Evaluation of Anticoagulants for Serologic Assays of Cholera Vaccination

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Blood collected with an anticoagulant is beneficial for simultaneous evaluation of immune cells and humoral components such as antibodies. However, it is critical that the anticoagulant does not affect quantitative and qualitative analyses of antibodies. In the present study, we examined the potential interference of the widely used anticoagulants heparin, EDTA, and acid citrate dextrose (ACD) on vibriocidal antibody activities and Vibrio cholerae lipopolysaccharide (LPS)-specific IgG, IgM, and IgA levels in the plasma and sera obtained from cholera patients or vaccinees. Serum vibriocidal antibody titer was inhibited in the presence of EDTA or ACD but not in the presence of heparin. Moreover, 100% (8/8) of the vibriocidal antibody titers of plasma samples obtained from the vaccinees in tubes containing heparin were identical to the titers of matched sera when compared with 37.5% (3/8) and 50% (4/8) of the plasma samples prepared with EDTA and ACD, respectively. Among LPS-specific Igs, the Pearson correlation coefficient (r) for IgA in serum and plasma was low (r = 0.716), and the coefficients for IgG and IgM were relatively high (r = 0.997 and r = 0.945, respectively) in heparinized plasma samples compared with the coefficient for IgG and IgM of EDTA- and ACD-treated plasma. Our results suggest that heparin is an appropriate anticoagulant for the collection of blood when measuring vibriocidal activities and antibody levels in plasma samples.

The serum vibriocidal antibody assay is a surrogate assay for the evaluation of immunogenicity induced by cholera vaccines against Vibrio cholerae (1, 2). Serum antibodies against V. cholerae bind to the bacterial surface and activate the complement pathway to induce bacterial lysis. Accumulating reports suggest an inverse correlation between vibriocidal antibody titer and susceptibility to cholera (3) and intestinal colonization of V. cholerae (4). The vibriocidal antibody titer has been used as a representative marker for the evaluation of cholera vaccine efficacy because it has a close relationship with protection from disease (5). However, it has some limitations; for example, it may not fully reflect protective immunity against cholera under certain conditions (such as in old people living in areas where cholera is endemic who have relatively high vibriocidal antibody titers even before the vaccination [6]), and individuals with a low vibriocidal antibody titer are often protected (4, 7).

In addition to the serum vibriocidal antibody assay, examination of cellular immunity through lymphocytes in the peripheral blood might be necessary for more accurate evaluation of cholera vaccine efficacy, especially for the analysis of long-lasting memory responses. Thus, phenotypic characteristics of peripheral blood mononuclear cells might be useful in the evaluation of memory B and T cell responses. To efficiently examine both humoral and cellular immune responses, blood collection using anticoagulants is necessary because it allows concurrent isolation of plasma and leukocytes. In particular, simultaneous preparation of plasma and leukocytes using anticoagulants might be advantageous in infants and young children with a limited quantity of blood.

Three anticoagulants, including heparin, EDTA, and acid citrate dextrose (ACD), have been commonly used in the clinical setting and they mainly act as thrombin inhibitors. Thrombin is a key enzyme that catalyzes the conversion of fibrinogen to fibrin, and therefore the fibrin promotes clot formation (8, 9). The heparin functions as an anticoagulant by activating antithrombin (10). ACD and EDTA prevent the formation of thrombin by chelating calcium required for the blood clotting cascade (11, 12). However, they are known to prevent activation of complement by blocking formation of the membrane attack complex (12–15). In addition, ACD and EDTA are widely used as chelating agents of divalent cations such as Ca2+ and Mg2+ and cause disruption of the outer membrane of Gram-negative bacteria (16).

Therefore, in order to use plasma for serologic assays such as the vibriocidal antibody assay, it is necessary to determine whether the anticoagulants used in the blood collecting tubes affect the assay results. In the present study, we examined the effects of heparin, EDTA, and ACD on vibriocidal antibody assays and quantitative analysis of antibody levels in plasma samples compared with corresponding serum samples.

MATERIALS AND METHODS

Bacteria and reagents. V. cholerae O1 El Tor Inaba (strain T19479) was kindly provided by Jan Holmgren (Gothenburg University, Sweden). Brain heart infusion (BHI) medium and guinea pig complement were purchased from Difco (San Jose, CA) and Rockland (Gilbertsville, PA), respectively. Heparin and ACD were obtained from Sigma-Aldrich (St. Louis, MO). EDTA and phosphate-buffered saline (PBS) were purchased from Gibco-BRL (Gaithersburg, MD).

