Published in final edited form as:

Tuberculosis (Edinb). 2020 January ; 120: 101901. doi:10.1016/j.tube.2020.101901.

Effects of anticoagulants and Ficoll on human serum antibody reactivities and functions against *Mycobacterium tuberculosis*

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Abstract

The ability to utilize leftover samples containing anticoagulants or Ficoll would provide substantial opportunities for future antibody and biomarker studies. Some anticoagulants might influence antibody reactivity against pathogens, but comprehensive studies investigating effects in the context of TB are lacking. We enrolled 24 individuals with and without history of *M. tuberculosis* and/or HIV-infection and investigated TB antibody reactivities, function, and other host protein biomarkers in simultaneously obtained serum and plasma from serum separation, EDTA, heparin, acid citrate dextrose (ACD), or mononuclear cell preparation (CPT™) tubes which contain heparin and Ficoll. Antibody isotype reactivities to two mycobacterial antigens, as well as phagocytosis of *M. tuberculosis*, correlated strongly and significantly between serum and plasma, irrespective of type of anticoagulant or Ficoll present ($r = 0.85$, $p < 0.0001$). However, the presence of ACD resulted in slightly lower values than those obtained with serum in both indirect (antibody reactivities to mycobacterial antigens) and Sandwich ELISAs (soluble CD14 measurements). Our data demonstrate that leftover plasma, regardless of containing anticoagulants or Ficoll, can be used in TB antibody or other host protein biomarker studies but suggest the value of a correction factor when using ACD plasma interchangeably with serum in antibody binding studies.

Keywords

EDTA; ACD; CPT; heparin; immunoglobulins; proteins

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Introduction

Investigating human serum antibodies and other non-antibody host proteins is important for both discovery of tuberculosis (TB) biomarkers and assessment of correlates of protection against TB. Increasing evidence now supports a role of human serum antibodies in the protection against TB (reviewed in [1–3]). However, knowledge of protective *M. tuberculosis* antigens as well as non-sputum-based biomarkers for the prediction and diagnosis of early TB are urgently needed. The ability to utilize leftover plasma from other studies, regardless of containing anticoagulants or Ficoll for blood separation, would greatly enhance opportunities for TB antibody and other host protein investigations.

The anticoagulants EDTA, heparin, and acid citrate dextrose (ACD) are commonly used for the separation of plasma and blood cells. In addition, the use of BD Vacutainer® Mononuclear Cell Preparation Tubes (CPT™), containing heparin sodium or sodium citrate and Ficoll-Paque, has greatly facilitated the separation and preparation of peripheral blood mononuclear cells (PBMCs) for research studies [4–6]. Ficoll, a neutral hydrophilic polysaccharide, dissolves in aqueous liquids. The use of CPT tubes is more convenient yet equally effective compared to the conventional Ficoll density gradient technique since the steps involved are easier to perform and are less time consuming. The less elaborate CPT isolation protocol also minimizes variations in processing and in specimen contamination, as well as eliminates the requirement for clinical sites with trained laboratory personnel or specialized laboratory equipment [4, 6]. For these reasons, CPT tubes are increasingly used for PBMC isolation in pediatric and adult populations.

Serum, which, unlike plasma, lacks fibrinogen and clotting factors, is traditionally used for antibody reactivity and functional assays. While prior studies by us and others show that the anticoagulants heparin and EDTA in plasma have little effect on antibody reactivities to *M. tuberculosis* and other mycobacterial antigens, little is known about the effects of ACD and CPT plasma on antibody reactivities, especially in the context of TB [7–9]. Furthermore, we and others previously showed that antibodies to *M. tuberculosis* antigens can have Fc-gamma receptor (FcγR)-mediated functions, such as the uptake of *M. tuberculosis* into macrophages [1–3]. Whether anticoagulants could influence such antibody functions is unclear. Prior studies have shown that blood treated with citrate has decreased activity of some immune functions, such as activation of the complement system, neutrophil degranulation, and cytokine production [10, 11]. Furthermore, serum antibodies from cholera patients have lower vibriocidal activity in the presence of ACD and EDTA compared to heparin or no anticoagulants [12]. Therefore, our objectives were to assess and to correlate the following with simultaneously obtained serum and plasma from heparinized, EDTA, ACD, and CPT tubes: i) IgG, IgM, and IgA antibody reactivities to two mycobacterial antigens, one polysaccharide and one protein; ii) antibody-mediated human macrophage phagocytosis of *M. tuberculosis*; and iii) levels of the human TB biomarker host protein soluble CD14 (sCD14).

