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Expression of Antimicrobial Defensins in the Male Reproductive Tract of Rats, Mice, and Humans[1]

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ABSTRACT

Defensins are antimicrobial peptides that play a major role in innate immunity. Using reverse transcriptase-polymerase chain reaction, immunochemistry, or both, we performed a search of all presently known defensins in rat testis, epididymis, and isolated testicular cells; in mouse testis and epididymis; and in human testis and ejaculates. In the rat, all α- and β-defensins except RNP-4 were expressed within the testis, whereas α-defensins RNP1–2, RNP-4, and β-defensins RBD-1 and RBD-2 were present within the epididymis. In the mouse, the cryptdin transcripts CRS1C, mBD-1, and mBD-2 were detected in the testis and epididymis, whereas mBD-3 and mBD-4 were expressed only in the epididymis, and CRS4C was absent in both organs. In the human testis, transcripts for four known defensins were expressed with the consistent exception of HBD-2 and HBD-3. In rat interstitial tissue, resident macrophages expressed most of the defensins studied, whereas Leydig cells produced only RBD-2. In contrast, all studied defensins except RNP-4 were present in the seminiferous tubules. Within these tubules, peritubular and Sertoli cells expressed most of the studied α- and β-defensins, whereas spermatogonia displayed only α-defensins, but at relatively high levels. Meiotic pachytene spermatocytes expressed only β-defensins, whereas postmeiotic spermatids and their cytoplasmic lobes displayed both types. In humans, the HBD-1 peptide was expressed mainly in the germ line from pachytene spermatocytes to late spermatids. The peptide was also present in ejaculated spermatozoa and seminal plasma, where multiple soluble forms were present. Finally, high salt concentration or dithiothreitol-sensitive cationic extracts from human seminal plasma were indeed found to display antimicrobial activity. We conclude that the male reproductive tract produces defensins that most probably assume an important, innate organ defense system against pathogens.

epididymis, gamete biology, sperm, spermatogenesis

INTRODUCTION

A number of microorganisms are able to infect the reproductive tract tissues and semen in humans and animals, with serious consequences for reproductive and endocrine function. Infecting microorganisms may penetrate the testis via the blood and lymphatic vessels in the interstitial compartment of the testis, the rete testis, or rarely, through the tunicae albuginea or the scrotum skin [1]. Retrograde infection of the epididymis may occur from microorganisms present in the vas deferens, or via the blood vessels supplying this organ. A common result of microbial infection of the testis and epididymis is orchitis or epididymal inflammation [1, 2], conditions that may lead to the destruction of the epididymal duct and transient or permanent sterility [3].

Whereas certain microorganisms represent a threat to fertility in men and semen is an essential vector in sexually transmissible diseases [3], the ability of the testis or other components in the male reproductive tract to react to a microbial attack has been explored only recently, when Dejucq et al. demonstrated that the rat testis possesses an efficient interferon-inducible antiviral protein system [4–7]. To broaden our exploration of the testicular host defense system, we have undertaken a study of a class of antimicrobial peptides known as defensins. These antimicrobial peptides have been the subject of numerous studies and are reported to participate in innate immunity of various multicellular organisms, including plants, insects, and mammals [8–10]. Defensins are 3- to 6-kDa cationic peptides containing six disulfide-paired cysteines. Their broad spectrum of microbicidal activity includes Gram-positive and Gram-negative bacteria, mycobacteria, many fungi, and several enveloped viruses such as herpes simplex virus, vesicular stomatitis virus, cytomegalovirus, influenza A/WSN, and human immunodeficiency virus [11, 12]. Defensins rapidly kill microorganisms by permeating the microbial membranes and impairing the microorganism's ability to carry out metabolic processes [13]. In mammals, defensins are divided into α-defensins and β-defensins, which differ in the placement and connectivity of their six conserved cysteines, and in their patterns of expression. α-Defensins have been isolated from neutrophils and Paneth cells of the small intestine [14–16], whereas β-defensins have been found principally in the epithelial cells in the kidneys, skin, and the female reproductive tract [17].

The aim of the present work was to study the expression of α- and β-defensins in the male reproductive tract of rats, mice, and humans. For this purpose, a systematic search of all known defensins was performed using reverse transcriptase-polymerase chain reaction (RT-PCR) experiments in total testes of all three species, in isolated rat testicular cells, in rat and mouse epididymides, and in human ejaculates. The in situ immunolocalization of a particular defensin, human beta-defensin-1 (HBD-1), was more specif-

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ically studied in human testicular biopsies, seminal plasma, and ejaculated spermatozoa using immunohistochemistry and cytochemistry. We show that the male urogenital tract in mammals expresses a broad range of defensins, suggesting the presence of innate defenses against infection or other yet-to-be-discovered functions in the reproductive tract.

MATERIALS AND METHODS

Animals, Human Tissues, and Reagents

Male Sprague-Dawley rats were purchased from Elevage Janvier (Le Genest Saint Isle, France). Male Swiss white mice were obtained from our in-house breeding stock. Human testes were obtained from patients undergoing therapeutic orchidectomy for metastatic prostate carcinoma (the protocol was approved by the Ethical Committee of the city of Rennes, Bretagne, France). Enzymes for cell preparations were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France).

Cell Isolation

Sertoli cells. Sertoli cells were isolated from 20-day-old rats as previously described [18]. Sertoli cell suspensions were seeded at a density of approximately 1×10^6 cells/ml in 75 cm^2 tissue culture flasks (NUNC, Copenhagen, Denmark). The cells were then incubated at 32°C in a humidified atmosphere of 5% CO_2 and 95% air in Ham F12/Dulbecco modified Eagle medium (DMEM; Life Technologies, Cergy Pontoise, France) supplemented with insulin (10 mg/ml), human transferrin (5 mg/ml), and gentamycin (10 mg/ml). The culture medium was changed daily until the end of the experiment. On the second day of culture, cells were exposed to a hypotonic treatment in order to eliminate the contaminating germ cells [19]. The purity of the isolated Sertoli cells was ≥98%. On Day 5 of culture, cells were recovered and frozen in liquid nitrogen and stored at −80°C until they were used for RNA extraction.

Peritubular cells. Isolation of peritubular cells from 20-day-old rats was carried out during the Sertoli cell preparation process as previously described [20]. Cells were cultured in the same culture medium as described for Sertoli cells above, supplemented with 10% fetal calf serum (FCS). Cells were seeded at a density of approximately 200 000 cells/ml in 75 cm^2 tissue culture flasks. After 4–5 days of culture the cells were removed by a brief treatment with 0.05% trypsin and 0.5 mM EDTA in PBS, washed, and then replaced at ¼ density in 175 cm^2 flasks. When the subcultured cells had grown to confluence, they were washed with PBS buffer and incubated with culture medium without FCS three times for 2 h and then for 24 h. The cells (≥99% purity) were then collected, snap-frozen in liquid nitrogen, and stored at −80°C until they were used for RNA extraction.

