Blood Collection Tubes Influence Serum Ficolin-1 and Ficolin-2 Levels

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The ficolins are members of a recently discovered family of host innate opsonins that can activate the lectin pathway of complement. The ficolins bind many ligands, although they are typically described as binding acetylated sugars. Ficolin-1 (M-ficolin) and ficolin-2 (L-ficolin) are known to bind Streptococcus pneumoniae serotypes 19C and 11A, respectively. While studying the binding of ficolins to pneumococci, we found variations in ficolin-2 binding among serum samples collected in different types of blood collection tubes. Plastic tubes, which contain a silica clot activator, yielded sera with reduced ficolin-2 binding and apparent ficolin-2 levels. We found that the silica clot activator eluted from plastic red-top tubes inhibited ficolin-2 ligand binding, while other related proteins, like mannose-binding lectin (MBL) and ficolin-1, were not affected. These tube types did not affect the concentrations of other related opsonins (C1q, MBL, or ficolin-3 [H-ficolin]). Interestingly, we also found that ficolin-1 levels were increased 2- to 3-fold in plastic serum separator tubes compared to the increases in other tube types. These findings have implications for future ficolin-1 and ficolin-2 studies, as proper sample collection and handling are essential.

MATERIALS AND METHODS

Microbial strains and serum. The pneumococcal serotype 11A strain (JC03) is a clinical strain that was made in our laboratory to be streptomycin resistant (16). The pneumococcal serotype 19C strain used for ficolin-1 binding assays in this study was obtained from Statens Serum Institut (Copenhagen, Denmark). The Saccharomyces cerevisiae strain used for the MBL binding assays in this study was a kind gift from the laboratory of Stephen Moser at the University of Alabama at Birmingham (Birmingham, AL). Unless otherwise specified, bacteria were cultured on blood agar plates (BD Biosciences, San Jose, CA) or in Todd–Hewitt liquid medium (BD Biosciences) plus 0.5% yeast extract (THY) at 37°C in 5% CO₂. Yeast was cultured on yeast extract-peptone-dextrose (YPD) (1% yeast extract, 2% peptone, 2% dextrose) agar plates or in YPD broth at 30°C.

Normal human serum samples were obtained from seven consenting healthy adult volunteer donors at the University of Alabama at Birmingham (approved by the institutional review board [IRB] at the University of Alabama at Birmingham) in each of the following tube types: (i) BD Vacutainer glass tubes without coating (catalog no. 366441; BD Biosciences), (ii) BD Vacutainer glass tubes with silicone-coated interiors (catalog no. 366430; BD Biosciences), (iii) BD Vacutainer plastic serum separator tubes (SSTs) with silicone-coated interiors and a silica clot activator (SQA) (catalog no. 367985; BD Biosciences), and (iv) BD Vacutainer plastic serum separator tubes (SSTs) with silicone-coated interiors and a silica clot activator (SQA) (catalog no. 367985; BD Biosciences), and (iv) BD Vacutainer plastic serum separator tubes (SSTs) with silicone-coated interiors and an SCA (catalog no. 367820; BD Biosciences). For simplicity, the four types of blood collection tubes are referred to throughout this report as glass red-top tubes, coated glass red-top tubes, plastic SSTs, and plastic red-top tubes, respectively. Of note, an SCA is present in the two types of plastic tubes but absent from the glass tubes. Additional serum samples were collected in glass red-top tubes from 20 consenting healthy adult volunteer donors at the University of Rochester (Rochester, NY) under its own IRB approval.

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The complement cascade, a critical component of host immune defense, is activated through three major pathways, alternative, classical, and lectin. The alternative pathway is an amplification loop primarily involving complement component 3 (C3) and factor B. The classical pathway is initiated through C1q recognition of antibodies, which in turn activates the associated serine proteases (C1r and C1s) (1, 2). The lectin pathway is activated by several innate opsonins, such as mannose-binding lectin (MBL) and the recently discovered ficolins, ficolin-1, ficolin-2, and ficolin-3 (also known as M-ficolin, L-ficolin, and H-ficolin, respectively). The binding of MBL and the ficolins to their respective ligands activates the mannose-binding lectin-associated serine proteases (MASPs), which cleave C4 and C2 (3).

The ficolins contain a fibrinogen-like domain that is involved in binding to patterns of acetylated targets. Crystalllography studies have shown that ficolin-1 and ficolin-3 have only one binding pocket each, while ficolin-2 has four binding pockets, which may make it a more promiscuous binder (4). Ficolin-2 is known to bind many acetylated ligands (5) and various biological ligands, such as apoptotic cells (6), and may be important in renal transplantation (7) and preeclampsia (8). It also binds to several significant human pathogens, like group B streptococcus (9) and Salmonella enterica serovar Typhimurium (10), and to many Gram-positive bacterial teichoic acids (11). Thus, it may be important in host defense against these pathogens as well.

