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# Antiphospholipid antibodies in 100 homozygous sickle cell patients (SSFA2) in Abidjan, Côte d'Ivoire.

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***Abstract :*** Structural changes in sickle red cell membranes appear to be the major factor for inducing increased levels for antiphospholipid antibodies. The aim of the study was to identify antiphospholipid antibodies in homozygous sickle cell patients and to investigate the relationship with the clinical status. Antiphospholipid antibodies were investigated in 100 homozygous sickle cell patients and in 50 healthy subjects. Lupus anticoagulants were detected by clotting tests. Total antiphospholipid antibodies including anticardiolipin were determined by a semi quantitative enzyme-linked immunosorbent assay. Positive patients and the controls were analysed for antibodies to deoxyribonucleic acid and rheumatoid factors with a slide latex agglutination test. We did not find antiphospholipid antibodies in the controls. The lupus anticoagulants and total antiphospholipid antibodies frequencies were respectively 9 and 44%. One patient was positive for both. We did not find anti deoxyribonucleic acid antibodies in antiphospholipid antibodies positive patients. None of the lupus anticoagulants patients was positive for rheumatoid factors. In 9/44 (20.4%) total antiphospholipid antibodies patients, rheumatoid factors were positive with a mean value of 40 UI/ml.
Antiphospholipid antibodies were not related to the clinical status at the time samples were drawn and may be a part of a wider autoimmune disorder.

***Key words :*** homozygous sickle cell disease, antiphospholipid antibodies, lupus anticoagulants, Abidjan

## Introduction

Antiphospholipid antibodies (APA) are a wide group of immunoglobulins (Ig), among which lupus anticoagulants (LA) and anticardiolipin antibodies (aCL) are the best known and characterized (1). The main antigens are β2-glycoprotein I and prothrombin. LA appear to be directed against β2 glycoprotein I or prothrombin (2). It was reported that aCL bind to β2-glycoprotein I (2). These antibodies are considered risk factors for arterial and venous thrombosis. APL do probably not cause thrombosis by themselves but rather influence the thrombotic process once negatively charged phospholipid becomes exposed (2).
APA were first described in patients with systemic lupus erythematosus. APA can occur in haemolytic disorders such as sickle cell disease (SCD). Repeated sickling produce a disruption and rearrangement of red cells membranes. The exposure of negatively charged phospholipids may result in the induction of antibodies against these cell membrane constituents (3).
Therefore we screened a large number of patients with SCD and control subjects with normal haemoglobin (Hb) AA for the presence of APA. The aim of the study was to identify APA in patients with SCD and to investigate the relationship between APA and the clinical status : steady states or painful vaso-occlusive crises.

## Patients, material and methods

The studied population consisted of one hundred Ivorian patients with homozygous SCD ($SSFA_2$). 50 were in steady state and 50 in painful vaso-occlusive crises. 54 were men and 46 women (sex ratio : 1.17). The median age was 16 (range 1-39 years) (Table I). All the patients were regularly attended by the haematological department of the Teaching Hospital of Yopougon, Abidjan.

No transfusion was given in the three months before the beginning of the study.

The control group consisted of 50 healthy subjects with normal AA Hb. 26 were men and 24 women (sex ratio : 1.08). The median age was 14 (3-30). They have the same repartition as the patients with SCD for sex and age ($p>0.5$) (Table I).

Patients and control group gave their oral agreement for their participation in this study.

Blood was drawn from patients and controls both into dry tubes, into 0.109 M trisodium citrate (1 volume for 9 blood volumes) and into EDTA. Serum and platelet- poor plasma samples were obtained by two cycles of centrifugation at 2.400g for 20 min. Serum and plasma samples were frozen in small aliquots and stored at - 80°C until use.

### Lupus anticoagulants (LA) tests (2, 4)

LA was diagnosed according to the revised criteria proposed by the Subcommittee for Standardization of lupus Anticoagulants / phospholipid-dependent Antibodies (1,4). LA were detected by mixing procedures which are based on the activated partial thromboplastin time (APTT) determination of a mixture of one part of the patient's plasma and one part of normal plasma. Results were expressed using the numerical Rosner Index calculated according to the following formula : 100 x (mixture clotting time - normal plasma clotting time) / patient's clotting time. A value of ≥ 15 being abnormal. Thrombin time (TT) was measured in order to exclude other causes of the prolonged APTT before going on to other LA tests.

The tissue thromboplastin inhibition (TTI) assay was performed using a 1:500 dilution of the thromboplastin reagent (Neoplastine, Diagnostica Stago, Asnières, France) in a solution of 25 mM $CaCl_2$. The test was run as a single reagent system using the prothrombin time mode. Results were expressed as the ratio of the patient to control clotting times, with a value of ≥ 1.2 being abnormal.

