FINE STRUCTURE OBSERVATIONS ON THE DISTRIBUTION OF ANTIGENIC SITES ON GUINEA PIG SPERMATOZOA

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INTRODUCTION

The antigenic properties of mammalian spermatozoa have been a topic of interest since the work of Landsteiner before the turn of the century, and the last decade has seen a considerable resurgence of interest in this phenomenon. As many as 16 distinct antigens have been reported to exist on or in mammalian spermatozoa (reviewed by Piko, 1967; Metz, 1972). Weil and Rodenburg (1962), and more recently Mancini et al. (1971), have shown that an antigen apparently derived from accessory glands "coats" the spermatozoa and may aid in sperm transport through the female tract. Katsch et al. (1972) have purified and partially characterized an antigen from guinea pig testes causing aspermatogenesis in guinea pigs and have shown it to be a glycoprotein. Antigenicity of acrosomal enzymes (see Metz, 1972) has also been demonstrated and Menge (1970) has shown that specific antienzyme antibody prevents attachment of rabbit spermatozoa to the zona pellucida.

A number of workers have utilized immunofluorescence techniques to demonstrate antigenic regions in spermatozoa. In a key study, Willson and Katsch (1965) showed differences in immunofluorescence patterns on guinea pig sperm derived from various portions of the reproductive tract. Johnson and Hunter (1970) have shown that capacitated rabbit spermatozoa display less immunofluorescence than noncapacitated sperm. A number of workers have demonstrated (see Bedford, 1972, for example) that mammalian spermatozoa attach to eggs at a medial position on the sperm head at the level of the equatorial segment. This region (in human spermatozoa) also shows immunofluorescence after treatment with immune sera from various donors (Hjort and Hansen, 1971). Thus, data on the antigenic characteristics of spermatozoa and changes therein not only may provide valuable insights into the basic immunological properties of these cells, but may also contribute to our understanding of reproductive processes as well.

Little information is available at the fine structural level pertaining to antigen location. The work of Singer (1959) initiated the development of techniques which allow direct visualization of antigenic sites on cell surfaces by utilizing topographical marker molecules coupled to antibodies. Such techniques have more recently been applied to a number of cellular systems, using a variety of molecular markers (Pinto da Silva et al., 1971; Smith and Revel, 1972; Karnovsky et al., 1972; Koo et al., 1973). This report presents preliminary observations on the distribution of antigenic sites on guinea pig spermatozoa treated with rabbit antiguinea pig globulin coupled to hemocyanin. Hemocyanin is a cylindrical molecule so that when viewed in profile fashion it has a square appearance (Van Bruggen et al., 1963). Negative staining of *Busycon* hemocyanin confirms this feature and shows particles approximately 300 μm on a side. This unambituous shape characteristic makes it possible to readily identify the loci of these markers even at modest magnifications.

MATERIALS AND METHODS

Preparation and Purification of Antiserum

Freshly collected guinea pig spermatozoa from the cauda epididymis and ductus deferens (usually from two animals) were mixed and injected intradermally into adult female rabbits. Five injections were given, equally spaced over a 6-wk period. The inocula (1-cm^3 volume) consisted of 22–42 $\times$ 10^6 cells in Hanks' salt solution mixed with an equal volume of Freund's complete adjuvant. The final injection contained 100 $\times$ 10^6 cells, and 12 days later the animals were exsanguinated by cardiac puncture. The antiserum was separated from the blood and decomplemented at 56°C for 30 min. Purification of the IgG fraction involved conventional methods including precipitation with saturated ammonium sulfate and *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) (DE52, Whatman) chromatography. Immunoelectrophoresis against goat antirabbit serum indicated that the collected fraction contained only IgG activity. In order to get an approximate titer of the antibody preparation, sperm from the cauda epididymis were treated with a dilution series ranging from 1,000 to 10 $\mu g/10^7$ cells. Treatment was carried out for 0.5 h at 37°C in the presence of complement. Cells treated with the higher concentrations of antibody showed gross agglutination and microscopically, loss of motility and acrosomes. At a concentration of 100 $\mu g/10^7$ cells, the degree of macroscopic agglutination was reduced and a significant number of cells retained acrosomes and motility. At 50 $\mu g/10^7$ cells, agglutination was slight and the majority of cells were mobile with intact acrosomes. Lower concentrations of antibody were without effect.

