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Research Article

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Shared Antigenic Determinants Between Spermatozoa and Bacteria: An Experimental Study

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Abstract

Sperm immobilization factor (SIF), the secretory protein of Staphylococcus aureus, is known to cause complete immobilization, death and morphological alterations in mouse spermatozoa in vitro. However, the present study aims to explore a newer dimension of SIF i.e., to bind to motile and non-motile bacteria and its ability to induce immobilization of motile bacteria in vitro. The results showed that 800µg of SIF caused complete immobilization of motile bacteria, however, death and morphological alterations could not be observed even with 1000µg of SIF. Furthermore, this SIF-mediated bacterial immobilization was reversed when each of the SIF-binding receptor from mouse spermatozoa and bacteria (Escherichia coli and Streptococcus pyogenes) was incubated with bacteria, thereby, providing an experimental evidence of similarity between the antigenic determinants present on spermatozoa and bacteria against a common ligand, SIF.

Introduction

Mimicry, a widely observed phenomenon, allows microorganisms to escape host defense by arming themselves with structures that are either identical or appear to be identical or nearly identical to those found on cell surface of the host [1]. Undoubtedly, the microorganisms employ "every possible trick in the book" to achieve this state of molecular mimicry *viz*. direct or indirect appropriation of host glycan, convergent evolution toward similar biosynthetic pathways, and even lateral gene transfer [2]. However, in some instances, the effect of the microbe is aggravated by autoimmune reactions to these host-like antigens [3]. Besides being involved in autoimmune diseases, this concept of mimicry is now being applied in case of infertility as well. The molecular mimicry between the invading bacteria and spermatozoa has been implicated in production of antisperm antibodies (ASAs), which has been touted as one of the reasons for immune infertility [4]. In this regard, some study groups have attempted to demonstrate mimicry between bacteria and spermatozoa; however, this association is mainly supported by indirect evidences *viz*. case reports/ in *silico* analysis/ small pilot studies [5]. The following are the various case studies that have attempted to prove an association between antisperm antibodies and bacteria *viz*. *Helicobacter pylori*,*Ureaplasma urealyticum, E. coli* and *Shigella* and *Salmonella* [6-9]. However, the present study provides a direct experimental evidence of the same.

In this regard, in our previous communication, we have reported that sperm immobilization factor (SIF), isolated from *Staphylococcus aureus*, was capable of causing impairment of mouse spermatozoa *in vitro* and infertility in female mice. Using SIF as a tool, the corresponding SIF-binding receptor from mouse spermatozoa (MS-SBR) was extracted and purified. This receptor efficiently blocked the SIF-induced sperm damage *in vitro* and infertility *in vivo*. Further, the binding studies carried out by using FITC-labelled SIF showed bright green fluorescence not only on mouse spermatozoa, but on various bacteria (motile and non-motile) as well. This further provided evidence regarding the presence of common SIF-binding receptors on both spermatozoa as well as bacteria.

The corresponding SIF-binding bacterial receptors were isolated and purified from *Escherichia coli* (a representative of motile and Gram negative bacteria) and *Streptococcus pyogenes* (a representative of non-motile, Gram positive bacteria). The mimicry between SIF-binding sperm and bacterial receptors was authenticated by employing the two bacterial receptors as a corrective measure against various negative influences of SIF on functional parameters of spermatozoa as well as fertility, which eventually presented them as an alternative for SIF-binding sperm receptor, and therefore, a putative therapeutic intervention against SIF-induced infertility [10,11]. Since, till now, the SIF-mediated negative influences have been studied w.r.t. spermatozoa only, hence, in the present study, we wanted to exploit an altogether newer aspect of SIF and examine if the detrimental effects of SIF were limited to spermatozoa only or it could compromise bacteria as well, thereby, providing an experimental evidence for mimicry between spermatozoa and bacteria. Also, it would be interesting to evaluate these receptors as an antidote against SIF-mediated bacterial impairment.

Materials and Methods

Microorganisms

S. aureus strain, capable of causing 100% immobilization of mouse

spermatozoa, was used for isolation and purification of the sperm immobilization factor (SIF).

Further, the standard bacterial strains *viz*. *Escherichia coli* (MTCC 1687), *Proteus mirabilis* (MTCC 425), *Pseudomonas aeruginosa* (MTCC 3542), *Salmonella enterica* Serovar Typhi (MTCC 733), *Vibrio cholerae* (MTCC 3904), *Shigella flexneri* (MTCC 1457) and *Streptococcus pyogenes* (MTCC 1924) used in the present study were procured from Microbial Type Culture Collection, IMTECH, Sector-39, Chandigarh, India.

