Immune Evasion by Pathogenic *Leptospira* Strains: The Secretion of Proteases that Directly Cleave Complement Proteins

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Leptospirosis is an infectious disease of public health importance. To successfully colonize the host, pathogens have evolved multiple strategies to escape the complement system. Here we demonstrate that the culture supernatant of pathogenic but not saprophytic *Leptospira* inhibit the three complement pathways. We showed that the proteolytic activity in the supernatants of pathogenic strains targets the central complement molecule C3 and specific proteins from each pathway, such as factor B, C2, and C4b. The proteases cleaved α and β chains of C3 and work in synergy with host regulators to inactivate C3b. Proteolytic activity was inhibited by 1,10-phenanthroline, suggesting the participation of metalloproteases. A recombinant leptospiral metalloprotease from the thermolysin family cleaved C3 in serum and could be one of the proteases responsible for the supernatant activity. We conclude that pathogenic leptospiral proteases can deactivate immune effector molecules and represent potential targets to the development of new therapies in leptospirosis.

**Keywords.** Leptospira; Leptospirosis; Immune Evasion; Innate Immunity; Complement System; Proteases.

Leptospirosis is a neglected infectious disease of public health importance [1, 2]. The disease is caused by spirochetes from the genus *Leptospira*, which includes pathogenic and saprophytic species [3]. To successfully establish an infection, leptospires must evade host immune defenses, including the complement system. Complement is a major arm of innate immunity, and one of its main functions is to recognize and destroy microorganisms (for a comprehensive review, see [4]). The three activation pathways of the complement system ensure that virtually any nonhost surface is recognized as hostile. However, pathogenic microorganisms have evolved multiple strategies to escape complement attack, which include: (1) the production of a capsule to prevent complement recognition, (2) the acquisition of host fluid phase complement regulatory proteins, such as factor H (FH) and C4b-binding protein, and (3) the secretion of proteases that inactivate complement molecules [5–7].

The ability of pathogenic leptospires to multiply and spread in the host reflects a low efficacy of complement against them [8]. Indeed, these bacteria are able to efficiently acquire the host regulators FH and C4b-binding protein, promoting complement inactivation on their surfaces. Diverse membrane proteins have been described as ligands for these regulators, including the leptospiral immunoglobulin-like (Lig) proteins A and B, which bind both regulators [9].

The secretion of proteases that cleave and inactivate complement proteins has been described as an effective immune evasion strategy for several human pathogens [10, 11]. Interestingly, pathogen-derived proteases target a wide range of substrates, including the central complement molecule C3, proteins involved in cascade initiation, such as C1q and immunoglobulins, as well as terminal components, such as C5 [12]. Illustrative examples of these proteases are elastase (PaE) and alkaline protease (AprA) from *Pseudomonas aeruginosa*,...
gelatinase (GelE) from Enterococcus faecalis, and aureolysin (Aur) from Staphylococcus aureus. All these proteins are secreted enzymes that belong to the metalloprotease class. PaE and AprA proteases target C1q and C3, promoting a functional impairment of all complement pathways [13]. The protease GelE cleaves C3 and the fragments C3b, C3a and iC3b, promoting a substantial reduction of the pathogen’s phagocytosis by human polymorphonuclear leukocytes [14, 15]. Furthermore, secreted Aur acts in synergy with host regulators to inactivate C3, effectively hampering the host innate immune response [16].

To our knowledge the secretion of proteases as an immune evasion strategy has not yet been investigated in Leptospira. Given the ability of these pathogenic microorganisms to disseminate fully in the host, we believe it would be quite relevant to examine complement inhibition in the fluid phase, which could create a favorable environment for leptospiral multiplication and spread. Therefore, the purpose of this work was to analyze the ability of pathogenic leptospires to secrete proteases that target complement molecules. The cleavage of these proteins could markedly impair complement activation and may constitute a new immune evasion mechanism in Leptospira.

**MATERIALS AND METHODS**

**Proteins, Antibodies and Sera**

The complement proteins purified from human plasma and the polyclonal antibodies against them were purchased from Complement Technology. The anti-goat immunoglobulin G (IgG) conjugated with peroxidase was purchased from KPL. Normal human serum was obtained from healthy volunteers, after informed consent.