Leydig cells and testicular macrophages. Highly enriched Leydig cells (≥98% purity) and testicular resident macrophages were prepared from adult rat testes according to the multistep isolation method described by Klinefelter et al. [21]. Testicular macrophages were plated for 15 min in medium supplemented with 10% FCS. Macrophages and Leydig cells were then cultured for 24 h in Ham F12/DMEM (1:1 v/v) supplemented with gentamycin (50 μg/ml), 0.1% BSA, and 10% FCS. After culture the cells were snap-frozen separately in liquid nitrogen and stored at −80°C until they were used for RNA extraction.

Germ cells. Germ cells were prepared from adult rat testes as previously described [22] except that enzymatic dissociation of cells was replaced by a mechanical dispersion. Pachytene spermatocytes, early spermatids, and the cytoplasmic lobes from late spermatids were prepared by centrifugal elutriation with a purity of greater than 85%–90% [22]. Upon isolation, cells were snap-frozen in liquid nitrogen and stored at −80°C until they were used for RNA extraction.

Spermatogonia. Testes of male Sprague-Dawley rats at 9 days postpartum were excised and decapsulated. Seminiferous epithelial cells were dispersed by enzyme treatment and separated with a purity of greater than 90%, as previously described [23]. Briefly, after enzymatic dissociation, the cells were separated by sedimentation velocity at unit gravity at 4°C using a 2%–4% BSA gradient in Ham F12/DMEM in an SP-120 chamber (STAPUT). After 2.5 h of sedimentation, 35 fractions were collected. Cell fractions 16 to 21 were pooled, washed with PBS, centrifuged, and the dry cell pellet was snap-frozen in liquid nitrogen and stored at −80°C until it was used for RNA extraction.

Semen Collection and Preparation

Semen was obtained by masturbation after 3 days of abstinence from patients with normal sperm characteristics who were undergoing in vitro fertilization protocols. After ejaculation, semen samples were allowed to liquefy at 37°C for 1 h. A fraction was used to prepare smears and the remaining semen was centrifuged at $1000 \times g$ for 10 min in order to separate spermatozoa from seminal plasma. Spermatozoa and seminal plasma samples were frozen and stored at −80°C until they were used for RNA extraction and cationic peptide purification, respectively.

Total RNA Isolation and RT-PCR

RT-PCR was performed to analyze the expression of defensins in the testes, epididymides, isolated rat testicular cell populations, and human ejaculated spermatozoa. Total RNA was purified from the various types of samples using the RNeasy Total RNA isolation kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. Two micrograms of RNA were ethanol-precipitated in the presence of 3 M sodium acetate pH 5.2 and resuspended in sterile water. A treatment with 5 U deoxyribonuclease I (Promega, Charbonnières Les Bains, France) for 1.5 h at 37°C in the presence of 20 U RNasin (Promega) was undertaken to ensure that contaminating genomic DNA in the RNA template was removed, RNA was then precipitated again as described above and resuspended in sterile water, and the cDNA synthesis was performed with 2 μg/ml of hexanucleotides and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies) in the reaction medium (50 mM Tris-HCl pH 8.5, 75 mM KCl, and 3 mM $MgCl_2$) containing 0.5 mM of each deoxy-NTP, 10 mM dithiothreithol (DTT), and 40 U RNasin (Promega) in a final volume of 20 μl. After incubation at 37°C for 1.5 h, the reaction volume was brought up to 100 μl. A negative control was performed at the same time using a similar reaction mixture but without MMLV reverse transcriptase.

RT-PCR was conducted in a 25-μl reaction volume containing *Taq* DNA polymerase buffer (Life Technologies), 0.2 mM of each deoxy-NTP, 1.5 or 3 mM $MgCl_2$, 0.2 mM of each sense and antisense primer, and 0.625 U *Taq* DNA polymerase. Four microliters of each cDNA mixture was used as a template. The PCR primers used to screen for all known defensins in rats, mice, and humans were designed from published sequences and are given along with the expected products sizes in Table 1. Amplification took place in a PTC-200 thermocycler (MJ Research, Watertown, MA) in 35 cycles for 1 min at 94°C, 1 min at the temperature chosen for annealing (Table 1), and for 2 min at 72°C. All RT-PCR products were subsequently separated by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and visualized with UV light. All amplification products detected in this study were sequenced and confirmed to correspond to the defensins of interest.

Complementary DNA Sequencing

PCR products were extracted from agarose gels with the QIAEX II Gel Extraction Kit (Qiagen) following the manufacturer's recommendations. Extracted DNA were quantified using a TM 950 Alpha Imager (Alpha Innotech Corporation, San Leandro, CA). Sixty nanograms of each cDNA template were sequenced on both strands using a thermal cycling method with fluorescent dye labeled-dideoxynucleotide terminators and *Taq* polymerase (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer, Courtaboeuf, France) in accordance with the manufacturer's instructions. The different sequences were analyzed by an automated DNA sequencer (373A, Applied Biosystems, Courtaboeuf, France). A database search was performed for the obtained sequences using the Basic Local Alignment Search Tool (BLAST) 2.0 program (available at http://www.ncbi.nlm.nih.gov).

Immunohistochemistry and Cytochemistry

Human testes were fixed in Bouin-Holland fixative and embedded in paraffin wax. Thin sections (5 μm) were deparaffined, rehydrated, and microwaved in a citrate buffer (10 mM pH 6.0) for 15 min for antigen retrieval [24]. Sperm smears fixed with alcohol/ether (50:50, v/v) were permeabilized for 5 min in methanol at −20°C. Testis sections and sperm smears were treated to inactivate endogenous peroxidases by incubation for 5 min in 3% hydrogen peroxide. Samples were then blocked for 10 min with 1% BSA in PBS (p/v) and subsequently incubated for 18 h at 4°C with the anti-HBD-1 rabbit polyclonal antiserum [17] used at a final dilution of 1:500 in PBS containing 0.1% Tween-20 (v/v) and 1% BSA (p/v) (TPBS-BSA). In parallel, a negative control was performed using

TABLE 1. PCR primers designed for the study of defensin expression in reproductive tracts of male rats, mice, and humans.