Preparation of Hemocyanin and Coupling to IgG

The methods utilized are similar to those employed by Karnovsky et al. (1972) and are briefly summarized here.

About 1 dozen *Busycon canaliculatum* (Woods Hole Biological Supply, Woods Hole, Mass.) were cut around the foot with a scalpel and allowed to drain into beakers. About 300 cm^3 of hemocoel fluid were collected, vacuum filtered, and centrifuged at 3,000 rpm in an International refrigerated centrifuge (International Equipment Company, Needham Heights, Mass.) to remove debris. The supernate was then centrifuged in a Spinco ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 20 min (10,000 *g*) to remove a fine white precipitate and the resultant supernate run at 100,000 *g* for 1 h to pellet the hemocyanin. This pellet was covered with 3% NaCl and allowed to slowly dissolve overnight at 4°C. The hemocyanin was dialyzed against phosphate-buffered saline (pH 6.9) and 4 cm^3 containing 384 mg protein were mixed with 5 cm^3 of purified IgG containing 40.5 mg protein in 40 cm^3 of 0.1 M phosphate buffer (pH 6.75). 1 cm^3 of 5% glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) was added dropwise and stirring continued for 2 h. The solution was dialyzed against phosphate-buffered saline and centrifuged at 1,700 *g* to remove precipitated material. The supernate was transferred to small vials and stored at +4°C.

Labeling of Spermatozoa

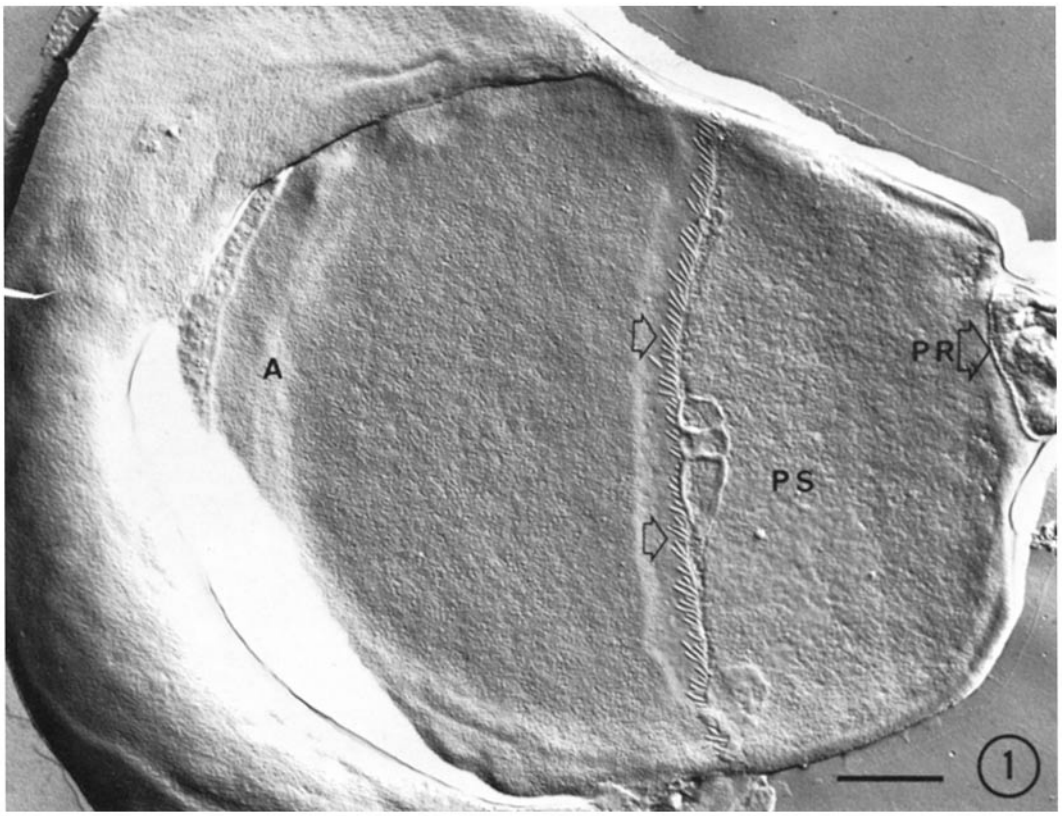
Freshly obtained guinea pig epididymal spermatozoa in Hanks' balanced salt solution were incubated at 4°C for 30 min with various concentrations of the rabbit antiguinea pig sperm globulin-hemocyanin complex (RAGPSG-hemocyanin) ranging from 14 to 63 $\mu g/10^7$ cells. After incubation, the cells were washed two or three times with cold (4°C) Hanks' salt solution. Fixation was carried out with Karnovsky's fixative (1965) for 1 h at 4°C, followed by two washes in cold cacodylate buffer. Controls going through the various media (not containing RAGPSG-hemocyanin) were also prepared for replication.

