Radioiodination of surface proteins of bull spermatozoa and their characterization by sodium dodecyl sulphate–polyacrylamide gel electrophoresis

M. Vierula and H. Rajaniemi*

*Department of Anatomy, University of Kuopio, 70101 Kuopio 10, and *Department of Anatomy, University of Oulu, 90220 Oulu 22, Finland*

Summary. Surface proteins of ejaculated bull spermatozoa were radioiodinated, solubilized and characterized by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The electron microscopic autoradiographs showed that the labelling was equally distributed to all parts of the spermatozoon and restricted to the sperm surface. The electrophoresis of solubilized radioactivity revealed 6 radioactive fractions with approximate molecular weights of 67 000–69 000, 47 000–50 000, 34 000–37 000, 25 000–28 000, and 14 000–16 000. The 6th fraction probably represented labelled lipids. The electrophoresis of radioiodinated seminal plasma proteins revealed only 2 radioactive protein peaks which coincided with the sperm surface protein fractions IV and V.

Introduction

The outer surface of the spermatozoon has been the subject of intensive investigation during the past few years. These studies have clearly indicated that the composition of the mammalian sperm surface undergoes molecular changes during epididymal transit and capacitation and that these changes are necessary for the spermatozoa to achieve fertilizing ability. The approaches used to investigate these macromolecular changes on the sperm surface have been numerous. The density and distribution of negative charges have been investigated at various developmental stages by means of a colloidal iron hydroxide labelling technique (Cooper & Bedford, 1971; Yanagimachi, Noda, Fujimoto & Nicolson, 1972; Bedford, Cooper & Calvin, 1972; Yanagimachi, Nicolson, Noda & Fujimoto, 1973; Fléchon, 1975). However, this technique gives only limited information of the chemical nature of the binding sites of colloidal iron hydroxide.

The distribution of glycoproteins on the sperm surface has been studied by using carbohydrate-specific lectins (Edelman & Millette, 1971; Nicolson & Yanagimachi, 1972; Nicolson & Yanagimachi, 1974; Gordon, Dandekar & Bartoszewicz, 1974, 1975; Nicolson, Usui, Yanagimachi, Yanagimachi & Smith, 1977). Olson & Hamilton (1978) labelled the surface carbohydrate residues of rat spermatozoa from the caput and cauda epididymidis by a galactose oxidase–sodium [^3H]borohydride or sodium metaperiodate–sodium [^3H]borohydride technique.

The surface composition of spermatozoa has not been analysed by using protein-specific biochemical probes. Lactoperoxidase-catalysed radioiodination has been successfully used to label tyrosine (and probably histidine) residues of soluble proteins (Marchalonis, 1969), but when this method was applied to intact human erythrocytes, haemoglobin was not labelled, indicating that lactoperoxidase could not penetrate the erythrocyte membrane (Phillips & Morrison, 1970). Marchalonis, Cone & Santer (1971) radioiodinated the surface proteins of mouse lymphocytes

in a single-cell suspension and also found that in electron microscopic autoradiographs the grains were associated only with the outer surfaces of the intact cells.

In this study we used the lactoperoxidase-catalysed radioiodination technique to label tyrosine-containing proteins and glycoproteins on the surface of ejaculated bull spermatozoa.

Materials and Methods

Semen collection and washing of spermatozoa

Spermatozoa were obtained from Finnish Ayrshire bulls by means of an artificial vagina. Semen samples were transported immediately after collection to the laboratory. Only samples in which the average concentration of spermatozoa was $1000\text{--}2000 \times 10^6/\text{ml}$ and the motility was $>60\%$ were accepted for radioiodination. The labelling of spermatozoa was always performed within 6 h after collection.

An aliquot of 1 ml semen was diluted in 2 ml sperm-Ringer solution, pH 7.4 (Mann, 1946). The spermatozoa were then washed in a saline-based medium containing 7.5% Ficoll (Pharmacia, Uppsala, Sweden) according to the procedure described by Harrison (1976). After centrifugation through Ficoll the spermatozoa were washed in sperm-Ringer solution (pH 7.0) by centrifugation at 500 g for 10 min to remove Ficoll. The final sperm pellet was resuspended in sperm-Ringer (pH 7.0).

Radioiodination of the surface proteins of spermatozoa

The lactoperoxidase-catalysed radioiodination procedure was used essentially as described by Marchalonis *et al.* (1971). The iodination was carried out at room temperature and the reaction mixture contained $300\text{--}600 \times 10^6$ spermatozoa, 20 μg (2.44 i.u.) lactoperoxidase (Calbiochem, San Diego, California; grade B), 0.5 mCi carrier-free Na^{125}I (Radiochemical Centre, Amersham, England) in total volume of 1 ml sperm-Ringer solution (pH 7.0). The iodination reaction was initiated and sustained by adding three 20 μl aliquots of 88 μM -hydrogen peroxide at 5 min intervals. The reaction was allowed to proceed for 15 min and thereafter stopped by adding 4 ml 1 mM-NaI prepared in sperm-Ringer solution. Labelled spermatozoa were then sedimented by centrifugation at 500 g for 10 min at 22°C and washed 4 times in 5 ml sperm-Ringer solution (pH 7.4). The final sperm pellet was suspended in 1 ml sperm-Ringer solution (pH 7.4) and the suspension was divided into aliquots of 250 μl , frozen and stored at -80°C until electrophoretic analysis. The morphology and motility of the spermatozoa were studied immediately after iodination and in the final suspension after washing.

