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Immunoexpression of testis-specific histone 2B in human spermatozoa and testis tissue

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During mammalian spermatogenesis, the chromatin of the spermatogenic cells is profoundly reorganized. Somatic histones are partly replaced by testis-specific histones. These histones are then replaced by transition proteins and finally by protamines. This series of nucleoprotein rearrangements results in a highly condensed sperm cell nucleus. In contrast to spermatozoa from other species, human spermatozoa still contain a significant amount of histones, including testis-specific histone 2B (TH2B). In the present study it is shown that an antibody targeting tyrosine hydroxylase, which has been found previously to cross-react with rat TH2B, also specifically immunoreacts with human TH2B on Western blots, in immunohistochemistry of human testis tissue, and in immunocytochemistry of decondensed human spermatozoa. In human testis tissue, TH2B immunostaining first apparent in spermatogonia, shows marked variation, especially at the pachytene spermatocyte stage, and then reaches an intense signal in round spermatids. Shortly before spermatid elongation, a portion of the spermatid nucleus, corresponding to the acrosomal region, loses its immunoreactivity. During condensation of the spermatid nucleus, the immunodetectability of TH2B disappears gradually, from the anterior region of the nucleus onwards. At the final stages of spermiogenesis, the immunostaining is completely absent. Immunocytochemical staining of spermatozoa revealed no TH2B immunosignal, but immunostaining was observed when spermatozoa obtained from semen were decondensed to make nuclear proteins accessible to the antibody. There was, however, a striking intercellular variability in the intensity of staining of spermatozoa within an ejaculate. In a population of 35 men attending our Andrology Clinic, we observed inter-individual differences in total sperm TH2B content, which showed a significant, although not very pronounced, negative correlation with normal morphology ($P = 0.05$).

Key words: histones/immunohistology/spermatozoa/testis/TH2B

Introduction

Spermatogenesis is a complex process in which diploid stem cells divide, differentiate and mature into haploid, highly specialized spermatozoa. Among the many changes, one of the intriguing developmental characteristics of spermatogenic cells is the replacement of somatic histones, first partly by testis-specific histones and then by transition proteins which are finally replaced by protamines (Meistrich *et al.*, 1978; Balhorn, 1982, 1989; Meistrich and Brock, 1987). This results in a compact nucleoprotamine complex, which is highly distinct from the somatic nucleohistone complex, with inter- and intra-protamine disulfide bridges resulting in a highly compact and stable supramolecular structure that fits into a small sperm nucleus and also may render the chromatin relatively resistant to harmful external influences (Marushige and Marushige, 1975; Hunter *et al.*, 1976; Ward, 1993). In rat and mouse spermatogenesis, the histones are completely replaced by protamines, so that mature spermatozoa are devoid of histones (Grimes, 1986). In human spermatozoa, however, about 15% of the basic nuclear proteins are histones (Tanphaichitr *et al.*, 1978).

It is as yet unclear whether the remaining histones in human spermatozoa have a specific role. It has been suggested that the sperm DNA that is complexed as nucleohistone may have a role in the designation of initiation sites for nuclear decondensation, or that the nucleohistone complement may contain genes poised for early expression after fertilization (Gatewood *et al.*, 1987). In contrast to this possibly beneficial effect of sperm nuclear histones, a number of studies have presented evidence that spermatozoa from infertile males contain more histones than those of fertile males, indicating that insufficient removal of histones might be involved in infertility (Chevaillier *et al.*, 1987; Hofmann and Hilscher, 1991; Foresta *et al.*, 1992). Among the histones present in human spermatozoa, testis-specific histone 2B (TH2B) is a major variant (Tanphaichtitr *et al.*, 1978).

Recently, an antibody targeting tyrosine hydroxylase (TH) was found to cross-react with TH2B (Unni *et al.*, 1995). Since the primary structure of TH2B is highly homologous between man and rat (Wattanaseree and Svasti, 1983), we evaluated whether the anti-TH antibody would also specifically bind to human TH2B. After having established that this was indeed the case, we characterized the immunoexpression pattern of TH2B in human testis tissue and in human spermatozoa, and investigated the TH2B content of spermatozoa in a number of males visiting our infertility clinic.

Materials and methods

Preparation of acid-extractable proteins

Testes and epididymides from adult Wistar rats were used. After homogenization of rat testis tissue, the homogenate was centrifuged at 10 000 *g* for 10 min. The pellet was stored at –25°C. Rat spermatozoa were obtained from caput and cauda epididymides. Epididymal tissue was minced and shaken in phosphate-buffered saline (PBS) to permit dispersal of luminal contents. After sedimentation of the tissue pieces, the supernatant was layered over a discontinuous Percoll gradient consisting of 10%, 40% and 65% Percoll; Pharmacia, Uppsala, Sweden (v/v in PBS). The gradient was spun at 500 *g* for 45 min. Spermatozoa were recovered at the interphase between the 40% and 65% Percoll.

Human semen was layered on top of 40% Percoll and centrifuged at 500 *g* for 20 min. Spermatozoa were collected from the bottom of the tube, washed and stored at –25°C.

Human peripheral blood mononuclear cells were isolated from heparinized blood of a healthy adult volunteer. A leukocyte-enriched cell suspension was obtained by lysis and removal of red blood cells. Ten ml of whole blood was added to 30 ml lysis buffer (containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA, at pH 7.4) at 4°C, and was shaken vigorously. The mixture was left on ice for 15 min, during which time it was periodically shaken. The non-lysed cells were pelleted by centrifugation at 3000 *g* at 4°C. The supernatant was removed and the pellet was resuspended in 10 ml lysis buffer at 4°C. After vigorous shaking, the remaining cells were pelleted by centrifugation at 3000 *g* and the supernatant was removed. The pellet was frozen in liquid nitrogen for storage until use.