	Peptide		Primer sequences	Temperature	Product size (bp)	GenBank accession no.	Reference
Rat	RNP 1/2	S	5′-GGACGCTCACTCTGCTTACC-3′	60°	304	U16686/U16685	[26]
		AS	5′-TGGATTCTTCTTGGTCGGAG-3′				
	RNP-3	S	5′-AAGAGCGCTGTGTCTCTTGC-3′	60°	320	U16683	[26]
		AS	5′-CAACAGAGTCGGTAGATGCG-3′				
	RNP-4	S	5′-TCTGCTCATCACCCTTCTCC-3′	60°	260	U16684	[26]
		AS	5′-AACAGAGACGGTAGATGCGG-3′				
	RD-5	S	5′-ACTTGTCCTCCTTTCTGCCC-3′	58°	231	AF115768	[27]
		AS	5′-ATCCCCATAATGCCTTCTCC-3′				
	RBD-1	S	5′-TACCTGGGAGTCTCACGTCC-3′	60°	300	AF068860	[28]
		AS	5′-CCCTTGCTGTCCTTTATGTC-3′				
	RBD-2	S	5′-ACCAGGCTTCAGTCATGAGG-3′	60°	233	AF068861	[28]
		AS	5′-CATCCCATTGGTTCTTGGTC-3′				
Mouse	Cryptdins	S	5′-ACTCCCAGCCATGAAGACAC-3′	55°	297	AH0053998/AH0053999 AH005400/AH005401 AH 005402	[29]
		AS	5′-CATGTTCAGCGACAGCAGAG-3′				
	CRS1-C	S	5′-ACTCCCAGCCATGAAGACAC-3′	55°	439	M33226.1	[30]
		AS	5′-TTGCAATTGACGCTAAGCAC-3′				
	CRS4-C	S	5′-ACTCCCAGCCATGAAGACAC-3′	55°	429	M33227.1	[30]
		AS	5′-GAAGCAAGAGCAATCAAGGC-3′				
	mBD-1	S	5′-TCCTCTCTGCACTCTGGACC-3′	60°	271	AF003524/5	[31]
		AS	5′-ATCGCTCGTCCTTTATGTCC-3′				
	mBD-2	S	5′-CCTTTCTACCAGCCATGAGG-3′	59°	215	AJ011800	[32]
		AS	5′-GCAACAGGGGTTCTTCTCTG-3′				
	mBD-3	S	5′-CTCCACCTGCAGCTTTTAGC-3′	62°	263	AF092929	[33]
		AS	5′-GCTAGGGAGCACTTGTTTGC-3′				
	mBD-4	S	5′-CTCCACTTGCAGCCTTTACC-3′	59°	202	NM_019728	[34]
		AS	5′-CATGGAGGAGCAAATTCTGG-3′				
Human	HNP 1/3	S	5′-AGCTAGAGGATCTGTGACCC-3′	58°	304	M21130/M23281.1	[54]
		AS	5′-GCAGAATGCCCAGAGTCTTC-3′				
	HD-5	S	5′-TGAGGCTACAACCCAGAAGC-3′	60°	197	M97925.1	[15]
		AS	5′-AGCAGAGTCTGTAGAGGCGG-3′				
	HD-6	S	5′-TAGCCATGAGAACCCTCACC-3′	60°	388	M98331	[16]
		AS	5′-TGGCAATGTATGGGACACAC-3′				
	HBD-1	S	5′-GTCAGCTCAGCCTCCAAAGG-3′	56°	310	U73945	[55]
		AS	5′-CTTCTGCGTCATTTCTTCTG-3′				
	HBD-2	S	5′-TTTGGTGGTATAGGCGATCC-3′	62°	253	NM_004942	[56]
		AS	5′-GAGGGAGCCCTTTCTGAATC-3′				
	HBD-3	S	5′-AGCCTAGCAGCTATGAGGATC-3′	60°	210	AJ23673	[57]
		AS	5′-CTTTCTTCGGCAGCATTTTC-3′				
	Actin	S	5′-GACTACCTCATGAAGACT-3′	55°	512		
		AS	5′-TTGCTGATCCACATCTTG-3′				

the preimmune serum at a final dilution of 1:500 in TPBS-BSA. After several washes in TPBS, slices and smears were incubated for 45 min with a secondary biotinylated goat anti-rabbit antibody at a final dilution of 1:500 in TPBS-BSA. Samples were subsequently washed in TPBS and incubated for an additional 30 min with a streptavidin-peroxidase complex (DAKO, Trappes, France) at a dilution of 1:500 in TPBS-BSA. Immunoreaction was revealed for 1 to 5 min with a diaminobenzidine solution (Sigma-Aldrich) or for 20 min with 3-amino-9-ethylcarbazole (AEC; DAKO). Finally, sections and cells were counterstained with Masson hemalun.

Purification of Cationic Peptides from Seminal Plasma and Conditioned Media from Caco-2 Cells

Cationic peptides from seminal plasma and human Caucasian colon adenocarcinoma conditioned media (Caco-2-CM) were extracted using the weak cation exchange matrix Macro-Prep CM support (Bio-Rad, Ivry-sur-Seine, France). Briefly, matrix Macro-Prep CM beads were added to the seminal plasma after its 30-fold dilution in PBS or to the Caco-2-CM at a ratio of 1:60 (v/v). Mixtures were incubated overnight at 4°C with gentle stirring, then sedimented at 1000 × *g* for 5 min, and washed three times for 5 min with 80 volumes of 25 mM ammonium acetate (pH 7.5). Peptides bound to the matrix beads were eluted with four matrix volumes of 10% acetic acid in water for 30 min, followed by two successive elutions with 5% acetic acid in water. Eluates were finally pooled and lyophilized for immediate use in AU-PAGE or antimicrobial activity assays.

Western Blot Analysis

Freshly lyophilized cationic extracts were resuspended in 5% acetic acid in water and fractionated by AU-PAGE [25]. Samples were subsequently transferred onto Immobilon-P^{SQ} (PVDF) membranes (Millipore) for 30 min with 0.7% acetic acid and 10% methanol at 0.18 A using the Hoefer mini-VE blot module (Amersham Biosciences, Orsay, France). Following transfer, blots were fixed for 30 min at room temperature with 0.5% glutaraldehyde in TBS, blocked for 1 h in 5% nonfat powdered milk in TBS containing 0.1% Tween-20 (TTBS), and then incubated for 18 h at 4°C in a rabbit anti-HBD-1 antiserum [17] at a final dilution of 1:2000 in TTBS supplemented with 1% nonfat powdered milk. In parallel, a negative control was performed using the preimmune serum at a final dilution of 1:2000 in TTBS supplemented with 1% nonfat powdered milk. Blots were washed several times in TTBS and the peroxidase-conjugated anti-rabbit antibody was added (final dilution 1:2000) for 2 h. Finally, membranes were washed several times in TTBS and the complexes were detected using the enhanced chemiluminescence detection method ECL$^+$ according to the manufacturer's recommendations (Amersham Biosciences), and subsequent exposure of the membranes to Biomax MR film (Kodak).

