An antiserum against protamines for immunohistochemical studies of histone to protamine transition during human spermiogenesis*

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Summary. Specific polyclonal antisera have been obtained against total human protamines isolated from purified sperm nuclei. The specificity of antibodies was assessed in immunodotting and immunoblotting assays. In this preliminary report, these specific antibodies were used as probes for in-situ determination of histone to protamine transition during human spermiogenesis. Protamine-containing sites were detected on sections of human testes by light microscopy using the immunoperoxidase technique. As in other mammals, protamines appear and concentrate in condensed nuclei of elongating spermatids, i.e. during the later steps of human spermiogenesis.

Keywords: human sperm protamines; immunohistochemistry; histone-protamine transition; spermiogenesis

Introduction

During spermiogenesis, morphological changes of spermatid nuclei are concomitant with modifications of the basic protein complement associated with deoxyribonucleic acid. Somatic-type histones present in the early steps of spermiogenesis are generally replaced by more basic proteins enriched in arginine, termed intermediate proteins or protamines, depending on their properties. Protamines have very low molecular weight and some of them are cystein-rich (Monesi, 1964; Geremia *et al.*, 1976; Loir & Lanneau, 1978; Meistrich *et al.*, 1978; Rodman *et al.*, 1979; Grimes, 1986).

In some species, biochemical studies of nuclear protein transitions have been carried out on purified fractions of germ cells at different steps of spermiogenesis (Fawcett *et al.*, 1971; Romrell *et al.*, 1976; Bouvier, 1977; Meistrich *et al.*, 1981; Balhorn *et al.*, 1984).

However, the difficulties in obtaining human testes and purified fractions of intermediate and old spermatids do not yet permit biochemical analysis of nuclear basic proteins during spermiogenesis. Several basic proteins have been extracted from human sperm heads (Tanphaichitr *et al.*, 1981; McKay *et al.*, 1985, 1986). Recently, histones, protamines and a third group of proteins with intermediate molecular properties have been isolated and characterized (Gusse *et al.*, 1986).

Protamines are generally considered as poor immunogens. Nevertheless, antibodies against human protamines can be detected in some autoimmune diseases, in infertile and vasectomized men (Samuel, 1978; Samuel *et al.*, 1978).

The purpose of this preliminary work was to produce an antiserum against purified human protamines whose specificity was ascertained by immunoblotting. Subsequently, this antiserum was used to localize protamines in human germ cells with immunohistochemical techniques.

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Materials and Methods

Purification of human sperm protamines. Ejaculates were collected from donors assessed as normal from their spermogram and spermocytogram: sperm counts were $>20 \times 10^6/\text{ml}$, $>50\%$ of spermatozoa were motile and $<50\%$ were morphologically abnormal. The samples were preserved at -80°C , pooled and washed three times in the following medium: 20 mM-Tris-HCl pH 7.5, 150 mM-NaCl, 0.25 M-sucrose, 2 mM-EDTA, 0.5 mM-phenylmethylsulphonyl fluoride, and 0.2% diisopropyl-fluorophosphate. Isolation and purification of basic nuclear proteins were performed as described previously (Gusse *et al.*, 1986).

Briefly, sperm heads were separated from tails by sonication and demembrated with CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate). Acid-soluble proteins were extracted after reduction and alkylation and purified on carboxymethylcellulose.

Preparation of the antisera. Lyophilized purified human protamines (900 μg), including HP1a, HP1b, HP2, HP3 and HP4 fractions, were dissolved in a sterile solution of 150 mM-NaCl at 0°C . Proteins were precipitated by adding three volumes of 0.01% (w/v) methylated bovine serum albumin in distilled water (Benoit *et al.*, 1982). The antigenic solution was emulsified with 3.5 ml Freund's complete adjuvant containing 1 mg killed *Mycobacterium tuberculosis*/ml (Sigma Chemical Company, St Louis, MO, U.S.A.) and electrically homogenized at 0°C . Three male rabbits, aged 3-5 months, were intradermally and subcutaneously injected at several sites with about 300 μg human protamines. At 31, 45 and 60 days after the first injection, rabbits received one booster injection of 100 μg protamines prepared in the same conditions as above. Antibody production was checked every week for 10 weeks by bleeding at the marginal ear vein. Undiluted antisera were stored below -70°C .