Streptococcus pneumoniae is a major human pathogen and has many different capsule types (12). The major host defense mechanism is complement deposition on the pneumococcal surface, which opsonizes pneumococci for host phagocytes. Capsules reduce complement deposition and increase virulence (13). A recent study found that ficolin-2 bound only pneumococcal serotypes 11A, 11D, and 11F (14). While studying its protective role in infections by serotype 11A, we discovered inconsistent ficolin-2 binding with different peripheral blood samples from one individual. We traced our discrepancies to a recent change in blood collection tubes from glass to plastic, which has been occurring in the last decade to improve workplace safety (15). Here, we show that different blood collection tube types affect ficolin-1 and ficolin-2 levels but not the MBL, ficolin-3, or C1q level.
We eluted the SCA from a 10-ml plastic red-top tube (catalog no. 367820; BD Biosciences) by adding 1 ml of water and rotating the tube for 3 h at room temperature (RT). The eluted SCA was reconstituted with 10× Hanks’ balanced salt solution (HBSS) (Gibco, Life Technologies), bovine serum albumin (catalog no. A7030; Sigma), and CaCl₂ to final concentrations of 1×, 0.5%, and 2.2 mM, respectively.

Detection of ficolin-2, ficolin-1, and MBL binding by flow cytometry. Bacteria (1×10⁷ CFU/ml) or yeast were incubated in ficolin-binding buffer (FBB) with 2.5% normal human serum for 1 h at 4°C. FBB contains 1× HBSS with 2.2 mM CaCl₂ and 0.5% bovine serum albumin (catalog no. A7030; Sigma). For the inhibition assays, normal human serum (at a final concentration of 2.5%) was first incubated with the SCA eluate for 10 min on ice and then added to the bacteria or yeast. After being washed, the bacteria were stained with an anti-ficolin-2 monoclonal antibody (MAb) (GN5, catalog no. HM2091; Hycult Biotech), an anti-ficolin-1 MAb (7G1, catalog no. HM2196; Hycult Biotech), or an anti-MBL MAb (3E7, catalog no. MA1-40145; Pierce) for 30 min at 4°C, followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (catalog no. 1030-02; Southern Biotech) for 30 min at 4°C. Stained bacteria were analyzed using a FACS Calibur flow cytometer. All flow cytometry data were analyzed using FCS Express V3 software (De Novo Software, Los Angeles, CA).

Detection of ficolin-2 by Western blotting. Serum from donor A was diluted 10-fold, and then 10 µl was run on a 10% SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane and blocked with 5% skim milk in Tris-buffered saline plus 0.05% Tween 20 (TBST). The membrane was then incubated with an anti-ficolin-2 biotinylated antibody (Ab) (catalog no. BA2428; R&D Systems) followed by streptavidin conjugated to alkaline phosphatase (catalog no. 43-4322; Invitrogen). The membrane was developed using 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (catalog no. BP1610; Fisher Scientific) plus nitroblue tetrazolium chloride (NBT) (catalog no. BP108; Fisher Scientific) in 1 M Tris (pH 8.8).

Determining ficolin-1, ficolin-2, ficolin-3, MBL, and C1q levels by enzyme-linked immunosorbent assays (ELISAs). Serum concentrations were determined using commercially available kits for each analyte, following the manufacturers’ suggested instructions. The ficolin-1 kit was from US Biologicals (catalog no. 025116), the ficolin-2 kit was from Hycult Biotech (catalog no. HK336), the ficolin-3 kit was from Hycult Biotech (catalog no. HK340), the MBL kit was from Hycult Biotech (catalog no. HK323), and the C1q kit was from Hycult Biotech (catalog no. HK356).

Statistics. An analysis of variance (ANOVA) was performed to compare the mean concentrations of C1q, MBL, ficolin-1, ficolin-2, and ficolin-3 in the different tube types using JMP10 software (SAS, Cary, NC).

RESULTS

Ficolin-2 activity depends on blood collection tube type. Different serum samples from one donor showed strikingly different ficolin-2 binding to S. pneumoniae serotype 11A. To investigate the effects of the blood collection tubes, peripheral blood samples were collected from 7 healthy human donors into the four different types of blood collection tubes (described in Materials and Methods), and the sera were tested for ficolin-2 binding to pneumococci. We found variabilities in ficolin-2 binding among the donors. The serum samples collected in the plastic Tubes showed lower ficolin-2 binding ability than the serum samples collected in the glass tubes (Fig. 1A). These findings suggest that ficolin-2 may have been degraded or absorbed in the plastic tubes or that ficolin-2 binding may be inhibited by a material(s) present only in the plastic tubes.

