

Frequency of specific coagulation inhibitors and antiphospholipid antibodies in Tunisian haemophiliacs

Fréquence des inhibiteurs spécifiques de la coagulation et des anticorps antiphospholipides chez les hémophiles tunisiens

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Abstract. Production of factor VIII or factor IX inhibitors is a major complication limiting the efficiency of substitutive therapy in haemophiliacs. Moreover, viral infections, the second serious complication of replacement therapy, may be associated to the occurrence of antiphospholipid antibodies which paradoxically lead to thrombosis. We investigated the prevalence of coagulation inhibitors (factor VIII and factor IX inhibitors, antiphospholipid antibodies) in Tunisian haemophiliacs, and we assessed concomitant coagulation factor deficiencies. Thirty-two previously treated haemophiliacs (20 haemophiliacs A; 12 haemophiliacs B) were screened for factor VIII and factor IX inhibitors by APTT mixing study, Bethesda test and modified Nijmegen method, and investigated for the presence of anticardiolipin, anti- β 2 glycoprotein I, lupus anticoagulant and associated coagulation factors deficiencies. The frequency of factor VIII and factor IX inhibitors was low (5%) in contrast to the high prevalence of antiphospholipid antibodies (28.1%). Four and nine patients were positive for anticardiolipin and anti- β 2 glycoprotein I, respectively. No lupus anticoagulant was detected. The prevalence of antiphospholipid antibodies was higher in patients with positive hepatitis C virus infection serology as compared to patients with negative serology (41.6% vs. 20%). Concomitant factor VII and/or factor V deficiency was found in 10 patients. In conclusion, the occurrence of factor VIII and factor IX inhibitors is rare among Tunisian haemophiliacs. The clinical relevance of antiphospholipid antibodies requires further investigations. We emphasize the importance of screening for concomitant deficiencies in haemophiliacs when the clinical presentation is suggestive.

Key words: *haemophilia, coagulation inhibitors, antiphospholipid antibodies, coagulation factor, Tunisia*

Résumé. Le traitement de l'hémophilie est à l'origine de deux complications majeures : l'apparition d'inhibiteurs anti-facteur VIII ou anti-facteur IX compromettant son efficacité et la transmission d'infections virales pouvant s'accompagner de l'apparition d'anticorps anti-phospholipides, lesquels sont paradoxalement associés aux thromboses. Nous avons étudié, dans une population d'hémophiles tunisiens, la prévalence des inhibiteurs pathologiques (anti-facteur VIII, anti-facteur IX, anti-phospholipides) et recherché d'autres déficits associés. Trente-deux hémophiles (20 hémophiles A et 12 hémophiles B) antérieurement traités ont été testés pour la recherche d'inhibiteurs anti-facteur VIII et anti-facteur IX par le temps de céphaline activé du mélange témoin-malade, le test Bethesda et la méthode de Nijmegen. Les patients ont également été testés pour la recherche d'anti-cardiolipine, d'anti- β 2-glycoprotéine I, de lupus anticoagulant, et d'un déficit associé de la coagulation. La prévalence

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des inhibiteurs anti-facteur VIII et anti-facteur IX est faible (5 %), alors que les anti-phospholipides sont fréquents (28,1 %). Quatre et neuf patients avaient respectivement des anticorps anti-cardiolipine et anti- β 2-glycoprotéine I. Aucun cas de lupus anticoagulant n'a été détecté. Les anti-phospholipides sont plus fréquents chez les patients séropositifs pour le virus de l'hépatite C que chez les patients séronégatifs (41,6 % vs 20 %). Des déficits associés en facteurs VII et/ou V ont été retrouvés chez 10 patients. En conclusion, les inhibiteurs anti-facteur VIII et anti-facteur IX sont rares chez les hémophiles tunisiens. La signification clinique des anti-phospholipides reste à préciser par d'autres études. Nous soulignons l'intérêt de rechercher des déficits associés chez les hémophilies devant un contexte clinique évocateur.

Mots clés : hémophilie, inhibiteur de la coagulation, anti-phospholipides, facteur de la coagulation, Tunisie

Haemophilia is a hereditary and severe coagulation disease due to deficiency in coagulation factor VIII (haemophilia A) or factor IX (haemophilia B). The advances in clotting factor replacement therapy have markedly improved the management of patients with haemophilia. However, multiple transfusions are responsible of major complications like immunization against factor VIII (FVIII) or factor IX (FIX) and viral pathogen transmission especially hepatitis B and C viruses (HBV and HCV, respectively) and human immunodeficiency virus (HIV).

