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Inhibitor(s) of natural anti-cardiolipin autoantibodies

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SUMMARY

IgG fractions were purified on Sepharose anti-human IgG column from eight sera of healthy donors, having no anti-cardiolipin (aCL) activity as measured by anti-cardiolipin ELISA assay (aCL-ELISA). All the IgG fractions, after elution with 4·9 M $MgCl_2$, reacted with CL. The antigen-binding characteristics of the IgG fractions purified from normal human serum (NHS) were similar to those of IgG fractions purified from sera of four patients with the anti-phospholipid syndrome (APLS). Competition assay confirmed the specificity of the binding of the purified IgG fractions to CL. The same results have been achieved with IgG fractions purified on Sepharose Protein-A column. The binding to CL was completely inhibited by either whole NHS and sera from various animal species, or by β_2-glycoprotein I (β_2-GPI). Our results support the notion of the existence of both natural anti-CL antibodies and serum inhibitor(s) in sera of healthy individuals. It is conceivable that in part the pathogenesis of APLS entails defects in the natural inhibitors of aCL antibodies.

Keywords anti-phospholipid antibodies cardiolipin aCL-ELISA autoantibodies β_2-GPI

INTRODUCTION

Anti-phospholipid antibodies (APLA) are autoantibodies detected in the sera or plasma of patients with autoimmune disorders or infectious diseases, such as syphilis [1]. APLA are detected in serum by ELISA assay, in which cardiolipin (CL) is most commonly used as an antigen (aCL-ELISA).

The presence of APLA characterizes the anti-phospholipid syndrome (APLS) [2,3], which consists of thrombocytopenia, thromboembolic phenomena and recurrent fetal loss. The pathogenic role of APLA has been recently shown in a series of experimental models [4,5].

Recent studies have reported that factors present in the serum or in the plasma can affect the binding of APLA to the negatively charged phospholipids. A cofactor β_2-glycoprotein I (β_2-GPI), which is required for APLA binding to negatively charged phospholipids, has been identified [6,7]. The exact role played by the cofactor β_2-GPI is still controversial [8]. While McNeil *et al.* [6] and Galli *et al.* [7] claim that the β_2-GPI is an absolute requirement for the binding of aCL antibodies, Harris *et al.* [9] state that although the cofactor enhances the binding of purified APLA, this enhancement differs between antibody preparations, and the cofactor is not an absolute requirement for the binding. Recently it has been shown by Gharavi [10] that high avidity IgG APLA hardly require the presence of the cofactor for their binding to phospholipids. Variability in β_2-GPI dependency has been found in different subclasses of immunoglobulins within a single patient's serum [11].

Hunt *et al.* [12] and Matsuura *et al.* [13] showed that infection-induced aCL antibodies can bind to anionic phospholipids in the absence of β_2-GPI, and the cofactor can even inhibit their binding. On the other hand, binding of aCL antibodies from patients with autoimmune disorders is significantly enhanced by β_2-GPI.

Sammaritano *et al.* [14] have described how placental anticoagulant protein-I (PAP-I), a calcium-dependent phospholipid binding protein, inhibits the binding of APLA in ELISA assay.

Heat inactivation of normal serum revealed specific aCl antibodies, as described by Cheng [15]. It has been suggested by this group that serum factor(s) mask these antibodies in the usual immunoassay.

In this study we have investigated the presence of aCL antibodies in normal human serum. We show that IgG fractions purified from sera of normal healthy donors bind specifically to CL in aCL-ELISA assay. This binding is inhibited by the addition of normal human sera (NHS) and sera from various animal species. We also found that binding of aCL from healthy donors was inhibited by β_2-GPI.

MATERIALS AND METHODS

Patients' sera

Blood was withdrawn by venepuncture from healthy donors (sera 116, 148, 149, 254, 252, 253, 255, 264) and from patients

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with APLS (sera 159, 257, 494, 484), centrifuged and the sera were separated and kept at −70°C until used.

