Journal of Immunological Methods 464 (2019) 114–118

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

The influence of EDTA Vacutainer blood collection tube on the level of blood interleukin-1 receptor antagonist

Najib Aziz[a,*], Anthony W. Butch[b], Timothy C. Ryner[a], Otoniel Martinez-Maza[a,c], Roger Detels[a,d]

[a] Department of Epidemiology, UCLA, Fielding School of Public Health, Los Angeles, CA 90095, USA
[b] Department of Intercollegiate Athletes, UCLA, Los Angeles, CA 90095, USA
[c] Departments of Obstetrics & Gynecology and Microbiology Immunology and Molecular Genetics, UCLA, USA
[d] Department of Medicine, UCLA, David Geffen School of Medicine, Los Angeles, CA 90095, USA

ARTICLE INFO

Keywords:
Biomarker
Cytokine
EDTA blood
IL-1ra
Stability

ABSTRACT

Background: The use of anticoagulants may influence the composition of blood cells and interfere with plasma levels of IL-1ra when unprocessed EDTA blood samples are stored for long periods of time.
Methods: Blood was drawn into EDTA and heparinized blood collection tubes from 11 HIV-1 negative men participating in the Multicenter AIDS Cohort Study (MACS) and 4 healthy volunteers. The blood was processed according to the experiments detailed in the method and after incubation; supernatants were collected and stored at -70 °C until batch testing using IL-1ra ELISA.
Results: There was no difference between the levels of IL-1ra in EDTA blood collected into plastic and glass tubes ($p = .911$). There were significant increases from baseline levels of IL-1ra ($p \leq .05$) after 24 h incubation for diluted whole blood and PBMC supernatants but not for granulocytes supernatants.
Conclusion: EDTA as an anticoagulant influences the blood concentrations of IL-1ra in unprocessed blood. Thus, EDTA blood is not a suitable specimen for measurement of IL-1ra. Other types of anticoagulated blood should be processed within one hour of draw whenever measuring plasma levels of IL-1ra.

1. Introduction

Arrays of cytokines and other biomarkers are released through cell-mediated immunological responses to various inflammatory and infectious disease stimuli. Elucidations of these responses have provided important insights into the biology of the immune system. The accurate quantification of those biomarkers will be a useful laboratory tool for monitoring of progression and treatment outcomes of numerous diseases. However, several pre-analytical factors such as specimen type, handling, processing and storage conditions, and age of blood before centrifugation can greatly influence the measurement of many blood biomarkers.

Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood has been used for several decades for hematology testing (Banfi et al., 2007). Neutrophils and monocytes appear to be the most sensitive to storage in EDTA, whereas lymphocytes are the most stable cell type (McColl et al., 1992). Interleukin-1 receptor antagonist (IL-1ra) cytokine is secreted by various cell types including monocytes, macrophages, neutrophils, leukemia cells, monocytic cell lines, adipocytes, and epithelial cells. Although neutrophils produce significant amounts of IL-1ra, monocytes produce 20 times more IL-1ra than neutrophils (Reardon et al., 1991).

By binding to IL-1 receptor without activation of the signaling pathway, IL-1ra inhibits the biological activities of IL-1α and IL-1β, both of which play an important pro-inflammatory role in various diseases (Arend et al., 1998).

Previously, we found blood levels of IL-1ra to be very unstable and increased in unprocessed EDTA anticoagulated blood (Aziz et al., 2016). The purpose of this communication is to further investigate the type of blood cell or blood collection tube responsible for the release of high concentrations of IL-1ra into EDTA anticoagulated blood that is left unprocessed for up to 24 h. We hypothesized that the increased concentrations of IL-1ra in EDTA plasma that occurs following a delay in blood processing might be due to the effects of EDTA on neutrophils.

2. Material and methods

2.1. Specimens

The UCLA institutional review board for human studies approved

* Corresponding author.
E-mail address: naziz@mednet.ucla.edu (N. Aziz).

https://doi.org/10.1016/j.jim.2018.10.009
Received 20 July 2018; Received in revised form 19 September 2018; Accepted 17 October 2018
Available online 19 October 2018

the protocols. After informed consent from each subject, blood was collected into 10 mL Vacutainer plastic tubes (Becton Dickinson Vacutainer Systems, New Jersey) containing either EDTA or sodium heparin.

