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Histones interact with anionic phospholipids with high avidity; its relevance for the binding of histone–antihistone immune complexes

L. F. PEREIRA, F. M. MARCO, R. BOIMORTO, A. CATURLA, A. BUSTOS, E. G. DE LA CONCHA & J. L. SUBIZA *Department of Immunology, Hospital Universitario San Carlos, Madrid, Spain*

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SUMMARY

Antibodies recognizing anionic phospholipids have been described in systemic lupus erythematosus (SLE) and other autoimmune diseases. Recent studies have shown that some of these antibodies may recognize a cardiolipin-binding protein (apolipoprotein H) rather than phospholipids. A similar possibility is conceivable for other cardiolipin-binding proteins that are targets of autoantibodies. In this study we have addressed whether this might be the case for histones, a set of highly cationic and widely distributed proteins that react in a well known autoantibody system. Our results indicate that: (i) histones bind to anionic phospholipids (cardiolipin and phosphatidylserine) with high avidity, but not to zwitterionic phospholipids (phosphatidylcholine); (ii) monoclonal and polyclonal antihistone antibodies recognize histones bound to cardiolipin; (iii) the addition of histones to serum samples containing antihistone antibodies often enhances their anticardiolipin reactivity. In addition, we have found that antihistone-producing hybridomas derived from MRL-*lpr* mice may show anticardiolipin activity due to the presence of histones in the cell culture supernatants with the resultant formation of immune complexes. Taken together, the results suggest a potential role for histones in the anticardiolipin activity detected in sera containing antihistone antibodies. These histone–phospholipid interactions should be taken into account when evaluating the pathogenic effects of antihistone antibodies or other autoantibodies reacting with nuclear components (e.g. nucleosomes) containing histones.

Keywords phospholipids histones anticardiolipin antibodies antihistone antibodies

INTRODUCTION

Anticardiolipin antibodies (ACA) have been found in patients with systemic lupus erythematosus (SLE) and other connective tissue or infectious diseases, in patients taking certain drugs and also in apparently healthy individuals. They have been associated with important clinical manifestations like thrombosis or fetal loss, and their pathogenic role has been reported. Therefore their detection, greatly facilitated since the introduction of the solid-phase anticardiolipin tests, has become of interest in many clinical situations (reviewed in [1]).

It is, however, unclear whether anticardiolipin antibodies comprise a homogeneous antibody population, and even whether cardiolipin or other anionic phospholipids are actually the target molecules to which these antibodies have the potential to bind. In this regard, recent studies have shown that a cardiolipin-binding protein (apolipoprotein H) may be required to detect anticardiolipin antibodies [1–3] and even to induce ACA in experimental animals [4]. Although the role of this so-called ACA-cofactor in the anticardiolipin assay is controversial [5], some authors have shown that at least some 'anticardiolipin' antibodies are actually anti-ACA-cofactor antibodies [2,6]. As these findings raise the possibility that other autoantibodies recognizing cardiolipin-binding proteins could behave as 'anticardiolipin antibodies' as well, we were prompted to assess whether this could be the case for antibodies reacting with histones. These antibodies are also found in SLE, certain autoimmune disorders and other conditions [7], and although their pathogenic role is still unknown, they have been associated with disease activity in SLE patients [8]. We reasoned that histones, which are highly cationic proteins, might have affinity for cardiolipin, as described for other anionic substances [9–11], and therefore might behave as 'ACA cofactors' in the presence of antihistone antibodies.

Here we show that histones bind to cardiolipin and other anionic phospholipids in such a way as to be still recognized by both monoclonal and polyclonal antihistone antibodies. Moreover, we present evidence that spontaneously formed histone–antihistone complexes in culture supernatants from antihistone-

L.F.P. current address: Hospital S. Pedro de Alcántara, Cáceres, Spain.

Correspondence: Dr J. L. Subiza, Department of Immunology, Hosp. Univ. San Carlos, E-28040, Madrid, Spain.

producing hybridomas may bind to cardiolipin, being detected with this reactivity.

MATERIALS AND METHODS

Serum samples

Blood was obtained from eight patients and eight healthy individuals. Six patients met the American Rheumatism Association revised criteria for SLE, and the other two had been diagnosed as primary antiphospholipid syndrome (APS). They were used as a source of antihistone and anticardiolipin antibodies respectively.

Monoclonal antibodies

ANA1 (hybridoma supernatant; kindly provided by Dr R. J. T. Smeenk, Central Laboratory, The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and MAB052 (ascites fluid; Chemicon, Temecula, CA) are IgG MoAbs reactive with H2B (ANA1) and all histone fractions (MAB052), respectively. 2F1 is a hybridoma culture supernatant containing an unrelated monoclonal IgG used as control. Other MoAbs used were produced during this study (see below).