IgG purification

IgG was purified from human sera on Sepharose anti-human IgG column (BioMakor, Rehovot, Israel) or on Sepharose Protein-A column (Sigma Chemical Co., St Louis, MO). The IgG was eluted by 4·9 M $MgCl_2$ (Sigma), and was dialysed against PBS (pH 7·4). IgG concentration was determined by the OD at 280 nm.

Detection of cardiolipin antibodies (aCL-ELISA)

Polysterene 96-well ELISA plates (Costar, Cambridge, MA) were coated with 100 μg/ml CL (Sigma) in ethanol, 50 μl/well. The plates were dried overnight at 4°C and were blocked with 0·5% gelatin/PBS for 2 h at room temperature. Gelatin was used as a blocker and a diluent, since the presence of inhibitory factors in sera was examined by the aCL-ELISA assay. After three washings with PBS, 50 μl of either the serum or the IgG fraction, diluted in 0·3% gelatin/PBS, were added and the plates were incubated for 2 h at room temperature. The plates were washed three times and an anti-human IgG conjugated to alkaline phosphatase (Sigma) diluted in 0·3% gelatin/PBS was added. The plates were incubated overnight at 4°C. The assay was developed by adding *p*-nitrophenyl phosphate (Sigma) in buffer carbonate pH 9·8 and the OD was read at 405 nm.

Competition aCL-ELISA

IgG fractions (25 μg/ml, concentration of about 50% of the maximal binding), diluted in 0·3% gelatin/PBS, were pre-incubated with 5–150 μg/ml CL for 2 h at room temperature with constant shaking. Control samples consisted of 25 μg/ml of each IgG fraction, pre-incubated with the buffer alone. Then 50 μl of the mixtures were added to CL-coated plates for aCL-ELISA assay. The percentage of inhibition was calculated as follows:

$$\frac{(\text{OD of control sample} - \text{OD of inhibited sample})}{\text{OD of control sample}} \times 100$$

Inhibition of the binding of natural aCL antibodies to CL with NHS

IgG fractions (25 μg/ml) diluted in 0·3% gelatin/PBS were mixed with serum dilutions 1:50–1:3200 (final dilutions). After 2 h of incubation at room temperature, 50 μl of the mixtures were added to ELISA plates for aCL-ELISA. The residual binding was calculated as the percentage of the binding of the same amount of the IgG in the absence of serum (100%).

The effect of β_2-GPI on the binding of aCL antibodies

IgG fractions (50 μg/ml) diluted in 0·3% gelatin/PBS were mixed with β_2-GPI (10 μg/ml, 30 μg/ml). After 30 min of incubation at room temperature, 50 μl of each mixture were added to ELISA plates for aCL-ELISA. The residual binding was calculated as the percentage of the binding of the same amount of the IgG in the absence of β_2-GPI. (β_2-GPI was the kind gift of Dr A. Tincani, Servio Immunologia Clinica Brescia, Italy.)