Antimicrobial Activity Assay of Human Semen

Freshly lyophilized cationic extracts were resuspended in Mueller Hinton Browth (MHB) culture medium (AES Laboratoires, Combourg, France) for antimicrobial activity assays. *Bacillus megaterium* (strain MA), *Micrococcus luteus* (strain A270), and *Escherichia coli* (strains D22 and 363), kindly provided by Dr. M.H. Metz-Boutigue (INSERM U.338, Stras-

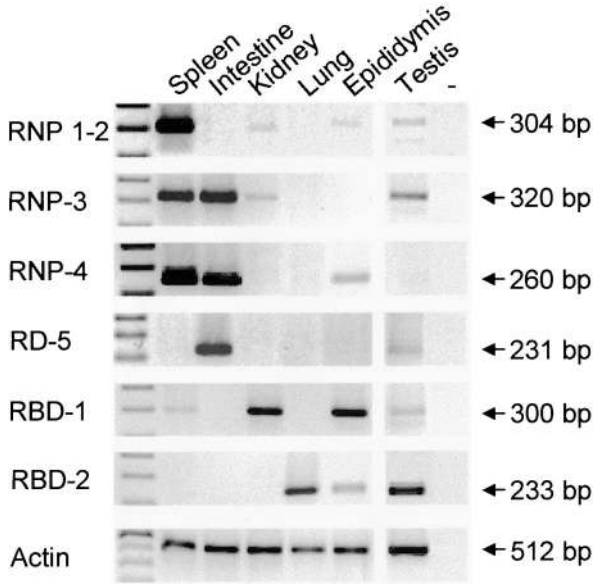

FIG. 1. RT-PCR analysis of defensins expression in rat epididymis and testis (negative view). Amplifications from total RNA of spleen (RNP1-2, RNP-3, and RNP-4), small intestine (RD-5), kidney (RBD-1), and lung (RBD-2) were performed as positive controls. Control amplification was performed with no cDNA as a template (−). The integrity of the cDNA was verified with β-actin amplification. Arrows indicate the size of each amplification product. The results presented are representative of three to five independent experiments performed with different batches of tissues.

bourg, France) were stored frozen in 20% glycerol at −80°C. Bacteria were grown to the stationary phase overnight in MHB at 225 rpm and 37°C. Exponential-phase cultures were further prepared by diluting the overnight culture at 1:100 in fresh MHB and incubating them at 225 rpm and 37°C until an A_{620nm} of 0.4 to 0.6 was reached. Then, 100 μl of a midlogarithmic phase culture of bacteria with a starting absorbance of 0.001 at 620 nm were incubated in microtiter plates in the presence of 0.01 to 10 μg of crude human seminal plasma or cationic extract from human seminal plasma previously resuspended in 10 μl of MHB. Microbial growth was assessed by the increase of A_{620nm} after 16 h of incubation at 37°C with gentle stirring at 190 rpm. The A_{620nm} value of control cultures growing in the absence of test mixtures was taken as 100% growth.

RESULTS

Identification of Defensin Transcripts in Rat Testis and Epididymis

RT-PCR analyses were performed to detect defensin mRNAs in rat testis and epididymis. Spleen, intestine, kidney, and lung were used as positive controls, respectively, for α-defensin rat neutrophil peptide 1–2, -3, and -4 (RNP1–2, RNP-3, and RNP-4) [26]; for enteric rat defensin-5 (RD-5) [27]; and for rat β-defensin-1 and -2 (RBD-1 and RBD-2) [28] (Fig. 1). The α-defensin RNP1–2 transcript was detected by an expected 304-base pair (bp) DNA fragment with a high intensity in the spleen. Much weaker signals were revealed in the kidney, epididymis, and testis. Expression of the α-defensin RNP-3 mRNA was visualized with high intensity in the spleen and intestine by the presence of an expected 320-bp DNA fragment. A signal of lower intensity was found in the kidney and the testis. The presence of the α-defensin RNP-4 transcript was evidenced by the detection of the expected 260-bp DNA fragment, with an intense signal in the spleen and intestine, and with a more discrete one in the epididymis. The expected 231-bp DNA fragment corresponding to the α-defensin RD-5 mRNA was detected in the intestine and with a lower intensity in the testis. The expected 300-bp DNA fragment

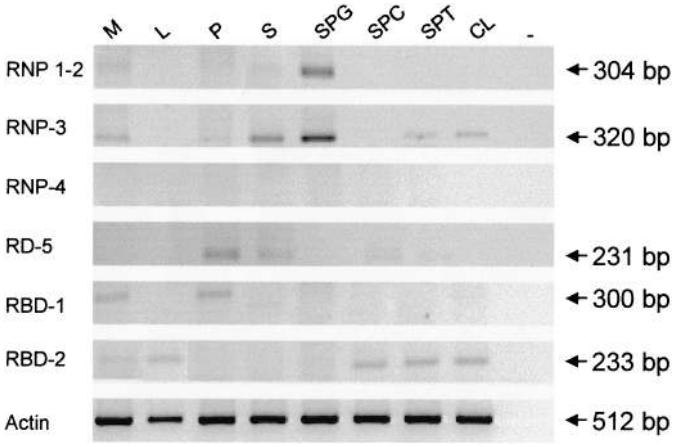

FIG. 2. RT-PCR analysis of defensin expression in rat isolated testicular cells (negative view). Amplifications of defensin transcripts RNP1-2, RNP-3, RNP-4, RBD-1, and RBD-2 were performed with total RNA from testicular macrophages (M), Leydig cells (L), peritubular cells (P), Sertoli cells (S), spermatogonia (SPG), pachytene spermatocytes (SPC), early spermatids (SPT), and cytoplasmic lobes (CL). Control amplification was performed with no cDNA as a template (−). The integrity of the cDNA was verified with β-actin amplification. Arrows indicate the size of each amplification product. The results presented are representative of three to five independent experiments performed with different batches of cells.

corresponding to the β-defensin RBD-1 transcript was evidenced at high levels in kidney and epididymis, and much weaker levels in spleen and the testis. Expression of the β-defensin RBD-2 mRNA was found in lung, epididymis, and testis by the detection of the expected 233-bp DNA fragment. The distribution of the different defensins in the nonreproductive organs used as positive controls is entirely consistent with the literature [26–28].