Phospholipids

Cardiolipin (diphosphatidylglycerol) from bovine heart; L-α-phosphatidylcholine type XIII-E from egg yolk; and L-α-phosphatidyl-L-serine from bovine brain (Sigma Chemical Co, St Louis, MO) were used.

Binding assays between phospholipids and histones

These were carried out by ELISA using phospholipids adsorbed to polystyrene as described to detect anticardiolipin antibodies [2]. U-wells of microtitre plates (Greiner, Frickenhausen, Germany) were filled with 50 μl of phospholipids 50 μg/ml diluted in ethanol (cardiolipin and phosphatidylcholine) or chloroform : methanol (1 : 4) (phosphatidylserine) and left to evaporate at 4°C. After washing with PBS pH 7·4, wells were neutralized with 0·3% gelatin in PBS (1 h at room temperature). Then 50 μl of total histones (Boehringer, Mannheim, Germany), unlabelled or biotin-labelled as described [12], diluted in PBS-0·3% gelatin were added and incubated during 2 h at room temperature. In those assays performed with biotin-labelled histones, a 1 : 5000 dilution of avidin-peroxidase was added to the wells once washed, and incubated for 30 min at room temperature. In the case of unlabelled histones, the wells were first filled with a dilution of ANA1 (1 : 100), MAB052 (1 : 1000) or control 2F1 (1 : 100) in PBS-0·3% gelatin (1 h at room temperature). After washing, bound IgG was detected with peroxidase-coupled goat antibodies to mouse IgG (Sera Lab Ltd., Sussex, UK) (1 h at room temperature). After a final washing, a chromogenic substrate (H_2O_2-OPD) was added and absorbance values were measured at 492 nm, once the enzymatic reaction was stopped with 1 N HCl. In all assays, negative controls (ethanol or chloroform : methanol-treated wells) and positive binding control (wells coated with methylated bovine serum albumin (BSA) plus DNA [9]), were included.

Avidity studies

The avidity of binding between cardiolipin and histones was studied assessing the effect of increasing the medium ionic strength [13]. Histones were diluted at 5 μg/ml in 0·3% gelatin-PBS, in the presence of various concentrations of NaCl (0·15–2·4 M), before adding to cardiolipin-coated wells. After incubation (2 h at room temperature), bound histones were detected by ELISA using ANA1 as above.

Binding of histone–antihistone complexes to cardiolipin

Monoclonal antibodies and serum samples, with or without antihistone activity, diluted in PBS-0·3% gelatin (1 : 50 and 1 : 30, respectively) were mixed (v/v) with increasing amounts of histones in the same medium. Mixtures were incubated for 1 h at 37°C plus an overnight incubation at 4°C, and then added to cardiolipin-coated wells following the same ELISA described above.

Production of monoclonal antibodies derived from MRL mice reacting with histones and/or cardiolipin

Cell fusions were carried out using spleens of 4-month-old female MRL-*lpr* mice (CBM, Universidad Autónoma, Madrid, Spain) and FO cells as described [14]. Culture supernatants were screened for IgG reacting with histones or cardiolipin by ELISA as described [14]. Selected hybridomas showing any of these activities were cloned twice by limiting dilution, picking up only from those wells where a single clone could be assured microscopically. Hybridoma supernatants (cell-free) were collected after 3–5 days of culture or daily to test the kinetics of antibody reactivity during 11 days of culture time. In some experiments, supernatants were tested for antibody reactivity after treatment under dissociating conditions [14]. Thus, 10 ml of a 5-day culture supernatant were dialysed against 3 M NaCl, 1·5 M glycine pH 8·9 or PBS (control) overnight at 4°C and tested for reactivity against cardiolipin and histones after a further dialysis with PBS, preserving the same final volume. The loss in activity was calculated establishing a reference curve with several dilutions of a control sample (dialysed with PBS).

Absorption to protein A beads and PAGE analysis

The volume (8–10 ml) of a given hybridoma supernatant (5-day culture) containing 1 mg of IgG as measured by ELISA [14] was mixed with 200 μl of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) previously washed with PBS. After an overnight incubation at 4°C, beads were washed three times with PBS and treated with 200 μl of running buffer (0·1 M Tris-HCL, 0·1% SDS, 10% glycerol, 0·01% bromophenol blue pH 6·8) in the presence of 0·1% β-mercaptoethanol. After boiling (3–4 min) the samples were centrifuged (1200 g; 5 min) and 50 μl of supernatant run on 0·1% SDS-20% PAGE [15]. In these experiments, a parallel assay using uncoated, i.e. protein-A-free, Sepharose beads (CL-4B; Pharmacia) was always included as control.