The production of inhibitors represents a serious complication that compromises treatment efficacy and exposes haemophilia patients to a major bleeding. In predominantly white populations, the rate of inhibitors to FVIII ranges between 3.6 and 27% [1]. The transmission of hepatitis or HIV infection is a major co-morbid condition in haemophilia patients and a leading cause of death in this population [2, 3]. It is well established that these viral infections may be associated with the occurrence of antiphospholipid antibodies (APA) which are a heterogeneous family of auto-antibodies recognizing target phospholipid antigens such as cardiolipin or the so-called phospholipid cofactors' proteins like β 2-glycoprotein I [4]. These antibodies were described in autoimmune diseases, in neoplasia but also in infectious diseases and other disorders [4-6]. The laboratory assessment of these antibodies is based on two methods: coagulation assays that screen for lupus anticoagulant (LA) and immunological assays [6]. These antibodies act *in vitro* as coagulation inhibitors sometimes making difficult the detection of FVIII and FIX inhibitors, but they paradoxically cause thrombosis *in vivo*.

Although the other coagulation factor deficiencies have been rarely studied in haemophilia patients, an associated deficiency can interfere with screening for inhibitors and render it more difficult. In addition, such simultaneous co-inheritance of clotting factor deficiency limits the efficiency of specific factor concentrates use.

Epidemiology of transfusion transmitted infections and inhibitors' production is available from Western world, but they are nearly lacking from developing countries. Worldwide, the availability of safe factor concentrates for all haemophiliacs is a real gap. In Tunisia, about 310 haemophiliacs are recorded. Our therapeutic strategy is based on curative treatment of declared bleeding. The cryoprecipitate has long been the major replacement product in haemophiliacs A. Since 1980, imported factor concentrates have become available, thus our haemophiliacs have been exposed to the HIV epidemic in mid-80. Now, the tendency is to abandon cryoprecipitates' use and make specific factor concentrates available for all haemophiliacs by fractionation of our own plasma or commercial imported products.

The aim of this study was to determine the prevalence of coagulation inhibitors (FVIII and FIX inhibitors and APA) and to screen for other haemostatic abnormalities in Tunisian haemophilia patients.

Patients and methods

Patients

We studied 32 haemophiliacs (20 haemophiliacs A and 12 haemophiliacs B) among a total of 50 patients originating from the Sahel (center of Tunisia) and followed in Clinical Hematology Service at Farhat Hached Teaching Hospital - Sousse. Informed consent of the patients or their parents was obtained with approval of the local ethic committee.

All the patients received on-demand treatment. Haemophilia A patients have received cryoprecipitates. Eleven of them were also treated with plasma-derived FVIII concentrates. Haemophilia B patients were treated either with the prothrombinic complex (PPSB) or plasma-derived FIX concentrates. The demographic characteristics of the patients are summarized in *table 1*.

Table 1. Demographic, clinical and viral serology status of patients with haemophilia.

	Haemophilia A	Haemophilia B
n (%)	20 (62.5)	12 (37.5)
Age (years)		
Mean	22.3	17.6
Range	12-55	8-27
Severity of haemophilia	Severe: n = 16 Moderate: n = 4	Severe: n = 4 Moderate: n = 3 Mild: n = 5
Age at the start of supply therapy		
Mean	4.2 years	4.5 years
Range	1 month - 16 years	6 months – 16 years
Viral serology status		
HIV (+)	1/20	1/12
HCV (+)	9/20	3/12
HBs (+)	5/20	0

HIV (+): positive serology for HIV infection; HCV (+): positive serology for HCV infection; HBs (+): positive hepatitis B virus surface antigen. Severe: FVIII or FIX, < 1%, moderate: FVIII or FIX, 1-4%, mild: FVIII or FIX, 5-40%.

Samples were drawn at least one week after the last clotting factor infusion. Blood was collected by venipuncture in sodium citrate tubes. Plasma was prepared after two centrifugations at 2500 g for 15 min. Coagulation factors were measured on fresh plasma and the screening for inhibitors was done on aliquots of plasma frozen at -80°C. Antiphospholipid determination was done on serum frozen at -80°C.

