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Topographical Location of H–Y Antigen on Mouse Spermatozoa by Immunoelectronmicroscopy

(hybrid antibodies/tobacco mosaic virus/gradient centrifugation/replicas)

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ABSTRACT H–Y (male) antigen was visually located on mouse sperm by electron microscopy, by use of the indirect hybrid antibody method and tobacco mosaic virus as the visual marker. Labeling was achieved by centrifugation of the sperm through a discontinuous gradient consisting of alternating layers of immune reagents and wash solutions. Treated sperm were examined topographically by preparation of platinum–carbon replicas. Antigen was located mainly on the acrosomal cap of the sperm head.

Among the most valuable methods for analyzing the organization of the cell surface is the use of visual labels attached to antibody as a means of locating surface antigens. To date, studies of this sort in mice have centered mainly on normal and malignant lymphoid cells; other cell types have received less attention, largely because of technical limitations. The antigenic organization of the sperm surface may be particularly relevant to studies of the relationship between the cell surface and morphogenesis: an antigen has recently been detected serologically on mouse sperm, but not on other adult cells, which is specified by a mutant gene at the *T* locus, one of a series of alleles resulting in lethal morphogenetic abnormalities during embryogenesis (1).

In order to study sperm effectively by visual labeling and electron microscopy, it is necessary to avoid the repeated centrifugations and resuspensions involved in the usual labeling procedures, because these are detrimental to sperm viability; it is also necessary to achieve more revealing views than those afforded by conventional thin sections. The procedure we describe is a one-step centrifugation through a gradient consisting of alternate layers of immune reagents and wash solutions, followed by preparation of platinum–carbon replicas of treated sperm.

The first antigen we studied in this way was the H–Y (male) antigen, which is responsible for rejection of male skin grafts by female recipients. Females rejecting male grafts produce H–Y antibody, and antiserum made in this way has recently been used in serological tests to show that H–Y antigen is present on the surface of mouse sperm (2). In the present study, H–Y antigen was located by use of the hybrid antibody method (3), with tobacco mosaic virus (TMV) chosen as the visual marker because its size and shape render it easily distinguishable from structural features of the sperm surface.

Abbreviation: TMV, tobacco mosaic virus.

MATERIALS AND METHODS

Antiserum. A pool of antiserum to H–Y antigen was obtained from C57BL/6 female mice 7–21 days after rejection of second-set skin grafts from C56BL/6 males.

Preparation of Cells. Epididymal sperm were obtained in suspension by cutting the entire epididymus into short segments in a few drops of phosphate-buffered saline, pH 7.0 containing 5% heat-inactivated (56° for 30 min) fetal bovine serum (immunoprecipitin-tested, free of IgG; Grand Island Biological Co., Grand Island, N.Y.), 5 mg/ml of fructose, and 0.1 mg/ml of $CaCl_2$ (4). After 5–10 min at room temperature (25°), medium containing swimming sperm was removed by pipette.

Isolated epidermal cells to be used for absorption of antiserum were obtained from tail skin by trypsinization (5) and were kept overnight at 4° before use.

Reagents. Rabbit $F(ab')_2$ hybrid antibody, anti-mouse IgG/anti-TMV (6), was used at a concentration of about 0.1 mg/ml with respect to anti-IgG. Purified TMV was used at a concentration of 0.5 mg/ml.

Preparation of Gradients. Tubes were made from 20-cm lengths of 1-ml disposable pipettes (inner diameter, 3 mm; outer diameter, 5 mm; Corning Glass Works, Corning, N.Y.), siliconized with 1% dimethyldichlorosilane in benzene (Bio-Rad Laboratories, Richmond, Calif.); one end was heat-sealed and blown to a conical tip.

Gradients of immune reagents and wash solutions were established in these tubes by adding sequentially, by micropipette, layers of decreasing specific gravity (Table 1 and Fig. 1). Fetal bovine serum was added to each layer in an amount determined empirically to prevent intermixing of layers during subsequent centrifugation of sperm. To ascertain that the gradient was preserved during centrifugation, layers were visually distinguished with Earle's balanced salt solution containing phenol red and 5 mg/ml of fructose for reagent layers, and phosphate-buffered saline supplemented with 5 mg/ml of fructose for wash layers (Fig. 1). Gradients were prepared and maintained at 4° and were used within 1–2 hr after preparation.

