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β_2-Glycoprotein I-dependence of anticardiolipin antibodies in multibacillary leprosy patients

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Summary This study was undertaken to investigate the influence of β_2-glycoprotein I (GPI) on anticardiolipin antibody (aCL) titration in leprosy. The study group consisted of 140 sera from patients with multibacillary leprosy (46 borderline, 94 lepromatous). The group included newly diagnosed, previously untreated patients, patients under treatment and patients released from treatment. GPI addition enhanced significantly the aCL titres in sera from lepromatous leprosy but not in those from borderline leprosy. Moreover, when the patients were classified according to their bacteriological status, aCL titres were found to be significantly higher in skin smear positive patients compared to bacteriologically negative patients. Thus, the present study demonstrates that aCL in multibacillary leprosy patients are mainly of the GPI-dependent type and emphasizes the importance of GPI addition for aCL titration in leprosy.

Introduction

Anticardiolipin antibodies (aCL) are immunoglobulins that react with negatively charged phospholipids. aCL have been frequently detected in blood samples from patients with systemic lupus erythematosus (SLE) and in patients with infectious diseases such as syphilis, HIV-infection and mycobacterioses.[1]

aCL have been reported in 50–80% of multibacillary leprosy patients.[2–4] Their production and clinical significance in leprosy is still unknown. We have previously reported that aCL titration is a useful tool to improve significantly the specificity of leprosy serodetection when it is combined with an anti-phenolic glycolipid-I antibody immunoassay.[5]

Recently, it has been shown that some aCL require β_2-glycoprotein I (GPI, also called

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apolipoprotein H), a plasma cofactor, for optimal detection in ELISA assays. The addition of GPI to aCL ELISA identifies two distinct types of aCL, GPI-independent aCL, which recognizes only CL, and GPI-dependent aCL, which binds to the CL/GPI complex.[6]

In view of these recent findings, we studied the influence of GPI on aCL binding to CL in blood samples from patients with multibacillary leprosy. We also attempted to establish whether the presence of aCL is related to the bacteriological status of leprosy.

Materials and methods

SERA

The study group consisted of sera from 140 patients with multibacillary leprosy. Sixty-three of these patients were from Italy, 46 from Eritrea and 31 from other areas (Latin America, Asia, North Africa). Leprosy was diagnosed on clinical and bacteriological grounds and confirmed by histopathology in a minority of cases. Patients were classified at the time of initial examination according to the Ridley-Jopling scale and grouped as borderline (including multibacillary BT, BB and BL) and lepromatous (LL). The disease status was established at the time of blood collection. The patients were classified as bacteriologically positive or negative on the basis of their skin smear status. The study group included newly diagnosed, previously untreated patients, patients under antileprosy treatment and patients released from treatment. Detailed information on the leprosy group is summarized in Table 1.

One hundred and four sera from SLE patients and 123 sera from healthy volunteers served as positive and negative controls.

ANTICARDIOLIPIN ASSAY

Serum from all subjects was assayed for aCL in the absence (CL-ELISA) and after addition of GPI (GPI-CL-ELISA) using a technique previously described.[7] Briefly, ELISA plates (ICN Linbro, #76-381-04)) were coated with cardiolipin (Sigma) in ethanol (2·5 μg/50 μl per well) at 4°C overnight. In order to evaluate the different aCL subpopulations, the even columns of each plate were incubated with a source of GPI (GPI-CL-ELISA). In a pilot experiment with autoimmune sera, postcoating with fetal calf serum (FCS) as a source of GPI produced similar

Table 1. Clinical classification, bacteriological status and drug history of leprosy patients

	Borderline ($n = 46$)	Lepromatous ($n = 94$)
Smear-positive		
untreated	22	16
under treatment	3	33
Smear-negative		
released from treatment	18	31
under treatment	3	14

