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Immune mediators associated to male infertility in a mouse model of DNA immunization with the sperm protease proacrosin

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ABSTRACT

The immune response has relevant physiological functions both in the male and female reproductive system, and must be tightly controlled to achieve a successful pregnancy. Several immune factors have been related to infertility, among them humoral and cellular immune responses triggered by sperm antigens. The present study was aimed at evaluating the immune profile induced by DNA immunization against the sperm protease proacrosin in CF1 male mice and its effect upon fertility.

Immunized animals exhibited higher anti-proacrosin antibodies levels than controls (indirect ELISA), both in serum ($p < 0.01$) and in seminal vesicle fluid (SVF; $p < 0.05$). IgG2a levels were higher than IgG1 in serum ($p < 0.01$) and similar in SVF. IL-10 and TGF-β1 mRNA levels were lower in testis ($p < 0.05$), whereas TNF-α and IFN-γ transcript levels were increased in SV tissue ($p < 0.05$). Immunized mice showed a trend toward higher IFN-γ concentration in serum and SVF than controls. Male fertility rate was diminished in immunized mice ($p < 0.01$) and inversely correlated with serum and SVF anti-proacrosin IgG levels ($p < 0.001$). Immunized animals also had fewer pups born than controls ($p < 0.01$).

To our knowledge, this is the first report on DNA immunization done in CF1 mice. Injection of proacrosin DNA induces an immune response in the male reproductive tract characterized by high levels of specific antibodies and cytokine changes. These factors may alter the crucial balance of the genital tract microenvironment required for adequate fertilization and pregnancy.

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1. Introduction

Numerous reports have associated the immune response to spermatozoa with fertility impairment, and several animal models have been designed to study this condition. AntiSperm Antibodies (ASA) play an important role in interfering with normal sperm functions, since they may affect sperm performance by blocking sperm transport and capacitation, Acrosome Reaction (AR), sperm binding and penetration of the ZP, sperm binding and fusion to the oolemma, early embryo development and implantation (Chamley and Clarke, 2007; Vazquez-Levin et al., 2014). In particular, agglutinating ASA can reduce sperm forward progressive motility and may affect sperm penetration through cervical mucus (Kremer and Jager, 1992). In addition to antibody production, immunity to sperm leads to the expression of other immune factors, among them cytokines, known to play multiple roles in male and female reproduction, by participating in intercellular communication and mediating inflammatory responses (Ingman and Jones, 2008; Fraczek and Kurpisz, 2015; Schumacher and Zenclussen, 2015). There are reports describing alterations in fertility due to inflammation or infection caused by an imbalance in cytokine production (Hedger and Meinhardt, 2003; Ochsenkuhn et al., 2006). In the testis, cytokines have two relevant functions in physiological conditions, as they mediate immune-endocrine interactions during inflammation and work as growth and differentiation factors in the regulation of cell–cell interactions (Hales et al., 1999). Regarding pro-inflammatory cytokines, TNF-α is produced by germ cells and plays a role in the maintenance of the Blood-Testis-Barrier (BTB) (Li

Abbreviations: AR, Acrosome Reaction; ASA, AntiSperm Antibodies; BTB, Blood Testis Barrier; INF-γ, interferon gamma; IL-4, interleukin 4; IL-10, interleukin 10; TGF-β, transforming growth factor beta; OD, optical density; h-proacrosin, human proacrosin; Rec-40, recombinant h-proacrosin; TNF-α, tumor necrosis factor alpha; SVF, seminal vesicle fluid; ZP, zona pellucida; RE, relative expression.

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et al., 2006), whereas INF-γ production has been linked to anti-viral protection (Hedger and Meinhardt, 2003). On the other hand, regulatory cytokines such as IL-4, IL-10 and TGF-β1, -β2 and -β3 are constitutively expressed in the rodent testis and could have a role in immunosuppression as well as other functions in testis physiology (Yotsukura et al., 1997; Robertson et al., 2002; Winnall et al., 2011).

Cytokines are also present in the seminal plasma, and their immunomodulatory activities are essential to protect spermatozoa from the female immune system and to prepare the uterus for pregnancy (Robertson et al., 2011, 2013). Although controversy still exists about their origin, the seminal vesicles seem to be the most important contributors of components with immunomodulatory properties, such as cytokines (Robertson et al., 1996) and prostaglandins (Skibinski et al., 1992). In particular, TGF-β1 is essential for induction of immune tolerance against sperm antigens (Robertson et al., 2002); moreover TGF-β1 and -β2 appear to mediate the pro-inflammatory cascade elicited in the female tract at insemination (Ingman and Robertson, 2002). In addition, an increased concentration of pro-inflammatory cytokines in seminal plasma, such as IFN-γ and TNF-α, has been associated with poor semen quality and male infertility as well as with recurrent pregnancy loss (Daher et al., 2004; Chaouat et al., 2007). Despite its relevance, data is still scarce about the induction of immune mediators in male genital tract after an immunization.