Identification of Defensin Transcripts in Rat Isolated Testicular Cells

To investigate the specific cellular distribution of rat defensins within the testis, RT-PCR analyses were performed on RNAs prepared from isolated rat testicular cells (Fig. 2). An amplification fragment corresponding to RNP1–2 was clearly visible in spermatogonia by the presence of an expected 304-bp DNA product. Extremely low amplification of these fragments were also consistently detected in testicular macrophages and Sertoli cells. The RNP-3 amplification product with an expected 320-bp DNA fragment was present in Sertoli cells, spermatogonia testicular macrophages, peritubular cells, early spermatids, and in the cytoplasmic lobes of elongated spermatids. In accordance with our previous results on the total testicular extracts, we failed to detect any amplification product corresponding to RNP-4 in isolated rat testicular cells. The testicular cells that express RD-5 were shown to be peritubular cells and Sertoli cells, as evidenced by the presence of low levels of an expected 231-bp DNA fragment. Signals generated by an expected 300-bp amplification product corresponding to RBD-1 mRNA were also consistently detected in testicular macrophages and peritubular cells, but not in any of the germ cells studied. A low abundance RBD-2 transcript was present in testicular macrophages, Leydig cells, pachytene spermatocytes, early spermatids, and cytoplasmic lobes with an expected DNA fragment of 233 bp.

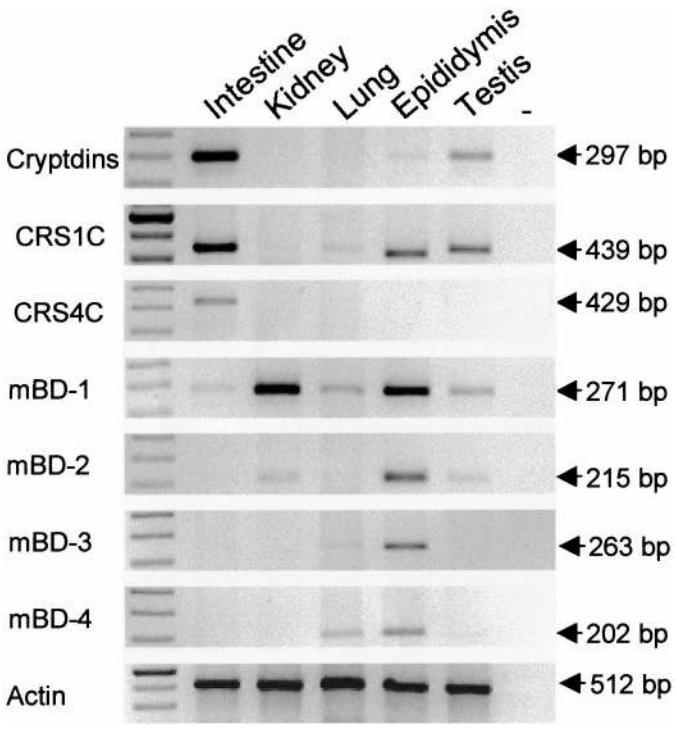

FIG. 3. RT-PCR analysis of defensin expression in mouse epididymis and testis (negative view). Amplifications were performed from total RNA of small intestine (cryptdins CRS 1-C and CRS 4-C), kidney (mBD-1 and mBD-2), epididymis (mBD-3), and lung (mBD-4) as positive controls. Control amplification was performed with no cDNA as a template (−). The integrity of the cDNA was verified with β-actin amplification. Arrows indicate the size of each amplification product. The results presented are representative of three to five independent experiments performed with different batches of tissues.

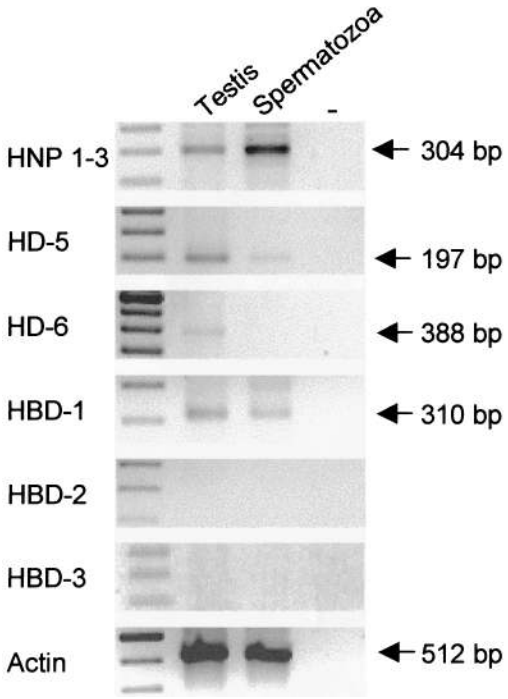

FIG. 4. RT-PCR analysis of defensin expression in human testis and ejaculated spermatozoa (negative view). Amplifications were performed from total RNA of testis and spermatozoa for all known human defensins (HNP1-3, HD-5, HD-6, HBD-1, HBD-2, and HBD-3). Control amplification was performed with no cDNA as a template (−). The integrity of the cDNA was verified with β-actin amplification. Arrows indicate the size of each amplification product. Control amplification was performed using total RNA from a Caco-2 cell line for the β-defensins HBD-1, HBD-2, and HBD-3 (data not shown). The results presented are representative of three to five independent experiments performed with different batches of tissues or cells.

Identification of Defensin Transcripts in Mouse Testis and Epididymis

The study of defensin expression in mouse testis and epididymis was performed using RT-PCR analysis (Fig. 3) using small intestine, kidney, epididymis, and lung as controls for the α-defensin cryptdins [29] cryptdin-related sequences 1C and 4C (CRS1C and CRS4C) [30], for mouse β-defensin-1 and -2 (mBD-1 and mBD-2) [31, 32], mouse β-defensin-3 (mBD-3) [33], and mouse β-defensin-4 (mBD-4) [34]. Cryptdin transcripts were detected at a high level in the small intestine, and much more weakly in the epididymis and testis with a signal of an expected size of 297 bp. The expression of CRS1C mRNA was visualized by the presence of the expected 439-bp DNA fragment in the intestine, lung, epididymis, and testis. The expected 429-bp amplification product corresponding to CRS4C was seen only in the intestine. A 271-bp product corresponding to mBD-1 was amplified at high levels from the kidney and epididymis, and at lower levels in the intestine, lung, and testis. Strong expression of mBD-2 was found by the presence of an expected 215-bp DNA fragment in the epididymis, whereas lower levels were seen in the kidney and testis. Finally, mBD-3 and mBD-4 transcripts were detected in lung and epididymis by the amplification of 263-bp and 202 bp products, respectively.

Identification of Defensin Transcripts in Human Testis and Ejaculated Spermatozoa

RT-PCR analysis was used to search for defensin transcripts in human testis and ejaculated spermatozoa (Fig. 4). All the human defensin transcripts investigated (except HBD-2 and HBD-3) were detected in the testis by the amplification of 304, 197, 388, and 310 bp DNA fragments, which correspond to the α-defensin human neutrophil peptide 1–3 (HNP1–3), human defensin-5 (HD-5), HD-6, and to the β-defensin HBD-1, respectively. In spermatozoa, we detected HNP1–3, HD-5, and HBD-1 amplification products but not HD-6, HBD-2, or HBD-3. Control RT-PCR experiments were performed for HBD-2 and HBD-3 using the unstimulated Caco-2 and phorbol 12-myristate 13-acetate (MPA)-stimulated A549 cell lines that validate our choice of primers (data not shown).