results to purified GPI (OD CL/FCS versus OD CL/GPI had $n = 12$, $t = 1{\cdot}081$ with $p = 0{\cdot}305$) as also previously reported by other authors.[8,9] When test samples were replaced by purified IgG, the results were unchanged. This indicated that endogenous GPI depending on sera dilution was irrelevant for antibody determination at the working serum dilution of 1:100. In view of this pilot study, the even columns of each plate were washed with phosphate buffered saline (PBS) and incubated with 50 μl of 10% FCS in PBS for measurement of GPI-dependent aCL and the odd columns were incubated in PBS alone for measurement of GPI-independent aCL. Incubation was at 20°C for 2 h. After washing with PBS (120 μl/well), the plates were blocked with 0·3% gelatin or 1% electrophoretically purified bovine serum albumin (BSA, Sigma) in PBS (100 μl/well) at 20°C for 1 h. Diluted control samples (normal human serum and aCL high-titre serum) and test sera (1:100 in 1% BSA/PBS) were then added in duplicate in odd and even columns in order to evaluate GPI-independent and GPI-dependent antibodies on the same plate. After incubation at 20°C for 3 h and three washings with PBS, 50 μl of peroxidase-conjugated rabbit anti-human IgG (DAKO1, 1:1000 on 1% BSA/PBS) were added in each well and incubated at 20°C for 1 h. After three washings with PBS the plates were incubated with freshly prepared ABTS (ICN-Flow, 2,2′-azino-di-[3-ethyl-benzthiazoline sulphonate]/H_2O_2, 100 μl/well). The reaction developed in the dark at 20°C and its strength was read when the 414-nm optical density (OD) of the standard high-titre wells reached 0·9–1·1 values. Blanks obtained from uncoated wells on the same plate were subtracted to account for non-specific binding.

Optical density values were converted into a range in which 30 IU/ml was the upper limit. Such a value corresponds to 100 GPL units when compared with a standard curve using commercial aCL standard sera (Antiphospholipid Associated, Louisville, KY, USA).[10]

Antibody titres follow a log-normal distribution and were transformed before analysis. Results below the lower limit of detection of the assay were assigned the value of the lower limit of detection (6 IU/ml) to allow more accurate comparison by parametric methods after log transformation of data. Laboratory ranges were determined from the healthy subject group. Normal values (< mean + 3 SD) were taken as <42·6 GPL for CL-ELISA and <38·6 GPL for GPI-CL-ELISA.

STATISTICAL ANALYSIS

The significance of the differences between the two ELISA assays was tested by Student's test for continuous variables, and McNemar's test for categorical values. Comparison of antibody titres within each ELISA assay was done by ANOVA.

Results

To characterize GPI dependency, sera from patients with multibacillary leprosy, patients with SLE and healthy subjects were compared for their antibody binding to CL in the presence and absence of GPI. Addition of GPI enhanced significantly antibody binding in both the leprosy group (mean 49·83, SD 55·75, 95% CI 10·23, −1·06, $p = 0{\cdot}0076$) and the SLE group (mean 36·4, SD 35·2, 95% CI 0·41, 7·10, $p = 0{\cdot}0283$) but not in the control group (mean 9·3, SD 11·1, 95% CI −3.78, 0·22, $p = 0{\cdot}0812$).

Table 2. Antibody titres in the leprosy subgroups, expressed as GPL ± SD

	n	CL-ELISA	GPI-CL-ELISA	95% CI for difference	*P*[a]
Borderline					
smear-positive	25	35·0 ± 41·0	37·2 ± 37·6	−8·87, 4·43	0·4978
smear-negative	21	46·6 ± 33·3	44·3 ± 30·7	−6·04, 10·78	0·5629
Lepromatous					
smear-positive	49	69·1 ± 43·8	84·4 ± 30·9	−24·5, −6·00	0·0018
smear-negative	45	38·5 ± 34·7	40·2 ± 31·4	−8·55, 5.12	0·6241

[a]Paired *t*-test (two-tailed) among assays within each group.

Comparison of categorical values was done on the leprosy group. Of the 140 leprosy sera, GPI-independent aCL were detected in 76 (54·3%) sera. When GPI was added to the assay system, 93 (66·4) sera resulted to be positive. These included 72/76 sera that were GPI-dependent aCL positive and 21 sera which became positive only after GPI addition.

For further evaluation, leprosy sera were divided in four subgroups according to clinical form and bacteriological status. We found that GPI addition enhanced significantly aCL titres (Table 2) and the proportion of aCL-positive patients (Table 3) only in sera from bacteriologically positive LL. In contrast, there was no significant enhancement in any of the other three leprosy subgroups.

