Biochimica et Biophysica Acta, 389 (1975) 69–83

BBA 76919

IMMUNOLOGICAL EVIDENCE FOR THE LOCALIZATION OF SIALOGLYCOSPHINGOLIPIDS AT THE CELL SURFACE OF SEA URCHIN SPERMATOZOA*

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(Received October 21st, 1974)

SUMMARY

The localization of sialoglycosphingolipids in the plasma membrane of sea urchin spermatozoa was studied by employing immunological methods including immunolysis of liposomal model membranes.

The antibodies produced against these complex lipids were found to agglutinate various sea urchin spermatozoa differently. Both species differences and species similarities in the agglutination were found in spermatozoa of the echinoderm, the sea urchin and the starfish. The agglutination of the sea urchin spermatozoa was inhibited specifically by certain carbohydrates.

Only a limited number of molecular species of sialoglycosphingolipid were localized at the surface of the plasma membrane of sea urchin spermatozoa cells. Moreover, topographical differences were found in the localization of the sialoglycosphingolipids at the cell surface of spermatozoa.

INTRODUCTION

In previous papers [1–3] we have shown that sialoglycosphingolipids of the sea urchin eggs and spermatozoa are composed of relatively simple chemical constituents. The major sialoglycosphingolipids have a common oligosaccharide backbone consisting of glucose, to which sialic acids are linked [4, 5]. Nevertheless, they exhibit a characteristic composition for species and gamete [4]. These individual differences in the chemical structure prompted us to examine the possible role of these lipids in the recognition function of cells in fertilization and early developmental processes. However, little attention has been given to these physiological roles.

In the sea urchin gametes, the carbohydrate structure of the jelly substances surrounding the egg cells has been studied by several workers [6–8] with a special interest in the acrosomal reaction. The potential importance in fertilization of the

* This is Part VII of the series Biochemistry of Lipids of Sea Urchin Gametes and Embryos.

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saccharides present on the cell surface of spermatozoa has only recently been recognized by some workers. Thus, Edelman and Millette [9] reported that mouse spermatozoa were specifically agglutinated by concanavalin A. Nicolson and Yanagimachi [10] using various lectins and a viral neuraminidase indicated that certain polysaccharides are localized at specified regions on the surface of the plasma membrane of rabbit and hamster spermatozoa.

In a previous paper Nagai and Ohsawa [11] have described a simplified immunization procedure for obtaining specific and high titer antisera against sialoglycosphingolipids. The present study demonstrates by using such antisera that there are antibodies against several, well-defined molecular species of sea urchin sperm sialoglycosphingolipids, specifically agglutinate sea urchin spermatozoa. Also, we show that the saccharide chain of a particular sialoglycosphingolipid is exposed in specific regions of the spermatozoa cell surface.

MATERIALS AND METHODS

Materials

The sea urchins *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus*, *Anthocidaris crassispina* and *Clypeaster japonicus* were obtained from the Misaki Marine Biological Station, the University of Tokyo. The starfish, *Asterias amurensis*, was kindly supplied by Dr. H. Kanatani, the Ocean Research Institute, the University of Tokyo. Lecithin was prepared from chicken egg yolk phospholipids according to the method of Rhodes and Lea [12] using aluminium oxide (M. Woelm Eschwege, Germany) column chromatography. Cholesterol (guaranteed reagent) was obtained from Koso Chemical Co. Ltd., Tokyo and recrystallized from ethanol. Methylated bovine serum albumin was kindly supplied by Dr S. Nojima, Department of Chemistry, National Institute of Health, Tokyo, which was prepared from bovine albumin powder (Fraction V from bovine plasma, Armour Pharmaceutical Co.) according to Mandel and Hershey [13]. Dicetyl phosphate was from K and K Laboratories, Inc., Plainview, New York. Glucose-6-phosphate dehydrogenase was obtained from Worthington Biochemical Corporation, New Jersey. Trypsin was purchased from Mochida Pharmaceutical Mfg. Co. Ltd., Tokyo. Trypsin inhibitor from soy bean was from Boehringer Mannheim, Japan. All organic solvents were redistilled before use.

Preparation of sialoglycosphingolipids

The preparation of the total sperm sialoglycolipid fraction was carried out as previously described [11]. Briefly, sperm were extracted with a chloroform/methanol solvent mixture. The residue obtained by evaporation of the extract was treated with acetone and repeatedly partitioned between aqueous and organic solvents according to the methods of Folch et al [14]. The crude sialoglycolipid fraction recovered from the aqueous phase after dialysis and lyophilization was subjected to a mild alkaline hydrolysis in chloroform/methanol to remove the ester-type lipids. The glycolipids which were recovered from the hydrolysate were further purified by silicic acid column chromatography. The column was first eluted with chloroform/methanol (9:1, v/v) to remove the remaining non-polar lipids and free fatty acids. The sialoglycolipids were then eluted with methanol. To examine the possibility of

contamination by protein materials the lipids thus obtained were hydrolysed with 6 M HCl at 105 °C for 24 h and analysed by a JLC-6AH amino acid automatic analyzer (Japan Electron Optics Co. Ltd., Tokyo). The sialoglycolipid fraction was found to contain not more than 0.1 % of protein-like materials. The total sialoglycolipids of *P. depressus* sperm were prepared similarly. These were further fractionated into sialoglycolipid-1, -2 and -3 by preparative thin-layer chromatography. The solvent system was chloroform/methanol/2.5 M ammonia (60:35:8,v/v/v) on precoated silica gel plate (DC-precoated silica gel plate, E. Merck, Germany) and the lipid bands were located with iodine vapor and then scraped off the plate. The powder was packed into a small column and eluted with chloroform/methanol (1:2,v/v) to obtain the sialoglycolipid. After removal of the solvent, the residue was treated with chloroform/methanol (2:1,v/v) in order to dissolve the sialoglycolipid, and the insoluble material was discarded. The homogeneity of the sialoglycolipids thus obtained was checked by thin-layer chromatography using chloroform/methanol/2.5 M ammonia (60:35:8,v/v/v) as a developing solvent.

