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Pathogenic Consequences in Semen Quality of an Autoimmune Response against the Prostate Gland: From Animal Models to Human Disease

Ruben D. Motrich,* Mariana Maccioni,* Andres A. Ponce, † Gerardo A. Gatti,* Juan P. Mackern Oberti,* and Virginia E. Rivero 2*

We have recently proposed an autoimmune etiology in ~35% of chronic nonbacterial prostatitis patients, the most frequent form of prostatitis observed, because they exhibit IFN-γ-secreting lymphocytes specific to prostate Ags. Interestingly, this particular group of patients, but not the rest of chronic nonbacterial prostatitis patients, also presented striking abnormalities in their semen quality.

In this work, we use an experimental animal model of autoimmune prostatitis on Wistar rats developed in our laboratory to investigate when, where, and how sperm cells from autoimmune prostatitis individuals are being damaged. As in patients, a marked reduction in sperm concentration, almost null sperm motility and viability, and an increased percentage of apoptotic spermatozoa were detected in samples from animals with the disease. Prostate-specific autoantibodies as well as elevated levels of NO, TNF-α, and IFN-γ were also detected in their seminal plasma. In contrast, epididymal spermatozoa remain intact, indicating that sperm damage occurs at the moment of joining of prostate secretion to sperm cells during ejaculation. These results were further supported by experiments in which mixture of normal sperm cells with autoimmune seminal plasma were performed.

We hypothesize that sperm damage in experimental autoimmune prostatitis can be the consequence of an inflammatory milieu, originally produced by an autoimmune response in the prostate; a diminished prostate functionality, evidenced by reduced levels of citric acid in semen or by both mechanisms simultaneously. Once more, we suggest that autoimmunity to prostate may have consequences on fertility.

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The past decade has seen a resurgence of interest and exciting new research on chronic prostatitis and related syndromes (1). One important reason for this enthusiasm is the recognition that chronic prostatitis syndromes represent an important worldwide health care problem (2). Chronic bacterial prostatitis is characterized by uropathogenic infections of the prostate gland that respond well to antimicrobial treatment (3). In contrast, chronic nonbacterial prostatitis (CNBP)3 (now classified as NAG syndrome) is characterized by uropathogenic infections of the prostate gland that respond well to antimicrobial treatment (3). In contrast, chronic nonbacterial prostatitis (CNBP)3 (now classified as NAG syndrome), which accounts for 90–95% of prostatitis cases, is of unknown etiology and is characterized by the presence of a mixture of pain, urinary, and ejaculatory symptoms with no uniformly effective therapy (4–6). A rationale treatment is urgently required, because until now the goal therapy for these patients has not had good results, and it has focused on amelioration of symptoms rather than on a cure (7). One reason for the lack of significant progress toward the understanding of its etiology, pathophysiology, and therefore the design of rationale therapies is the absence of adequate animal models in which to study the disease process.

Recently, we have analyzed a group of patients with CNBP to search for the presence of a possible autoimmune response to prostate Ags. We demonstrated the existence of IFN-γ-secreting lymphocytes that proliferate in response to known human prostate Ags such as prostate-specific Ag and prostatic cancer phosphatase (PAP) in 34% of patients with CNBP (8). Only this group of patients showed significantly elevated levels of cytokines such as IFN-γ, IL-1, and TNF-α in their seminal plasma, arguing for a local inflammation of noninfectious cause.

The relationship between chronic prostatitis and infertility has been controversial for many years with some reports arguing for a positive relationship between chronic prostatitis and altered semen quality (9, 10), whereas others showed no alterations (11, 12). One possible explanation of such contradictory findings could be that CNBP comprises an heterogeneous group of patients and, as we and others have described (8, 13, 14), that the presence of autoimmune response against prostate Ags is detected only in a subset of patients with CNBP. We have also reported major abnormalities in some of the ejaculate parameters only in CNBP patients who presented signs of an autoimmune response against the prostate gland (15). When the comparative analysis of sperm parameters was done considering all patients enrolled (CNBP patients with and without an autoimmune response against prostate), nonsignificant differences were found. These findings allowed us to assume that these significant differences could have been masked in the other mentioned studies. Therefore, we hypothesize that the autoimmune response to prostate Ags and the inflammatory environment elicited could have detrimental consequences on sperm quality.

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3 Abbreviations used in this paper: CNBP, chronic nonbacterial prostatitis; PAP, prostatic acid phosphatase; EAP, experimental autoimmune prostatitis; PSBP, prostate steroid-binding protein; SI, stimulation index; HOS, hypoxosmotic swelling; PL, prolidium iodide; ROS, reactive oxygen species.
From the results obtained in chronic autoimmune prostatitis patients, some questions arose such as when, where, and how the sperm cells are being damaged and which are the putative mediators and pathogenic mechanisms involved.

