The binding of labelled basic proteins by boar spermatozoa

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Summary. Seminal plasma basic proteins were labelled with ^{131}I . The efficiency of the labelling was studied by superimposing protein density traces on a radioactive fractionation plot. These labelled proteins were incubated with spermatozoa and shown to bind more readily to spermatozoa from boars after the removal of the vesicular glands than to spermatozoa obtained from their normal litter mates. Most of the labelled protein became bound to the membranes which were isolated by sucrose density gradient centrifugation. The membranes were separated into two bands which equilibrated at the relative densities of 1.150 and 1.165. These fractions consisted of membrane vesicles of different size; the smaller band on the gradient, which equilibrated at 1.165, consisted of denser membrane material.

Introduction

The proteins of the accessory sex gland secretions from several mammals have been shown to coat epididymal spermatozoa (Weil & Finkler, 1958; Matousek, 1964; Hathaway & Hartree, 1963). These sperm-coating antigens, which are mainly of seminal vesicle origin, are not readily removed once they have been adsorbed by the spermatozoa. Bournsnel & Coombs (1966) have evidence that a proportion of boar seminal plasma proteins is firmly adsorbed on the sperm surface and only partly removed by washing. There are indications that basic proteins can increase the permeability of biological membranes (Ryser & Hancock, 1965; Drew & McLaren, 1970; Hibbitt & Benians, 1971), and it is possible that seminal vesicle basic proteins may have a similar effect on the membranes of spermatozoa. Boar seminal plasma has a high concentration of basic proteins of seminal vesicle origin (Lavon & Bournsnel, 1971), which could make the sperm membrane susceptible to damage during the cooling and freezing processes required for long-term semen storage. Boar spermatozoa cooled to temperatures below 10°C show an irreversible loss of motility (Polge, 1956), which may be associated with the haemagglutinin basic protein produced by the seminal vesicles (Roberts *et al.*, 1974). By using boars which have had their seminal vesicle glands surgically removed (Davies *et al.*, 1975), a preparation of semen can be obtained free of seminal vesicle secretion. In the present study, a fraction containing most of the basic proteins in boar seminal plasma was labelled with iodine-131 to establish whether binding between boar spermatozoa and the seminal vesicle basic proteins was occurring on contact.

Materials and Methods

Vesiculectomy

Large White boars of good breeding potential were selected from the Institute minimal disease herd. Surgery was performed at 4–8 months of age as described by Davies *et al.* (1975) and the boars were then allowed to recover fully before being trained for semen collection. Intact boars from the same litter, and therefore of similar age and genetic background, were retained as controls. Two intact and three vesiculectomized boars were used.

Semen collection

Semen was collected by the gloved-hand technique (Hancock & Hovell, 1959) through gauze into a prewarmed vacuum flask at 30°C. Collections were made from all boars at weekly intervals.

To obtain seminal plasma samples, the spermatozoa were removed from the semen immediately after collection by centrifugation at 10,000 *g* for 30 min at 25°C. The seminal plasma was carefully removed from the sedimented spermatozoa, filtered and stored at -20°C. Regeneration of the seminal vesicle glands was monitored by measuring the citrate (Moellering & Gruber, 1966) and protein (Layne, 1957) concentrations in the seminal plasma.

Separation of the basic protein fraction

Nelson & Bournsnel (1966) and Bournsnel & Briggs (1969) have demonstrated that boar seminal plasma proteins may be separated by gel filtration into two main fractions, a large molecular weight acidic fraction and a smaller molecular weight fraction containing the majority of basic proteins. To obtain a basic protein fraction to label with iodine-131, intact boar seminal plasma was dialysed overnight in 0.1 M acetate buffer, pH 4.0, and 3 ml fractionated on a G-200 Sephadex column (Pharmacia, Uppsala), which had been previously equilibrated with the same buffer, and run at a flow rate of 10 ml/hr. Fractions (2.5 ml) of the eluate were collected and the protein monitored at 280 nm in a Uvicord II (LKB Instruments Ltd, Croydon). The initial higher molecular weight acidic protein peak was discarded while the whole of the basic protein peak was collected, the fractions combined and dialysed against 0.1 M-tris-HCl buffer, pH 7.5 (Text-fig. 1). To remove any acidic proteins still present this fraction was loaded on a DEAE-Sephadex column (Pharmacia) equilibrated and eluted with more 0.1 M-tris-HCl buffer, pH 7.5. The protein collected in the eluate was then used for labelling with iodine-131.

Text-fig. 1. Protein profile of boar seminal plasma separated on G-200 Sephadex, showing the position of the protein peak subsequently run on DEAE-Sephadex and then labelled with iodine-131 (see text).