![FIG 1](http://cvi.asm.org/) FIG 1 Ficolin-2 levels vary by blood collection tube type. (A) Serum ficolin-2 binding to serotype 11A pneumococci for serum samples collected from 7 healthy human adult donors using glass tubes (black bars), coated glass tubes (white bars), coated glass tubes (gray bars), or plastic tubes (striped bars). MFI, mean fluorescence intensity. The inset shows representative flow cytometry histograms for ficolin-2 binding to serotype 11A pneumococci (black line) for serum samples collected from glass tubes (G), coated glass tubes (CG), plastic separator tubes (SST), or plastic tubes (P) from donor B. The gray-shaded areas represent bacteria stained with an isotype control antibody. (B) Serum ficolin-2 levels (µg/ml) for 7 healthy human donors using glass tubes (black bars), coated glass tubes (white bars), plastic SSTs (gray bars), or plastic tubes (striped bars). M indicates molecular mass markers (lane 1), *, not determined; serum was unavailable for the coated glass tube type from donor F. In panel A, data represent the means ± SEM for an experiment run in triplicate. In panel B, data represent the averages of duplicate results, and error bars represent the ranges.

Tube types affect ficolin-2 levels determined by ELISA but not by Western blotting. To further investigate the possibilities described above, we determined the concentrations of ficolin-2 in the serum samples collected in the four tube types using a commercially available ELISA kit. Again, as expected, there were variabilities in the ficolin-2 levels among the donors. The serum samples collected in the glass red-top tubes contained the expected levels of ficolin-2, with a median of 3.4 µg/ml (range, 0.9 to 5.7 µg/ml) (Fig. 1B). However, the serum samples collected in the plastic tubes contained strikingly less ficolin-2 than those collected in the glass tubes (P < 0.0001) (Fig. 1B). In some cases, the serum samples collected in the plastic tubes contained ficolin-2 levels below the limit of detection (<0.3 µg/ml for donors B, E, and G) (Fig. 1B).

To examine if ficolin-2 had been degraded in the plastic tubes, we determined the molecular sizes and the levels of ficolin-2 by performing Western blot analyses. In contrast to the ELISAs, the Western blots showed no differences in the sizes or levels of ficolin-2 among the four different types of serum samples (Fig. 1C).
Thus, despite the apparent decrease in the ficolin-2 levels by ELISAs, the serum samples collected in the plastic tubes contained ficolin-2 that had not been degraded. Therefore, we hypothesized that ficolin-2 was being inhibited by materials, like the silica clot activator (SCA), that are uniquely present in the plastic tubes.

**SCA sequesters ficolin-2 and acts as an inhibitor.** To investigate the hypothesis described above, we mixed a serum sample collected in a glass red-top tube with a serum sample from one of the two plastic tubes and studied the mixture for ficolin-2 binding to pneumococci. Mixing the sera significantly decreased the ficolin-2 levels but not the levels of similar complement pathway proteins. Therefore, we investigated the effects of tube type on the levels of C1q and MBL using sera from 7 donors. Accordingly, we found no differences in C1q levels among the different tube types, although their levels were slightly lower than those previously published (14, 19). The levels for several donors were below the limit of detection for the MBL assay. This striking variation was not surprising, as MBL deficiency is common and occurs in 5 to 30% of the population (20–22).

We also investigated the effects of collection tube type on the other ficolins. There were no significant differences in serum ficolin-3 levels across the different blood collection tube types (P = 0.991) (Fig. 3C). Also, the ficolin-3 levels were consistent with those in previously published reports (23). Unexpectedly, the ficolin-1 levels were at least 2-fold higher in the sera collected in the plastic SSTs (Fig. 3D). This also corresponded to an increase in ficolin-1 binding to serotype 19C pneumococci with the sera from the plastic SSTs compared to sera collected with the other tube types (data not shown). There were no differences in the ficolin-1 levels in the samples from the glass or plastic red-top tubes (Fig. 3D).