Animal models of many human diseases have been studied for many years and have provided a great deal of information about mechanisms involved in the autoimmune response. Our laboratory has developed over the past decade a rat and a mouse model of experimental autoimmune prostatitis (EAP) that is characterized by an organ specific mononuclear infiltration and a cellular and humoral autoimmune response against prostate Ags (16–19). Prostate steroid-binding protein (PSBP) has been described as the major autoantigen involved in the autoimmune response (19, 20).

Although very little is known about the immunology of human disease, most of the features already reported on it, are shared with those of our animal model. The type of infiltration characterized by intra-acinar mononuclear cells and the presence of IFN-γ-secreting T cell response against prostate Ags are the main aspects shared by human and animal diseases. In contrast, the Ab response, which reaches important levels in the animal model, is not detected in the human disease.

Moreover, as further characteristics are being described in human patients, the validity of EAP as an animal model of the disease is being corroborated. In the present work, we used the animal model to respond some unanswered questions and to advance on the underlying mechanisms and pathogenic consequences of autoimmunity against prostate Ags.

### Materials and Methods

**Animals and immunization**

Sexually mature Wistar rats were bred and maintained under specific pathogen-free conditions in the vivarium of the Center of Immunology and Biochemical Chemistry Research of the National University of Cordoba (Cordoba, Argentina). Fifty-five 2-mo-old male rats were included in our study. Animals were maintained in a 16-h light, 8-h dark cycle at 20°C. Animals and immunization were achieved by the injection of saline solution with food and water ad libitum. The experimental protocol and all the performed tests were carefully reviewed and approved by the institutional review board.

Animal immunization was achieved by the injection of saline solution plus CFA (Sigma-Aldrich; group I; n = 17), 1 mg of prostate extract plus CFA (group II, n = 20) or 120 µg of purified PSBP plus CFA (group III; n = 18), on days 0, 20, and 35. Animals received four intradermal injections in different places: right footpad; left footpad; base of the tail; and shoulders.

**Ags and reagents**

Prostate extracts were prepared from Wistar rat prostate glands. Pooled glands from 50 rats were homogenized in 0.01 M PBS, pH 7.2, with protease inhibitors in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 100,000 × g for 30 min, and the supernatant was used as Ag. Protein concentration was determined using the Folin phenol reagent. The preparations were aliquoted and kept frozen at −20°C.

PSBP was purified following the procedure described previously by Chen et al. (21). Briefly, ventral prostates were removed in a sterile manner from Wistar male rats (body weight, 250–300 g) and homogenized in 20 mM Tris-HCl using an Ultra-Turrax homogenizer. The cytosolic fraction was obtained after 60 min of centrifugation at 100,000 × g and 4°C. The resultant supernatant was applied onto a Mono-Q FPLC column. Proteins were eluted with a linear 0–60% NaCl gradient in 20 mM Tris-HCl. Protein concentration of each fraction was evaluated by measuring its OD_{280}. Each fraction was run under nondenaturing conditions in 15% polyacrylamide gels (SDS-PAGE); then, the gels were stained with Coomassie blue. Fractions containing two bands of 18 and 20 kDa (corresponding to both amino acids, HEPES buffer, penicillin/streptomycin, 50 mM 2-ME, and 10% FCS (Tecghen) and were distributed at a concentration of 1.5 × 10^{3} cells/well in a 0.1-ml volume into flat-bottom 96-well microtiter plates (BD Biosciences). Syngeneic naive spleen cells irradiated at 1500 rad served as APCs. These cells were preincubated for 2 h at 37°C with prostate Ags: prostate extract at 130 µg/ml or purified PSBP at 20 µg/ml or PAP at 20 µg/ml. APCs were then washed twice and added to 0.1 ml of responder cells at 4 × 10^{3} cells/well final cell concentration. All cell combinations were set up in triplicate. Plates were incubated for 4 days at 37°C in water-saturated 7.5% CO2 atmosphere and pulsed for the final 18 h with 1 µCi of methyl-[3H]TdR. Labeled material was automatically harvested and counted in a β plate scanner (Pharmacia). The response was expressed as stimulation index (SI) and as Δcpm. Stimulation indexes were calculated from cpm incorporated in Ag-pulsed cultures/cpm incorporated in cultures with nonpulsed APC. Threshold value for positivity for each Ag assayed (prostate extract, PSBP, and PAP) was set up above the mean SI plus 3 SDs of the SI observed in the control group. Δcpm was calculated from cpm incorporated in Ag-pulsed cultures minus cpm incorporated in cultures with nonpulsed APC.

**Abs against prostate Ags in serum and in semen**

Abs against PE, PAP, or PSBP were titrated by conventional ELISA in multiwell plates (Costar) precoated with 50 µl/well prostate extract at 100 µg/ml, PSBP at 20 µg/ml or PAP at 5 µg/ml in 0.05 M carbonate buffer, pH 9.6. After overnight incubation at 4°C, microwells were washed twice and blocked with 5% BSA (Sigma-Aldrich) in PBS for 2 h at 37°C, rinsed once with PBS-Tween 20 at 0.05%, and then filled with 0.1 ml of serum (obtained after cardiac puncture) or seminal plasma dilution (1/50 or 1/6 respectively) for 1 h at 37°C. To detect specific IgG, plates were again washed and incubated with HRP-conjugated goat anti-rat IgG (Sigma-Aldrich) for 1 h at 37°C. The reaction was developed with o-phenylenediamine-H2O2. OD was measured at 490 nm in a Bio-Rad Plate Reader.