Six individual sialoglycolipids were isolated from *H. pulcherrimus* sperm by the same preparative procedure using thin-layer chromatography. *H. pulcherrimus* sperm sialoglycolipid-1 to -4 and -6 were chromatographically pure. However, the sialoglycolipid-5 preparation was slightly contamined with sialoglycolipid-3 and -4.

Preparation of antisera against the sialoglycosphingolipids

Antisera against the sialoglycolipids were prepared by the method described previously [11]. Briefly, mixed micelles consisting of sialoglycolipids, egg lecithin and cholesterol in a ratio of 1:4:10 (w/w/w) were prepared and mixed with methylated bovine serum albumin solution. In the case of *P. depressus* sperm sialoglycolipid-1, -2 and -3, antigenic micelles consisting of the glycolipid, egg lecithin and cholesterol in a ratio of 1:4:30 (w/w/w) were complexed with methylated bovine serum albumin prior to use. The antigen complex thus prepared was incorporated into an equal part of Freund's complete adjuvant to make an emulsion. 2 ml of the emulsion containing 2 mg of the individual sialoglycolipid and 2 mg of mycobacteria (human type virulent strain, Aoyama B) was injected into the four foot pads of a rabbit intradermally. Boosters of intradermal injection into the back of the rabbit were given at two-weeks' intervals. The antisera were collected after 4 weeks. Titration of the antisera was performed according to the microfloculation technique of the Venereal Disease Research Laboratory Slide Test [15].

Agar double-diffusion test

1 % agar in aqueous 0.15 M NaCl solution was used. The antigen saline solution at a final concentration of 0.1 % (w/v) was placed in the central well in the absence of any auxiliary lipids and undiluted antisera were placed in the other outer surrounding wells. The agar plate was kept at room temperature for about 18 h.

Sperm agglutination with antiserum

In this experiment spermatozoa of the sea urchin, *H. pulcherrimus*, *A. crassispina*, *P. depressus* and *C. japonicus*, and of the starfish, *A. amurensis* were used. Sperm were shed into a petri dish by injection of 0.5 M KCl solution into the body cavity. Sperm thus obtained were suspended in 10 vols of sea water and sedimented

by centrifugation at 10 000 $\times g$ for 20 min. The sperm collected were diluted with a 100-fold excess of sea water and added to 1 ml of the test solution of antiserum which had been prepared for the agglutination test by diluting the original antiserum with the indicated amount of sea water. The agglutination of sperm was observed under a phase-contrast microscope with 200-fold magnification about 1 min after mixing.

Inhibition test of sperm agglutination with various sugars

The carbohydrate to be used for the inhibition test was dissolved in 0.9 ml of sea water to give the final concentration of 100 mM and to this solution 0.1 ml of antiserum prepared by dilution with sea water was added. The mixture was incubated at 37 °C for 1 h. After the preincubated mixture was cooled to room temperature, a drop of sperm suspension diluted with sea water to 100 times was added. The sperm agglutination was observed within 5 min under a phase-contrast microscope with 200-fold magnification.

Absorption of antisera with sperm cells

Both anti-*H. pulcherrimus* sperm sialoglycolipids and anti-*P. depressus* sperm sialoglycolipid-3 sera were absorbed by either intact or trypsinized spermatozoa. The term intact sperm refers to the sperm cells that were packed at the bottom of a centrifuge tube by centrifuging the shed sperm suspension in 10 vol of sea water at 10 000 $\times g$ for 20 min. Trypsin was dissolved in 1.5 ml of sea water and to this solution 0.5 ml of the intact sperm suspension was added. After the mixture was incubated at room temperature for 30 min, 2 mg of soy bean trypsin inhibitor was added to stop the reaction. The trypsinized sperm were collected by centrifugation at 10 000 $\times g$ for 20 min after diluting the digestion mixture in 10 volumes of sea water.

For an absorption test 0.5 ml of the packed intact or trypsinized sperm thus prepared was added to 1 ml of antiserum and the mixture was incubated at room temperature for 1 h. The incubated mixture was centrifuged at 10 000 $\times g$ for 20 min and the supernatant removed. To this supernatant 0.5 ml of the packed sperm was again added. This absorption procedure was repeated 3 times until the ability to agglutinate sperm was completely removed from the antiserum.

Absorption of anti-H. pulcherrimus sperm sialoglycolipids serum with homogenized spermatozoa

1 ml of the packed, intact *H. pulcherrimus* sperm which were prepared as described above was mixed with 3 ml of saline and homogenized with 10 strokes of a Potter glass homogenizer and then by sonication at 20 *K* cycles for 5 min. To this homogenate was added 0.05 ml of inactivated antiserum to *H. pulcherrimus* sperm sialoglycolipids. This inactivated serum was obtained by initial heating at 56 °C for 30 min. The mixture was incubated at room temperature for 1 h and finally by leaving at 4 °C overnight. The mixture was then centrifuged at 7000 $\times g$ for 60 min. The supernatant was removed and dialysed against saline for 1 h.