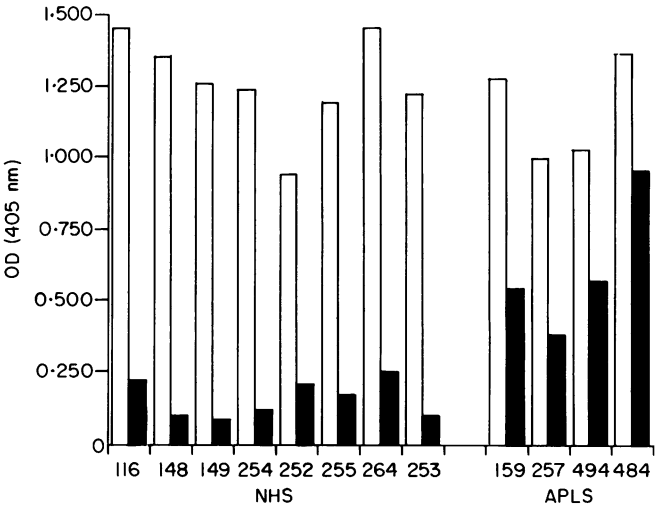

Fig. 1. Binding of IgG and sera in anti-cardiolipin (aCL)-ELISA. Binding of IgG fraction (□, 50 μg/ml), and serum (■, 1:200) from eight healthy individuals (116, 114, 149, 252, 253, 254, 255, 264) and four patients with anti-phospholipid syndrome (APLS) (159, 257, 494, 484) to CL in aCL-ELISA assay. The IgGs were purified on a Sepharose anti-human IgG column and eluted by 4·9 M $MgCl_2$.

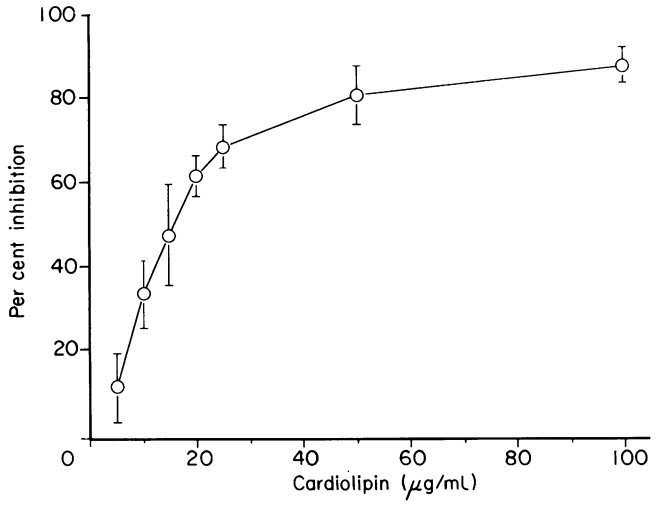

Fig. 2. Specificity of IgG fraction binding by anti-cardiolipin (aCL) competition assay. IgG fractions (25 μg/ml) from six normal healthy individuals (NHS) were pre-incubated with increasing concentration of CL (5–100 μg/ml). The mixture (50 μl) was then added to aCL-ELISA assay. Each point represents the mean ± s.d. of the inhibition percentage of six different IgGs. Each sample was tested three times.

RESULTS

IgG fractions were purified on Sepharose anti-human IgG column, from the sera of eight healthy donors having no anti-cardiolipin activity, as measured by aCL-ELISA (Fig. 1), and from four APLS patients all having high aCL titres. The purity of the IgG fractions was confirmed by SDS-PAGE.

All the purified IgG fractions, including those derived from NHS, reacted with CL in aCL-ELISA (Fig. 1). Similar CL binding characteristics of the IgG fractions from the healthy donors and from the patients with APLS were found. Prelimin-

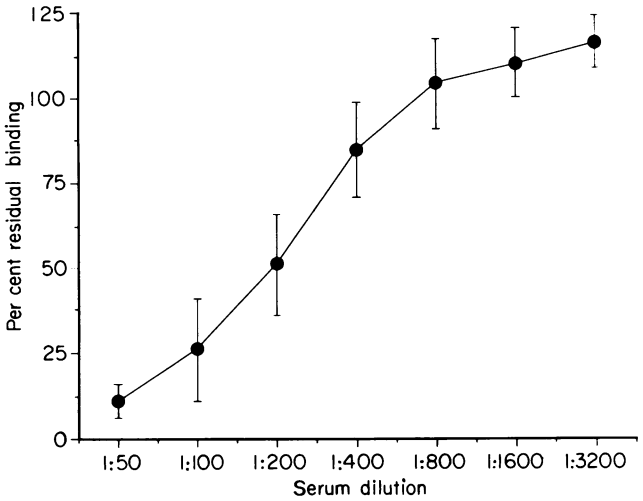

Fig. 3. Serum inhibition of the binding of purified IgG fractions to cardiolipin. IgG fractions (25 μg/ml) from eight human sera were pre-incubated with their respective unfractionated sera (diluted 1:50–1:3200). Each mixture (50 μl) was then added to the anti-cardiolipin (aCL)-ELISA assay. The residual activity was calculated as the percentage of the activity of the same amount of the IgG pre-incubated with buffer alone (100%). Each point represents the mean $\pm$ s.d. of eight IgG fractions. Each sample was tested four to five times.