Coagulation study

Factor VIII and FIX activities were measured by one-stage assays using, respectively, FVIII and FIX deficient plasmas (Diagnostica Stago, Paris, France). Prothrombin time (PT) and thrombin time (TT) were determined by standard techniques (Neoplastin CI plus, Thrombin; Diagnostica Stago). Factors II, V, VII and X were determined by one-stage assays using factor deficient plasmas (Diagnostica Stago). Fibrinogen was assessed by using the Clauss method (STA-fibrinogen, Diagnostica Stago) while the von Willebrand factor antigen was measured by an immunoturbidimetric method (Liatest FvW, Diagnostica Stago).

Screening for FVIII and FIX inhibitors

Screening and quantitative measurement of FVIII and FIX inhibitors were done according to the WHO recommendations [7-10]. Our screening strategy included two steps. First, FVIII and FIX inhibitor screening was based on activated partial thromboplastin time (APTT) which was determined on 1:1 mixture of patient plasma with normal pooled plasma (NPP). The APTT was determined with and without incubation at 37°C for 60 min to detect

immediate-acting inhibitors and time-dependent inhibitors, respectively. An inhibitor is suspected if the APTT of the mixture which was determined using four different reagents [CK-Prest, PTT-A and PTT-LA (Diagnostica Stago); Pathromtin (Dade Behring, Marburg, Germany)] shows a little or no correction. Time-dependent inhibitor was defined if the APTT of the incubated mixture was greater than the APTT of a reference mixture prepared from NPP and physiologic buffer and greater than the APTT of the non incubated mixture. Second, all patients were tested for time-dependant FVIII and/or FIX inhibitors using two methods for dosage of residual FVIII or FIX activity: classical Bethesda assay [9, 10] and modified Nijmegen method [8]. In the latter assay, Unicalibrator (Diagnostica Stago) was used as buffered NPP, and STA-deficient VIII as FVIII deficient plasma. The calculation of the inhibitor level was based on the dilution of test plasma that neutralises 50% of added FVIII. The threshold for positive level was defined as 0.4 Bethesda Units (BU).

Antiphospholipid determination

Coagulation tests were used to screen for LA and ELISA test with commercial kits (Orgentec Diagnostica, Mainz, Germany) to detect IgG, IgM, IgA anti-cardiolipin (aCL) and anti-β2 glycoprotein I (anti-β2GPI). Lupus anticoagulant characterization was based on the four steps according to the recommendations of the Scientific and standardization committee of the International society on thrombosis and haemostasis (ISTH) [11]: i) detection of prolonged phospholipid dependant coagulation tests; ii) identification of the inhibitory effect by performing mixture study; iii) confirmation that the inhibitor is phospholipid-dependent

by performing the same assay in the presence of excess phospholipids; iv) exclusion of other coagulation defects. The screening for LA was based upon three different assays: i) APTT test using four different commercial reagents [CK-Prest, PTT-A and PTT-LA (Diagnostica Stago); Pathromtin (Dade Behring)]; ii) Tissue Thromboplastin Inhibition test (TTI) at 1:500 dilution (Néoplastine CI plus, Diagnostica Stago); iii) and dilute Russel Viper Venom Test (dRVVT) (LA1, Dade Behring).

The level of APA was determined using an ELISA assay according to the recommendations of the "European APL Forum" [12]. The commercial kits provide calibrators, positive and negative controls that are polyclonal sera. The results of IgG, IgM, IgA aCL measurement were expressed as GPL, MPL and APL U/mL, respectively. The concentration of anti- β 2GPI is given as U/mL. The cut-off levels of positivity were defined by the manufacturer as 10 and 8 U/mL for aCL and anti- β 2GPI, respectively; these values correspond to the 95th percentile ranges of 100 healthy Tunisian blood donors.

To check whether APA are related to viral infections, all haemophiliacs were screened by ELISA technique for the presence of HBV surface antigen (Monolisa HBs Ag ultra; Bio-Rad; Marnes-la-Coquette; France) and for the detection of HCV (Monolisa HCV Ag-Ab Ultra; Bio-Rad) and HIV (Genscreen Ultra HIV Ag-Ab; Bio-Rad) markers. Positive sera for HCV and HIV were confirmed by immunoblotting.

Statistical analysis

Statistical analysis was performed using the SPSS-13.0 software. Chi-Square tests were used to compare frequencies of APA. The significant level was set at $p < 0.05$.