Absorption of antiserum with antigenic micelles

1.5 mg of *H. pulcherrimus* sperm sialoglycolipids, 6 mg of egg lecithin and 45 mg of cholesterol were dissolved in 0.5 ml of tetrahydrofuran. The solution was shaken vigorously with 0.5 ml of saline and then 5 ml of saline was added with vigor-

ous shaking to form antigen micelles. The antigen micelles thus prepared were collected by centrifugation at 10 000 × *g* for 30 min in the cold and added to 2 ml of anti-*H. pulcherrimus* sperm sialoglycolipids serum diluted with saline to 1:64. The mixture was incubated at 4 °C overnight with occasional shaking. The incubation mixture was centrifuged at 10 000 × *g* for 30 min to separate the micelles from the antiserum.

An antigen-containing liposomal model membrane was used to detect antibody still present in the antiserum absorbed by antigenic micelles. As described in a previous paper [11], liposomes were prepared from a mixture of egg lecithin, cholesterol and dicetyl phosphate in a molar ratio of 2:1.5:0.22, respectively, together with sufficient amounts of antigen. Glucose trapped within the liposome was used as a marker in these experiments. The antigen-containing liposome would undergo immune lysis in the presence of antibody and complement and result in the release of the trapped glucose. The released glucose was assayed spectrophotometrically by the change in the 340-nm absorbance indicating reduction of NADP in the presence of hexokinase, glucose-6-phosphate dehydrogenase and the necessary cofactors. The "% trapped glucose released" was calculated according to the method of Kinsky et al. [16].

RESULTS

Sperm agglutination with antiserum

The antibodies against total sialoglycolipids of *H. pulcherrimus* sperm which were produced in rabbits were active at a titer of 1:2048 or 1:4096, as described in the previous paper [11].

Immunoelectrophoretograms indicated that the antibody against the total sialoglycolipids of *H. pulcherrimus* sperm might be γ-immunoglobulin and could react with antigen to precipitate the antigen-antibody complex.

Sperm prepared from the sea urchin, *H. pulcherrimus*, *A. crassispina* and *P. depressus*, were agglutinated by anti-*H. pulcherrimus* sperm sialoglycolipids serum at dilutions of 1:2048, 1:1024 and 1:2048, respectively, although sperm of *C. japoni-*

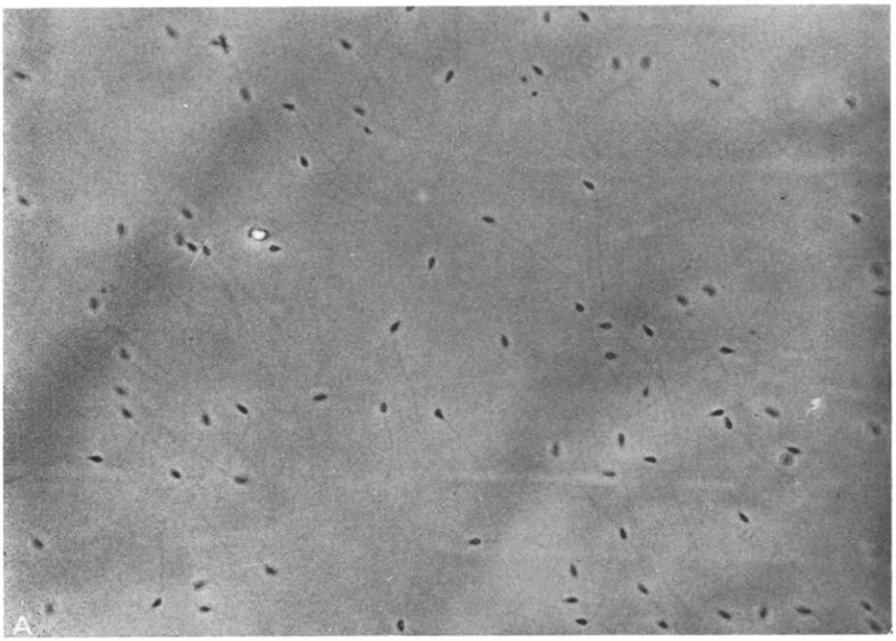
TABLE I

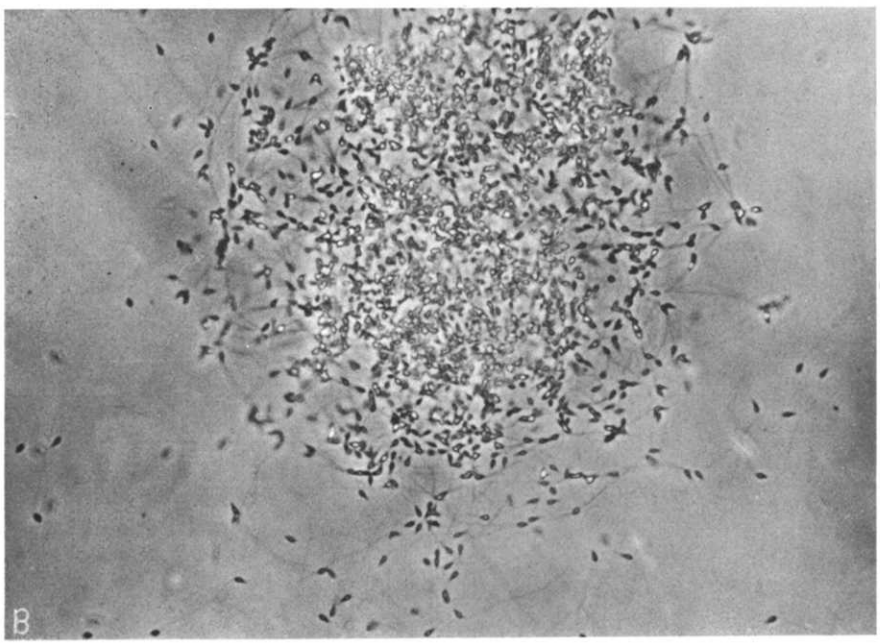
SPERM AGGLUTINATION OF VARIOUS SEA URCHIN SPECIES BY ANTISERUM TO *H. PULCHERRIMUS* SPERM SIALOGLYCOSPHINGOLIPIDS

