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Original article

Immunoelectron microscopic visualization of intermediate basic proteins HPI_1 and HPI_2 in human spermatids and spermatozoa

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Abstract – In humans, intermediate basic proteins HPI_1 and HPI_2 are considered as common precursors of the P_2 protamine family, according to data provided by structural studies of these proteins. The occurrence and fate of proteins HPI_1 and HPI_2 were investigated in nuclei of human spermatids and spermatozoa by means of immunoelectron microscopy. A specific polyclonal antibody against a synthetic peptide overlapping the N-terminus of HPI_1 and HPI_2 was prepared and used to detect these proteins on sections of testis and ejaculated sperm. A quantitative analysis of labelling density was performed on micrographs using an interactive image analysis system. The first signs of labelling of intermediate basic proteins appeared in spermatid nuclei at steps 4–5 of spermiogenesis, i.e. during the chromatin condensation process. The nuclear labelling density strongly increased in elongating spermatids (steps 5 and 6) and then sharply decreased from step 6 to step 8 of spermiogenesis. However, weak labelling persisted in the nuclei of mature spermatids and ejaculated spermatozoa. The present results show that the intermediate basic proteins HPI_1 and HPI_2 are synthesized in large amounts in human spermatids during elongation phase and disappear almost totally in mature spermatids when deposition of protamines is completed in condensed nuclei. © Inra/Elsevier, Paris

intermediate basic proteins / protamines / male gamete / spermiogenesis / immunocytochemistry

Résumé – Mise en évidence par immunocytochimie ultrastructurale des protéines basiques intermédiaires HPI_1 et HPI_2 dans les spermatides et les spermatozoïdes humains. Chez l'homme, les protéines basiques intermédiaires HPI_1 et HPI_2 sont considérées comme les précurseurs communs des protamines P_2, selon les données fournies par les études structurales de ces protéines. La mise en place et le devenir des protéines HPI_1 et HPI_2 ont été étudiées dans les noyaux des sperma-

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tides et des spermatozoïdes humains par immunocytochimie ultrastructurale. Un anticorps polyclonal spécifique dirigé contre un peptide de synthèse recouvrant le domaine N-terminal des protéines HPI_1 et HPI_2 a été préparé et utilisé pour détecter ces protéines sur des coupes de testicule et de spermatozoïdes éjaculés. Une analyse quantitative de la densité du marquage a été effectuée sur les micrographies à l'aide d'un système d'analyse d'image interactif. Le marquage des protéines intermédiaires basiques se manifeste dans les noyaux des spermatides aux stades 4–5 de la spermiogenèse, durant la condensation de la chromatine. La densité du marquage nucléaire augmente fortement dans les spermatides en élongation (stades 5 et 6) et décroît ensuite brusquement du stade 6 au stade 8 de la spermiogenèse. Cependant, un faible marquage persiste dans les noyaux des spermatides matures et des spermatozoïdes éjaculés. Ces résultats montrent que les protéines basiques intermédiaires HPI_1 et HPI_2 sont synthétisées en grande quantité dans les spermatides humaines durant la phase d'élongation et disparaissent presque totalement dans les spermatides matures, au terme de la mise en place des protamines dans les noyaux condensés. © Inra/Elsevier, Paris

protéines basiques intermédiaires / protamines / gamète mâle / spermiogenèse / immunocytochimie

1. INTRODUCTION

In mammals, the spermatid nucleus is completely reorganized during mid-spermiogenesis. Concomitantly with structural changes in the spermatid chromatin, histones are replaced by protamines [21]. In man, histone-to-protamine replacement occurs at the beginning of the spermatid maturation phase [22]. Human sperm nuclei contain two families of protamines, P_1 and P_2, which differ in their molecular mass, amino acid composition and sequence. The P_1 family is represented by HP_1 (50 residues) with different degrees of phosphorylation, and is related to other mammalian P_1 protamines. Protamines HP_2 (57 residues), HP_3 (54 residues) and HP_4 (58 residues) belong to the P_2 family. All three proteins contain high amounts of arginine, cysteine and histidine, and differ only in their N-terminal sequence [3, 4, 10, 13, 20]. Protamines P_1 and P_2 appear in human spermatid nuclei at steps 4–5 of spermiogenesis. [17, 18, 22]. However, it can be inferred from biochemical findings in the mouse [6, 9, 12, 33] and the description of genomic sequences of both protamine genes in man [10, 11] that protamine P_1 is synthesized as a mature protein, whereas protamine P_2 derives from a precursor form.