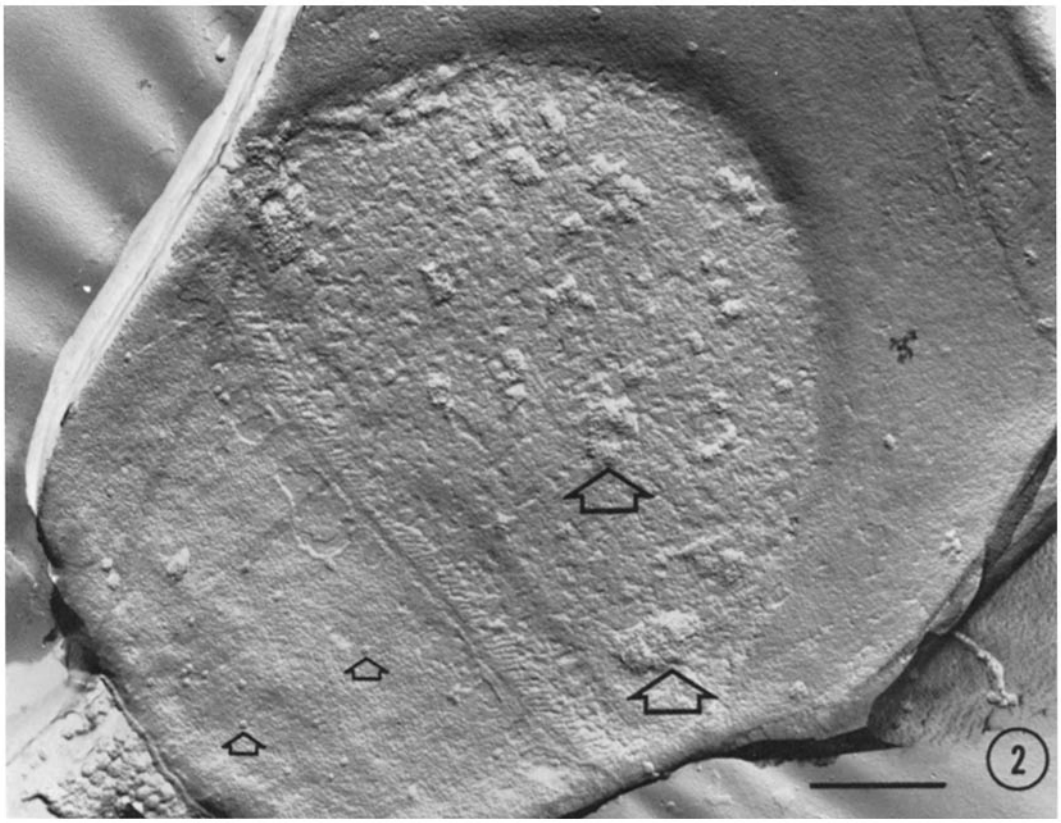
Replica Preparation

The fixed spermatozoa were rinsed twice with distilled water and allowed to dry (air) on parlodion-coated grids. Carbon platinum replicas were prepared and the grids cleaned by removing the parlodion in acetone and floating on a Clorox solution, followed by several rinses with distilled water. The

FIGURE 1 Guinea pig spermatozoon, treated with supernate from twice absorbed (with guinea pig sperm) RAGPSG-hemocyanin complex. Note acrosomal region (*A*) including an expanded peripheral portion, "stitching" at the posterior border of the acrosome (arrows), postacrosomal sheath (*PS*), and posterior ring (*PR*). $\times$ 14,000.

