Anti- 2 glycoprotein I and anticardiolipin antibodies in leptospirosis, syphilis and Kala-azar

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Abstract

Objective

Reports have shown that anticardiolipin (aCL) antibodies present in patients with autoimmune diseases are dependent on the cofactor 2 glycoprotein I (2 GPI), as opposed to aCL antibodies seen in infectious diseases such as syphilis, HIV, hepatitis C, etc. The assay for anti- 2GPI antibodies has been reported to be more specific for antiphospholipid syndrome (APS). However, the prevalence of these antibodies in diseases such as leishmaniasis and leptospirosis remains unknown. The aim of the present study was determine the prevalence of antibodies to cardiolipin and to 2GPI in patients with different infectious diseases, including leptospirosis, syphilis and leishmaniasis.

Methods

Samples from patients with Kala-azar (visceral leishmaniasis), syphilis or leptospirosis were tested for IgG and IgM anticardiolipin and IgG anti- 2GPI antibodies by ELISA.

Results

In patients with Kala-azar the prevalence of IgG aCL, IgM aCL and anti- 2GPI was 6% (2/30), 3% (1/30) and 53% (16/30), respectively. In syphilis the prevalence was 18% (14/74), 13% (10/74) and 10% (8/70), respectively. In leptospirosis the frequency of these antibodies was 23% (9/39), 10% (4/39) and 17% (6/34), respectively. There was no statistical correlation between aCL and anti- 2GPI antibodies in these diseases.

Discussion

This study clearly shows a significant prevalence of anti- 2GPI antibodies in leptospirosis and leishmaniasis and syphilis. This indicates that the assay for anti- 2GPI antibodies should be thoroughly validated before it is introduced as a definitive tool for the diagnosis of APS, testing a larger number of sera from patients with a wider range of clinical conditions.

Key words

Antiphospholipid antibodies, anticardiolipin antibodies, anti- 2 glycoprotein I, syphilis, leptospirosis, Kala-azar.

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Introduction

Antiphospholipid (aPL) antibodies have been associated with the presence of thrombotic complications and fetal loss in patients with autoimmune diseases, particularly systemic lupus erythematosus (SLE) (1, 2) and primary antiphospholipid syndrome (APS) (3). Previous reports have shown the dependence of anticardiolipin (aCL) antibodies on a cofactor, 2 Glycoprotein I (2GPI), in autoimmune diseases in contrast to infectious diseases where such antibodies would not depend on this cofactor (4). It has been suggested that the inhibition of 2GPI (a glycoprotein with anticoagulant properties *in vitro*) activity in plasma by aCL antibodies is responsible, at least in part, for their prothrombotic activity in patients with autoimmune diseases (5). Moreover, the detection of antibodies directed to this cofactor has been indicated as a more specific marker for APS than anticardiolipin antibodies (6, 7), and to be particularly associated with thrombotic manifestations.

On the other hand, aCL antibodies present in infectious diseases are not associated with clinical manifestations of APS or with a positive lupus anticoagulant test. However, some reports have recently indicated that anti- 2GPI antibodies can be found in patients with infectious diseases such as parvovirus B 19, AIDS , HTLV-1 etc, challenging the belief of a better specificity of the anti- 2GPI test (8-11) A false positive test for APS could lead to the dangerous misdiagnosis of patients and to unnecessary treatment. Hence, the importance of specific tests for diagnosis of APS.

The prevalence of aCL antibodies in a wide variety of infectious diseases, including syphilis, tuberculosis, mycoplasma, Lyme disease, Q fever, EBV, Mediterranean spotted fever, malaria, etc. has been reported in numerous publications (6, 12-17), but their prevalence as well as the prevalence of anti- 2GPI antibodies in leptospirosis (a spirochetal disease affecting the skeleton muscle, heart, and kidney) and in visceral leishmaniasis or kala-azar (a parasitic disease affecting the liver, spleen, and bone marrow) has not been determined. This study addresses those questions.

Material and methods

Patients

Three groups of patients with infectious diseases were studied. Thirty patients with a diagnosis of visceral leishmaniasis (Kala-azar) were included, before treatment. They came from an area in the northeast of Brazil in which this disease is endemic. The diagnosis was based on serologic tests and myelogram. Seventy-four consecutive syphilis patients being followed at Sexually Transmitted Diseases Unit were studied. The inclusion criterion was positivity of both VDRL and FTA-ABS tests in serum. The majority of the patients had already been treated with penicillin. Concomitant HIV infection was observed in 7 cases. Thirty nine cases of leptospirosis were studied. The diagnosis was based on clinical features and serologic tests. None of the patient in these 3 groups had a history of thrombotic events or repeated spontaneous fetal losses.