The basic nuclear proteins of spermatozoa and white blood cells were extracted as described previously (Platz *et al.*, 1977). In short, the sample was suspended in 1 ml of cold distilled water. Subsequently 0.33 ml of 1 N HCl was added, followed by 1 ml of 0.25 N HCl. The sample was left to stand on ice for 20 min, during which time the mixture was shaken frequently. The suspension was centrifuged at 12 000 *g* for 10 min. The supernatant was removed and proteins were precipitated by adding an equal volume of trichloroacetic acid. The precipitate was recovered by centrifugation (12 000 *g* for 10 min) and washed with acidified acetone (200 ml acetone + 0.1 ml 12 N HCl). After subsequent washing in acetone, the sample was dried under vacuum.

Preparation of human sperm proteins

Semen samples were obtained from patients attending the Andrology Outpatient Clinic of the University Hospital Dijkzigt, Rotterdam. Thirty-five consecutive semen samples, with more than 1×10^6 sperm/ml and a volume of at least 1 ml, were included. All semen samples were first routinely analysed using the World Health Organization methodology (WHO, 1992). Subsequently, 1 to 2 ml of each sample were layered on top of 2 ml 60% Percoll (v/v in PBS) and centrifuged at 500 *g* for 20 min. The pellet was washed twice in 1 ml PBS. The final pellet was resuspended in 1 ml PBS and the sperm concentration was determined in an improved Neubauer counting chamber. A portion containing 0.5×10^6 spermatozoa was centrifuged in a microfuge (10 000 *g*) for 10 min, and the pellet was stored at –80°C until analysis.

Since the basic nuclear proteins could be efficiently extracted by the Laemmli buffer without previous acid extraction (data not shown), the acid extraction step was omitted. The sperm pellets were suspended in 25 µl double strength Laemmli electrophoresis lysis buffer (Laemmli, 1970), containing 0.125 M Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS) (w/v), 20% glycerol (v/v), and 130 mM dithiothreitol (DTT). From one semen sample with a high sperm concentration (170×10^6 spermatozoa/ml), pellets of 0.5×10^6 Percoll-purified spermatozoa were stored, and used as a standard by running one sample per gel alongside the other semen samples.

Each sample was boiled for 3 min in the sample buffer, and was run on SDS–PAGE (Laemmli, 1970) using 15% polyacrylamide gels. The separated proteins were transferred to a nitrocellulose membrane and immunostained as described below. The intensity of the signal was quantified with a Hewlett Packard ScanJet IIcx and analysed using DeskScan II version 2.0 for Windows software. The results obtained for the actual measurements were all within the linear portion of our standard dose–response curve.

Sperm decondensation

Semen samples used for immunocytochemistry were also obtained from the Andrology Outpatient Clinic. After routine analysis, spermatozoa present in 1 ml of semen were washed twice in 4 ml Ham's medium, after which the remaining pellet was resuspended in PBS to obtain a concentration of $50–100\times10^6$ spermatozoa per ml. Smears of spermatozoa were fixed in methanol for 5 min and subsequently incubated for 20 min in Tris–HCl pH 7.2, containing 0.3 mM DTT and 0.1% SDS. The smears were washed in Tris–HCl and fixed again with methanol for 5 min.

Testis biopsy samples

Samples were obtained from normogonadotrophic patients undergoing testis biopsy for evaluation of azoospermia. The tissue was immediately immersed in Bouin's fixative and left for 24 h, after which the samples were thoroughly washed in 70% ethanol. The sections were dehydrated in ethanol, cleared in xylene and embedded in paraffin, and stored at room temperature.

Electrophoresis and Western blotting

The anti-TH antibody (mouse monoclonal anti-tyrosine hydroxylase immunoglobulin G; Boehringer GmbH, Mannheim, Germany) is known to specifically cross-react with rat TH2B, because of sequence homology at the N-termini of TH and rat TH2B (Unni *et al.*, 1995). In order to assess whether anti-TH antibody could recognize human TH2B, we performed two types of gel electrophoresis. First, basic nuclear proteins of human leukocytes, human spermatozoa, rat testis, and rat caput and cauda epididymal spermatozoa were extracted by boiling for 3 min in double strength Laemmli buffer. The samples were run on a 15% polyacrylamide SDS gel (Laemmli, 1970). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Non-specific antibody binding was blocked by incubating the membrane in 5% (w/v) bovine serum albumin (BSA; Sigma, St. Louis, USA) in PBS–Tween 0.1% (v/v) for 1 h. After washing four times in PBS–Tween, the membrane was incubated with the primary antibody (anti-TH) diluted 1:1600 in 5% BSA (w/v) in PBS–Tween 0.1% (v/v) for 1 h. The membrane was then again washed four times in PBS–Tween, and subsequently incubated with biotinylated goat anti-mouse antibodies (Dako, Glostrup, Denmark) diluted 1:2000 in 5% BSA (w/v) in PBS–Tween 0.1% (v/v) for 1 h. The membrane was again washed four times in PBS–Tween and incubated for 1 min with chemiluminescent reagent (Pierce, Rockford, IL, USA). Excess reagent was removed with blotting paper. The specifically bound antibody was visualized using Hyperfilm™ β-max paper (Amersham, CEA AB, Sweden).

The above samples were also run on a 15% polyacrylamide acid–urea–Triton (AUT) gel to provide further evidence that the observed immunoreactive band was indeed a histone (Wattanaseree and Svasti, 1983). Samples were dissolved in 0.4% (v/v) Triton X-100, 1% (v/v) mercaptoethanol and 0.9 M acetic acid. The AUT gels contained 0.4% (v/v) Triton X-100, 1.5 M urea and 0.9 M acetic acid, while the electrode buffer contained 0.4% (v/v) Triton X-100, 0.1% (v/v)

mercaptoethanol and 0.9 M acetic acid. Gels were pre-run for 4 h at 130 V, until a consistent current was obtained, and run for 30 h. The separated proteins were transferred on a nitrocellulose membrane in 50 mM acetic acid. Immunodetection of TH2B was performed as described above.