Serum and plasma samples. All experiments using human samples were performed with the approval of the institutional review board of the

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International Vaccine Institute. Human convalescent-phase sera were obtained from three cholerapatients and pooled for testing vibriocidal activity in the presence or absence of anticoagulants. To examine the direct effect of heparin on vibriocidal activity in serum, 10 human serum samples with low, medium, and high vibriocidal antibody titers were chosen from subjects who had received bivalent, killed oral cholera vaccine (Shanchol) in clinical trials in Kolkata, India. In addition, four healthy volunteers (two males and two females 33 to 40 years old and taking no medication) were orally administered Dukoral (consisting of killed V. cholerae and cholera toxin B subunit) twice at day 0 and day 14. Blood (3 ml) was collected twice at day 0 and day 28 using tubes containing EDTA, ACD, or heparin or without anticoagulant (BD Vacutainer; Becton, Dickinson, NJ, USA).

**Vibriocidal antibody assay.** The vibriocidal antibody assay was carried out as previously described (17). Briefly, human serum or plasma samples were serially diluted 2-fold with 0.85% saline from an initial 1:2.5 dilution, and 25 μl of diluent was transferred to 96-well plates. V. cholerae was cultured in BHI medium for 2 to 3 h at 37°C to mid-log phase. The cultured bacteria were collected by centrifugation, resuspended in saline, and adjusted to a concentration of 1 × 10⁸ CFU/ml with saline containing 10% guinea pig complements. Then, 25 μl of the bacterial culture was added to microplates containing serum or plasma diluents. After incubation at 37°C with shaking for 1 h, 150 μl of sterilized BHI medium per well was added and the plate was incubated at 37°C without shaking for an additional 4 h. The bacterial growth was measured as optical density at 600 nm (OD₆₀₀), with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The vibriocidal antibody titer was expressed as the reciprocal of the highest dilution completely inhibiting bacterial growth.

**Enzyme-linked immunoabsorbent assay.** Serum and plasma IgG, IgM, and IgA levels against lipopolysaccharide (LPS) of V. cholerae were measured by enzyme-linked immunoabsorbent assay (ELISA) as previously described (18). Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 μl of 2.5 μg LPS in PBS at 4°C overnight. After blocking with 300 μl of 1% bovine serum albumin (BSA) in PBS, serum or plasma samples diluted in PBS containing 1% BSA and 0.05% Tween 20 were incubated at room temperature for 2 h. The plates were washed and incubated with alkaline phosphatase-conjugated anti-human IgG (1:1,000), anti-human IgM (1:1,000), or anti-human IgA (1:500) (Southern Biotechnology, Birmingham, AL, USA) at room temperature for 2 h. After washing with PBS containing 0.05% Tween 20, the wells were incubated with 100 μl of 1 mg/ml 4-nitrophenylphosphate for 30 min, and optical density was read at 405 nm using a microplate reader (Molecular Devices). Endpoint titers were defined as the reciprocal of the highest dilution that gave an absorbance value of >0.1.

**Statistical analysis.** To compare vibriocidal antibody titer and the level of LPS-specific antibody isotypes in serum and plasma samples, the results were plotted against each other and the relationship between the two values was examined by use of the Pearson correlation coefficient (r). Statistical analysis was performed and P values were obtained using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

**RESULTS**

Vibriocidal antibody activity in serum is inhibited by ACD and EDTA but not affected by heparin. To examine the effects of anticoagulants on the vibriocidal antibody activity of serum, human convalescent-phase sera with high vibriocidal activity were initially diluted 1:1,000 with 5 mM EDTA, 15 USP units of heparin, or 0.18 ml ACD/ml of sera, because these are the concentrations used in commercially available blood collection tubes. The sera were serially diluted 2-fold with saline to 1:512,000. As shown in Fig. 1, the vibriocidal activity of sera diluted with saline or heparin showed typical kinetics with a sigmoidal curve. However, sera diluted with EDTA showed lower bactericidal activity than those diluted with saline. In addition, sera containing ACD showed an atypical vibriocidal pattern similar to a prozone effect at high concentrations of sera. These results indicated that heparin was the only anticoagulant that did not alter the vibriocidal activity of human sera. Next, clinical human sera obtained from 10 vaccinees who were orally administered a bivalent, killed oral cholera vaccine (Shanchol) were divided into three groups based on their vibriocidal antibody titer: low titer (<2.5 to 20), medium titer (80 to 320), and high titer (640) (Table 1). The 10 serum samples were initially diluted 1:2.5 with saline containing 15 USP units of heparin/ml of sera followed by further dilution with saline to 1:10,240 for the vibriocidal antibody titer. As shown in Table 1, vibriocidal antibody titers were identical regardless of the presence of heparin, suggesting that heparin hardly affected the vibriocidal activity of antibodies in human clinical samples.