Four basic proteins of intermediate size between histones and protamines (HPI_1, HPI_2, HPS_1, HPS_2) are also present in low amounts in testis and human sperm nuclei, and they are immunologically related to P_2 protamines [23]. Intermediate basic proteins the sequences of which are now known share a common C-terminal domain identical to the amino acid sequence of protamines HP_2, HP_3 and HP_4 [1, 19, 25]. From these structural studies, it can be assumed that protein HPI_1, the largest of the intermediate basic proteins (101 residues), may represent a common precursor of human sperm P_2 protamines. However, the localization and expression pattern of putative P_2 family precursors during spermiogenesis have not been described.

The aim of the present work was to investigate the occurrence of intermediate basic proteins HPI_1 and HPI_2 during human spermiogenesis and to follow their fine structural distribution in spermatids and spermatozoa by means of a specific purified peptide antibody.

2. MATERIALS AND METHODS

2.1. Peptide synthesis and purification

A cysteinyl peptide (HD3) was synthesized. Its amino acid sequence corresponded to the

sequence of 15 residues located at the N-terminal domain of the human sperm intermediate protein HPI_1 (sequence 17–31). A cysteine residue was added at the C-terminus of the peptide sequence for coupling to a carrier protein (*figure 1*).

The 16 amino acid HD3 peptide was prepared according to standard Fmoc procedures on an automatic solid-phase synthesizer and cleaved from the solid support by trifluoroacetic acid treatment. The crude peptide was purified by high performance liquid chromatography. Peptide purity was estimated at higher than 90 % by analytical HPLC and by amino acid analysis.

2.2. Peptide-carrier conjugation

The carrier was keyhole limpet hemocyanin (KLH: Pierce product, Ref.: 77100) because of its large molecular mass (MW: 6.5×10^6). A heterobifunctional cross-linker, (sulfo-MBS: m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, Pierce product, Ref.: 22312) was chosen to react toward carrier primary amines and peptide sulfhydryls. The carrier was first activated by conjugating to the active ester end of the cross-linker sulfo-MBS via lysine amino groups (molecular ratio: linker/carrier = 4 000, reaction buffer: potassium phosphate 20 mM pH: 7.0, total reaction volume: 1.5 mL, reaction time: 30 min at room temperature with constant stirring). This activated carrier was then isolated by gel filtration on a PD1 pre-packed column (Pharmacia Biotech, Ref.: 17-0851-01) to remove excess reagents and possessed, via cross-linker molecules, a large number of reactive maleimide groups on its surface (equilibration and elution buffer: sodium, phosphate 50 mM/NaCl 0.15 M pH: 6.0). The reaction product was an opalescent solution (2 mL). The HD3 peptide, which was synthesized with a C-terminal cysteinyl residue, had a sulfhydryl group that provided a highly specific conjugation site for reacting with the cross-linker sulfo-MBS maleimide group to form a stable thio-ether bond (molecular ratio: peptide/activated carrier = 2 000, peptide solubilization and reaction buffer: sodium phosphate 100 mM/NaCl 0.15 M, pH: 7.5, total reaction volume: 1.5 mL, reaction time: 3 h at room temperature with constant stirring).

