

Desialylation of Spermatozoa and Epithelial Cell Glycocalyx Is a Consequence of Bacterial Infection of the Epididymis*

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Farhad Khosravi^{‡§}, Vera Michel[‡], Christina E. Galuska[§], Sudhanshu Bhushan[‡], Philipp Christian[§], Hans-Christian Schuppe[¶], Adrian Pilatz[¶], Sebastian P. Galuska^{§||1,2}, and Andreas Meinhardt^{‡¶1,3}

From the Institutes of [‡]Anatomy and Cell Biology and [§]Biochemistry and [¶]Department of Urology, Pediatric Urology, and Andrology, Faculty of Medicine, Justus Liebig University, 35392 Giessen, Germany and the ^{||}Department of Reproductive Biology, Leibniz Institute for Farm Animal Biology, 18196 Dummerstorf, Germany

Urinary tract infections caused by uropathogenic *Escherichia coli* (UPEC) pathogens belong to the most frequent infections in humans. In men, pathogens can also spread to the genital tract via the continuous ductal system, eliciting bacterial prostatitis and/or epididymo-orchitis. Antibiotic treatment usually clears pathogens in acute epididymitis; however, the fertility of patients can be permanently impaired. Because a premature acrosome reaction was observed in an UPEC epididymitis mouse model, and sialidases on the sperm surface are considered to be activated via proteases of the acrosome, we aimed to investigate whether alterations of the sialome of epididymal spermatozoa and surrounding epithelial cells occur during UPEC infection. In UPEC-elicited acute epididymitis in mice, a substantial loss of *N*-acetylneuraminic acid residues was detected in epididymal spermatozoa and epithelial cells using combined laser microdissection/HPLC-ESI-MS analysis. In support, a substantial reduction of sialic acid residues bound to the surface of spermatozoa was documented in men with a recent history of *E. coli*-associated epididymitis. *In vitro*, such an UPEC induced *N*-acetylneuraminic acid release from human spermatozoa was effectively counteracted by a sialidase inhibitor. These findings strongly suggest a substantial remodeling of the glycocalyx of spermatozoa and epididymal epithelial cells by endogenous sialidases after a premature acrosome reaction during acute epididymitis.

Acute epididymitis or a combined epididymo-orchitis in men represents a relevant entity in urological practice. Epididymitis is usually the result of an infection starting in the urethra that ultimately ascends to the epididymis and testis (1). Uropathogenic *Escherichia coli* (UPEC)⁴ belongs to the most com-

mon microbes associated with the condition, particularly in men over the age of 35 (2). Although antibiotic treatment is usually successful in clearing the pathogens, about 40% of patients with UPEC epididymitis are subsequently diagnosed with impaired semen parameters causing sub- or infertility (3). Biopsies from patients with a past history of epididymitis showed drastic structural alterations such as epithelial cell damage, fibrosis, and absence of spermatozoa further distally (4). Rodent UPEC epididymitis models could replicate the damage seen in men and pointed to a role of the UPEC virulence factor α -hemolysin in an extensive premature acrosome reaction occurring in the epididymal duct, implying the untimely release of acrosomal enzymes in infected epididymis prior to reaching the female reproductive tract (5).

The acrosome contains various digestive enzymes, among them proteases as well as various glycosidases, such as hyaluronidases and sialidases, that are essential for penetration of the zona pellucida and glycan remodeling of the gametes as a prerequisite for spermatozoa-oocyte binding (6–11). Recent data revealed that, during capacitation, a biochemical process in the female reproductive tract in which spermatozoa accomplish full fertilizing “capacity,” two sialidases (the neuraminidases NEU1 and NEU3) are shed from the surface of spermatozoa, likely by the activity of proteases, which could be efficiently counteracted by protease inhibitors (10). This process led to the release of sialic acid residues from the surface of spermatozoa (10). The acrosome appears to contain a further reservoir of sialidases, which are released physiologically during the acrosome reaction prior to fertilization (8, 12). Because NEU1 and NEU3 together are able to desialylate several different glycoconjugates (α 2,3-, α 2,6-, and α 2,8-sialylated), a significant restructuring of the glycocalyx can be expected (13–15).

Sialic acids are acidic nine-carbon backbone α -keto sugars and represent one of the most important molecules of life (16, 17). *N*-acetylneuraminic acid (Neu5Ac) as well as *N*-glycolylneuraminic acid (Neu5Gc) are the most abundant species in mammals. These sugar residues are commonly linked to the outermost position of the largest share of glycoproteins, which places them in an ideal position for their indispensable role in a diverse range of cellular processes such as intercellular adhe-

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¹ Both authors contributed equally to this work.

² To whom correspondence may be addressed: Dept. of Reproductive Biology, Leibniz Institute for Farm Animal Biology, Germany. Tel.: 49-38208-68-751; E-mail: galuska.sebastian@fhn-dummerstorf.de.

³ To whom correspondence may be addressed: Institute of Anatomy and Cell Biology, Faculty of Medicine, University of Giessen, Germany. Tel.: 49-641-99-47024; Fax: 49-641-99-47029; E-mail: andreas.meinhardt@anatomie.med.uni-giessen.de.

⁴ The abbreviations used are: UPEC, uropathogenic *Escherichia coli*; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; siglec, sialic

acid-binding immunoglobulin-type lectin; SNA, *Sambucus nigra*; LCMD, laser capture microdissection; KDO, 3-deoxy-D-manno-oct-2-ulosonic acid; DANA, *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid; KDN, 2-keto-3-deoxynononic acid; HTF, human tubal fluid; PSA, *Pisum sativum*; DMB, 4,5-methylene dioxibenzene.

Desialylation of Glycocalyx during Epididymitis

sion and communication. However, sialic acids also play an important role during infection of many viruses, fungi, as well as bacteria (18–20). For instance, during invasion, pathogenic bacteria use sialic acid residues on the surface of epithelial cells for the initiation of infection by mediating bacterial adherence. On the other hand, detachment of sialic acid residues from host cells represents a further powerful means of bacteria to manipulate the immune response, *e.g.* by influencing the recognition of specific sugar moieties by sialic acid-binding immunoglobulin-type lectins (siglecs) on immune cells (21, 22). For example, several pathogenic bacteria secrete sialidases as a virulence factor, leading to sepsis as a consequence of an uncontrolled stimulation of the immune system. In this regard, bacterial sialidase inhibitors can efficiently counteract the dramatic progression of the disease in a mouse model, demonstrating their clinical potential during infection (23, 24). In consideration that UPEC infection of the epididymis results in a premature release of acrosomal content (5), we aimed to investigate whether this affects the sialome of spermatozoa and surrounding epididymal epithelial cells using a mouse epididymitis model and ejaculates of men with a recent history of the disease.

