

Antibodies to anionic phospholipids and cofactors in kala-azar. Comparative study with malaria, toxoplasmosis and “autoimmune diseases”

H. Skouri¹, R. Gandouz¹, I. Kraiem¹, I. Harrabi², M. Ben Said³

¹Laboratoire d'hématologie et banque du sang (03/UR/0818). CHU Sahloul. 4000 Sousse. Tunisia;
²Service d'épidémiologie. CHU Farhat Hached. 4000 Sousse. Tunisia; ³Laboratoire de parasitologie.
CHU Farhat Hached. 4000 Sousse. Tunisia.

Abstract

Objective

To investigate the distribution of various anti-anionic and anti-cofactor phospholipid antibodies in children with kala-azar, and to compare them to malaria, toxoplasmosis and auto-immune disease.

Patients and Methods

The frequency and the concentration of antibodies against cardiolipin (aCL), phosphatidylserine (aPS), phosphatidylinositol (aPI), phosphatidic acid (aPA), β 2GPI (anti- β 2GPI), prothrombin (aPT), annexin V (aAnnV), protein C (aPnC) and protein S (aPnS) were studied in sera from 103 children with kala-azar and compared with malaria (n=32), toxoplasmosis (n=31), systemic lupus erythematosus (SLE) (n=40) and antiphospholipid syndrome (APS) (n=35).

Results

The prevalence of aCL, aPS, aPI, aPA, anti- β 2GPI, aPT, aAnnV, aPnC and aPnS was 54%, 56%, 43%, 28%, 73%, 67%, 55%, 30%, 25%, respectively. Ninety-three per cent of children with kala-azar (96/103) had one or more aPL specificities, but none had thromboses. The spectrum of aPL was quite similar to that found in patients with SLE and APS.

Conclusion

Antiphospholipid antibodies are a frequent finding in kala-azar. The aPL produced mimic those found in autoimmune disease. However, further studies are required to assess the exact role of these aPL during leishmaniasis.

Key words

Anti-anionic phospholipid antibodies, anti-cofactor antibodies, antiphospholipid syndrome, kala-azar, Tunisia, thrombosis.

Hadeef Skouri, MD, PhD
 Raoudha Gandouz, MD
 Imen Kraiem, MD
 Imed Harrabi, MD
 Moncef Ben Said, MD, PhD

Please address correspondence and
 reprint requests to:

Dr. Moncef Ben Said,
 Laboratoire de Parasitologie,
 CHU Farhat Hached,
 4000 Sousse, Tunisia.

E-mail: moncef.bensaid@rns.tn

Received on October 17, 2007; accepted in
 revised form on April 14, 2008.

© Copyright CLINICAL AND
 EXPERIMENTAL RHEUMATOLOGY 2008.

Introduction

Antiphospholipid antibodies (aPL) are a heterogeneous family of autoantibodies recognizing various phospholipid antigens (1-3). It is well established that some aPL, particularly those found in patients with autoimmune disorders like systemic lupus erythematosus (SLE) or antiphospholipid syndrome (APS), may lead to thromboses, multiple abortions, thrombocytopenia and a wide variety of other complications (1-3). However, aPL were also reported in many infectious diseases including viral, bacterial and parasitic infections, where, in contrast to autoimmune diseases, they do not seem to be associated with thromboses (4). This led to distinguish pathogenic and non-pathogenic aPL. Why these aPL have different behaviour remains unknown. It was suggested that the binding target of aPL differs in these two conditions (1-3). Indeed, in autoimmune disorders, aPL require phospholipid proteins which are their main target, and thus are referred to as being cofactor dependent, whereas those generated in response to infection generally are cofactor independent. However, the demonstration of anti-cofactor antibodies in patients with viral, bacterial and parasitic infections makes this dichotomy controversial (5). Furthermore, accumulating reports have established that many infections may not only trigger the production of aPL but also appear to be associated with APS clinical manifestations (6-8).

In infectious diseases, most studies have focused on IgG and IgM isotypes of aCL and anti- β 2GPI; whereas IgA isotypes as well as other aPL specificities were less commonly studied (9-11). Moreover, studies on aPL were carried out in predominantly white populations but data about other groups and ethnics are scarce. The reported prevalence of aPL varies widely between studies, and ranges between 0-90% (5, 9-11). In patients with kala-azar, the occurrence of aPL was only reported in a single Brazilian study (n=30), where the reported frequencies of IgG aCL, IgM aCL and IgG anti- β 2GPI were 6%, 3% and 53%, respectively (9).

In this study, we aimed to assess the frequency of various antigenic specificities of aPL, including different anti-anionic

phospholipid and anti-cofactor antibodies, in Tunisian patients with kala-azar, and to compare them with malaria, toxoplasmosis, SLE and APS patients.

Visceral leishmaniasis is endemic in Tunisia, where it is caused by *leishmania infantum* and mainly affects children. It is well known that this infection is accompanied by a depth immune disturbance characterized by the production of a large variety of autoantibodies together with various autoimmune perturbations (12-14), and hence, theoretically high risk of developing aPL.

Patients and methods

Patients and sera

Sera from 103 children suffering from visceral leishmaniasis (42 females, 61 males; mean age = 3.5 years, range 8 months to 11 years) were retrospectively investigated for aPL antibodies. The diagnosis of kala-azar was based up on the presence of amastigotes of *leishmania infantum* in the bone marrow aspiration and significant titer of anti-leishmaniasis antibodies in the indirect immunofluorescence test. Blood samples were collected just before initiation of treatment. Medical records during the hospital stay, the treatment and the period of control, which lies between 3 to 10 months, were retrospectively reviewed for all these children in order to identify a possible documented history of thrombosis. In addition, among the 103 children studied, the persistence of aPL were studied in 35 patients for whom subsequent serum samples after treatment were available. These samples were obtained between 1 to 12 months (mean = 20 weeks) after the initiation of treatment.

