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Research Article

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Immune Status and Red Cell alloimmunization among SCD Patients in Côte d'Ivoire

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Abstract

Sickle cell disease is the most popular genetic disease in the world. In Côte d'Ivoire, the SCD prevalence is 12%. As such, SCD is a public health problem. Few studies are really investigating the relative levels of Th1, Th2 and Th17 types in black Africans and in the occurring of alloimmunization.

This works attempts to identify the cytokine pattern produced by these patients during the course of the disease as Th1 cytokines, Th2 cytokines and Th17 and the relationship with alloimmunization.

***Patients and Methods:** This is a prospective study followed at the National blood Transfusion Center in Abidjan, Côte d'Ivoire. Cytokines were measured by using Bio Legend's LEGEND plex™ Human Inflammation Panel assays. We used Grifols DG gel® system to perform the immune hematology tests.*

***Results:** We recruit 50 patients with a diagnosis of sickle cell disease (SCD). Of these, only 31 have benefited from the research of irregular agglutinins (62%), comprising 14males (45.16%) and 17 females (54.84 %). The overall alloimmunization prevalence of 16.12%. The prevalence of alloimmunization was significantly greater in males than in females (60%). According to alloimmunization, we note an increased levels of IL-10 in non alloimmunized patients with SCD, when compared with alloimmunized and the levels of IL-4 was higher in alloimmunized patients compared to non alloimmunized. Lower IFN-γ levels were detected in non alloimmunized and alloimmunized SCD patients. Concerning IL-17, there was a small increase in patients without alloantibodies compared to those with. However no significant differences were noted in the 2 groups.*

***Conclusion:** The impact of biomarkers in the occurrence of alloimmunization is a constant preoccupation of researchers. Due to lack of financial aspect, small number of patients was enrolled. Even, we cannot draw any definitive conclusion; however our study brings data regarding the functioning of the immune system in SCD giving valuable insight.*

Keywords: Th1, Th2, Th17, Sickle Cell Disease, Alloantibodies, Cytokines

Abbreviations

IL: Interleukin
Th: T helper
SCD: Sickle cell disease
HO-1: Heme oxygenase
INFγ: Interferon gamma

Introduction

Sickle cell disease is the most popular genetic disease in the world and reaches its highest prevalence in countries around the equator and the prevalence is between 20 to 30% [1,2]. In Côte d'Ivoire, the SCD prevalence is 12% [3]. As such, SCD is a public health problem. Moreover, SCD is a chronic inflammatory disease exhibiting increased levels of proinflammatory cytokines with chronic hemolysis, recurrent clinical and subclinical occlusion, organ damage and high mortality [4,5].

Two clinical status are well known, sickle cell steady state and sickle cell crisis. The steady state is characterized by a situation which can degenerate at any time into crisis e.g the vasoocclusive crisis when triggers factors are present [6]. The mechanisms is complex and multifaceted and involved factor such as cytokines [6,7].

Many evidences show that type I, type II and type 17 are significantly altered in SCD patients. Type 1(Th1) cells typically produce IL-2, TNF β, IFNα, while type 2(Th2) cells produce IL-4, IL-5, IL-6, IL-9 and IL-10 and Th17, IL-17. A predominance in Th1 response

results in a vigorous mediated immunity while a predominant Th2 results in a stronger humoral immunity [8].

While there are published reports of individual cytokines, few studies are really investigating the relative levels of Th1, Th2 and Th17 types in black Africans and in the occurring of alloimmunization.

This works attempts to identify the cytokine pattern produced by these patients during the course of the disease as Th1 cytokines, Th2 cytokines and Th17 and the relationship with alloimmunization contributing therefore to identify triggers that can explain mechanism of alloimmunization in sickle cell patients.

Patients and Methods

Study population

This is a prospective study with 50 SCD patients from 4 to 55 years old followed at the transfusion therapeutic unit located in National blood Transfusion Center in Abidjan, Côte d'Ivoire. It was conducted from October 2016 to February 2017 after approval from the national ethics board. Informed consent was obtained from the patients' parents and each subject was prospectively enrolled in the study.

Patients were assigned in two groups: steady state group and crisis group. The steady state was defined by no acute illness, no crisis or infection during the last three months and the crisis group was patients admitted for vasoocclusion with infection or anemia.

Documentation of homozygous or heterogynous sickle cell patients had been determined by hemoglobin electrophoresis on cellulose acetate strips (PH 9.2).