The densitometry was standardized by comparing the TH2B content of patient samples with that of a standard sample. The standard samples were made by dividing one sperm sample with a high sperm number into a number of aliquots with 0.5×10^6 spermatozoa. Each time patient samples were run, at least one standard sample was run alongside. The density measurements of the patient samples were divided by the density measurement of the standard sample. This resulted in a arbitrary unit of 1, if the measurements of a patient sample and a standard sample were the same. The reliability of the method was demonstrated on control blots by a linear dose–response relationship, using samples with increasing numbers of spermatozoa.

Immunohistochemistry and immunocytochemistry

Immunolocalization of TH2B was performed using the mouse anti-TH antibody. Sections of 5 μm were mounted on slides treated with 3-aminopropyltriethoxysilane (Sigma Chemical Company, St Louis, MO, USA), deparaffinized and rehydrated. Endogenous peroxidase was blocked by a 20 min incubation in 3% (v/v) H_2O_2 in methanol. Non-specific antibody binding was blocked with normal goat serum (Dako), diluted 1:10 in 5% (w/v) BSA in PBS (pH 7.4). The tissues were then placed in a Sequenza immunostainer (Shandon Scientific Ltd., Runcorn, UK) and incubated at 4°C overnight with the primary antibody (anti-TH), diluted 1:100 in 5% (w/v) BSA in PBS. Immunostaining was performed using biotinylated goat anti-mouse immunoglobulin (Dako) for 30 min, streptavidin–peroxidase (Dako) for 30 min, and metal-enhanced diaminobenzidine substrate kit (Pierce, Rockford, IL, USA) for 7 min. The sections were counterstained for 15 s with Mayer's haematoxylin, and viewed with a Zeiss Axioscop 20 light microscope at magnifications of 100× and 400×. Control sections were incubated with 5% BSA (w/v) in PBS without the primary antibody and subsequently processed as described above.

Immunocytochemistry was performed as described above, excluding the deparaffinization step.

Statistics

All statistical calculations were performed using Statistical Package for Social Studies (SPSS Inc., Chicago, USA) software (version 6.0 for Windows). The relationship between semen parameters and sperm histone content was calculated according to Spearman's rank correlation.

Results

Identification of TH2B in human spermatozoa

In order to determine if anti-TH could be used to identify human TH2B, acid-extractable proteins from human spermatozoa, human white blood cells, rat testis tissue and rat epididymal sperm were separated by SDS–PAGE, blotted and immunostained with anti-TH. As can be seen in Figure 1a, an immunoreactive protein of 17 kDa was present in human spermatozoa (lane B) but not in human leukocytes (lane A). The other signal in the lane corresponding to the human leukocytes, was non-specific (see figure legend). In Figure 1b, it is apparent that the anti-TH antibody reacted specifically with a 17-kDa protein that was present in rat testis tissue (lane A) but that was not found in rat caput and cauda epididymal spermatozoa (lanes B and C). Lane D corresponds to human spermatozoa, which produced a signal of electrophoretic mobility identical to that of rat testis tissue. Acid-extractable proteins of rat testis, human spermatozoa and human leukocytes were further analysed by AUT electrophoresis followed by immunoblotting. It appeared that the anti-TH antibody recognized a protein band co-migrating with an H2B marker (standard samples of H2A and H2B were obtained from Boehringer Mannheim) in the extracts from human spermatozoa and rat testis, while no leukocyte protein was recognized (Figure 2). The TH-reactive band in rat testis extract migrated slightly slower than the TH-reactive band in human sperm extract.

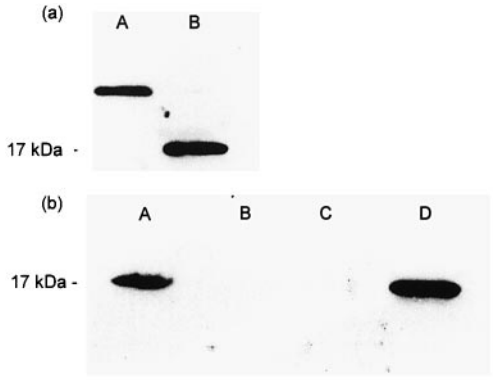

Figure 1. Immunodetection of proteins, using an antibody targeting tyrosine hydroxylase (TH) on SDS–PAGE and Western blot. (**a**) shows a clear 17-kDa band in lane (B) corresponding to human spermatozoa. This band is not present in lane (A) corresponding to human leukocytes, which are known to contain somatic type H2B but not TH2B. In lane (A) a non-specific band appears, which is also seen in negative controls where no anti-TH is added, indicating non-specific binding of the second antibody (biotinylated goat anti-mouse immunoglobulin G) to a human leukocyte protein (not shown). (**b**) shows a clear 17 kDa band in lanes (A) and (D) corresponding to rat testis tissue and human spermatozoa, respectively. Lanes (B) and (C), corresponding to rat caput and caudal epididymal spermatozoa, respectively, do not show a detectable immunoreactive signal.