To 1 ml of antiserum prepared against *H. pulcherrimus* sperm sialoglycolipids diluted with sea water as indicated a drop of the sperm suspension diluted with a 100-fold excess of sea water was added. Sperm agglutination was observed under a phase-contrast microscope with 200-fold magnification at about 1 min after mixing, The term + means any occurrence of discernible agglutination, while the term − means 0 % agglutination.

Sperm	Dilution ratio						
	1: 4096	1: 2048	1: 1024	1: 512	1: 256	1: 16	1: 3
H. pulcherrimus	−	+	+	+	+		
A. crassispina		−	+	+	+		
C. japonicus					−	−	−
P. depressus	−	+	+	+	+		
A. amurensis					−	−	−

cus and of the starfish, *A. amurensis*, were not, as shown in Table I. There was no agglutination by normal rabbit serum (Fig. 1A) or by rabbit serum obtained by injection of Freund's complete adjuvant alone. When anti-*H. pulcherrimus* sperm sialoglycolipids serum was added to fresh motile spermatozoa of either *H. pulcherri-*





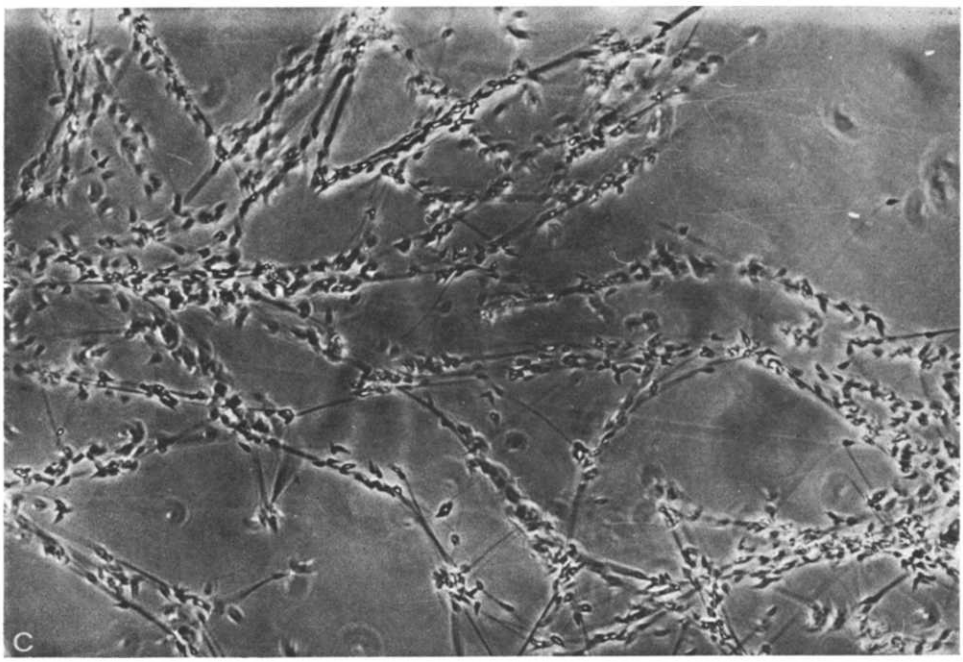


Fig. 1. Sperm agglutination with antiserum against sialoglycosphingolipids of *H. pulcherrimus* sperm. A drop of sperm suspension diluted with sea water to 100 times was added to 1 ml of antiserum diluted with sea water in the indicated ratio and sperm agglutination was observed within 1 min under a phase-contrast microscope with 200-fold magnification. A: *H. pulcherrimus* spermatozoa were treated with normal rabbit serum (1 : 3 dilution); B: *H. pulcherrimus* spermatozoa, with antiserum to *H. pulcherrimus* sperm sialoglycosphingolipids (1 : 2048 dilution); C: *P. depressus* spermatozoa, with antiserum to *H. pulcherrimus* sperm sialoglycosphingolipids (1 : 2048 dilution).

mus or *P. depressus*, agglutinations of the sperm occurred rapidly. This reaction was head to head, head to tail and tail to tail, resulting in the formation of large rafts, as shown in Fig. 1B. On the other hand, *A. crassispina* spermatozoa were agglutinated tail to tail by the addition of anti-*H. pulcherrimus* sperm sialoglycolipids serum (Fig. 1C).

Antiserum prepared against the sialoglycolipid-1 and -2 of *P. depressus* sperm were active at antibody titers of 1:512 and 1:64, respectively. In the case of *P. depressus* sperm sialoglycolipid-3, antisera were produced in two rabbits (A) and (B), which were active at antibody titers of 1:32 and 1:1024, respectively. The dilution ratios of these antisera to agglutinate spermatozoa of *P. depressus* and *H. pulcherrimus* is shown in Table II. *P. depressus* sperm could be agglutinated readily by both anti-*P. depressus* sperm sialoglycolipis-3 sera (A) and (B), only a little by anti -2 serum, and not by anti -1 serum. *H. pulcherrimus* sperm could be agglutinated also by both anti -3 sera (A) and (B), less effectively by anti -2 serum and not by anti -1 serum.

