Evasion of Complement-Mediated Lysis and Complement C3 Deposition Are Regulated by <i>Francisella tularensis</i> Lipopolysaccharide O Antigen

Corey D. Clay, Shilpa Soni, John S. Gunn and Larry S. Schlesinger

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Evasion of Complement-Mediated Lysis and Complement C3 Deposition Are Regulated by Francisella tularensis Lipopolysaccharide O Antigen

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The bacterium Francisella tularensis (Ft) is a potential weapon of bioterrorism when aerosolized. Macrophage infection is necessary for disease progression and efficient phagocytosis by human macrophages requires serum opsonization by complement. Microbial complement activation leads to surface deposition of a highly regulated protein complex resulting in opsonization or membrane lysis. The nature of complement component C3 deposition, i.e., C3b (opsonization and lysis) or C3bi (opsonization only) fragment deposition, is central to the outcome of activation. In this study, we examine the mechanisms of Ft resistance to complement-mediated lysis, C3 component deposition on the Ft surface, and complement activation. Upon incubation in fresh nonimmune human serum, Schu S4 (Ft subsp. tularensis), Ft (Ft subsp. novicida), and LVS (Ft subsp. holarctica live vaccine strain) were resistant to complement-mediated lysis, but LVSG and LVSR (LVS strains altered in surface carbohydrate structures) were susceptible. C3 deposition, however, occurred on all strains. Complement-susceptible strains had markedly increased C3 fragment deposition, including the persistent presence of C3b compared with C3bi, which indicates that C3b inactivation results in survival of complement-resistant strains. C1q, an essential component of the classical activation pathway, was necessary for lysis of complement-susceptible strains and optimal C3 deposition on all strains. Finally, use of Francisella LPS mutants confirmed O Ag as a major regulator of complement resistance. These data provide evidence that pathogenic Francisella activate complement, but are resistant to complement-mediated lysis in part due to limited C3 deposition, rapid conversion of surface-bound C3b to C3bi, and the presence of LPS O Ag. The Journal of Immunology, 2008, 181: 5568–5578.
which may affect vaccine utility. The genetic basis for phase variation, which also occurs for type A isolates, is also known (10). In fact, based on changes in LPS structure, multiple gray variant phenotypes have been described including variants that completely lack O Ag and variants that express antigenically distinct O Ag compared with LVS (11, 12).

Tularemia results from exposure to infected animal tissue, bites from arthropod vectors, or the direct ingestion or inhalation of Francisella (13). Pneumonic tularemia is the most serious form because untreated cases result in 30–60% mortality compared with 5–6% mortality associated with cutaneous disease (14, 15). Pneumonic tularemia develops upon inhalation of <10 CFU or via hemogenous spread of bacilli from peripheral sites to the lung (13, 16). Because of its highly infectious and lethal nature, Ft is designated as a category A select agent by the Centers for Disease Control and Prevention and is a potential weapon of bioterrorism. Francisella predominantly infects and replicates within macrophages and spreads systemically via the reticuloendothelial system (17).

Considering the extremely low infectious dose required to cause disease, it is paradoxical that in vitro studies examining macrophage infection by Ft require high experimental multiplicities of infection of at least 100:1 without opsonization. However, phagocytosis of type A Francisella, LVS, and Fn by human monocyte-derived macrophages increases dramatically in the presence of serum in a C3- and CR-dependent manner (18, 19). Complement-mediated opsonization has also been shown for the phagocytosis of LVS by human monocyte-derived dendritic cells (20). Given the importance of complement-mediated opsonization for uptake by phagocytes and a previous report that LVS does not fix C3 (21), we studied the nature of complement interactions with Francisella in nonimmune human serum. In this study, we examined several Francisella subspecies and variant strains to determine 1) whether complement activation leads to bacterial lysis, 2) the nature of C3 deposition and subsequent fragmentation, 3) complement activation pathways, and 4) resistance mechanisms to complement-mediated lysis.

Materials and Methods

Bacterial strains

Ft subsp. tularensis strain Schu S4, a Centers for Disease Control and Prevention clinical isolate, was provided by R. Lyons (University of New Mexico, Albuquerque, NM). Ft subsp. holarctica LVS (ATCC 29684) was provided by K. Elkins (Center for Biologics Research and Evaluation, U.S. Food and Drug Administration, Bethesda, MD). Fn (U112), LVSg, and LVSr were provided by F. Nano (University of Victoria, British Columbia, Canada). LVSg is a spontaneous gray phase variant that rarely lack O Ag and variants that express antigenically distinct O Ag phenotypes have been described including variants that completely lack O Ag and variants that express antigenically distinct O Ag compared with LVS (11, 12).

Human sera, complement components, and reagents

Serum was isolated from healthy adult volunteers with no known exposure to Francisella according to a protocol approved by the Ohio State University Medical College Internal Review Board. The sera were processed to maintain optimal complement activity (23). Briefly, isolated nonheparinized whole blood was kept at room temperature to allow clot formation and then at 4°C to allow for clot contraction. The clot was removed by centrifugation at 500 × g for 15 min at 4°C. The serum fraction was collected, filtered sterilized, aliquoted, and stored at −80°C. Heat inactivation (HI) was performed at 56°C for 30 min. C5-depleted (d), C8d, Clq, and factor B (FB)-depleted (FBD) sera, and purified Clq and FB were purchased from Complement Technology and stored at −80°C. On the day of use, fresh sera were thawed at room temperature and diatomically chilled on ice until needed. A concentrated serine protease inhibitor mixture (containing AEBSF, aprotinin, elastatin, and GGACK) was purchased from Calbiochem. Fifty percent hydroxyamine was purchased from Alfa Aesar. Other chemicals were purchased from Sigma-Aldrich.