**Serum vibriocidal antibody titers are completely concordant with those of heparinized plasma.** To test whether plasma can be used to determine vibriocidal antibody titers, we prepared plasma samples using commercially available blood collection tubes con-

**FIG 1** Serum vibriocidal activity was not changed in the presence of heparin but was decreased in the presence of ACD and EDTA. Pooled convalescent-phase sera obtained from three human cholera patients were diluted in 1:1,000 with saline in the absence of anticoagulant or in the presence of heparin (15 USP units/ml), ACD (0.18 ml ACD/ml), or EDTA (5 mM) and then serially diluted 2-fold with saline in the absence of anticoagulant to a dilution of 1:512,000. Values are mean ± standard deviation of two independent assays.
taining EDTA, heparin, or ACD from four healthy volunteers vaccinated with Dukoral. Significantly different vibriocidal antibody titers of the plasma were observed depending on the types of tube. As shown in Fig. 2A, eight plasma samples prepared with heparin-containing tubes showed vibriocidal antibody titers identical to those of the corresponding sera (r = 1.000, P < 0.01). In contrast, only 50% of plasma samples (4/8) prepared from ACD-containing tubes showed coincident results with matched sera (r = 0.907, P < 0.01) (Fig. 2B), and only three out of eight plasma samples (37.5%) prepared with EDTA-containing tubes exhibited the same vibriocidal antibody titers as the corresponding sera (r = 0.803, P < 0.05) (Fig. 2C). Interestingly, five out of eight plasma samples prepared with tubes containing EDTA showed a prozone effect in which bacterial growth was completely inhibited at high concentrations (1:2.5 dilution) of the plasma samples, although the prozone effect was not observed at 1:5 or 1:10 dilutions (data not shown). Taken together, our data indicate that plasma samples should be prepared with tubes containing heparin for the determination of vibriocidal antibody titer.

Serum antibody level is not significantly affected by anticoagulants. To examine the effect of anticoagulants on the quantitative analysis of serum antibody levels, V. cholerae LPS-specific IgG, IgA, and IgM titers were compared in serum and plasma samples obtained from the vaccinees. As shown in Fig. 3, the serum IgG and IgM levels were highly correlated with those of plasma. The correlation coefficients ranged from 0.957 to 0.997 for LPS-specific IgG, from 0.928 to 0.945 for LPS-specific IgM, and from 0.716 to 0.917 for LPS-specific IgA. Statistical analysis indicated that the P values of correlations from all antibody titers were significant (P < 0.05). Although the IgA titers from heparin-prepared plasma samples displayed relatively low correlations with the matched sera (r = 0.716) compared to ACD- and EDTA-treated plasma samples, the IgG and IgM titers from heparin-prepared plasma samples showed noticeably high correlations (r = 0.997 and r = 0.945, respectively) compared with ACD- and EDTA-treated samples, suggesting that heparin is an appropriate anticoagulant when plasma samples are to be used to determine antibody levels induced by V. cholerae.

DISCUSSION

Simultaneous acquisition of blood cells and plasma from anticoagulated blood is useful for the concurrent measurement of humoral and cellular immune responses. In the present study, we examined the effects of anticoagulants on vibriocidal antibody titers and antigen-specific antibody levels in serum and plasma samples from cholera patients or vaccinees. Vibriocidal antibody titers of serum and plasma were decreased in the presence of EDTA or ACD but not heparin. Moreover, the vibriocidal antibody titers of plasma isolated from blood collected with heparin-containing tubes, but not EDTA- or ACD-containing tubes, were 100% concordant with the titers of matched serum. In addition, LPS-specific IgG and IgM titers in sera were highly correlated with titers in the corresponding plasma samples as determined by Pearson correlation coefficient (r = 0.997 and r = 0.945, respectively), compared with ACD- (r = 0.957 and r = 0.936, respectively) and EDTA-treated plasma (r = 0.977 and r = 0.928, respectively). Taken together, our data indicate that among the anticoagulants currently used for blood collection, heparin has the advantage over others for testing vibriocidal activity and quantity of antibodies in plasma.