**Semen collection**

Semen samples were obtained by electroejaculation as described previously by Ponce et al. (22). Briefly, nonanesthetized rats were placed in a box that covered the cephalic half of the body while the rest laid on a metallic grid support. After the genital area was cleaned, the prepuce was retracted, and the penis was introduced into an Eppendorf plastic tube. The rectal probe was lubricated, and the bronze bipolar electrode was inserted into the rectum to a depth of 20–30 mm and held by the technician. The alternating current (220 V, 50 cycles/s) was applied for 5 of every 10 s. The current was controlled by a rheostat and was calculated from an oscilloscope reading of the voltage drop across a resistor placed in the circuit. A series of one to five pulses was normally given at the 6- to 6.8-V setting. Immediately after electrostimulation, a white viscous plug was obtained. Immediately after, a series of 1–10 pulses was given at 13–15 V, and the remaining portion of the ejaculate was collected and diluted with 150 µl of Tyrode’s buffer medium without BSA (pH 7.4, 280 mOsm/kg) to avoid coagulation. Functional parameters of spermatozoa were determined within 3–10 min by subjective evaluation in fresh semen.
following standard methods in our laboratory. Seminal plasma was obtained by centrifuging semen at 300 × g for 7 min at room temperature and kept at −80°C until use.

**Fructose, citric acid, and α-glucosidase levels in semen**

Citrate, fructose, and α-glucosidase seminal levels were measured as tests of function of the prostate, seminal vesicles, and epididymis, respectively. Neutral α-glucosidase, fructose, and citric acid were determined according to the World Health Organization semen analysis manual (23). Briefly, for determining neutral α-glucosidase, 4-nitrophenyl-α-D-glucopyranoside was converted into 4-nitrophenol and α-glucosidase and 4-nitrophenol was photometrically measured. The acid isoenzyme was inhibited by addition of 1% SDS and phosphate buffer, pH 6.8, so that only the neutral isoform was measured. Fructose was determined according to the hexokinase method, and citric acid was measured with the UV method using the citrate lyase-catalyzed reaction.

**IFN-γ and TNF-α in seminal plasma**

TNF-α and IFN-γ quantities present in seminal plasma were determined by solid phase sandwich ELISA protocol according to the manufacturer’s instructions (BD Biosciences). Briefly, 96-well plates were coated with primary anti-TNF-α or anti-IFN-γ capture Abs and blocked with PBS-5% BSA. Seminal plasma samples were incubated overnight at 4°C followed by the addition of biotinylated anti-TNF-α-detecting mAb or biotinylated anti-IFN-γ-detecting mAb. Plates were developed by adding avidin peroxidase and its substrate (TMB), and the OD was measured at 492 nm in a microplate reader (Bio-Rad). The amounts of TNF-α or IFN-γ were extrapolated from the standard curve, which was generated in 1/2 dilutions. Results are expressed in picograms per milliliter.

**Measurement of NO levels in seminal plasma**

Nitrite concentration in seminal plasma was measured by using the Griess reagent. Briefly, 0.10 ml of seminal plasma was mixed with 0.2 ml of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) and incubated for 15 min at room temperature in the dark. OD was measured at 540 nm, and nitrite concentration was calculated using a calibration curve. Results are expressed as micromols per milliliter.

**Epididymal sperm obtainment**

Animals were killed by CO₂ asphyxiation followed by cervical dislocation. The epididymis was removed, trimmed of fat, and clamped at the corpus-cauda junction. Rat spermatozoa were obtained by chopping cauda epididymis with a sharp pair of scissors, allowed to disperse, and homogenized in 5 ml of a microplate reader (Bio-Rad). The amounts of TNF-α or IFN-γ were extrapolated from the standard curve, which was generated in 1/2 dilutions. Results are expressed in picograms per milliliter.

**Assays to determine sperm concentration, motility, viability, and sperm response to the hypoosmotic swelling (HOS) test**

Sperm concentration and motility were assessed at 23 ± 2°C in a Makler counting chamber (Seif Medical Instruments) under an inverted microscope at ×200 magnification. The results are expressed as the percentage of motile cells (progressive plus nonprogressive spermatozoa). No fewer than 200 gametes were examined.

The viability parameter was evaluated by supravital staining with Hoechst 33258 (Calbiochem). Using the appropriate UV fluorescence optics (AxioLab; Zeiss), spermatozoa showing brightly fluorescence nuclei were scored as dead, and sperm cells that excluded the Hoechst 33258 and were not fluorescent were scored as viable. The viability of 200–250 cells was assessed.