This peptide/carrier conjugate was then purified by gel filtration on a PD10 column (equilibration and elution buffer: sodium phosphate 100 mM/NaCl 0.15 M pH: 7.5) The conjugate product was always an opalescent solution (2 mL). After hydrolysis of 100 µL of conjugate solution with 6N hydrochloride acid (1 h,

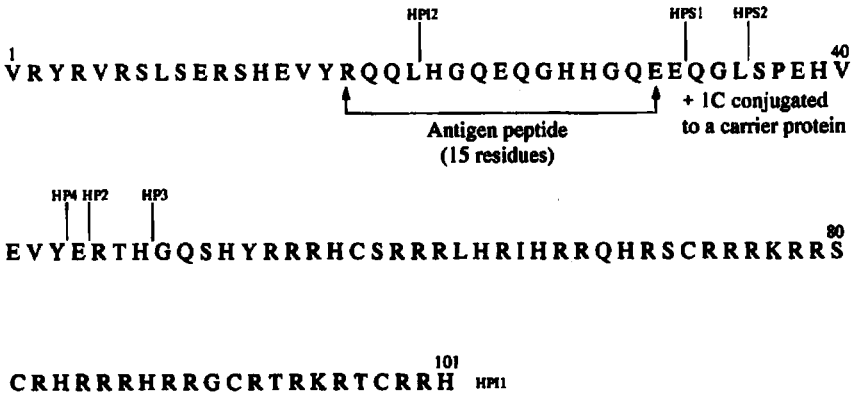


Figure 1. Structural relationships between the three human protamines HP_4 (58 residues), HP_2 (57 residues) and HP_3 (54 residues) and the intermediate basic proteins HPI_1, HPI_2, HPS_1 and HPS_2. The amino-sequence of HPI_2 (81 residues) is identical to the sequence of the C-terminal region of the intermediate basic protein HPI_1 (101 residues) which may be considered as a precursor of three other intermediate basic proteins HPI_2, HPS_1 and HPS_2 as well as of human sperm protamines HP_2, HP_3 and HP_4 [1, 4, 19]. The 15 amino acids synthetic peptide (HD_3) overlaps the N-terminal domain of HPI_1 and HPI_2.

150 °C), amino acid analysis was performed to estimate the number of haptens coupled to the carrier prior to immunization [26]. A coupling molecular ratio was calculated at 1 800.

2.3. Immunizations and immunoassays

Two male rabbits aged 3–5 months were immunized by intradermal injection of 150 µg equivalent peptide in complete Freund's adjuvant on day 0. Boots (intradermal injections) were performed with 25 µg equivalent peptide in incomplete Freund's adjuvant on days 21, 42 and 63. Bleedings were performed 7 days after each injection. Sera were tested at various dilutions in immunoassays (ELISA procedure) to test their capacity to react with the HD3-peptide.

2.4. Electrophoresis and immunoblotting

The specificity of the antibody was assessed by western blotting of basic nuclear proteins extracted from human sperm nuclei as reported previously [13]. Total basic nuclear proteins were separated on 17 % acid-urea polyacrylamide gel electrophoresis. Proteins were transferred onto 0.45 µm nitrocellulose sheets (Schleicher and Schuell) at 2 mA/cm2 for 30 min at room temperature using 1 % acetic acid buffer. The blots were blocked for 2 h at room temperature in tris-buffered saline (TBS), pH 7.2, containing 10 % low fat milk (w/v). The sheets were incubated with the anti-peptide antibody (1:200 dilution) in 0.1 M TBS, pH 7.2, containing 0.1 % Tween 20 (v/v) for 2 h at room temperature followed, after washing, by a peroxidase-conjugated anti-rabbit antibody (ECL, Amersham Inc.) (1:5 00) for 1 h at room temperature. Visualization was carried out using the luminescent substrate of the ECL system (ECL, Amersham Inc.).