To determine whether the presence of either GPI-independent aCL or GPI-dependent aCL is related to leprosy activity, antibody binding in bacteriologically positive and negative leprosy was compared. There was a significant difference of antibody titres between smear-positive LL and smear-negative LL in both the CL-ELISA (mean 69·1, SD 39·7, $F = 13·949$, $p = 0·0003$) and the GPI-CL ELISA (mean 84·4, SD 31·1, $F = 47·189$, $p < 0·0001$). The significant difference resulted also by comparing categorical values ($p = 0·00151$). By contrast, there was no difference between sera from smear-positive borderline leprosy and sera from smear-negative borderline leprosy.

Table 3. Results of the two aCL assays in the leprosy subgroups

	n	CL-ELISA positive	GPI-CL-ELISA positive		*p*[a]
Borderline					
smear-positive	25	9	11	(8)[b]	0·3085
smear-negative	21	11	11	(10)[b]	0·1572
Lepromatous					
smear-positive	49	35	47	(35)[b]	0·0015
smear-negative	45	21	24	(10)[b]	0·2248

[a]McNemar's test.
[b]Sera in which CL-ELISA and GPI-CL-ELISA were both positive.

Discussion

For solid-phase immunoassays of aCL, optimal concentration of GPI is critical for recording valid and consistent results. Most of the previous studies on aCL in leprosy did not describe in detail the ELISA method they employed. It is quite likely that they used adult bovine serum (ABS) to block the plates and to dilute the serum samples as recommended by Harris *et al.*[11] If this was the case, the assays may have detected GPI-dependent aCL. Binding of GPI-dependent aCL, however, requires the presence of GPI in a dose-dependent fashion; in these assays, GPI concentration was neither established nor optimized.

In our study, GPI influence on aCL titration was investigated by using two ELISA assays, CL-ELISA and GPI-CL-ELISA. Each serum was tested by the two assays on the same plate, in order to exclude errors due to inter-plate variations. The first intention of our study was to investigate whether GPI is relevant for enhancing antibody binding in leprosy patients. Analysis of variance of antibody titres and comparison of categorical values demonstrated that optimal addition of GPI enhances significantly aCL titration in sera from multibacillary leprosy and in those from SLE. This is not surprising, because lepromatous leprosy has been associated with serological features traditionally linked to autoimmune disorders, such as polyclonal B cell activation with multiple antibodies including antinuclear antibodies.[12]

Our findings, however, are different from those reported by Hojnik *et al.*[13] In their study, these authors reported a surprisingly high frequency of aCL antibodies in leprosy sera. In addition, they found that sera that gave increased aCL levels in the CL-ELISA without GPI, displayed increased levels also in the modified aCL-ELISA with GPI. Accordingly, they concluded that leprosy-induced aCL do not show any consistent dependency on GPI. The discrepancy between their results and our study may be due to differences in the sampling of the study group and/or in the technical procedures (e.g. blocking agent, diluting buffer).

An important question is whether GPI, not CL/GPI complex, is the true antigen for aCL. In a previous study,[7] we reported that sera that were positive in our GPI-CL-ELISA showed no binding to plates coated with purified GPI, as confirmed by immunoblotting using purified GPI. This indicates that both GPI and CL are necessary to form the epitope to which GPI-dependent aCL are directed. Nevertheless, whether the epitope is a complex epitope formed by both GPI and CL or a neoepitope exposed on either of the components by their mutual interaction remains unknown.

The second aim of our study was to investigate whether the presence of aCL is related to the form and the bacteriological status of the disease. For this purpose, the leprosy group was divided in four subgroups: smear-positive LL, smear-negative LL, smear-positive borderline, smear-negative borderline. Our study demonstrates that GPI addition is significantly relevant only in those patients with bacteriologically positive LL. Of interest was the observation that aCL titres were significantly higher in skin smear positive LL compared with smear-negative LL in paired tests in both assays. This difference was even more evident in the GPI-CL-ELISA.

In conclusion, the present study demonstrates that aCL in multibacillary leprosy patients are mainly of the GPI-dependent type. This emphasizes the importance of optimal addition of GPI in the immunoassay for aCL detection in leprosy. Although the clinical significance of GPI-dependent aCL in leprosy, if there is any, is not known, the relationship between aCL and bacteriologically positive lepromatous leprosy strengthens our previous report[7] on the

potential use of aCL titration, in association with species-specific serological tests, to improve serodetection of subclinical lepromatous leprosy.

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