EFFECT OF MODIFIED MEMBRANE VESICLES FROM SEMINAL PLASMA ON THE FERTILIZING CAPACITY OF RABBIT SPERMATOZOA[1]

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SUMMARY: Partial extraction of cholesterol and phospholipid from membrane vesicles in rabbit seminal plasma decreased their inhibitory effect on fertilizing capacity in rabbit spermatozoa. Pronase digestion, to remove surface proteins, had no pronounced effect on vesicle decapacitation activity. Evidence of fusion between these vesicles and spermatozoa was obtained using [^{3}H] galactose labelled vesicles. The results are consistent with addition of vesicle lipid (cholesterol) to the sperm plasma membrane causing an inhibition of fertilizing capacity.

Spermatozoa from several divergent species of mammals have been found to require a period in the female reproductive tract before gaining the capacity for fertilization. Membrane vesicles in seminal plasma appear to reverse molecular changes occurring in the sperm cell during capacitation (1). Evidence has also been obtained that indicates sperm fertilizing capacity is influenced by cholesterol levels in the sperm cell plasma membrane (2,3). During capacitation, the plasma membrane of this cell changes in some way that facilitates its fusion with the outer acrosomal membrane (4). Membrane fusion has been reported to be inhibited by cholesterol (5,6). Decapacitation by membrane vesicles in seminal plasma therefore could involve addition of vesicle cholesterol to the sperm plasma membrane. The present study was undertaken in an attempt to: (a) broadly characterize the lipid

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and protein components of membrane vesicles from seminal plasma, (b) determine decapacitation activity in vesicles after digestion with pronase and partial extraction of lipid, and (c) assess the nature of the interaction between vesicles and spermatozoa. The experiments described were conducted in the rabbit.

MATERIALS AND METHODS Rabbits, of mixed strain, were purchased from a local breeder for use in these experiments. Seminal plasma was obtained by centrifugation of fresh semen, collected with an artificial vagina from fertile bucks, at 10,000 g for 30 min. Membrane vesicles were isolated from seminal plasma by sedimentation on discontinuous sucrose density gradients (1). Rabbit seminal plasma contains vesicles with densities of 1.20 and 1.16 g/cc and they are referred to as heavy and light vesicles, respectively. Vesicle protein was estimated colorimetrically (7). Lipids were extracted with chloroform and methanol (2:1) and the dry extract was weighed. The crude lipid extract was acetylated and then fractionated into neutral lipids, glycolipids and phospholipids using a Florisil (Fisher) column (8). Cholesterol in the neutral lipid fraction was measured with o-phthalaldehyde (9). Glycolipids were identified, after deacetylation, by co-chromatography with standard glycolipids on silica gel plates (Brinkman) using chloroform, methanol and water (100:42:6) as the developing solvent. Gangliosides were quantitated by multiplying by 2.8 the amount of sialic acid measured (10). Phospholipids were identified by comparing Rf values following chromatography on silica gel plates developed with chloroform, methanol, acetic acid and water (75:25:15:8). The amount of phospholipid was estimated by multiplying by 25 the inorganic phosphorus level (11). Dry weight was determined after placing vesicle preparations in an oven at 54°C for 24 hrs. Electrophoresis of vesicle proteins was performed using sodium dodecyl sulfate and 7.5 (w/v)% polyacrylamide gels (12). A gel scanner (Helena) determined the distribution of polypeptide bands in gels stained with Coomassie Brilliant Blue (Mann). Periodic acid-Schiff stain located bands containing sugar.

Vesicles suspended in 0.1 M KCl and 0.01 M Tris, pH 7.0, were digested with 10 (w/w)% pronase (Nutritional Biochemicals) for 4 hrs. at 37°C. They were subsequently isolated by sedimentation on 5 (w/v)% sucrose at 105,000 g for 3 hrs. Lipids were partially depleted from the vesicle preparations by extraction with dry ethyl ether (13) and with acetone, ethyl ether and water (30:10:1). The extracted vesicles were lyophilized and assayed for protein, phospholipid and cholesterol. Samples of these preparations were negatively stained with 1 (w/v)% uranyl acetate and examined under an electron microscope (Zeiss) to assess vesicle structure.