Results

Desialylation of Spermatozoa and Epithelial Cells Is a Consequence of UPEC Infection in the Epididymitis Mouse Model—To elucidate whether the UPEC-induced acrosome reaction leads to an activation of endogenous sialidases, initiating a subsequent desialylation of spermatozoa, an established epididymitis mouse model was utilized (25). Histopathology in Masson-Goldner-stained epididymis demonstrated fibrotic remodeling and interstitial leukocytic infiltration as the most obvious consequences 3 days post-UPEC infection, as shown previously (Fig. 1A) (4, 26). Acrosomal staining with FITC-labeled peanut agglutinin lectin indicated a premature acrosome reaction in the majority of spermatozoa in the lumen of the epididymis following UPEC infection (Fig. 1B), similar to previous findings (5). In contrast, the acrosome was visible in most spermatozoa in untreated and sham controls.

To gain a first insight into the sialylation status, sialic acid residues were visualized on epididymal sections using FITC-conjugated SNA lectin (Fig. 2). SNA prevalently binds α 2,6-linked sialic acid residues; however, α 2,3-linked sialic acid residues will also be bound by this lectin but to a lesser degree. Fluorescence microscopy revealed diffusely distributed and weaker SNA labeling in spermatozoa of UPEC-infected mice, whereas in controls, prominent staining of sialic acid residues was evident in the acrosome and sperm tail (Fig. 2). The reduced SNA staining in UPEC-treated mice suggested that Neu5Ac residues were cleaved during infection.

Because qualitative as well as quantitative lectin analyses of sialic acids are often misleading, and SNA lectin visualize only a proportion of all sialic acid residues on glycoconjugates (27), a recently developed combination of LCMD and DMB-HPLC-ESI-MS analyses was applied (Fig. 3) (28). Starting from murine paraffin-embedded tissue samples, epididymal spermatozoa were isolated via LCMD after nuclear staining using Mayer's hematoxylin (Fig. 3, A and B). Thereafter, sialic acids were released under acidic conditions, fluorescently labeled, and

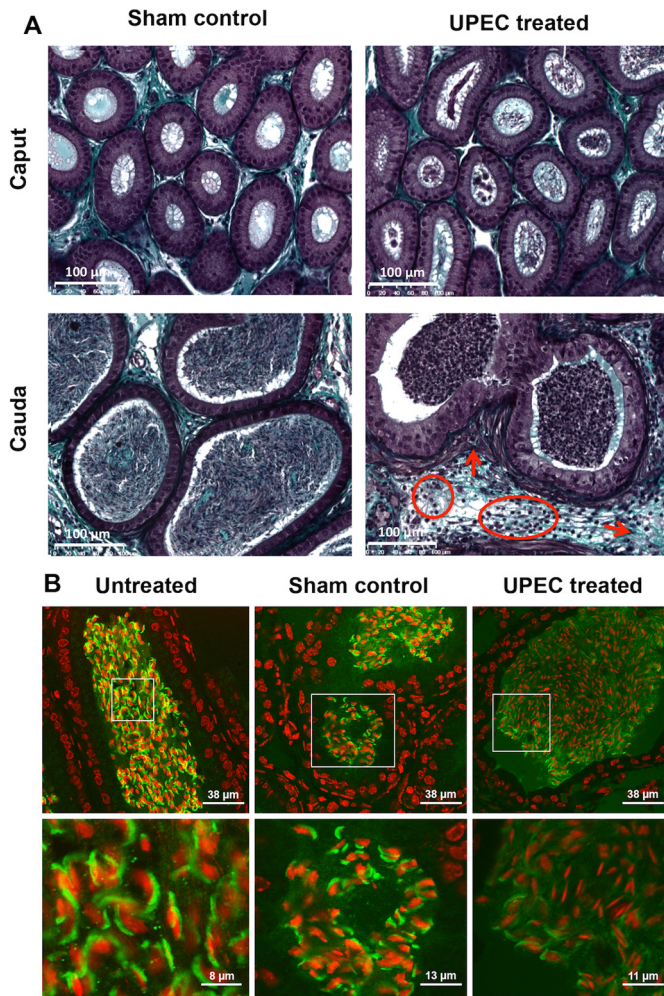


FIGURE 1. UPEC infection causes pathological alterations in the cauda epididymis and a premature acrosome reaction in epididymal spermatozoa. A, Masson-Goldner-stained sections of cauda epididymal tissues from mice 3 days post-UPEC infection and PBS sham controls were assessed by light microscopy. Arrows indicate fibrotic remodeling (thickening of the smooth muscle cell layer, collagen accumulation). Circles demonstrate interstitial infiltrating leukocytes. B, sperm acrosomes were fluorescently stained with peanut agglutinin-FITC lectin (green), and nuclei were counterstained using TO-PRO-3 (red) in the cauda epididymides from mice 3 days post-UPEC treatment, PBS sham controls, and untreated controls. The insets in the second row were magnified digitally.

subjected to a HPLC-ESI-MS system. The obtained chromatograms showed smaller peaks of DMB-labeled Neu5Ac in infected material (Fig. 3C). The respective DMB-Neu5Ac mass at m/z 448 $[M + Na]^+$ in the ESI-MS spectrum verified the presence of DMB-Neu5Ac during the corresponding retention time of an DMB-Neu5Ac standard (Fig. 3C). Calculation of corresponding peak areas demonstrated a significant reduction of Neu5Ac (~19%) in spermatozoa in UPEC epididymitis (Fig. 3D).