For confirming that blood tube material was not affecting IL-1ra levels, EDTA blood was collected into tubes from 11 HIV-1 negative healthy men participating of the Multicenter AIDS Cohort Study (MACS) (Detels et al., 2012).

Separately, blood was collected into EDTA Vacutainer and heparinized Vacutainer tubes from 4 apparently healthy UCLA employee volunteers (one female and three male) for evaluation of anticoagulant (Heparin and EDTA) effect on IL-1ra levels in whole blood cells, peripheral blood mononuclear cells (PBMC), and granulocytes (three diverse cell types).

2.2. Reagents

The following reagents were used for the experiments: 1.077 g/mL Ficoll-paque Plus (GE Healthcare Bio-Sciences AB, Sweden), red blood cell (RBC) lysis buffer (Roche GmbH, Mannheim, Germany), 1.113 g/mL Lymphocyte-poly cell separation media (cat# CL5070, Cedarlane, North Carolina, USA), Hanks' balanced salt solution 1× (HBSS), Phosphate buffer saline 1× (PBS) (HBSS and PBS are both without Ca^{++} and Mg^{++}), EDTA 2% solution, and lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO USA).

2.3. Methods

To check whether tube structure material had an effect on IL-1ra levels, 8 mL of blood was drawn into a single EDTA-containing Vacutainer blood collection tube per subject from 11 male HIV-1 negative MACS participants. Within one hour of blood draw, 2 mL of well-mixed blood per subject was centrifuged. After centrifugation, 1 mL of EDTA plasma was collected as a baseline sample into a labeled cryovial and then frozen at -70 °C for batch testing. Also within one hour of blood draw, 2 mL of well-mixed blood per subject was split evenly (1 mL each) into pre-labeled glass and plastic culture tubes and those tubes were incubated at room temperature (21-22 °C) for 24 h. After incubation, the glass and plastic culture tubes were centrifuged and 0.5 mL separated EDTA plasma per tube was aliquoted into a pre-labeled cryovial and stored at -70° C until batch analysis of IL-1ra.

The effect of anticoagulants (EDTA and sodium heparin) on the levels of IL-1ra of diluted whole blood, PBMC, and granulocytes was investigated. Blood (10 mL/tube) was drawn from each of four healthy volunteer subjects, into two plastic tubes containing sodium heparin and two plastic tubes containing EDTA. The laboratory experiments are detailed as follows.

2.3.1. Diluted whole blood

1200 µL of well-mixed blood (heparinized or EDTA) from each subject of four healthy volunteers was diluted with 4800 µL of 1× PBS and 1 mL of the diluted blood aliquoted into each of 6 separate round bottom polypropylene culture tubes (six for heparinized and six for EDTA blood). Tube #1 served as the **baseline** and was incubated for 1 h; tube #2 for 6 h; tube #3 for 15 h; tube #4 for 24 h; **tube #5** received 4 mg of additional EDTA for monitoring the effect of EDTA on IL-1ra production and was incubated for 24 h; and **tube #6** received 100 pg of LPS which served as a positive control and was incubated for 24 h.