2.5. Ultrastructural immunocytochemistry

Human testicular biopsies were obtained from four healthy 20–25-year-old men with proven brain death. Ejaculated spermatozoa were collected from four voluntary donors assessed as normal after spermogram and spermocytogram according to WHO criteria [32]. Pieces of testes and the pellets of ejaculated spermatozoa were fixed by immersion for 1 h in 0.1 M Sorensen buffer, pH 7.4, containing 2 % paraformaldehyde and 0.1 % glutaraldehyde. Some sperm pellets were immersed for 60 min at 37 °C in 0.05 M borate buffer, pH 9.0, containing 1 % SDS and 6 mM EDTA to modify the chromatin structure and then were washed twice in the same buffer before fixation. Samples were dehydrated sequentially in 50, 75 and 90 % dimethylformamide, and then processed for embedding in Lowicryl K4M medium (Polysciences Paris, France) [2]. Ultrathin sections (80 nm) collected on uncoated nickel grids were incubated successively with the anti-HD3 antibody (1:500) for 2 h, followed by a gold-labelled (15 nm particle size) goat anti-rabbit antibody (1:100) (Biocell, Cardiff, UK). As control, some sections of ejaculated spermatozoa were incubated in the same conditions with another monoclonal antibody recognizing the P_2 protamine family (h BNP C_4 p) which was previously obtained in the laboratory [17]. Sections were counterstained with aqueous uranyl acetate. Controls of the immunostaining specificity included incubations with pre-immune serum, increasing dilutions of first and second antibody and omission of first antibody. Ultrathin sections were examined under a JEOL 120 CX electron microscope at 60 kV (Jeol Ltd., Tokyo, Japan).

2.6. Quantitative evaluation of gold particle distribution

Quantitative evaluation was carried out on four tissue blocks per subject. The spermatids were divided into eight steps according to the classification of Holstein and Roosen-Rünge [16]. Three categories of spermatids could be distinguished: 1) young spermatids with round nuclei (steps 1 and 2); 2) intermediate spermatids with elongating nuclei (steps 3–5); and 3) mature spermatids with condensed nuclei (steps 6–8). To avoid bias in subsequent analysis, only nuclei of spermatids exhibiting visible anterio-posterior structures (cap, acrosomal vesicle, mature acrosome sliced longitudinally, neck structures) were selected. Gold particles were recorded over the spermatid nuclei at the different steps of spermiogenesis and the ejaculated sperm nuclei. For each cell type, 50 sections selected at random were counted. The EM micrographs were all taken at standard magnification X 8000. Gold grain counting was performed on negative prints using an interactive image analysis system (Samba, Alcatel, France). The nuclear samples consisted of the number of gold particles per μm^2. Background subtraction was realized taking into consideration adjacent tissue-free resin regions.

2.7. Statistical analysis

Statistical analysis of the data was carried out by one-way analysis of variance (ANOVA) to determine significant labelling between the different steps of spermiogenesis (Macintosh Statview II Program). The Scheffé multiple range test was used to study specific comparisons.

3. RESULTS

The presence of antibodies against HD3 peptide in sera of the two rabbits immunized (7375 and 7376) was shown by the ELISA procedure on the 7th day after the third booster injection (*figure 2*). Only serum 7376 was used for the study. Following acid-urea PAGE of proteins extracted from human sperm nuclei, the immunoblot assays revealed that antiserum bound to proteins corresponding to the molecular weight of intermediate basic proteins HPI_1 and HPI_2. By contrast, neither histones nor protamines showed any immunoreactivity (*figure 3*).

In immunoelectron microscopy, no labelling was found in the nucleus or cytoplasm of young spermatids. The labelling first appeared in nuclei of spermatids at steps 4–5 (*figure 4*), but only a few grains were distributed in these cells. It then increased and was much more intense in step 6 spermatids (*figure 5*). Colloidal gold particles progressively became scarce in mature spermatids with condensed nuclei (steps 6–8) and a few gold particles were still present in the nuclei of mature spermatids (*figure 6*) and ejaculated spermatozoa (*figure 7*). Comparatively, a strong labelling of ejaculated