The HOS test evaluates whether an intact membrane is biochemical and functionally active. The procedure used for the HOS test was similar to the one described by Jeyendran et al. (24). The sperm suspension was mixed with a hypoosmotic solution (100 mOsm/L; Fiske Osmometer G-52), pH 7.4, for 45 min at 37°C. Evaluations were made by phase contrast microscopy at a magnification of ×400; 200 cells or more were observed, and the percentage of spermatozoa that showed tail swelling was determined by dividing the number of reacted cells (×100) by the total number of spermatozoa counted in the same area.

**Assessment of apoptosis by annexin V staining**

Staining for annexin V was performed using an apoptosis detection kit II (BD Pharmingen) containing annexin V conjugated with FITC. Sperm samples were resuspended in binding buffer supplemented with 4 mg/ml BSA (fraction V; A-4503; Sigma-Aldrich) at a concentration of 10 × 10⁶ spermatozoa/ml. From this solution, an aliquot of 100 μl was stained with 5 μl of annexin V-FITC solution and 5 μl of propidium iodide (PI) solution. Samples were incubated in polypropylene tubes for 15 min in the dark, and then 400 μl of binding buffer were added. Samples were analyzed within 1 h. The samples were analyzed using a FACS Star Plus-Flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with standard optics. An argon ion laser (INNOVA 90; Coherent) tuned at 488 nm and running at 200 mW was used as light source. From each cell, forward light scatter (FSC), orthogonal light scatter (SSC), A-FITC fluorescence, and PI fluorescence were evaluated using CellQuest version 3.3 (BD Biosciences). A gate was applied in the FSC-SSC dot plot to restrict the analysis to spermatozoa. For the gated cells, the percentages of annexin V-negative or -positive (A− or A+), and PI-negative or -positive (PI− or PI+), as well as double-positive cells were evaluated, based on quadrants determined from single-stained and unstained control samples.

**Analysis of sperm motility in mixture experiments**

The working medium for analysis of sperm kinetics was modified Krebs-Ringer bicarbonate solution containing 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄, 25.07 mM NaHCO₃, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 4.0 mg/ml BSA, 50 μg/ml streptomycin sulfate, and 75 μg/ml penicillin (Gentamicin). They were equilibrated overnight to pH 7.4 at 37°C in a 5% CO₂ atmosphere and kept at 37°C all through the protocol. Sperms were collected from normal untreated adult rats by electroejaculation. The epididymal spermatozoa were used for the analysis to spermatozoa. For the gated cells, the percentages of annexin V-negative or -positive (A− or A+), and PI-negative or -positive (PI− or PI+), as well as double-positive cells were evaluated, based on quadrants determined from single-stained and unstained control samples.

**Statistics**

Statistical analysis was performed with the Wilcoxon paired test, paired t test, one-way ANOVA followed by Dunnet’s test when the ANOVA test yielded statistical differences (p < 0.05) among experimental groups, and Fisher’s exact test as appropriate. p <0.05 was considered statistically significant.

**Results**

Autoimmune response can be detected systemically and locally in rats bearing experimental autoimmune prostatitis

Autoimmune prostatitis in animals immunized with either prostate extract or PSBP was evidenced by different methods (Fig. 1). As expected, histopathological studies revealed lesions of varying intensity in the prostate gland of almost all experimental animals irrespective of whether the immunizing Ag was prostate extract (group II) or PSBP (group III) (Fig. 1A). Observed lesions were mainly characterized by moderate perivascular cuffing, mononuclear infiltration accompanied by hemorrhage, and edema. Histological alterations were not detected either in prostate samples from animals of group I or in seminal vesicles, testis, and epididymis samples from animals of the three groups under study (Fig. 1A). A positive proliferative response against prostate Ags was also detected only in draining LN cells from prostate extract- and PSBP-immunized animals (Fig. 1B). Indeed, lymph node cells from control animals of group I exhibited low cpm values in response to prostate Ags (group I: Δcpm, 118.32 ± 109.76 against prostate extract; Δcpm, 177.25 ± 111.89 against PSBP; and Δcpm, 165.50 ± 136.95 against PAP). On the contrary, autoimmune animals from groups II and III presented high cpm values and therefore positive stimulation indexes.
group II: Δcpm, 3141.66 ± 722.26 against prostate extract; Δcpm, 3865 ± 877.70 against PSBP; and Δcpm, 2244.66 ± 1148.09 against PAP. Autoantibodies against prostate Ags were also detected in circulating blood (Fig. 1C) and as immune complex depositions in the prostate gland (16). To investigate whether these autoantibodies generated against prostate Ags could somehow be part of the prostatic fluid and thus appear in the ejaculate, we searched for its presence in seminal plasma from animals under study. Indeed, PSBP-specific IgG could be locally detected in seminal plasma of animals from groups II and III.

Taken together, our results clearly indicate that male rats immunized with prostate Ags develop inflammatory lesions in prostatic tissue and produce both a cellular and humoral specific autoimmune response, which can be detected systemically and locally.