There are several possible reasons why plasma samples containing ACD or EDTA were unsuitable for the measurement of complement-mediated vibriocidal activity. First, both reagents are known to chelate divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ in the outer membrane of Gram-negative bacteria, resulting in bacterial lysis, release of LPS, and increased permeability of the bacteria to antimicrobial agents (19–21). Indeed, in the present study, bacterial growth was completely inhibited in the sample prepared with 5 mM EDTA, even with highly diluted sera (data not shown). Although a high concentration of ACD (7.5%) was previously reported to have antimicrobial activity against Gram-negative bacteria and yeast (22), ACD did not show direct antimicrobial activity in our study, probably due to the use of a low concentration (0.38%). Second, both reagents have been reported to block classical and alternative complement pathways by interfering with C3 activation and formation of the membrane attack complex (12). It has been reported that Ca$^{2+}$ and Mg$^{2+}$ are necessary for full activation of the complement pathway to kill target bacteria because Ca$^{2+}$ and Mg$^{2+}$ are required for the C1 complex (C1r2C1s2) and C4bC2 complex, respectively, in the classical complement activation pathway, which is an antibody-dependent pathway (23, 24). In contrast, only Mg$^{2+}$ is required for activation of complement components (C3b-factorB) in the alternative pathway, an antibody-independent pathway, that is initiated by bacterial surface components and C3 (18, 24, 25).
The results of this study showed that heparin did not affect vibriocidal antibody activity or anti-\textit{V. cholerae} antibody titers in the plasma and serum samples. This implies that collection of blood using heparinized tubes might be suitable for both quantitative and qualitative analysis of antibodies in the blood of human subjects immunized with cholera vaccines. However, heparin has been shown to increase resistance of \textit{Neisseria meningitidis} in serum (26); therefore, the use of heparinized blood in the evaluation of vaccine efficacy should be carefully considered for the clinical evaluation of other vaccines.

Although plasma is a useful analytic specimen for serologic immunomonitoring of vaccines, it is not identical with serum in the protein profile and content. For example, cytokines such as interleukin-1B (IL-1β), IL-2, and tumor necrosis factor alpha (TNF-α) have been detected at higher concentrations in ACD-prepared plasma and heparin-prepared plasma than in serum (27). In contrast, some proteins, such as beta-2 microglobulin and histidine-rich protein 2, were detected at higher levels in sera than in plasma (28, 29), whereas a close relationship between serum and plasma levels was shown for IgG and IgA levels to mycobacterial antigens or 3-nitrotyrosine (30, 31). Although platelets are relatively stable in heparin and ACD anticoagulants, the protein profiles of plasma extracted from EDTA-treated blood are very different from those of plasma prepared with ACD or heparin because EDTA causes platelet clumping and aggregation, resulting in changes in plasma protein content (32, 33).

Besides anticoagulants, the blood collection tube contains multiple components such as clotting activator, silicones, a silica clot activator, and polymeric surfactants (34, 35), all of which may affect the assays. Appropriate blood collection is crucial for accurate immunomonitoring of human clinical samples. The results of this study suggest that plasma prepared with heparinized blood can be substituted for serum in the analysis of vibriocidal antibody activity and quantitative analysis of vaccine-specific antibodies.

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**FIG 3** Titers of antibodies to \textit{V. cholerae} LPS in serum were concordant with those in heparinized plasma. Eight samples each of sera and plasma prepared from vaccinees with Dukoral pre- and postvaccination were analyzed for \textit{V. cholerae} LPS-specific antibody levels. The serum IgG (A, D, and G), IgM (B, E, and H), and IgA (C, F, and I) titers were compared with titers of plasma obtained using blood collection tubes containing heparin (A, B, and C), ACD (D, E, and F), or EDTA (G, H, and I). Diagonal lines indicate regression between serum and plasma containing anticoagulants. The Pearson coefficient ($r$) for the correlation between plasma and serum was obtained from three independent assays and $P$ values on each panel were analyzed by using GraphPad Prism 5 software.
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