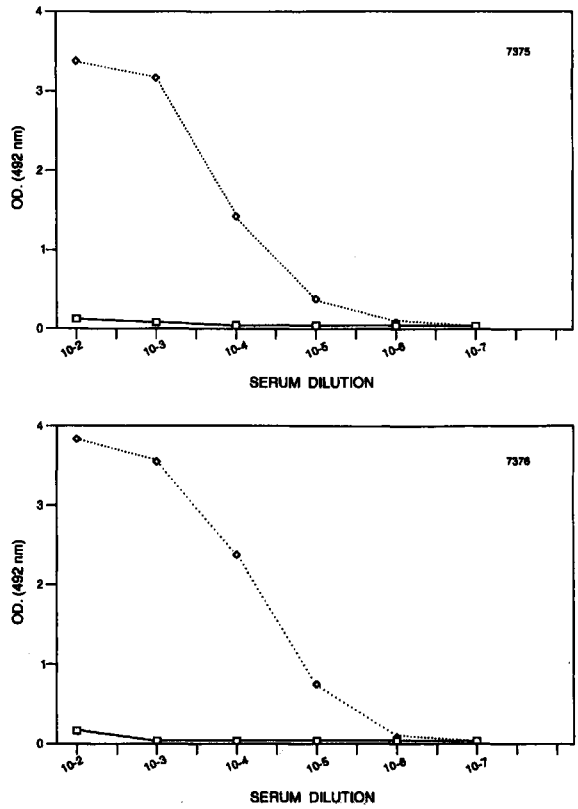


Figure 2. ELISA titration curves of the sera anti-HD_3 peptide from the rabbits 7375 and 7376.

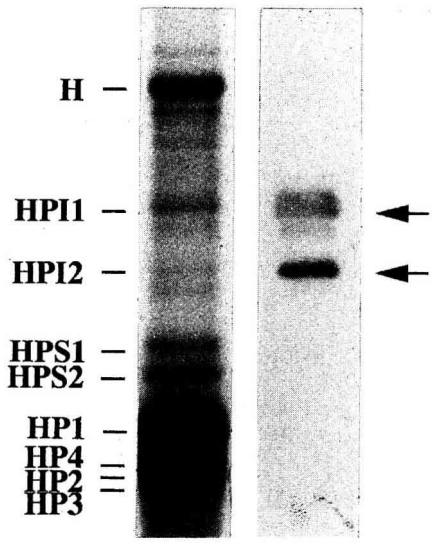


Figure 3. Acid-urea polyacrylamide gel electrophoresis and immunoblotting analysis of basic proteins extracted from human sperm nuclei. Coomassie blue staining (A). After transfer onto a nitrocellulose sheet, only HPI_1 and HPI_2 reacted with the serum anti-HD_3 peptide (B). H = histone; HPI_1, HPI_2, HPS_1, HPS_2 = intermediate basic proteins; HP_1 = protamine P_1 family, $HP_{2,3,4}$ = proteins of the protamine P_2 family.

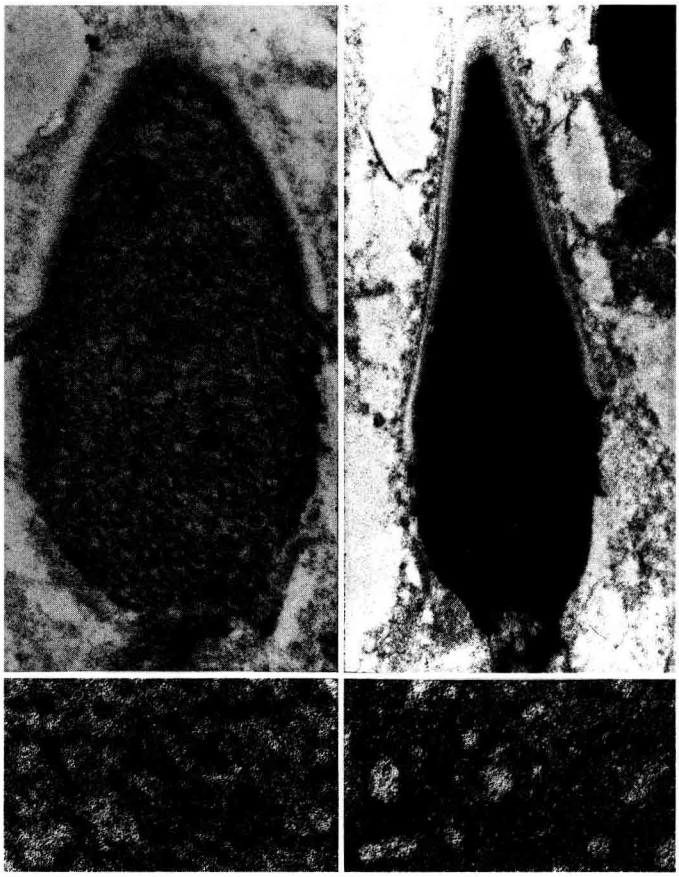
sperm nuclei was obtained with the anti-P_2 protamine family antibody (*figure 8*) while the immunolabelling with the anti-HD3 antibody was not modified after chromatin decondensation by SDS-EDTA treatment (*figure 9*). The nucleus and the cytoplasm of spermatogonia and spermatocytes as well as of testicular somatic cells were all unlabelled. Control grids incubated with preimmune serum or in the absence of the primary antibody did not show any labelling (data not shown).