Immunolocalization of HBD-1

Immunolocalization of HBD-1 in human testis and ejaculated sperm was determined using a previously validated polyclonal antibody raised against HBD-1 [17]. In testicular sections, specific staining was observed in the testicular interstitial compartment and in seminiferous tubules (Fig. 5, A and B). In interstitial tissue, the specific staining was clearly located in Leydig cells (Fig. 5A), where in seminiferous tubules the signal was observed in pachytene spermatocytes, early spermatids, and with a particularly high intensity in elongated spermatids (Fig. 5B). No signal was detected in sections incubated with preimmune serum instead of the anti-HBD-1 polyclonal antibody (Fig. 5C).

In sperm smears, specific HBD-1 staining was observed

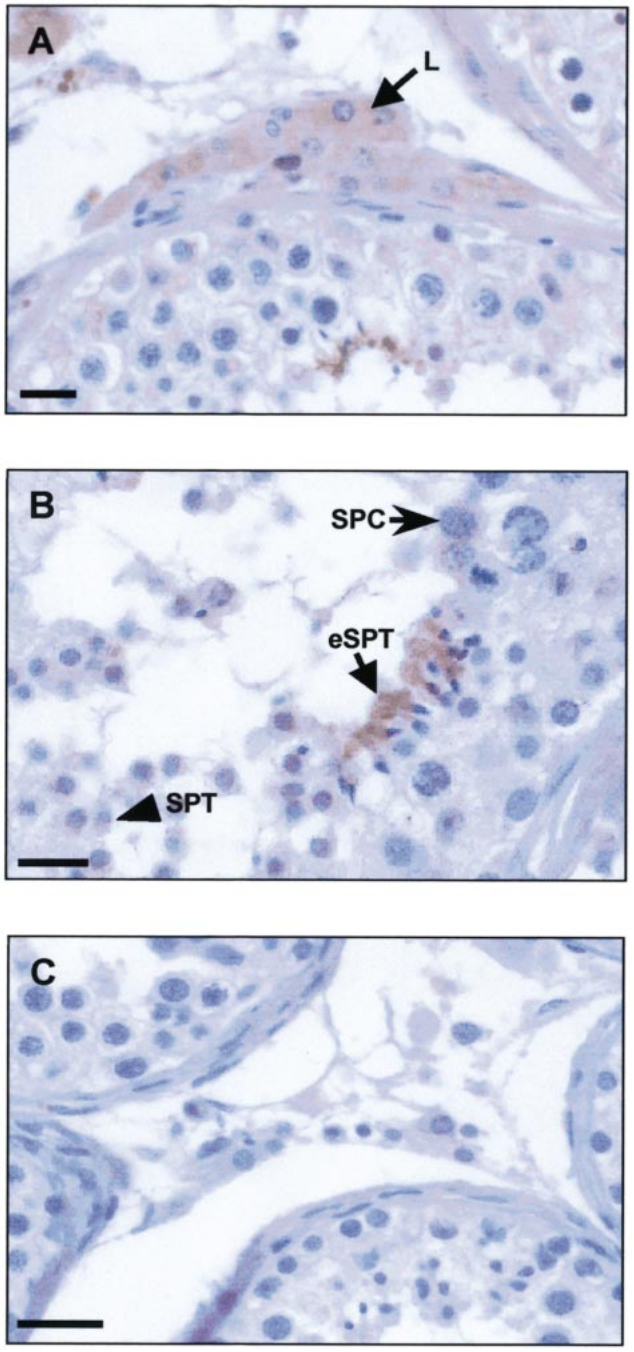

FIG. 5. Immunolocalization of the beta-defensin HBD-1 in human testis sections. An anti-HBD-1 rabbit polyclonal antiserum was used at a dilution of 1:500 and immunoreaction was revealed with diaminobenzidine. Sections were counterstained with Masson hemalun. **A**) In the interstitium, immunostaining was also visualized in Leydig cells (L). Bar = 25 μm. **B**) Discrete immunolabeling was detected within the seminiferous tubule in pachytene spermatocytes (SPC), whereas early spermatids (SPT) and elongated spermatids (eSPT) displayed a strong signal. Bar = 25 μm. **C**) Negative control was performed using the corresponding preimmune serum. Bar = 50 μm.

on the lower head portion of spermatozoa, and diffuse specific staining was also seen in seminal plasma (Fig. 6, A vs. B).

In parallel experiments to detect HBD-2 using a specific polyclonal antibody [35], no testicular labeling was observed (data not shown).

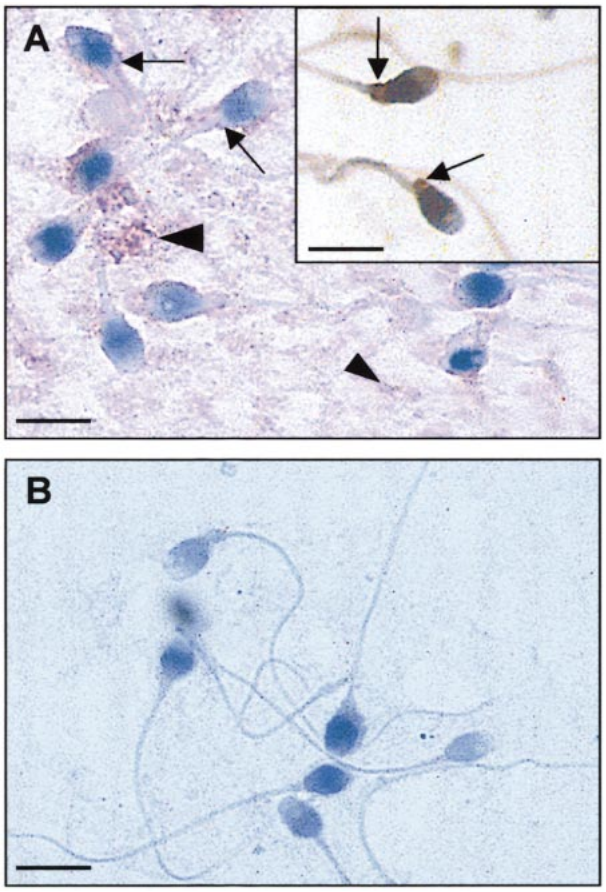

FIG. 6. Immunolocalization of the beta-defensin HBD-1 in human sperm smears. An anti-HBD-1 rabbit polyclonal antiserum was used at a dilution of 1:500 and immunoreaction was revealed with AEC or diaminobenzidine (insert). Smears were counterstained with Masson hemalun. **A**) Very localized immunolabeling was detected in spermatozoa (arrow), whereas the seminal plasma was strongly stained (arrowhead). Bar = 5 μm. Insert, close view of positive immunolabeling on the lower head portion of spermatozoa (arrow). Bar = 5 μm. **B**) Negative control was performed using the appropriate preimmune serum. Bar = 5 μm.