Although, in UPEC-treated samples, a peak for DMB-Neu5Gc was apparent in the HPLC chromatogram, in the sham control, hardly any signal was detectable. However, no mass corresponding to DMB-Neu5Gc was obtained by ESI-MS in untreated as well as infected mice (data not shown). Because of the fact that DMB reacts with all α -keto acids, possible contamination in infected mice by α -keto acids originating from the pathogen was examined by separate analysis of bacteria only. In

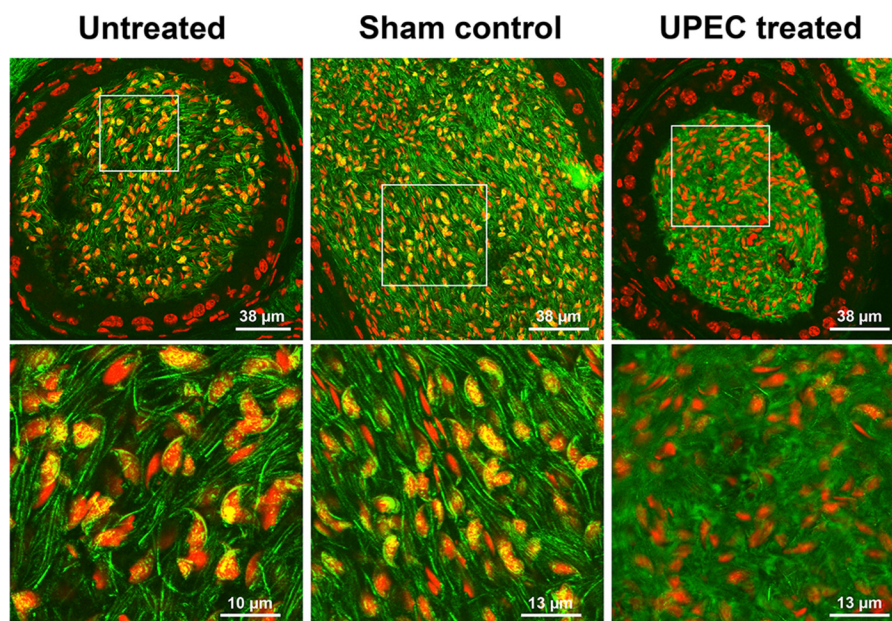


FIGURE 2. In UPEC epididymitis, sialic acid labeling is decreased in spermatozoa. α 2,6-linked sialic acid residues were visualized by SNA-FITC lectin (green), and nuclei were counterstained with TO-PRO-3 (red) in sections from mouse epididymis 3 days post-UPEC and a PBS sham control as well as an untreated control. In contrast to untreated and sham controls, in UPEC infection, a more diffuse sialic acid labeling is evident. The images in the second row were magnified digitally.

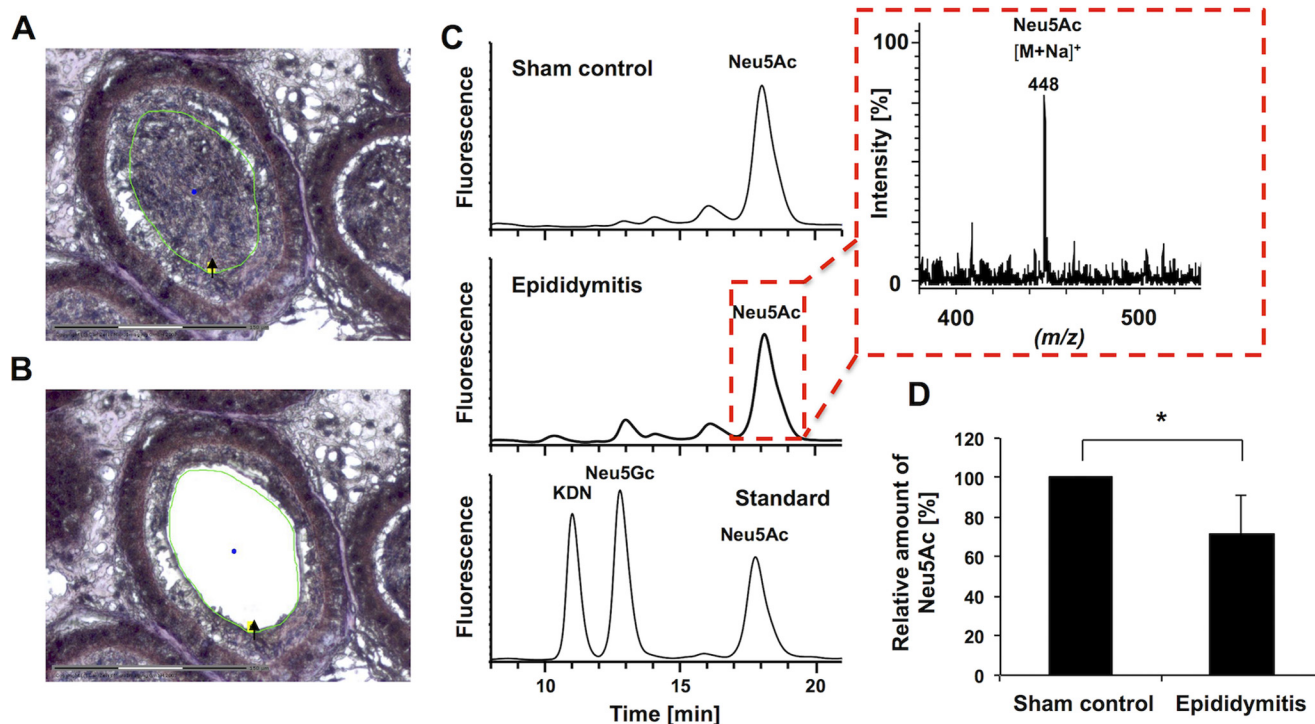


FIGURE 3. Epididymal spermatozoa are desialylated following UPEC infection. *A*, spermatozoa from the epididymal lumen were isolated by laser microdissection from hematoxylin-stained, paraffin-embedded tissue sections. The excised area is indicated by the black arrow and green circle. Scale bar = 150 μ m. *B*, the same section after the excision of luminal content. *C*, the obtained spermatozoa were subsequently used for sialic acid quantification. After hydrolysis and fluorescent labeling, DMB-sialic acid residues were separated using reverse-phase HPLC. A set of sialic acid standards was used for the generation of a calibration line and to control the retention times. ESI-MS spectra were recorded during the HPLC runs to verify the presence of different sialic acid species, as shown exemplary for Neu5Ac in UPEC-treated and PBS sham control epididymides. *D*, the amounts of Neu5Ac and the respective standard deviations were calculated from four epididymides obtained from PBS sham control and UPEC-injected mice, respectively ($n = 6$ animals). Given mean values were set to 100% for the sham control. The statistical evaluation was performed using Student's *t* test (unequal variances, two-tailed). *, $p \leq 0.05$.