Immunohistochemical localization of TH2B in the human testis

Immunohistochemical studies using anti-TH antibodies on human testis tissue displaying normal spermatogenesis demonstrated strong immunoreactivity in the nuclei of spermatogenic cells (Figure 3). The nuclei of the somatic cells of the testis (Sertoli cells, peritubular myoid cells, interstitial cells, endothelial cells) remained invariably negative. A clear signal was apparent in the spermatogonia lining the basement membrane of the seminiferous tubules. Primary spermatocytes, especially the pachytene spermatocytes, showed a marked variation in nuclear immunodetectability of TH2B (Figure 3D). The early round spermatids displayed a uniformly intense immunosignal. During early spermiogenesis, a region corresponding to the acrosomal cap of the round spermatid nucleus displayed reduced TH2B immunostaining as compared with that of the rest of the nucleus (Figure 3B). During spermatid

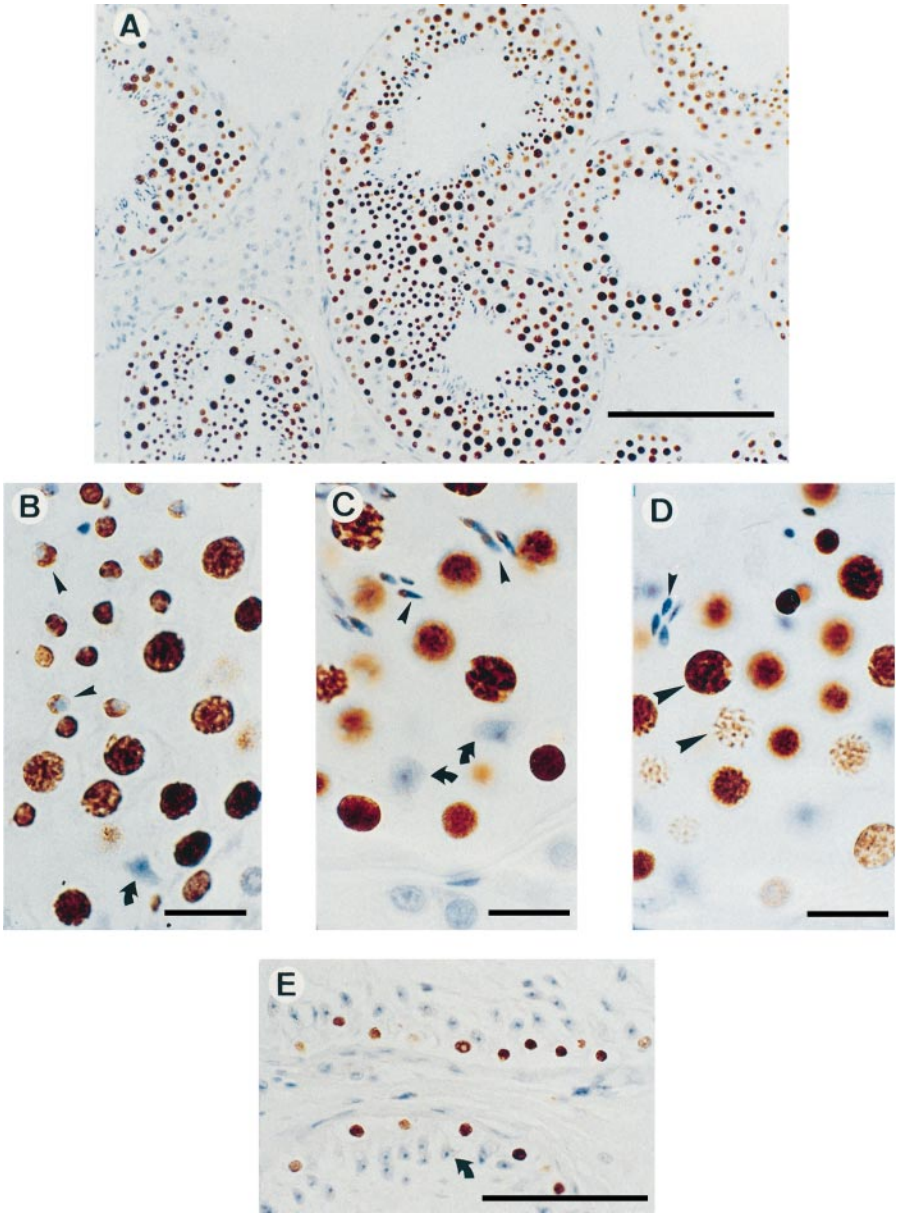

Figure 3.

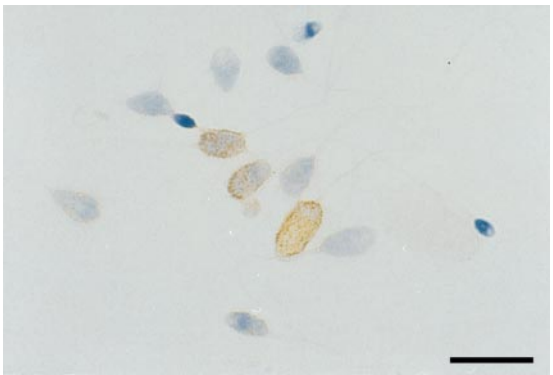

Figure 4.

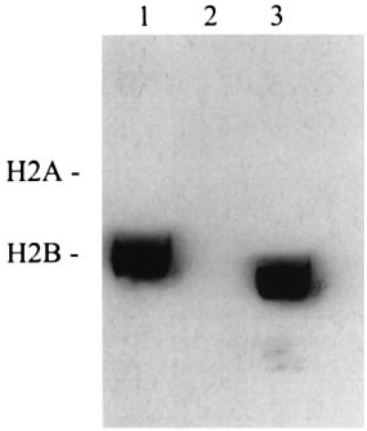

Figure 2. Detection of proteins that immunoreact with antibodies targeting tyrosine hydroxylase (TH), after acid–urea gel electrophoresis and Western blotting. The standard calf thymus H2A and H2B were obtained from Boehringer Mannheim. Aliquots of 3 μg of standard H2A and H2B were loaded on to the polyacrylamide acid–urea–Triton gels and run alongside the samples containing human spermatozoa, human leukocytes and rat testis tissue. A clear band representing TH2B is seen in lanes 1 and 3 corresponding to rat testis tissue and human spermatozoa, respectively, but is absent in lane 2 corresponding to human leukocytes. Both immunoreactive bands co-migrate with the standard H2B, although the immunoreactive rat testis protein migrates slightly slower that the immunoreactive human sperm protein, indicating that the observed protein is indeed a histone of the 2B type.

elongation, this region of reduced immunostaining increased, concomitant with expansion of the acrosome. In the final stages of spermatid nuclear condensation, a small region of immunodetectable TH2B could be discerned in the posterior region of the spermatid nucleus (Figure 3C), after which TH2B became immunologically completely undetectable (Figure 3D).