The pattern of nuclear labelling at the different steps of spermiogenesis is shown in *figure 10*. No significant differences were found between the mean grain counts obtained from each subject. The differences in the number of gold particles per nucleus area were statistically significant between the different steps of spermiogenesis ($P < 0.001$). The nuclear labelling density of proteins HPI_1 and HPI_2 strongly increased in intermediate spermatids (steps 5 and 6) (Scheffé test, $P < 0.01$) and then sharply decreased from stage 6 to stage 8 of spermiogenesis (Scheffé test, $P < 0.01$). However, it remained detectable in nuclei of mature spermatids and ejaculated spermatozoa.

4. DISCUSSION

In mouse, as in man, protamines P_1 and P_2 are encoded by single-copy genes (PRM_1 and PRM_2) closely linked on chromosome 16 [15, 31]. However, mouse protamine P_2 (mP_2; 63 residues) is derived from a precursor form, pmP_2 (106 residues), whereas mouse protamine P_1 is synthesized as a mature protamine [33]. In the later steps of spermiogenesis, the N-terminal part of pmP_2 is processed by limited proteolysis generating a series of polypeptides (pmP2/5, pmP2/11, pmP2/16, pmP2/20, pmP2/26 and pmP2/32) which differ in the length of their N-terminal extension [6, 12]. With the exception of the largest precursors, pmP_2 and pmP2/5, the intermediates of proteolysis generated from pmP2 inside spermatid nuclei persist in mature sperm [9]. P_2 protamines from mouse (mP_2) and man (HP_2, HP_3, HP_4), as well as pmP_2 and the intermediate basic protein HPI_1, share strong structural homology [4, 7]. In humans, a unique start codon (ATG) which is localized 110 nucleotides downstream from the transcription initiation site has been determined from the nucleotide sequence of the P_2 gene [11]. Therefore, all proteins related to human P_2 protamines must arise from HPI_1, which can be considered as their common precursor. The latter seems to be post-translationally processed in several steps (HPI_2, HPS_1, HPS_2, HP_4, HP_2 and HP_3, successively) via proteolytic cleavages at specific sites leading from HPI_1 to protamine HP_3 [1, 4, 19, 24].

