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The role of Lsa23 to mediate the interaction of *Leptospira interrogans* with the terminal complement components pathway

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ABSTRACT

Leptospirosis is a severe worldwide zoonotic disease caused by pathogenic *Leptospira* spp. It has been demonstrated that pathogenic leptospires are resistant to the bactericidal activity of normal human serum while saprophytic strains are susceptible. Pathogenic strains have the ability to bind soluble complement regulators and these activities are thought to contribute to bacterial immune evasion. One strategy used by some pathogens to evade the complement cascade, which is not well explored, is to block the terminal pathway. We have, thus, examined whether leptospires are able to interact with components of the terminal complement pathway. ELISA screening using anti-leptospires serum has shown that the pathogenic, virulent strain *L. interrogans* L1-130 can bind to immobilized human C8 (1 μg). However, virulent and saprophyte *L. biflexa* strains showed the ability to interact with C8 and C9, when these components were employed at physiological concentration (50 μg/mL), but the virulent strain seemed more competent. Lsa23, a putative leptospiral adhesin only present in pathogenic strains, interacts with C8 and C9 in a dose-dependent mode, suggesting that this protein could mediate the binding of virulent *Leptospira* with these components. To our knowledge, this is the first work reporting the binding of *Leptospira* to C8 and C9 terminal complement components, suggesting that the inhibition of this pathway is part of the strategy used by leptospires to evade the innate immunity.

1. Introduction

Leptospirosis is an important, but neglected, disease with worldwide distribution. Pathogenic species of the spirochete *Leptospira* are the causal agent of this bacterial zoonotic disease [1]. The illness is prevalent in developing countries where the lack of appropriate sanitary conditions is a real problem. Severe leptospirosis, called Weil's Syndrome, is characterized by kidney and liver failure, and multisystem bleeding, presenting a mortality rate of 4–50% [2]. Humans are considered accidental and terminal hosts and can acquire leptospiral infection by direct or indirect contact with urine from infected animals.

Virulent *Leptospira* are invasive bacteria, which have several strategies to disseminate through blood circulation until reaching and colonizing target organs. There is a consensus that complement system evasion is important for leptospiral virulence, since only pathogenic species are able to survive bactericidal activity from human serum [3,4].

The complement system is a major effector of the innate immunity, the first line of defense. It comprises more than 15 soluble plasma proteins that are activated as a tightly regulated cascade resulting in opsonization (C3b surface deposition), generation of inflammatory anaphylatoxins C3a and C5a, and formation of the cytolytic membrane attack complex (MAC) [5,6]. Terminal complement pathway starts after C5 cleavage by C5 convertase, generating C5a and C5b. The last fragment forms a complex with soluble C6, binding reversibly to cellular membranes. Assembly of transmembrane pores occurs after associations between C5b-6 and C7, C8 and C9. The final complex, the MAC, is formed when multiple C9 molecules polymerized to C5*b*-8, 9.

The ability of leptospires to interact with fluid-phase

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complement regulators factor H and C4BP, blocking activation of alternative and classical pathways at C3-convertase level has been shown [4,7,8]. A number of leptospiral binding proteins for factor H and C4BP have been described, as Lsa24/LenA/LfhA [7], LigA and LigB [9], Lsa30 [10], Lsa33 and Lsa25 [11] and Lsa23 [12]. However, pathogenic microorganisms usually develop many routes to evade the complement system, expressing different receptors that can interfere in one or more distinct level of the complement cascade.

One strategy explored for some pathogens, but not well studied in pathogenic *Leptospira*, is the blockage of terminal pathway by direct binding with proteins of MAC. Inhibitors of the terminal pathway were described for several microorganisms including parasites [13,14] and pathogenic bacteria [15–19]. Involvement of leptospira with the terminal complement pathway was recently suggested by interaction with the regulatory protein vitronectin and the surface protein LcpA [20]. However, the involvement of pathogenic leptospires with proteins of terminal complement pathway has not been evaluated.

Thus, we decided to investigate whether leptospires by interacting with terminal complement components control this pathway of the host immune system so as to survive in human serum. We show that virulent low-passage *L. interrogans* L1-130 and saprophyte *L. biflexa* strains bind the terminal complement C8 and C9, when these components are at physiological concentration. Furthermore, we report the surface protein Lsa23 as C8 and C9 binding protein, which may mediate MAC inhibition. To our knowledge, these are the first data showing direct interaction of *Leptospira* and the human complement components C8 and C9.