Bactericidal assays

Complement-mediated killing was conducted using fresh nonimmune or HI (negative control) sera. Bacteria were suspended in gelatin veronal buffer (GVB; 0.1% gelatin, 5.5 mM barbitol, 142 mM NaCl, 0.5 mM MgCl₂, and 0.15 mM CaCl₂; pH 7.3) at equalized concentrations by measuring the OD at 600 nm. For each assay, 2 × 10⁶ bacteria were incubated with various serum concentrations for 1 h in microcentrifuge tubes (final volume of 200 μl in reaction buffer) at 37°C with slow agitation. For some experiments, 10 mM EDTA or 10 mM EGTA with 7 mM MgCl₂ was added to fresh serum to determine the potential contribution of pathways. In other experiments, Clq, Clq-replete (r); (achieved by adding purified Clq to Clq at a final concentration of 200 μg/ml), FBD, and FB (achieved by adding purified FB to FBD at a final concentration of 400 μg/ml) sera were used. Reactions were stopped by placing tubes on ice for 5 min. For some experiments, a serine protease inhibitor mixture diluted in ice-cold PBS was used to stop the reaction, and the results were the same as those using iced tubes. Ten-fold serial dilutions were plated to determine surviving CFU.

C3 deposition assays and Western blotting

Fresh donor, HI donor, C5d, Clq, and Clq-r sera were used to evaluate complement component C3 deposition on Ft strains. After preblocking microcentrifuge reaction tubes in PBS containing 0.1% human serum albumin (ZLB Plasma) for 30 min at 37°C, 5 × 10⁶ bacteria were incubated in 10% fresh serum or serum containing 10 mM EDTA for 5 or 30 min at 37°C. Reactions were stopped and samples were washed as described above for Western blotting experiments. To ensure equal loading, aliquots were taken from the final PBS wash and plated to count CFU. After final resuspension in Laemmli’s sample buffer, samples were boiled for 2 min and separated by minigel (7% T, 7% C) PAGE (25). The gel was transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked overnight at 4°C in advanced ECL blocking buffer at 2% (v/v), which was also used for Ab dilution (Amersham Biosciences). Goat antiserum to human C3 (diluted to 1/20,000; Quidel) served as the primary Ab for 1-h incubations at room temperature. Advanced ECL reagent was used for detection (Amersham Biosciences).

ELISA to detect complement component deposition on Francisella strains

C8d serum was used to evaluate complement component C3, C5, and C7 deposition on Ft strains. After preblocking microcentrifuge reaction tubes as described for Western blotting experiments above, 3 × 10⁶ bacteria/reaction were incubated in 10% fresh serum or serum containing 10 mM EDTA for 5 or 30 min at 37°C. Reactions were stopped and samples were washed as described above for Western blotting experiments. To ensure equal loading, aliquots were taken from the final PBS wash and plated to count CFU. Bacteria (3 × 10⁷) in suspension were added to medium-binding polystyrene wells in triplicate (Costar/Corning) and left to dry overnight. Wells were blocked overnight at 4°C with 3% OVA. After extensive washing with PBS, primary Abs were added for 1-h incubations at room temperature, which included goat antiserum to human C3 (Quidel), human C5 and human C7 (CompTech) each diluted to 1/10,000 in blocking buffer. HRP-conjugated rabbit anti-goat IgG (H + L) Ab (diluted to 1/20,000; Bio-Rad) was used as the secondary Ab for 1-h incubations at 37°C. Sera were pooled from several donors and were used at 1/20,000 dilution. To control for Ab reactivity in the absence of Ft, each well was incubated with 10% serum from eight different healthy donors (1:10,000 dilution) and further incubated with HRP-conjugated goat anti-human IgG (H + L) Ab (1:20,000).
room temperature. Substrate was added for 10 min at room temperature (Bio-Rad), and the reaction was stopped with 2% oxalic acid. Absorbance at 415 nm was measured on a 96-well plate reader ( Molecular Devices).

Values obtained from samples containing EDTA (≤0.2 in each case) were subtracted out in each case.

**Determination of the nature of C3 bound to Francisella**

C3-bound bacterial pellets were obtained as described above for Western blotting. To examine the nature of the C3 fragments bound to each strain, hydroxylamine (NH$_4$OH) was used to cleave thioester bonds formed between C3 and bacterial acceptor molecules as described (24), with modifications. Briefly, after completing C3 deposition reactions and removal of unbound C3, samples were solubilized by boiling in 1% SDS for 5 min. Control samples were prepared immediately for Western blotting and paired samples were first incubated in 2 M NH$_4$OH in 20 mM Tris-buffered H$_2$O (pH 10.5) for 1 h at 37°C. Western blotting was performed as described above except that 16.5-cm gels were used to create better separation between fragments and to allow for increased sample loading. Also, 5% milk was used to block and dilute Abs (primary at 1/2000 and secondary at 1/1000). Ab incubations were done for 1 h at room temperature. ECL reagents were used for detection (Amersham Biosciences). Band densitometry was analyzed using Image J software available through the National Institutes of Health web site (http://rsb.info.nih.gov/ij).