UPEC, the presence of DMB-labeled KDO, a well known bacterial α -keto acid (16), was measurable as a prominent peak at the retention time of DMB-Neu5Gc (Fig. 4A). In addition, KDO-related monoisotopic pseudomolecular masses of DMB-

KDO at m/z 337 ($[M + H]^+ - H_2O$), 355 ($[M + H]^+$), and 377 ($[M + Na]^+$) were monitored in the ESI-MS spectrum at this particular time (Fig. 4B). To verify this finding, KDO was applied to the DMB-HPLC analysis, demonstrating that

Desialylation of Glycocalyx during Epididymitis

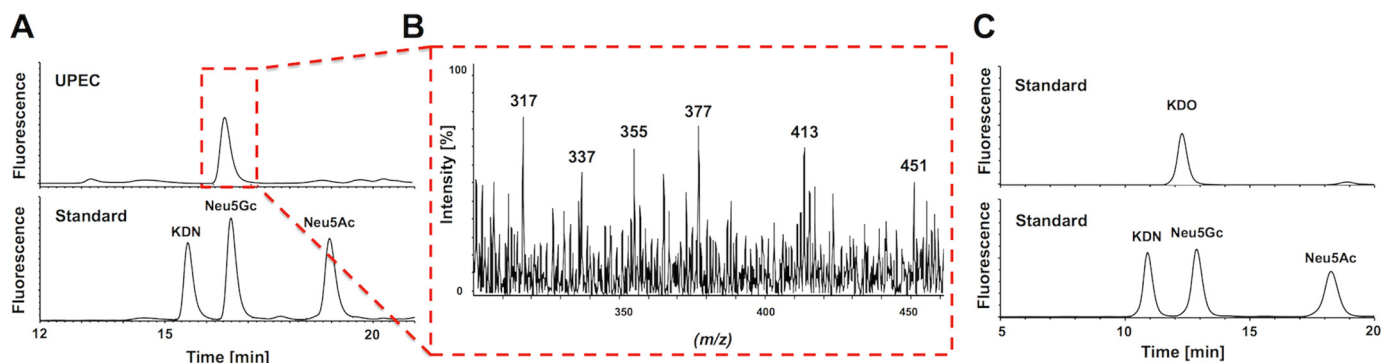


FIGURE 4. **Analysis of α -keto acids in UPEC.** A, sialic acid standards and UPEC samples were hydrolyzed, and α -keto acids were fluorescently labeled with DMB for HPLC separation. B, the ESI-MS spectrum recorded during the elution time of the peak is highlighted by the red dotted frame. C, comparison of the retention time of KDO and Neu5Gc standards.

Neu5Gc and KDO show overlapping peak areas (Fig. 4C). Thus, in UPEC-treated mice, bacterial KDO was obviously recorded at the retention time of DMB-Neu5Gc.

Release of sialic acids from the spermatozoa to the luminal fluid following UPEC infection could be enzymatically mediated by soluble sialidases, a hypothesis supported by an observation of Gagneux and co-workers (10), who described that the sialidases NEU1 and NEU3 are released from the cell surface of spermatozoa after proteolytic activation. Consequently, soluble neuraminidases would be also able to reach the cell surface of surrounding cells. To test this hypothesis, the sialylation status of epididymal epithelial cells (whose apical surface borders on the luminal fluid) was analyzed using the abovementioned combined LCMD/DMB-HPLC-ESI-MS strategy (Fig. 5). In support, epididymal epithelial cells showed a reduction of sialic acid residues ($\sim 49\%$) 3 days post-infection with UPEC (Fig. 5, C and D). Reduction of Neu5Ac from epithelial cells is substantially stronger in comparison with spermatozoa (Fig. 3). Thus, the obtained results demonstrate that the sialylation status of epididymal spermatozoa in the lumen as well as that of the lumen lining epithelial cells decreases in the epididymis after bacterial infection.

In Vitro Desialylation of Human Spermatozoa Can Be Counteracted with Sialidase Inhibitors—To investigate whether UPEC can also induce hyposialylation in human spermatozoa, motile spermatozoa of healthy donors collected after swim-up were subsequently infected with UPEC (Fig. 6). DMB-HPLC analysis pointed to a reduction of Neu5Ac to approximately half the amount measured in an untreated control (Fig. 6A). Correspondingly, PSA-FITC lectin staining detected a substantially higher number of acrosomally reacted spermatozoa compared with controls (Fig. 6B).

To examine whether the investigated hyposialylation of spermatozoa is mostly the consequence of a general loss of glycoconjugates, *e.g.* because of the degradation of the glycoprotein backbone or the activation of neuraminidases during the acrosome reaction, motile human spermatozoa were treated with UPEC in the presence of the sialidase inhibitor DANA (Fig. 6A). Here, co-incubation of DANA with UPEC completely prevented hyposialylation of human spermatozoa (Fig. 6A). Bacterial sialidase activity could be excluded by *in silico* analysis of the UPEC genome. Consequently, the decreased sialylation sta-

tus in infected spermatozoa is likely based on the enzymatic activity of released sialidases.

Hyposialylation Is Detected in Spermatozoa of Men with a Recent History of Epididymitis—In addition, semen samples of patients suffering from *E. coli* epididymitis were included in our study. For this purpose, spermatozoa were obtained by swim-up from ejaculates of men 14 days after diagnosis and treatment of *E. coli*-based unilateral epididymitis. The analyzed samples showed malformation (sperm head, acrosome visualized by PSA-FITC staining, Fig. 7A) and much lower levels of Neu5Ac ($\sim 75\%$ reduction) compared with healthy control spermatozoa (Fig. 7B). Thus, in agreement with the determined loss of sialic acid residues in the epididymitis mouse model as well as in the *in vitro* experiments, spermatozoa of epididymitis patients are hyposialylated.

Discussion

Glycans play an essential role in fertilization, particularly in mediating the species-specific interaction between spermatozoa and the zona pellucida of the egg (9, 11). Binding of spermatozoa to the zona pellucida is thereby strongly induced by terminal Neu5Ac residues of sialyl-Lewis^X oligosaccharides on the surface of the zona pellucida. In addition, successful interaction of both gametes requires previous desialylation of the spermatozoal surface (10) (Fig. 8). For this purpose, spermatozoa are equipped with sialidases (8, 10, 12, 29). Under normal conditions, neuraminidases are only activated in the female reproductive tract as a consequence of capacitation and/or acrosome reaction (8, 10, 12, 29). Premature release of neuraminidases prior ejaculation is usually avoided, as untimely desialylation could result in elimination of spermatozoa by phagocytic cells (30). Under pathophysiological conditions such as bacterial epididymitis, considerable premature exocytosis of the acrosome has been observed *in vivo* and *in vitro*, which contributes substantially to the loss of fertilizing capacity, as shown by subsequent *in vitro* fertilization experiments in mice (5). This prompted us to examine whether an undue acrosome reaction could affect the sialome of epididymal spermatozoa and surrounding epithelial cells by applying an established acute mouse bacterial infectious epididymitis model and ejaculates of men with a recent history of the disease.