2.3.2. PBMC and granulocytes

The cells were isolated within one hour of the draw from the remainder of the heparinized and EDTA whole blood (from the four healthy volunteers) based on the double density gradient technique followed by RBC lysis (Rondelli et al., 2013). In summary, a double density gradient (DDG) was prepared in 15 mL conical polypropylene tubes by layering 3.0 mL of Ficoll with a density 1.077 g/mL (Ficoll-paque Plus, GE Healthcare) over 3.0 mL of Ficoll with a density of 1.113 g/mL (Lympholyte-Poly, Cedarlane). 7 mL of whole blood was diluted with 14 mL of sterile 1× PBS to make a 1:3 dilution. Using three DDG tubes per subject, 7 mL of the diluted blood (heparinized or EDTA) was carefully layered onto the Ficoll in each tube and centrifuged at 500 ×g for 30 min with no centrifuge brake. After centrifugation, PBMC (buffy coat) were found at the plasma and 1.077 g/mL density Ficoll interface, granulocytes were found at the 1.077 g/mL density and 1.113 g/mL density Ficoll interface, and erythrocytes were found at the bottom of the tube. PBMC and granulocytes were collected in that order from each tube via gentle pipetting and placed into separate new 15 mL conical tubes (1 tube for granulocytes and 1 for PBMC per subject). The samples were then washed by adding 10 mL of sterile 1× HBSS to each of the tubes and centrifuging them for 350 ×g for 10 min. After wash, 2 mL of RBC lysis buffer was added into the tubes containing granulocytes only (not into the PBMC tube) for removal of RBC and the tubes were held at 4 °C for 10 min. After centrifugation, PBMC and granulocyte concentrations were adjusted with 1× PBS (Sigma) to 10^6 cells/mL and separated into two sets of 6 tubes per subject (6 tubes containing PBMC and 6 tubes containing granulocytes). For both sets, 4 mg of EDTA was added to tube #5 and 100 pg of LPS was added to tube #6.

All three sets (diluted whole blood, PBMC, and Granulocytes) of tubes were then incubated at 37 °C with 5% CO_2 as follows: tube #1 for 1 h, tube #2 for 6 h, tube #3 for 15 h, and tubes #4–6 for 24 h. After each incubation period, the supernatant was collected from each sample and stored at -70 °C until batch analysis of IL-1ra. This process enabled us to recover about 75 ± 10.4% of granulocytes from EDTA blood and 55.0 ± 26.4% from heparinized blood. The purity of isolated granulocytes was checked by staining 5×10^5 granulocytes with CD11b FITC and CD45 PerCP and analysis done by FACScaliber flow cytometer and CellQuest™ Software (DB Biosciences, USA).

Interleukin-1 receptor antagonist (IL-1ra) concentrations were measured using a sandwich enzyme immunoassay technique from R&D Systems (Minneapolis, MN 55413). Based on manufacturer data, the minimum detectable dose (MDD) was 18.3 pg/mL and the intra-assay coefficient of variation (CV) was determined to be 7.3% and 5.0% for control samples with mean concentrations of 64.9 pg/mL ($n = 20$) and 538 pg/mL ($n = 20$), respectively.

3. Statistical analysis

A four-parameter curve-fitting by Microsoft Excel was used to generate a calibration curve of the IL-1ra enzyme immunoassay and to calculate the unknown level of IL-1ra in a sample. The results of IL-1ra were adjusted to one million WBC counts per mL and for diluted whole blood the result was multiplied by the dilution factor of five.

The differences in IL-1ra levels between baseline and 24 h incubation of EDTA whole blood into plastic tubes, EDTA whole blood into glass tubes, and mean difference of IL-1ra levels between the two tube types in 11 samples were investigated by STATA statistics data analysis of paired *t*-test.

The rates of change in levels of IL-1ra over 24 h were evaluated using the Generalized Estimating Equation (GEE) of SAS. The dependent variable was measured blood biomarker concentrations and was modeled as a linear function based on the incubation time in hours. EDTA plasma levels of IL-1ra exhibited a significant difference in the rate of increase after 6 h, a linear spline with a knot at 6 h was used for the rate of change and was calculated as follows: If time was ≤6 h then the concentration = intercept + (coefficient 1) x (time). If time was > 6 h then the concentration = intercept + (coefficient 1) x (time) + (coefficient 2) x (time-6). Coefficients are the rate of change in concentration with time being measured in hours. To account for the correlation between repeated time point measures in a given subject, generalized estimating equations with a first-order autoregressive covariance pattern were specified to reduce the number of parameters

in the model. A *p*-value ≤.05 was considered to be statistically significant. In addition to a p-value of ≤0.05, the predicted concentration change per hour was required to be at least 10% or more than baseline in order to be considered significant to account for measurement uncertainty of the immunoassays. Data analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC 2013) and Stata Statistics Data Analysis 14.2 (StataCorp College Station, Texas 77845). Graphs were produced using SigmaPlot software version 13 (Jandel Scientific, San Rafael, CA 2016).

