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Autoantibodies Against All the Phospholipids: A Comparative Systematic Study with Systemic Lupus Erythematosus and Healthy Sera

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Summary: The sera of systemic lupus erythematosus patients were tested by ELISA for the presence of autoantibodies against all the phospholipids: cardiolipin, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylserine. The quantity of phospholipid coated (in comparison with that initially deposited) on the microtitration plates was precisely evaluated in order to determine if the results (absorbance values) obtained for each phospholipid could be compared directly. The systemic lupus erythematosus sera tested gave positive results for all the phospholipids. The highest level of autoantibodies was observed with phosphatidic acid followed by phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylglycerol, phosphatidylcholine and phosphatidylethanolamine. The sera seemed to contain antibodies directed either against all the 8 phospholipids tested or more specifically against one or two phospholipids. The results were compared with those obtained with 17 healthy sera. Much lower values were obtained for the sera of healthy subjects, the majority of which showed a weak binding, similar for all the phospholipids. These results suggest that the anti-phospholipid autoantibodies present in systemic lupus erythematosus sera are significantly higher than those of healthy subjects. It is concluded that in the investigation of anti-phospholipid antibodies, tests should be carried out for all the phospholipids.

Introduction

Numerous papers have reported the presence of high levels of anti-phospholipid antibodies in human serum and their association with clinical disorders, especially systemic lupus erythematosus. However, these "anti-phospholipid" antibodies are detected either by the solid-phase Anti-Cardiolipin Test or the Standard Test for syphilis; the antigen of both tests is cardiolipin. This means that the term "anti-phospholipid" is most frequently employed in place of "anti-cardiolipin" antibodies. It has nevertheless been reported that anti-phospholipid autoantibodies also react with other phospholipids, particularly the anionic ones. Harris et al. (1) showed in 1985 that 90% and 85% of the anti-cardiolipin activity can be inhibited with phos-

phatidic acid and phosphatidylglycerol respectively, whereas only 80% of inhibition is obtained with the cardiolipin. In 1987, Gharavi et al. suggested that these antibodies are more specific for phosphatidylserine and phosphatidylinositol rather than for cardiolipin (2). Colaco & Male (3), Norberg et al. (4) and Costello & Green (5) reported the presence of anti-phosphatidylserine antibodies in systemic lupus erythematosus sera. Pengo et al. (6) found that affinity-purified anti-cardiolipin antibodies reacted with cardiolipin, phosphatidylserine, phosphatidylinositol and phosphatidic acid. Branch et al. (7) suggested that anticoagulant activity is always associated with the presence of anti-phosphatidylserine antibodies but also detected antibodies to cardiolipin, phosphatidylinositol, phospha-

tidylglycerol and phosphatidylethanolamine. McNeil et al. (8) compared the lupus anticoagulant and the anti-cardiolipin antibodies of two patient sera for their specificity towards all the phospholipids, and found reactivity towards the anionic phospholipids.

These comparatively rare reports deal with the presence, in systemic lupus erythematosus sera, of autoantibodies directed against phospholipids other than cardiolipin (for review, see *l.c.* (9)). Even though investigators agree that anionic rather than zwitterionic phospholipids (phosphatidylethanolamine, phosphatidylcholine) are involved, it is evident that the results reported are contradictory on the importance of one or another phospholipid antigen in this affection. This question is complicated by the fact that most investigators have used the same test, i.e. an ELISA in which the binding to antigen-coated wells is measured, to detect anti-phospholipid antibodies.

We suggest that the solution lies in the answers to the following questions:

- 1) are the phospholipids coated on the wells in a quantitative and reproducible manner?
- 2) are the different anti-phospholipid antibodies present in all systemic lupus erythematosus sera or are they only detected in particular cases?

To answer the first question, we titrated the coating of phospholipids on ELISA plates; to answer the second question, a systematic comparative study of systemic lupus erythematosus sera with all the phospholipids was undertaken.

Materials and Methods

The ELISA was developed for the study of anti-phosphatidylserine antibodies raised in rabbits (10). Phospholipids (from Sigma) were dissolved (100 mg/l) in hexane and 200 μ l were deposited in each well. The hexane was evaporated under a gentle air stream at room temperature and the wells were saturated for 30 min at 37 °C with a solution of 10 g/l bovine serum albumin in 0.01 mol/l phosphate buffer, pH 7.2, 9 g/l NaCl (PBS) and 0.5 g/l Tween 20. The plates were washed three times with PBS and incubated overnight at 4 °C with serum diluted (1/100) in PBS, 0.5 g/l Tween 20. After three further washes with PBS, the plates were incubated with anti-IgG-peroxidase conjugate (Immunotech) diluted in PBS, 0.5 g/l Tween 20 for 1 h at 37 °C. The plates were again washed three times with PBS and incubated with 1 g/l *o*-phenylene diamine in 0.1 mol/l citrate buffer pH 5.5 containing 0.25 ml/l H₂O₂ for 6 min in the dark. The reaction was stopped by the addition of 50 μ l of 4 mol/l H₂SO₄ and the absorbance at 496 nm was measured by a Dynatech MR610 plate spectrophotometer.