Thin-layer chromatography indicated that *P. depressus* sperm sialoglycolipid -1, -2 and -3 gave a single spot with differing R_F values (Fig. 2.).

The agar double-diffusion test revealed that antigen-specific antibody was produced against each of the *P. depressus* sperm sialoglycolipid-1, -2 and -3 antigens as shown

TABLE II

AGGLUTINATION OF *P. DEPRESSUS* AND *H. PULCHERRIMUS* SPERM BY ANTISERA TO *P. DEPRESSUS* SPERM SIALOGLYCOSPHINGOLIPIDS

All antibody titers were adjusted with saline to the same level as the titer (1 : 32) of antiserum (A) to *P. depressus* sperm sialoglycolipid-3. The adjusted antisera were then serially diluted with saline. To 1 ml of the antisera diluted as indicated a drop of the diluted sperm suspension was added. Sperm agglutination was observed under a phase-contrast microscope at 200-fold magnification at about 1 min after mixing. The term + means any occurrence of discernible agglutination, while the term − means 0 % agglutination.

Antiserum*	Dilution ratio					
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
	P. depressus sperm					
−1	−	−	−	−	−	−
−2	+	−	−	−	−	−
−3 (A)	−	−	+	+	+	−
−3 (B)	+	+	−	−	−	−
Normal rabbit serum	−	−	−	−	−	−
	H. pulcherrimus sperm					
−1	−	−	−	−	−	−
−2	+	+	−	−	−	−
−3 (A)	+	+	−	−	+	−
−3 (B)	+	+	−	+	−	−
Normal rabbit serum	−	−	−	−	−	−

* Antiserum, −1, −2 and −3: antiserum to *P. depressus* sperm sialoglycolipid −1, −2 and −3, respectively (see the text); (A) and (B): antisera obtained from different rabbits immunized with the same antigen.

in Fig. 3. Each antigen placed in the central well was found to react only with its own antiserum present in the surrounding wells to yield a precipitin band, but not with the other antigens.

Inhibition test of sperm agglutination by various sugars

The agglutination of *H. pulcherrimus* sperm and *P. depressus* sperm by anti-*H. pulcherrimus* sperm sialoglycolipids serum was specifically inhibited in the presence of 100 mM glucose or 100 mM methyl α-glucoside, while agglutination of *A. crassispina* sperm by anti-*H. pulcherrimus* sperm sialoglycolipid serum could be inhibited with 100 mM galactose but not with glucose (Table III). Smaller concentrations of glucose or galactose did not result in inhibition. Neither *N*-acetylgalactosamine nor sialic acid 100 mM were found to be inhibitory.

Absorption tests of antiserum by spermatozoa

Antiserum to *H. pulcherrimus* sperm sialoglycolipids was repeatedly absorbed by either intact or trypsinized spermatozoa of *H. pulcherrimus* until the ability to agglutinate sperm was completely lost as described in the text. Changes in the antibody titer of the antiserum before and after this absorption treatment were assessed

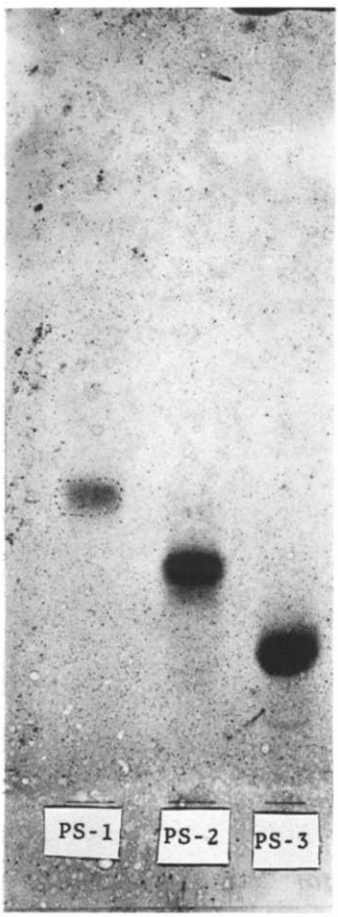


Fig. 2. Thin-layer chromatography of sialoglycosphingolipids of *P. depressus* spermatozoa. PS-1: *P. depressus* sperm sialoglycolipid-1; PS-2: *P. depressus* sperm sialoglycolipid-2; PS-3: *P. depressus* sperm sialoglycolipid-3; solvent system: chloroform/methanol/2.5 M NH_4OH (60 : 35 : 8, v/v/v); detection : resorcinol spray reagent.

by the immunolysis of liposomes containing antigen, in the presence of guinea pig serum as a source of complement.

Little suppression of immunolysis was observed by this absorption treatment. However, the antiserum absorbed by the homogenate of *H. pulcherrimus* sperm, which lost the ability to agglutinate sperm, caused a significant decrease in immunolysis (Fig. 4). The antiserum absorbed by the antigenic micelles consisting of *H. pulcherrimus* sperm sialoglycolipids, lecithin and cholesterol (1:4:30, w/w/w) also resulted in a complete suppression of the immunolysis of the liposome containing the antigen, indicating nearly quantitative removal of antibodies from the serum. This absorbed antiserum did not exhibit the sperm agglutinability.