In human testis tissue from an infertile male, where only Sertoli cells and spermatogonia were present in the seminiferous tubules, again the nuclei of spermatogonia, but not Sertoli cell nuclei, stained positive (Figure 3E). All other somatic cell nuclei did not immunoreact.

Immunocytochemical localization of TH2B in human spermatozoa

Immunostaining of TH2B was absent in all morphologically normal and in most abnormal spermatozoa in human semen

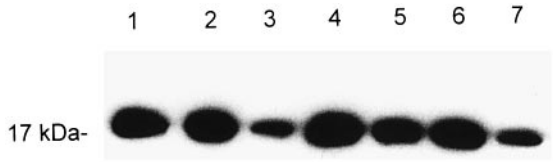

Figure 5. Immunodetection of TH2B in spermatozoa from human infertility patients. Protein was extracted from 0.5×10^6 spermatozoa from seven different patients attending our Andrology Clinic, separated on SDS–PAGE and transferred on a nitrocellulose membrane. Note the diversity of signal intensity between different lanes. The gel shown in this figure was exposed slightly longer than normal in order to highlight the patient-to-patient variation in sperm TH2B content. A less exposed version was used for the actual measurements (Figure 6) but was less suitable for photography.

samples. However, some of the abnormal spermatozoa and most of the round cells stained intensely (results not shown). When the spermatozoa were subjected to decondensation treatment, a larger number of spermatozoa stained positive (Figure 4). However, the spermatozoa that remained in the condensed form remained negative. Among the spermatozoa that became decondensed, the intensity and pattern of immunostaining was variable, but was not correlated with the size of the decondensed sperm nucleus, however. After decondensation, the original morphology of the spermatozoa was lost, making it impossible to ascertain which spermatozoa might have been morphologically abnormal prior to decondensation. Therefore it was not possible to detect a putative correlation between sperm morphology and sperm TH2B immunostaining at the single cell level.

TH2B level in semen samples

The amount of TH2B present in a fixed (0.5×10^6) number of spermatozoa of 35 males visiting our Andrology Outpatient Clinic was determined by measuring the intensity of the TH2B immunosignal on Western blot. Figure 5 shows the TH2B immunoblots for several of these patients. There clearly was a large inter-individual variation in sperm TH2B content. It should be noted that these data represent the total amount of TH2B per 0.5×10^6 spermatozoa, and do not give information about the variation of TH2B content among individual spermatozoa. Quantitative analysis of the TH2B level in sperm samples confirmed the qualitative observations (Figure 6). In order to assess if the variation in total sperm TH2B content

Figure 3. Immunohistochemical localization of TH2B in human testis tissue, which is stained with anti-TH antibody. The histological sections were counterstained with Mayer's haematoxylin. Immunostaining is localized in the nuclei of spermatogonia, primary and secondary spermatocytes, and round and elongating spermatids. Sertoli cells (curved arrows in panels **C** and **E**), peritubular myoid cells, interstitial cells, and cells associated with arterioles were not stained. Panels **B**, **C**, and **D** show human testis tissue at a higher magnification. Note the variation in pachytene spermatocyte immunostaining, especially in panel **D** (large arrowheads). There is also a change in TH2B immunostaining in elongating spermatids; panel **B** shows a decrease in TH2B immunostaining at the acrosomal region of the round spermatid nucleus (small arrowheads), and panel **C** shows a small region of immunodetectable TH2B at the posterior region of the spermatid nucleus (small arrowheads), whereas in panel **D**, no immunodetectable TH2B is seen in the elongated spermatid nuclei (small arrowhead). Panel **E** shows a detail of a testis biopsy sample from an infertile patient showing only Sertoli cells (curved arrow) and spermatogonia in the tubular lumen; note the presence of TH2B immunoreactivity in all spermatogonia. Bars = 100 μm (panel **A**, original magnification ×200), 10 μm (panels **B**, **C**, **D**, original magnification ×1000), 50 μm (panel **E**, original magnification ×400).

Figure 4. Immunocytochemical localization of TH2B in human spermatozoa. The spermatozoa were treated (see Materials and Methods) to induce decondensation. There is a clear signal in some, but not all, of the decondensed sperm nuclei. All non-decondensed sperm nuclei remain negative. Bar = 10 μm; original magnification ×1000.

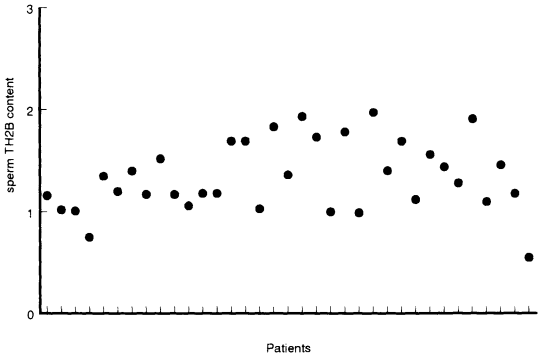

Figure 6. Variation of total sperm TH2B content in 35 infertile males. The total TH2B content of 0.5×10^6 spermatozoa isolated from a single ejaculate from each of the subfertile males was estimated. All values are relative to the average amount of TH2B in a standard sperm sample obtained from an ejaculate with a high number of spermatozoa. Samples were first washed in Percoll 60%. Proteins were separated on SDS–PAGE, blotted and immunostained. The intensity of the resulting signal was quantified (see Materials and Methods).