Acrosin (EC 3.4.21.10) is a trypsin-like protease and localized to the sperm acrosome as an inactive zymogen (proacrosin; 53 kDa) that is converted to the active enzyme and released during the AR (Zahn et al., 2002). Several functions have been attributed to the proacrosin/acrosin system, mainly binding to glycoproteins of the *zona pellucida* (ZP) and participation in the release of acrosomal content (Vazquez-Levin et al., 2005) in mammals. Specific antibodies towards human (h-)proacrosin were shown to impair some of its functions in *in vitro* assays (Veaute et al., 2010) and antiacrosin antibodies were found in sera of women consulting for infertility (Veaute et al., 2009a). Moreover, the proacrosin/acrosin system has been found immunogenic after DNA immunization with the coding sequence of h-proacrosin of BALB/c female and male mice, an inbred strain with a low fertility rate. Mouse anti-h-proacrosin antibodies have been shown to bind to the mouse proenzyme and to inhibit *in vitro* fertilization, early embryonic development, sperm-ZP binding, and Ca^{+2}-ionophore-induced AR; moreover, immunized animals had diminished their fertility (Veaute et al., 2009b; Garcia et al., 2012).

Based on this background information, the present study was aimed at evaluating the ability of DNA immunization with proacrosin to modulate male fertility in the outbred strain CF1, known to have a high fertility rate (Katz-Jaffe et al., 2013). In addition to the assessment of presence and levels of antibodies towards proacrosin/acrosin in serum and SVF of immunized and control animals, the expression of a subset of pro-inflammatory and regulatory cytokines in the male gonad and seminal vesicles as well as its presence in serum and SVF was evaluated.

2. Materials and methods

2.1. Materials

A plasmid containing the h-proacrosin coding sequence was kindly donated by Prof. Dr. Wolfgang Engel (Institut für Humangenetik, Göttingen, Germany) to MVL, as previously described (Furlong et al., 2000) and subcloned in the pSF2-CMV eukaryotic expression vector for DNA immunization kindly provided to MVL by Dr. Stephen Johnston (Texas University, Southwestern Medical Center, Dallas, TX, USA).This construction was named pSF2-Acro (Veaute et al., 2009b). Plasmid DNA was obtained from bacterial cultures and purified by anion exchange chromatography using a commercial kit (Jetstar, Genomed, Löhne, Germany) following the manufacturer's instructions. The concentration of purified DNA was determined by optical density (OD) reading at 260 nm, and its quality was assessed by agarose gel electrophoresis and by the ratio of OD at 260 nm and at 280 nm (Sambrook and Russell, 2001).

Recombinant h-proacrosin was cloned and expressed using the pET22b expression vector as previously reported, and recombinant proacrosin (Rec-40: 42–44 kDa, aminoacids 1-402) was purified by preparative SDS-PAGE as previously described (Furlong et al., 2000).

2.1.1. Animal immunization protocol

For the immunization protocol, six to eight weeks old CF1 male mice were used. Animals were bred in a colony established at the IBYME animal facility. All experimental procedures were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals (Ilar, 2010). Adult male mice were inoculated in the quadriceps muscle with 50 μg of pSF2-Acro (n = 12) or pSF2 (n = 8) (plasmid without insert, control) in 50 μL of sterile phosphate-buffered saline (PBS). Injections were repeated four times, every three weeks. Blood samples were taken by tail vein puncture one week before each inoculation. Three weeks after the last dose (week 12), animals were bled by cardiac puncture and sacrificed, one serum aliquot was stored at −80 °C for cytokine determination, and another was kept at −20 °C for antibody analysis.

2.1.2. Assessment of immune response

The humoral immune response to proacrosin was evaluated by a previously optimized indirect ELISA (ELISA-Acro (Garcia et al., 2012)). Dilution of sera, as well as dilution of secondary antibodies, time of incubation, blocking agent and amount of antigen, was previously optimized in order to have an adequate positive to negative discrimination. Briefly, 96 well-polystyrene plates (Costar, Corning, NY, USA) were coated with 0.8 μg/well of recombinant h-proacrosin (Rec-40). After blocking non-specific binding sites with PBS supplemented with 3% skimmed milk, 1:200 dilutions of sera in PBS-1%-skimmed milk or pure seminal vesicle fluid (SVF) were tested. Anti-proacrosin IgG, IgG1, and IgG2a were assessed by incubation with peroxidase conjugated anti-mouse IgG (Jackson, Baltimore, MD, USA), anti-mouse IgG1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) or anti-mouse IgG2a (Abcam Inc. Cambridge, MA, USA), respectively, followed by incubation with hydrogen peroxide and tetramethylbenzidine (Zymed, San Francisco, CA, USA). In order to find the dilution of secondary antibodies giving a comparable OD between both subtypes of IgG, the ELISA was optimized by immobilizing different amounts of monoclonal antibodies from each subtype. OD was measured using a microplate reader (Emax Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). For serum samples, results are expressed as the ratio between the sample OD and the preimmune sera OD (OD/OD preimmune). For SVFs, results are expressed as the ratio between the sample OD value and the average OD value of samples from pSF2 inoculated mice (OD/OD control).

2.1.3. Assessment of male mice fertility

Immunized male mice were mated with non-treated 14 to16 week-old female mice with 1 male per 3 females in a cage, starting at week 9 of the immunization protocol. Fertility of female mice was proven by their mating with untreated males prior to the initiation of the immunization protocol; only female mice that had offspring

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