**LPS expression analysis by silver stain and Western blot**

Bacteria, cultured overnight on chocolate II agar plates, were suspended in PBS at a concentration of 3 × 10$^8$ CFU/ml, as determined by OD to equalize the amount of bacteria, and subsequently washed twice in PBS by centrifugation at 12,000 × g for 4 min with resuspension. The final pellet was resuspended in 200 μl of Laemmli loading buffer, boiled for 10 min, and then incubated with proteinase K (Invitrogen) at a final concentration of 10 mg/ml for 2 h at 65°C. Samples were boiled again for 10 min and stored at −20°C until used. LPS was separated by 12% SDS-PAGE and silver stained as described (25). Briefly, gels were fixed overnight in solution containing 40% EtOH and 5% acetic acid. Gels were then incubated in 0.7% periodic acid in fixing solution for 7 min and subsequently washed with multiple exchanges of water. The staining solution (0.013% concentrated ammonium hydroxide, 0.02 N NaOH, and 0.67% silver nitrate (w/v)) was applied with vigorous agitation for 10 min, then gels were washed three times (each 10 min) in water. Gels were developed in solution containing 0.275% monohydrous citric acid (w/v) and 0.0025% formaldehyde. Upon completion, development was stopped using 5% acetic acid.

For Western blotting, strains were grown overnight on chocolate II agar plates. Bacteria were suspended at a concentration of 1 × 10$^8$ CFU/ml (as determined by OD to equalize the amount of bacteria), pelleted, boiled in Laemmli’s sample buffer for 10 min, then incubated with 10 mg/ml proteinase K at 65°C for 2 h. The samples were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked overnight with 5% dehydrated milk and immunoblotting was performed with anti- F. tularensis FB-11 Ab (diluted to 1/1000; Abcam) or with anti-Fn 5 mAb (diluted to 1/1000; ImmunoPrecise) for 4 h at room temperature using goat anti-mouse IgG (diluted to 1/4000; Bio-Rad) for 2 h at room temperature as the secondary Ab. Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich) as the substrate.

**Statistics**

To determine associations of significance between or within groups where indicated, data were analyzed using unpaired (one-tailed) or one-sample Student’s t tests or one-way ANOVA followed by either Bonferroni’s or Dunnett’s multiple comparison posttests (using GraphPad Prism 4 software). CFU data were converted to log values to equalize variances. Differences between groups were considered statistically significant for p values <0.05.

**Results**

**Complement-mediated lysis of Francisella in human serum**

Since complement-mediated opsonization is necessary for efficient phagocytosis of Francisella by macrophages and dendritic cells, we compared the ability of five strains to survive complement-mediated lysis in healthy nonimmune human serum (Table I). Strains included type A virulent Ft subspp. tularensis (Schu S4), Fn, Ft subspp. holarctica live vaccine strain (LVS), a gray phase variant of LVS (LVSG), and a putative capsule-negative strain derived from LVS (LVSR). We also included DH5α, a laboratory E. coli strain, as a positive control for complement activity in serum because of its marked sensitivity to complement-mediated lysis. Bacteria were incubated with 5 or 50% (fresh or HI) donor serum for 1 h, the reaction stopped, and the bacteria subsequently plated to count surviving CFU (Fig. 1). Because complement components C2 and FB are irreversibly denatured by mild heat, HI serum served as a negative control for complement-mediated lysis. In each experiment, CFU obtained from incubations in GVB + + alone (reaction buffer) or in HI serum were equivalent. Results are presented as the ratio of surviving CFU in fresh vs HI serum. Data show that virulent strains known to cause disease in either humans or animal models (Schu S4, Fn, and LVS) are resistant to lysis in both 5 and 50% serum. By comparison, LVSG and LVSR were both susceptible to complement-mediated lysis as survival decreased by at least 100- and 100,000-fold, respectively.

**Fixation of complement components C3, C5, and C7**

Complement component C3 is the central component of the complement cascade. Since C3 fragment deposition is necessary for complement-mediated lysis and for opsonization, we examined strain, as a positive control for complement activity in serum because of its marked sensitivity to complement-mediated lysis. Bacteria were incubated with 5 or 50% (fresh or HI) donor serum for 1 h, the reaction stopped, and the bacteria subsequently plated to count surviving CFU (Fig. 1). Because complement components C2 and FB are irreversibly denatured by mild heat, HI serum served as a negative control for complement-mediated lysis. In each experiment, CFU obtained from incubations in GVB + + alone (reaction buffer) or in HI serum were equivalent. Results are presented as the ratio of surviving CFU in fresh vs HI serum. Data show that virulent strains known to cause disease in either humans or animal models (Schu S4, Fn, and LVS) are resistant to lysis in both 5 and 50% serum. By comparison, LVSG and LVSR were both susceptible to complement-mediated lysis as survival decreased by at least 100- and 100,000-fold, respectively.