For a comparative purpose, the following sera were also investigated:

31 sera from females with recently acquired toxoplasmosis infection (mean age = 17.3 years, range 8 to 28 years); 32 sera from patients with falciparum malaria (21 males, 11 females; mean age = 27.7 years, range 20 to 35 years); 40 sera from females with SLE (mean age = 33.2 years, range 20 to 56 years). The diagnosis of SLE was established according to the American Rheumatology Association criteria (15) and 35 sera from patients with APS (26

Competing interests: none declared.

females, 9 males; mean age = 27.1 years, range 18 to 47 years) including 27 primary APS (PAPS) cases and 8 secondary APS (SAPS) cases associated to SLE. The diagnosis of APS was made according to the updated APS criteria (16). Twenty-five patients had venous or arterial thrombosis and 10 patients had multiple unexplained fetal loss beyond the 10th week of gestation. The biological criteria were the presence of aCL, anti- β 2GPI antibodies and/or lupus anticoagulant (LA) on two occasions at 12 weeks apart. LA was determined according to the recommendations of the International Society for Thrombosis and Haemostasis Standardization Subcommittee (ISTH) (17), aCL antibodies were tested by a home-made ELISA or commercial kit, and anti- β 2GPI antibodies were tested by commercial kit.

Laboratory investigations

Detection of anti-anionic phospholipid antibodies: Antibodies against cardiolipin (aCL), phosphatidylserine (aPS), phosphatidylinositol (aPI) and phosphatidic acid (aPA), were detected by commercial ELISA kits (Orgentec Diagnostica, Mainz, Germany) which distinguish between IgG, IgM and IgA isotypes for aCL and between IgG and IgM for aPS, aPA and aPI antibodies. This commercial ELISA system allows the detection of both β 2GPI-dependent and independent anti-anionic phospholipid antibodies.

Detection of anti-cofactor antibodies: IgG, IgM and IgA isotypes for anti- β 2GPI and anti-prothrombin antibodies (aPT), and IgG and IgM anti-annexin V antibodies (aAnnV) were measured by commercial ELISA kits (Orgentec Diagnostica, Mainz, Germany) according to the manufacturer's recommendations.

Detection of anti-protein S and anti-protein C antibodies: All sera, except those from patients with APS, were screened for IgG and IgM isotypes of anti-protein S (aPnS) and anti-protein C (aPnC) antibodies by a home-made ELISA method as previously reported (10,18). Briefly, irradiated polystyrene plates (Nunc-Intermed, Raskilde, Denmark) were coated overnight with 5 μ g/

mL of protein C or protein S (Diagnostica Stago, Asnieres, France) in TBS (50 mmol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4). The plate wells were blocked by incubation for 1 hour at 25°C with 50 μ L of TBS containing 1% bovine serum albumin (BSA, Sigma). After washing three times with TBS-0.1% Tween 20, 50 μ L of diluted serum or controls (1:100) in TBS-Tween-BSA 1% were added in each well and plates were incubated for 60 min at room temperature. The plates were washed three times with TBS-Tween and horseradish peroxidase-conjugated goat anti-human IgG or IgM (Sigma) added for one hour at 25°C. Plates were again washed three times and the assay developed using TMB solution. The reaction was stopped by addition of 50 μ L of HCl (1M) and the absorbance measured at 450 nm. All samples were tested in duplicate and control wells (coated only with TBS) were systematically used for each serum to ensure specific binding. Interpretation of results: All aPL ELISAs using commercial kits were performed according to the "minimal requirements" proposed by the European APL Forum (19). Each commercial ELISA kit contains polyclonal calibrators and control samples allowing the calibration of the assay and the measurement of aPL concentration. The aCL results were expressed as GPL, MPL and APL units/mL for IgG, IgM and IgA, respectively, whereas concentrations of the other aPL were expressed in U/mL. The threshold for negative and positive values were defined by the manufacturer as concentrations below and more than 10 U/mL, respectively, with the following exceptions: cut-off value for IgM aCL was 7 MPL/mL; cut-off value for IgG, IgM and IgA anti- β 2GPI was 8 U/mL; IgG and IgM aAnnV results were reported as negative (<5U/mL), borderline (5-8 U/mL) and positive (>8 U/mL). In our laboratory, these values were checked to correspond approximately to the 95th percentiles of a healthy Tunisian blood donors group. Anti-PnC and anti-PnS cut-offs were calculated by the method of percentiles. The threshold for positive values was defined by optical density >95th

percentile from a group of 100 healthy blood donors. The calculated aPnC and aPnS cut-off values were 0.24 for IgG aPnC, 0.47 for IgM aPnC, 0.64 for IgG aPnS and 0.75 for IgM aPnS.

Statistical analysis

Statistical analyses were performed with SPSS for Windows version 10.0. Because the distribution of aPL levels was not Gaussian, their concentrations were presented as median and inter-quartile range. Comparative analyses were performed using *t*-test and χ^2 method for quantitative and qualitative variables, respectively. Pearson's correlation coefficient (*r*) was used to analyse association between pairs of aPL in patients with kala-azar. The threshold for statistical significance was set at *p* < 0.05.

Results

Prevalence of aPL antibodies

Ninety-six out of the 103 patients with visceral leishmaniasis (93%) were positive for at least one type of aPL, versus 20 of 31 (64%) in toxoplasmosis, 14 of 32 (43%) in malaria, 27 of 41 (65%) in SLE and 19 of 26 (73%) in APS. The frequencies and the isotypes of various anti-anionic phospholipid and anti-cofactor antibodies in the 5 groups studied are summarized in Tables I and II, respectively.