The vaso-occlusive crisis (VOC) patients were admitted to the unit and those who were non-symptomatic, steady state sickle cell patients coming at the unit for routine controls. Vaso-occlusive (VOC) was defined as an episode of diffuse acute pain and clinical investigations were assesses by hematologist. Steady state SCD patients were matched with crisis SCD patients with chronic transfusion.

All subjects were coming most from Côte d'Ivoire and West African countries (Nigeria, Togo, Ghana, Mali, and Guinea).

Cytokines assays

Blood sample were collected by veinopuncture in EDTA for the determination of the basic hematological indices and for the cytokine assay. Complete blood counts were obtained with an electronic cell counter (Sysmex XN 550 Hematology analyzer). The plasma was separated from the tube sample at 1,000g at 4°C for 10 min, aliquoted and stored at -30°C for cytokine assays. Prior to use, the samples were thawed completely, mixed and centrifuged. Serums were measured by using Bio Legend's LEGEND plex™ Human Inflammation Panel assays which is bead-based immunoassays, using fluores¬cence–encoded beads and The Bio Legend LEGEND MAX™ Human IL-4 ELISA Kit. These panels allow simultaneous quantification of many human inflammatory cytokines and che¬mokines, but we had focused on Th1 cytokines (IFN γ), Th2 cytokines (IL-4, IL-10) and Th17 (IL-17A). The assay was performing using ELISA for IL-4 and a filter plate for the others cytokines in Bio legend laboratory in San Diego, US. A minimum of 3000 positive beads for these cytokines was acquired with a cytometer type BD FACS Calibur™. The Bio Legend LEGEND MAX™ Human IL-4 ELISA Kit, Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a capture antibody was used to detect IL-4. All samples were run and analyzed on the same day after being thawed completely. Manufactured supplied controls were used to monitor coefficients of variation, which were <10%. The minimum detectable concentration for Th1, Th2 and Th17 cytokines were respectively IFN γ: 1.5pg/mL, IL-1β: 0.9pg/mL, TNF α: 1.0 pg/mL, IL-12 p70: 0.6pg/mL, IL-4: 0.6 pg/mL, IL-6: 1pg/mL, IL-10: 0.8pg/mL, IL-17 A: 1.9pg/mL. Data analysis was done using LEGEND plex™ Data Analysis Software when data acquisition is completed.

Alloantibodies screening and identification

We used Grifols DG gel® system with 8 columns was used to perform the immunohematology tests. The plasma was screened first for the presence of alloantibodies with a standardcommercial 3RBCs group O from Diagast®. When it's positive, the antibodies identification was done using 12cells extended commercial (Diagast®) panel but due to lack of reagents this step was not process systematically.

Statistical analysis

All results are expressed as mean+/- SD. Data were analyzed using the SPSS, version 22.0. Statistical significance was calculated using Student's unpaired t test, the Mann-Witney U test, Chi-square and Fisher's exact test. Statistical test results with a P value ≤0.05 were considered to be significant.

Results

Demographic characteristics in the sickle cell patients

General characteristic between alloimmunized and nonalloimmunized are given in table1. We recruit 50 patients with a diagnosis of sickle cell disease (SCD). Of these, only 31 have benefited from the research of irregular agglutinins (62%), comprising 14males (45.16%) and 17 females (54.84 %).

Table 1: Demographics characteristics of SCD alloimmunized patients versus non alloimmunized in Côte d'Ivoire

	Alloimmunized	Non alloimmunized	p value
Sex ratio(Female/Male)	0,67	1,36	
Age (years) mean± SD	30,20 ± 14,39(19-55)	33,56 ± 10,33(7-50)	0,3
Number of transfusion(mean± SD)	3,33 ± 2,51	6,23± 5,18	0,3
Number of crisis(mean± SD)	3,33 ±2,51	3,18 ±2,80	0,8
Chronic transfusion(%)	40	60	0,3
Crises(%)	80	52	
ABO blood group (%)			
A+	50	23,08	

B+	0	15,38	
O+	50	50,00	
AB+	0	3,85	
O-	0	7,69	
RH Phenotype (%)			
ccdeek		8,00	
ccDeek	100	56,00	
CcDeek		20,00	
ccDEek		12,00	
ccDeeK+		4,00	
BMI(mean± SD)	19,94± 3,61	20,96± 3,98	0,5
Hemoglobin g/dl (mean± SD)	8,87± 1,89	8,19± 2,26	0,5
WBC count (mean± SD)	9375± 2672,46	11736± 4703,58	
Hemoglobine type(%)			
SSFA2	100	53,85	
SFA2	0	26,92	
SC	0	15,38	
AFA2	0	3,85	
Antibodies specificities	antiC+antiE, anti Lea		