**Normal levels of ficolin-2 in normal sera collected with glass red-top tubes.** Because plastic tubes have replaced glass tubes recently without knowledge of the impact of such a change on ficolin-2 levels, it is possible that previously reported reference ranges of ficolin-2 were affected by the type of collection tube used. Therefore, we determined a reference range for ficolin-2 levels using sera collected from 27 healthy adults in glass red-top tubes. The ficolin-2 levels ranged from 0.9 μg/ml to 7.8 μg/ml, with a median concentration of 3.9 μg/ml (Fig. 4). Our results are similar to those previously reported (23, 24).
DISCUSSION

To improve workplace safety, plastic blood collection tubes have been used to replace glass tubes in the last decade (15). Because glass can naturally activate blood coagulation, glass tubes designed to collect sera have no additives and have been widely used for research and patient care. However, plastic tubes for obtaining sera contain various SCA materials, such as kaolin, silica particles, or silica gels (25), because plastic tubes do not activate blood clotting. The SCA is quite inert, and both types of tubes yield comparable results for many analytes. Thus, both types are used interchangeably to obtain human sera.

We now show that the two tube types yield sera that are strikingly different in ficolin-2 binding capacities. Unlike glass tubes, plastic tubes yield sera with almost no ficolin-2 binding capacity. We also demonstrated that the SCA in plastic tubes is responsible since the materials eluted from the plastic tubes strongly inhibited the binding of ficolin-2. Indeed, activities of other ficolin-2-like molecules (e.g., MBL, C1q, ficolin-3, and ficolin-1) were not affected. This selective inactivation may occur because ficolin-2 binding is more promiscuous than the binding of other molecules. Interestingly, the binding of ficolin-2 does not lead to complement activation and consumption, since both tube types yield sera with comparable CH50 titers (the CH50 measures the total hemolytic activity of a test sample and is the reciprocal of the dilution of serum complement needed to lyse 50% of a standardized suspension of sheep erythrocytes coated with antierythrocyte antibody) (data not shown). Thus, the silicate material in the plastic tubes may impact only ficolin-2, and it may be useful as a selective inhibitor of ficolin-2.

In addition to reduced binding to pneumococci, we found that the ficolin-2 level measured by a commercially available ELISA kit was also dependent on the tube type. Our study confirmed a recent finding by a European group, which was published while we were preparing our report (26). However, we found that the effect of tube type on the serum level is dependent on the assay type, since we observed that equivalent levels of ficolin-2 were demonstrated by Western blotting for ficolin-2 from sera collected in four different tube types (Fig. 1C). Taken together, our results showed that the silicate materials do not degrade ficolin-2 but may change its conformation or sequester it. Also, sequestered ficolin-2 is not available by ELISA or for ligand binding but may be released under the denaturing conditions used during SDS-PAGE.

We also noted that ficolin-1 levels tended to be 2- to 3-fold higher in sera obtained with plastic SSTs (Fig. 3D) than in those from other tube types. Ficolin-1 is present at high levels in neutrophils and monocytes (27). SSTs have been associated with the adherence of blood cells to tubes and cell lysis (28). Indeed, levels of potassium, which is typically intracellular, can be higher in sera obtained with SSTs (28, 29). Thus, it is likely that the use of SSTs may lead to the liberation of ficolin-1 from monocytes or neutrophils. This may explain some of the discrepancies in the reported levels of serum ficolin-1 in the literature (23, 30, 31).

We studied tubes from Becton, Dickinson because it is the dominant manufacturer of blood collection tubes in the United States. However, there are additional blood collection tube manufacturers in the United States (28) and around the world. The performance characteristics of blood collection tubes may be manufacturer dependent (32), and different silicate materials may be used as SCAs. Furthermore, not all silicate materials can bind ficolin-2 since we observed that a silicate material from a commercial source (catalog no. 44054; Sigma) did not bind ficolin-2 (A. Brady and M. Nahm, unpublished data). Thus, one must evaluate glass and plastic tubes from each manufacturer. However, the European group reported results similar to ours, and we anticipate that the effects of plastic tubes commonly apply worldwide.

Although plastic tubes have been in use for a decade, their impact on ficolin-2 measurements is being discovered only now. In fact, instructions for the commercially available ELISA kits for ficolin-2 are unclear as to which tube type to use. The same is true for the ficolin-1 ELISA kit used for this study and other commercially available kits. Previous study results might have been impacted by the tube types. Fortunately, our ficolin-2 levels are comparable to those in the literature (23, 24); thus, the tube type change probably did not affect the previously reported ficolin-2 levels. However, one must be aware that a seemingly trivial change can greatly impact the study of ficolin-2, a new innate opsonin that requires much more investigation.

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FIG 4 Ficolin-2 levels (μg/ml) in 27 normal adult serum samples collected in glass red-top tubes. The black horizontal line represents the median.
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