Labelling of the basic protein fractions

A labelled basic protein fraction was prepared by the method of Hunter & Greenwood (1962). Aliquots (5 µg in 20 µl) of basic protein were reacted with 5 mCi (125 µl) of iodine-131 (Radiochemical Centre, Amersham) using chloramine T (88 µg in 20 µl) as an oxidizing agent. Unreacted iodine-131 was separated from labelled protein by passing the mixture down a small column packed with G-50 Sephadex (Pharmacia) equilibrated and run with 0.1 M-sodium barbitone buffer, pH 8.6. The protein was recovered in the void volume in two 1-ml fractions, stored in 0.1-ml aliquots at -20°C, and used within 1 week. The efficiency of iodine-131 incorporation was between 15 and 20%, giving a specific activity for the labelled protein of 100–150 µCi/µg.

Characterization of labelled protein

Labelled and unlabelled protein fractions were compared by disc electrophoresis on 7.5% polyacrylamide gels buffered at pH 7.5 with 0.1 M-tris-HCl containing 6 M-urea. The samples of

protein, labelled and unlabelled, were placed at the anode of the gels and subjected to electrophoresis for 60–90 min at 5 mA/tube. At the end of the run the gels were immediately scanned at 280 nm (Gilford Instruments, Ohio). The gels containing the labelled proteins were frozen on solid CO₂ and sliced into 2-mm sections which were then placed in vials containing 10 ml double-distilled water. After diffusion for 2 hr the radioactivity of the sections was counted (Philips Gammamatic, Holland). The gels containing unlabelled protein were fixed in 5% TCA at 60°C and stained with amido black.

Labelling of seminal plasma

The ¹³¹I-labelled basic protein (6 µCi in 0.1 ml) was added to 10 ml normal seminal plasma to give a minimum mean specific activity of 15 nCi/mg protein.

Experimental procedure

Incubation of spermatozoa. Duplicate 5-ml aliquots of semen collected from either a normal boar or from a vesiculectomized boar were centrifuged at 700 g for 10 min while a determination of the sperm number was made (Neubauer double counting chamber). The seminal plasma supernatant was removed by aspiration, and the sedimented spermatozoa gently resuspended in labelled normal seminal plasma at 30°C with a Pasteur pipette. The volume of seminal plasma added was such that the sperm concentration was 4×10^8 /ml. The resuspended spermatozoa were incubated with shaking for 30 min at 30°C in small bijou bottles placed in a metabolic water bath. At the end of the incubation, the resuspended semen was washed five times at 25°C in successive 10-ml vols of 0.25 M-buffered sucrose solution, pH 7.5. Between the washes the spermatozoa were centrifuged at 700 g and resuspended as gently as possible to prevent damage. The number of spermatozoa lost in the washings was counted. After the fifth and final wash the sperm pellet was resuspended in 0.25 M-sucrose solution. These spermatozoa and each successive washing were counted for radioactivity (Philips Gammamatic). The specific activity (ct/10⁸ spermatozoa) was calculated and corrected for decay of the isotope.

Separation of membranes. Spermatozoa from boars without seminal vesicles were incubated with labelled normal seminal plasma as described above. The final washed spermatozoa were centrifuged at 10,000 g for 10 min and then resuspended in a sucrose solution of relative density 1.13. This sperm suspension was then frozen and thawed twice before being homogenized with twenty up-and-down strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 10 min and the supernatant removed and placed on a continuous sucrose gradient, relative density 1.13 to 1.19. The gradient was centrifuged for 17 hr at 80,000 g on a Beckmann 25.1 swing-out rotor. After centrifugation the membrane bands could be seen and these were removed on 0.5-ml aliquots. From these fractions the refractive index (Abbey refractometer) and radioactivity (Philips Gamma-matic) were determined. The fractions containing the membrane bands were fixed in glutaraldehyde buffered at pH 7.0 for 1 hr at room temperature, and then post-fixed in osmium tetroxide for 1 hr at 5°C before being processed for electron microscopy.

Results

Polyacrylamide gel electrophoresis of labelled/unlabelled proteins

Labelled and unlabelled protein fractions showed a similar distribution of bands on polyacrylamide gels suggesting that labelling with ¹³¹I did not produce an obvious change in the protein characteristics. A protein density trace and a radioactive fractionation plot for a gel loaded with labelled protein are shown in Text-fig. 2. The radioactivity maximum corresponded with the main protein peak shown by the 280 nm density trace and the staining pattern. There was no protein at the anode of the gel, indicating that the fraction was composed of only basic components.

Text-fig. 2. Radioactive fractionation (●) of a polyacrylamide gel, 7.5%, pH 7.5, loaded with boar seminal vesicle basic protein labelled with iodine-131, and a protein density trace (---) and staining pattern for gels run under identical conditions with unlabelled basic protein.