Prior Incubation of Sperm with Antiserum. 5×10^6 sperm in phosphate-buffered saline were incubated in 0.1-ml volumes with H–Y antiserum or control serum, diluted 1:3, for 20 min at 22°. At this dilution, H–Y antibody was present in large excess, since a similar aliquot of antiserum absorbed with 10^7

sperm still contained H–Y antibody detectable by immunoelectronmicroscopy. This cell suspension was then diluted to 0.5 ml with phosphate-buffered saline and 0.10–0.15 ml was layered onto a prepared gradient, above the first wash layer (Fig. 1).

Centrifugation of Gradients. Centrifugation was at 1000 rpm for 15 min followed by 1200 rpm for 5 min, at 4° in a PR-2 refrigerated centrifuge (269 head; International Equipment Co., Boston, Mass.); this resulted in a steady flow of sperm through the gradient at a rate we know to afford optimal labeling. After centrifugation, sperm were generally slightly less viable (about 75% alive) than untreated sperm (about 85% alive) as indicated by trypan blue exclusion, and about half of the treated sperm were motile at room temperature.

Preparation of Replicas. For topographical examination of treated sperm, platinum–carbon replicas were prepared. Sperm were removed from pellets at the bottom of the centrifuged gradients and gently resuspended in a few drops of Ringer's solution (Cutter Laboratories, Berkeley, Calif.) containing 5 mg/ml of fructose. A drop of this sperm suspension was placed on a formvar-coated 75-mesh grid (prepared with 0.5% formvar in ethylene dichloride). After 5–10 min were allowed for sperm to attach to the formvar, excess suspension was removed by pipette and the grid was floated on a drop of 1% glutaraldehyde in 0.15 M cacodylate buffer (7) for 10 min. In a similar manner, the grid was rinsed briefly with Ringer's solution and dehydrated progressively in 70%, 95%, and 100% ethanol, and finally air-dried from 100% ethanol. Postfixation with osmium tetroxide after fixation with glutaraldehyde did not improve sperm preservation, and was therefore not used. All procedures were performed at 4°, except for dehydration in 100% ethanol at 22°. The grid was shadowed at a 45° angle with platinum–carbon, and at a 90° angle with carbon, in a Balzers BA360M freeze-etch apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.). The formvar support film was dissolved by immersing the grid in chloroform for 1 min; the replica was cleaned by floating the grid on 50% clorox in distilled water for 30 min, air-dried, and examined with a Zeiss EM 9S-2 electron microscope (Carl Zeiss, Inc., New York, N.Y.).

RESULTS

Serology of H–Y antiserum in relation to sperm

As there is no available counterpart of sperm in females for purposes of control, the specificity of H–Y antiserum reactions with sperm cannot be confirmed by the general method of demonstrating absence of reaction with a comparable antigen-negative cell. Instead, specificity is assured by use of two aliquots of the same H–Y antiserum (B6 ♀ anti-B6 ♂) as follows: aliquot *A* is absorbed with male epidermal cells, and aliquot *B* is absorbed with female epidermal cells. These absorptions were performed by incubation of 0.2 ml of H–Y antiserum (diluted 1:3) with 0.2 ml of packed epidermal cells for 30 min at 4°. We have ascertained that *A* gives no reaction with sperm above the background seen with normal (virgin B6 ♀) mouse serum, whereas *B* (see below) is positive. The positive reaction in these circumstances can be due only to H–Y antigen.

The use of epidermal cells for this absorption is essential because skin grafting (even skin autografting) induces autoantibodies that are cytotoxic for male and female epidermal cells of all mice and for sperm (M. Scheid and L. Flaherty, personal communication). These autoantibodies in H–Y antiserum are not completely absorbed by hemopoietic cells from males or females.

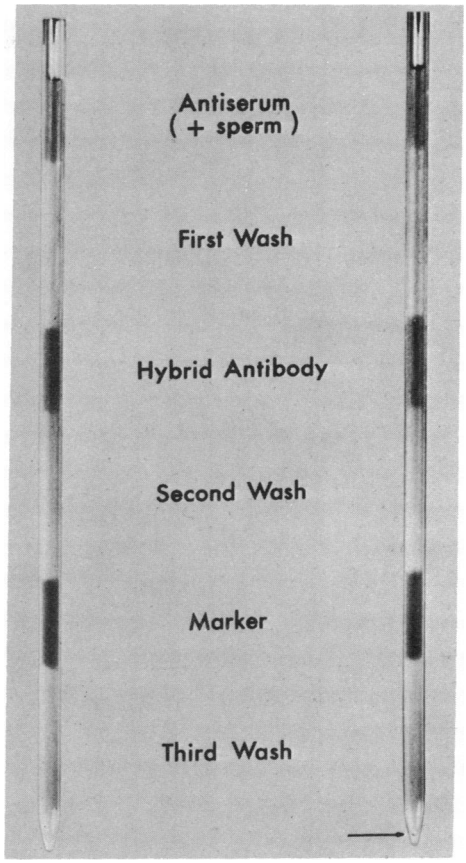

FIG. 1. Gradient consisting of alternate layers of immune reagents and wash solutions before (*left*) and after (*right*) centrifugation of sperm. Layers are discernible due to the presence of phenol red in the reagent layers. A pellet of sperm is indicated (*arrow*).