High levels of nitric oxide, TNF-α, and IFN-γ are detected in seminal plasma from autoimmune rats

To analyze the presence of local proinflammatory mediators, the levels of NO, TNF-α, and IFN-γ were studied in seminal plasma from control and autoimmune rats as a reflection of the prostate environment. High levels of NO were detected in seminal plasma from autoimmune rats (groups II and III) compared with the levels observed in the control group (group I; p < 0.05; Fig. 2A). Moreover, higher levels of TNF-α were also detected in seminal plasma from autoimmune rats of groups II and III when compared with the levels observed in rats from group I, although only with a significant increase in samples from group II (p < 0.05). When the levels of IFN-γ were evaluated, similar data were obtained (Fig. 2C), evidencing significantly increased levels of this cytokine in seminal plasma of autoimmune animals (p < 0.05). These results suggest that an inflammatory process is taking place in the prostate in prostate extract- and PSBP-immunized animals due to an autoimmune response against prostate Ags.

Prostate biochemical markers are altered in seminal plasma from autoimmune rats

Citric acid and zinc are classical biochemical markers of prostate gland function, as well as fructose for seminal vesicles and neutral α-glucosidase for epididymis (23). These markers are usually assayed...
as sensors of the functionality of the respective glands in human diseases, and many reports show that an inflammatory response can cause impairment of the functions of these glands. Because the existence of abnormalities in these marker levels has not been demonstrated in our model, we investigated whether the inflammatory process that is taking place in the prostate of autoimmune animals could affect some biochemical functional markers. For that reason, we measured citric acid, fructose, and neutral α-glucosidase levels in seminal plasma. No differences were found in the levels of fructose and neutral α-glucosidase when measured in the different groups under study, showing that the immunization performed in groups II and III does not affect the seminal vesicle and epididymis function (Fig. 2D). In contrast, the levels of citric acid, which mirrors prostate gland functionality, were significantly decreased in samples from autoimmune rats from groups II and III (Fig. 2E), indicating an altered function of the gland in autoimmune animals.

These results corroborate our previous data on the specificity of the autoimmune response in this model (19), confirming that only the prostate gland is affected. In other words, the immunization procedure generates an immune response that preserves the normal function of seminal vesicles and epididymis but induces an inflammatory state that to some extent alters the prostate gland function.

**Sperm quality parameters are altered in semen from animals with autoimmune prostatitis whereas epididymal spermatozoa remain intact**

We previously demonstrated that chronic prostatitis patients who evidenced autoimmune response to prostate Ags have altered sperm quality (15). To evaluate whether this also occurs in the experimental autoimmune model, semen samples from animals under study were obtained by electroejaculation, and different parameters such as sperm concentration, total sperm motility, sperm...
viability, and the HOS test were analyzed. For a complete overview of the sperm quality during the development of the prostate-specific autoimmune response, the tests were performed after the second immunization (on day 27; data not shown) and after the third immunization (on day 42; data shown in Fig. 3) of the experimental protocol. In general, all these evaluated parameters were impaired in the autoimmune animals, and they worsened as long as the autoimmune response had progressed and become established. For example, group II (animals immunized with prostate extract that develop a florid autoimmune prostatitis) was the first to show a decrease in sperm concentration on day 27, but, as the immune response progressed (day 42), both groups of autoimmune animals (groups II and III) demonstrated a marked reduction in sperm concentration compared with control animals ($p < 0.01$). At the same time, sperm motility and viability in semen samples from animals under study were assayed. Almost null sperm motility and viability levels were detected in both autoimmune animal groups (groups II and III) when compared with the control group (Fig. 3, B and C). These huge alterations in sperm motility and viability were seen in an earlier stage (after the second immunization) as well as in a later stage (after the third immunization) of the autoimmune response against prostate ($p < 0.01$). Finally, the HOS test, which analyzes whether sperm plasma membrane is intact and also whether it is biochemically and functionally active, was performed. Very low levels of reactive spermatozoa were detected in both groups of autoimmune animals (groups II and III) compared with the control group (Fig. 3D, $p < 0.05$). Our results suggest that the stronger the autoimmune response against prostate the worse are the alterations observed in semen. Certainly, animals from group II, which showed the most severe infiltration and lesions and highest seminal TNF-α and IFN-γ levels, evidenced the most striking alterations in semen quality.

To investigate which is the sperm death process taking place, sperm apoptosis in ejaculate samples was studied. The Annexin V-PI assay, which measures early and late apoptosis, was performed in semen samples obtained on day 42 of the experimental protocol. The annexin V-PI assay identified four categories of cells (Fig. 3E). Higher levels of apoptotic spermatozoa were detected in semen from both autoimmune animal groups (groups II and III) compared with the control group ($p < 0.05$). The frequency of...
early apoptotic cells (A+/PI--) was significantly higher in group III than in group II. However, seminal spermatozoa from group II showed significantly higher percentages of late apoptotic, early necrotic (A+/PI+), and necrotic (A-/PI+) spermatozoa than those in group III (p < 0.05) (Fig. 3E).