Table I. Results of routine semen analysis (mean ± SEM) and correlation with sperm testis-specific histone 2B (TH2B) content in 0.5×10^6 spermatozoa of 35 subfertile men. The sperm TH2B contents of patients were compared with the TH2B content of a standard sperm sample

		Correlation[a] with histone content	*P* value
Total sperm count ($\times$ 10^6)	98.8 (± 15.1)	−0.144	0.41
Concentration ($\times10^6$/ml)	27.4 (± 3.1)	−0.021	0.91
Motility[b] (%)	38.1 (± 3.2)	−0.138	0.43
Morphology[c] (%)	19.9 (± 2.1)	−0.328	0.05
Histone content[d]	1.3 (± 0.06)		

[a]Spearman.
[b]Grades a+b (WHO).
[c]WHO criteria.
[d]Arbitrary units.

might be associated with semen sample characteristics, the TH2B concentration was compared with the results of the corresponding semen analyses. As is shown in Table I, there was no correlation between total sperm TH2B content and sperm concentration of the initial sample (r = –0.210; P = 0.91) or sperm motility (r = –0.138; P = 0.43). In this group of patients, total sperm TH2B content showed a marginally significant correlation with morphology (r = 0.328; P = 0.05); a higher TH2B concentration was found in ejaculates with more abnormal spermatozoa. Greater than 1 $\times10^6$ round cells per ml were present in only six samples, thus this factor was not included in the statistical calculations.

Discussion

In this study we have shown that the anti-TH antibody, known to cross-react with rat TH2B, can also be used to detect human TH2B in testis tissue and in spermatozoa. A clear and prominent 17-kDa band was detected on immunoblots of acid-soluble proteins extracted from rat testis tissue and human spermatozoa, but not from human leukocytes and rat epididymal spermatozoa. Tyrosine hydroxylase, a 57-kDa protein (Mayerhofer and Russell, 1990), was not encountered in any of the extracts. Additional evidence that the protein recognized by the anti-TH antibody is indeed a histone of the 2B type and not H2A or a totally unrelated protein of 17 kDa was obtained by electrophoresis on AUT gels. The mobility of the immunoreactive protein was almost similar to rat TH2B and the bovine H2B standard.

In histological sections of human testis, only the nuclei of spermatogenic cells showed immunostaining: spermatogonia, spermatocytes, and round spermatids reacted positively with varying intensity. The immunodetectability of TH2B in elongating spermatids was confined to the post-acrosomal region of the nucleus, whereas the nuclei of fully condensed spermatids did not stain. All somatic cell nuclei (Sertoli cells, Leydig cells, peritubular myoid cells, and endothelial cells of arterioles) were clearly negative. The absence of immunodetectability of TH2B in the anterior part of the nuclei of elongating spermatids and in spermatozoa was due to the highly condensed condition of the nuclei and not to the absence of TH2B, as Western blotting and immunostaining of acid-extractable proteins from human spermatozoa revealed a positive signal corresponding to TH2B.

Previous studies have shown that about 15% of human sperm nuclear proteins are histones (Tanphaichitr *et al.*, 1978), including TH2B (Wattanaseree and Svasti, 1983; Gusse *et al.*, 1986; Gatewood *et al.*, 1990; Prigent *et al.*, 1996). Using immunocytochemistry, we could not detect TH2B in untreated human and rat spermatozoa. Protein separation on SDS–PAGE followed by immunoblotting, however, confirmed the presence of TH2B in human spermatozoa, and also showed that TH2B was absent in rat spermatozoa. It appears that immunocytochemical detection of TH2B in morphologically normal mature human sperm cells is hampered by inaccessibility of the epitope to the antibody. When the human spermatozoa were decondensed, immunocytochemistry showed the presence of TH2B, albeit with a remarkably diverse pattern of staining. Whether the observed intercellular variation in TH2B immunostaining indeed reflects a difference in sperm TH2B content is a point of concern. During the course of this study, it became clear that too vigorous a decondensation of spermatozoa resulted in loss of immunostaining from all spermatozoa (results not shown). Presumably all TH2B is then removed from the nucleus. The heterogeneous staining pattern of the decondensed spermatozoa can probably not be attributed to loss of TH2B from some cells because many well-decondensed and large nuclei stain intensely while other nuclei, also decondensed but smaller, stain lightly or not at all. Conversely, one might argue that the smaller nuclei contain TH2B that is still masked; however, there was no correlation between the size of the decondensed spermatozoa and the signal intensity. Therefore the observed heterogeneity in immunostaining probably is a true reflection of an intercellular variation in sperm TH2B content.

Very little is known about the regulation of expression of testis-specific histones in the human testis. A recent report (Prigent *et al.*, 1996) indicates that, during spermiogenesis, the immunodetectability of histones H2B and H3 is constant in the nuclei of early round spermatids, and then increases in

intermediate spermatids (step 3–4). The histone H3 immunosignal decreases at the end of the elongation phase (step 5), while the H2B labelling decreases in mature spermatids (step 6). Previous studies in the rat have shown that a number of histones (H1a, H2AX, TH3) are expressed early during the mitotic stages of spermatogenesis, while others (TH2A, TH2B, H1t) are first expressed at a later time when the spermatogenic cells have entered the meiotic phase (Meistrich and Brock, 1987; Meistrich *et al.*, 1985). In the rat, TH2B is first synthesized in the preleptotene or leptotene spermatocytes, and its synthesis continues until the mid or late pachytene stage. TH2B is the major form of H2B in rat round and elongating spermatids, although somatic H2B is still present (Brock *et al.*, 1980; Meistrich *et al.*, 1985; Unni *et al.*, 1995). Our studies show a strong TH2B immunosignal in spermatogonia in human testis tissue, indicating that, contrary to the situation in the rat, first expression of TH2B in the human testis occurs at the early mitotic stages of spermatogenesis.