These results suggested either that the surface structure of spermatozoa responsible for the sperm agglutination differs somewhat from the antigenic structure exposed on the surface of the liposome, or that the amount of the anti-glycolipid

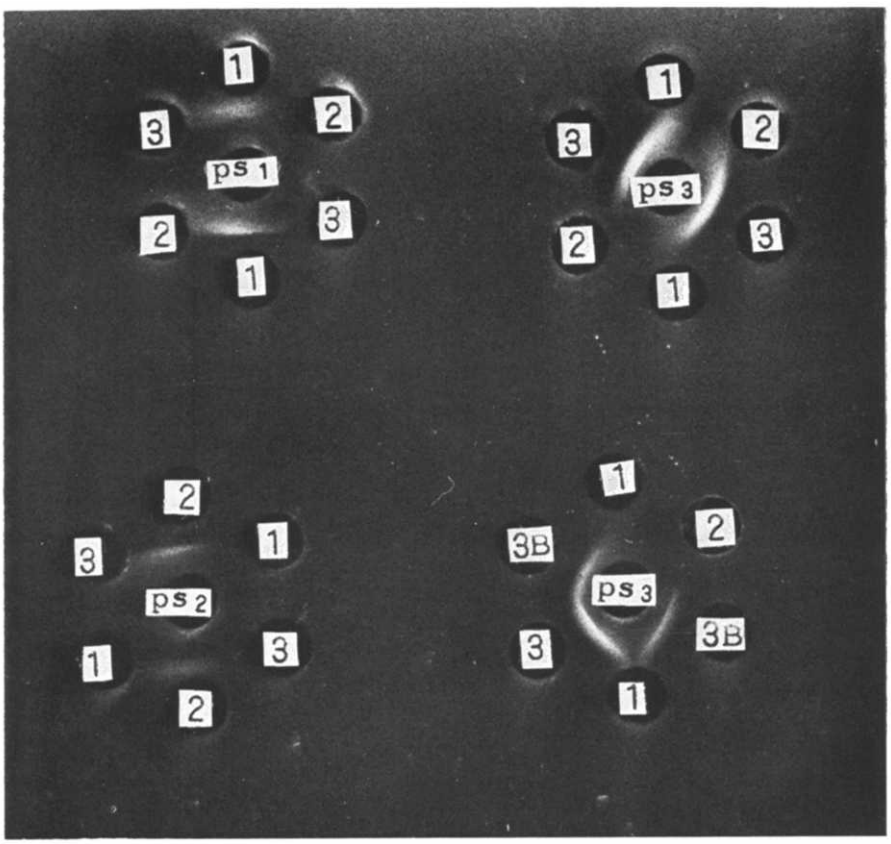


Fig. 3. Ouchterlony's double-diffusion test in agar. The central well contains 0.1 % (w/v) of saline solutions of antigen sialoglycosphingolipids, isolated from *P. depressus* spermatozoa. The outer surrounding wells contain undiluted antiserum to *P. depressus* sperm sialoglycosphingolipid. 1: antiserum to *P. depressus* sialoglycolipid-1, 2: antiserum to *P. depressus* sialoglycolipid-2, 3: antiserum (A) to *P. depressus* sialoglycolipid-3; 3B: antiserum (B) to *P. depressus* sialoglycolipid-3.

antibody responsible for the sperm agglutination is so dilute in the antiserum produced that the present liposomal assay system consisting not only of the total *H. pulcherrimus* sperm sialoglycolipids antigen mixture but of a variety of antibodies is not sufficiently sensitive for mesauring the absorption effect. The following experiments showed that the latter is the case.

Six different liposomes containing each sialoglycolipid, of *H. pulcherrimus* sperm, -1 to -6, isolated by preparative thin-layer chromatogram, were prepared and assayed for the immunolysis of the liposomes by antiserum to *H. pulcherrimus* sperm sialoglycolipids.

Lysis occurred predominantly in the liposomes containing antigen-1, -3 and -5, while with the antiserum absorbed by intact *H. pulcherrimus* spermatozoa, the immunolysis was remarkably suppressed in the liposomes containing antigen-1, -4 and -5.

In the case of antisera against *P. depressus* sperm glycolipids, almost complete

TABLE III

INHIBITION OF SPERM AGGLUTINATION WITH VARIOUS SUGARS

0.9 ml of sugar solution and 0.1 ml of antiserum toward *H. pulcherrimus* sperm sialoglycolipids diluted with sea water in the indicated ratio were mixed and preincubated at 37 °C for 1 h. After the preincubation, a drop of the diluted sperm suspension was added and agglutination was observed with 5 min after mixing under a phase-contrast microscope at 200-fold magnification. The term + means any occurrence of discernible agglutination, while the term − means 0 % agglutination.

Sugar (100 mM)	*H. pulcherrimus*			*P. depressus*			*A. crassispina*		
	1 : 2560	1 : 1280	1 : 640	1 : 2560	1 : 1280	1 : 640	1 : 1280	1 : 640	1 : 320
No addition	+	+	+	+	+	+	+	+	+
Glucose	−	+	+	−	+	+	+	+	+
Methyl α-D-glucoside	−	+	+	−	+	+	+	+	+
Methyl β-D-glucoside	−	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	−	+	+
GalNAc	+	+		+	+		+	+	
GlcNAc	+	+		+	+		+	+	
AcNeu	+	+		+	+		+	+	

Abbreviations: AcNeu, *N*-Acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine.