2. Materials and methods

2.1. Biological components

Complement components from terminal pathway C7, C8 and C9, purified from normal human serum (NHS) and the corresponding goat polyclonal antiserum were purchased from Complement Technology, INC. (Tyler, TX, USA). Monoclonal HRP-conjugated anti-His tag antibody, bovine serum albumin (BSA) and the highly glycosylated protein fetuin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Bacterial strains

Low-passage virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF), pathogenic culture-attenuated *L. interrogans* serovar Copenhageni strain M20 and saprophytic *L. biflexa* serovar Patoc strain Patoc 1 were cultured at 28 °C, under aerobic and static conditions, in liquid EMJH medium (Difco®, BD, Franklin Lakes, NJ, USA) with 10% (vol/vol) *Leptospira* enrichment EMJH (Difco®, BD) enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vo.: 0,001%), calcium chloride (wt/vol: 0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol: 0.03%) and meat extract (wt/vol: 0.02%) [21]. Virulence of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and *L. interrogans* serovar Kennewicki (LPF) is maintained by continuous passage in recently weaned male Golden Syrian hamsters.

2.3. Antiserum production against whole cell leptospires and antibodies titration

L. interrogans serovar Kennewicki (LPF) cultures were harvested by centrifugation at 11,500 g. Washed pellets were heat-inactivated at 56 °C for 20 min and resuspended in PBS. Recently weaned male Golden Syrian hamsters were subcutaneously injected with a suspension of 10^9 inactivated leptospires mixed with 12.5% Allydrogel (2% $Al(OH)_3$; Brenntag Biosector, Frederikssund, Denmark) as adjuvant. One booster injection was given after 15 days with the same mixture. The animals were bled from the retro-orbital plexus 30 days after the first injection and the pooled serum were stored at −20 °C. Antibodies titration was performed against *L. biflexa* serovar Patoc (Patoc 1), *L. interrogans* serovar Copenhageni (M20) and *L. interrogans* serovar Copenhageni (Fiocruz L1-130) by ELISA. The titers were considered the highest dilution in which the obtained absorbance values at 492 nm were 1.0.

2.4. Attachment assay between leptospires and immobilized human components of terminal complement pathway

C7, C8 and C9 complement proteins (10 μg/mL, Complement Technology) were immobilized onto microplates wells for 16 h at 4 °C. BSA and fetuin (Sigma-Aldrich) were used as negative controls for nonspecific binding (10 μg/mL). The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked, for 2 h at 37 °C, with 3% BSA diluted in PBS. Then, leptospires (10^7 bacteria/well) were allowed to attach to different complement components for 2 h at 37 °C. Bound leptospires were detected by adding an appropriate dilution ($OD_{492nm} = 1.0$) of anti-leptospires whole serum raised in hamsters as follows: *L. biflexa* serovar Patoc (Patoc 1) - 1:50; *L. interrogans* serovar Copenhageni (M20)–1:500; *L. interrogans* serovar Copenhageni (Fiocruz L1-130)–1:100. After 1 h at 37 °C, wells were washed and 100 μL PBS containing HRP-conjugated anti-hamster IgG (1:5000–Sigma-Aldrich) were added to each well. Peroxidase reactions were detected adding the *o*-phenylenediamine dihydrochloride substrate (OPD–1 mg/mL) and H_2O_2 (1 μL/mL) diluted in citrate-phosphate buffer (pH 5.0). Sulfuric acid (2 M) was used to stop the reactions and absorbance measurements were taken at 492 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific). For statistical analysis, the binding of leptospires strains to complement proteins was compared to their binding to BSA and fetuin using one-way ANOVA followed by Tukey post-test. Comparison among leptospiral strains was also performed.

2.5. Incubation of Leptospira spp. to soluble human C7, C8 and C9 complement components

Leptospires (10^9/mL) were centrifuged at 11,500 g for 30 min, washed twice with low-salt PBS (lsPBS – containing 50 mM NaCl) and incubated with 10 μg/mL of C7, C8 or C9, the same concentration used for the screening ELISA with immobilized components. Bacteria were allowed to interact with the soluble components C7, C8 and C9 for 2 h at 37 °C. Then, the mixtures were centrifuged for 15 min at 11,500 g. Supernatants were collected for further analysis. Pellets were resuspended in lsPBS or washed once with the same buffer before being resuspended. Leptospires incubated at the same conditions in absence of any component were used as blank control.

2.6. ELISA assay for evaluation of leptospires interaction with soluble human C7, C8 and C9

As described above, mixtures of leptospires and complement proteins were centrifuged after 2 h of incubation. Supernatants, resuspended pellets and resuspended pellets after one washing with lsPBS were adsorbed on microplates wells for 16 h at room temperature. After three washes with PBS-T, wells were blocked with *Super Block™ (TBS) Blocking Buffer* (Thermo Fisher Scientific). Terminal complement components were detected by adding corresponding polyclonal antiserum anti-C7 (1:32,000), anti-C8