Physiologically, spermatozoa genesis and differentiation occur in the testis. In the last steps, spermatozoa mature and are stored in the epididymis. The most mature spermatozoa are kept in the caudal region of the epididymis until the ejaculation, when they join the sex accessory gland fluids to form the semen/ejaculate. Regarding this fact, we wished to investigate where the sperm alterations observed in semen originated. There were two possibilities: 1) that these alterations were the result of epididymal damage as a result of a satellite effect of the prostate inflammation (although we had not previously detected any histological alteration in epididymis or autoantibodies against epididymal or testicular Ags, (data not shown)); 2) that these alterations were a result of damage originated during the encounter of sperm with the prostate fluids during ejaculation. To answer these questions, we also analyzed epididymal sperm quality parameters in immunized animals.

Animals were sacrificed on days 27 and 42, and the same sperm analyses previously performed in semen samples were done in epididymal sperm. Epididymal sperm concentration, motility, and viability revealed no differences between autoimmune animals and control animals either on day 27 (data not shown) or on day 42 (Fig. 4, A–C). In accordance with these results, when the HOS assay was performed, no alterations were observed, revealing normal epididymal sperm cell membrane integrity and functionality in both autoimmune groups compared with the control group (Fig. 4D). As shown in Fig. 4E, when epididymal sperm apoptosis was analyzed, no increase in the percentage of apoptotic or necrotic spermatozoa was found in samples from both autoimmune animal groups (groups II and III) when compared with the control group.

These findings evidenced that the striking alterations observed in semen originated after sperm genesis in testis and its storage in epididymis, suggesting that the inflammation in the prostate, the consequence of an autoimmune response against prostate Ags, could be involved in sperm cell damage. The kinetics of this damage appeared to be quick, and its detrimental effect could seriously compromise the seminal quality of an individual.

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**FIGURE 4.** Epididymal sperm functionality in rats with autoimmune prostatitis. Sperm concentration (A), sperm motility (B), sperm viability (C), HOS (D), and apoptosis by annexin V staining (E) were conducted in epididymal sperm samples of immunized animals. Different parameters of spermatozoa were determined immediately after epididymal sperm obtainment by evaluation following standard methods. All cases, samples were processed individually, and five animals per group were analyzed. *, p < 0.01 vs control group.

**Apoptosis in epididymal sperm**

<table>
<thead>
<tr>
<th>Spermatozoa (%) ± S.E.</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live (A+/PI-)</td>
<td>98.60 ± 0.41</td>
<td>98.92 ± 0.21</td>
<td>98.60 ± 0.20</td>
</tr>
<tr>
<td>Live, early apoptotic (A+/PI-)</td>
<td>0.70 ± 0.31</td>
<td>0.70 ± 0.07</td>
<td>0.55 ± 0.05</td>
</tr>
<tr>
<td>Dead, late apoptotic/early necrotic (A+/PI+)</td>
<td>0.01 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Dead, late necrotic (A-/PI+)</td>
<td>0.69 ± 0.12</td>
<td>1.27 ± 0.15</td>
<td>0.89 ± 0.10</td>
</tr>
</tbody>
</table>
Sperm alterations observed in autoimmune rats occur in a short time and are caused at least in part by cytokines locally produced by the autoimmune response.

The results presented here supported the hypothesis that the alterations observed in the sperm cells occurred once the prostate secretions, loaded with the inflammatory mediators produced as a consequence of the autoimmune response, meet the spermatozoa during ejaculation. However, one can wonder what mechanism is involved in the production of these alterations and whether the damage could occur in such a short time.

To answer this question, the time course of sperm motility was evaluated in semen samples from different groups. As shown in Fig. 5A, sperm motility diminished slowly in semen samples from animals of group I, showing a sperm motility of 60% immediately after ejaculation with a slow drop after 8–9 min. However, marked loss of sperm motility was observed in semen samples from autoimmune animals (groups II and III); this effect was more drastic in samples from group II. This loss in motility was evident both immediately after sperm collection and during the time course of the analysis.

To assay whether some metabolite present in seminal plasma from autoimmune rats could produce these alterations, mixture experiments were performed. As can be seen in Fig. 5B, when sperm cells from normal nontreated animals were mixed with seminal plasma from autoimmune rats, a marked drop in sperm motility was observed. However, when seminal plasma from animals of group I (control group) was added to normal sperm cells, the same slow drop on sperm motility seen in animals from group I was observed, indicating that the immediate contact between sperm cells and an inflammatory seminal plasma can reproduce the detected abnormalities. To investigate the mechanisms involved in the observed alterations, we performed experiments adding rTNF-α to normal sperm at the concentrations detected in seminal plasma from autoimmune rats. The addition of recombinant TNF-α was able to significantly diminish the sperm motility and increase sperm apoptosis of normal spermatozoa. The addition of recombinant TNF-α was able to significantly diminish the sperm motility and increase sperm apoptosis of normal spermatozoa (Fig. 6, A and B). Moreover, when TNF-α-neutralizing Abs were added, this effect was partially blocked (Fig. 6A). Because IFN-γ is a crucial mediator of an inflammatory process, and its presence had been detected in seminal plasma samples from autoimmune CNBP patients (8) and autoimmune animals (Fig. 2C), its direct effect on sperm motility was also investigated. The addition of rIFN-γ was able to significantly reduce the sperm motility and enhance sperm apoptosis of normal spermatozoa (Fig. 6, C and D). Moreover, when IFN-γ-neutralizing Abs were added, this effect was partially blocked (Fig. 6C).