2GPI purification

Human 2GPI was purified by the perchloric acid treatment of normal serum followed by heparin-sepharose affinity purification as described previously (18) with some modifications. Perchloric acid 70% (1.25 ml) was added drop by drop under constant stirring to 50 ml of normal serum at 4°C and left stirring for 30 min. After centrifugation for 30 min at 10.000 g, the supernatant was neutralized to pH 7 and dialyzed overnight against 30 mM NaCl, 20 mM Tris HCl (pH 7.2) with two changes of buffer. This preparation was passed through two columns of affinity chromatography: The contaminating IgG were removed first with protein G (Pharmacia-Biotech) and then 2GPI was bound to a heparin-sepharose CL-6B (Pharmacia-Biotech) column. The heparin-sepharose column was washed with increasing molarities of NaCl (30-150 mM), 20 mM Tris pH 7.2. The 2GPI was eluted with NaCl 350 mM, Tris 20 mM pH 7.2. All of these steps were carried out at 4°C. The fractions with the highest optical density were

collected and, after concentration with PEG, analyzed by sodium dodecyl sulfate polyacrylamide gel electroforesis (SDS-PAGE) and immunobloting using rabbit anti-serum to 2GPI (19).

Antigenic specificity of anti- 2GPI: ELISA

The purity of the 2GPI was analyzed by SDS PAGE as described above and the specificity of this antigen in the ELISA system was determined using a rabbit anti-human 2GPI anti-serum and a known preparation of human 2GPI (19) as the positive control. Furthermore, goat anti-human IgG, IgM and IgA was used in this ELISA in order to search for immunoglobulin contamination in the preparation.

Anti- 2GPI assay

This assay was performed as described previously by Roubey *et al.* (20) with some modifications. The best antigen concentration for coating the plates as well as the conjugate concentration were determined before performing the ELISA. Microtitration high binding plates (Costar 3590) were coated with 50 µl/well of 2GPI in PBS (10 µg/ml) and left overnight at 4°C. These plates, which are gamma-irradiated by the manufacturer, were used because they had been shown to be suitable for detecting the anti- 2GPI antibodies in sera from patients with antiphospholipid syndrome in the absence of anionic phospholipid (20). The plates were blocked (100 µl/well) for 1 hour with 2% ovoalbumin (Sigma) in PBS. After washing with PBS the samples diluted 1:100 in 1% ovoalbumin 0.5% Tween 20 were added in duplicate (50 µl) to the plate and incubated for 2 hours at room temperature. The alkaline phosphatase conjugated goat anti-human IgG diluted 1:5000 in diluent solution was added (50 µl/well) and incubated for 1 hour. The reaction was developed using substrate p-nitrophenyl phosphate diluted in diethanolamine buffer and the optical density at 405 nm was read using a microplate reader. Furthermore, a positive control (patient with high aCL titer and APS) was used in each plate at three different dilutions: 1/100, 1/200 and 1/400. The color reaction in each plate was stopped when OD of the 1/100 dilution of the positive control reached 1.0 OD (this was approximately 15-20 min in the experimental conditions under which we ran this assay).The results were considered positive when the OD obtained exceeded that of the mean value plus 3 standard deviations of 50 sera from normal healthy individuals. Different runs were normalized by using 10 normal sera in each plate of anti- 2GPI ELISA. In order to express the results in units, the OD of each sample was subtracted from the cut-off value and multiplied by 100.

Anticardiolipin assay

The ELISA for anticardiolipin was performed as described elsewhere (21) with some modifications: Plates were coated overnight at 4°C with a 50 µg/ml solution of cardiolipin (Sigma) in ethanol. After evaporation of the solvent, the wells were blocked with 10% adult bovine serum (ABS) in phosphate buffered saline (PBS). The samples diluted in 10% ABS (1:50) were added to wells in duplicate and incubated for 2 hours at room temperature. Alkaline phosphatase conjugated goat anti human IgG and IgM (Sigma) was used as second antibody. The reaction was developed as described above. International calibrators (Louisville APL Diagnostics, Doraville, GA) were used to construct a calibrator curve and to express the results in GPL and MPL units for IgG and IgM aCL, respectively. Degrees of positivity were considered as follows: High positive >80 GPL/MPL units, Medium positive ≥ 20-80 units, low positive ≥10, < 20 units.