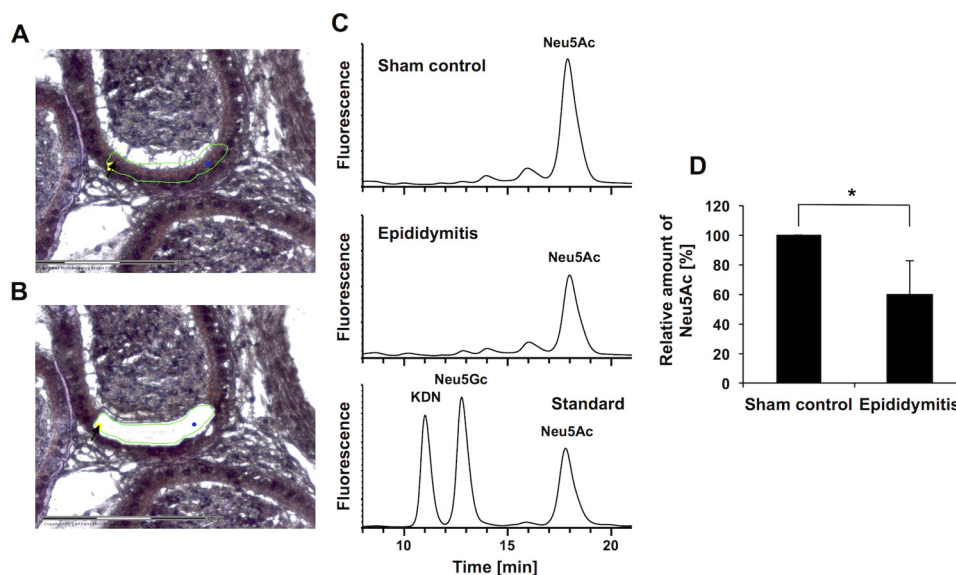


FIGURE 5. **Reduction of sialic acid content in epithelial cells in UPEC epididymitis.** *A*, epithelial cells were isolated from paraffin-embedded mouse epididymis by laser microdissection. The excised area is indicated by the *black arrow* and *green line*. *B*, the same section after excision of epithelium. Scale bar = 150 μm . *C*, DMB-labeled sialic acid residues of the isolated cells were separated using RP-HPLC. A set of sialic acid standards was used for quantification and to control the retention times. The ESI-MS spectrum was recorded during the HPLC runs (data not shown). *D*, the amounts of Neu5Ac and the respective standard deviations of six different controls and infected mice (one epididymis from six animals each). Given mean values were set to 100% for the sham control. The statistical evaluation was performed using Student's *t* test (unequal variances, two-tailed). *, $p \leq 0.05$.

Both in murine epididymitis as well as in *in vitro*-treated human spermatozoa, UPEC stimulates a premature acrosome reaction, leading to a significant desialylation of spermatozoa as well as adjacent cells (illustrated in Fig. 8). Surprisingly, the investigated desialylation was much more pronounced in epididymitis patients. The desialylation could be efficiently blocked with a neuraminidase inhibitor *in vitro*, indicating that the loss of sialic acid residues is particularly a consequence of endogenous neuraminidases (Fig. 8).

A limitation in the analysis of human samples in this study is the relatively small number of semen samples from patients/controls suitable for glycoanalysis because of the need to clean ejaculates by the swim-up method. Because semen samples of patients with acute epididymitis are usually characterized by a largely reduced semen quality and leukocytospermia, the swim-up technique was employed to select the viable and motile sperm, thereby removing contaminating non-sperm cells (e.g. leukocytes) to avoid bias between patient and control samples.

Because sialic acids have multifactorial roles in the host response to infection, the massive release of sialic acid residues from the cell surface of epithelial cells and spermatozoa may contribute to the observed tissue damage in epididymitis (Fig. 8). This view is supported by the fact that bacterial lipopolysaccharide triggers complement activation and that hyposialylation promotes the perforation of host cell membranes by the membrane attack complex by counteracting a protective mechanism conveyed by complement factor H (31). Complement factor H exhibits sialic acid-specific binding domains that are necessary for efficient cell surface binding (32). Factor H on cell membranes is essential to remove activated complement factor 3b from cellular surfaces to prevent perforation of host cell membranes and limit tissue damage. As shown recently, complement factor H is an important component of seminal

plasma, as it protects spermatozoa against such a complement attack in the female reproductive tract (33). Thus, hyposialylation of spermatozoa and other host cells may result in a deficit of sialic acid-dependent factor H binding and, thus, less protection against the activated complement system.

Moreover, many sialylated and polysialylated glycoproteins are integrated into the plasma membrane of spermatozoa during epididymal transit (34, 35). These glycoproteins inhibit, *inter alia*, in a sialic acid-dependent way the phagocytosis by leukocytes as seen in murine spermatozoa (30, 36). In this regard, the substantial loss of sialic acid residues during epididymitis could prompt phagocytosis of spermatozoa during transit through the epididymis and/or the female reproductive tract, as already indicated (30, 37).

The presence of leukocytes, mainly macrophages, is a frequent observation in the lumen of the epididymal duct of healthy men and rodents. Together with neutrophils and T cells, their numbers increase strongly in infection (25, 38, 39). It appears therefore likely that soluble neuraminidases may also detach sialic acid residues from the cell surface of immune cells during infection. However, their terminal sialic acids play an important role in counteracting excessive immune response via an interaction with siglecs in *cis* (40). As an example, the anti-inflammatory signaling of siglec-10 is activated by the interaction with sialic acid residues on CD24 in *cis* (24). This complex represses tissue damage-induced immune responses (41) similar to those seen in infected epididymis (4). Some bacteria, like *Clostridium perfringens*, secrete microbial sialidases targeting the described sialic acid mediated CD24·siglec-10 complex. As a consequence, mice have an increased risk of septicemia, as shown previously (22, 24). Interestingly, sialidase inhibitors suppress the inflammatory response and septic symptoms. Thus, in epididymitis patients, activated endogenous sialidases may act as an indirect virulence factor similar to microbial siali-