For electron microscopic autoradiography the spermatozoa were radioiodinated identically.

Radioiodination of seminal plasma proteins

Semen diluted in sperm-Ringer (1:2 v/v) was centrifuged at 1200 g for 45 min at 4°C. The reaction mixture contained 100 μl seminal supernatant, 20 μg lactoperoxidase and 0.2 mCi carrier-free Na^{125}I in a total volume of 1 ml sperm-Ringer solution (pH 7.0). The iodination reaction was initiated and sustained by adding three 20 μl aliquots of 88 μM -hydrogen peroxide at 5 min intervals. After incubation for 15 min the reaction was terminated by adding 2 ml sperm-Ringer solution (pH 7.4). The reaction mixture was then divided into aliquots of 250 μl , frozen and stored at -80°C until analysed by electrophoresis.

Electron microscopic autoradiography

Labelled spermatozoa were fixed in PFG-cacodylate, which contained 11% picric acid, 1% formaldehyde, 1.25% glutaraldehyde and 150 mM-sodium cacodylate prepared as described by

Ito & Karnovsky (1968) and postfixed in osmium-cacodylate containing 40 mM-osmium tetroxide, 75 mM-glucose and 150 mM-sodium cacodylate following the procedure described by Jones (1973). After fixation the spermatozoa were centrifuged at 1200 g for 30 min and the pellet was then cut into small pieces, which were dehydrated in ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections, silver or gray, were cut and stained with uranyl acetate and lead citrate for 30 min and 5 min respectively. The sections were processed for autoradiography by the loop method described by Caro & Van Tubergen (1962) using Ilford L₄ emulsion (Ilford Limited, Ilford, Essex, England). The samples were exposed for 4 weeks and developed with Kodak Microdol-X for 5 min and fixed with Ilford Hypam for 5 min at 20°C. The specimens were examined in a Jeol 100 B electron microscope.

The distribution of the grains in relation to the plasma membrane was analysed by measuring the distances of the grain centres from it in HD units (Salpeter, McHenry & Salpeter, 1978). The half distance (HD) for ¹²⁵I when Ilford L₄ nuclear emulsion is used is 90 nm (Salpeter *et al.*, 1978). The centre of the grain, determined by punching the centre of the smallest possible circle around the grain was considered as the location of the grain. Forty-six micrographs were analysed and 400 grains were counted.

Disc electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS)-containing gels (10% acrylamide, 0.37% N,N'-methylene-bis-acrylamide, 0.1% SDS) was performed according to the method of Zahler (1974). Labelled sperm suspension (250 µl) was diluted to 0.5 ml so that the final concentrations of SDS, urea and β-mercaptoethanol in the sample were 1%, 4 M, and 1% respectively. The sample suspension was heated at 100°C for 4 min and then centrifuged at 20 000 g for 30 min. Aliquots of 30–50 µl of the supernatant were layered on to polyacrylamide gels (0.5 × 9.5 cm). Pyronine-Y was used as the marker stain. Running buffer contained 0.1% SDS in 0.1 M-sodium phosphate (pH 7.0). After running for 11 h (5 mA/gel) at room temperature the gels were removed from the tubes and frozen in a freezing box. The frozen gels were sliced into 1 mm sections, which were counted for ¹²⁵I radioactivity (efficiency for ¹²⁵I, 52%).

The standard proteins included in the electrophoreses were bovine serum albumin (mol. wt 68 000), ovalbumin (mol. wt 45 000), chymotrypsinogen A (mol. wt. 25 000), and cytochrome c (mol. wt 12 500) (Combithek; Boehringer Mannheim GmbH, Western Germany). The approximate molecular weights of labelled surface proteins were estimated from their respective relative mobilities.

Some gels were also stained with Coomassie Brilliant Blue for proteins.

Results

Radioiodination of spermatozoa and localization of radioactivity

The morphological integrity and motility of bull spermatozoa was preserved well during the iodination and subsequent washings. Immediately after termination of the iodination the spermatozoa were morphologically intact (Pl. 1, Fig. 1) and 20–30% of them were still motile at room temperature. After the subsequent washings some loose heads and tails appeared but over 90% of the washed spermatozoa were morphologically intact (Pl. 1, Fig. 2). The average radioactivity in the final labelled sperm suspension was $3\text{--}4 \times 10^6$ c.p.m./300–600 × 10⁶ spermatozoa. The electron microscope autoradiographs showed that the grains of the iodine label were mostly associated with the plasma membrane and that they were equally distributed to every part of the spermatozoon (Pl. 1, Figs 3–6).