Results

Frequency of FVIII and FIX inhibitors

Among the 32 studied patients, only one had an inhibitor. This patient was 18 years old and suffered from severe haemophilia A. He was treated on demand by either cryoprecipitates or plasma derived FVIII concentrates; he never received recombinant FVIII. Bleeding episodes were managed with high doses of FVIII concentrates but didn't require the use of bypassing agents. Factor VIII inhibitor was detected by APTT in the non incubated mixture of the patient's plasma and NPP and also after incubation at 37°C for 60 min, using the four reagents. The inhibitor was time-dependant and its concentration using Bethesda and Nijmegen modified assays was 10 BU. Screening for antiphospholipid antibodies, especially LA, was negative.

Screening for FVIII and FIX inhibitors using the two methods, APTT on the mixture without and after incubation at 37°C and the Bethesda assay, was negative in all the remaining patients.

Associated coagulation abnormalities

Eight patients showed an associated factor VII deficiency with a level ranging between 32 and 62% (normal: 70-120%). Five of them were haemophiliacs A (two brothers and their first cousins) and three were haemophiliacs B. Combined factor V deficiency was detected in three patients with haemophilia A, one of them had also a factor VII deficiency. The measured factor V levels were 51%, 53% and 64% (normal: 70-120%).

Four patients with severe haemophilia A had a slight decrease in the FIX level: 53% in two brothers, 48% and 51% in two brothers among their first cousins, respectively (normal: 60-150%). Thrombin time, levels of fibrinogen, factor II, factor X and von Willebrand factor were in the normal range for all patients.

Frequency of antiphospholipid antibodies

No LA was detected in any of our patients. APA were detected in nine patients (28.1%) by ELISA method (table 2). APA levels ranged between 15 and 30 UI/mL. The prevalence of aCL and anti- β 2GPI was 12.5% (4/32) and 28.1% (9/32), respectively.

Among the nine patients with APA, three were HCV positive and two had HCV and HIV co-infection. APA frequency was higher in the HCV positive group as compared to patients with negative serology (25% vs 5% for aCL; 41.6% vs 20% for anti- β 2GPI), but the difference was not statistically significant.

Discussion

To our knowledge, this is the first report on Tunisian hemophiliacs' immunization. Inhibitors to FVIII and FIX were found to be unfrequent (5%) in our patients, in contrast to the high prevalence of APA and the frequent combined coagulation factor deficiency.

According to a meta-analysis published in 2003, the overall prevalence of FVIII inhibitors ranged from 3.6 to 27%. In severe haemophilia A, the rate of inhibitors may reach 44% [1]. Studies concerning haemophilia B are more limited and the reported prevalence of FIX inhibitors ranged from 1.8 to 2% and from 2.8 to 4% in severe forms [13, 14]. The reported immunization disparity is in part related to the methodologies used: study design, patient's selection (severity, low or high responders), transfusion history,

Table 2. APA specificities and isotypes in relation to viral serology status.

APA positive patients	Anticardiolipin (n = 4; 12.5%)			Anti- β 2GPI (n = 9; 28.1%)			Viral serology
	IgG	IgM	IgA	IgG	IgM	IgA	
1	+	-	+	+	-	+	HCV+/HIV+
2	-	-	-	-	-	+	HCV-/HIV-
3	+	-	-	+	-	-	HCV-/HIV-
4	-	-	-	-	+	-	HCV+/HIV-
5	-	+	-	+	-	-	HCV+/HIV-
6	-	-	-	+	-	-	HCV-/HIV-
7	-	-	-	-	+	-	HCV+/HIV-
8	-	-	-	-	+	-	HCV-/HIV-
9	+	-	-	-	-	+	HCV+/HIV+

APA: antiphospholipid antibodies; HCV (+): positive serology for HCV infection; HIV (+): positive serology for HIV infection.

definition of severe haemophilia, follow up period, frequency of inhibitors screening. . . [1]. Inhibitors production is also influenced by haemophilia severity and genetic factors such as the mutation type, positive family history for inhibitors, immunogenetic factors, ethnic origin and race [15-17]. Additional factors like early start of replacement therapy, treatment modality, type of factor concentrate (plasma-derived vs. recombinant), activation of the immune system (infection, vaccination, surgery. . .) may also contribute to the risk of inhibitors occurrence. However the actual contribution of each factor remains controversial [15, 18-23].