Location of H–Y antigen on sperm

The labeling sequence used to visually locate H–Y antigen was: (*a*) H–Y antiserum (previously absorbed with female cells); (*b*) hybrid antibody; and (*c*) TMV. Although replicas

TABLE 1. *Gradient composition*

Layer	Composition*	Total volume (ml)	Specific gravity (g/cm³)
Antiserum	0.1 ml of antiserum, diluted (plus sperm)	0.1–0.15	<1.0045
First wash	0.02 ml of FBS	0.2	1.0050
Hybrid antibody	0.05 ml of hybrid antibody + 0.02 ml of FBS	0.1	1.0060
Second wash	0.05 ml of FBS	0.2	1.0070
Marker	0.01 ml of TMV + 0.03 ml of FBS	0.1	1.0080
Third wash	0.08 ml of FBS	0.2	1.0100

* Diluted to total volume with phosphate-buffered saline plus 5 mg/ml of fructose for wash layers, or with Earle's balanced salt solution (with phenol red) plus 5 mg/ml of fructose for reagent layers, to visually distinguish adjacent layers. FBS, fetal bovine serum.

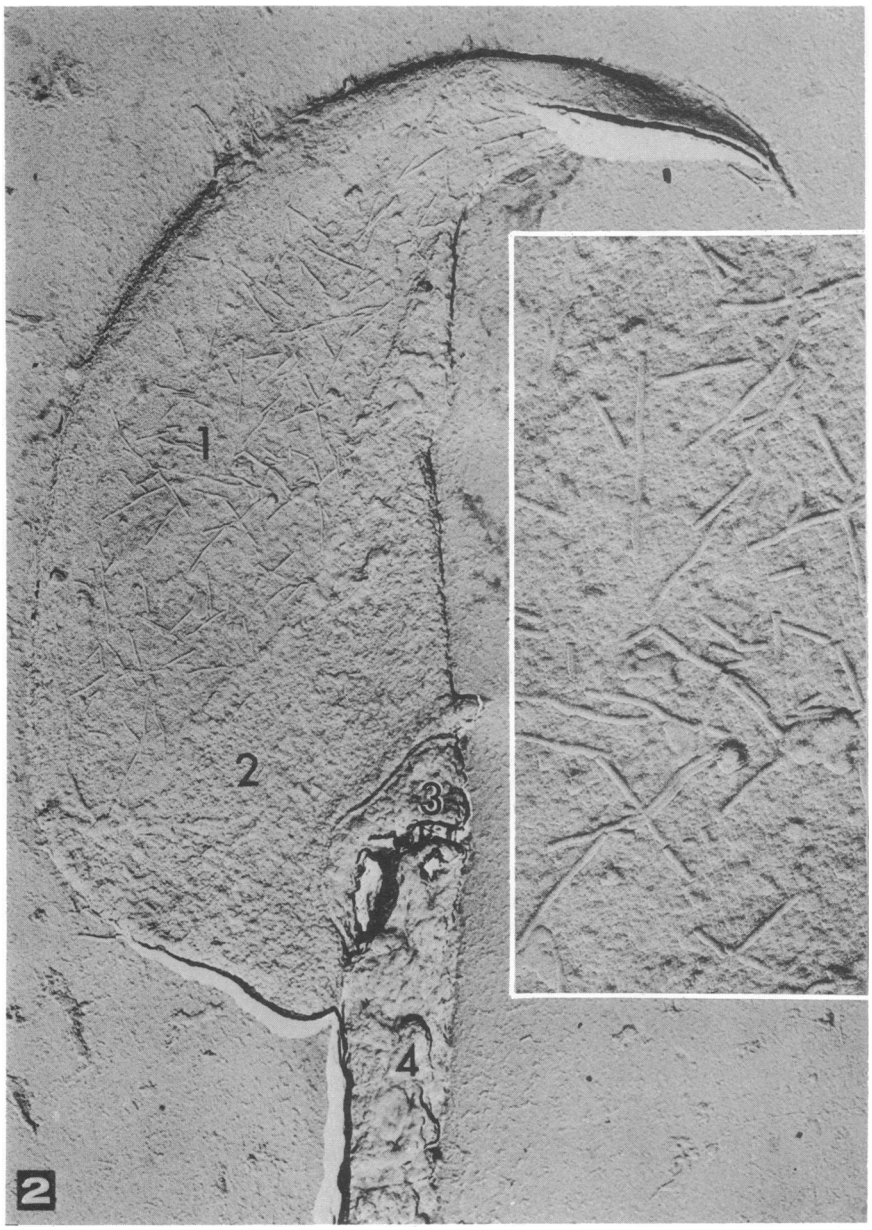

FIG. 2. Replica of a mouse sperm head labeled for H-Y antigen with TMV. Label is confined to the acrosomal cap (*1*), and is absent from the post-acrosomal region (*2*), the connecting piece (*3*), and the midpiece (*4*). ×17,100. *Inset:* a higher magnification of the labeled acrosomal cap region, marked by a regular periodicity of the surface. ×50,540. Direction of shadowing: upper right corner to lower left corner.

provide a topographical view of one side of the entire sperm, label specifically associated with H-Y antigen was confined to the head. In 59 of 65 (90%) labeled sperm, TMV was located primarily on the acrosomal cap of the head (Fig. 2) [terminology according to Fawcett (8)], the surface of which is marked by an ordered periodicity of 125 Å. Little or no label was present on other morphologically identifiable regions of the sperm head, the post-acrosomal region, or the connecting piece. In the remaining 10% of labeled sperm, a more uniform distribution of label over the entire head was observed. In each preparation studied, from 20–40% of sperm were either totally devoid of label or labeled below control levels (less than 20 TMV per sperm), while 30–40% were labeled with 20–60 TMV, and 20–30% were labeled with 60–200 TMV.

DISCUSSION

The topographical location of H-Y antigen on mouse sperm primarily on the acrosomal cap of the head may be particularly noteworthy because the acrosomal cap is the only part of the sperm surface that obviously displays a highly ordered structure. Ordered arrays of particles have previously been observed in the acrosomal region of the head of rabbit (9, 10) and human (11) sperm, but whether these periodicities were in the surface membrane or were part of the acrosome proper was not clear. Although the replicas in the present study are of intact sperm, the periodic structure observed could reside either within the plasma membrane or within the underlying acrosome, being rendered visible in the latter case because of the close apposition of the plasma membrane to the acrosome