Five patients had alloantibodies with an overall alloimmunization prevalence of 16.12%. Among our patients, 40 % was alloimmunized, while 60% was men. The prevalence of alloimmunization was significantly greater in males than in females. Patients ranged in age from 4 to 55 years old. The mean age of alloimmunized patients was 30.20 ±15.54 years (min/max= 19/50 years), while that of non alloimmunized patients was 24.92 ± 14.06 (min/max= 7/50 years). There was no significant difference in the age range between the two groups (p value= 0.4). The mean number of transfusion of red cells bags per year was 3.33 ± 2.51 in alloimmunized SCD patients versus 6.23±5.18 for non alloimmunized.

Regarding hemoglobin type, in alloimmunized 5 were HbSS homozygous type (100%). In non alloimmunized, 14 were HbSS homozygous type (53.85%), 7 Sβ+ Thal heterozygous type (26.92%), 4 HbSC (15.38%) and 1 βThal homozygous type (3.85%).

Sickle cell disease was associated with lower level of hemoglobin in steady state and crisis group (respectively 8.87 ± 1.89 and 8.19 ± 2.26 g/l). 80% of alloimmunized patients were in crisis compared to 52% in the non alloimmunized group.

Chronic transfusion was done in 40% of patients with alloantibodies versus 60% in non alloimmunized patients. Difference was not significative.

Link between transfusion and studied variables

Mean age, sex distribution, hemoglobin type and level, WBC count and alloimmunization rate according to number of blood transfusion is shown in table 2.

Table 2: Number of Transfusion according to sex, age, hemoglobin type hemoglobin level, white blood cells and presence or absence of alloantibodies

	Sex		Age (Years)	hemoglobin type				hemoglobin level(g/dl)	WBC	alloimmunization		Chronic transfusion
Number of Transfusion	**Female**	**Male**	**mean± SD**	**SSFA2**	**SFA2**	**SC**	**AFA2**	**mean± SD**	**mean± SD**	**Yes**	**No**	**%**
0	1(6,67%)	3(20%)	20,75 ± 7,63	25%	0	50%	25%	8,3 ± 1,37	10133,33 ± 645,91	0	1(4,76%)	0
1-5	6(40%)	7(46,67%)	20,61 ± 13,75	61,54%	30,77%	7,69%	0	8,6 ± 1,31	8841 ± 199,45	2(40%)	9(42,86%)	0
5-10	4(26,67%)	3(20%)	24,28 ± 8,71	42,86%	28,57%	14,29%	14,29%	7,95 ± 2,8	13600 ± 5973,55	1(20%)	5(23,81%)	44,44
> 10	4(26,67%)	2(13,33%)	21,33 ± 14,34	66,67%	33,33%	0	0	6,66 ± 1,42	14783,33 ± 4753,70	2(40%)	6(28,57%)	55,56

Hemoglobin level was lower in patients receiving more than 10 RBC blood bag and WBC count greater in patients receiving more than 5 blood bags.

The prevalence of patients receiving blood was greater in females than in males. 53.34% women receiving more than 5 RBC bags versus 33.33% of men (table 2).

The proportion of patients who had been transfused was significantly smaller in the group of patients with a diagnosis of Hb SC or AFA2 than in the group of HbSS or SSFA 2. 40 % of patients were alloimmunized with 1 to 5 transfusions while 42.86% are not alloimmunized even when receiving 1 to 5 transfusions.

Plasma levels of cytokines

The median concentrations of cytokines measured are shown in table 3. The data show the levels of IFN-γ, IL-4, IL-10 and IL-17A in the sera of patients.

According to the number of transfusion received, the differences were not significative.

Increased levels of IFNγ, IL-17 and IL-10 were found in patients without transfusion. IL-4 is lower when the patient is not transfused. (Table3)

We calculated the IFN-γ to IL-4 ratio in view of the fact that the ratios of Th1 to Th2 cytokines are more relevant and pertinent than the levels of these cytokines alone. The IFN-γ to IL-4 ratio is 6.80, 0.89, 3.62, 2.88 respectively for those receiving none transfusion, 1 to 5 transfusion, 5 to 10 transfusion and more than ten transfusion.