The effect of these vesicles on sperm fertilizing capacity was established from the rate of fertilization observed after oviductal insemination with treated sperm cells (1). Capacitated sperm were obtained by flushing the uterus of a doe 12 hrs. after mating. Ovulation was induced by injection of 70 I.U. human chorionic gonadotrophin and inseminated females were autopsied after 24 hrs. Fertilized eggs were at the 4 or 8 cell stage when recovered from the oviducts.

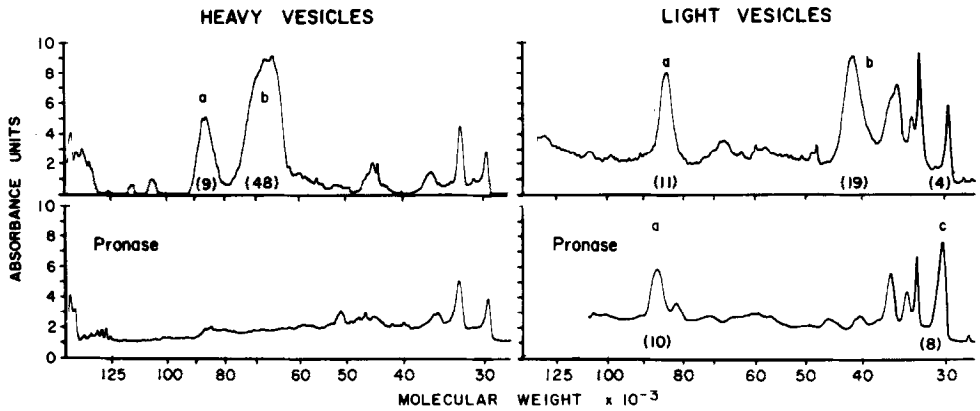

Fig. 1.- Heavy and light vesicle polypeptide patterns showing effects of pronase digestion. The scans were obtained after electrophoresis using sodium dodecyl sulfate and 7.5% polyacrylamide. Stain, Coomassie Brilliant Blue. Numbers in parenthesis are relative amounts (%).

[^{3}H] Galactose labelled vesicles, containing 8.5 x 10^5 cpm/mg protein, were prepared by incubation with galactose oxidase (Kabi) and [^{3}H] $NaBH_4$ according to the method of Saito and Hakomori (14). Washed ejaculated rabbit spermatozoa, at a concentration of 16 x 10^6 sperm/ml, were incubated with [^{3}H] galactose labelled vesicles at 20°C in Hanks solution (Difco). Following incubation for 30 min. sperm cells were recovered by sedimentation through 2 (w/v)% albumin in Hanks. Radioactivity in the cells was assayed in a liquid scintillation counter (Packard) using a water miscible phosphor (New England Nuclear), after dissolution of the sperm in 50 (v/v)% Biosolv (Beckman). The fraction of sperm radioactivity in glycolipid was estimated following extraction with cholorform and methanol, and it has been compared with the fraction of radioactivity in vesicle glycolipid.

RESULTS AND DISCUSSION Heavy and light vesicles can be seen from Fig. 1 to have distinct and complex polypeptide compositions. They both possess a major polypeptide (component a) with a molecular weight of 9.5 x 10^4. Component b, quantitatively the most significant polypeptide, reacted positively for sugar indicating it is a glycoprotein. Since heavy and light vesicle component b differ in electrophoretic mobility, they are clearly different proteins. These glycoproteins appear to be located on the outer vesicle surface, as they were attacked by pronase (Fig. 1). In

pronase-digested light vesicles component c increased indicating it is a cleavage product of another protein. Vesicle proteins accounted for approximately one fifth of vesicle wet weight and 70% and 54% of heavy and light vesicle dry weight, respectively.