Seminal vesicle proteins binding to spermatozoa

Significantly ($P < 0.01$) more labelled protein bound to spermatozoa from vesiculectomized boars than to spermatozoa from intact boars (Table 1). Some radioactivity was present in the washings but this decreased to a constant level after three or four washes. The sperm concentration in the washings (10^5 spermatozoa/ml) was low compared with the total sperm number (10^9 spermatozoa). Most of the spermatozoa present in the washings were damaged; thus the radioactivity present in the final washing was probably due to disintegrating spermatozoa.

Table 1. Differential binding of labelled basic protein fraction to boar spermatozoa

	Intact boars	Vesiculectomized boars
No. of boars	2	3
Samples incubated	8	10
Mean specific radioactivity of spermatozoa (ct sec ⁻¹ /10 ⁸ spermatozoa $\pm$ S.D.)	49.6 $\pm$ 7.9*	96.9 $\pm$ 10.4*
Radioactivity ratio (final wash supernatant sperm pellet)	0.10	0.13
Protein level in seminal plasma of ejaculated semen (mg ml ⁻¹)	39.7	4.3

* $P < 0.01$.

Proteins binding to sperm membranes

The radioactivity of the labelled proteins was mainly associated with two bands which equilibrated at relative densities of 1.150 and 1.165 (Text-fig. 3). Electron micrographs of these bands revealed that they consisted mainly of membrane vesicles (Plate 1).

Electron micrographs of membrane material of boar spermatozoa from the radioactive peaks of the density gradient, associated with the radioactivity at relative densities of 1.150 (Fig. 1) and 1.165 (Fig. 2).

(Facing p. 74)

Text-fig. 3. Radioactive plot of the density gradient fractionation of incubated sperm membrane from a vesiclectomized boar.

Discussion

Twice as much (197%) labelled seminal plasma protein became bound to spermatozoa from boars without seminal vesicles than to normal boar spermatozoa after incubation at 30°C. This probably reflects the greater number of cationic binding sites available on spermatozoa from vesiclectomized boars. In a normal ejaculate, spermatozoa are already coated with seminal vesicle proteins, thereby reducing the available binding sites. The association of the radioactively labelled proteins with the membrane vesicles suggests that the outer membranes of the spermatozoa (plasma and acrosomal) are areas of attachment of the seminal plasma basic protein.

It is unclear why two separate membrane bands, both associated with a label, were isolated by the methods used. The homogenization technique was devised to remove plasma and acrosomal membranes from the spermatozoa while leaving the nucleus intact. Electron micrographs of the sperm pellets after centrifugation revealed that most cells had an intact nuclear membrane. This would suggest that the two bands consisted of plasma and acrosomal membrane attached to labelled protein, although one cannot entirely discount the presence of some nuclear membrane material in the preparation. Detergents were not used to specifically remove acrosomes from the spermatozoa (Lunstra *et al.*, 1973), as it was felt that these chemicals could lead to configurative changes of the membrane proteins.

Binding of labelled seminal plasma proteins to the membranes accords with the results of Bournell & Coombs (1966) who suggested that seminal plasma proteins were being absorbed by the spermatozoa. If the membrane surface charge of epididymal spermatozoa is negative (Yanagimachi *et al.*, 1972), then basic proteins would be absorbed by electrostatic attraction. Isoelectric focusing studies (Moore & Hibbitt, 1975) have shown that the isoelectric point (i.e.p.) of normal ejaculated boar spermatozoa is higher than that of spermatozoa from vesiclectomized boars even after washing (i.e.p. pH 6.5 and 4.5 respectively). The change in isoelectric points suggests that considerable 'sperm coating' occurs at ejaculation.

Several workers have shown that seminal plasma has a detrimental effect on spermatozoa of different mammals during cooling procedures and cold shock. Fulka *et al.* (1965) showed that bovine spermatozoa from the ampulla became sensitized to cold shock after treatment with the fluids of the seminal vesicles. Antisera to seminal vesicle fluid reduced this effect when added to ampullary spermatozoa before contact with seminal vesicle fluid, but had no effect on ejaculated spermatozoa (Matousek, 1964). In our study, a certain amount of protein was bound to the boar spermatozoa and could not be removed even after extensive washing, indicating a probable irreversible attachment

of the protein to the membrane. The iodinated basic protein fraction contained more than one component, as revealed by gel electrophoresis (Text-fig. 2) and isoelectric focusing (unpublished results), and it was not possible to ascribe the 'sperm-coating' to one particular protein.

Boar spermatozoa are also susceptible to cold shock (Polge, 1956) and seminal plasma could be detrimental to them on cooling to 5°C (Pursel *et al.*, 1972). If basic proteins from the seminal vesicle glands bind to the spermatozoa, as the results would suggest, then damage to the spermatozoa on cooling may be due to membrane breakdown induced by stresses of cooling enhanced by basic proteins.

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