Western Blot Analysis of HBD-1 in Human Seminal Plasma

Western blots of cationic extracts concentrated from human seminal plasma were performed in order to ascertain the presence of soluble HBD-1 (Fig. 7). Four isoforms of HBD-1 were detected. The predominant band appeared to correspond to the 44-amino acid (aa) form of HBD-1 as assessed by comparison with a recombinant HBD-1 peptide resolved in parallel. The upper band most probably corresponds to the 47 aa form of HBD-1 by comparison with the two HBD-1 isoforms (44 and 47 aa, respectively) produced by the control Caco-2 cell line [17].

Antimicrobial Activity Assay of Human Semen

In preliminary studies, antimicrobial activity of human seminal plasma was assessed using *E. coli* D22 and 363 strains, *M. luteus* A270 strain, and *B. megaterium* MA strain (data not shown). *B. megaterium* was chosen for detailed studies because it appeared to be the most sensitive to antimicrobial activity in seminal plasma. Human seminal plasma was found to be microbicidal to *B. megaterium* in a concentration-dependent manner (Fig. 8A). This activity

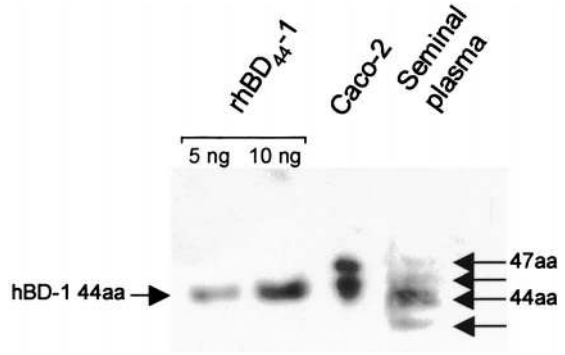

FIG. 7. Detection of hBD-1 in the cationic fraction of human seminal plasma. Cationic peptides from seminal plasma were subjected to AU-PAGE analysis followed by immunoblotting and probing with anti-hBD-1 antibody. A recombinant 44 amino acid form of hBD-1, $rhBD_{44}$-1 at 5 and 10 ng, was used as a reference. HBD-1 forms in seminal plasma are indicated by arrows on the right.

was at least 10 times augmented in the cationic extracts prepared from this material compared with the activity generated by the crude fraction of seminal plasma. The antimicrobial activity of the cationic extract was much lower in the presence of 20 mM DTT, a reducing agent for disulfide bonds (Fig. 8B), whereas it was partially diminished in the presence of 150 mM NaCl, a classical inhibitor of β-defensin antimicrobial peptides (Fig. 8B).

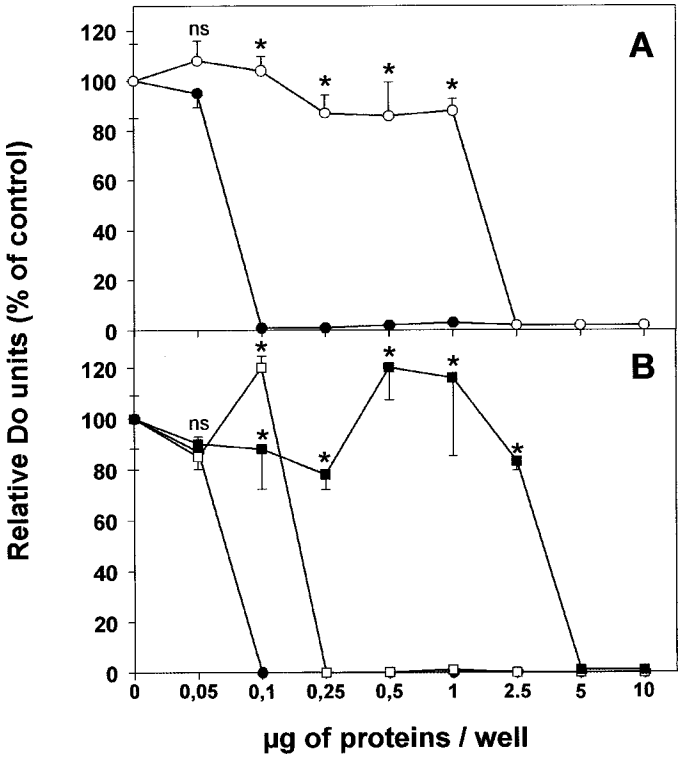

FIG. 8. An antibacterial assay of human seminal plasma and its cationic extract activity against *B. megaterium* (MA strain). **A**) Growth inhibition was determined in triplicate after 16 h of incubation with different amounts of protein extracts from crude human seminal plasma (open circles), and the cationic extract of seminal plasma (closed circles). **B**) Growth inhibition was also determined for the cationic extract of seminal plasma (closed circles) in the presence of 150 mM NaCl (open squares) or 20 mM DTT (closed squares). Each plot is the mean ± SEM of 3 replicate A_{620nm} determinations. ns, nonsignificant; $*P < 0.001$ compared with the respective control as determined by the Student *t*-test.

DISCUSSION

Innate immunity constitutes a first line of defense against an initial challenge by pathogens [36]. Increasing evidence has accumulated for the major role played by cationic antimicrobial peptides in preventing the onset of infection in many organisms [37, 38]. Defensins in mammals, and particularly in humans, have been studied in great detail over the past 10 years, and their presence and bioactivity has now been established in several organs and fluids [10]. In this context, it is particularly noticeable that no systematic study has been reported to search for such peptides in the male reproductive tract and in particular in the testis, an organ known to have developed a potent anti-infectious defense system [3].

To our knowledge, a systematic approach involving the study of the expression and distribution of all known defensins in a given species and in three different species such as the present study has never before been undertaken, either in the testis or epididymis, or in any other tissue or organ. Our RT-PCR results show that in rat testis, all known α- and β-defensins are expressed with the exception of RNP-4, whereas rat epididymis expresses α-defensins RNP1–2, RNP-4, and β-defensins RBD-1 and RBD-2.

In mice, our results are consistent with those of Grandjean et al. who found the cryptdin-1, -2, -3, and -6 group in mouse testis using immunohistochemistry [39]. Our study also reveals that CRS1C, mBD-1, and mBD-2 are present in the testis as well as in the epididymis, whereas the murine β-defensins mBD-3 and mBD-4 were expressed only in the epididymis and the α-defensin CRS4C was absent in the testis and epididymis.