Immunodetection procedures and specificity controls. Dot-immunobinding assays were carried out according to the method of Hawkes *et al.* (1982): 0.5 μl of a 0.01-0.05% (w/v) antigenic solution of total basic nuclear proteins, of sperm-specific histones or of purified protamines was spotted on a 0.45 μm nitrocellulose filter (Schleicher & Schuell, Dassel, West Germany) by using a 10 μl Hamilton syringe. After drying, sheets of nitrocellulose were stored at -20°C . Strips of nitrocellulose were neutralized by blocking non-specific antibody-binding sites with PBS-gelatin-Tween (PGT) containing 0.3% (w/v) gelatin (Sigma type II), 0.2% (v/v) Tween 20 in PBS pH 7.2, for 20 min with mechanical shaking. The dots were covered with 1:2 to 1:200 diluted rabbit antiserum in PGT for 2 h to overnight, washed three times with PGT, and incubated in horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (Institut Pasteur, Paris, France) at a dilution of 1:500 for 90 min. Peroxidase activity was revealed by 3,3'-diaminobenzidine (Sigma) or *o*-phenylenediamine (Sigma) staining.

The immunoblotting assays were performed according to the procedures described previously by Towbin *et al.* (1979) and Glass *et al.* (1981). Total basic nuclear proteins or carboxymethylcellulose-purified fractions were electrophoresed on acid urea 17% polyacrylamide gels (Gusse *et al.*, 1983). Proteins were then electrotransferred onto two superimposed nitrocellulose sheets at 30 V/200 mA for 1 h in 1% acetic acid. After saturation in PGT for 1 h at 37°C or overnight at 4°C , the first sheet was used for immunodetection by incubation for 2 h to overnight with 1:100 rabbit antiserum and then washed several times in PGT. Peroxidase-conjugated goat antirabbit IgG (Institut Pasteur) was added to a dilution of 1:200 for 90 min. After several washes in PGT, peroxidase activity was revealed using 3,3'-diaminobenzidine. The second sheet was stained with 0.2% Red Ponceau S in 0.3% trichloroacetic acid for accurate localizations of protein fractions. To control antisera specificity, some dots and blots containing human sperm proteins were incubated either with preimmune serum or with anti-protamine immunoglobulins previously precipitated with an excess of protamines.

Immunohistochemistry. Testis samples were obtained from 25-40-year-old men with proven brain death. Some tissue blocks were sliced using a cryostat without fixation or after previous fixation by immersion in a solution of 4% paraformaldehyde in 0.12 M-phosphate buffer pH 7.2 and rinsed overnight in the same buffer containing 8% sucrose. Other samples were fixed in 10% neutralized formal or in aqueous Bouin fixative and then embedded in paraffin wax (Cytoparaffin Microcolor 54°C). Smears of sperm cells were air-dried or fixed in Carnoy's solution or methanol-acetone (1:1 v/v). Some smears of cells were immersed for 3 min in the following solution to modify the chromatin structure: 2 M-guanidinium chloride (GuCl), 0.25 M-Tris-HCl pH 8.5, 0.5% β -mercaptoethanol (Rodman *et al.*, 1979). Sections and smears were sequentially incubated with the following antisera or reagents (Nakane & Pierce, 1967): (1) 1:50 to 1:200 diluted rabbit antiserum in PBS for 1 h or overnight; (2) HRP-labelled goat anti-rabbit IgG (Institut Pasteur) at a dilution of 1:100 for 1 h; and (3) a freshly prepared solution of 0.05% diaminobenzidine and 0.01% hydrogen peroxide in PBS for 5 min. After each incubation, the slides were rinsed in PBS for 15 min with three changes of the buffer. After the last wash, they were dehydrated, mounted in Eukitt and observed by light microscopy without counterstaining or after aqueous eosin staining for 1 min before dehydration. The indirect immunofluorescence technique was also used (Coons, 1958), and the second antibody was a fluorescein-isothiocyanate-conjugated goat antirabbit IgG at a dilution of 1:50 for 30 min. Sections were mounted in buffered glycerine at pH 7.6 and observed under a fluorescence microscope in epifluorescence configuration.

As immunohistochemical controls, some sections were incubated with labelled goat anti-rabbit antibody alone or with antiserum against human protamines previously treated with an excess of protamines.