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deposition on each of the five Ft subspecies/variant strains described above by Western blotting. Bacteria were incubated in 10% fresh or HI donor serum for 30 min and then washed extensively to remove unbound C3 before sample preparation. Native C3 is a heterodimer composed of a 120-kDa $\alpha$-chain and a 75-kDa $\beta$-chain. Upon activation, C3a is cleaved from the $\alpha$-chain leaving $\text{C3b}_{\alpha}$, a 110-kDa fragment. Upon fixation, $\text{C3b}_{\alpha}$ covalently binds acceptors on the bacterial surface and, depending on the acceptor, will migrate as a band greater than 110 kDa. Also, both the binding of multiple acceptors of varying molecular mass and the further degradation of $\text{C3b}_{\alpha}$ can result in a ladder-type banding pattern. The $\beta$-chain, however, is released by reduction of C3b cysteine bonds in sample buffer and is seen as a 75-kDa band.

C3 deposition was apparent on complement-resistant Ft strains (Schu S4, Fn, and LVS) indicative of complement activation (Fig. 2A). C3 deposition on complement-susceptible strains (LVSG and LVSR) was performed using C5d serum (Fig. 2B). C5 is a component of the terminal lytic pathway and its absence does not affect C3 deposition, but prevents bacterial lysis. To confirm that C3 deposition is unaffected, assays using resistant strains were performed with both C5d and fresh donor sera for comparison (Fig. 2A). C3 associated with LVSG and LVSR was markedly increased compared with resistant strains. Also, we found that LVSG bound less C3 compared with LVSR.

We performed ELISAs to better quantify total C3 deposition and to examine fixation of the downstream complement components C5 and C7. C8 is a component of the MAC and binds to the microbial surface subsequently to C7 deposition. Thus, we incubated each strain in 10% C8d serum for 5 or 30 min at 37°C to assess fixation of the upstream components. The above Western blotting results are supported by the finding that LVSR bound significantly greater amounts of total C3 (Fig. 3). LVSG bound an intermediate amount, but still more than each wild-type strain. At 5 min, compared with LVSR, wild-type strains did not bind C5 and LVSG bound significantly less than LVSR. At 30 min, C5 that bound to wild-type strains increased, but the amount bound to LVSG increased dramatically and was comparable to the amount fixed by LVSR. For C7 (a component of the MAC), a similar trend occurred in that there was little binding at 5 min except on LVSR. Then, at 30 min, binding to wild-type strains remained low and binding to LVSG increased to approach the level fixed by LVSR.
Together, these findings provide strong evidence that the regulation of C3 deposition, which results in a marked reduction in the deposition of downstream components, is crucial for Francisella resistance to complement-mediated lysis.

Temporal analysis of the nature of C3-derived fragments that bind to Francisella

The nature of bound C3 fragmentation can determine the outcome of complement activation. C3b is necessary for activation of the terminal lytic pathway of complement, but smaller cleavage fragments (including C3bi, C3dg, and smaller fragments) do not initiate lysis. Of particular interest is C3bi because, like C3b, it mediates opsonophagocytosis. On Ft, inactivation of C3b to C3bi would account for both resistance to lysis and C3-mediated opsonization. We assessed the nature of C3 fragmentation by Western blot analysis (Fig. 4). Importantly, the study of fragment deposition by immunoblotting is confounded by the fact that C3 forms complexes via covalent bonds, thereby affecting band migration. At physiological pH, C3α, 120-kDa native C3 α-chain; C3bαi', 110-kDa C3 b-chain; C3β, 75-kDa native C3 β-chain; and C3biαi', 68-kDa C3bi α-chain fragment. C. Densitometry ratios of C3biαi' to C3bαi at early time points are compared for each strain. For variant strains, ratios ≤1 indicate a higher rate of C3b deposition compared with the rate of C3b to C3bi conversion. Mean ratios ± SEM for the densitometry of independent blots are given (n = 2 for Schu S4 and LVSG and n = 3 for LVS, Fn, and LVSR).

FIGURE 4. The nature of bacteria-bound C3 fragments for different Francisella strains over time. In brief, 7.5 × 10^8 wild-type (A) or variant strain (B) bacteria/reaction were incubated in 10% C5d serum from 1 to 60 min at 37°C. After washing in ice-cold PBS containing protease inhibitors, bacterial pellets were lysed by boiling in 1% SDS. To allow for the quantitative assessment of fragmentation, hydroxylamine (NH₂OH) was used to release C3bαi' (and smaller α-chain fragments) from covalent thioester bonds to bacterial acceptor molecules. Immunoblotting for C3 was performed as in Fig. 2, except that each lane contains 1 × 10^8 bacteria and a 16.5-cm gel was used for SDS-PAGE to allow for greater band separation. Control lanes contain 2 ng each of native C3 and C3b. Each blot is representative of at least three independent experiments. C3α, 120-kDa native C3 α-chain; C3bαi', 110-kDa C3 b-chain; C3β, 75-kDa native C3 β-chain; and C3biαi', 68-kDa C3bi α-chain fragment. C. Densitometry ratios of C3biαi' to C3bαi at early time points are compared for each strain. For variant strains, ratios ≤1 indicate a higher rate of C3b deposition compared with the rate of C3b to C3bi conversion. Mean ratios ± SEM for the densitometry of independent blots are given (n = 2 for Schu S4 and LVSG and n = 3 for LVS, Fn, and LVSR).