Statistical analysis

Quantitative variables were expressed as the mean ± SD. Pearson's coefficient of correlation was used whenever appropriate. The chi square test was used to determine the association between qualitative variables. Results were considered statistically significant when $p < 0.05$.

Results

A total of 143 patients were included in the present study. Thirty of them had the diagnosis of visceral leishmaniasis, 74 had syphilis and 39 had leptospirosis. Other demographic data are presented in Table I.

The frequency of anticardiolipin and anti- 2GPI antibodies in the three groups of patients is presented in Table II: IgG aCL were detected in the serum of two patients with Kala-azar (6%), one of them at low levels and the other one at moderate levels. A low level of IgM aCL was detected in the serum of only one patient (3%), who also had IgG aCL antibodies. Among the syphilis patients, IgG aCL were detected in 14/74 (18%) as follows: 4 with low, 9 with medium and 1 with high levels, respectively. IgM aCL were detected in 10/74 (13%), 3 with low levels and 7

Table I. Demographic characteristics of the three groups of patients with infectious diseases (n=143).

	Kala-azar (n=30)	Syphilis (n=74)	Leptospirosis (n=39)
Male/female	16/14	40/34	36/3
Mean age ± SD	11 ± 14	28 ± 9	36 ± 13
Median age (range)	6 (1-65)	26 (8-53)	35 (13-73)

Table II. Frequency of aCL (IgG and IgM) and IgG anti- 2 GPI in infectious diseases.

	IgG aCL	IgM aCL	Anti- 2 GPI
Kala-azar	2/30 (6%)	1/30 (3%)	16/30 (53%)
Syphilis	14/74 (18%)	10/74 (13%)	8/70 (10%)*
Leptospirosis	9/39 (23%)	4/39 (10%)	6/34 (17%)*

*A few samples were not available for anti- 2 GPI detection.

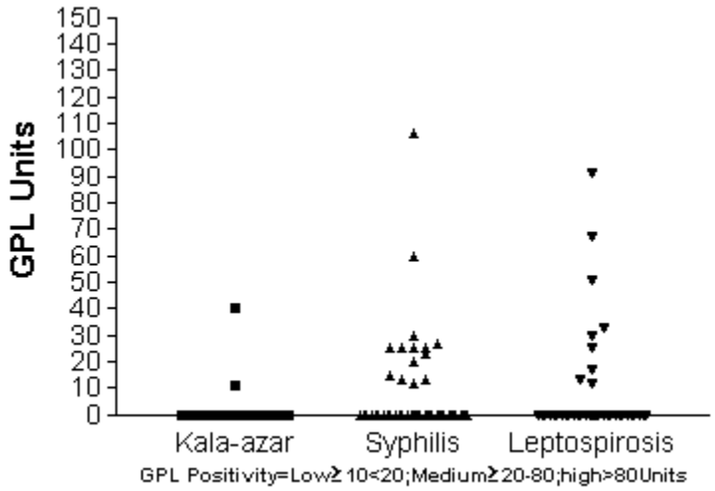

Fig. 1. IgG aCL in the 3 groups of infections diseases. Kala-azar; Syphilis; Leptospirosis.

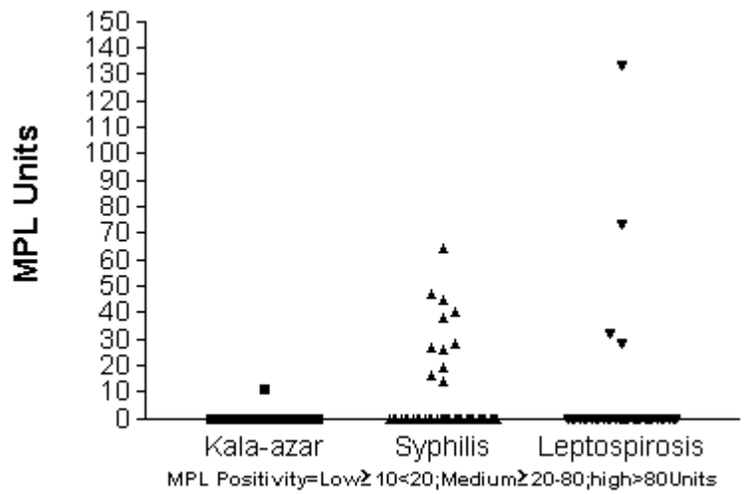

Fig. 2. IgM aCL in the 3 groups of infections diseases. Kala-azar; Syphilis; Leptospirosis.

