperatures (0°C) yielded predominantly postacrosomal sheath labeling. An experimental regimen including 0°C, room

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**FIGURE 2** Carbon-platinum surface replica of epididymal rabbit sperm incubated in Hanks' balanced salt solution for 0.5 h with no further treatment before fixation. The typical regions and landmarks of the sperm head are clearly demonstrated. × 19,000.

**FIGURE 3** Surface replica of a control sperm cell incubated for 0.5 h at 0°C with rabbit IgG coupled to hemocyanin (70 μg IgG/10^7 cells). Only a few widely scattered hemocyanin markers (arrows) are evident. × 20,000.
temperature (22°C), and 37°C incubation with RARSG confirmed that the intensity of labeling and its spread over the anterior end of the sperm head was enhanced the higher the temperature of incubation (Figs. 9-11). With this particular activity of antibody (46 μg/10⁷ cells), labeling at 0°C resulted in label over the postacrosomal sheath and some markers over the equatorial segment (Fig. 9). Cells labeled at room temperature showed hemocyanin markers distributed over the acrosomal region as well as increased labeling of the postacrosomal sheath (Fig. 10) and 37°C incubation resulted in heavy labeling of the entire sperm head (Fig. 11). Some clusters of markers can be seen in these preparations, but the higher temperatures did not appear to enhance “cluster formation.”

Spermatozoa exposed to very high (100 μg/10⁷ cells) and low (20 μg/10⁷ cells) concentrations of labeled antibody were compared as to hemocyanin localization. At low levels of antibody it was considerably more difficult to determine that the localization of label was temperature dependent. Both 0°C- and room temperature-treated cells showed primarily postacrosomal sheath labeling (Figs. 12, 13), whereas in the case of high antibody concentrations the 0°C-incubated cells had considerable dispersion of label over the acrosomal region (Fig. 15), and at room temperature this region was quite uniformly labeled (Fig. 16). In these two series of experiments an additional sample was initially incubated with antibody at 0°C, washed several times, and then incubated at room temperature for a further 0.5 h in the absence of labeled antibody. It was hoped that any “migration” of initially attached label could be detected in this way. Cells incubated at low antibody levels with this regimen showed about the same distribution (Fig. 14) as their control counterparts labeled in the standard manner. The sperm treated with high antibody concentrations retained the postacrosomal sheath labeling density (Fig. 17).

A number of observations of antibody labeling distribution were also made on ejaculated spermatozoa to determine if any differences could be detected in comparison to the epididymal samples. These cells behaved in a similar manner regarding antibody distribution, including the relative absence of acrosomal labeling at low temperatures and its saturation at higher temperatures.

**DISCUSSION**

A number of light microscope studies employing fluorescent antisperm antibodies indicated that antigen may be localized in a segregated fashion over the head and tail of spermatozoa. Metz (18) showed differences between head and tail localization in sea urchin spermatozoa. Mancini et al. (17) and Hansen and Rebbe (10) have shown regional differences in staining patterns in human spermatozoa. Furthermore, Russo and Metz (22) have shown that treatment of rabbit sperm with antisperm antibody inhibits fertilization in vitro.