4. Results

The mean concentration of IL-1ra in plasma that was immediately separated from EDTA whole blood samples was 213 pg/mL. As expected, incubation of EDTA whole blood samples for 24 h at room temperature resulted in significant increases in IL-1ra concentration and the mean levels of IL-1ra in EDTA whole blood samples stored in plastic and glass tubes ($n = 11$) were 2046 pg/mL and 2000 pg/mL, respectively. There was a statistically significant difference between the mean values of IL-1ra at baseline and plastic tubes ($p < 0.001$) and baseline and glass tubes ($p < 0.001$). The mean value of IL-1ra between plastic and glass tubes was not significant ($p = 0.911$). The differences in the mean and median of plasma IL-1ra levels in EDTA whole blood stored in plastic and glass tubes were 46 and 184 pg/mL respectively (Fig. 1).

The source of the IL-1ra (pg/mL) increase in the supernatants of diluted whole blood, separated PBMC, and separated granulocytes for four healthy volunteers was investigated within 24 h of incubation. Intercepts (mean baseline concentrations), coefficients (predicted changes in the measured concentration per hour), SE (standard errors associated with the coefficients), and *P* values ($Pr > |z|$) of IL-1ra levels for diluted whole blood, PBMC, and granulocytes supernatants are presented in Table 1.

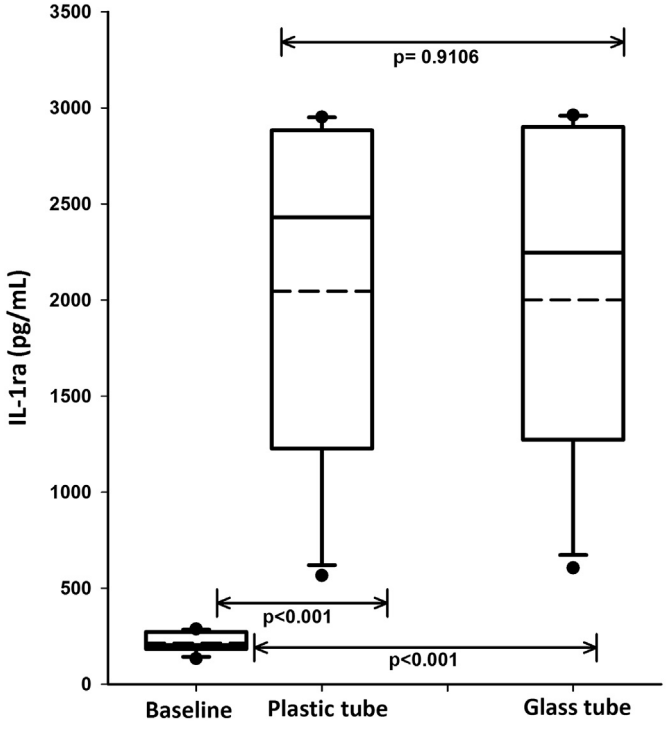

Fig. 1. Mean plasma concentrations of IL-1Ra (medium dash) in EDTA blood samples stored in plastic and glass tubes for 24 h at room temperature were 2046 pg/mL and 2000 pg/mL respectively. The baseline mean concentration of IL-1Ra (plasma separated within 3 hours post collection) was 213 pg/mL. The solid and dashed horizontal lines inside the box represent the median and mean value respectively. The lower and upper lines of the box represent the 25th and 75th percentiles, respectively. Lower and upper whisker caps represent the 5th and 95th percentiles, respectively. The difference in the mean between baseline and plastic tube were significant ($p < 0.001$). The difference in the mean between plastic tube and glass tube was not significant ($p = 0.9106$).

There were statistically significant ($p < 0.001$) increases from baseline values in the concentration of IL-1ra after 24 h of 1187 pg/mL and 548 pg/mL (an increase of 5.12 and 0.16 fold) for diluted EDTA and heparin whole blood, respectively (Fig. 2 A, B). The mean increases of IL-1ra in the supernatants of diluted EDTA and heparinized whole blood after 24 h in samples where EDTA was added (tube #5) were 1284 pg/mL and 1340 pg/mL, respectively.