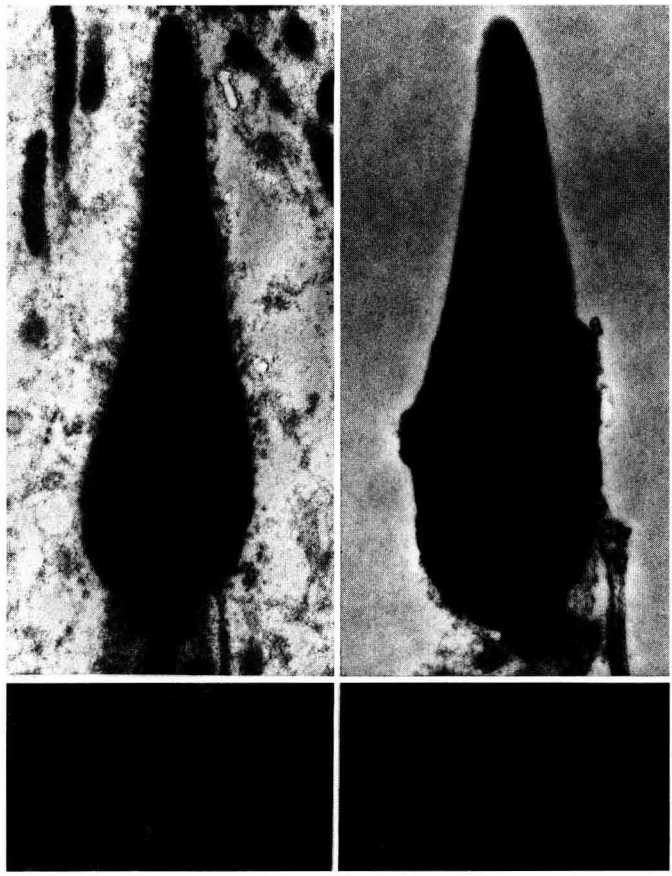
The specificity of the polyclonal anti-HD3 antibody used in this study was asses-



Figures 4 and 5. Anti-HD_3 peptide antibody labelling in an intermediate spermatid at steps 4–5 (**4**) and in a mature spermatid at step 6 of spermiogenesis (**5**). Note the increase in labelling density in mature spermatid nuclei (× 28 000). Inserts show immunolabelling at high magnification (× 50 000).

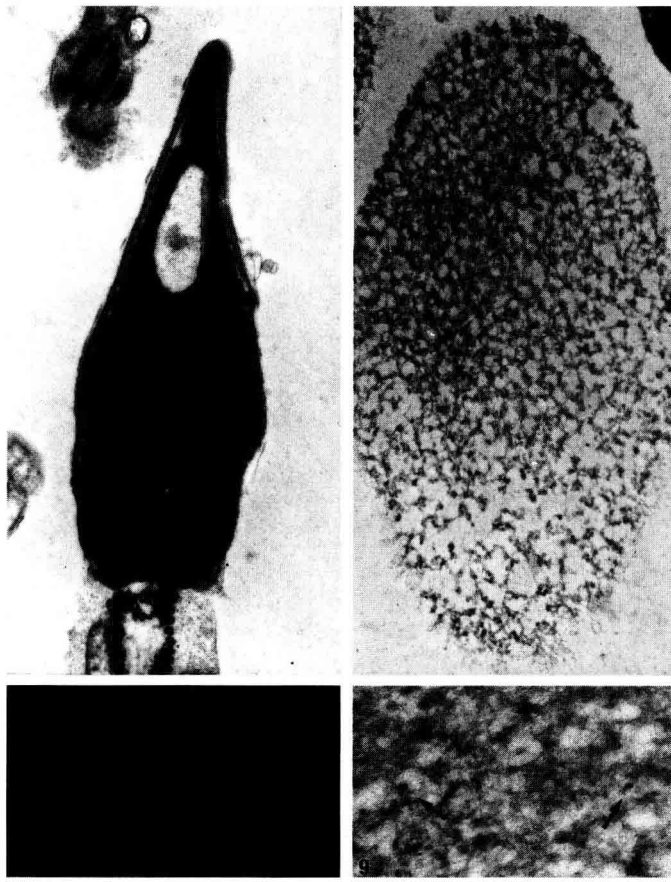
sed in ELISA and western blot: it recognized only the two intermediate basic proteins HPI_1 and HPI_2. Ultrastructural controls showed that the accessibility of chromatin to the antibody was not affected by the compaction state of nuclei. The deposition of proteins HPI_1 and HPI_2 inside human spermatid nuclei appeared to occur at the end of elongation phase (steps 4–5). At that time, as shown by previous quantitative immunocytochemical data [22], the amount of somatic-type histones H_2B and H3 was decreasing, whereas the first signs of labelling of protamines were detectable in the nucleus. It is likely that, in elongating spermatids, histones are displaced by spermatid-specific transition proteins (TP_1 and TP_2), as suggested by prior cytochemical findings [8]. Transition proteins, in turn, are subsequently removed from the condensing chromatin at the end of the spermatid elongation phase and are replaced by protamines (reviewed by Balhorn [5] and Hecht [14]).