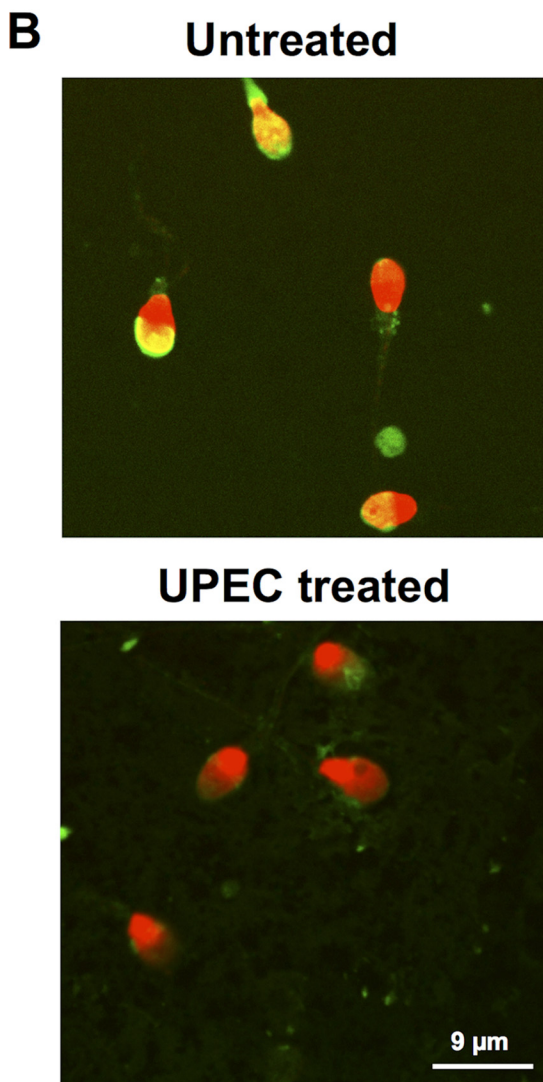
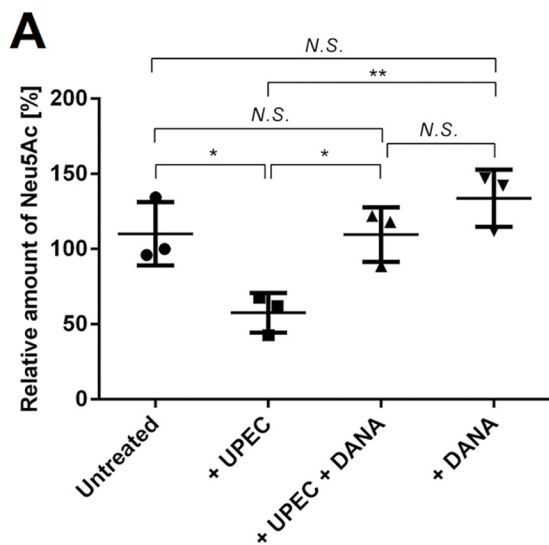


FIGURE 6. UPEC-dependent desialylation of human spermatozoa can be counteracted by sialidase inhibitor *in vitro*. A, spermatozoa from healthy donors prepared by swim-up were incubated with UPEC for 3 h, and the amount of Neu5Ac was compared with untreated samples using the DMB-HPLC approach as shown in Fig. 3. In parallel, UPEC-treated as well as untreated spermatozoa were co-incubated with the sialidase inhibitor DANA.

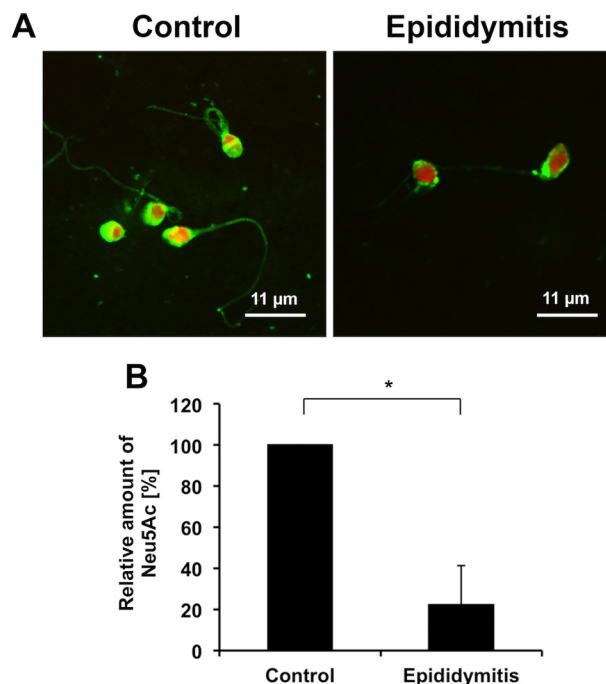


FIGURE 7. Reduced levels of Neu5Ac in patients with a history of acute epididymitis. A, acrosomes of swim-up spermatozoa obtained from healthy men (control) and patients 14 days after diagnosis and treatment of acute epididymitis were visualized by PSA-FITC lectin (green). Nuclei were counterstained with TO-PRO-3 (red). B, sialic acid residues of isolated spermatozoa from these patient groups ($n = 3$, respectively) were released and fluorescently labeled with DMB for quantification. The statistical evaluation was performed using Student's *t* test (unequal variances, two-tailed). Given mean values were set to 100% for all sham controls. *, $p \leq 0.05$.

dases of *C. perfringens*, *Vibrio cholerae*, and *Streptococcus pneumoniae* (42).

Furthermore, the altered glycocalyx of epididymal epithelial cells could influence adhesion of UPEC. Because of the desialylation and the resulting loss of negatively charged sialic acid residues, type 1 fimbriae like FimH may better reach potential binding partners such as $\text{Mannose}\alpha 1-3\text{Man}$ or $\text{Man}\alpha 1-3\text{Man}\beta 1-4\text{GlcNAc}$ (43). In contrast, sialic acid-dependent UPEC binding to epithelial cells was described (44). Accordingly, the adhesion of UPEC to epididymal epithelial cells would be inhibited by epithelial desialylation.

Taken together, UPEC infection causes hyposialylation in mouse epididymal spermatozoa and epithelial cells as well as in ejaculated spermatozoa of men with a history of epididymitis. Likely this happens mechanistically through activation of endogenous sialidases. Hyposialylation has the potential to negatively affect the course and magnitude of infection in patients suffering from UPEC-elicited epididymitis, providing a possible explanation for the characteristic long-term impairment of fertility associated with this pathovar (5, 45). However, the precise consequences of the examined hyposialylation and the potential of sialidase inhibitors as therapeutic agents during UPEC infec-

One control was set as 100%, and the other controls were set in relation to it. Treatments were calculated relative to the mean of controls. Significance levels were calculated using one-way analysis of variance. *, $p \leq 0.05$; **, $p \leq 0.01$; *N.S.*, not significant ($n = 3$). B, acrosomes were labeled by PSA-FITC lectin (green), and nuclei were counterstained with TO-PRO-3 (red). The presence of acrosomes was compared with the untreated control.