The phospholipid neutralization test was carried out using the Staclot LA test procedure (Diagnostica Stago, Asnières, France) which uses phosphatidylethanolamine in hexagonal phase as a source of phospholipids for neutralizing LA activity and APTT reagent sensitive to LA. Results were expressed as the decrease of the clotting time of the plasma containing phosphatidylethanolamine as compared with that of plasma without phosphatidylethanolamine. A decrease of the clotting time ≥ 8 s being considered corrected.

### ELISA test

APA directed against both cardiolipin, phosphatidic acid and phosphatidylserine were measured by a semi quantitative enzyme-linked immunosorbent assay (ELISA), using Asserachrom APA Kit (Diagnostica Stago, Asnières, France). According to the kit, values exceeding 15 UI / ml were regarded as positive.

Only positive patients and controls were analysed for antibodies to native desoxyribonucleic acid (DNA) and rheumatoid factors (RF) with a qualitative and semi-quantitative slide latex agglutination test (Visualine ADNn and Visualine Waaler Rose, Diagnosphere bioControl, Courbevoie, France). The presence of visible agglutination indicates antibodies anti DNA concentration equal or higher than 40 UI/ml. According to the kit, the lack of agglutination indicates a RF level lower than 6 UI/ml in the sample. Results above 20 UI/ml were considered positive.

### Statistical analyses

All the studied parameters were expressed as the arithmetical mean and standard error of the mean (m ± SD). Comparisons between groups were analyzed by chi squared test ; p values less than 0.05 were considered statistically significant.

## Results

Patients' characteristics are shown in Table I.

Homozygous $SSFA_2$ patients' profile in painful crisis and in steady state were similar. The degree of haemolytic anaemia was the same in the 2 groups. Mean cell volume and platelets counts were within the normal range. However, white blood cells were higher in painful crisis ($p=0.02$, Table I).

LA and ELISA tests were negative in the control group. LA and total APA frequencies were respectively 9 and 44% (Table II). The prevalence of total APA was 52% because one patient was positive for LA and ELISA tests (Table II). Semi quantitative ELISA test didn't allow the

**Table I : Characteristics of the homozygous $SSFA_2$ patients**

| | Painful crisis | Steady state | p |
|---|---|---|---|
| Age years | 15 (4 - 39) | 16 (1 - 36) | > 0.05 |
| Sex M/F | 28/22 | 26/24 | > 0.05 |
| Sex ratio | 1.27 | 1.08 | |
| S (%) | 85.82 ± 7.36 | 87.07 ± 6.57 | > 0.05 |
| F (%) | 12.28 ± 7.17 | 12.78 ± 6.75 | > 0.05 |
| $A_2$ (%) | 1.92 ±1.1 | 2.32 ± 1.53 | > 0.05 |
| Haemoglobin (g/l) | 8.35 ± 1.53 | 8.22 ± 1.07 | > 0.05 |
| Mean cell volume (fl) | 92.25 ± 13.03 | 88.35 ± 9.64 | > 0.05 |
| White cells (x $10^9$/l) | 20.4 ± 15 | 14.63 ± 12.22 | 0.02 |
| Platelets (x $10^9$/l) | 298.49 ± 133.46 | 389.52 ± 131.71 | 0.001 |

determination of the aCL isotypes.

APA were not related to the clinical status steady state or painful crisis at the time the samples were drawn (p > 0.05, Table II).

We did not find anti DNA antibodies in all APA positive patients. Of the 52 APA patients, 9 (17.3%) had increased values of RF (> 20 UI/ml). In these RF positive patients, LA were negative but total APA measured by semi quantitative ELISA method were increased. The frequency of RF in ELISA positive patients was 20.4% (9/44). RF were positive with a mean value of 40 (24-96) UI/ml. Six patients were in painful crises and 3 in steady states. Mean value of RF was 48 UI/ml in the SCD patients in painful crises and 24UI/ml for the $SSFA_2$ patients in steady states.

## Discussion

Some authors (4,5) have worked on APA in SCD.

However, these populations were heterogeneous with several variants of SCD like sickle cell thalassaemia (SFA (2) or SAFA(2), sickle cell trait (AS), sickle cell haemoglobin C (SC). Our study was confined only to homozygous SSFA (2) patients in painful crises or in steady states but with the same percentage of Hb S and foetal Hb (Hb F) (Table I). Polymerization of Hb S can be hindered by Hb F. High or low concentration of Hb F

**Table II : Frequency of APA in 100 homozygous $SSFA_2$ patients**

| | Control group | Patients | | | |
|---|---|---|---|---|---|
| | | Clinical status | | | p |
| | | Painful crisis | Steady state | Total | |
| LA | 0/50 (0%) | 4/50 (8%) | 5/50 (10%) | 9/100 (9%) | > 0.05 |
| ELISA | 0/50 (0%) | 27/50 (54%) | 17/50 (34%) | 44/100 (44%) | > 0.05 |
| Total | | 31/50 (62%) | 22/50 (44%) | 52/100 (52%) | |