These results allow us to affirm that an inflammatory environment, the result of an autoimmune response directed against the prostate gland, can seriously affect the sperm, having a negative impact in the semen quality of an individual and compromising his fertility.

**Discussion**

Witebsky et al. (27) proposed rationales for the autoimmune basis of clinical disease. They were consciously modeled on Koch's postulates and required an autoimmune response to be recognized.
as either autoimmune- or cell-mediated immunity, the correspond-
ing Ag to be identified, and an analogous autoimmune response to
be induced in an experimental animal. In addition, the immunized
animal must also develop a similar disease. Some years later, Rose
and Bona (28) proposed novel criteria to consider a disease as an
autoimmune disease, based on new information gained from the
use of molecular biology and hybridoma techniques; these include
direct evidence of the transfer of a pathogenic Ab or pathogenic T
cells, indirect evidence based on the reproduction of the autoim-
mune disease in experimental animals, and circumstantial evidence
from clinical clues. These authors emphasized the importance of
the animal models to corroborate the autoimmune nature of a given
pathology. Indeed, animal models of numerous human diseases
have been studied for many years and have provided a great deal
of information about mechanisms involved in autoimmune re-
sponses (29).

The etiology of the most frequent form of chronic prostatitis,
CNBP (National Institutes of Health category III), has proved elusive
despite years of investigation (7). Recently, we have proposed that
CNBP is a heterogeneous syndrome and that the etiology of this dis-
goal involves an autoimmune response against pros-
te of these patients exhibited IFN-\(\gamma\)-secreting lymphocytes
against prostate Ags such as PAP, prostate-specific Ag, and other Ags.
present in prostate homogenates and seminal plasma (8, 13, 14, 30). High levels of proinflammatory cytokines and striking abnormalities in semen parameters were also described in CNBP that exhibited this autoimmune component (15). We consider that Witebsky's postulates can be readily applied in this particular group of patients, arguing for an autoimmune etiology of their disease: they have cellular autoimmune response against prostate Ags; and the disease can be reproduced in rodents. Indeed, our animal model of autoimmune prostatitis shows almost all the previously mentioned characteristics of human chronic autoimmune prostatitis: specific lymphocytes against prostate Ags; the presence of Th1 cytokines in the lymphocyte supernatants; and the type of infiltration and histological lesions seen in the gland (observed in both the animal models and the autoimmune prostatitis patients; Refs. 8, 18, and 31). In this work we demonstrate that many of the abnormalities seen in the semen of chronic autoimmune prostatitis patients are also reproduced in the animal model: markers of local inflammation (increased proinflammatory cytokatoes in seminal plasma); reduction of biochemical markers of prostate gland; and marked alterations of sperm quality, abnormalities detected in both the animal model and in this subset of CNBP patients that evidenced an autoimmune response to prostate gland.

The use of our animal model of autoimmune prostatitis also allowed us to address questions otherwise difficult to answer in the human patients. Among them, it was important to determine when, where, and how sperm cells from autoimmune prostatitis individuals were being damaged. Two possibilities were evaluated: either these alterations were a result of an epididymal damage as a consequence of a satellite effect of the prostate inflammation; or they were the result of a rapid damage originated during the joining of sperm cells with the prostate fluids at the moment of ejaculation. Our findings demonstrate that only ejaculated spermatozoa are being damaged and that apoptosis is involved.

Numerous studies have now shown the presence of nuclear DNA strand breaks and apoptosis in human ejaculated spermatozoa from infertile patients and the abnormal persistence of apoptotic marker proteins (32). Why human spermatozoa possess these abnormalities is still not clear (33). Two processes that have been linked to the presence of DNA strand breaks in spermatozoa are anomalies in apoptosis during spermatogenesis or abnormalities after spermatogenesis.

Many recent studies indicate that oxygen-derived free radicals induce apoptosis to spermatozoa. The excessive generation of these reactive oxygen species (ROS; superoxide, hydroxyl, NO, peroxide, peroxynitrite) by spermatozoa and by contaminating leukocytes associated with genitourinary tract inflammation has been identified in idiopathic male infertility (34). Henkel et al. (35) reported that DNA fragmentation was strongly correlated with ROS production. However, oxidative stress is the result of elevated ROS, depressed total antioxidant capacity, or both. Mammalian spermatozoan membranes are rich in polyunsaturated fatty acids, making them very susceptible to oxygen-induced damage, which is mediated by lipid peroxidation. In a normal situation, the antioxidant mechanisms present in the reproductive tissues and their secretions are likely to quench these ROS and protect against oxidative damage to gonadal cells and mature spermatozoa. The prostate gland contribute with its secretion to the antioxidant capacity present in semen (34), and significant oxidative stress in the semen or prostatic fluid from patients with chronic noninfectious prostatitis has been reported, comparable with levels reported in men with recognized infertility (36). In concordance with these previous reports, we evidenced elevated levels of NO in semen from animals with autoimmune prostatitis. This finding clearly shows that spermatozoa from these animals are exposed to an oxidative stress that could be a cause of the many alterations observed in semen.