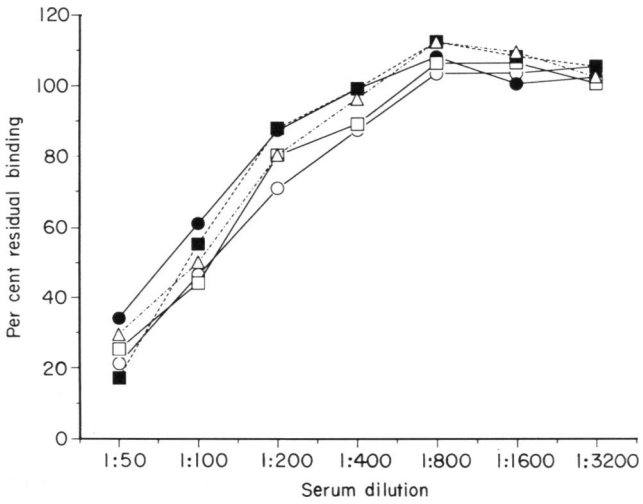

Fig. 4. Inhibition of the binding of IgG fraction from healthy subjects to cardiolipin (CL) by various animal sera. IgG fractions of six human sera (25 μg/ml) were pre-incubated with dilutions of 1:50–1:3200 of sera from various animals. The pre-incubation conditions, the anti-cardiolipin (aCL)-ELISA assay and the calculation of the residual activity, were the same as in Fig. 3. Each point represents the mean of six different IgG fractions pre-incubated with one dilution of the same serum (s.d. = 5 $\pm$ 13). ○, Bovine; ●, chicken; □, horse; ■, mouse; △, rabbit.

ary experiments indicated that the IgG fractions which were purified on a Sepharose-Protein A column and eluted by 4·9 M $MgCl_2$ gave similar results.

Competition assays confirmed the specific binding of the IgG fractions to CL (Fig. 2). Pre-incubation of the IgG fractions with increasing concentrations of CL (5–100 μg/ml) led to a nearly total inhibition of the binding to CL in the aCL-ELISA.

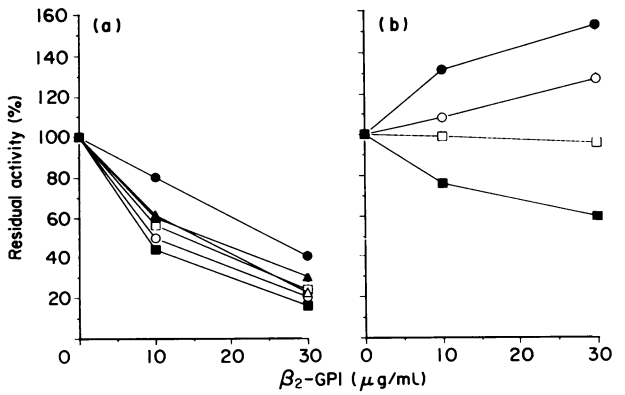

Fig. 5. The effect of β_2-glycoprotein I (β_2-GPI) on anti-cardiolipin (aCL) antibodies. IgG fractions (50 μg/ml) from normal healthy individuals (NHS) (a) and from serum of an anti-phospholipid syndrome (APLS) patient (b) were pre-incubated with β_2-GPI (10 μg/ml, 30 μg/ml) for 30 min. Each mixture (50 μl) was then added to aCL-ELISA. The caculation of the residual binding was the same as in Fig. 3.