In one patient, LA and ELISA tests were positive, so the prevalence of total APA was 52%.

p > 0.05 : there were no differences between patients in painful crisis or in steady state.

has a relationship with clinical severity of SCD (6).
The elevated white blood cells during painful crisis were surely related to infectious complications (Table I).
Infections are a major cause of morbidity or mortality in SCD. Even if thrombocytopenia is often associated with APA syndrome (1), we found that the platelets counts were normal like in other study (3). However platelets counts were lower in the painful crisis than in the steady state ($p = 0.001$, Table I). SCD is associated with inflammatory illness (6). In painful crisis, patients received anti-inflammatory treatment which could explain this result.
We did not find, according to De Ceulaer (3), APA in the 50 subjects of the control group. Our study showed that APA were frequently increased in SSFA (2) patients with a prevalence of 52% (Table II). De Ceulaer (3), Nsiri (4) and Kucuk (5) had respectively a prevalence of APA of 8%, 44.4% and 68%. The differences between all these frequencies could have a relationship, on the one hand with the nature of the variants of SCD in each population and, on the other hand, with the nature of the APA they screened : LA, aCL or total APA. Nsiri (4) investigated APA on 37 patients including 18 homozygous $SSFA_2$, 8 S/_ thalassemia and 11 sickle trait. APA were explored by LA and Ig G and Ig M aCL. Kucuk (5) studied 25 patients with SCD. Nineteen of the 25 patients had homozygous SCD, 3 had SC disease and 3 sickle thalassemia. He detected Ig G, Ig A and Ig M aCL. De Ceulaer 3 worked on 108 patients SSFA2 and screened the presence of Ig G aCL. The LA test was positive in 9/100 (9%) patients (Table II). Nsiri found that the frequency of LA was 62.2%. This difference may be related to the clotting tests.
Indeed, it is very hard to give precise recommendations on which assays to use for detection of LA because of the lack of a golden standard (a well-defined LA-positive patient population) (2).
Total APA including aCL were detected in 44/100 patients (Table II). This result was very similar to Nsiri (44.4%) (4). Asserachrom APA kit contains not only cardiolipin, but also other type of phospholipids such as phosphatidylserine, phosphatidylinositol, phosphatidic acid. This ELISA test didn't allow us to determine aCL (Ig G, Ig A, IgM) isotypes.
The cause of the increase of antibodies production against phospholipids in these patients is not known. A probable source for antibody generation is the sickle red cell, since the membrane of the red cell has increased hexagonal phase II content (7), which Rauch and Jan (8) have shown to be the critical factor for generation of APA.
APA formation can be induced in mice by phospholipids in a hexagonal phase II but not by phospholipids in a bilayer phase (5). Although structural changes in sickle red cell membranes appear to be the major factor for inducing increased levels of APA in patients with SCD, mechanisms of the antibodies formation against phospholipids are to date still not well established.
There was no relationship between the increase of the level of APA and the clinical status : steady states or painful vaso-occlusive crises ($p > 0.05$, Table II). It could be due to the timing of the APA measurements which are known to fluctuate (9). Membrane alterations on the sickle red cell induce phospholipids changes and the appearance of a procoagulant activity. All these factors are responsible for a procoagulant status in patients with SCD that may contribute to the vaso-occlusive process (10).
There is a growing consensus that LA is a stronger risk factor for thrombosis than aCL (1, 2). APA do probably not cause thrombosis by themselves but rather influence the thrombotic process once negatively charged phospholipids become exposed (2). There is indirect clinical support for the concept of a double-hit scenario: following mild endothelial damage, a small platelet thrombus develops (first hit) ; the slightly activated platelets expose negatively charged phospholipids ; this leads to patchy deposition of bivalent _2 glycoprotein I-antibody complexes; theses complexes cause further platelet activation and thrombus growth (second hit) (2).
Whatever the aetiopathogenesis of APA in SCD was, patients with APA did not show any more serious disease course contrary to patients without APA. This was in agreement with the observation that APA induced complications only at levels much higher than those observed in SCD (3). APA appear either in the context of a generalized immune deregulation as observed in, for example, systemic lupus

erythematosus (so called secondary APA syndrome) or without clear features of an associated immune disorder (so called primary APA syndrome) (1, 9). None of our patients was positive for anti-DNA antibodies (Table II). RF were present in 9 of the 52 (17,3%) APA positive patients (Table II). In these 9 patients, the production of APA may be a part of a wider autoimmune disorder, present in some patients with SCD (3-5, 10).

## Conclusion

APA were associated with SCD. Their identification is useful to recognize patients having a higher risk to develop arterial and venous thrombotic complications, but not to predict treatment outcome and disease prognosis.

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