Isolation and purification of sperm immobilization factor (SIF) from S. aureus

SIF was extracted and purified from sperm immobilizing strain of *S. aureus* by the method previously standardized in the laboratory [10]. Briefly, supernatant of *S. aureus* culture was mixed with powdered ammonium sulfate until 80% saturation and dialyzed. It was purified to apparent homogeneity by sequential column chromatography using Sephadex G-100 and DEAE-Cellulose columns.

Evaluation of bacterial impairment by SIF

The detrimental effect of SIF was evaluated on two bacteria, *E. coli* (a representative of motile and Gram negative bacteria) and *S. pyogenes* (a representative of non-motile, Gram positive bacteria).

Motility

An aliquot of 100µl of 4-6h old culture (when bacteria were actively motile) of *E. coli* was incubated with 100µl purified SIF at different concentrations (100-1000µg) for different time intervals (0, 1, 2 and 4h). After completion of each incubation period, a hanging drop preparation was made and the slide was observed under light microscope at magnification 400X. A control sample lacking SIF was also simultaneously processed. All the results were compared with those of control samples and the minimum concentration of SIF causing 100% immobilization of bacteria was determined. Immobilization was indicated by absence of movement of bacteria.

Since *S. pyogenes* is a non-motile bacteria, hence, this parameter was not evaluated in this case.

Viability

To determine the effect of different concentrations of SIF on bacterial death, an aliquot of 100µl from each reaction mixture (prepared as described above) was subsequently cultured on nutrient agar plates. Death was indicated by absence of growth on the media plates.

Morphology

Topographical imaging of bacterial membrane was done with the help of scanning electron microscopy (SEM) to check the consequence of incubation of SIF on the bacterial morphology. For this, bacterial cultures grown for 4-6h in nutrient broth were pelleted and washed twice in PBS (50mM, pH 7.2) and finally suspended in 1ml of PBS. The bacterial suspension was mixed with either PBS or purified SIF (1000µg) and incubated at 37°C for 30min. The samples were processed for SEM according to the protocol of Watson et al., [12].

Binding studies with FITC-labelled SIF

The binding studies of FITC-labeled SIF with bacteria were carried out by conjugating SIF (200µg) with FITC according to F/P ratio, as per the instructions given in FITC-Protein Labeling Kit (Bangalore Genei Pvt. Ltd., India). The spermatozoal and bacterial suspensions were prepared as follows:

1. The sperm preparation was washed twice with PBS and the pellet was finally suspended in 500µl of PBS (50mM, pH 7.2).
2. The bacterial cultures were grown for 6-8h in Nutrient broth, centrifuged (10,000rpm, 10min), washed twice in PBS (50mM, pH 7.2) and finally suspended in 1ml of PBS.

Same concentration of labelled SIF (100µl) was added to 100µl of each suspension (bacteria or spermatozoa) and incubated at 37°C for 1h (It is to be mentioned here that spermatozoa and bacteria were kept as separate entities and they were never mixed). Following this, 100µl of 3% formaldehyde was added to the reaction mixture and incubated at 37°C for 1h. Post completion of incubation period, the reaction mixture was washed thrice with PBS. The pellet was finally suspended in 50µl of PBS (50mM, pH 7.2). A wet mount was prepared and observed under fluorescent microscope (Nikon, Japan). Similarly, a control consisting of spermatozoa/bacteria and unlabeled SIF was set up to rule out auto fluorescence.

Isolation and purification of SIF-binding receptor from (a) Mouse spermatozoa (MS-SBR)

The receptor was extracted and purified from mouse spermatozoa by the method previously standardized in the laboratory [11]. Briefly, sperm suspension (10^8 spermatozoa) was centrifuged and the pellet was washed twice with PBS. After centrifugation at 5000rpm, the pellet was treated with 4M NaCl for 2h at 37°C under shaking conditions. The salt treated mixture was then centrifuged at 1500rpm for 10min. The supernatant was dialyzed against distilled water, concentrated against PEG 6000. The protein was purified by gel filtration chromatography using Sephadex G-200 and the molecular weight, as determined by SDS-PAGE, was found to be ~44 kDa.

(b) *Escherichia coli* (E-SBR)

Following the standardized protocol, the cell pellet of 72h old *E. coli* culture was sonicated at low frequency i.e. 15 cycles of 30 seconds each with 1-minute interval. Following centrifugation, cell debris was treated with 1M solution of NaCl under shaking conditions for 12h to extract the maximal receptor. The receptor was further purified by gel permeation column chromatography and the molecular weight was determined by SDS-PAGE (Thaper et al., 2018).