FIGURE 2 Guinea pig spermatozoon treated with RAGPSG-hemocyanin complex. Large clumps of the marker protein are seen on the acrosomal surface (large arrows). Individual markers are seen on the postacrosomal sheath surface (small arrows). $\times$ 18,000.


A
PR
PS
1


2

dried grids were examined in a Philips 201 electron microscope. The figures are printed directly from the negatives so that the shadows appear white.

RESULTS

Control guinea pig spermatozoa, that is, those carried through a 0.5-h incubation at 4°C in media not containing tagged antibody as well as antibody-treated cells, showed regions typical of other mammalian spermatozoa (Fig. 1). Included among these structures are a broad "inflated" acrosomal cap, postacrosomal sheath, and posterior ring. So-called "basal striations," oriented cords of material noted just anterior to the posterior ring of sperm from other mammalian species, were not observed on guinea pig spermatozoa. The posterior border of the acrosome is ornamented by a fine periodic "stitching" (Fig. 1). The surface membrane of both control and experimentally treated sperm sometimes appeared to be partially ruptured or blebbed, particularly over the acrosomal region.

To insure specificity of the guinea pig sperm antibody, a number of absorption experiments were conducted. In one such experiment, hemocyanin-labeled antibody was twice absorbed with guinea pig lymphocytes. Absorbed antibody was then reacted with guinea pig spermatozoa. The results clearly demonstrated that the pattern of labeled antibody on the spermatozoa was similar to that of those treated with nonabsorbed antibody. In a similar fashion, antibody was twice absorbed with guinea pig spermatozoa. Sperm treated with this preparation showed essentially no markers (Fig. 1). Cells briefly (15 min) fixed with glutaraldehyde and then exposed to labeled antibodies were also found to be unlabeled. Procedures of this sort indicate the high specificity of the antibody preparation.

Cells labeled with antibody do not appear to display a completely random distribution of the hemocyanin markers (Fig. 2). Single or small groups of markers can be seen primarily over the postacrosomal sheath (Figs. 2, 3), whereas the membrane overlying the thin, nonexpanded portion of the acrosome is heavily labeled with large clusters of hemocyanin molecules (Figs. 2, 4). The outermost margin or expanded region of the acrosome appears much less heavily labeled. These impressions are gained primarily from the incubations with high concentrations of labeled antibody (63 μg per 1 $\times$ 10^7 cells). It is conceivable that such clustering occurred due to mobility of membrane components during labeling. Since the procedure was carried out at 4°C, however, this would seem to be a remote possibility. More uniform and lighter labeling resulted after incubation with a fivefold lower concentration of labeled antibody. A significant fraction of both control and experimentally treated cells showed a partial or complete loss of acrosomal components. This loss most probably is the result of a false acrosome reaction incurred during the incubation of cells in the various media before fixation. Such damage may also be caused by cold shock of sperm at the incubation temperatures used. In such cases the equatorial segment region remains attached to the cells and often exhibits hemocyanin labeling.