A number of investigators have shown that ejaculates of subfertile men and/or ejaculates containing a large number of morphologically abnormal cells contain a relatively large amount of histones (Terquem and Dadoune, 1983; Chevaillier *et al.*, 1987; Foresta *et al.*, 1992). Most studies have used indirect methods to detect the persistence of histones in spermatozoa, such as staining with toluidine blue to test the availability of DNA phosphates (Andreetta *et al.*, 1995) and with aniline blue to stain all remaining histones (Terquem and Dadoune, 1983), differential nuclear chromatin decondensation using detergents (Calvin and Bedford, 1971; Bedford *et al.*, 1973), the acridine orange test for single-stranded DNA (Evenson *et al.*, 1980; Tejada *et al.*, 1984), and the use of chromomycin A_3 to test for guanosine-rich sequences of DNA (Bianchi *et al.*, 1996). We are now in a position to determine the presence of a single histone variant in human spermatozoa, and to correlate its persistence with routine semen analysis parameters. In our patient population, we found no correlation between total sperm TH2B content and total number of spermatozoa per ejaculate, sperm concentration, and sperm motility. There was a marginally significant negative correlation between total sperm histone content and sperm morphology ($P = 0.05$).

Although fertile men produce a substantial amount of morphologically abnormal spermatozoa, abnormalities of sperm morphology are encountered more often in subfertile men (Bostofte *et al.*, 1985; Kruger *et al.*, 1988). Sperm morphogenesis is a complex process, which includes the successful compaction of the DNA. An association between defects in DNA packaging and sperm morphology abnormalities has been reported previously (Gledhill, 1970; Bedford *et al.*, 1973; Dadoune and Alfonsi, 1986). Recently it was observed that the knock-out of the gene encoding ubiquitin-conjugating enzyme HR6B in the mouse was associated with morphological abnormalities of spermatozoa (Roest *et al.*, 1996). These morphological abnormalities may result from impaired chromatic reorganization during spermatogenesis, which may require ubiquitination of histones. Disruption of the normal dense chromatin structure of spermatozoa in transgenic mice expressing a gene construct that encodes galline, the avian homologue of protamine, which is under the control of a protamine-1 promoter, causes sperm abnormalities and infertility (Rhim *et al.*, 1995). This is probably explained by impaired DNA compaction in chromatin containing both protamine and galline. Both mouse models demonstrated that a disturbance in chromatin remodelling could lead to marked morphological abnormalities of spermatozoa and to infertility.

The presence of histones in human sperm is also regarded as a consequence of a derailment in the process of chromatin remodelling during spermatid condensation, and is considered an ominous sign with respect to fertility (Terquem and Dadoune, 1983; Chevaillier *et al.*, 1987; Foresta *et al.*, 1992). Our results and those of others (Gatewood *et al.*, 1987), however, show that in virtually all human ejaculates, spermatozoa can be shown to contain histones. It should be kept in mind that, in somatic cells, histones not only play a structural role in organizing nuclear DNA; evidence is accumulating that histones might play a crucial role in the regulation of transcription (Ura *et al.*, 1997). Histones in spermatozoa are possibly interacting with a specific part of the genome (Gatewood *et al.*, 1987), and there are indications for early post-conception paternal-specific expression of several genes (Sawicki *et al.*, 1981). From this, it is tempting to suggest that sperm histones might be involved in marking the genes that are poised for early expression after fertilization.

In addition to the role of nuclear proteins, mRNAs found in human ejaculated spermatozoa have been the focus of a considerable amount of speculation. A number of recent studies have demonstrated the presence of mRNA coding for c-*myc*, β-actin, β_1-integrin, protamine 1, protamine 2 and transition protein 2 in mature human spermatozoa (for review see Kramer and Krawetz, 1997). Whether the highly condensed chromatin within the head of the spermatozoa is actually (partially) transcriptionally active, or whether the observed mRNAs are stable and dormant remnants of past haploid synthetic and equilibrative activity in earlier stages of germ cell differentiation (Miller, 1997), which persist because of lack of degradative mechanisms within the spermatozoa, remains to be elucidated.

In conclusion, we have shown that an antibody targeting tyrosine hydroxylase can be used to specifically identify TH2B in human spermatozoa and testis tissue. Our results indicate that TH2B is first expressed at an early mitotic stage of human spermatogenesis, and is present throughout spermatogenesis and in mature spermatozoa. There appears to be a marked variation in total sperm TH2B content among subfertile males that may correlate, to some extent, with abnormalities of sperm morphology. Finally, the present results indicate sperm-to-sperm variation in TH2B immunoexpression within a single ejaculate. The significance of this observation, and the fertilizing potential of human spermatozoa containing varying amounts of TH2B, are the subject of ongoing research.

References

Andreetta, A.M., Stockert, J.C. and Barrera, C. (1995) A simple method to detect sperm chromatin abnormalities: cytochemical mechanism and possible value in predicting semen quality in assisted reproductive procedures. *Int. J. Androl.*, **18**, Suppl 1, 23–28.

Balhorn, R. (1982) A model for the structure of chromatin in mammalian sperm. *J. Cell Biol*., **93**, 298–305.

Balhorn, R. (1989) Mammalian protamines: structures and molecular interactions. In Adolph, K.W. (ed.), *Molecular Biology of Chromosome Function*. Springer Verlag, New York, pp. 366–395.

Bedford, J.M., Bent, M.J. and Calvin, H.I. (1973) Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. *J. Reprod. Fertil*., **33**, 19–29.

Bianchi, P.G, Manicardi, G.C., Urner, F. *et al.* (1996) Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. *Mol. Hum. Reprod.*, **2**, 139–144.

Bostofte, E., Serup, J. and Rebbe, H. (1985) The clinical value of morphological rating of human spermatozoa. *Int. J. Fertil.*, **30**, 31–37.

Brock, W.A., Trostle, P.K. and Meistrich, M.L. (1980) Meiotic synthesis of testis histones in the rat. *Proc. Natl. Acad. Sci. USA*, **77**, 371–375.

Calvin, H.I. and Bedford, J.M. (1971) Formation of disulfide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J. Reprod. Fertil.*, **13** (Suppl.), 65–75.