Subjects and Methods

Subjects.

We enrolled 24 asymptomatic volunteers and patients; 11/24 (46%) had a history of a positive tuberculin skin-test, 18/24 (75%) had a history of receiving the *M. bovis* Bacillus Calmette Guerin (BCG) vaccination, and 9/24 (38%) had a history of microbiologically proven TB, four of whom were HIV co-infected. Subjects' median age was 42 years (range 25 to 69) and 14/24 (58%) were male. Blood samples were obtained using BD Vacutainer® collection tubes: Serum Separation Tubes (SST™), Heparin (lithium heparin), ACD, K2 EDTA, and Mononuclear Cell Preparation Tubes containing sodium heparin and Ficoll (CPT™; BD Life Sciences: Preanalytical Systems, Franklin Lakes, NJ). Of the 24 subjects, 16 had blood simultaneously obtained with all tubes, 7 with all except for ACD tubes, and one with all except for ACD and EDTA tubes. To allow for simultaneous processing of samples, the assessments of *M. tuberculosis* phagocytosis and sCD14 were performed only with samples for the 16 subjects that had all plasma types available. Serum and plasma tubes were centrifuged for 10 minutes at 3000 revolutions per min (rpm) for cell separation. CPT tubes were centrifuged for 30 minutes at 2660 rpm (~1500 relative centrifugal force [rcf]), and PBMCs were separated from CPT plasma as per manufacturer's instructions (BD Life Sciences: Preanalytical Systems, Franklin Lakes, NJ). Samples were stored at -80°C until tested. Approval for human subjects' research was obtained from the institutional review board of the Albert Einstein College of Medicine, Bronx, New York. All subjects signed informed written consent prior to enrollment and blood draw.

Antibody detection assays.

The purified native mycobacterial protein PstS1, also referred to as 38 kDa protein (Rv0934, purified from H37Rv) was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources), NIAID, NIH. The mycobacterial capsular polysaccharide arabinomannan (AM) was isolated from the *M. tuberculosis* strain H37Ra as described [13, 14]. Enzyme-linked immunosorbent assays (ELISAs) were performed using 96-well microtiter plates (Immulon 2HB; Fisher Scientific) as described [13, 14] and repeated on separate days to confirm reproducibility of data. Briefly, the plates were coated with PstS1 at 4 µg/ml or AM at 10 µg/ml. Serum and plasma samples were diluted (1:50 and 1:100) and added in duplicates to the antigen-coated wells. Negative controls were processed as described above with the addition of buffer solution instead of a serum or plasma. Positive controls included murine monoclonal antibodies (mAbs; 1:200) obtained from BEI Resources, NIAID, NIH: an IgG1 to PstS1 (IT-15), and an IgG3 to AM (CS-35). Antibodies were detected with Protein G alkaline phosphatase (AP; Calbiochem; 1:2,500), goat anti-human IgA-AP (Sigma; 1:1000), mouse anti-human IgM-AP (Southern Biotech; 1:1,000), or, goat anti-mouse IgG3-AP for the positive control of AM and goat anti-mouse IgG1-AP for the positive control of PstS1, using p-nitrophenyl phosphate substrate. The optical densities (OD) were measured at 405 nm.

Phagocytosis assay.

Assessment of antibody-mediated effects on *M. tuberculosis* phagocytosis of human macrophages was performed as described [13]. Briefly, the *M. tuberculosis* strain H37Ra

was conjugated with fluorescein isothiocyanate (FITC). Human monocytic cells (THP-1), differentiated into macrophages, were co-incubated with either heat-inactivated sera or one of the four types of plasma samples (10% dilution) and FITC-labeled H37Ra at a multiplicity of infection (MOI) of 20 for 3 hours. Fetal bovine serum (FBS) and serum (10% dilution) from a healthy TST- donor without history of BCG vaccination or *M. tuberculosis* exposure were included as negative controls. Cells were treated with trypan blue to quench the fluorescence from noninternalized FITC-labeled bacteria, and phagocytosis was then evaluated by flow cytometry.

Detection of sCD14.