There were also significant increases ($p < 0.001$) from baseline values in the concentration of IL-1ra after 24 h of 80.09 pg/mL and 109.83 pg/mL (increase of 5.03 and 3.47 fold) in supernatants of PBMC of EDTA and heparinized blood, respectively (Fig. 2C, D). The mean increases of IL-1ra after 24 h in samples where EDTA was added (tube #5) for PBMC supernatants of EDTA and heparinized blood were 50.0 pg/mL and 63 pg/mL, respectively.

In the supernatants of EDTA and heparin granulocytes, increases of 8.2 pg/mL and 20.5 pg/mL per hour ($p \leq 0.05$) were seen in the concentration of IL-1ra up to 6 h peaks concentrations of 80.09 pg/mL and 109.83 pg/mL, respectively; after 6 h these concentrations began to decline, with decreases of 9.2 pg/mL ($p = .103$) and 26.8 pg/mL ($p = 0.018$) per hour, respectively. (Fig. 2 E, F). The mean increases of IL-1ra after 24 h in samples where EDTA was added (tube #5) for granulocyte supernatants of EDTA and heparinized blood were 286.0 pg/mL and 350.0 pg/mL, respectively.

Comparing supernatant IL-1ra levels after 24 h of incubation, both with and without added EDTA (4 mg/mL), the concentration of IL-1ra was the highest in both EDTA and heparin diluted whole blood.

IL-1ra levels were almost double in the granulocyte supernatants spiked with EDTA compared to the supernatants of non-spiked granulocytes in both types of anticoagulated blood.

PBMC samples spiked with EDTA showed the lowest increase in IL-1ra levels compared to the non-spiked samples in both types of blood.

5. Discussion

Common anticoagulants such as EDTA, sodium heparin, and sodium citrate are routinely used for preserving whole blood for a wide variety of clinical laboratory tests. Unfortunately, anticoagulants have the potential to interfere with the quantification of certain biomarkers and, in some cases, can even stimulate the cell to secrete unwanted protein. EDTA is the most commonly used anticoagulant and it inhibits the clotting process by chelating calcium in blood while also serving as a stabilizing agent for unstable blood molecules such as cytokines and hormones (Banfi et al., 2007).

EDTA has been shown to inhibit both in vitro and in vivo effects of IL-1 (McColl et al., 1992; Hannum et al., 1990). Its effect on blood calcium may also interfere with neutrophil activity since free intracellular Ca^{++} concentrations appear to be important in modulation of the neutrophil oxidative burst and degranulation (Mandell, 1987). Neutrophils represent the first line of defense against bacterial infection and have complex biological actions such as sensing of chemotactic factors, migration, phagocytosis, and microbial killing (Forehand et al., 1989).

The source and reason for the increased IL-1ra concentration seen in unprocessed blood that is kept for long periods of time was not established (Aziz et al., 2016).

There was no influence of the type of tube used for blood collection and no significant difference ($p = 0.911$) was observed between levels of IL-1ra of blood collected and stored in glass or plastic tubes, but as anticipated (Aziz et al., 2016) there were statistically significant ($p < 0.001$) increases of 8.6 (plastic tube) and 8.3 (glass tube) fold in IL-1ra levels compared to baseline IL-1ra concentrations (Fig. 1).

The cellular source of IL-1ra increase release was investigated. We

Table 1
Stability of IL-1ra in different cells types stored in an incubator (37 °C and 5% Co_2) for 24 h.

IL-1ra (pg/mL)	Intercept[a]	Coefficeint-1	SE	Pr > |z|	Coefficeint-2	SE	Pr > |z|
EDTA blood							
Diluted blood	194	13.63	1.510	< 0.0001	37.02	7.633	< 0.0001
PBMC	13.26	0.399	0.0001	< 0.0001	3.182	0.414	< 0.0001
Granulocyte	106	8.234	4.629	0.0753	−9.211	5.642	0.1026
Heparinized blood							
Diluted blood	212	8.80	0.727	< 0.0001	6.96	0.870	< 0.0001
PBMC	24.54	1.958	0.599	0.0011	2.13	0.811	0.0087
Granulocyte	142	20.490	10.990	0.0623	−26.76	11.259	0.0175

[a] The intercept represents the mean concentration of IL-1ra of diluted blood, PBMC supernatants and granulocyte supernatants at baseline; coefficient 1 is the predicted value change in concentration for each hour from baseline to 6 h; coefficient 2 is the predicted value change in concentration (after six hours) per hour for 24 h; SE is the standard error of the coefficient; and Pr > |z| is the calculated P value using the generalized estimating eq. *P* values ≤ .05 are considered statistically significant.