**Leptospira Strains and Culture Supernatants**

The Leptospira strains used are listed in Figure 1. All strains were cultivated for 7 days in modified Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 29°C under aerobic conditions [17]. The supernatants containing the secreted proteins were obtained as follows: freshly harvested leptospires were washed twice in phosphate-buffered saline (PBS; pH 7.4), counted by dark-field microscopy using a Petroff-Hausser chamber, and suspended in a final volume of PBS to obtain 1 × 10^9 leptospires. To allow the secretion of proteases, bacterial suspensions were incubated at 37°C for 4 hours. The supernatants were collected after centrifugation, passed through a 0.22-mm filter, and used in functional assays.

**Cloning, Expression and Purification of Leptospira interrogans Thermolysin**

*Escherichia coli* DH5α was the cloning host strain, and *E. coli* BL21 (SI) was chosen for expression of the recombinant proteins, using a T7 promoter-based expression plasmid pAE [18]. The leptospiral thermolysin (gene ID, 2770759; chromosome, 1; open reading frame, nucleotides 4076605–4078989) fragments PepSY-M4 and M4 were amplified by polymerase chain reaction from genomic DNA of *Leptospira interrogans* serovar Copenhageni strain M10 using the following primers: CTCGAGGATGCTAGAGATAC (fragment M4, F), CGCAA GCTCTAAATACTGT (fragment M4, R), CTCGAGCAGTT CCAGAGAA (fragment PepSY-M4, F), and CGCAAGCTTC TAAATACGT (fragment PepSY-M4, R). Cloning, expression, and purification were performed as described elsewhere [17].

**Electrophoresis and Protein Dosage**

Leptospiral culture supernatants or recombinant thermolysins were solubilized in reducing loading buffer, heated for 3 minutes at 96°C, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [19]. Protein concentration was determined using Pierce BCA Kit. All protein preparations were free of lipopolysaccharide according to the limulus amebocyte lysate test (<0.125 endotoxin units/mL).

**Proteolytic Activity and Inhibition Assays**

Leptospiral culture supernatants (3 µg of total secreted proteins) or recombinant thermolysins (1 µg of the fragments PepSY-M4 or M4) were incubated with purified complement proteins (0.25 µg) or serum (amount corresponding to 0.25 µg of the complement protein studied), for different times at 37°C. Proteolytic specificity was further confirmed with reactions using 5, 10, and 50 times less leptospiral supernatant (corresponding to 0.6, 0.3, and 0.06 µg of total protein, respectively). To analyze C3b cleavage, reactions were performed with the regulatory proteins factor I (FI) (0.2 µg) and/or FH (0.5 µg), in the presence or absence of the leptospiral supernatants. The chemical nature of the proteases was accessed by preincubation of leptospiral supernatants with inhibitors of serine, metallo-, cysteine, or aspartyl proteases (5 mmol/L Phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L 1,10-phenanthroline, 28 µmol/L E-64, or 5 µmol/L pepstatin, respectively) for 30 minutes.

The cleavage products were analyzed by means of Western blot [20]. The reactions were subjected to SDS-PAGE and transferred to nitrocellulose membranes [21]. After blocking with 10% skimmed milk in PBS-T (PBS containing 0.05% Tween 20), complement proteins were detected by goat anti-human polyclonal antibodies, followed by peroxidase-conjugated anti-goat antibodies. Positive signals were detected by enhanced chemiluminescence (West Pico, Pierce).