The antisperm antibodies used in this study appear to have a high affinity for the membrane overlying the postacrosomal sheath of rabbit spermatozoa. As the temperature of incubation is increased from 0°C to 22°C or 37°C, label is also seen over the acrosomal region. This “spreading” effect is also enhanced if very high concentrations (~100 μg/10⁷ cells) of antibody are used. The fact that incubation at room temperature of previously labeled cells (at 0°C) does not significantly change the distribution of label indicates that “acrosomal” labeling is not the result of migration of postacrosomal membrane sites toward the anterior part of the cell. The special nature of the postacrosomal sheath plasma membrane is further demonstrated when common antigens are first absorbed out with rabbit lymphocytes. In such experiments, the postacrosomal sheath retains a heavy complement of label whereas acrosomal labeling is markedly diminished. The recent observations of Fellous et al. (7) using immunofluorescent...
FIGURES 6, 7, and 8 The results of absorption of the RARSG with rabbit lymphocytes are depicted in this series of micrographs. Typical heavy labeling results with nonabsorbed antibody (65 μg/10⁷ cells) after room temperature incubation (Fig. 6). ×16,500. After two serial absorptions with lymphocytes from a female rabbit, a reduction in label over the acrosome can be seen (Fig. 7). ×18,000. A similar reduction results after treatment with RARSG absorbed twice with lymphocytes from a male animal (Fig. 8). × 19,000.
FIGURES 9, 10, and 11 Epididymal rabbit sperm incubated with RARSG-hemocyanin (46 µg/10⁶ cells) at temperatures of 0°C (Fig. 9), 22°C (Fig. 10), and 37°C (Fig. 11). Labeling over the acrosomal region rises from virtually nil at 0°C to a rather heavy level at 37°C. The labeling density over the postacrosomal sheath also steadily increases with rising temperature. × 18,000.
FIGURES 12, 13, and 14  Epididymal rabbit sperm treated with RARSG-hemocyanin (19 μg/10^7 cells) at 0°C (Fig. 12) and 22°C (Fig. 13). Note that after treatment with this low concentration of antibody the 0°C and 22°C incubations result in rather similar antibody distributions primarily localized over the postacrosomal sheath. When cells originally incubated at 0°C were washed and further incubated at 22°C (Fig. 14), label was still localized to the postacrosomal sheath. × 19,000.
Figures 15, 16, and 17  Epididymal rabbit sperm treated with RARSG-hemocyanin at a high antibody concentration (93 μg/10⁷ cells). When labeled at 0°C, the postacrosomal sheath is heavily labeled (Fig. 15), and some label can also be seen over the equatorial segment of the acrosome. At 22°C (Fig. 16), label is more uniformly spread over the sperm head with heaviest density over the postacrosomal sheath. When cells initially incubated at 0°C were washed and further incubated at 22°C (Fig. 17), no significant shift of label from the postacrosomal sheath could be seen. Fig. 15-17, × 18,000.
ence indicate that an antibody to teratocarcinoma cells can be localized on the postacrosomal sheath of both human and mouse spermatozoa, suggesting some commonality of antigenic properties in this region.

Surface differences in the membrane overlying the acrosome as compared to the postacrosomal sheath have been noted by using a ferric colloid binding technique (reviewed by Bedford, reference 3). Colloidal iron hydroxide binding was shown to be discontinuous over the sperm and tail membrane of a number of mammalian species, including the rabbit, by Yanagimachi et al. (25). These authors found similar sharp differences between acrosomal and postacrosomal sheath labeling patterns. Studies on the localization of histocompatibility antigens (16) indicated that labeled antibody was associated exclusively with the membrane region overlying the acrosome. Kerek et al. (13) using ferritin-labeled HL-A antibodies found irregular clusters or patches over the entire sperm head surface. However, these latter observations are limited by the difficulties of obtaining overall patterns from thin sections.

In contrast to the work done with lymphoid cells (12, 24), there was no evidence of polarized migration of label due to temperature or other influences. The distribution of marker often showed some heterogeneity of a minor sort. Small clusters of hemocyanin molecules were often seen and larger patches were occasionally present, particularly on the most heavily labeled cells. Such distributions, however, were also present on cells fixed with glutaraldehyde before incubation with antibody as well as on cells incubated at 0°C. The overall results indicate a relative lack of long-range mobility of antigenic sites over the sperm head, although small local variations of a random nature would not be detectable by these methods. Differences in labeling pattern between the plasma membrane overlying the acrosome and postacrosomal sheath may be related to intrinsic structural properties of the membranes or, conceivably, to underlying components of the acrosome or postacrosomal sheath which exert restrictive forces across the membrane. Transmembrane restrictions inhibiting lateral movement in the lipid layer have been suggested by Nicolson and Yanagimachi (20) as a possible factor in explaining differences in lectin-induced aggregation of Ricinus agglutinin receptors on rabbit sperm membranes. These authors also find marked differences in labeling patterns between the acrosomal region and the postacrosomal sheath, the latter having a higher degree of receptor shifting and aggregation after treatment with labeled lectin. Elgsaeter and Branton (5) have shown that the removal of spectrin (associated with the cytoplasmic surface of the red cell membrane) causes intercalated membrane particles to be more easily aggregated by inducing agents, demonstrating that restrictive forces can operate at some distance from the centrally oriented plane of the membrane particles. The present observations that the intramembranous particles of the plasmalemma overlying the acrosome are, on the average, larger than those of the postacrosomal sheath membrane further indicate a degree of regional specialization in this otherwise continuous membrane.

Capacitation has been thought to involve subtle transformations of the plasma membrane overlying the acrosome culminating in fusion with the outer acrosomal membrane (see review by Bedford [1]). The postacrosomal sheath membrane, on the other hand, remains stable during this period of time, but is believed to be the initial site of sperm attachment and membrane fusion with the egg (Bedford [2]). These studies are in the process of being extended to capacitated spermatozoa in order to determine possible changes in antigenic properties associated with these dynamic membrane transformations.

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