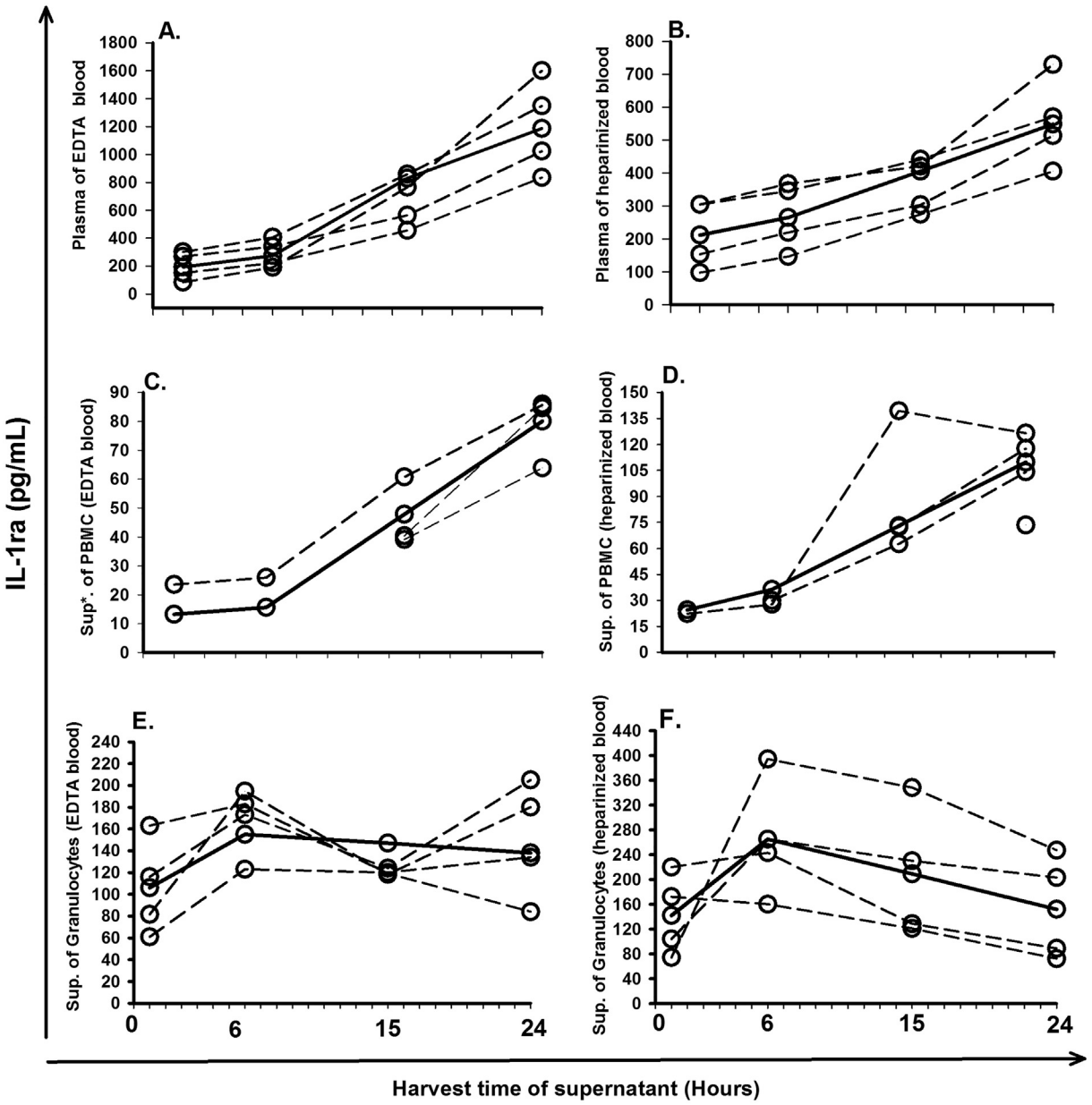

Fig. 2. Increases in IL-1Ra concentration that occurred over time at baseline (1), 6, 15, and 24 h (circle) when unprocessed EDTA and heparinized diluted blood (one million WBC per ml), respectively, (A, B), one million of PBMC of EDTA or heparinized blood into one ml of 1x PBS without Ca^{++} and Mg^{++}, respectively (C, D), and one million of granulocytes of EDTA or heparinized blood into one ml of 1x PBS without Ca^{++} and Mg^{++}, respectively (E, F) were incubated at 36.5 °C and 5% CO_2 for 24 h. Dashed line represent data for each subject and the solid line represents the predicted average change for all subjects. *Sup; supernatant.

observed that the spontaneous release of IL-1ra into diluted whole blood was higher after 24 h incubation when compared to levels of IL-1ra in supernatants of isolated granulocytes or PBMC. The reason behind the higher levels of IL-1ra in whole blood may be due to the intact networking or communication among blood cells, including both monocytes and granulocytes.

The lowest spontaneous release levels of IL-1ra within 24 h were seen for PBMC (monocytes and lymphocytes); this may be due to a lack of granulocytes or other factors. Separated PBMC from EDTA anticoagulated blood released less IL-1ra than the separated PBMC from heparinized blood (3 out of 4 subjects have undetectable levels of IL-1ra up to 15 h of incubation). This may be a sign of the interference of EDTA through its effect on metal cations (Ca^{++}, Mg^{++}, Zn^{++}, etc.) which has been seen in a number of biologic systems including interaction between cells or nucleic acid synthesis (Alford, 1970).

Mean levels of IL-1ra in the supernatants of separated granulocytes from EDTA anticoagulated and heparinized blood were 106 and 142 pg/mL after one hour of incubation and were 169 and 266 pg/mL after 6 h incubation respectively. Granulocytes are fragile, short-lived, and activate very easily during separation from whole blood. One possible explanation for these high levels of IL-1ra release at 1 and 6 h could be the activation of cells during separation, followed by a stabilizing period, and then decline of IL-1ra levels due to neutrophil death and a lack of interaction with other supporting blood cells during overnight incubation (Table 1).