(c) *Streptococcus pyogenes* (S-SBR)

According to the protocol described in our previous communication (Thaper et al., 2019) [11], the stationary-phase culture of *S. pyogenes* was centrifuged, washed and lysed using a sonicator at low frequency. Following centrifugation, cell pellet was treated with 4M solution of NaCl under shaking conditions for 12h. After dialysis of the supernatant, the receptor was purified by using Sephadex G-200 column and the relative molecular weight of the protein was estimated to be ~96 kDa using SDS-PAGE.

Evaluation of receptors as ameliorating agents against SIF-induced bacterial impairment

Before putting up each experiment, SIF was pre-incubated with each receptor (MS-SBR/E-SBR/S-SBR) for 30min at 37°C. When the pre-incubation period was over, similar series of experiments were performed as in case of SIF (motility and binding studies with FITC-label led SIF), except that an additional set of reaction mixture containing different concentrations of each receptor pre-incubated with SIF was also prepared. Since concentration of SIF as high

as 1000µg failed to cause any bacterial death or morphological alteration, hence, the amelioration of both these parameters was not performed with any of the receptors.

Receptor-mediated amelioration of SIF-induced impairment was evaluated in case of:

Motility
For this, the different concentrations (100-1000µg) of MS-SBR/ E-SBR/ S-SBR were tested for complete blockage of bacterial immobilization within 4h.

Binding studies with FITC-labelled SIF
Each receptor was evaluated for its ability to block the binding to spermatozoa and various motile as well as non-motile bacteria upon incubation with labelled SIF. For this, same concentration of labelled SIF (100µl) along with MS-SBR/E-SBR/S-SBR was added to 100µl of each suspension (bacteria or spermatozoa) and then processed as described above.

Results and Discussion
From the onset, it was apparent that besides being spermiostatic and spermicidal, SIF, afflicted various other functional parameters of spermatozoa. Moreover, the intravaginal administration of SIF (5µg) in female mice rendered them infertile. The FITC-labelled SIF was found to bind to mouse spermatozoa and various motile and non-motile bacteria, indicating the presence of similar SIF-binding sites on spermatozoa and bacteria. Based on these interesting observations, we performed a series of simple experiments to determine what effect SIF might have on bacteria viz. *E. coli* and *S. pyogenes*.

Evaluation of bacterial impairment by SIF
Motility
The SIF-induced immobilization of *E. coli* was assessed as a function of time. The examinations repeated at hourly intervals for 4h revealed that SIF at 800µg induced 100% immobilization of *E. coli* after 2h of incubation. However, immediate immobilization of bacteria by SIF was not observed even at a concentration as high as 1000µg. This observation seemed to fit the general expectation that both *E. coli* and spermatozoa, being motile, must possess flagella; and proteins that compose structures with the similar functions with bacterial flagella are highly conserved in the evolution. In this regard, a report by Figura et al., describes the partial linear homology between tubulin (main constituent of spermatozoon flagella) and flagellin protein of bacteria [6]. It is worth mentioning here that although SIF completely blocks the motility of spermatozoa and bacteria, yet the concentration and time required to completely immobilize spermatozoa were less than that for bacteria (50µg and 30min vs 800µg and 2h). This difference in concentration and time might be due to the fact that both spermatozoa and bacteria are unrelated cell types (i.e. bacteria are prokaryotic and spermatozoa are eukaryotic), indicating that bacterial and sperm receptors might be similar, but not homologous.

Viability
SIF, being spermicidal, was evaluated for its bactericidal effect as well. The results showed that death was not induced in *E. coli*/*S. pyogenes* even after 4h of incubation with SIF at 1000µg. However, the possibility of induction of bacterial death by a higher concentration of SIF (>1000µg) or prolonged incubation period (>4h) with bacteria cannot be ignored.

Morphology
SIF is known to cause morphological alterations in head, mid-piece and tail of mouse spermatozoa. However, when scanning electron microscopy (SEM) was carried out to determine the consequence of incubation of SIF (1000µg) on the bacterial morphology, the results revealed absence of any morphological alterations, as evident by appearance of intact normal rods in case of *E. coli* and spherical cells in case of *S. pyogenes* (Figure 1). It is possible that a concentration higher than 1000µg might be successful in afflicting morphological changes in bacteria.

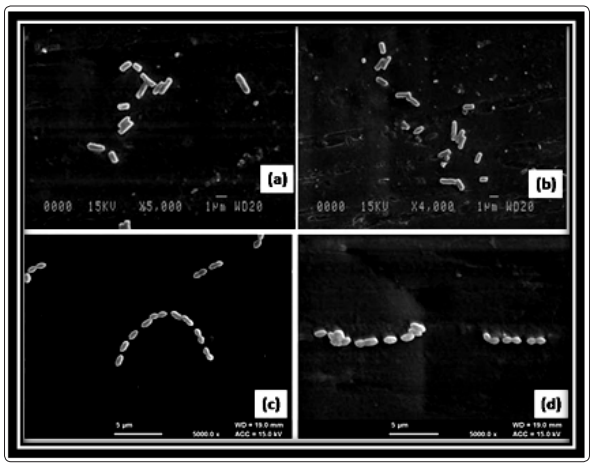

Figure 1: Representative scanning electron micrographs showing *E. coli* (a,b) and *S. pyogenes* (c,d) upon incubation with PBS (a, c) or SIF (b,d).