Soluble CD14 levels were determined with a commercially available sandwich ELISA (R&D systems, Minneapolis, MN, USA) as described [15]. Briefly, wells of 96-well microtiter plates (Costar, Corning, NY) were coated with sCD14 capture antibody and incubated overnight at 25°C. Diluted serum or plasma samples (1:600) were added in duplicates and sCD14 detected with an HRP-labeled detection antibody. The ODs were determined as per the manufacturer's instructions. Human sCD14 standards (Sigma-Aldrich, St. Louis, MO, USA) were performed in parallel on all of the plates, and concentrations were calculated based on the generated standard curve.

Statistical analysis.

Because of the small sample size, in which Pearson correlations can be greatly impacted by outliers/extreme values, we used Spearman correlation as a more robust measurement in our primary analysis of all associations. To further assess linear relationships between continuous variables, we also performed the Pearson test for all associations. To assess the degree of increase between values obtained from serum versus plasma containing the various anticoagulants and/or Ficoll, we further obtained the slope coefficients for all associations assessed by linear regression. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc.).

Results

Antibody reactivities in the presence of different anticoagulants and Ficoll.

We found strong and highly significant correlations for antibody isotype reactivities to both mycobacterial antigens assessed in serum versus CPT plasma or ACD plasma (Figure 1). When comparing tests performed on different days with the same sample types, the correlation coefficients were all ≥ 0.9 , indicating a high reproducibility of our ELISA values. Due to the small sample size, values in figures are reported for Spearman correlations but were similarly strong when assessed by the Pearson correlation test (Supplemental Table 1). Because IgA and IgM responses to PstS1 were overall low, the correlation for reactivity to PstS1 was restricted to IgG. Equally strong correlations were also found for antibody reactivities to both antigens between CPT plasma and all three other plasma types: heparinized, EDTA, and ACD ($r \geq 0.85$, $p < 0.0001$ for all correlations), as well as between ACD plasma and the other plasma types ($r \geq 0.89$, $p < 0.0001$ for all correlations). We further obtained the slope coefficients assessed by linear regression for associations between serum and plasma containing the various anticoagulants and/or Ficoll (Figure 1 and

Supplemental Table 2). These data indicate slightly lower values obtained with ACD plasma compared to serum (slope coefficients 0.61 – 0.87) in both indirect (antibody, especially IgA, reactivities to mycobacterial antigens; Figure 1) and Sandwich ELISA (sCD14; Figure 3).

Antibody-mediated *M. tuberculosis* macrophage phagocytosis in the presence of different anticoagulants and Ficoll.

We next assessed whether anticoagulants or Ficoll could have an effect on antibody FcγR-mediated function, such as the uptake of *M. tuberculosis* into macrophages. We found strong and highly significant correlations for antibody mediated *M. tuberculosis* phagocytosis between human macrophages (THP-1 cells) co-incubated with serum compared to plasma containing the various anticoagulants and/or Ficoll (Figure 2 and Supplemental Table 1). When comparing experiments performed on different days with the same sample types, the correlation coefficient was 0.95, indicating a high reproducibility of our phagocytosis assay.

Measurements of non-antibody host proteins in the presence of different anticoagulants and Ficoll.

Because host proteins, such as C-reactive protein (CRP) and sCD14, have shown value as diagnostic TB biomarkers, we further assessed sCD14 levels by Sandwich ELISA and compared levels in serum to plasma containing the various anticoagulants or CPT plasma. While not quite as strong as for antibody reactivities, concentrations nevertheless correlated highly and significantly between serum and CPT plasma ($r = 0.78$, $p < 0.001$; Fig. 3A), between serum and ACD plasma ($r = 0.75$, $p = 0.001$; Fig. 3B), and between serum and heparinized or EDTA plasma ($r = 0.79$ and 0.75 , $p < 0.001$ and $p = 0.001$, respectively; Fig. 3C&D and Supplemental Table 1). The slightly lower correlation coefficients were also seen when comparing tests performed on different days with the same sample types ($r = 0.76$), indicating a higher sensitivity to slight day to day variation of the sCD14 Sandwich ELISA.

Discussion

The ability to utilize leftover plasma from other studies without concern for interference with antibody reactivities or functions would open opportunities for antibody and biomarker research opportunities. We here show that values obtained with all types of plasma correlated strongly and significantly with serum values. The anticoagulants heparin and EDTA, and/or the polysaccharide Ficoll have little effect on the binding of the antibody variable region or the antibody constant region in FcγR-mediated phagocytosis of *M. tuberculosis*. While ACD appeared to have little effect on FcγR-mediated phagocytosis, it did result in lower values than those obtained with serum in both indirect (antibody reactivities to mycobacterial antigens) and Sandwich ELISAs (sCD14) with slope coefficients ranging from 0.61 – 0.87. Our data demonstrate that leftover plasma, regardless of the presence of anticoagulant or Ficoll, can be used in TB antibody or other host protein biomarker studies. However, when using ACD plasma interchangeably with serum in antibody binding studies, our data suggest the value of a correction factor.