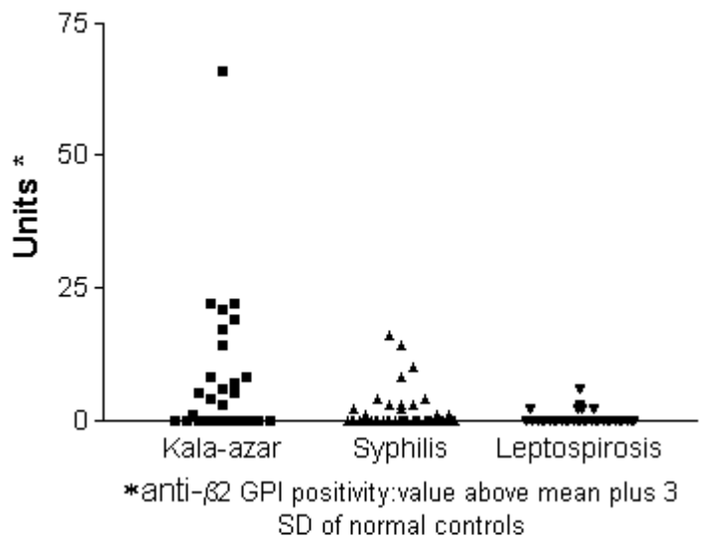

Fig. 3. Anti- 2 GPI in the 3 groups of infections diseases. Kala-azar; Syphilis; Leptospirosis.

with moderate levels. Two sera were positive for IgM and negative for IgG aCL. Thus, a total of 16 patients (21%) with syphilis had either IgG or IgM aCL antibodies. There was a correlation between the titers of VDRL and IgG aCL ($r = 0.49$, $p < 0.05$) and between VDRL and IgM aCL ($r = 0.51$, $p < 0.05$). IgG aCL were also detected in 9 patients (23%) with the diagnosis of leptospirosis (low levels in 3, moderate in 5, and high levels in one sera) and IgM aCL were demonstrated in 4 patients (10%), 3 with moderate and one 1 high levels. A total of 11 patients (28%) with leptospirosis had aCL antibodies. The levels of IgG and IgM aCL expressed in GPL and MPL are illustrated in Figures 1 and 2, respectively.

Anti- 2GPI were found in 16 of 30 patients (53%) with Kala-azar, in 8 of 70 patients (11%) with syphilis, and in 6 of 34 patients (17%) with leptospirosis. The serum concentration of anti- 2GPI antibodies in the 3 groups of patients is shown in Figure 3. No association between IgG anti- 2GPI antibodies and IgG aCL antibodies was found ($p = 0.16$). Similarly, no correlation was found between IgG anti- 2GPI antibodies and titres of IgG aCL ($r = 0.12$, $p = 0.17$).

Discussion

2GPI is a normal plasma protein with five domains and a molecular weight of approximately 50 kD. Interest in this protein has increased in the last few years because it has been described as a cofactor for anticardiolipin binding (4, 22, 23). It has been suggested that the phospholipid binding site on 2GPI is located in the fifth domain (24). 2GPI has a negative effect on coagulation, inhibiting the contact activation of the intrinsec pathway (25), and can diminish platelet aggregation *in vitro* (26). Antibodies against this protein may theoretically have a prothrombotic effect, as has been suggested by studies demonstrating an association between anti- 2GPI detected by ELISA and thrombotic events in SLE or primary antiphospholipid syndrome (PAPS) (5, 27-29). Moreover Sanmarco *et al*. (6) reported an anti- 2GPI prevalence of 54% in primary or secondary APS, 9%

in SLE without APS and only 0.7 % in patients with different infectious diseases including hepatitis C, HIV, Q fever, Mediterranean spotted fever and syphilis. They concluded that the anti-2GPI assay had more specificity and predictive value than anticardiolipin for the APS, although with less sensitivity. Similarly, Guerin *et al*. (7) observed significantly elevated levels of anti-2GPI antibodies in patients with "putative antiphospholipid syndrome" and only "marginally elevated levels" of these antibodies in patients with SLE, HIV, infectious mononucleosis or stroke. A search for anti- 2GPI in patients with syphilis resulted negative. The authors concluded that the sensitivity and specificity of anti- 2GPI for definite or probable antiphospholipid syndrome (APS) was 83% and 95%, respectively.