The comparison of the frequency of aPL positive patients between the different groups showed significant higher frequencies in kala-azar as compared to toxoplasmosis and malaria for IgG isotype of aCL (*p*=0.003 and <0.0001), anti- β 2GPI (both *p*<0.0001), aPT (both *p*<0.0001), aPI (*p*=0.04 and 0.03), aPS (both *p*<0.0001) and aAnnV (both *p*<0.0001); and as compared to SLE and APS for IgG anti- β 2GPI (*p*<0.0001 and 0.006), aPT (both *p*<0.0001) and aAnnV (*p*=0.001 and 0.02). In contrast, there was no difference between kala-azar and SLE and APS groups for IgG aCL and the remaining anti-anionic phospholipid antibodies. IgM isotypes of aCL, aPI and anti- β 2GPI were statistically more frequent in kala-azar than in toxoplasmosis (aCL, *p*=0.03; aPI, *p*=0.03; anti- β 2GPI, *p*=0.002) and malaria (aCL, *p*=0.008; aPI, *p*=0.01;

Table I. Frequencies of anti-anionic phospholipid antibodies and their isotypes in the 5 studied groups.

	Malaria (n=32)						Toxoplasmosis (n=31)						Kala-azar (n=103)						LES (n=40)						APS (n=35)					
	aCL	aPS	aPI	aPA	aCL	aPA	aPS	aPI	aPA	aCL	aPA	aPS	aPI	aPA	aCL	aPA	aPS	aPI	aPS	aCL	aPA	aPS	aPI	aPS	aCL	aPA	aPS	aPI	aPA	
Total (%)	2(6)	4(12)	4(12)	1(3)	8(26)	2(6)	8(26)	5(17)	2(6)	56(54)	29(28)	58(56)	44(43)	29(28)	16(40)	18(45)	14(35)	15(38)	22(62)	4(12)	4(12)	4(12)	4(12)	4(12)	4(12)	4(12)	4(12)	4(12)	4(12)	
IgG alone	1	1	1	0	4	1	1	2	1	28	12	25	13	12	0	3	0	0	5	0	0	0	0	0	0	0	0	0	0	
IgM alone	0	2	2	0	2	3	7	3	1	5	10	13	20	10	2	6	6	10	4	0	0	0	0	0	0	0	0	0	0	
IgA alone	0	-	-	-	0	-	-	-	-	0	-	-	-	-	2	-	-	-	3	-	-	-	-	-	-	-	-	-	-	
IgG+IgM	0	1	1	1	0	0	0	0	0	15	7	20	11	7	6	9	8	5	6	4	4	4	4	4	4	4	4	4	4	
IgG+IgA	0	-	-	-	2	-	-	-	-	3	-	-	-	-	0	-	-	-	1	-	-	-	-	-	-	-	-	-	-	
IgM+IgA	0	-	-	-	0	-	-	-	-	0	-	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	
IgG+IgM+IgA	1	-	-	-	0	-	-	-	-	5	-	-	-	-	5	-	-	-	2	-	-	-	-	-	-	-	-	-	-	

Table II. Frequencies of anti-cofactor antibodies and their isotypes in the 5 studied groups.

	Malaria (n=32)						Toxoplasmosis (n=31)						Kala-azar (n=103)						LES (n=40)						APS (n=35)					
	aβ2GPI	aPT	aAnnV	aPnC	aPnS	aβ2GPI	aPT	aAnnV	aPnC	aPnS	aβ2GPI	aPT	aAnnV	aPnC	aPnS	aβ2GPI	aPT	aAnnV	aPnC	aPnS	aβ2GPI	aPT	aAnnV	aPnC	aPnS	aβ2GPI	aPT	aAnnV	aPnC	aPnS
Total (%)	8(25)	3(9)	6(19)	5(15)	3(9)	11(35)	1(3)	7(23)	2(6)	1(3)	75(73)	69(67)	56(55)	31(30)	26(25)	15(38)	15(38)	9(23)	14(35)	18(45)	28(80)	7(20)	7(20)	7(20)	7(20)	7(20)	7(20)	7(20)	7(20)	7(20)
IgG alone	0	3	5	2	1	2	1	2	1	0	30	48	25	14	8	1	3	3	6	3	3	3	3	3	3	3	3	3	3	3
IgM alone	2	0	1	2	1	1	0	5	1	1	5	2	3	8	12	1	9	3	6	12	3	1	0	0	0	0	0	0	0	0
IgA alone	5	0	-	-	-	6	0	-	-	-	3	1	-	-	-	4	0	-	-	-	9	0	-	-	-	-	-	-	-	-
IgG+IgM	0	0	0	1	1	0	0	0	0	0	18	18	28	9	6	3	3	3	2	3	8	3	0	0	0	0	0	0	0	0
IgG+IgA	0	0	-	-	-	1	0	-	-	-	6	0	-	-	-	1	0	-	-	-	1	0	-	-	-	-	-	-	-	-
IgM+IgA	0	0	-	-	-	1	0	-	-	-	3	0	-	-	-	2	0	-	-	-	2	0	-	-	-	-	-	-	-	-
IgG+IgM+IgA	1	0	-	-	-	0	0	-	-	-	10	0	-	-	-	3	0	-	-	-	2	0	-	-	-	-	-	-	-	-