Results and Discussion

For the coating of ELISA plates, phospholipids are generally dissolved in ethanol or in chloroform/meth-

anol (1 + 4, by vol.) (9). Usually, cardiolipin is dissolved in ethanol and the other phospholipids in chloroform/methanol. The use of a single solvent should be more convenient and should give more comparable results. However, ethanol does not dissolve all the phospholipids and chloroform is known to attack plastic surfaces. We used hexane because it dissolves all the phospholipids, does not damage our plates (Nunc, Maxisorp) and can be evaporated rapidly (about 5 min for a plate containing 200 μ l/well) under a gentle air stream.

The temperature of incubation during the ELISA is also of importance. In fact, the ELISA tests reported are carried out at 20 °C or 37 °C (especially the incubation of the plates with the serum to be tested). However, when studying the thermodynamics of the affinity of antigen-antibody interactions, it can be shown that the affinity (association constant K_{ass}) varies with temperature as follows:

$$\frac{d \ln K_{\text{ass}}}{dT} = - \frac{\Delta H^\circ}{RT^2},$$

where ΔH° is the variation of the enthalpy of the reaction, R is the gas constant and T is the absolute temperature. This implies that for an interaction that is primarily exothermic (i.e. negative ΔH° by the formation of polar bonds), the affinity decreases with increasing temperature (11). Thus, many antigen-antibody interactions have a higher affinity at 4 °C than at 20 °C or 37 °C. In the case of phospholipids, there could be ionic interactions with the antibodies. Consequently, the incubation with the serum should be carried out at 4 °C.

The absorbance measured at the end of the ELISA is proportional to the amount of antigen-antibody complexes formed. It is then important to know precisely the quantity of antigen (phospholipid) contained in the well after the coating procedure. Such a control has never been reported. The different kinds of ELISA plates as well as the different solvents used, could be responsible for the differences observed in the results reported.

We evaluated precisely the quantity of each phospholipid coated on the plates. The supernatants resulting from the washes of the wells were tested for their phospholipid content by quantitative thin-layer chromatography (12). The exact quantity of coated phospholipid was then defined as the quantity deposited minus the loss during the washes. It was found that for 20 μ g of phospholipids deposited, 95.6% of cardiolipin, 96.9% of phosphatidic acid, 98.1% of phosphatidylcholine, 96.8% of phosphatidylethanolamine, 96.7% of phosphatidylglycerol, & 7.8% of phosphati-

dylinositol and 99.5% of phosphatidylserine were coated. These results show that the quantities of phospholipids coated (with hexane, in Nunc Maxisorp plates) are very close so that the absorbances obtained on the different phospholipids can be directly compared.

For each ELISA, a quadruple assay was carried out for every phospholipid. At least three experiments were performed (at different times) for each serum. The incubation time for the reaction was 6 minutes and controlled carefully. Representative results with standard deviations are given in table 1, showing that the intra-assay as well as the inter-assay variations are low, thus permitting the direct comparison of the results without using standards.

The totality of the results obtained with 38 systemic lupus erythematosus sera are presented in figure 1. An important binding of auto-antibodies is observed for all the phospholipids and especially for phosphatidic acid and phosphatidylserine. Much lower values were obtained for the healthy sera; furthermore, these values were very close for all healthy subjects, so that they are represented as a mean value $\pm$ standard deviation for each phospholipid (fig. 1).

The number of patients with a significantly higher level of autoantibodies than the healthy subjects were: 31 for phosphatidic acid (81.6%), 27 for phosphatidylserine (71%), 14 for phosphatidylethanolamine (36.8%), 12 for phosphatidylglycerol (31.6%), 10 for cardiolipin (26.3%), 7 for phosphatidylinositol (18.4%) and 7 for phosphatidylcholine (18.4%).

It should be noted that 36.8% of the systemic lupus erythematosus patients have a significantly higher level of autoantibodies to phosphatidylethanolamine

Fig. 1. ELISA on phosphatidic acid (PA), phosphatidylserine (PS), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Each point represents the mean value of absorbances obtained with one phospholipid for each systemic lupus erythematosus serum. $\bar{x}$ represents the mean value and the standard deviation of the absorbance obtained with one phospholipid for all healthy sera ($n = 17$).

compared with healthy subjects. The binding to zwitterionic phospholipids (phosphatidylethanolamine, phosphatidylcholine) is a subject of debate, as it has often been reported that they are not recognized by the autoantibodies. Our results demonstrate that both phosphatidylcholine and phosphatidylethanolamine can bind specifically anti-phospholipid autoantibodies.