Results

Human sperm nuclei contain several basic proteins which can be classified into three families according to their molecular properties and electrophoretic mobilities (Gusse *et al.*, 1986). Sperm histones are either somatic-like histones or testis-specific variants. Four molecular species of protamines called HP1, HP2, HP3 and HP4 have been purified, some of them being phosphorylated. The

Fig. 1. Dot immunoassay for serum antibodies against total human sperm protamines at a concentration of 0.01% (w/v). A serial dilution assay of the serum (1:25 to 1:200) at weekly intervals is shown (1 to 10).

Fig. 2. Analytical polyacrylamide gel electrophoresis and immunoblotting of human sperm protamines: (a) electroblot on nitrocellulose (second sheet) stained with Red ponceau S; (b) immunoblot (first sheet of nitrocellulose). Lane 1: total human protamines used as antigenic mixture; lanes 2–6: purified human protamines. Equal amounts of proteins were loaded onto the polyacrylamide gel (20 µg per slot).

Fig. 3. Electrophoresis (20 μ g proteins per slot) and immunoblotting of basic proteins extracted from human sperm nuclei. Lanes 1–7 represent immunoblots of the major fractions obtained during the purification of basic proteins by carboxymethylcellulose chromatography. Lane 8 is an immunoblot of total acid-soluble nuclear proteins. Lane 9 shows the electrophoretic pattern of total acid-soluble proteins from sperm nuclei stained with Coomassie brilliant blue.

third family of sperm-specific nuclear proteins is a group of five proteins (HP-I1, I2, I3, S1, S2) which have intermediate basicity and electrophoretic mobility between those of histones and those of protamines.

The presence of antibodies against human protamines in rabbit serum was shown by immunodots with immunoperoxidase staining. On the 7th day after the first booster injection, a positive reaction was detected as a coloured dot against the white filter background after peroxidase staining. The staining of dots became more and more intense with time up to the last booster injection, thus suggesting an increase in the rabbit antibody titres. The antiserum reacted with pooled purified protamines (HP1, HP2, HP3, HP4) (Fig. 1), as well as with total basic nuclear proteins, but not with sperm-specific histone-like proteins. Reactions became negative after prior incubation of antiserum pretreated with human protamines. No reaction was observed with preimmune serum.

The immunoblot assays revealed that basic nuclear proteins extracted from human spermatozoa can be easily transferred from polyacrylamide gels to nitrocellulose. Antiserum bound specifically to all four protamines (Fig. 2). By contrast, neither histones nor intermediate proteins showed any immunoreactivity, except for one animal which developed antibodies reacting both with human protamines and with the intermediate protein HPS2 (Fig. 3). The detection of immuno-complexes was more sensitive after diaminobenzidine than after phenylenediamine staining.

The indirect immunoperoxidase technique on paraffin-wax sections revealed that only elongated spermatids with condensed nuclei presented strongly stained nuclei (Fig. 4). No preferential location in any part of the nuclei could be detected. Young spermatids with round nuclei remained unstained (Fig. 4). No staining was detected in the cytoplasm of any of these cells, in the other cells of the seminiferous epithelium (spermatogonia, spermatocytes, spermatozoa and Sertoli cells) or in the Leydig cells (Fig. 4). The indirect immunofluorescence technique gave similar results. On control sections, nuclei of intermediate and elongated spermatids were never stained. Without previous treatment, the same immunohistochemical methods applied to smears did not show any

Fig. 4. Human testis, Bouin-fixed, stained by the indirect immunoperoxidase method with antihuman protamine antiserum. Slides were counterstained with aqueous eosin. In the seminiferous tubules, only late spermatids (→) with elongated nuclei are immunoreactive. All other cell structures which are observed on this figure are revealed only by their staining with eosin. No reaction is detected in the interstitial space (IS). Sg = spermatogonia; Sc = primary spermatocytes; Sd = round spermatids; Sp = spermatozoa; S = Sertoli cells; Lp = lamina propria. a, $\times 500$; b, $\times 750$; c, d, $\times 1500$.

nuclear staining in ejaculated spermatozoa. The guanidinium chloride and β -mercaptoethanol solution induced sperm head swelling and these swollen sperm nuclei were stained by indirect immunohistochemical techniques (Fig. 5).

Fig. 5. Indirect immunoperoxidase method with antihuman protamine antiserum on smears of sperm cells fixed in Carnoy's solution. (a) Only ejaculated spermatozoa with swollen nuclei (treated by guanidinium chloride and β -mercaptoethanol) are immunoreactive. $\times 2000$. (b) Sperm cells without previous treatment of chromatin are unstained. $\times 2000$.