Table 1 shows that phospholipids and cholesterol comprise nearly three fourths of vesicle lipid. As expected, light vesicles contained about twice as much lipid as heavy vesicles. They also had more cholesterol. It is interesting to note that light vesicles have been attributed with higher decapacitation activity (15). Extraction with dry ethyl ether (13) decreased cholesterol and phospholipid by comparatively small amounts (Table 2). With acetone, ethyl ether and water (30:10:1) as solvent, approximately two thirds of the cholesterol and half the phospholipid was eluted. Vesicle structure did not persist after this treatment, however, the remaining suspension was stable.

Sperm cells treated with native vesicles, dry ether extracted vesicles and pronase-digested vesicles gave fertilization rates of 23% (14/61), 26% (9/34) and 36% (8/22), respectively, after oviductal insemination about 3 hrs. post-ovulation (Table 3). By comparison, sperm cells exposed to vesicles extracted with acetone, ether and water were capable of fertilizing 64% (32/50) of the eggs recovered and this is not significantly different ($\chi^2_1 = 1.45$) from the fertilization rate of 76% (48/63) achieved with untreated spermatozoa. Fertilization was significantly ($\chi^2_1 = 43.42$, $0.01 \gg P$) more frequent in these two groups than in the other three groups. These results indicate that while surface proteins of the vesicles are probably not implicated in decapacitation, vesicle lipids are important. This finding is in agreement with a recent observation in this laboratory showing that synthetic phospholipid vesicles containing cholesterol reversibly inhibit sperm fertilizing ability (3).

TABLE 1

LIPID COMPOSITION OF MEMBRANE VESICLES FROM RABBIT SEMINAL PLASMA

Lipid	Heavy vesicles	Light vesicles
Total lipids (μg/mg protein)	422	865
	Per cent	
Phospholipid	41	41
Sphingomyelin	(28)	(15)
Phosphatidylethanolamine	(6)	(15)
Phosphatidylcholine	(<1)	(5)
Phosphatidylserine	(3)	(3)
Ganglioside	7	5
Cerebroside (2 gal, 1 glu; 1 gal)	8	13
Cholesterol	33	30
Other neutral lipids	10	12

TABLE 2

CHOLESTEROL AND PHOSPHOLIPID LEVELS IN EXTRACTED MEMBRANE VESICLES FROM SEMINAL PLASMA

Vesicles	Heavy vesicles		Light vesicles	
	Phospho-lipid	Cholesterol	Phospho-lipid	Cholesterol
	(μg/mg protein)			
Native	173	139	354	260
Extracted				
Dry ether	156	118	243	225
Acetone, ether and water	91	58	177	106

Rabbit sperm cells became radioactively labelled during incubation with [^{3}H]galactose containing vesicles. Glycolipid appeared to represent similar fractions of sperm and vesicle

TABLE 3

EFFECT OF PRONASE-DIGESTED AND EXTRACTED MEMBRANE VESICLES FROM SEMINAL PLASMA ON THE FERTILIZING CAPACITY OF RABBIT SPERM CELLS

Vesicles*	No. of insemn**	No. of eggs	Fertilized eggs No.	Fertilized eggs %
Nil	13	63	48	76
Native	13	61	14	23
Pronase-digested	7	22	8	36
Extracted				
Dry ether	8	34	9	26
Acetone, ether and water	12	50	32	64

* Vesicle concentration was 1 mg protein/ml (0.5 mg protein/ml from heavy and light vesicles).
**There were 1.25 to 5.0 x 10^4 sperm cells deposited in each oviduct.

TABLE 4

FRACTION OF [^{3}H] GALACTOSE IN GLYCOLIPID FROM VESICLES AND SPERM CELLS INCUBATED WITH THE VESICLES

Source	Total radio-activity (cpm)	Glycolipid fraction (%)
Vesicles	450	40
Sperm cells	214	48

radioactivity (Table 4). A cerebroside containing 2 galactose and 1 glucose residue was found in both types of vesicles. Heavy vesicles have in addition a cerebroside with a single galactose. Non-extractable radioactivity in these preparations can be

attributed largely to labelled glycoproteins (8). Hence, these results imply that decapaciation results from vesicle fusion with the sperm cell.

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