The EDTA spiked tubes for granulocytes released more IL-1ra at 24 h compared to PBMC; this may be due to the binding of metal cations by EDTA, causing granulocyte priming and increased production of IL-1ra. Malyak, et al. confirmed that peripheral neutrophils are capable of being stimulated and producing more IL-1ra which may explain why we saw a higher concentration of IL-1ra in LPS stimulated whole blood containing both monocytes and granulocytes versus isolated PBMC (Malyak et al., 1994).

6. Conclusion

We can conclude that the use of EDTA as an anticoagulant dramatically influences the measurement of blood concentrations of IL-1ra in unprocessed blood. Our data suggest that alterations of calcium homeostasis by EDTA, activation of granulocytes during blood processing, as well as supportive interaction among blood cells in whole blood may cause the increased secretion of IL-1ra. For this reason, EDTA anticoagulated blood will not be a suitable specimen for measurement of blood levels of IL-1ra. Other types of anticoagulated blood should be processed within one hour of draw whenever measuring plasma levels of IL-1ra.

Conflict of interest

The authors declared no conflicts of interest.

Acknowledgements

We thank the men who participate in the MACS, who make this and many other studies possible. This work was supported by grants from the National Institutes of Health (U01-A1-35042); The MACS is funded by the National Institute of Allergy and Infectious Diseases, with additional supplemental funding from the National Cancer Institute (U01-AI35042, 5-M01-RR00722 (GCRC), U01-A135043, U01-AI37984, U01-A135039, U01-AI35040, U01-AI37613, and U01-AI35041). This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. N01-CO56000.

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