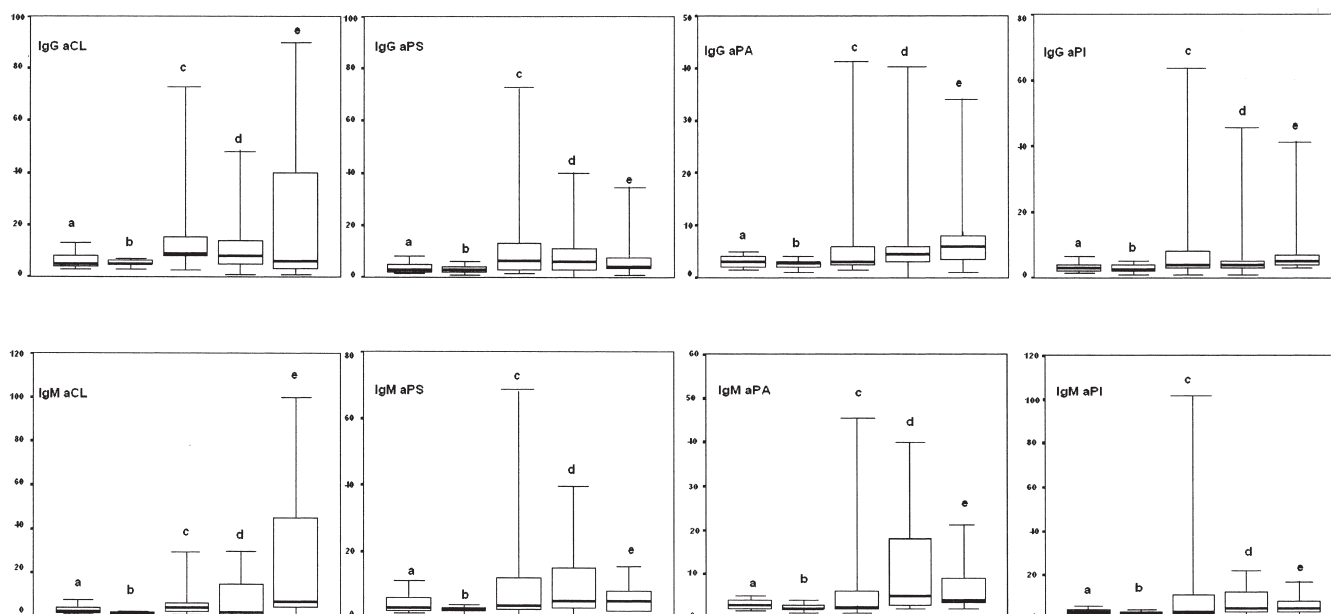


Fig. 1. Concentrations of IgG and IgM anti-anionic aPL in the different populations studied: Malaria (a); Toxoplasmosis (b); Kala-azar (c); LES (d) and APS (e). Results are expressed as GPL/mL and MPL/mL for IgG and IgM aCL, respectively, and as UI/mL for the other anti-anionic aPL. Bar and box represent median and interquartile range. Whisker represents range of values.

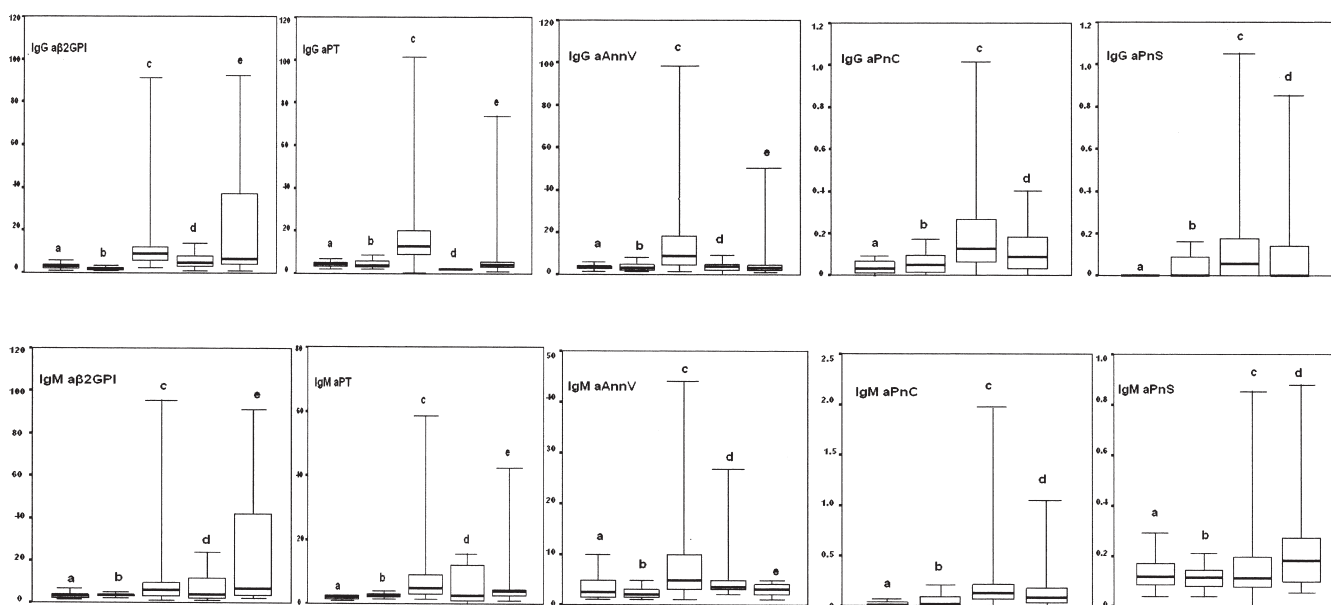


Fig. 2. Concentrations of IgG and IgM anti-cofactor aPL in the different populations studied: Malaria (a); Toxoplasmosis (b); Kala-azar (c); LES (d) and APS (e). Results are expressed as UI/mL for aβ2GPI, aPT and aAnnV and as optical density for aPnC and aPnS. Bar and box represent median and interquartile range. Whisker represents range of values.

anti-β2GPI, $p=0.005$), but not than in SLE and APS groups. However, the frequencies of IgG and IgM isotypes of aPnC, aPnS and aPA did not demonstrate any difference between the kala-azar and the other groups. Moreover, we did not find statistical differences between the 5 groups studied concerning the frequency of the IgA isotypes of aCL, anti-β2GPI and aPT.

Concentrations of aPL antibodies

The median and interquartile range of anti-anionic phospholipid and anti-cofactor antibodies levels in the 5 groups studied are summarized in figures 1, 2 and 3. In kala-azar, aPL concentrations >40 U/mL were demonstrated for the following specificities: IgG aCL (n=3); IgG and IgM aPI (n=3 and n=6); IgG and IgM aPS (n=4 and n=3); IgG and

IgM aPA (n=1 for both); IgG, IgM and IgA anti-β2GPI (n=6, n=5, n=4); IgG and IgM aPT (n=11, n=1); IgG and IgM aAnnV (n=14, n=1). Among children with kala-azar, 33 had at least one aPL >40 U/mL (32%). In addition, concentrations more than 99th percentiles were found in 4 and 3 sera for IgG and IgM aPnC, respectively; and in 3 sera for IgG aPnS.