Binding studies with FITC-labelled SIF
The results of binding studies carried out with FITC-labelled SIF revealed the presence of bright green fluorescence on whole of the mouse spermatozoa/ bacteria *viz*. *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa, Salmonella enterica* serovar Typhi, *Vibrio cholerae, Shigella flexneri* and *Streptococcus pyogenes* which depict the presence of common SIF-binding receptors on surface of spermatozoa (Fig. 2b) and bacteria (Fig. 2c-2i). The control with spermatozoa/bacteria and unlabeled SIF in all the cases showed no fluorescence (Fig. 2a).

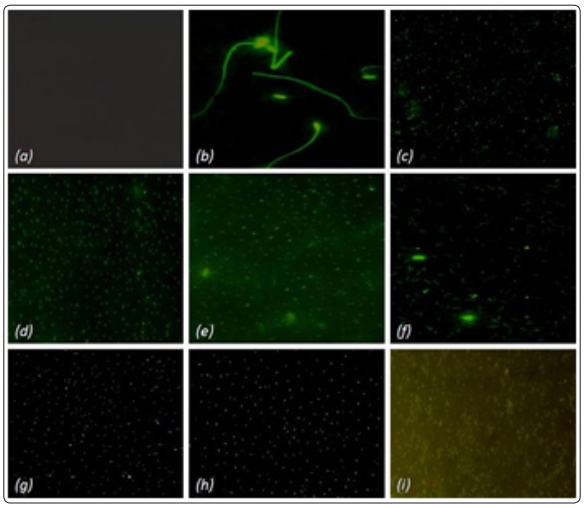

Figure 2: Fluorescent microscopy

Fluorescent microscopy of
(A) Control with unlabelled SIF and spermatozoa/bacteria/PBS
(B) FITC labelled SIF incubated with mouse spermatozoa
(C-I) FITC labeled SIF incubated with (C) *Proteus mirabilis*
(D) *Pseudomonas aeruginosa*
(E) *Salmonella enterica* serovar Typhi
(F) *Escherichia coli*
(G) *Streptococcus pyogenes*
(H) *Vibrio cholerae*
(I) *Shigella flexneri*

Evaluation of receptors as ameliorating agents against SIF-induced bacterial impairment

Using SIF as a tool, the corresponding receptors from mouse spermatozoa and bacteria (motile: *E. coli*; non-motile: *S. pyogenes*) have already been isolated and purified. Interestingly, these receptors successfully block the SIF-induced impairment of mouse spermatozoa. The next aim was to evaluate the efficacy of these receptors as ameliorating agents against SIF-induced impairment of bacteria, which was assessed on the following parameters:

Motility

When *E. coli* was incubated with SIF (800µg) in the presence of receptors *viz*. MS-SBR (1000µg), E-SBR (400µg), S-SBR (600µg) separately for 2 hours, all bacteria were 100% motile and demonstrated complete blockage of immobilization. However, bacteria incubated only with SIF (800µg), serving as the control, showed 100% immobilization.

Binding studies

The fluorescent microscopy studies carried out with FITC-labelled SIF in presence of either of the receptors revealed that the binding to mouse spermatozoa and bacteria was completely blocked when labelled SIF was incubated with either of the receptor (Figure. 3), thereby, providing an evidence for annulment of effect of SIF.

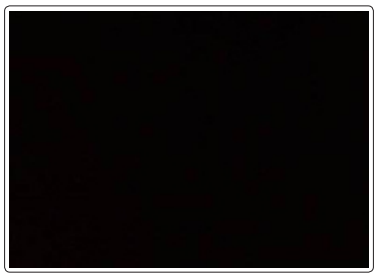

Figure 3: Receptor mediated blockage of binding of FITC-labelled SIF with spermatozoa/bacteria induced by SIF

Conclusion

The evidence provided in this study suggests that the SIF cell surface receptors are expressed on sperm cells as well as on several bacteria, thereby, proving mimicry between bacteria and spermatozoa. The future studies must focus on characterizing these receptors in order to improve the knowledge of function of these receptors and for their exploitation as an intravaginal therapy to treat the infertility induced by SIF producing microorganisms.

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