Curiously, immunization seems to be rare in our haemophilia patients eventhough most of them present a severe form (20/32). We assume that the exclusive use of plasma-derived products as well as on-demand treatment and probably the absence of genetic predisposition are likely to limit immunization in our patients. Among Tunisian haemophiliacs, the FVIII gene polymorphism was recently characterized by Elmahmoudi *et al.* [24]. The haplotype patterns showed some differences with other populations. Interestingly, the polymorphisms described were suggested to be associated with a high risk of inhibitor development, since they are localized in regions targeted by inhibitors. The discrepancy between this genetic polymorphism profile and the low frequency of inhibitors may suggest the contributive role of other protective factors. It should be mentioned however that the precise regimen of replacement therapy in our haemophiliacs could not be determined because the patients were treated in different sites. Our findings need to be confirmed by additional trials. The association of haemophilia to other coagulation factor deficiencies is poorly documented. Deficiencies of factor VII and factor V are the most described associated coagulation defects in haemophilia patients [25-30]. A systematic screening of additional coagulation abnormalities, particu-

larly when a context of consanguinity exists, would allow a better evaluation of the bleeding risk and therefore a more appropriate therapeutic care of haemophilia patients.

Such combined deficiencies, may interfere in clotting tests used for diagnosis and inhibitors screening, and may limit the efficiency of replacement therapy. Associated clotting factor deficiencies may be acquired (chronic hepatitis due to viral hepatitis infection) or inherited. On the other hand, they may result from an independent segregation of several genetic abnormalities or from a common functional genetic defect [26]. In our series, the associated factor deficiencies are moderate and might be favoured by the strong consanguinity that may lead to the transmission of more than one genetic abnormality. The low level of FIX observed in four patients with haemophilia A remains unexplained.

Antiphospholipid antibodies were relatively frequent in our study (28.1%). The prevalence of aCL and anti- β 2GPI was 12.5% (4/32) and 28.1% (9/32), respectively, but there was no case of LA. Studies focusing on APA in haemophiliacs are limited. The reported prevalence varies from 0 to 92% for aCL [31-36] and from 0 to 58% for LA [31, 32, 34, 37]. Only two studies evaluated the frequency of anti- β 2GPI in patients with haemophilia, the first did not find any case with positive anti- β 2GPI [31] and the second reported a frequency of 4.1% in a series of 72 severe haemophiliacs [36]. The occurrence of APA in haemophilia has been associated to hepatitis C infection [33] and to HIV infection [32]. However, this association remains controversial [34, 38]. In our study, among the nine APA positive patients, five (55.5%) had a HCV infection or a HCV/HIV co-infection. The occurrence of APA seems to be related to HCV infection. Indeed, the prevalence of APA was higher in the group of HCV positive patients as compared to the patients with negative serology: 25% vs 5% for aCL, 41.6% vs 20% for anti- β 2GPI. However, these results need to be confirmed in larger series.

Although LA and inhibitors to FVIII or FIX have opposite clinical behaviour as specific factor inhibitors are associated with a risk of bleeding while LA is paradoxically associated with thrombosis, the coexistence of the both antibodies is possible in haemophiliacs and may lead to practical diagnosis difficulties. Indeed, both of them prolong phospholipid-dependent coagulation tests and it may be difficult to confirm or rule out the diagnosis of LA in haemophiliacs with inhibitors. So that the use of two or more methods is highly recommended for the diagnosis of LA [31, 37]. Moreover LA might interfere with the clotting tests used to measure intrinsic coagulation factors leading to false results.

The pathogenic role of APA in haemophilia patients remains unknown. Haemophiliacs are theoretically protected against thrombotic risk, but rare cases of thrombotic events have been reported. In these cases an associated risk factor is often found: surgery, venous catheter, anti-haemophilic factor infusion or a pre-existent prothrombotic state [39, 40]. So APA may contribute in combination with other hereditary or acquired risk factors to the occurrence of thrombosis.

Conclusion

The development of specific inhibitors to FVIII or FIX is rare in our haemophilia patients in contrast to APA. However, further prospective studies are necessary to elucidate the pathologic role of APA in haemophilia patients. In addition, measurement of all coagulation factors is helpful when the patient does not respond to replacement therapy, especially if no inhibitor is detected and/or when consanguinity occurs.

Conflicts of interest: none.

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