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Bacteria were incubated with 10% C5d serum from 1 to 60 min and we compared hydroxylamine-treated vs untreated samples. For resistant strains (Schu S4, LVS, and Fn), overall C3 fragment deposition occurred rapidly and increased over time (Fig. 4A). In hydroxylamine-treated groups, the appearance of C3biαi' (68-kDa C3biαi' fragment) occurred in excess of C3bαi' (110 kDa). For susceptible strains (LVSG and LVSR), the overall rate of C3
fragment deposition was increased compared with resistant strains (Fig. 4B). Although C3bi deposition also occurred on LVSG and LVSR, the persistent and increasing appearance of C3bα’ was in stark contrast to its relative absence on resistant strains. Also, the greater amounts of C3bα’ on LVSR at early time points, compared with LVSG, correlate with its greater susceptibility to lysis. At early time points, ratios of C3bα’ band densities to C3bα band densities illustrate more rapid conversion of C3b to C3bi on resistant strains (Fig. 4C). Unfortunately, densitometry is less quantitative as the band intensities for C3 deposition become saturating. Ratios of C4bi deposition were also analyzed, with LVSG, correlate with its greater susceptibility to lysis. At early time points, before the saturation of band intensities, indicate that new C3 deposition occurs more rapidly than conversion of bound C3b to C3bi. For Francisella, these results indicate the importance of rapid conversion of C3b to C3bi for resistance to complement-mediated lysis. Also, we conclusively show that complement activation by Schu S4, LVS, and Fn leads to opsonization characterized by persistent C3bi deposition.

Complement-mediated lysis of susceptible Francisella strains occurs by more than one activation pathway

Once we confirmed that Francisella activates complement by demonstrating C3 deposition, we explored mechanisms of upstream activation. With the use of chelators in serum viability assays, we began to differentiate the relevant complement activation pathways targeting Ft. Initiation of the classical pathway is typically dependent upon surface recognition by IgM or IgG and subsequent recruitment of C1 (a complex formed by C1q, C1r, and C1s). The interaction between C1q and C1r is stabilized by Ca²⁺ and the serine protease activity associated with C1s requires Mg²⁺ (27). Similarly, lectin pathway-associated serine proteases require Ca²⁺ for activity. There is no Ca²⁺ requirement associated with the alternative pathway; however, Mg²⁺ is necessary for the interaction between FB and C3b. We used EDTA, which chelates both Ca²⁺ and Mg²⁺, to block all three activation pathways and EGTA, which chelates Ca²⁺, to specifically block the classical and lectin-mediated pathways (Fig. 5). The inclusion of EDTA and EGTA inhibited complement activity (restored viability) in serum concentrations as low as 1% where the classical and lectin, but not alternative, pathways are active. Bacterial lysis at concentrations higher than 10% was not completely inhibited by EGTA, indicative of some alternative pathway activity. Compared with the viability in concentrations of fresh serum above 10%, however, markedly less killing occurred in the presence of EGTA, suggesting the involvement of more than one pathway at these higher concentrations of serum. The implications of these data are that C3bBb (alternative pathway C3 convertase) formation occurs on both LVSG and LVSR, but that C4b2a (classical and lectin pathway C3 convertase) formation is predominant. Once C4b2a initiates C3b deposition, amplification of C3b deposition via C3bBb likely occurs.

To further explore mechanisms of complement activation, we used C1q-depleted serum (C1q-depleted serum deficient of classical pathway activity; Fig. 6A) or FBd serum (serum deficient of alternative pathway activity; Fig. 6B) to test survival of complement-susceptible strains. Lysis of LVSG did not occur in 5, 10, or 20% C1q-depleted serum, but was restored in C1qr serum. Lysis of LVSR did not occur in 5% C1q-depleted serum and occurred to only a small extent in 10% and 20% C1q-depleted serum, but was completely restored in C1qr serum. In FBd and FB sera, lysis occurred that was comparable to C1qr serum for both strains. Also, compared with FBd serum, lysis appeared to increase in 20% FB serum (significant only for LVSR), which supports a minor role for alternative pathway activation. Taken together, results illustrated in Figs. 5 and 6 demonstrate two important findings. First, the classical pathway, but not the alternative pathway, is necessary for the optimal lysis of complement-susceptible strains at both low and high serum concentrations. Second, in the absence of an active classical pathway, either by Ca²⁺ chelation or by depletion of C1q, complement-susceptible strains can activate the alternative pathway to a small extent.