The recent identification in the rat epididymis of a new peptide—Bin1b—that exhibits structural characteristics and antimicrobial activity similar to that of β-defensins [40], together with the present data on defensin expression in rat and mouse epididymis indicates that this secretory organ is an important site of antimicrobial peptide production in the male reproductive system. In addition to the defensins identified here and to Bin1b, the human cationic antimicrobial protein-18 (hCAP-18) was identified in the epithelium of human epididymis [41]. Finally, a recent work suggests that the human EP2 gene codes at least nine mRNA variants that are specifically expressed in the epididymis, among which are β-defensin-like variants [42]. That the epididymis could be a key site for antimicrobial activity is compatible with its function of protecting spermatozoa during their transit and storage.

Transcripts for all known human defensins were shown to be expressed by the human testis, with the consistent exception of HBD-2 and HBD-3. The absence of the latter contrasts with the results of Bals et al. [35], who noted a very faint expression of HBD-2 mRNA within the testis. HBD-2 peptide is well known to be an inducible defensin (for a review see [43]). Because the suppliers offer no information about the origin and physiological status of the tissue samples used to generate RNA master blots, it cannot be excluded that the testicular sample analyzed by Bals et al. was inflamed, thus explaining the presence of low levels of the HBD-2 transcript. The HBD-1 mRNA was recently localized at very low levels in the human prostate and testis, together with those of α-defensins HNP1–3 [44]. The present data on HNP1–3 is in accordance with this latter study. However, in contrast to our findings, the authors failed to detect HD-5 and HD-6 defensins in the human

testis. The discrepancy may result from the high interindividual variability of testicular defensin expression that was consistently observed in the present study for HBD-1. This variability was observed using immunohistochemistry, Western blot analysis, and even RT-PCR, as our results originate from multiple experiments performed on numerous independent testicular and semen samples. In addition, our own positive RT-PCR results concerning the expression of HNP1–3 transcripts in the whole human testis, together with the data of Zhao et al. [44] may be reconciled with our immunohistochemistry experiments using a commercial anti-HNP1–3 (Bachem, Bubendorf, Switzerland) that failed to detect the peptides in any testicular cell, whereas a strong specific signal was observed within blood vessels irrigating the interstitial compartment (data not shown).

Due to the lack of availability of human testes for preparing isolated cells, we focused on rat testes in order to study the cellular distribution of the testicular defensins. In the interstitium, resident macrophages express most of the defensins studied, whereas the testosterone-producing Leydig cells express only the β-defensin RBD-2, suggesting the preeminence of the former in the antimicrobial defense within this compartment of the testis. Compared with the latter compartment, the seminiferous tubule appears to be very well equipped to react to a bacterial attack because its different cellular constituents express all studied defensins with the unique exception of α-defensin RNP-4. In fact, peritubular cells and Sertoli cells express most of the α- and β-defensins studied, whereas the germ cell line expresses only a limited number of defensins. Thus, spermatogonia express only α-defensins, but at relatively high levels. Pachytene spermatocytes express only β-defensins, whereas spermatids and their cytoplasmic lobes express both types. The variety of defensins expressed by the Sertoli and peritubular cells most probably has a self-defense function but could also play a role in the protection of the germ cells against pathogens, which is indeed of prime importance because their infection could result in the total destruction of the germ line. We hypothesize that the complementary pattern of expression of defensins with different spectra of activities between the different somatic and germ cells is likely to generate an efficient antimicrobial defense network system within the seminiferous tubule.

We also demonstrate that HBD-1 is expressed in the human germ line from pachytene spermatocytes to late spermatids. Furthermore, the expression of HBD-1 was also located in the human Leydig cell cytoplasm, strongly suggesting that a key function such as testosterone production occurs under direct antimicroorganism protection, an observation previously made by us with antiviral proteins [5]. Regardless of human or rat tissues, the resurgence of expression of a strong antimicrobial system in late spermatids is probably indicative of the need for spermatozoa, which are no longer protected by testicular somatic cells, to contribute to their own defense while being transported within the male excurrent ducts and then in the female reproductive tract. In this context it is important to note the demonstration of the presence of the transcripts for defensins HD-5, HBD-1, and HNP1–3 in human ejaculated spermatozoa. Because human semen from normal donors often contains leukocytes (for a review see [45]), we cannot exclude the possibility that the HNP1–3 transcripts evidenced here in the spermatozoa fraction resulted from the presence of neutrophils that constitutively express HNP1–3 [11]. Indeed, neutrophils were traced by RT-PCR in the HNP1–3 positive ejaculates at our disposal using a couple of probes specific from the leukocyte common antigen CD45 (data not shown). In contrast, the presence of HBD-1, the only defensin for which we could access an antibody, was unequivocally demonstrated in spermatozoa by immunocytochemistry, more precisely at the lower head portion of the cells. Of note is that neutrophils present in the ejaculates also displayed a marked labeling for HBD-1 (data not shown). A strong specific labeling for HBD-1 was also observed in the seminal plasma where multiple soluble forms of the defensin were evidenced. This most probably reflects variable N-terminal proteolytic processing of the peptide [17, 46].

That human seminal plasma displays antibacterial properties has been known for decades, although the active agent or agents had not been identified [47, 48]. That activity of the cationic extract of seminal plasma prepared here was partially reduced by NaCl probably indicates that HBD-1 and other possible β-defensins present in the cationic extract contribute to their antibacterial activity, as the latter are salt-sensitive [49, 50]. The moderate salt concentration that prevails in the semen [51] is highly favorable to defensin-type activities. However, our data also suggest that other molecules with antibacterial properties that have not yet been characterized are present in the enriched cationic extract of the sperm. In this respect, the present study clearly demonstrates the presence of another defensin transcript (HD-5) in ejaculated sperm.

What could be the origin of the seminal HBD-1 forms? Because HBD-1 is present in spermatozoa and less mature germ cells, and because HBD-1 is known to be secreted [17], the germ line could represent a significant source of this defensin. Furthermore, a recent study demonstrates the widespread expression of HBD-1 transcript in various human organs, mostly secretory glands (kidney, salivary glands, trachea, placenta, prostate, testis . . .) and epithelial cells derived from trachea, bronchi, small airways, and the mammary gland [44]. It is therefore quite possible that the peptide is also produced along the male reproductive tracts by at least other accessory glands besides the prostate, whose secretions contribute to the constitution of seminal plasma. Moreover, the presence of antibacterial substances in prostatic fluid was also suggested [52, 53]. In order to clarify this, the possible production of HBD-1 by the epididymis, seminal vesicles, and prostate is currently under investigation in our laboratory. In any case, the antimicrobial activity demonstrated in the seminal plasma ensures a complementary protection to the self-protecting equipment of spermatozoa.

Beside their antimicrobial activities, additional roles of defensins, such as recruitment of inflammatory cells and wound healing [36], could be significant in the physiology and pathology of testicular function.

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