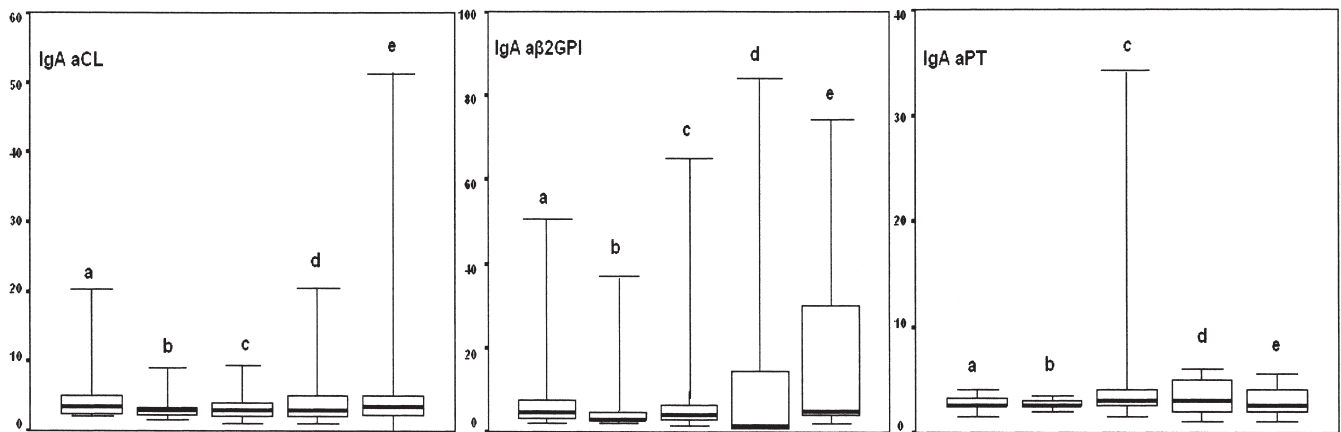


Fig. 3. Concentrations of IgA aPL in the different populations studied: Malaria (a); Toxoplasmosis (b); Kala-azar (c); LES (d) and APS (e). Results are expressed as APL /mL for IgA aCL and as UI/mL for IgA aβ2GPI and aPT. Bar and box represent median and interquartile range. Whisker represents range of values.

Correlation between aPL antibodies

Antiphospholipid antibodies of IgG isotype demonstrated highly significant correlations ($p < 10^{-3}$) between the following specificities: aCL with aPI ($r=0.62$), aPA ($r=0.7$), aPS ($r=0.65$) and anti-β2GPI ($r=0.52$); aPI with aPA ($r=0.91$), aPS ($r=0.83$) and anti-β2GPI ($r=0.81$); aPA with aPS ($r=0.74$) and anti-β2GPI ($r=0.79$); aPS with anti-β2GPI ($r=0.49$), aPT ($r=0.41$) and aAnnV ($r=0.48$); aAnnV with aPnC ($r=0.34$). For the IgM isotypes, highly significant correlations ($p < 10^{-3}$) were found between the following specificities: aCL with aPI ($r=0.67$), aPA ($r=0.83$), aPS ($r=0.63$) and anti-β2GPI ($r=0.85$); aPI with aPA ($r=0.84$), aPS ($r=0.87$), anti-β2GPI ($r=0.69$) and aPT ($r=0.36$); aPA with aPS ($r=0.79$), anti-β2GPI ($r=0.77$) and aPT ($r=0.4$); aPS with anti-β2GPI ($r=0.56$), aPT ($r=0.51$) and aAnnV ($r=0.4$); aAnnV with aPnC ($r=0.61$). However, there was no correlation between the following pairs: IgG aPS with aCL, aPI, aPA, anti-β2GPI, aPT and aAnnV; IgG aPnC with aCL and anti-β2GPI; IgG anti-β2GPI with aAnnV and aPT; IgM aPnC with aCL, aPI, aPA and anti-β2GPI. The remaining associations demonstrated moderate correlations ($p < 0.05$). For IgA isotypes, highly significant correlations were found between aCL and anti-β2GPI ($r=0.75$; $p < 10^{-3}$), as well as between aCL and aPT ($r=0.3$; $p=0.002$). However, the concentration of IgA anti-β2GPI showed no significant correlation with aPT.

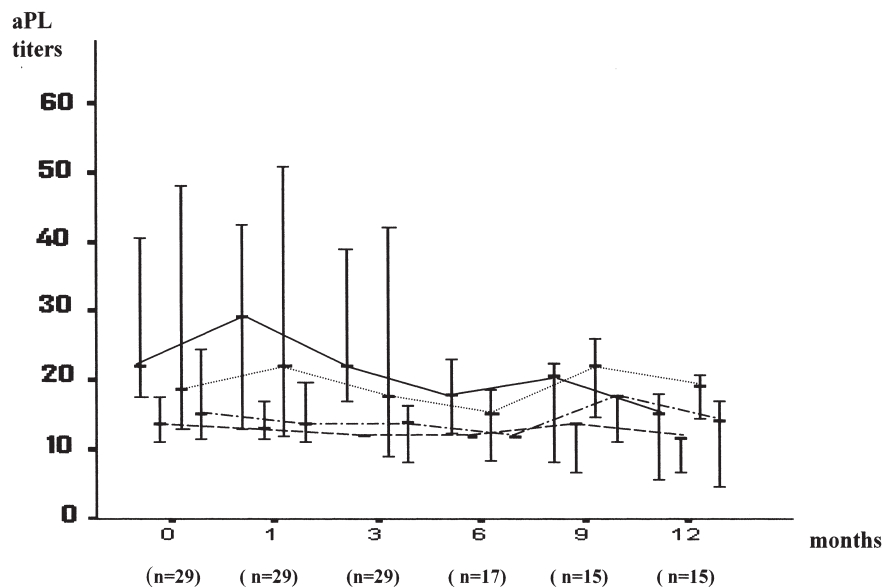


Fig. 4. Follow-up study of IgG and IgM isotypes of aCL and aβ2GPI in 29 children with kala-azar. (—): IgG aCL; (---): IgM aCL; (.....): IgG aβ2GPI; (-.-.-): IgM aβ2GPI. Results are expressed as GPL /mL and MPL/mL for IgG and IgM aCL, respectively, and as UI/mL for IgG and IgM aβ2GPI. Bar and whisker represent median and range of values, respectively.