The role of C1q in mediating C3 deposition on complement-resistant and -susceptible strains

After showing that C1q is required for the optimal lysis of LVSG and LVSR, we determined its role in mediating C3 deposition on both complement-resistant and -susceptible strains. As described above, C3 deposition was analyzed by Western blotting, but here we used 10% C5d, C1q, and C1qr sera as sources of complement (Fig. 7). Since susceptible bacteria are lysed in C1q-sufficient serum within 1 h (Fig. 6), we tested survival at earlier time points and found that significant lysis did not occur in 15-min assays (data not shown). Thus, 15-min serum incubations were performed to evaluate C3 deposition on complement-susceptible strains in C1qr serum. Results demonstrate the requirement of C1q for
press structurally altered LPS O Ag compared with LVS (11). In this study, we used an LVS wbtA mutant (LVSΔwbtA), with deletion of a gene within the O Ag operon and devoid of LPS O Ag, to directly test the effect of LPS O Ag expression on complement activation (Table I and Fig. 8). Survival assays were performed as described above to examine effects on complement-mediated lysis. In 5 and 50% fresh donor serum, lysis of LVSΔwbtA did not differ significantly from lysis of LVSR (Fig. 8A). Survival assays using fresh serum with the addition of chelators (data not shown) and using component deficient sera (Fig. 8D) were repeated using LVSΔwbtA to assess important activation pathways. Results correlated with those previously described for LVSR in that a similar degree of lysis occurred for each serum group and that the classical pathway was necessary for optimal lysis. Next, Western blots of C3 deposition were performed to determine the effect of O Ag on the temporal nature and mechanism of C3 fixation. Like LVSR, and unlike LVS, LVSΔwbtA rapidly fixed total C3-derived fragments over time and specifically fixed a markedly increasing amount of C3βc (Fig. 8C). Note that even at 1 min, when amounts of C3β are similar between the three strains, amounts of C3βc are greater on the susceptible strains. The use of C5d, C1q, and C1q r sera as complement sources demonstrated that optimal C3 fixation by LVSΔwbtA requires functional classical pathway activity (Fig. 8D).

Based on the observed effects of wbtA deletion on complement activation by the mutant bacteria, we characterized O Ag expression by both wild-type and variant strains examined in our studies. Using whole cell Fn, LVS, LVSG, LVSR, and LVSΔwbtA lysates, we analyzed O Ag expression by Western blot (Fig. 8E) and silver stain. Using an anti-F. tularensis-type O Ag Ab for immunoblotting, a typical laddering pattern is demonstrated for LVS with a grouping of full-length chains near the top of the membrane and clearer delineation of bands representing progressively shorter chains. For LVSG, an identical pattern is evident, but total O Ag expression is reduced compared with LVS. This implies that LVS and LVSG express O Ag of similar length, but that the amount of O Ag per bacterium (or the amount of O Ag expressing bacteria in a population) is reduced for LVSG. O Ag was not detected for LVSR or, as expected, LVSΔwbtA. These results were duplicated by silver stain (data not shown) in that O Ag banding was evident for LVS, was less evident for LVSG, and was absent for LVSR and LVSΔwbtA. Using an anti-F. novicida-type O Ag Ab, we detected full-length O Ag expression by Fn, but not by LVS or any of the LVS-derived variant strains (data not shown). Thus, wild-type strains, which resist complement-mediated lysis and rapidly mediate conversion of surface-bound C3b to C3bi, express abundant, full-length O Ag. LVSR and LVSΔwbtA, which are highly susceptible to complement-mediated lysis and rapidly fix high amounts of C3βc, do not express O Ag. LVSG, which is moderately susceptible to lysis and fixes persistent C3βc (albeit less

optimal C3 deposition on each strain. Our cumulative data provide strong evidence that the classical pathway has a dominant role in mediating complement activation by Francisella.

O Ag expression is a major determinant of susceptibility to complement-mediated lysis and C3b to C3bi conversion

Thus far, our examinations of complement activation by LVS variant strains used LVSG and LVSR. Both have been shown to ex-
rapidly compared with LVSR and LVS\texttt{wbtA}), expresses a relatively intermediate amount of O Ag.

To confirm the regulatory role of O Ag in mediating resistance to complement-mediated lysis and in mediating conversion of C3b to C3bi, we used an additional mutant strain devoid of \textit{wbtM} (LVS\texttt{wbtM}), which is a gene downstream of \textit{wbtA} in the O Ag operon. In addition, we used the complemented strain containing the pFTNAT plasmid expressing functional \textit{wbtM} as previously described (22). We found that compared with wild-type LVS and with the complemented mutant (LVS\texttt{wbtM:pFTNAT-wbtM}), LVS\texttt{wbtM} and LVS\texttt{wbtM} that contained an empty plasmid (LVS\texttt{wbtM:pFTNAT}) were susceptible to complement-mediated lysis in both 5 and 50% fresh donor serum at 1 h (Fig. 8F). The degree of susceptibility strongly correlates with both LVS\texttt{wbtA} and LVSR. By ELISA, we also found that in 10% C5d serum, LVS\texttt{wbtM} fixed relatively high amounts of C3, C5, and C7 (comparable to wild-type strains in Fig. 3; data not shown).
not shown). Finally, we determined the nature of C3-derived fragments fixed in 10% C5d serum at 10 min. We found high amounts of C3bi on LVS\textsubscript{wbtM} and LVS\textsubscript{wbtM:pFTNAT}, but much higher amounts of C3bi\textsuperscript{\gamma}, compared with C3bi\textsuperscript{\alpha} fixed to LVS and to LVS\textsubscript{wbtM:pFTNAT-wbtM} (Fig. 8G). We conclude that relative O Ag expression correlates strongly with the degree of complement activation by each strain.