In the present study, we demonstrated a positivity of anti- 2GPI in the serum of 53% of patients with visceral leishmaniasis (Kala-azar), 17% of those with leptospirosis and 10% of those with syphilis. However, we did not find an association between anticardiolipin and anti- 2GPI antibodies in the sera of patients with infectious diseases. Similarly Faghiri *at al*. (9) found anti-2GPI in 12% of HTLV-1 associated myelopathy/tropical spastic paraparesis and no correlation between anticardiolipin and anti- 2GPI antibodies in these sera. Interestingly, in a recent letter published in *Lupus*, Guérin *et al*. (10) found anti- 2GPI antibodies in the plasma of 17% (7/41) patients with different acute infectious diseases selected by the positivity of lupus anticoagulant. After recovery, the anti-2GPI antibodies were no longer detected. A transient positivity of the anti- 2GPI in our patients could not be excluded since we did not repeat the test in subsequent samples.

Characteristically the detection of antiphospholipid antibodies in infectious diseases is not associated with thrombotic complications. The reason why these antibodies would have different behavior in infectious diseases is not known. The concept that no dependence of 2GPI for these antibodies has been defended by most authors. Should this concept be true, one would not expect to find anti- 2GPI antibodies in infectious diseases sera. Forastiero *et al*. (30) found anti- 2GPI in only one of 55 syphilis patients. In contrast, in their study 82.8% of samples with anticardiolipin antibodies from patients with SLE or APS were anti-2GPI positive. However, in other studies described above, as in ours, antibodies to 2GPI were found to be present in larger percentages in infectious diseases. Similarly, Loizou *et al*. (31) demonstrated the presence of 2GPI-dependent anticardiolipin antibodies in 6/12 patients with parvovirus B19 and Weiss *et al*. (11) demonstrated anti-2GPI in 8/107 (7.4%) HIV-infected patients, none of them with evidence of arterial or venous thrombosis. These authors also did not find a correlation with anticardiolipin antibodies. These data may challenge the specificity of anti- 2GPI as a marker for thrombotic complications. Alternatively, it suggests that there may be anti- 2GPI antibodies with different functional activity. On the other hand, the divergent prevalence of anti- 2GPI in infectious diseases may be due to methodological reasons. It has been suggested that antibody binding to 2GPI in the absence of phospholipid occurs when irradiated plastic plates are used (32), because irradiation is able to increase the anionic charges in the surface of the plate. New epitopes (cryptic) are formed by the interaction between 2GPI and those anionic surface that are recognized by anti- 2GPI antibodies. The utilization of irradiated or non-irradiated plates, may detect anti- 2GPI antibodies with different specificity. Alternatively, such differences in the plates could discriminate between antibodies with different affinities to 2GPI or yet allow binding of different amounts of 2GPI.

Presently, the origin of the induction of these autoantibodies in infectious diseases is not known. It could be secondary to a cross-reaction between protein from the microorganism and 2GPI or, alternatively, be due to a polyclonal increase in immunoglobulin levels seen in these infections.

In previous studies we have demonstrated the presence of anticardiolipin antibodies in infectious diseases such as tuberculosis, Hansen's disease, infectious endocarditis and syphilis (14, 15). In the present study we confirm this positivity in patients with syphilis, and add visceral leishmaniasis and leptospirosis to the list of infectious diseases associated with aCL positivity. To our knowledge this represents the first description of the presence of antiphospholipid antibodies in leishmaniasis. In a broad review of the English literature we were able to find only one study of anticardiolipins in leptospirosis (33). In that study, the authors found IgG aCL antibodies in 8 out 16 patients with leptospirosis, all of them with severe complications of the disease.

In conclusion, this study shows that anticardiolipin and anti- 2GPI are present in leptospirosis, syphilis and leishmaniasis sera. The clinical significance, if any, of anti- 2GPI antibodies in these diseases is not understood. We propose that before the anti- 2GPI ELISA is adopted for confirmation of the diagnosis of APS, further studies including standardization of the method and the utilization of a wider range of clinical conditions, be carried out. As recommended at the 8th International Symposium on Antiphospholipid Antibodies in Sapporo, Japan (34), the anticardiolipin and lupus anticoagulant tests remain the first line of tests to be carried out to help in the diagnosis of APS.

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