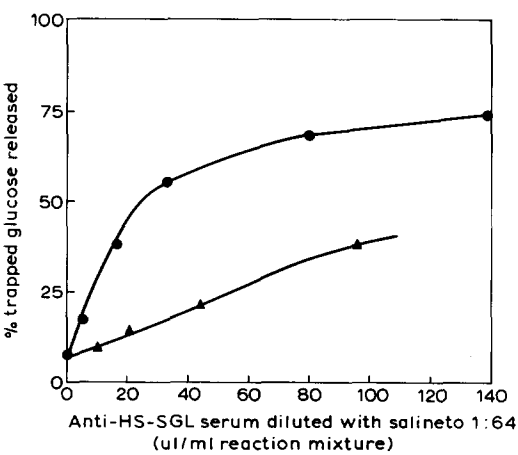


Fig. 4. Effect of absorption of antiserum to *H. pulcherrimus* sperm sialoglycosphingolipids (HS-SGL) by homogenates of *H. pulcherrimus* spermatozoa on immunolysis of liposomes sensitized with *H. pulcherrimus* sperm sialoglycosphingolipids. Liposomes were prepared from mixtures containing egg lecithin, cholesterol and dicethyl phosphate in a molar ratio of 2 : 1.5 : 0.22, respectively, and 31 μg of *H. pulcherrimus* sperm sialoglycosphingolipids per μmol of egg lecithin. Glucose release was determined by spectrophotometric assay after a 30-min incubation in the presence of complement. ●—●: control, ▲—▲: absorbed with homogenate.

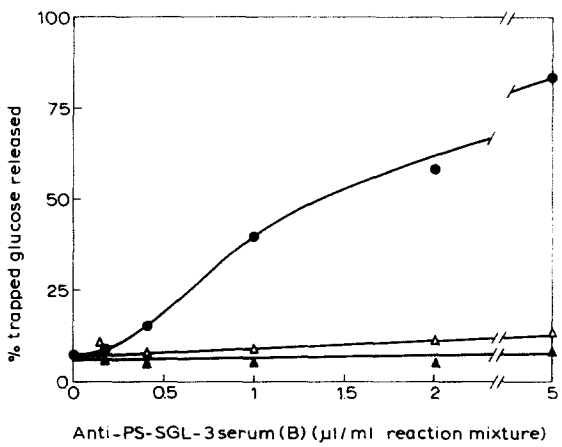


Fig. 5. Effect of absorption of antiserum to *P. depressus* sperm sialogglycosphingolipid-3 (PS-SGL-3) serum with intact or trypsinized spermatozoa of *P. depressus* on immunolysis of the liposomes sensitized with *P. depressus* sperm sialoglycolipid-3. Liposomes were prepared from mixtures containing egg lecithin, cholesterol and dicethyl phosphate in a molar ratio of 2 : 1.5 : 0.22, respectively, and 23 μg of *P. depressus* sperm sialoglycolipid-3 per μmol of egg lecithin. Glucose release was determined by spectrophotometric assay after a 30-min incubation in the presence of complement. ● — ●: control; ▲ — ▲: absorbed with intact spermatozoa; △ — △: absorbed with trypsinized spermatozoa.

suppression of immunolysis occurred in the liposome containing *P. depressus* sperm sialoglycolipid-3 exposed to the antiserum to *P. depressus* sperm sialoglycolipid-3 which was absorbed in advance by either the intact or the trypsinized *P. depressus* spermatozoa until there was a complete loss in the ability to cause sperm agglutination (Fig. 5).

DISCUSSION

The experiments described in this paper demonstrated that antiserum to *H. pulcherrimus* sperm siloglycosphingolipids could agglutinate *H. pulcherrimus* spermatozoa. This agglutination is caused by antibodies produced to these sialoglycosphingolipids, since normal rabbit sera and the serum from rabbits injected only with Freund's complete adjuvant alone, did not agglutinate spermatozoa. This is substantiated by the finding that the sperm agglutinability was completely removed from the serum by absorption with antigen micelles.

Interestingly, antiserum to *H. pulcherrimus* could also agglutinate *P. depressus* and *A. crassispina* spermatozoa which are similar species but not spermatozoa of *C. japonicus* or of the starfish, *A. amurensis* which comes under the same Echinodermata which are different species. Chemical characterization of the sialoglycolipids of sea urchin egg and spermatozoa had revealed that the sialoglycolipids are relatively simple in their constituents. However, they show a characteristic composition for species and gamete, that is, different sialoglycolipids are found not only in gametes of different species (species specificity) but also in egg cells and spermatozoa within the same species (gamete specificity) [4, 5]. In the case of *A. amurensis*, however, we could not find sialoglycolipids in testis or ovary harvested in the breeding season (Nagai, Y.,

unpublished), although the occurrence of *N*-glycolyl-8-*O*-methylneuraminic acid had been reported in viscera of the starfish, *Asterias forbesi* by Warren [18].