According to alloimmunization, we note an increased levels of IL-10 in nonalloimmunized patients with SCD, when compared with alloimmunized patients (table 3), and levels of IL-4 was higher in alloimmunized patients compared to non alloimmunized. Lower IFN-γ levels were detected in non alloimmunized and alloimmunized SCD patients. Concerning Th17, there was a little increase of Il-17 in patients without alloantibodies compared to those with. However no significant differences were noted in the 2 groups. The IFN-γ to IL-4 ratio is 0.70 in alloimmunized subjects and 2.7 in non alloimmunized subjects indicating a trend toward a Th2 bias in alloimmunized patients (Table 3).

Table 3 : IL-4, INFγ, IL-10 and IL-17 A according to number of transfusion and presence of alloantibodies in SCD patients

Number of transfusion					alloimmunization	
Cytokines	**None**	**1-5**	**5-10**	**>10**	**presence of alloantibodies**	**No alloantibodies detected**
INFγ	12,11 ± 12,64	4,12 ± 4,17	6,85 ± 07,42	11,38 ± 13,39	5,7± 18,9(0-21,31)	7,02 ± 9,02(0-31,67)
IL-4	1,78 ± 0,51	4,6 ± 8,35	1,89 ± 0,31	3,95 ± 4,54	8,12 ± 13,53(1,4-13,2)	2,6 ± 2,25(1,3-32,31)
INFγ/IL-4	6,80	0,89	3,62	2,88	0,70	2,7
Il-17A	24,60 ± 10,58	8,52 ± 10,31	14,81 ± 10,77	4,33± 2,82	7,04± 8,21(0-20,37)	8,74± 11,15(0-38,93)
IL-10	30,55 ± 43,72	6,13 ± 9,68	17,17 ± 19,88	10,50± 21,53	6,80± 7,26(1,75-19,23)	10,86± 15,93(0-57,85)
IL-17A/IL-10	0,81	1,39	0,86	0,41	1,04	0,80

Discussion

In SCD, transfusion of red blood cells is a therapeutic that cannot be ignored in the management of patients with sickle cell disease (SCD).Despite the benefit of the transfusion, alloimmunization remain a major factor responsible for morbidity and mortality in most African countries. Unfortunately, there are poor and sometimes no alloimmunization prevention program following potentially sensitizing events. History of records [9]. In our study, only 62% of the SCD have benefited from the research of irregular agglutinins.

Five patients have alloantibodies with an overall alloimmunization prevalence of 16.12%. This is lower to the prevalence in others studies done in Ivory Coast found by Akré (62.8 % in 2008) and similar to the prevalence found by Kaboré and Sekongo respectively in SCD patients (15.2%,28.2%) This variation may be due first to the implementation of Rh Kell and extended phenotyping, second to the antibodies screening in polytransfused patients and third to the improvement of technology as mentioned by Kanigawa in Nigeria [10-13].The rate of alloimmunization is very variable and several factors are implicated (genetic background, inflammatory status, number of blood units transfused etc…) and results are conflicting.

With the regard to the influence of sex on antibody formation, numerous studies have reported a high risk for women because of their specific acting immune system, because they are more often in contact with foreign antigens through pregnancy and transfusion [14,15]. Regarding history of pregnancy among the alloimmunized patients, 2 were female without gesture. Our results did not demonstrate a higher immunization rates in females due to the small number of patients. Thus, males have the highest rate with 60%, this trend was also observed by Hussein in Egypt [16].

What triggers the alloantibodies formation is still a great interest because it's not currently not clear [17]. Several factors are known including at least four main contributing followed points: the number of transfusion received the differences in red blood cell antigens between the donor and the recipients, the recipient's immune status and the immunomodulatory effect of the blood transfusion [18].

Some individuals will not become immunized to any antigens despite multiple red cells transfusion. The number of transfusions however, seems most likely to have a major influence [14,17,18]. In our study, others factors seem to be implicated and the number of transfusion is not significative, may be due to the small number of studied patients [19]. Indeed patient receiving an average of 6.23± 5.18 bags per years are not immunized compared to those receiving less (table 1).Another point is the type of blood products received by the patients. It can also affect the rate of alloimmunization. For example leukoreduction of red blood cells decreases the occurrence of alloimmunization [20]. Unfortunately, in our study we don't have records on the type of blood but we can say that leukoreduced product are not used frequently and most of the patients still receiving non

leukoreduced products (*Rapport d'activités 2016 CNTS*)

Considering the recipient's immune system status, identification of biomarkers such as cytokines is therefore a great interest and can help identify in patients most likely to make antibodies after a blood transfusion [21].