**Complement Activation Assays**

Complement assays were performed as described elsewhere [22] with modifications. Microtiter plates were coated overnight with *E. coli* lipopolysaccharide (1 µg per well), human IgG (400 ng per well), or mannan (1 µg per well) in 0.1 mol/L sodium carbonate buffer (pH 9.6). Plates were washed with PBS-T between each of the following steps. Plates were blocked with PBS–3% bovine

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*Leptospira* Proteases Cleave Complement Proteins • *JID* 2014;209 (15 March) • 877
serum albumin for 2 hours at 37°C. For the alternative pathway (AP), serum was diluted in AP buffer (144 mmol/L sodium chloride, 0.96 mmol/L sodium barbital, 2.48 mmol/L barbituric acid, 1.4 mmol/L magnesium chloride, and 10 mmol/L ethylene glycol tetraacetic acid), and for the classic and lectin pathways, it was diluted in gelatin veronal buffer with Ca++ and Mg++ (GVB++) buffer (144 mmol/L sodium chloride, 0.96 mmol/L sodium barbital, 2.48 mmol/L barbituric acid, 0.83 mmol/L magnesium chloride, and 0.25 mmol/L calcium chloride). To analyze the inhibitory effect, diluted serum was preincubated with leptospiral supernatants (3 µg of total secreted proteins) or PBS for 1 hour at 37°C, and added to the plates for 1 hour at 37°C. Complement activation was measured by deposition of C3b (alternative pathway) or C4b (classical and lectin pathways) with anti-C3 and anti-C4 polyclonal antibodies, respectively, followed by peroxidase-conjugated anti-goat IgG. Substrate reaction was performed with o-phenylenediamine dihydrochloride, and absorbance was measured at 492 nm.

**N-terminal Sequencing**

The supernatant of *L. interrogans* serovar Kennewicki strain Fromm (2 µg of total secreted proteins) was incubated with C3 (150 µg) for 4 hours at 37°C. Proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Cleavage products were visualized with 0.1% Coomassie blue in 40% methanol, excised, and analyzed in a Shimadzu PPSQ-23 protein sequencer.

**RESULTS**

**Production and Electrophoretic Characterization of Leptospiral Secreted Proteins**

The first step of this work was to obtain an electrophoretic profile of the proteins secreted by the nine *Leptospira* strains studied, of which seven are pathogenic and two saprophytic (Figure 1). The strains were cultivated for 7 days at 29°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. The leptospires were then washed, resuspended in PBS (pH 7.4), and allowed to secrete proteins for 4 hours at 37°C. These conditions were selected to mimic the host environment regarding osmolarity (300 mOsm), pH (7.4) and temperature (37°C) [23].

The leptospiral supernatants were collected and analyzed by electrophoresis. The secreted proteins from the 9 *Leptospira* strains differ in composition, number, and intensity of bands. The majority of the components have molecular weights lower than 95 kDa (Figure 1). The two saprophytic strains share a similar protein profile, which differs from those presented by the pathogenic species.

**Inhibition of three Complement Pathways by Supernatant of Pathogenic *Leptospira***

To analyze if the supernatant of pathogenic *Leptospira* was able to inhibit the complement cascade, we tested its activity in a well-described assay where the three pathways can be assessed separately [22]. The alternative, classical and lectin pathways were specifically activated in microtiter plates coated with lipopolysaccharide, IgG, or mannan, respectively. To test for inhibitory activity, human serum was preincubated with the supernatant of pathogenic *L. interrogans* serovar Kennewicki strain Fromm or saprophytic *Leptospira biflexa* serovar Andaman strain CH11 before complement activation. Interestingly, we observed that only the supernatant of pathogenic *Leptospira* inhibited the deposition of C3b (alternative pathway) and C4b (classical and lectin pathways) (Figure 2). In contrast, proteins secreted by the saprophytic strain did not cause any inhibition of complement activity.
Direct Cleavage of C3b by Pathogenic Leptospiral Secreted Proteases

The complement inhibition produced by the supernatant of pathogenic Leptospira prompted us to investigate whether secreted proteases could target key complement molecules, thereby promoting a downregulation of complement activation. To investigate this hypothesis, different amounts of bacteria and culture incubation times were analyzed (Supplementary Figure 1). The tests revealed that the proteolytic activity of the leptospiral supernatant increases with both the total amount of bacteria present and incubation time. We also observed that the C3b molecule is rapidly cleaved by the secreted proteases. Indeed, after only 5 minutes of reaction a cleavage band of approximately 46 kDa is observed (Figure 3A). A more pronounced cleavage occurred after 40 minutes of incubation, with the production of additional fragments ranging from 47 to 28 kDa.