Persistence and clinical significance of aPL antibodies

All 35 children investigated for post-treatment persistence of aPL were found positive for IgG and/or IgM aCL and anti-β2GPI before initiation of treatment, and in 29 of them these antibodies persisted over the follow-up period, even though in some of them the aPL titers showed a slight decrease (Fig. 4). Of note, all the aPL positive children, ultimately healed. Despite the high frequency of aPL occurrence, the review of patients' medical records did not identify any children with a history of documented thromboembolism.

Discussion

In this study, we demonstrated the frequent occurrence of aPL in a large series of 103 cases of visceral leishmaniasis. Antiphospholipids were directed against both anionic phospholipid antigens and phospholipid binding proteins, and in some cases, their levels were significantly elevated with strong correlations between the concentrations of pairs of aPL. However, none of children with aPL had thromboses. In infectious diseases, aCL and anti-β2GPI were by far the most studied among aPL. As far as kala-azar is considered, only a prior study, conducted in

Brazil, assessed IgG and IgM isotypes of aCL and anti- β 2GPI (9). Santiago *et al.* reported a 53% anti- β 2GPI frequency in 30 Brazilian children with kala-azar (16/30). This result is quite similar to that shown in our study (75/103). However, aCL were only detected in 6% (2/30) of Brazilian patients as opposed to the high proportion of aCL positive patients in our study (54%). In both Brazil and Tunisia, visceral leishmaniasis is caused by the same species of leishmania, thus the parasite species itself can not explain the difference in the aCL frequency observed.

In our study, we showed that antibodies to PS, PI and PA were frequent in kala-azar as well as in malaria, toxoplasmosis and autoimmune diseases. Our results were quite similar to the findings of Petrovas *et al.*, who reported aPS and aPI in 56% and 34% of 44 HIV infected patients, respectively; whereas, anti- β 2GPI were only detected in 5% of them (20). In contrast, in a series of 46 patients with infectious mononucleosis and 50 with *Helicobacter pylori* infection, Sorice M *et al.* did not find aPS antibodies, whereas aCL and anti- β 2GPI were detected in 30.4 and 8%, respectively (10). In patients with autoimmune disease, anti-anionic phospholipids were reported in about 5 to 60%, but the diagnosis value and the clinical significance of these aPL subsets remain controversial (21-23).

Anti-prothrombin antibodies were demonstrated in a high proportion of patients with SLE (50-60%) and with APS (45%), and in about 16% of women with recurrent pregnancy loss (24-26). However, the clinical usefulness of aPT is controversial, and subsequently they are not included in the APS classification criteria (16). Anti-prothrombin antibodies were also found in 4 to 43% of patients suffering from various infectious diseases without thrombosis (10-11). In our study, the prevalence rates of aPT were 68% in patients with kala-azar, 9% with malaria, 3% with toxoplasmosis, 38% with SLE and 20% with APS.

Many studies showed that aAnnV were an independent risk factor for miscarriages, and its frequency lies between 5.5% and 17% in women with recurrent

abortion (24, 27). In addition, aAnnV were found in 20 to 30% of patients with SLE and APS; but in both situations, no association between aAnnV and thrombotic risk could be demonstrated (18, 24). Antibodies to PnC and PnS were found in about 20-30% of patients with SLE, and shown to be closely related to venous thrombosis (18). Only a single previous study assessed the prevalence of aAnnV and aPnS in patients with infectious diseases (10). In this study, the reported frequencies were 26% and 21.7% in infectious mononucleosis and 2% and 4% in *Helicobacter pylori* infection, respectively. In our series of patients, we found that aAnnV were more frequent in kala-azar than in the other groups, and that the concentration of these antibodies correlated strongly with all other aPL specificities. In addition, we found raised levels of aPnC and aPnS in 30% and 25% of patients with kala-azar, respectively, a figure somewhat similar to that found in LES.

However, the aPL frequencies found in our study need cautious interpretation due to the age difference between children with kala-azar and control patients; indeed, it was demonstrated that aPL may be common in children (28). Our results and others concluded to the existence of a very broad range in the reported aPL frequency during infectious diseases. These conflicting results may be due to the type of infection itself, indeed viral agents seem to be more involved in the aPL production than parasitic or bacterial agents (4, 8). However, additional factors may be involved. First, the lack of standardization in assays used together with methodological problems regarding the definition of positivity threshold, the calibration and the type of assay used (commercial vs. home-made), are a major drawback in assessing assay reproducibility and comparisons (19, 29-32). Second, the disease stage and the coexistence of dual infection may constitute a serious bias in the analysis of the aPL frequency. Indeed, aPL may be transient during the course of the infection, and in co-infected patients it may be difficult to assess the relative contribution of each infection in

aPL production (4, 11). Third, the occurrence of aPL may be influenced by genetic and ethnic factors (33-35). In this respect, the high frequency of IgA isotype of aCL and anti- β 2GPI found in our study together with prior reports in African patients with SLE, APS and infectious diseases but not in Europeans suggest some link with ethnic factors and argue for a genetic role in aPL production (11, 36-38).