The mode of the sperm agglutination by antiserum to *H. pulcherrimus* sperm sialoglycolipids differs among spermatozoa from different species. Agglutination in spermatozoa of *H. pulcherrimus* and *P. depressus* involved both head and tail, resulting in large rafts, while that in *A. crassispina* spermatozoa occurred only tail to tail, resulting in the formation of rows. Agglutination in *H. pulcherrimus* and *P. depressus* was specifically inhibited by methyl-α-D-glucoside, whereas in *A. crassispina* by galactose. In another experiments the major carbohydrates in the sialoglycosphingolipids of the sea urchin gametes were found to be glucose and sialic acid [4]. The following structures were established for the two sialoglycosphingolipids of *A. crassispina* spermatozoa, *N*-acetylneuraminyl(2 → 8)*N*-acetylneuraminyl (2 → 6) glucosyl ceramide and *N*-acetylneuraminyl (2 → 6) glucosyl ceramide [5]. Monosialodiglucosyl ceramide was found to be the major sialoglycosphingolipid of eggs of the same species [5]. On the other hand, galactose was also present in the gametes although in much smaller concentrations. For example, the molar ratio of galactose to glucose in spermatozoa sialoglycosphingolipids was found to be 0.04 in *A. crassispina*, 0.05 in *H. pulcherrimus* and 0.04 in *P. depressus* [4]. These results suggest that sialoglycosphingolipids may be present at the surface of the plasma membrane of spermatozoa, and that their topographical localization differs among species of the sea urchin. The specific inhibition by galactose of agglutination in *A. crassispina* indicates the possibility that the galactoside type of glycolipids may be principally present at the spermatozoa surface of this species.

In order to obtain further information about the localization of these complex lipids at the cell surface, it was necessary to use individual sialoglycosphingolipids. Six different ones isolated from *H. pulcherrimus* sperm were incorporated as antigens into lecithin-cholesterol liposomes. The liposomes were exposed to either the antiserum to *H. pulcherrimus* sperm sialoglycolipids or the antiserum absorbed by intact *H. pulcherrimus* sperm. It was shown that antibodies in the antiserum to *H. pulcherrimus* sperm sialoglycolipids were elicited predominantly against antigen-1, -3 and -5, respectively. Absorption by *H. pulcherrimus* spermatozoa of antiserum prepared towards *H. pulcherrimus* sperm sialoglycolipids indicated preferential removal of antibodies of *H. pulcherrimus* sperm sialoglycolipid-1, -4 and -5. These results suggest that each of the *H. pulcherrimus* sperm sialoglycolipid do not exist on the cell surface. Apparantly these are limited to the molecular species of the sialoglycolipids as -1, -4 and -5.

This conclusion is further strengthened by the experiments using three kinds of antisera which were produced against each of the individual isolated *P. depressus* sperm sialoglycosphingolipids-1, -2 and -3. Specific antibody production was confirmed by the agar double-diffusion techniques. Only antiserum of *P. depressus* sperm sialoglycolipid-3 could effectively agglutinate spermatozoa of *P. depressus* and *H. pulcherrimus* with a high titer. Immunolysis of the liposome containing *P. depressus* sperm sialoglycolipid-3 was almost completely suppressed by absorption of this serum by either the intact or the trypsinized spermatozoa of *P. depressus*. These experiments suggest that *P. depressus* sperm sialoglycolipid-3 is specifically localized on the surface of *P. depressus* spermatozoa.

Total sialosphingolipids of *H. pulcherrimus* used in the present experiment

contained not more than 0.1 percent of proteinaceous material. This may point to the possible contribution of contaminating protein in these serological reactions. However, this is highly unlikely due to the activity of the isolated sialoglycosphingolipid.

It has been shown in sphingolipid immunology that antibody activity is principally directed towards the carbohydrate portion of the lipid. In fact, we have shown in the present experiments that the sperm agglutination by antisera is suppressed in the presence of a high concentration of specific hexoses. The contribution of the ceramide portion of the lipid molecule to the antigenic determinant is still unclear. Moreover, cross reaction by the oligosaccharide portion of glycoproteins has sometimes been observed in antiserum prepared against glycosphingolipids. A possibility still remains that our results may be due in part by a contribution from the carbohydrate residues of glycoproteins or polysaccharides which have similar carbohydrate residues to those present in the glycolipids.

The present studies, however, represent the first evidence for the localization of glycolipid saccharides on the cell surface of spermatozoa. Preliminary experiments using fluorescein-labeled antibodies showed that the fluorescein was located at the acrosomal region and middle piece rather than at the other regions of the cell surface of *H. pulcherrimus* spermatozoa (Ohsawa, T. and Nagai, Y., unpublished). More precise identification of the specific regions may be achieved by an immuno-electron microscopic technique using ferritin- or peroxidase-labeled antibody.

Edelman and Millette [9] have recently shown by using fluorescein-labeled and ^{125}I-labeled concanavalin A that the majority of concanavalin A molecules were bound to mouse sperm head, especially to the region of the acrosome rather than to the sperm tail. Ericsson et al. [19] have reported the head to head agglutination for rabbit spermatozoa with Sendai virus, suggesting predominant localization of sialic acid in this region. Nicolson and Yanagimachi [10] have recently demonstrated that species differences and similarities are found in the surface oligosaccharides of rabbit and hamster spermatozoa by using five kinds of lectins with different specificities in affinity for sugar residues and influenza virus.

Structures of the determinant group in the present glycolipid antigen molecules and of the surface-localized sialoglycosphingolipids are currently under investigation.

ACKNOWLEDGEMENTS

We are indebted to the Misaki Marine Biological Station, the University of Tokyo for the supply of sea urchins and Dr H. Kanatani, the Ocean Research Institute, the University of Tokyo, for the generous gift of starfish. We wish to thank Dr M. Hoshi, Division of Biochemistry, the Institute of Low Temperature Science, the Hokkaido University, Hokkaido, for his valuable help. We also thank Dr Julian N. Kanfer, Biochemical Research, Eunice K. Shriver Center for Mental Retardation, Inc., Waltham, Mass. for helpful criticism. This work was supported in part by a grant from the Ministry of Education, Japan.

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