Chevaillier, P., Mauro, N., Jouannet, P. and David, G. (1987) Anomalous protein complement in some infertile men. *Lancet*, **2**(8562), 806–807.

Dadoune, J.P and Alfonsi, M.F. (1986) Ultrastructural and cytochemical changes of the head components of human spermatids and spermatozoa. *Gamete Res*., **14**, 33–46.

Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R. (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, **210**, 1131–1133.

Foresta, C., Zorzi, M., Rossata, M. and Varotto, A. (1992) Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int. J. Androl*., **15**, 330–337.

Gatewood, J.M., Cook, G.R., Balhorn, R. *et al.* (1987) Sequence specific packaging of DNA in human sperm chromatin. *Science*, **236**, 962–964.

Gatewood, J.M., Cook, G.R., Balhorn, R. *et al.* (1990) Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. *J. Biol. Chem.*, **265**, 20662–20666.

Gledhill, B.L. (1970) Changes in nuclear stainability associated with spermateliosis, spermatozoal maturation, and male fertility. In Wied, G.L. (ed.) *Introduction to Quantitative Cytochemistry.* Vol 2. Academic Press, New York, pp. 125–151.

Grimes, S.R. (1986) Nuclear proteins in spermatogenesis. *Comp. Biochem. Biophysiol*., **3**, 495–500.

Gusse, M., Saurière, P., Bélaiche, D. *et al.* (1986) Purification and characterization of nuclear basic proteins of human sperm. *Biochim. Biophys. Acta*, **884**, 124–134.

Hofmann, N. and Hilscher, B (1991) Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. *Hum. Reprod.*, **6**, 979–982.

Hunter, J.D., Bodner, A.J. and Hatch, F.T. *et al.* (1976) Single strand nuclease action on heat denatured spermiogenic chromatin. *J. Histochem. Cytochem.*, **24**, 901–907

Kramer, J.A. and Krawetz, S.A. (1997) RNA in spermatozoa: implications for the alternative haploid genome. *Mol. Hum. Reprod*., **3**, 473–478.

Kruger, T.F., Acosta, A.A., Simmons, K.F. *et al.* (1988) Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil. Steril*., **49**, 112–117.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **277**, 680–685.

Marushige, Y. and Marushige, K. (1975) Enzymatic unpacking of bull sperm chromatin. *Biochim. Biophys. Acta*, **403**, 180–191.

Mayerhofer, A. and Russell, L.D. (1990) Characterisation of a transiently expressed nuclear protein localized in elongating spermatids. *J. Cell Biol.*, **111**, 108a (Abstract).

Meistrich, M.L. and Brock, W.A. (1987) Proteins of the meiotic cell nucleus. In Moens, P.B. (ed.), *Meiosis*. Academic Press, Orlando, Florida, pp. 333–353.

Meistrich, M.L., Brock, W.A., Grimes, S.R. *et al.* (1978) Nuclear protein transition during spermatogenesis. *Fed. Proc*., **37**, 2522–2525.

Meistrich, M.L., Bucci, L.R., Trostle-Weige, P.K. and Brock, W.A. (1985) Histone variants in rat spermatogonia and primary spermatocytes. *Dev. Biol.*, **112**, 230–240.

Miller, D. (1997) RNA in the ejaculate spermatozoon: a window into molecular events in spermatogenesis and a record of the unusual requirements of haploid gene expression and post-meiotic equilibration. *Mol. Hum. Reprod.*, **3**, 669–676.

Platz, R.D., Meistrich, M.L. and Grimes, S.R. (1977) Low-molecular-weight basic proteins in spermatids. *Methods Cell Biol*., **16**, 297–316.

Prigent, Y., Muller, S. and Dadoune, J.P. (1996) Immunoelectron microscopical distribution of histones H2B and H3 and protamines during spermiogenesis. *Mol. Hum. Reprod.*, **2**, 929–935.

Rhim, J.A., Connor, W., Dixon, G.H. *et al.* (1995) Expression of an avian protamine in transgenic mice disrupts chromatin structure in spermatozoa. *Biol. Reprod*., **52**, 20–32.

Roest, H.P., van Klaveren, J., de Wit, J. *et al.* (1996) Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes a defect in spermatogenesis associated with chromatin modification. *Cell*, **86**, 799–810.

Sawicki, J.A., Magnuson, T. and Epstein, C.J. (1981) Evidence for expression of the paternal genome in the two-cell mouse embryo. *Nature*, **294**, 450–451.

Tanphaichitr, N., Sobhon, P., Talupphet, N. and Chalermisarachai, P. (1978) Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. *Exp. Cell Res.*, **117**, 347–356.

Tejada, R.I., Mitchell, J.C., Norman, A. *et al.* (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil. Steril*., **42**, 7–91.

Terquem, A. and Dadoune, J.P. (1983) Aniline blue staining of human spermatozoa chromatin: evaluation of nuclear maturation. In André J. (ed.) *The Sperm Cell*. Martinus Nijhoff Publishers, The Hague, pp. 249–252.

Unni, E., Mayerhofer, A., Zhang, Y. *et al.* (1995) Increased accessibility of the N-terminus of testis-specific histone TH2B to antibodies in elongating spermatids. *Mol. Reprod. Dev.*, **42**, 210–219.

Ura, K., Kurumizaka, H. and Dimitrov, S. *et al.* (1997) Histone acetylation: influence on transcription, nucleosome mobility and positioning, and linker histone-dependent transcription repression. *EMBO J*., **16**, 2096–2107.

Ward, W.S. (1993) Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. *Biol. Reprod*., **48**, 1193–1201.

Wattanaseree, J. and Svasti, J. (1983) Human testis-specific histone TH2B: fractionation and peptide mapping. *Arch. Biochem. Biophys.*, **225**, 892–897.

WHO (1992) *Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*, 3rd edn. Cambridge University Press, Cambridge.

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