Synergistic Inactivation of C3b by Pathogenic Leptospiral Proteases and Host Factors

Previous studies have shown that pathogenic leptospires are able to bind the host complement regulatory protein FH. At the bacteria surface, bound FH acts as a cofactor of FI in C3b cleavage [9, 24]. Considering this well-established evasion mechanism in Leptospira, we asked whether the secreted proteases could act together with host complement regulators in C3b cleavage. To answer this question, proteolytic assays were performed with C3b and the supernatant of pathogenic L. interrogans serovar Kennewicki strain Fromm, in the presence or absence of FI and/or FH (Figure 3B). We observed that a complete degradation of the α’ chain occurred when FI and FH were added to the reactions, suggesting that the leptospiral proteases work in synergy with these regulators to effectively inactivate C3b.
Cleavage of Complement Proteins From three Pathways by Proteases Secreted by Pathogenic Leptospira Strains

In the assays described above, we showed that pathogenic *L. interrogans* serovar Kennewicki strain Fromm secretes proteases that cleave C3b. To investigate whether this activity is restricted to pathogenic strains, and also to analyze if these proteases target other complement molecules, we repeated the experiments using a panel of nine *Leptospira* strains (Figure 4). Interestingly, we observed that all pathogenic strains were able to secrete proteases that cleave complement proteins from the three pathways. In contrast, the saprophytic strains did not present a significant proteolytic activity. The leptospiral proteases target central complement molecules, such as C3 and its fragments C3b and iC3b, and also proteins from the alternative (factor B [FB]), classical, and lectin pathways (C4b and C2) (Figure 4). To further confirm this activity, reactions with reduced amounts of leptospiral supernatants were performed (Supplementary Figure 2). In these assays, we demonstrated that the complement molecules are still cleaved, even when 5 to 50 times less supernatant was used, which reinforces the presence of enzymatic activity in the samples.

The leptospiral proteases were not able to cleave all proteins tested. C1q, C4, FD, properdin, and human IgG were not degraded by the leptospiral culture supernatant (data not shown). This indicates that these secreted enzymes have some degree of specificity for their substrates. Interestingly, the leptospiral proteases were not able to degrade purified C4 (data not shown). However, the C4b molecule, which is derived from C4, has its α' chain extensively degraded (Figure 4 and Supplementary Figure 2). A possible explanation would be the presence of proteolytic sites in the C4 α chain that are exposed only when this molecule is cleaved, generating the C4b α' chain.

Proteases Secreted by Pathogenic *Leptospira* and Degradation of α and β Chains of C3

To determine the cleavage sites of the leptospiral proteases, we performed an analysis of the C3 fragmentation by these enzymes. C3 was incubated with the leptospiral supernatant and the cleavage products were submitted to N-terminal sequencing by Edman degradation (Figure 5). Despite being unsuccessful in sequencing all of the cleavage products, the fragments that were sequenced revealed that both α and β chains of C3 are degraded. A schematic representation of the cleavages shows that the 43-kDa (SLQLPS↓RSSKI), 47-kDa (LNEQRY↓YGGGYG), and 68-kDa fragments may originate from α-chain degradation and that the 28-kDa (KLSINT↓HPSQKPL) and 46-kDa fragments are produced by β-chain cleavage. An analysis of the C3 structure (PDB number 2A73) showed that the proteolytic sites are located in hydrophilic, antigenic, and surface-exposed regions, which can be accessed by the leptospiral proteases (data not shown).

Inhibition of Complement-Directed Proteases Secreted by Pathogenic *Leptospira* by 1,10-Phenanthroline

To identify the classes of proteases involved in the cleavage of the complement proteins, inhibition assays were performed.