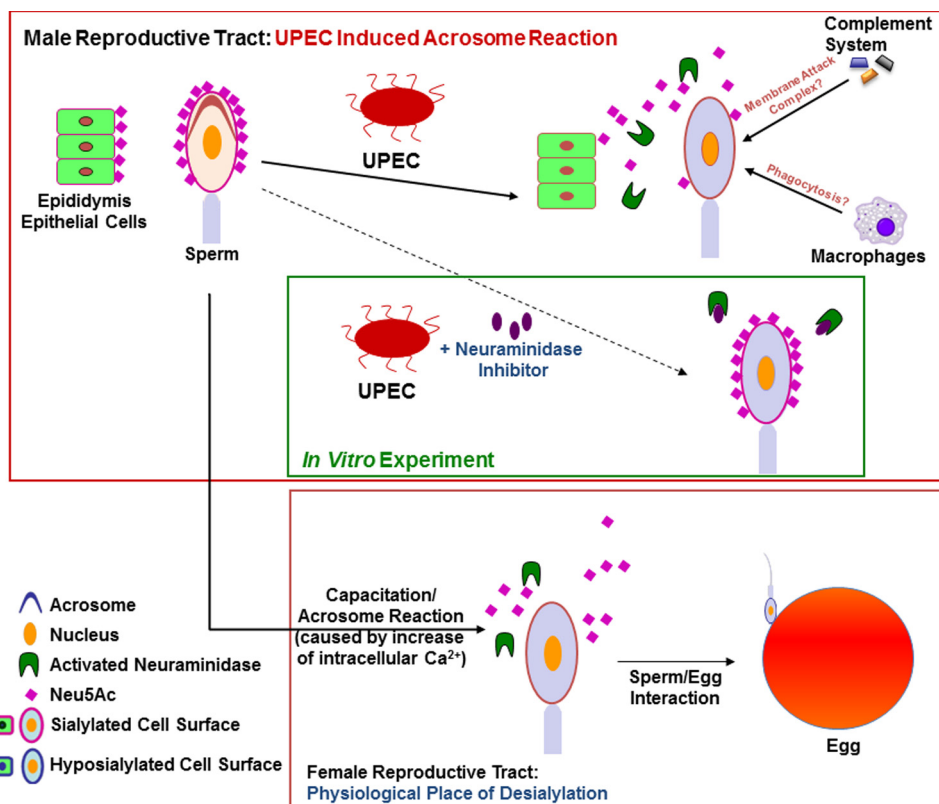


FIGURE 8. **Summary and working model.** Blue box, in the female reproductive tract, a capacitation/acrosome reaction leads to proteolytic activation and release of neuraminidases, resulting in desialylation of spermatozoa to allow sperm/egg interaction. Red box, the UPEC-induced acrosome reaction triggers premature desialylation of spermatozoa and the release of sialic acid residues from adjacent cells in the male reproductive tract. Consequently, pathological hyposialylation may negatively influence the protection of host cells against phagocytosis and the complement system. Green box, *in vitro* UPEC-induced hyposialylation of spermatozoa could be counteracted using neuraminidase inhibitors. Putatively, a further level of intervention could be upstream by blocking the proteolytic activation and release of neuraminidases using suitable protease inhibitors according to Ma *et al.* (10).

tion need to be further investigated. A better understanding is also needed in the comparative glycome analysis of human and mouse epididymal epithelial cells and spermatozoa to better judge how well the animal model reflects the situation in men.

Experimental Procedures

Materials—Neu5Ac, Neu5Gc, 2-keto-3-deoxynononic acid (KDN), and 3-deoxy-D-manno-octulosonic acid (KDO) were purchased from Sigma-Aldrich (Taufkirchen, Germany). All lectins were purchased from Vector Laboratories (Burlingame, CA). All reagents and chemicals used were of analytical grade.

Mouse and Human Ethics Statement—Adult inbred C57BL/6J mice (10–12 weeks old) were purchased from Charles River Laboratories (Sulzfeld, Germany). All studies were performed according to a protocol approved by the Animal Ethics Committee of the Regierungspräsidium Giessen (permit no. GI 18/17-No. 124/2012).

All patients and volunteers provided written informed consent for semen analysis. All samples were anonymized. As controls, three healthy men of reproductive age (median, 40 years; range, 19–54 years; ethics review board no. 32/11) were recruited during examination in the andrological/urological outpatient clinic. In addition, ejaculates of three patients (median, 40 years; range, 19–54 years) suffering from acute unilateral *E. coli*-related epididymitis were analyzed within the first 2 weeks after diagnosis. Patients who were part of the Giessen

Epididymitis Study (institutional review board no. 100/7, German Clinical Trials Register no. DRKS00003325) and treated according to current guideline recommendations (2). Of 22 patients providing semen, only the samples of three patients (aged 19, 54, and 47) had a suitable sperm concentration (>5 million/ml) to undergo further analysis. All patients underwent a comprehensive microbiological analysis for classical uropathogens by culture- and species-specific PCR analysis to detect *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, and *Chlamydia trachomatis*. Only patients suffering from *E. coli* epididymitis were part of the study. Empiric therapy was initiated with levofloxacin (500 mg once a day orally for 10 days). Sexually active men were asked to provide a semen sample 14 days after first presentation. Semen samples were collected by masturbation into a sterile container. Semen analysis was performed according to World Health Organization 2010 recommendations (46). To select motile sperm, the swim-up procedure was applied (47). Briefly, 1 ml of fresh semen was placed in a sterile centrifuge tube, and 1 ml of human tubal fluid (HTF) medium layered on top. Following 1 h of incubation at 37 °C, 1 ml of upper medium containing highly motile sperm was removed, diluted with 2 ml of HTF medium, and centrifuged at $500 \times g$ for 5 min.

Bacterial Strains and Propagation—UPEC strain CFT073 was kindly provided by Prof. Chakraborty (Institute of Medical

Desialylation of Glycocalyx during Epididymitis

Microbiology, Justus Liebig University, Giessen, Germany). UPEC was propagated in Luria-Bertani medium as described previously (48). The concentration of bacteria was calculated using standard growth curves (48, 49). For *in vivo* and *in vitro* experiments, *E. coli* was diluted in PBS and RPMI 1640 medium (Invitrogen), respectively.

Experimental Mouse Epididymitis Model—5–10 μl of PBS suspension containing between $4\text{--}8 \times 10^4$ bacteria was injected bilaterally close to the epididymis into the vas deferens, which was previously ligated to prevent retrograde ascent of the bacteria (25). For sham controls, PBS alone was injected into the vas deferens of control animals. Three days post-infection, mice were sacrificed, and the epididymides were removed. Bacterial ascent from the injection site to the epididymis has been demonstrated previously (5). The epididymis from one side was snap-frozen in liquid nitrogen after cryo-embedding in optimum cutting temperature medium for lectin staining, whereas the contralateral organ was immediately fixed in Bouin's solution and embedded in paraffin. Subsequently, sections were used for histological staining and laser capture microdissection.