Indeed, even a subcutaneous injection of foreign protein, which is a highly immunogenic route of administration, is not immunogenic in the absence of inflammation [22]. Consistent with this, rates of alloimmunization is strongly in relation with clinical history of patients who are receiving transfusions, more specially when there is inflammation [20]. Many evidences show that sickle cell disease is a chronic inflammatory disease. The patients with sickle cell anemia have high inflammation level and innate immune system activated. This led the authors hypothesis that the recipient inflammation regulate alloimmunization [20].

This inflammation may also come from blood product when there is a contamination or due to cytokines produced during storage of blood products [20]. Our study wants to give his contribution on how the immune status through INF γ, IL-4, IL-10 and IL-17 influence the occurrence of red blood cells alloimmunization among SCD patients in Côte d'Ivoire.

Classically, Th1 are for cellular immunity characterized by INF γ production, while Th2 cells with IL-4 regulate humoral immunity [23].The recently identified Th17 cells, are defined by the expression of IL-17 acting as an inflammation mediator [24]. We compared cytokine levels between patients who had red blood cell antibodies and those who were non alloimmunized. Patients with no detected alloantibodies had higher expression of IL10 when compared with alloimmunized patients. These results were consistent with Bao' study who find increased rate of IL-10 in non alloimmunized patients with SCD.

On the other hand, lower levels of IFNγ are detected either in non alloimmunized and alloimmunized SCD patients. This may be due to the anti- inflammatory activity of IL-10. Indeed, IL-10 is one of the most important cytokine with anti- inflammatory properties, act as a Th2 cytokine inhibiting IFN-γ production of T cells, with stimulatory effects on B cell differentiation and antibody production and antigen presentation suggesting, therefore IL-10 may play a role in disturbing antigen presentation and T cell activation preventing thus, production of alloantibodies [25]. A new approach based on genetic polymorphisms of this cytokine has been studied and suggest a possible role of IL-10 as an inflammatory marker, therefore having the capability to stimulate the occurrence of [22].

IL-4 was higher in alloimmunized patients and lower in non alloimmunized (table 3). Th1 (IFNγ)/ Th2 (IL-4) balance show a Th1 trend when none transfusion. The ratio change then, indicating a Th2 response with 1-5 transfusions and a Th1 bias for more than five transfusions (table 3). This variability doesn't allow us to identify any definitive Th trend andmay be due to the small number of our patients. Increased frequency of IL-4 expressing CD4+ cells, in a subset of alloimmunized patients may be consistent with skewed Th2 humoral responses in this subgroup [26].

Considering alloimmunization in our study (table 3), no significant differences in IL-17 levels were noted in the 2 groups, however the high levels in the 2 groups suggests an underlying inflammatory process [24]. IL-10 and Il-17 have opposite immune activities. IL-17 is pro inflammatory whereas IL-10 has anti-inflammatory and immune suppressive [23]. In non alloimmunized patients, the immune suppressive activity of IL-(10) is higher and there is enhanced IL17 activity resulting in a small imbalance of the IL-17 /IL-10 (27) (Table3). This small imbalance may be due to many others factors. For example, it is possible that geographical aspects in lymphocytes function and environmental exposures in sub-Saharan Africa, including malaria and other infections may impact the functioning of the immune system [26]. During the vaso occlusive crisis with hemolysis, the heme is free circulating in the peripheral blood. The function of HO-1 oxygenase is to remove the heme. If this enzyme is low, the heme removal following RBC transfusion is ineffective, resulting in a pro-inflammatory state and increasing risk of alloimmunization [19].

Our study has some limits. First the small number of patients, second the alloantibodies were already present at the recruitment step and the medical record and particularly the transfusion history was not always available. It is necessary to conduct a real prospective study that could strongly identify biomarkers and phenotype patients to improve the management of the SCD patients and reduce morbidity and mortality in our areas.

Conclusion

The impact of biomarkers in the occurrence of alloimmunization is a constant preoccupation of researchers; many studies in vitro have highlighted some factors involved in the occurrence of alloimmunization.Due to lack of financial aspect, small number of patients was enrolled. Even, we cannot draw any definitive conclusion;our study brings data regarding the functioning of the immune system in SCD giving valuable insight of what happens on the immune versant in these patients. The challenge in the future for a better care of SCD patients is important.These informations need to be checked in larger cohorts to reach these biomarkers and improve comprehension of the risk of alloimmunization in transfused SCD patients.

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