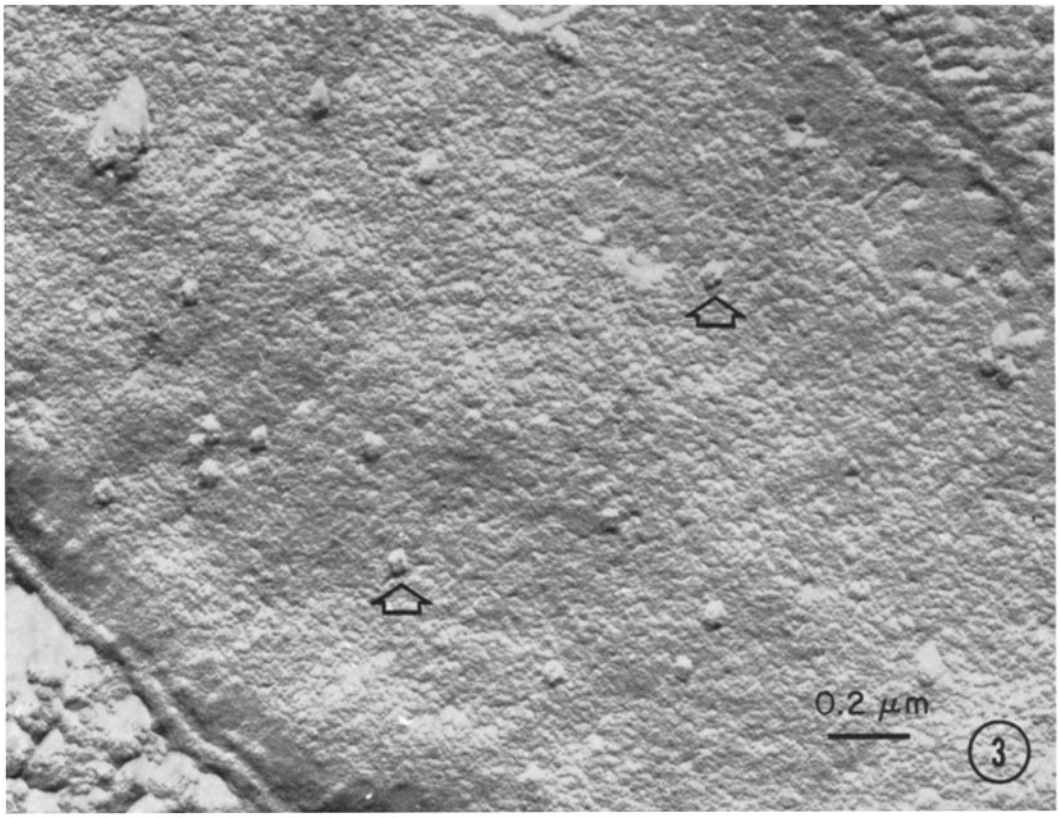
Although no systematic effort was made to determine the extent of label attached to the sperm tails, it can be stated that such labeling was very light and that few marker molecules were observed on the neck region.

