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High multi-cytokine levels are not a predictive marker of alloimmunization in transfused sickle cell disease patients

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ABSTRACT

Patients with sickle cell disease (SCD) receive multiple red blood cell (RBC) transfusions for both prevention of and therapy for disease-related complications. In some patients, transfusion results in development of both allo- and auto-antibodies to RBC antigens. What precipitates the antibody formation is currently unclear. It has been hypothesized that a pro-inflammatory state preceding the therapeutic transfusion may be a predisposing factor. Plasma levels of ten cytokines were evaluated upon recruitment to the study of 83 children with SCD undergoing therapeutic RBC transfusions. The levels of cytokines were correlated with development of anti-RBC antibodies prior, or during seven years post recruitment. Twelve subjects displayed significantly higher levels of all cytokines examined, with pro-, as well as anti-inflammatory properties. Surprisingly, the elevated levels of cytokines were preferentially found in patients without anti-RBC allo- and/or auto-antibodies. Further, presence of high cytokine levels was not predictive of anti-RBC antibody development during the subsequent seven year follow up. These data suggest that the increased concentration of multiple cytokines is not a biomarker of either the presence of or susceptibility to the development of RBC alloimmunization.

1. Introduction

Red blood cell (RBC) transfusions are used to both treat and prevent the complications of SCD. Standard practice is that donors and recipients are matched for AB0 groups and RhD type. However, there are over 300 blood groups on the RBC surface and it is not surprising that SCD patients develop alloimmunization to RBC antigens [1]. Anti-RBC antibodies (both allo- and auto-antibodies) are detected in 18–47% of SCD patients [2–6] as compared to 2.6–5% in patients who receive transfusion for treatment of other diseases [4,7]. One important factor in alloimmunization is antigen disparity between blood donor and recipient populations, especially pronounced in SCD patients, as recipients are mostly African American whereas donors are mostly Caucasian [8]. Alloimmunization rates remain high (5–11%) even when RBCs are phenotypically matched for C/c, E/e, and K/k [9–12]. While discrepancies in RBC antigens are important, additional immunologic determinants must be considered. Identifying those factors that contribute to alloimmunization could significantly improve the management of transfusion, and also inform about the regulation of immune responses.

The immune system can ignore, develop tolerance or respond to an antigen, depending on how the antigens are presented to the immune system. The innate immune system is designed to recognize pathogen-associated molecular patterns and in so doing, discriminate between self and infectious non-self [13]. Blood and blood products in general do not readily display ligands that activate the innate immune system. However, pre-existing conditions unrelated to transfusion may substitute for the absence of costimulatory signals. Several studies have documented increased inflammation and activation of innate immunity in SCD patients [14–20]. However, no consistent pattern of cytokine expression

Abbreviations: RBC, red blood cells; SCD, sickle cell disease.

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has been identified. For example, both elevated [21,22] and normal [23–25] plasma levels of the proinflammatory cytokine TNFα have been reported. Other studies suggested reduced production of IFNγ [26], or high circulating levels of Th2-type cytokines in the symptom-free state [15,17]. The role of inflammation has also been strongly suggested in an animal model of alloimmunization [27]. However, some additional factors likely play a role in this model, as distinct effects were achieved by inflammation induced by two different Toll Like Receptor ligands [28].

We here show that high levels of a number of different circulating cytokines can be found in a subset of SCD patients irrespective of the presence of anti-RBC alloantibodies. The seven years follow-up of these patients suggests that the increased concentration of multiple cytokines is not a biomarker of either the presence of or susceptibility to the development of RBC alloimmunization.

2. Materials and methods

2.1. Study population

In accordance with the Declaration of Helsinki, patients were enrolled after informed consent under an Institutional Review Board approved protocol at Children's National Medical Center to review medical records and retain patient samples to study alloimmunization. Patients' medical records were reviewed from birth to July 2013. At time of consent, blood samples were collected from 83 patients homozygous for hemoglobin S of both genders regardless of age who had received more than 2 Units of RBC transfusions in their lifetime.