To examine the possibility that the natural aCL autoantibodies in normal serum are inhibited by the existence of a natural serum inhibitor, we have pre-incubated a constant concentration of the IgG fractions (25 μg/ml, 50% of the maximal binding) with increasing dilutions of whole NHS. Each IgG was pre-incubated with its respective serum. NHS inhibited the binding of aCL antibodies in an aCL-ELISA assay in a dose-dependent manner (Fig. 3). The binding of the IgG fractions was almost totally inhibited by 1:50 dilution of the respective sera, about 10–15% of the binding of the same amount of IgG pre-incubated with the buffer (100%). There was no inhibition of binding when sera were diluted 1:800–1:1600. Paradoxically, higher dilutions enhanced the binding of the purified IgG fraction to CL. The non-respective human sera gave similar inhibition results. Bovine, chicken, horse and mouse animal sera were found to have similar inhibitory properties to NHS (Fig. 4).

The effect of β_2-GPI on the binding of aCL antibodies was examined (Fig. 5). β_2-GPI inhibited the binding of all the IgG fractions from NHS in a dose-dependent manner. In the presence of 30 μg/ml β_2-GPI the binding of these aCL antibodies was only 20–25% of the binding without the glycoprotein. The binding of the aCL from APLS patients was not affected in the same way by the β_2-GPI. The binding of two of the aCL antibodies was enhanced, one was inhibited and one remained unchanged.

DISCUSSION

In this study we have shown the presence of 'hidden' natural aCL autoantibodies in normal human sera. These antibodies are detected in the sera of healthy individuals and can be found in IgG fraction purified on Sepharose anti-human IgG column or Protein-A column and eluted with 4·9 M $MgCl_2$.

The binding of these aCL antibodies in aCL-ELISA is inhibited by factor(s) present in sera of healthy human as well as of various animal species. These results are in accordance with previous reports by Cheng [16,17], who showed that normal

human sera, if heat-inactivated, had detectable APLA in aCL-ELISA. The IgG purification, as well as the heat inactivation, may eliminate different inhibitory factors in the serum.

We found that β_2-GPI inhibited the binding to CL of all the aCL antibodies from NHS in a dose-dependent manner. The binding of two of the aCL antibodies from APLS patients was enhanced, one was inhibited and one was unchanged.

β_2-GPI was shown to enhance the binding of aCL antibodies from patients with systemic lupus erythematosus (SLE), but to inhibit the binding of such antibodies from patients with syphilis and other infectious diseases. The opposite interaction of β_2-GPI with aCL from sera of patients with autoimmune diseases and infectious diseases, and as shown here with natural autoantibodies, may suggest that we are dealing with different antibody populations. These antibodies may carry different idiotypes [18,19], and may differ in their pathogenic potential.

β_2-GPI may not be the only serum factor that affects the binding of aCl antibodies. Another possibility is the placenta anticoagulant protein-I. This protein, present in serum and other extracellular fluids, was shown by Sammaritano [20] to inhibit the binding of aCL antibodies to their antigen in aCL-ELISA.

The actual detection of APLA by aCL-ELISA in the sera of patients with autoimmune diseases such as SLE or APLS may not indicate an abnormal raised antibody titre, but may rather reflect a lack or reduction of normal regulatory inhibitory factor(s) in the sera.

The APLA present in the sera of healthy individuals could represent production of natural autoantibodies. These antibodies are indistinguishable from pathogenic antibodies, and their production is usually limited to minute amounts by suppressor mechanisms. A failure of this mechanism would lead to the production of autoantibodies in large amounts, which in combination with environmental factors or with a specific HLA can lead to the development of an overt disease [16,20]. Indeed, it has been reported that APLA, among other autoantibodies, can be triggered by various endotoxins [17].

It is important to emphasize that the sera of APLS patients gave higher levels of binding to CL than those of NHS, which can be routinely used to measure aCL antibodies of suspected APLS patients.

The nature of the aCL antibodies as well as their interaction with serum inhibitory factor(s) may be important in the pathogenesis of APLA complications, such as thrombosis or recurrent abortions, in the patient with APLS, and make the major difference between health and disease.

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