**Discussion**

The rapid onset and disease progression of pneumonic tularemia, despite low inoculation doses, implicates a failure of innate immune responses to control infection with *Francisella*. That Ft survives and replicates within phagocytes is particularly indicative of an ineffective microbicidal reaction to infection. However, bacilli must also survive exposure to extracellular mediators of innate immunity, including complement, as a prerequisite for cellular invasion. It has been established that Ft survives in whole blood both in vivo and ex vivo (28, 29). Importantly, pneumonic tularemia, the most severe form of tularemia, can develop secondary to cutaneous or mucosal infections. Clearly, Ft-complement interactions in serum or interstitial fluid would impact the outcome of secondary pneumonia disease. Complement is also abundant in bronchoalveolar fluid and, thus, likely affects primary pneumonia disease as well (30–32). Direct evidence of potent classical pathway activity in isolated human bronchoalveolar lavage fluid was demonstrated by C3 fixation by *Mycobacterium tuberculosis* and group B streptococcus (33, 34). The importance of complement in pulmonary immunity is exemplified in patients who are genetically deficient in either complement components or complement receptors, since they are at significantly increased risk for respiratory infection (35).

In addition to the direct mediation of pathogen lysis, complement modulates innate immune responses via the release of component fragments with anaphylactic activity and via opsonophagocytosis, which would affect cell-mediated responses. Matrix metalloproteinase 9-deficient mice have increased resistance to Schu S4 infection, likely due to its role in recruiting highly active neutrophils to the lung (36). Anaphylactic component fragments may have a similar role, and since the rate of *Francisella* phase variation increases upon intracellular or in vivo growth, increased complement activation by gray variants might compound local inflammation. Unfortunately, the role of complement in mediating disease in animal models caused by type A strains or Fn (the most virulent strains for mice) has not been studied. Opsonization is influential beyond simply increasing the rate of particulate uptake. Depending on the identity of the opsonin (e.g., IgG vs C3bi) and associated surface receptors, downstream signaling events differ. Phagosomal trafficking, cytokine responses, and production of reactive oxygen intermediates are all influenced by the exact nature of receptor-ligand interactions (37).

Our data demonstrate complement activation by each *Francisella* strain tested. Several studies previously addressed susceptibility of Fn, LVS, and respective derivative strains to complement-mediated lysis (discussed below). We extended these studies to include Schu S4 and we provide an analysis of complement activation at the level of surface component deposition. Since it was shown that CR3 and CR4 have a major role in opsonophagocytosis of Ft by human monocyte-derived phagocytes (18–20) and the primary ligand for these receptors is C3bi, we hypothesized that *Francisella* would fix C3b and that conversion to C3bi would ensue. Conversion to C3bi and smaller fragments would also account for the ability of bacilli to survive extracellularly in vivo. We characterize the nature of Ft-bound C3-derived fragments and show directly that opsonization with C3bi occurs. Our finding that LVS fixes C3 contradicts an earlier study, which reported that LVS did not bind radioactively labeled C3 when both were added to human serum (21). Since we found that rapid C3b conversion occurs on the surface of LVS, it is possible that C3 cleavage adversely affected radioactive labeling in that study. We also compared the nature of C3 deposition on strains resistant to complement-mediated lysis to its nature on susceptible strains. We found that, on resistant strains, conversion of C3bi\textsuperscript{\gamma} to C3bi\textsuperscript{\alpha} occurred more rapidly than deposition of new C3bi\textsuperscript{\gamma}. We extended our studies to show that the classical pathway is required for optimal complement activation by each strain tested, a finding that has previously been limited to serum-susceptible variant strains. Finally, we found that O Ag expression is a major determinant of complement activation. The strength of this finding is increased by the fact that multiple LVS variant strains, generated by distinct methodologies, were used. These include the selection of a spontaneous colony morphology variant (LVSG), the creation of a strain by random mutagenesis of LVS followed by the selection of a rough colony morphology variant (LVSR), and the construction of targeted gene mutants (LVS\textsubscript{wbtA} and LVS\textsubscript{wbtM}).

A role for capsule has previously been implicated in the serum resistance of *Francisella* (38). We chose to study LVSR, a putative capsule negative (Cap\textsuperscript{−}) strain derived from LVS by Sandstrom et al. (21) and subsequently studied by Cowley et al. (11), to characterize the effect of capsule production on complement component deposition. For other bacteria, encapsulation has been shown to reduce C3 deposition and to protect against subsequent lysis (39–41). The specific nature of the Ft capsule has not been determined conclusively. Hood (42) reported that decapsulation of Ft occurs in hypertonic saline and that capsular material is biochemically distinct from the cell wall of decapsulated bacilli. Recently, a putative capsule locus in the LVS genome, containing orthologous genes to capB and capC of *Bacillus anthracis*, was reported (43). In some studies, an electron-lucent material typical of a loose capsule can clearly be seen surrounding bacilli grown in defined medium (44, 45). Despite these reports, several laboratories, including ours, have been unable to conclusively identify capsular material by microscopy, possibly due to the use of rich medium for culturing (19, 46). In studies of serum resistance, Cap\textsuperscript{−} strains (including LVSR) were shown to activate complement (21, 47). Sorokin et al. (47) reported, however, that the Cap\textsuperscript{−} strain used in their study exhibited a truncated O Ag, which may have been the true determinant of complement susceptibility. To substantiate the designation of LVSR as a Cap\textsuperscript{−} mutant, electron micrographs comparing LVSR to LVS were presented by Sandstrom et al. (21). However, the represented LVS capsule does not resemble the images of *Francisella* capsule shown in the above-cited studies. Furthermore, O Ag expression by LVSR was not studied. We conclude, based on our studies, that mutations in LVSR affect O Ag expression and not capsule production. However, that O Ag is a constituent of capsular polysaccharide cannot be ruled out. Also, the possibility exists that encapsulation occurs only under specific growth conditions and provides an additional measure of protection against complement.