From step 5 to step 6 of spermiogenesis, the sharp increase in the nuclear labelling density of HPI_1 and HPI_2 paralleled the labelling which was observed for protamines



Figures 6 and 7. Anti-HD_3 peptide antibody immunolabelling in a mature spermatid at step 8 of spermiogenesis (**6**) and in an ejaculated spermatozoon (**7**). Note the persistence of a few gold particles on the nucleoplasm (× 28 000). Inserts show immunolabelling at high magnification (× 50 000).

localized by means of a specific monoclonal antibody (hBNP I_2 C_3) against both P_1 and P_2 families. However, the labelling of HPI_1 and HPI_2 sharply decreased in step 6 spermatid nuclei, in contrast with the protamine rate that was found to remain high and stable in mature spermatids and ejaculated spermatozoa [22]. We previously showed that monoclonal antibody hBNP I_2 C_3 and another monoclonal antibody recognizing the P_2 protamine family (hBNP C_4 p) gave the same results in immunoelectron microscopy [17]. This finding was not surprising, since antigenic sites common to the different protamines had been detected by polyclonal antisera against purified protamine HP_1b [23] and by monoclonal antibodies [27]. Elsewhere, on western blots of human intermediate basic proteins separated by acid-urea gel electrophoresis, both hBNP I_2 C_3 and hBNP C_4p cross-reacted with the four proteins HPI_1, HPI_2, HPS_1 and HPS_2 [17]. Therefore, the present results, together with preceding data on immunolabelling of protamines, are in good agreement with structural studies showing that the intermediate



Figures 8 and 9. Anti-P_2 protamine family antibody immunolabelling in an ejaculated spermatozoon (**8**). Anti-HD_3 peptide antibody immunolabelling in an ejaculated spermatozoon after chromatin decondensation by SDS-EDTA treatment (**9**) (× 28 000). Inserts show immunolabelling at high magnification (× 50 000).

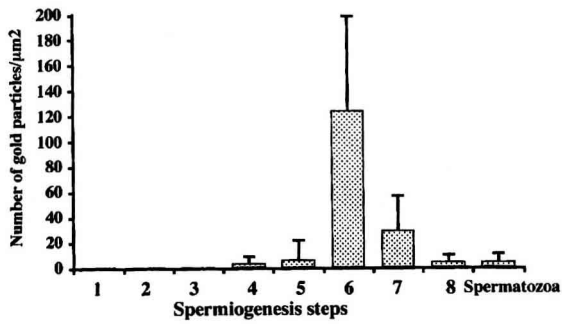


Figure 10. Pattern of nuclear labelling density for proteins HPI_1 and HPI_2 in spermatids at different steps of spermiogenesis and in ejaculated spermatozoa. Each value represents the mean number (± S.D.) of gold particles counted in 50 cells from four different men and expressed as the number of particles per μm^2.

basic proteins HPI_1 and HPI_2 represent precursors (pro-protamines) of the P_2 protamine family.

The lack of cytoplasmic labelling suggests that intermediate proteins do not accumulate inside the spermatid cytoplasm. This observation reinforces the hypothesis that the proteases implicated in the maturation of pro-protamines are closely associated with nuclei [9].

The persistence of low amounts of proteins HPI_1 and HPI_2 in human sperm nuclei, as revealed by immunoelectron microscopy, is in accordance with earlier biochemical data indicating that the four basic proteins of intermediate size between histones and protamines (HPI_1, HPI_2, HPS_1 and HPS_2) represent about 10 % of the amount of basic proteins isolated from sperm nuclei [13, 28–30].

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