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Figure 3. Pathogenic leptospiral proteases directly cleave C3b and work in synergy with host factors to inactivate this molecule. A, The supernatant of pathogenic *Leptospira interrogans* serovar Kennewicki strain Fromm was incubated with C3b for 5–120 minutes at 37°C. The cleavage of C3b by factor I (FI) using factor H (FH) as a cofactor was used as a control. B, The leptospiral supernatant was incubated with C3b, in the presence or absence of FI and/or FH, for 2 hours at 37°C. All cleavages were analyzed by Western blot with anti-C3 polyclonal antibodies. Asterisk indicates complete degradation of C3b α’ chain. Abbreviation: PBS, phosphate-buffered saline.
In these experiments, before the addition of the substrates, the leptospiral supernatant was treated with inhibitors of serine, metallo-, cysteine, or aspartyl proteases (Figure 6). We observed that only 1,10-phenanthroline was able to inhibit the proteolytic activity, which points toward the participation of metalloproteases. However, it does not completely exclude the contribution of other classes of enzymes, in particular serine proteases, some of which have been shown to be inhibited by 1,10-phenanthroline [25].

Cleavage of Complement Proteins in Human Serum by Pathogenic Leptospiral Proteases

Our data clearly demonstrate that leptospiral proteases are able to cleave various purified complement molecules. However, we
should consider that to constitute an immune evasion strategy, the secreted proteases must also have activity in serum. To address this issue, we performed further proteolytic assays, which revealed that the leptospiral proteases are able to cleave C3, C4, C2, and FB in NHS (Figure 7A).

These cleavages were less pronounced than those observed with the purified proteins, which could be partially explained by the presence of protease inhibitors in serum, such as α2-macroglobulin. Alternatively, it is also possible that the cleavages were affected by a competition among the different substrates, which are present all together in serum.

Another interesting observation was the cleavage of C4, which occurred only when NHS was used as a complement source (Figure 7A) but is not observed when the purified protein is used. We speculate that proteins present in human serum could act as cofactors of the leptospiral proteases, helping them cleave C4 in this context.

Cleavage of C3 in Human Serum by Recombinant Leptospira Thermolysin

The proteases inhibition assays indicate that metalloproteases could be among the enzymes responsible for the cleavages of the complement proteins. This observation prompted us to perform a detailed search in the Leptospira genomes to find possible candidates to this function. To our surprise, most of the proteases genes present in pathogenic Leptospira share some degree of identity and/or similarity with genes of the saprophytic strains. One of the few exceptions are those related to thermolysins, which are metalloproteases from the peptidase M4 family whose genes are present only in pathogenic strains. Among these proteins, the thermolysin encoded by the gene LIC13322 of L. interrogans serovar Copenhageni was selected to be produced as a recombinant protein in E. coli. This leptospiral thermolysin has a predicted signal peptide for secretion (Figure 7B) and catalytic domains that are 20%–25% identical and 32%–35% similar to metalloproteases from other pathogens that have already been described to cleave complement proteins (Supplementary Table 1).

Our attempts to express the full-length thermolysin LIC13322 were unsuccessful (data not shown). As an alternative, two different portions of the protein were produced: the M4 fragment, which contains the catalytic M4 domains, and the PepSY-M4 fragment, which includes the propeptide PepSY plus the catalytic domains (Figure 7C). To confirm the presence of thermolysin-like proteins in the Leptospira supernatants, we produced polyclonal antibodies against the M4 fragment in mice and used them in Western blot assays. The anti-M4 antibodies recognized proteins with molecular weights between 75 and 55 kDa (Figure 7D), which is in accordance with the predicted size of the native thermolysin fragments PepSY-M4 (71 kDa) and M4 (57 kDa).

The proteolytic activity of the thermolysin fragments PepSY-M4 and M4 was analyzed. Only the PepSY-M4 construction was able to cleave C3 in serum (Figure 7E). Indeed, it has been reported that the PepSY domain may act either as an inhibitor, preventing premature activation of the enzyme, or as a chaperone, promoting proper folding of the catalytic domain [27]. It seems that in the case of the recombinant leptospiral thermolysin, the PepSY domain may act as a chaperone, facilitating proper folding necessary for the proteolytic activity of the M4 domain.
DISCUSSION

In this work, we demonstrated that the supernatant of pathogenic Leptospira is able to inhibit the activation of the complement system (Figure 2). This inhibition can be directly correlated with the secretion of proteases that cleave and inactivate key complement proteins. The three pathways were inhibited, which reinforces the participation of proteases, since these enzymes target both α and β chains of C3, a central complement molecule, and also specific proteins from the alternative (FB), classical, and lectin pathways (C4 and C2) (Figures 4 and 5; Supplementary Figure 2).