Because we did not detect encapsulation on serum-resistant wild-type strains, we explored alternative mechanisms of resistance, including the effect of LPS expression. Compared with other Gram-negative pathogens, *Francisella* LPS is unique (48). The *Francisella* LPS O Ag is composed of a repeating carbohydrate tetramer that is identical for all subspecies tested thus far except for Fn, which has two novel sugars in the tetramer (49, 50). It is unlikely, however, that this structural difference in O Ag affects complement activation since Schu S4 and LVS do not differentially fix C3 fragments compared with Fn (Fig. 2). Previously, O
Ag mutant strains derived from both LVS and Fn have been found to be susceptible to lysis in serum, unlike the parent strains (46, 51–53). Also, LVS gray variants that lack O Ag are similarly susceptible to lysis in serum (12). We repeated these results using LVSΔwbtA (Fig. 8A) and LVSΔwbtM (Fig. 8F). Furthermore, we show similarities relating to serum resistance and C3 fixation between LVSR, LVSΔwbtA, and LVSΔwbtM (Fig. 8). Since the absence of O Ag expression is also similar for each strain (Fig. 8E) and since complementation of the LVSΔwbtM mutant restores resistance to lysis and efficient C3b to C3bi conversion (Fig. 8, F and G), we conclude that loss of O Ag expression is the primary cause of serum susceptibility.

In addition to identifying a role for O Ag, we show that complement resistance is due, in part, to a reduction of C3 on wild-type strains. Our data indicate that this is the result of rapid conversion of fixed C3b to C3bi, which would limit C3 convertase-mediated amplification of C3b deposition (Fig. 4). C3b inactivation is a common resistance mechanism employed by Gram-negative bacteria that are human pathogens and C3b fixation enables efficient uptake by host phagocytes (26, 54–56). We are currently evaluating two hypotheses to explain C3b cleavage based on known microbial mechanisms of complement evasion. The first involves direct bacterial protease expression and the second involves the recruitment of host-derived negative regulators of complement.

Importantly, a sufficient characterization of the mechanisms of C3b inactivation would include the evaluation of both hypotheses, since neither is mutually exclusive and either might be affected by O Ag expression. O Ag expression is reduced or absent on each complement-susceptible strain used in the present study and each was derived by a distinct methodology. Thus, the existence of unidentified Ft serum resistance factors, unaffected by O Ag, is unlikely. We do not propose that O Ag acts as an acceptor for serum-derived negative regulators (involved in C3b cleavage) because C3b to C3bi conversion occurs on LVSΔwbtA, and LVSΔwbtM (Fig. 8), that C3b conversion occurs on complement-susceptible strains, albeit at a slower rate than C3b deposition, signifies the relative importance of O Ag expression for survival. Other potential mechanisms of resistance to complement-mediated lysis cannot be ruled out such as steric hindrance to the formation of the MAC or the fixation of complement components by moieties distal to the outer membrane. However, we show that limited C5 and C7 (a component of the MAC) fixation occurs in the presence of O Ag (Fig. 3), which is consistent with its importance in regulating C3 deposition which then secondarily affects components downstream in the complement cascade.

The important role of the classical pathway was previously demonstrated by Sorokin et al. (47) who reported lysis of variant strains in serum. Consumption of complement activity, indicative of activation, in pooled human serum was mediated by whole bacteria, isolated outer membrane, and purified LPS. Significantly less complement consumption occurred in preabsorbed serum, which was depleted of specific bactericidal Abs by incubating pooled sera with acetone-dried bacteria at 4°C. Repletion of preabsorbed serum with anti-whole cell or anti-LPS Abs restored complement consumption. With respect to LVSΔ, Sandstrom et al. (21) reported that depletion of both IgM and C4 in human serum dramatically decreased lysis. In the present study, results using C1qD serum confirm the importance of the classical pathway not only for the lysis of variant strains, but also for complement activation by virulent strains (Figs. 6–8). Previously, we reported the identification of natural anti-Francisella Abs (using Fn) in human non-immune serum, which might have a role in complement activation by the classical pathway (18).

In summary, we show that the classical pathway, and to a lesser extent the alternative pathway, is activated by Francisella wild-type and LPS variant strains. We present conclusive evidence that complement is activated, based on C3 fixation, by virulent Francisella strains including Schu S4, LVS, and Fn. Rapid conversion of C3b to C3bi on these strains contributes to their ability to resist complement-mediated lysis. LPS variant strains derived from LVS were susceptible to complement-mediated lysis, due in part to limited C3b to C3bi conversion that led to striking increases in C3b deposition and to increased binding of components of the terminal lytic pathway (including C5 and C7). Finally, we identify O Ag expression as a key determinant for the outcome of complement activation by Francisella because it regulates deposition of terminal lytic pathway components due to an increased rate of C3b to C3bi conversion.

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Disclosures

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