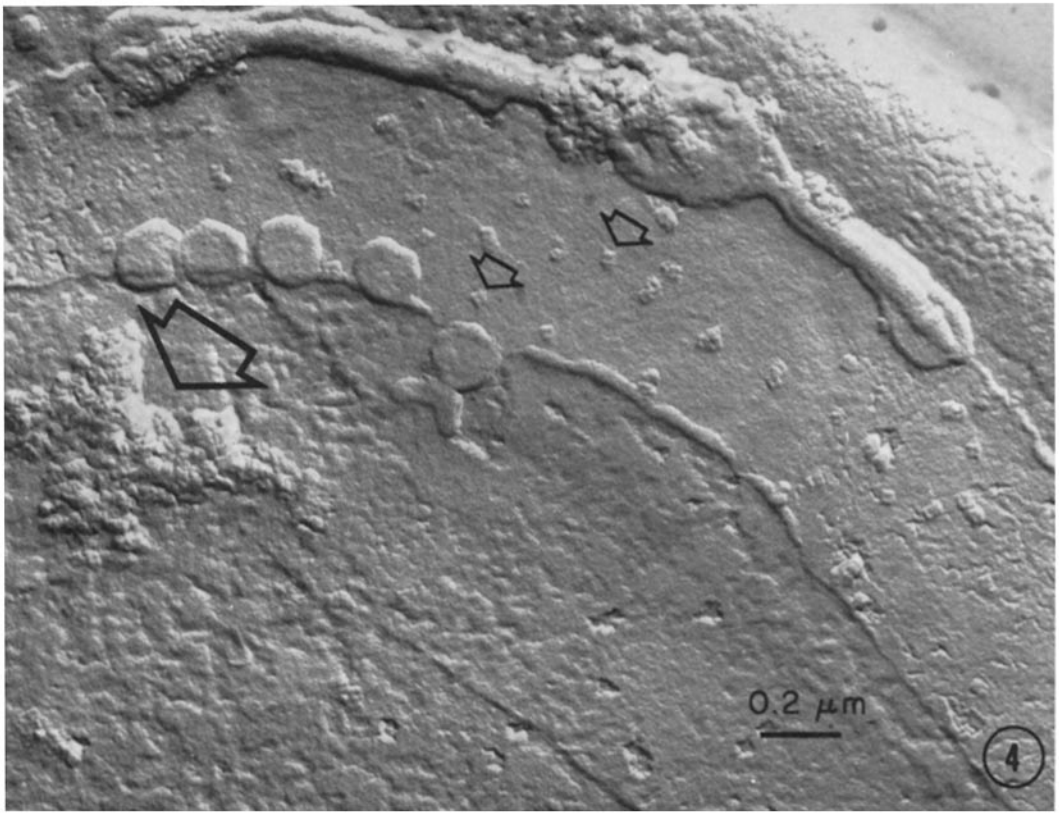
DISCUSSION

Previous immunohistochemical and fluorescence observations at the light microscope level have shown that mammalian spermatozoa bind specific antibody in a nonuniform manner (see Metz, 1972, for review). Mancini et al. (1971), working with a variety of antisera against human spermatozoa, showed that the acrosome, postacrosomal sheath, "intermediate segment" (apparently synonymous with an extended neck region), and neck

FIGURE 3 Higher magnification of a portion of Fig. 3 in the region of the postacrosomal sheath. The "square" configuration characteristic of hemocyanin can easily be detected at various sites (arrows). $\times$ 53,000.

FIGURE 4 Guinea pig spermatozoon treated with RAGPSG-hemocyanin complex. Shown is an area near the anterior margin of the acrosome including a surface membrane fold or fragment. Numerous individual profiles of the square hemocyanin markers can be seen (small arrows) together with a large clump of marker material (large arrow). $\times$ 51,000.


0.2 μm
3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are the main reactive areas, and that the intenseness of reaction varies with the segment of the reproductive tract from which the sperm were derived. The most intense reactions on epididymal cells were noted on the acrosome and postacrosomal sheath. Johnson and Hunter (1970) found the most intense fluorescence associated with the acrosomes of rabbit sperm treated with cow antirabbit sperm serum. A very recent study by Koo et al. (1973) employing a unique gradient labeling procedure showed that specific graft rejection antigen (H-Y) could be localized on the acrosomes of mouse spermatozoa, using tobacco mosaic virus as a marker. This antibody system may have quite different specificities, functions, and distributions than the specific antisperm antibody employed in the present study. To our knowledge, the study of Koo et al. (1973) is the only other study showing localization of such materials at the ultrastructural level.

Although it is conceivable that the "clustering" of labeled antibody occurring over the acrosomal area in some of our preparations is related to site motility on or in the membrane, a number of workers have shown that such rearrangements do not occur at 4°C (Taylor et al., 1971; Karnovsky et al., 1972). Furthermore, our preliminary experiments with cells labeled at room temperature indicate that there are no dramatic differences in labeling patterns at the higher temperature.

In a study also employing guinea pig sperm, Willson and Katsch (1965) found that the "outer" (expanded portion) acrosome stained less intensely than the "inner" (thin portion) acrosome in testicular sperm and that epididymal cells gave a uniform intense acrosomal staining. The postacrosomal sheath, neck, and tail gave a more diffuse but detectable fluorescence. The present results indicate that even in epididymal sperm there may still be a differential binding in various acrosomal regions. The finding of uniform fluorescence in epididymal sperm acrosomes by Willson and Katsch (1965) may be due to the lower sensitivity of light-fluorescence microscopy.

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