Despite the high frequency, the persistence and the high titer of aPL, which are supposed to be strong criteria for the thrombogenic character of aPL, we did not find any thromboembolic complication in our kala-azar patients records, even though infraclinical thrombosis may have been overlooked and the period of follow-up not long enough to confirm the absence of thrombosis. Studies on the pathogenesis of aPL generated by infectious agents have mainly focused on their thrombotic role (39-41). Surprisingly, some reports have demonstrated that aPL may modulate inflammatory cytokines and/or enhance apoptosis, and hence play a protective role. In placental malaria, Owens *et al.* found that high levels of aPL correlated with a reduced disease severity, and showed that changes in the aPL profile during pregnancy constitute a protective response to placental parasitemia (42). The aPL protective role in malaria was also demonstrated in non-pregnant women and children (43). Moreover, in HIV patients, Silvestris *et al.* found that *in vitro* apoptosis of T lymphocyte was increased in patients with high titers of aPS, and suggested that these antibodies contribute in the clearance of dead cells, and hence in the protection against thromboses (44). In leishmaniasis, the surface phospholipid antigens exhibited by the parasite play a crucial role in the ability to evade immune response and to increase its invasive capacity (45). In a recent study, it was demonstrated that the exposure of PS inhibits macrophage inflammatory activity in a way similar to apoptosis death cells (46). Therefore a critical question may arise. Does antibodies produced against these phospholipids play a protective role in such situation? We hypothesize that aPL produced in

the course of infections, by a molecular mimicry and/or disturbance of immune response, might play a protective role by limiting extension and invasiveness of the parasitic infection; but, paradoxically, aPL production may induce a hypercoagulable state which may lead, in the presence of favourable conditions as genetic background, environmental factors, vessel state ... to thrombosis events. The likely protective role of aPL may constitute an additional field in aPL pathogenesis research and requires further investigation.

In conclusion, aPL production may be a frequent feature during kala-azar. Further prospective trials are required to assess the clinical relevance of the aPL produced.

Acknowledgements

The authors would like to thank Dr. Eric Solary and Dr. Ali Bettaieb (INSERM U517, Faculté de Médecine, Dijon, France) for their critical reading of the manuscript.