Figure 6. The proteases secreted by pathogenic Leptospira are inhibited by 1,10-phenanthroline. Before the addition of complement proteins, the leptospiral supernatant was incubated with inhibitors of serine proteases (5 mmol/L PMSF; lane 4), cysteine proteases (28 µmol/L E-64; lane 5), metalloproteases (5 mmol/L 1,10-phenanthroline; lane 6), or aspartyl proteases (5 µmol/L pepstatin; lane 7). The vehicles of the protease inhibitors were used as controls, including phosphate-buffered saline (lane 1), ethanol-water solution (1:1; lane 2), and ethanol (lane 3). The cleavages of C3, C3b, iC3b, factor B (FB), C2, and C4b were analyzed by means of Western blot assays with polyclonal antibodies.
Interestingly, apart from the leptospiral proteases, only AprA from *S. aureus* was demonstrated to cleave C2 [28]. This protein was able to effectively inhibit the classical and lectin pathways of complement. Despite the high degree of homology between C2 and FB, AprA targets only C2. In contrast, the leptospiral proteases are able to cleave both of these proteins (Figure 4). This
could be a result of the action of different enzymes present in the supernatant or the activity of a single protease less specific than AprA.

One of the negative regulators of the alternative pathway is the serine protease FI. This enzyme, in the presence of the cofactors FH or membrane cofactor protein (MCP), cleaves C3b in fragments that are unable to continue the complement cascade. However, some of these fragments, such as iC3b, are still immunologically active. Both C3b and iC3b are important opsonins and facilitate phagocytosis through CR1 and CR3 receptors, respectively [29]. The leptospiral proteases cleave C3b into products that are distinct from those produced by FI (Figure 3A). The C3b fragment is extensively degraded by the bacterial enzymes, generating additional bands that are not obtained with the host serine protease. The secreted leptospiral proteases are also able to cleave purified iC3b, promoting an efficient inactivation of this molecule (Figure 4). The cleavage of both C3b and iC3b by the leptospiral proteases could result in a functional impairment of complement-mediated phagocytosis. Indeed, a similar effect was observed with the metalloprotease GelE from E. faecalis, which cleaves C3b and iC3b and promotes a substantial reduction in the pathogen’s phagocytosis by human polymorphonuclear leukocytes [14, 15].

We also showed in this study that the leptospiral proteases were able to work synergistically with the host regulators FI and FH in C3b inactivation (Figure 3B). This synergy between pathogenic proteases and host factors has also been described for other pathogens, such as E. faecalis [14, 15] and S. aureus [16]. The metalloproteases GelE and Aur from E. faecalis and S. aureus, respectively, cleave C3 in a specific α-chain site, generating a C3b-like molecule, which is further degraded by host FI and FH.

Another interesting finding in this study was that these proteases involved in complement inactivation are probably metalloproteases (Figure 6). A detailed search in the National Center for Biotechnology Information GenBank database [30] allowed us to find potential candidates that are metalloproteases from the thermolysin family. This family of enzymes comprises proteases that are considered virulence factors for diverse pathogens. Thermolysins are able to degrade various biologically important substrates, such as extracellular matrix components, interleukins, and proteins from the complement system [31]. To study the activity of these proteins, we expressed and purified two recombinant fragments of the thermolysin encoded by the gene LIC13322. The PepSY-M4 fragment was able to cleave C3 in human serum, suggesting that thermolysin could be one of the proteases responsible for the effects previously observed with the whole leptospiral supernatant. However, this protein was not able to cleave C2, C4, and FB (data not shown), which indicates that other leptospiral proteases are also involved in complement degradation. The purification and characterization of the native proteases secreted by pathogenic leptospires are currently underway.

From this work, we can conclude that the proteases secreted by pathogenic Leptospira are able to deactivate immune effector molecules. These proteases represent potential targets for the development of new treatments and prophylactic approaches in leptospirosis.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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