Our results are consistent with data by us and others showing that the anticoagulants heparin and EDTA have little effect on antibody reactivities to *M. tuberculosis* and other antigens [7–9]. By contrast, our data suggest a slight reduction of antibody binding, especially for IgA, in the presence of ACD. Hodgkinson et al., using different comparison methods, report little to minor effects of ACD on binding to cancer associated pathogens, such as papillomaviruses, Epstein-Barr virus, and *H. pylori* [8]. Our results are consistent with findings by Yang et al. reporting lower vibriocidal activity of serum antibodies from cholera patients in the presence of ACD (and EDTA) compared to heparin or no anticoagulants [12]. However, in contrast to Yang et al., we did not observe such effect for EDTA. We further show that *M. tuberculosis* phagocytosis by human macrophages, a FcγR-mediated antibody function, is unaffected by heparin, EDTA, ACD and Ficoll. These data are important because the various anticoagulants as well as CPT tubes are commonly used for PBMC-based studies with remaining archived leftover plasma available for future research.

Our study was limited by the relatively small sample size of 24 subjects, of whom 16 had simultaneously obtained plasma types containing all anticoagulants as well as Ficoll in CPT plasma. Because of the small sample size, in which Pearson can be greatly impacted by outliers/extreme values, we used Spearman as a more robust measurement of association. Nevertheless, when assessing linear relationships, we found similar highly significant p values with high r values using both methods, confirming strong correlations between serum and the different plasma types for the various readouts.

In conclusion, we show that heparin, EDTA, and Ficoll have little effect on the binding of the antibody variable region to *M. tuberculosis* antigens, while ACD appears to reduce antibody binding slightly. Neither the type of anticoagulant nor Ficoll appears to considerably affect Fc-γ receptor-mediated function, such as *M. tuberculosis* phagocytosis by macrophages. Our results demonstrate that leftover serum and plasma from heparin, EDTA, ACD and CPT tubes can be used for TB antibody studies and suggest the value of a correction factor when ACD plasma is used interchangeably with serum for antibody binding studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by grants from the National Institutes of Health (NIH/NIAID) AI117927, AI127173, and AI146329 to J.M.A. All authors declared no conflict of interest. The following antigens and murine mAbs were obtained through BEI Resources, NIAID, NIH: native PstS1 (Rv0934), purified from *M. tuberculosis*, Strain H37Rv, NR-14859; mAb to PhoS1/PstS1 (IT-15, NR-13605); and mAb to LAM (CS-35, NR-13811).

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Fig. 1. Antibody binding to *M. tuberculosis* antigens is not influenced by anticoagulants and/or Ficoll.

Correlations between antibody reactivity measured in CPT plasma and serum (n = 24) for IgG (A), IgA (B), or IgM (C) to arabinomannan (AM), and IgG to PstS1 (D), and between ACD plasma and serum (n = 16) for IgG (E), IgA (F), and IgM to AM (G), and IgG to PstS1 (H). Spearman rank correlation. Values for slope coefficients were assessed by linear regression.

Fig. 2. Antibody-mediated macrophage phagocytosis of *M. tuberculosis* is not influenced by anticoagulants and/or Ficoll.

Correlations of H37Ra (MOI 20) phagocytosis (% FITC-positive cells) by THP-1 derived human macrophages co-incubated with sera and CPT plasma (A), ACD plasma (B), heparinized plasma (C), or EDTA plasma (D) (n = 16 for all correlations). All sera and plasma samples were heat-inactivated and added at 10% dilution. Spearman rank correlation. Values for slope coefficients were assessed by linear regression.

Fig. 3. Detection of the human host protein sCD14 is not influenced by anticoagulants and/or Ficoll.

Correlations between sCD14 levels in serum and CPT plasma (A), ACD plasma (B), heparinized plasma (C), or EDTA plasma (D) ($n = 16$ for all correlations). Spearman rank correlation. Values for slope coefficients were assessed by linear regression.