References

- HANLY JG: Antiphospholipid syndrome: an overview. *CMAJ* 2003; 168: 1675-82.
- ASHERSON RA, FRANCES C, IACCARINO L *et al.*: The antiphospholipid antibody syndrome: diagnosis, skin manifestations and current therapy. *Clin Exp Rheumatol* 2006; 24 (Suppl. 40): S46-S51.
- GHARAVI AE, WILSON W, PIERANGELI S: The molecular basis of antiphospholipid syndrome. *Lupus* 2003; 12: 579-583.
- ASHERSON RA, CERVERA R: Antiphospholipid antibodies and infections. *Ann Rheum Dis* 2003; 62: 388-93.
- BLANK M, SHOENFELD Y: Beta-2-glycoprotein-I, infections, antiphospholipid syndrome and therapeutic considerations. *Clin Immunol* 2004; 112: 190-99.
- CERVERA R, ASHERSON RA, ACEVEDO ML *et al.*: Antiphospholipid syndrome associated with infections: clinical and microbiological characteristics of 100 patients. *Ann Rheum Dis* 2004; 63: 1312-17.
- ASHERSON RA, CERVERA R, PIETTE JC *et al.*: Catastrophic antiphospholipid syndrome: clues to the pathogenesis from a series of 80 patients. *Medicine* 2001; 80: 355-77.
- UTHMAN IW, GHARAVI AE: Viral infections and antiphospholipid antibodies. *Semin Arthritis Rheum* 2002; 31: 256-63.
- SANTIAGO M, MARTINELLI R, KO A *et al.*: Anti- β 2 glycoprotein I and anticardiolipin antibodies in leptosporosis, syphilis and Kala-azar. *Clin Exp Rheumatol* 2001; 19: 425-30.
- SORICE M, PITTONI V, GRIGGI T *et al.*: Specificity of anti-phospholipid antibodies in infectious mononucleosis: a role for anti-cofactor protein antibodies. *Clin Exp Immunol* 2000; 120: 301-06.
- LOIZOU S, SIGH S, WYPKEMA E *et al.*: Anticardiolipin, anti- β 2-glycoprotein I and antiprothrombin antibodies in black South African patients with infectious disease. *Ann Rheum Dis* 2003; 62: 1106-11.
- BOGDAN C, ROLLINGHOFF M: The immune response to leishmania: mechanisms of parasite control and evasion. *Int J Parasitol* 1998; 28: 121-34.
- HANDMAN E: Cell biology of Leishmania. *Adv Parasitol* 1999; 44: 1-39.
- MAKNI S, AYED K, BEN SAID M *et al.*: Study of circulating immune complexes during the evolution of visceral Mediterranean leishmaniasis. *Ann Trop Med Parasitol* 1989; 83: 349-55.
- HOCHBERG MC: The American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
- MIYAKIS S, LOCKSHIN MD, ATSUMI T *et al.*: International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295-306.
- BRANDT JT, TRIPLETT DA, ALVING B *et al.*: Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. *Thromb Haemost* 1995; 74: 1185-90.
- NOJIMA J, KURATSUNE H, SUEHISA E *et al.*: Association between the prevalence of antibodies to β 2-glycoprotein I, prothrombin, protein C, protein S, and annexin V in patients with thrombotic and thrombocytopenic complications. *Clin Chem* 2001; 47: 1008-15.
- TINCANI A, ALLEGRI F, BALESTRIERI G *et al.*: Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies. *Thromb Res* 2004; 114: 553-58.
- PETROVAS C, VLACHOYIANNPOULOS PG, KORDOSSIS T *et al.*: Antiphospholipid antibodies in HIV infection and SLE with and without anti-phospholipid syndrome: comparisons of phospholipid specificity, avidity and reactivity with beta2-GPI. *J Autoimmun* 1999; 13: 347-55.
- LOPEZ-SOTO A, CERVERA R, FONT J *et al.*: Isotype distribution and clinical significance of antibodies to cardiolipin, phosphatidic acid, phosphatidylinositol and phosphatidylserine in systemic lupus erythematosus: prospective analysis of a series of 92 patients. *Clin Exp Rheumatol* 1997; 15: 143-49.
- AMOROSO A, MITTERHOFER AP, DEL PORTO F *et al.*: Antibodies to anionic phospholipids and anti- β 2-GPI: association with thrombosis and thrombocytopenia in systemic lupus erythematosus. *Hum Immunol* 2003; 64: 265-73.
- BERTOLACCINI ML, ROCH B, AMENGUAL O *et al.*: Multiple antiphospholipid tests do not increase the diagnostic yield in antiphospholipid syndrome. *Rheumatology* 1998; 37: 1229-32.
- BIZZARO N, TONUTTI E, VILLALTA D *et al.*: Prevalence and clinical correlation of antiphospholipid-binding protein antibodies in anti-cardiolipin-negative patients with systemic lupus erythematosus and women with unexplained recurrent miscarriages. *Arch Pathol Lab Med* 2005; 129: 61-8.
- NOJIMA J, KURATSUNE H, SUEHISA E *et al.*: Anti-prothrombin antibodies combined with lupus anticoagulant activity is an essential risk factor for venous thromboembolism in patients with systemic lupus erythematosus. *Br J Haematol* 2001; 114: 647-54.
- VON LANDENBERG P, MATTHIAS T, ZAECH J *et al.*: Antiprothrombin antibodies are associated with pregnancy loss in patients with the antiphospholipid syndrome. *Am J Reprod Immunol* 2003; 49: 51-6.
- MATSUBAYASHI H, ARAI T, IZUMI S *et al.*: Anti-annexin V antibodies in patients with early pregnancy loss or implantation failure. *Fertil Steril* 2001; 76: 694-9.
- AVCIN T, AMBROZIC A, KUCHAR M *et al.*: Anticardiolipin and anti- β 2-glycoprotein I antibodies in sera of 61 apparently healthy children at regular preventive visits. *Rheumatology* 2001; 40: 565-73.
- AUDRAIN MAP, COLONNA F, MORIO F *et al.*: Comparison of different kits in the detection of autoantibodies to cardiolipin and beta2glycoprotein 1. *Rheumatology* 2004; 43: 181-5.
- AUDRAIN MAP, EL KOURI D, HAMIDOU MA *et al.*: Value of autoantibodies to β 2-glycoprotein 1 in the diagnosis of antiphospholipid syndrome. *Rheumatology* 2002; 41: 550-3.
- REBER G, SCHOUSBOE I, TINCANI A *et al.*: Inter-laboratory variability of anti-beta2-glycoprotein I measurement. A collaborative study in the frame of the European Forum on Antiphospholipid Antibodies Standardization Group. *Thromb Haemost* 2002; 88: 66-73.
- PELLIGRINO NM, CACCAVO D: Variability in anticardiolipin antibody detection: role of nonspecific IgG binding and different microtiter plates. *Clinical and Applied Thrombosis/Hemostasis* 2007; 13: 404-9.
- UTHMAN I, KHAMASHTA M: Ethnic and geographical variation in antiphospholipid (Hughes) syndrome. *Ann Rheum Dis* 2005; 64: 1671-76.
- SANCHEZ ML, KATSUMATA K, ATSUMI T *et al.*: Association of HLA-DM polymorphism with the production of antiphospholipid antibodies. *Ann Rheum Dis* 2004; 63: 1645-8.
- HORITA T, MERRILL JT: Genetics of antiphospholipid syndrome. *Curr Rheumatol Rep* 2004; 6: 458-62.
- CUCURULL E, GHARAVI AE, DIRI E *et al.*: IgA anticardiolipin and anti- β 2-glycoprotein I are the most prevalent isotypes in African American patients with systemic lupus erythematosus. *Am J Med Sci* 1999; 318: 55-60.
- DIRI E, CUCURULL E, GHARAVI AE *et al.*: Antiphospholipid (Hughes's) syndrome in African Americans: IgA aCL and α 2glycoprotein-I is the most frequent isotype. *Lupus* 1999; 8: 263-8.
- GRECO TP, AMOS MD, CONTI-KELLY AM *et al.*: Testing for the antiphospholipid syndrome: importance of IgA anti-beta2-glycoprotein I. *Lupus* 2000; 9: 33-41.
- MERONI PL: Pathogenesis of the antiphospholipid syndrome: an additional example

- of the mosaic of autoimmunity. *J Autoimmun* 2008; 30: 99-103.
40. SHERER Y, BLANK M, SHOENFELD Y: Antiphospholipid syndrome (APS): where does it come from? *Best Pract Res Clin Rheumatol* 2007; 21: 1071-78.
41. SHOENFELD Y, BLANK M, CERVERA R *et al.*: Infectious origin of the antiphospholipid syndrome. *Ann Rheum Dis* 2006; 65: 2-6.
42. OWENS S, CHAMLEY LW, ORDI J *et al.*: The association of anti-phospholipid antibodies with parity in placental malaria. *Clin Exp Immunol* 2005; 142: 512-8.
43. DANIEL-RIBEIRO CT, ZANINI G: Autoimmunity and malaria: what are they doing together? *Acta Trop* 2000; 76: 205-21.
44. SILVESTRIS F, FRASSANITO MA, COFFORIO P *et al.*: Antiphosphatidylserine antibodies in human immunodeficiency virus-1 patients correlate with evidence of T-cell apoptosis and mediate antibody-dependent cellular cytotoxicity. *Blood* 1996; 87: 5185-95.
45. WANDERLEY JLM, MOREIRA MEC, BENJAMIN A *et al.*: Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of *Leishmania (L) amazonensis* in mammalian hosts. *J Immunol* 2006; 176: 1834-9.
46. SPATH GF, GARRAWAY LA, TURCO SJ *et al.*: The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. *PNAS* 2003; 100: 9536-41.