Our findings suggest that mediators present in seminal plasma from autoimmune animals were responsible for these alterations. In fact, when seminal plasma from autoimmune rats was added to normal ejaculated spermatozoa, reduction of sperm motility was evident, suggesting that humoral mediator(s) present in seminal plasma from autoimmune animals was/were responsible for the observed effects. ROS may be involved in the effects observed, and indeed, high levels of NO were detected in the seminal plasma of autoimmune rats. Other putative candidates to produce these effects are TNF-α and IFN-γ; certainly, seminal plasma from autoimmune animals contained high levels of these cytokines. When rTNF-α or rIFN-γ were added to normal seminal plasma and then to normal sperm cells, they were able to mirror the observed effects, diminishing motility and enhancing apoptosis. In concordance, their effects could be blocked, at least in part, by neutralizing Abs. However, the decrease in sperm quality observed after recombinant cytokine addition (at doses measured in seminal plasma from EAP animals) does not reach the levels observed when total EAP seminal plasma was added. When both cytokines were put together simultaneously, at doses measured in seminal plasma from EAP animals, neither additive nor synergic effects were observed compared with the single addition (data not shown). Therefore, TNF-α, IFN-γ, NO, and probably other proinflammatory mediators play a role in the induction of the damage described.

TNF-α exerts its effects via two TNF-α-specific membrane-bound receptors, TNFR1 and TNFR2, which are coexpressed in most tissues (37, 38). TNFR1 activation is associated with the activation of kinases and NF-κB and the induction of apoptosis (38). Spermatozoa may be exposed to abnormal levels of TNF-α in the male reproductive tract or during their passage into the female reproductive tract in pathological conditions (39, 40). Some researchers have demonstrated that exposing human spermatozoa to pathological concentrations of TNF-α and IFN-γ can result in a significant loss of their functional and genomic integrity (41). Apoptosis may be a potential mechanism for the occurrence of TNF-α toxic effects. Upon engagement with its receptor, TNF-α activates the TNF-α receptor-associated factor, TNFR-associated death domain, and receptor-interacting protein kinase 1. In turn, caspase-2 and caspase-8 become activated with the end result of effector caspase-3 activation followed by cell death (42). Infliximab, a humanized mAb that blocks specifically TNF-α, reverses the toxic effects induced by TNF-α in human spermatozoa (43). These toxic effects that TNF-α exerts on spermatozoa could possibly be mediated by ROS because TNF-α has the potential to stimulate spermatozoa to generate ROS. In turn, ROS-related sperm membrane peroxidation may occur (44).

It has been reported that sperm cells from human, rabbit, and pig species express IFN-γ receptors, which seem to develop during spermatogenesis in the testes (45). Moreover, signaling members of the IFN-γ pathway like the JAK/STAT proteins, TYK2, STAT1, and STAT4, are present and active in human sperm (46). Dimitrov et al. (47) studied the effects of recombinant cytokines on spontaneous and ionophore-induced acrosome reaction and demonstrated that spermatozoa that underwent capacitation in medium with rIFN-γ showed a significant increase in spontaneous and induced acrosome reaction compared with the control. They postulated that some cases of infertility might result from a defective acrosome reaction caused by products of activated lymphocytes and macrophages that are released into the male and female reproductive tracts. Indeed, increased levels of IFN-γ have been reported in seminal plasma of infertile men and CNBP patients (8, 15, 48). In the present work, we demonstrate the detrimental effect of IFN-γ in sperm motility and viability, evidencing its capability
of inducing sperm apoptosis. Further work is needed to elucidate the mechanisms involved in these phenomena.

Thus, sperm damage in experimental autoimmune prostatitis can be the consequence of several causes; an inflammatory milieu, originally produced by an autoimmune response in the prostate gland, characterized by an enhancement of ROS levels and proinflammatory cytokines in seminal plasma; and a diminished anti-oxidant capacity caused by an impairment of prostate function; or both mechanisms simultaneously.

Our results argue in favor of the importance of prostate functionality and suggest that besides the clinical manifestations in chronic prostatitis patients (pelvic pain, irritating voiding symptoms, effects on sexual function), a group of them who have an autoimmune response against prostate could also have diminished fertility.

Utilization of animal models of autoimmune prostatitis will provide important approaches, which may help to identify the pathogenic mechanisms and also possible therapies for human disease.

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The Disclosures
The authors have no financial interest of interest.

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