The grain distribution analysed in relation to the plasma membrane is shown in Text-fig. 1. The distribution fits very well with that expected for a radioactive line source. The grain counting showed that 68% of the grains were located within 2HD units of the plasma membrane.

Text-fig. 1. Histogram of the grain distribution analysed in relation to the plasma membrane of the spermatozoa.

Fractionation of radioiodinated surface proteins of spermatozoa

To analyse the molecular composition of the labelled surface proteins the sperm samples ($7-10 \times 10^5$ c.p.m.) were solubilized and fractionated by SDS–polyacrylamide gel electrophoresis. Heating the sperm suspension in SDS, urea and β -mercaptoethanol solution effectively solubilized the spermatozoa and only nuclei could be identified by phase-contrast microscopy after the treatment. The nuclei were pelleted by centrifugation and the supernatant, which contained over 85% of the total radioactivity in the sample, was subjected to electrophoresis. Several acrylamide concentrations ranging from 5 to 15% were tested. The best separation of radioactive proteins was achieved with 10% polyacrylamide gels containing 0.1% SDS. The electrophoresis repeatably revealed 6 radioactive peaks (Text-fig. 2a). The R_F values and approximate molecular weights of the first 5 peaks (I–V) are presented in Table 1. Sperm

Table 1. The approximate R_F values and molecular weights of the first 5 labelled surface proteins of bull spermatozoa separated by SDS–polyacrylamide gel electrophoresis

Proteins	R_F values	Molecular weights
I	0.09–0.10	67 000–69 000
II	0.17–0.20	47 000–50 000
III	0.30–0.34	34 000–37 000
IV	0.40–0.45	25 000–28 000
V	0.64–0.69	14 000–16 000

PLATE 1

Fig. 1. Spermatozoa immediately after radioiodination. Incubation of the reaction mixture did not cause any morphological damage to the spermatozoa. $\times 1000$.

Fig. 2. Labelled and washed spermatozoa in the final suspension. Some loose heads and tails were present, but the majority of spermatozoa were still intact. $\times 1000$.

Figs 3–6. Electron microscopic autoradiographs prepared from the radioiodinated spermatozoa show that the grains are associated mostly with the plasma membrane in every part of the spermatozoon. $\times 11\,500$.

PLATE 1

(Facing p. 486)

Text-fig. 2. Distribution of radioactivity of the labelled spermatozoa (a) and seminal plasma (b) of the same bull in SDS-polyacrylamide gel electrophoresis. The localization of standard proteins with known molecular weights (see 'Methods') and pyronine-Y (P) are shown. The usual positions of the first 5 labelled protein fractions (I-V) are indicated.

samples from 15 different bulls have been labelled and virtually the same radioactive peaks have been repeatably obtained, within and amongst bulls (Text-fig. 3). Coomassie Brilliant Blue staining displayed at least 15 protein bands with R_F values between 0.02 and 0.75 and molecular weights between 100 000 and 12 000.

Fractionation of the radioiodinated seminal plasma proteins

The proteins labelled in the seminal plasma were distributed between two peaks with approximate molecular weights of 25 000–28 000 and 14 000–16 000 (Text-fig. 2b), which seemed to coincide with peaks IV and V of the sperm surface proteins (Text-fig. 2a).

Discussion

The present results show that spermatozoa, like erythrocytes (Phillips & Morrison, 1970) and lymphocytes (Marchalonis *et al.*, 1971), can be labelled by lactoperoxidase-catalysed radioiodination. Analysis of the grain distribution over the spermatozoa in relation to the plasma membrane showed that most of the grains were associated with the plasma membrane, suggesting that the radioiodine label is located at the surface of the spermatozoon. Although the distribution of the radioactive labelling in the SDS-electrophoresis was consistent within and among bulls, fractions often showed two or more subpeaks, indicating that each probably consists of more than one protein component. The 6th peak of radioactivity moved near the tracking dye and probably represented labelled membrane lipids (Mersel, Benenson & Doljanski, 1976).

The electrophoresis of labelled seminal plasma displayed two fractions of radioactivity which

Text-fig. 3. Distribution of radioactivity of the labelled spermatozoa from 3 different bulls. The main protein fractions (I–V) in each sample are identified. The sample volumes applied were 50 μ l for (a) and 30 μ l for (b) and (c). The radioactivity in (a) was higher than that in the other two samples.

coincided with the fractions IV and V of the spermatozoa. This observation suggests that the surface of ejaculated bull spermatozoa and seminal plasma may have some protein components in common. This is further evidence for the fact that there may be an interaction between the surface of the spermatozoon and surrounding seminal plasma proteins (Voglmayr, Fairbanks, Jackowitz & Colella, 1979). The functional significance of the labelled surface proteins detected by SDS-electrophoresis remains to be elucidated. However, some may play a role in the development of the fertilizing capacity of the spermatozoa during their maturation process in the accessory sex organs. Olson & Hamilton (1978) have shown that a new glycoprotein appears on the sperm surface during transit through the epididymis and the technique of radioiodination of intact spermatozoa can now be used to characterize the protein structure of the sperm surface and its changes at different developmental stages.

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