as a result of specimen preparation. We are currently attempting to resolve this by the technique of freeze-cleavage.

Because H–Y antigen is a product of the Y chromosome expressed on the cell surface, it offers a system for determining whether the constitution of the sperm surface reflects to any degree the haploid genome of the sperm. Haploid genome effects in sperm have not been demonstrated convincingly (12). Such effects are likely to be only partial, if they occur at all, because: (*a*) haploid stages of spermatogenesis are marked by low levels of RNA synthesis, and most protein synthesis occurring then is probably directed by RNA transcribed from the previously diploid genome (13); (*b*) in heterozygotes, crossingover between a gene and its centromere would result in retention of the heterozygous condition until the spermatid stage, when RNA synthesis would be extremely low (13), although for H–Y antigen this is presumably not a factor because crossingover is thought not to occur between X and Y chromosomes (14); and (*c*) the rate of turnover of surface-membrane components, probably slow for sperm, would affect the appearance of haploid genome products on the surface.

Although the pronounced variation we observed in the amount of label bound to different sperm from the same preparation might suggest a partial 'haploid effect' for H–Y antigen, it is not known how much variation of a similar kind might be found in a system in which haploid representation could be excluded, such as an autosomally-coded antigen like H-2 in a homozygote.

Our primary purpose in this study was to observe the distribution of any given antigen on a cell with a highly ordered morphology. For H-Y antigen, it seems we have a clear case of regional differentiation in that the antigen is confined to a morphologically delineated portion of the sperm surface. Although secondary antibody can cause redistribution of surface immunoglobulins and antigen–antibody complexes on lymphoid cells (15), redistribution of label is not a likely explanation for the regional representation of H–Y antigen observed because the secondary antibody and marker were added at low temperature. It will be important to determine whether other sperm-surface antigens are also regionally represented, and if so, whether they are also confined to the acrosomal cap or are present in other regions.

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