Discussion

Few reports have been published on the immunogenicity of protamines. Mouse sperm protamines (Rodman *et al.*, 1982, 1984) as well as ram protamines (Courstens *et al.*, 1983) appeared to be antigenic. Samuel (1978) and Samuel *et al.* (1978) showed that human protamines can also elicit the production of specific antibodies. The present data are in agreement with these conclusions. Immunodotting procedures which were used for qualitative and semiquantitative evaluation of antibody production after dilution of antisera (Hawkes *et al.*, 1982) showed the specificity of rabbit antiserum against human sperm protamines. Immunostaining was negative when rabbit antisera previously immuno-absorbed on purified human sperm protamines, or preimmune sera were used. Immunoassays showed that the major protamines (HP1a, HP1b, HP2, HP3, HP4) are antigenically distinct both from the acid-soluble histone-like proteins and from the basic intermediate proteins HPI1, I2, I3 and S1. This lack of cross-reactivity between protamines and the other classes of nuclear basic proteins can be explained by their structural differences (Gaastra *et al.*, 1978; McKay *et al.*, 1985, 1986; Ammer *et al.*, 1986; Gusse *et al.*, 1986). The binding of HPS2 to an immunoserum was only obtained for one of the animals studied. In this case, binding might be related to cross-reactivity with one of the major protamines or to a contamination of antigenic protamines by the HPS2 fraction which displays similar chromatographic characteristics. Complete or partial amino acid sequences of protamines HP2, HP3 and HP4 show strong homologies in their primary structure (McKay *et al.*, 1985; 1986; Ammer *et al.*, 1986; Gusse *et al.*, 1986) whereas HP1 protamines appear structurally distinct. Therefore, although our antiserum contains a mixture of antibodies, we suggest that the major protamines (HP1, HP2, HP3, HP4) probably share antigenic sites. Purification of individual protamine fractions in large amounts would allow selective depletion of immunosera and production of specific antibodies against each protamine.

Staining was satisfactory with the indirect immunoperoxidase technique on paraffin-wax sections after fixation in aqueous Bouin. This fixative, which is usually used in immunoperoxidase methods (Baker & Jaffe, 1975), contains picric acid which is known to precipitate small nuclear basic proteins (Bloch & Hew, 1960; Bols *et al.*, 1986).

Immunocytochemical observations proved that the immunosera against protamines from ejaculated human spermatozoa reacted with nuclear proteins of elongated spermatids in human testis. Resolution by light microscopy is insufficient to identify accurately intermediate spermatids with elongating nuclei which appeared to be weakly stained. The precise steps of spermiogenesis at which antisera react will only be detected by immunocytochemical staining at the ultrastructural level. The increase of nuclear labelling at the late steps of spermiogenesis indicates that chromatin in spermatids is enriched in protamines, as has been shown by previous biochemical (Geremia *et al.*, 1976; Loir & Lanneau, 1978; Grimes, 1986) and autoradiographic (Monesi, 1964, 1967; Mayer & Zirkin, 1979) studies in several mammals. These observations are in agreement with ultrastructural and cytochemical data suggesting that, in man, protamines first appear in condensed nuclei of spermatids at steps 5–6 (Dadoue & Alfonsi, 1986). The lack of cytoplasmic labelling reinforces the hypothesis that protamines are not accumulated inside cytoplasm before their migration to the cell nucleus (Loir & Lanneau, 1978).

The increase of immunoreactivity appeared to be concomitant with chromatin condensation in maturing spermatids. In contrast, nuclei of ejaculated spermatozoa from untreated semen smears were not labelled, although the antigenic material was derived from sperm cells. It is well known that a number of disulphide bridges are formed between cysteine-rich nuclear proteins during epididymal transit, ensuring a stronger stability of DNA–protein complexes (Bedford *et al.*, 1973). Consequently, intranuclear sites able to bind specific nuclear antibodies only become accessible by denaturation of nucleoproteins and chromatin decondensation, as has been shown previously (Samuel *et al.*, 1978; Samuel, 1978; Rodman *et al.*, 1979, 1982, 1984).

Our results show that specific antibodies against human sperm protamines can be used to demonstrate that the nuclear basic protein transition occurs during late spermiogenesis. The next step of the investigation will be purification of antibodies against individual protamine fractions to obtain more information on the timing of protamine deposition, screening human testis c-DNA libraries and examination of nuclear protein anomalies.

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