2.2. Transfusion protocol

Patients have received the 3rd generation leukodepleted RBC transfusions according to the standard protocol at Children's National Medical Center [29]. Patients had a complete RBC phenotype performed using both serological and molecular methods. Data were stored in a computerized database. Patients not on specific research protocols received cross-match compatible ABO and Rh matched RBCs. After the development of a first clinically significant RBC alloantibody, subsequent RBCs were additionally matched for C/c, E/e and K/k. After the development of a second clinically significant antibody, full phenotypically matched units were provided. Three patients in this cohort had anti-RBC alloantibodies identified prior being placed on this matching protocol. These patients were excluded from some of the analyses, as indicated, because their full transfusion history was not available. Demographic characteristics of the patient population are shown in Table 1.

2.3. Sample acquisition and plasma preparation

All patients are tested for the presence of new or additional allo/auto antibodies 72 h prior to the RBC transfusions. The lag time between the last transfusion and time of specimen collection was at least 4 weeks. Five milliliter (mL) of peripheral blood from each patient was collected in EDTA anticoagulant tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma was isolated immediately following the blood withdral by centrifugation for 10 min at 1000 rpm at 22 °C. Plasma was immediately divided into 100 μl aliquots and stored into 0.5 ml Nunc cryoTubes (Sigma–Aldrich Co. MO) at −80 °C. Each aliquot was thawed only once for the cytokine analysis.

2.4. Multiplex analysis

An aliquot of the plasma was thawed and tested in duplicates for the canonical Th1/Th2 and inflammatorycytokine/chemokines [Interleukin(IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10], IFNγ, tumor necrosis factor-α (TNFα), granulocyte macrophage colony-stimulating factor (GM-CSF), using Human Cytokine Multi-plex Panel (Life Technologies, San Diego, CA) following manufacturers' instructions. All patients, regardless of their clinical and transfusion outcome were randomly distributed on Cytokine Multi-plex plates. Data were analyzed using Bio-Plex Manager 3.0 software (Bio-Rad, Hercules, CA). In brief, the assay is based on conventional sandwich assay technology. The antibody specific to each cytokine is covalently coupled to Biosource (San Diego, CA) microspheres, with each antibody coupled to a different microsphere uniquely labeled with a fluorescent dye. The microspheres are incubated with standards, controls, and samples (50 μl) in a 96-well microtiter filter plate for 2 h at room temperature. The plate is then washed to remove excess reagents, and biotin-labeled detection antibody. After a 2-h incubation at room temperature, streptavidin–RPE conjugate is added for an additional 30 min. After a final wash step, the beads are resuspended in a buffer and analyzed by the Bioplex Instrument (Bio-Rad, Hercules, CA) to determine the concentration of the cytokines of interest. All specimens were tested in triplicates wells. Results were reported as the mean of the triplicates. Data analysis was performed using Bioplex Manager software v 2.0. A five parameter logistic curve fit was applied to each standard curve and sample concentrations were interpolated from the standard curve. These results concluded overall coefficients-of-variation (CVs) values for the various analytes of <20%. Results were expressed as average of two replicates.

2.5. Transfusion history data review

The 7-year RBC transfusion history from January 2006 to December 2012 was extracted from the Children's National Blood Bank database using Sunquest software (Sunquest Information Systems, Tuscon, AZ). The transfusion history data includes the total number of transfused RBC units, number and specificity of RBC alloantibodies, the date of first and subsequent alloantibody formation, and the number of transfused RBC units from the first alloantibody to each subsequent alloantibody along with RBC attributes.

2.6. Medical chart review

Medical history of all patients 90 days prior to specimen acquisition for evidence of inflammatory conditions warranting inpatient or ER admissions was abstracted from the hospital

Table 1
Demographic characteristics of SCD patient population.

Anti-RBC antibodies	Total	Females	Males	Chronic transfusion[a]	Age range (median)
Yes	28	12	16	11	8–21 (14)
No	55	25	30	14	3–22 (14)
Total	83	37	46	25	1–22 (14)

[a] Chronic transfusion is indicated for the prevention of primary and secondary stroke, acute cerebrovascular accidents and severe acute chest syndrome. Patients receive RBC transfusions (simple transfusion, partial exchange transfusion or erythrocytapheresis) every 4–5 weeks, depending on quantitative hemoglobin S concentration.