Tab. 1. Mean values of absorbances with the intra-assay standard deviation (4 assays/phospholipid) and mean values of absorbances with the inter-assay standard deviation (at least 3 experiments/serum).

Patient Gao	1st assay	2nd assay	3rd assay	Mean value of the 3 assays
Phosphatidylserine	0.910 $\pm$ 0.061	0.988 $\pm$ 0.048	0.991 $\pm$ 0.052	0.963 $\pm$ 0.046
Phosphatidic acid	1.134 $\pm$ 0.166	1.055 $\pm$ 0.041	1.336 $\pm$ 0.015	1.17 $\pm$ 0.144
Cardiolipin	0.604 $\pm$ 0.068	0.593 $\pm$ 0.054	0.567 $\pm$ 0.024	0.587 $\pm$ 0.015
Phosphatidylglycerol	0.776 $\pm$ 0.039	0.702 $\pm$ 0.038	0.673 $\pm$ 0.038	0.717 $\pm$ 0.057
Phosphatidylinositol	0.83 $\pm$ 0.043	0.748 $\pm$ 0.029	0.731 $\pm$ 0.06	0.77 $\pm$ 0.053
Phosphatidylethanolamine	0.5 $\pm$ 0.011	0.51 $\pm$ 0.032	0.354 $\pm$ 0.057	0.453 $\pm$ 0.09
Phosphatidylcholine	0.453 $\pm$ 0.06	0.67 $\pm$ 0.05	0.557 $\pm$ 0.055	0.56 $\pm$ 0.11
Patient Bau	Phosphatidylserine	Phosphatidic acid	Cardiolipin	
1st assay	1.34 $\pm$ 0.098	1.07 $\pm$ 0.055	0.88 $\pm$ 0.081	
2nd assay	1.34 $\pm$ 0.06	1.57 $\pm$ 0.166	1.38 $\pm$ 0.069	
3rd assay	1.22 $\pm$ 0.043	1.16 $\pm$ 0.04	1.02 $\pm$ 0.063	
4th assay	1.3 $\pm$ 0.054	1.17 $\pm$ 0.086	1.41 $\pm$ 0.08	
5th assay	1.23 $\pm$ 0.043	1.33 $\pm$ 0.133	0.88 $\pm$ 0.089	
Mean value of the 5 assays	1.255 $\pm$ 0.084	1.248 $\pm$ 0.613	1.032 $\pm$ 0.283

Fig. 2. ELISA on phosphatidic acid (PA), phosphatidylserine (PS), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Mean value and standard deviation of absorbances obtained on each phospholipid for the serum studied.

a) healthy subjects (upper part)

b) systemic lupus erythematosus patients (lower part)

The results obtained with each phospholipid were rather similar for the 17 healthy sera, as shown by the examples in figure 2a. For some healthy subjects, the binding to phosphatidylinositol was slightly higher

than to other phospholipids; this explains the increased standard deviation of the mean value for phosphatidylinositol (fig. 1).

For the systemic lupus erythematosus sera, different patterns were observed. Some sera had similar absorbances for all phospholipids, other had increased binding to only some of them. Representative results are detailed in figure 2b. Of the phospholipids tested, the highest values were observed for phosphatidic acid in 28 patients (73.7%), for phosphatidylserine in 5 patients (13.1%), for phosphatidylinositol in 2 patients, for cardiolipin in only 2 patients and for phosphatidylcholine in one patient. In all the 5 sera containing a higher level of anti-phosphatidylserine antibodies, the levels of anti-phosphatidylserine and anti-phosphatidic acid antibodies were the same (fig. 2b, CHE or DUB). An exceptionally increased binding of antibodies to phosphatidic acid was observed for the patient MALL (fig. 2b, MALL).

Conclusion

In this study, we measured the level of autoantibodies to each of the phospholipids (cardiolipin, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylserine) in the serum of systemic lupus erythematosus patients and compared their level with that of healthy subjects.

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Our results demonstrate that

- 1) anti-phospholipid autoantibodies are present in healthy subjects;
- 2) their level is significantly increased in systemic lupus erythematosus patients and
- 3), they are directed against not only cardiolipin but also against phosphatidic acid, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine.

The ELISA for anti-cardiolipin antibodies is more and more used as a complementary clinical test. However, it is generally accepted that this assay is less specific for predicting the clinical features than the lupus anticoagulant test (13). The development of an ELISA for all the phospholipids will give more information about the role played by the anti-phospholipid antibodies. It has been reported that fluctuation of the level of anti-cardiolipin autoantibodies can be observed within weeks (14) or even years (5). This problem should be further examined for all the phospholipids.

Our study is currently extended to the analysis of a larger number of sera as well as to the possible association of the different anti-phospholipid antibodies with the clinical features of the patients.

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