Masson-Goldner Staining—Deparaffinized and rehydrated sections of epididymides were stained by incubating sections consecutively in Weigert's hematoxylin (nuclei, 5 min), xylydine Ponceau (cytoplasm, 5 min), Orange G (erythrocytes, 20 s), and Light Green (connective tissue, 5 min). Dehydrated stained sections were subsequently embedded in Entellan[®] mounting medium (Merck, Darmstadt, Germany) and assessed with a Leica DM750 microscope.

Lectin Fluorescence Staining—After swim-up, freshly isolated human spermatozoa ($\sim 50,000$) were smeared and air-dried on SuperFrost glass slides (R. Langenbrinck). Serial cryosections (8 μm) were prepared from frozen mouse epididymides. Human spermatozoa and mouse epididymis sections were fixed with 2% paraformaldehyde for 30 min, washed, and blocked with 0.1% BSA (Roth, Karlsruhe, Germany) for 1 h. FITC-conjugated peanut agglutinin (binds particularly galactosyl ($\beta 1,3$) *N*-acetylgalactosamine glycan structure), *Pisum sativum* (PSA) (detects α -linked mannose-containing oligosaccharides with an *N*-acetylchitobiose-linked α -fucose), and *Sambucus nigra* (SNA) (binds prevalently sialic acid residues attached to terminal galactose in $\alpha 2,6$ and, to a lesser degree, $\alpha 2,3$ linkage) were used at a final concentration of 10 $\mu\text{g}/\text{ml}$ in PBS containing 0.1% BSA and incubated for 30 min. All FITC-labeled lectins were purchased from Vector Laboratories (Burlingame, CA). Nuclei were visualized with TO-PRO[®]-3 (Life Technologies, dilution 1:1000, 1 min). Images were acquired using a confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany). Excitation filters were set to 488 nm and 633 nm to detect FITC (lectin-bound glycostructures) and TO-PRO (nuclei), respectively.

Laser Capture Microdissection—Paraffin-embedded murine epididymides were sectioned (5 μm) and mounted on membrane slides for laser capture microdissection (LCMD) (Zeiss, Munich, Germany). As described previously, sections were stained with Mayer's hematoxylin, dehydrated, and air-dried (28). Tissue areas were marked using a $\times 20$ objective and dissected in Robo-LPC-mode. The number of isolated cells was calculated by counting stained nuclei. Dissected tissue was col-

lected in adhesive caps (Zeiss, Munich, Germany). The caps were cut and transferred into glass vials for sialic acid analysis (28).

Release and DMB Labeling of Sialic Acids—Washed spermatozoa collected after swim-up as well as collected tissue samples were subjected to sonication (10 min) and hydrolyzed in 500 μl of 2 N acetic acid for 90 min at 80 $^{\circ}\text{C}$, transferred into glass vials, and dried. Fluorescent labeling of sialic acids was performed using 80 μl of 4,5-methylene dioxybenzene (DMB) reaction buffer (1 M β -mercaptoethanol, 9 mM sodium hydrosulfite, 20 mM TFA, and 2.7 mM DMB (Dojindo, Kumamoto, Japan)) for 2 h at 55 $^{\circ}\text{C}$ as already described (50–53). Labeling was terminated by adding 20 μl of 0.2 M NaOH. To calculate the amount of Neu5Ac, a four-point calibration line was generated.

HPLC and Online HPLC-ESI-MS—DMB-labeled sialic acids were separated for quantification by a reverse-phase column (Superspher 100 C-18e-RP, 250 \times 4 mm, Merck-Hitachi, Darmstadt, Germany) at 40 $^{\circ}\text{C}$ using an Ultimate LC system that was directly coupled with an Esquire 3000 ESI-ion trap-MS (Bruker Daltonik) (28, 54). Fluorescently labeled sialic acids were separated by applying mobile phases (A and B) containing acetonitrile (Chem Solute, Th. Geyer, Renningen, Germany)/methanol (Merck)/water/TFA (Promochem, Wesel, Germany) (4:4:92:0.1) (A) and acetonitrile/methanol/water/TFA (45:45:10:0.1) (B). The LC method consisted of a linear gradient from 0% to 15% (B) over 30 min. The flow rate was 250 $\mu\text{l}/\text{min}$ over 60 min. Fluorescence detection settings were $\lambda_{\text{excitation}} = 372$ nm and $\lambda_{\text{emission}} = 456$ nm. Typical ESI source conditions were as follows: spray voltage, 1.4 kV; capillary temperature, 250 $^{\circ}\text{C}$; end plate offset, -500 V; capillary exit, 140 V (28).

Infection of Human Spermatozoa with UPEC in Vitro—Isolated spermatozoa ($\sim 7.5 \times 10^6$) were mixed in 1 ml HTF medium with approximately 5×10^5 bacteria (multiplicity of infection ~ 0.07) for 3 h at 37 $^{\circ}\text{C}$ and 5% CO_2 . Subsequently, the pellet (700 \times g, 10 min) was collected by centrifugation after two washing steps using PBS for DMB-HPLC analysis.

Inhibition of Spermatozoal Sialidase—As a control, UPEC-induced desialylation of isolated human spermatozoa was inhibited by adding 1 mM *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA, Sigma) to HTF medium for 3 h in 37 $^{\circ}\text{C}$ and 5% CO_2 . After treatment, spermatozoa were collected by centrifugation (700 \times g, 10 min) after two washing steps using PBS.

Statistical Analysis—HPLC data were analyzed by utilizing two-tailed, paired Student's *t* test. One-way analysis of variance was used for sialidase inhibitor experiments (Fig. 6A). All indicated values are mean \pm S.E. Sample size was estimated using a post hoc G*Power analysis.

Author Contributions—S. P. G. and A. M. designed the study and analyzed the data. F. K., S. P. G., and A. M. wrote the paper. V. M. performed LCMD as well as Masson-Goldner staining, and S. B. applied the mouse model. F. K. performed all lectin staining. F. K., P. C., and C. E. G. performed the DMB-HPLC analysis. C. E. G. and F. K. interpreted the MS spectra. H. C. S. and A. P. recruited and examined patients and provided andrological laboratory data. All authors reviewed the results and approved the final version of the manuscript.

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Desialylation of Glycocalyx during Epididymitis

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