Statistical analysis

Data were analysed using Microstat, an Ecosoft computer program. Experimental differences from controls were analysed comparing means by the t-test. Differences between independent groups were analysed by Fisher's exact test. $P > 0{\cdot}05$ was considered non-significant.

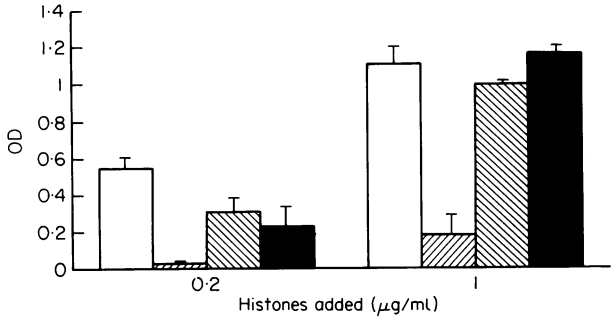

Fig. 1. Binding of histones to phospholipids. Biotin-labelled histones were added to wells precoated with different phospholipids or dsDNA (■) as a positive control. Bound histones were detected by means of avidin-peroxidase. Results are the mean of duplicates + s.d. CL (□), cardiolipin; PC (▨), phosphatidylcholine; PS (▧), phosphatidylserine. Binding was significant in all cases ($P < 0.05$) except when 0·2 μg/ml histones were added to PC-coated wells.

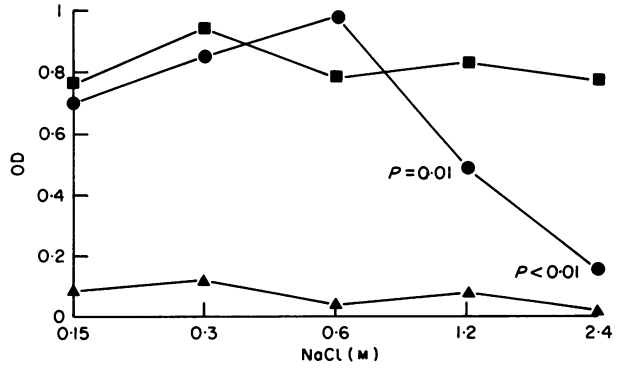

Fig. 3. Effect of ionic strength on cardiolipin–histone interaction. Histones (5 μg/ml) were incubated in cardiolipin-coated wells in the presence of increasing concentrations of NaCl. After washing, bound histones were detected by ELISA using ANA1 (●). Cardiolipin-coated wells treated with the same salt concentrations in the absence of histones were used as controls to detect background levels of ANA1 (▲) and reactivity of anticardiolipin antibodies (■). Results are the mean of duplicates.

RESULTS

Histones bind to anionic phospholipids

The binding of histones to cardiolipin was assayed by adding labelled histones to microtitre plates coated with different phospholipids (Fig. 1). As shown, binding was readily seen using wells precoated with anionic (cardiolipin, phosphatidylserine), but not with zwitterionic (phosphatidylcholine) phospholipids. Binding to the former was similar to that obtained with DNA-coated wells, used as positive controls. However, binding to phosphatidylcholine was low and only slightly elevated over background levels, obtained with ethanol-coated or methylated BSA (mBSA)-coated wells as negative controls (not shown), at the highest histone concentration tested (Fig. 1).

Antihistone antibodies react with histones bound to phospholipids

To demonstrate that histones could be recognized by antihistone antibodies when bound to phospholipids, an ELISA was performed as described in Materials and Methods. As shown in Fig. 2, histones were readily detected by ANA1 and MAB052 (which recognize H2B or all histone fractions, respectively) in wells precoated with cardiolipin or phosphatidylserine, as occurred in those wells precoated with mBSA plus DNA used as positive controls. Again, no histones were detected when they were incubated in phosphatidylcholine-coated wells (Fig. 2), or in wells precoated just with mBSA (not shown). In all cases, results were negative when instead of ANA1 or MAB, an unrelated MoAb (2F1) was used as control.

We took advantage of this ELISA system to test the effect of the ionic strength on the histone–cardiolipin interaction. As shown in Fig. 3, the amount of histones adsorbed onto cardiolipin-coated wells, as detected by ANA1, was not modified when binding between cardiolipin and histones was carried out in the presence of 0·3 M or 0·6 M NaCl, decreasing at higher salt

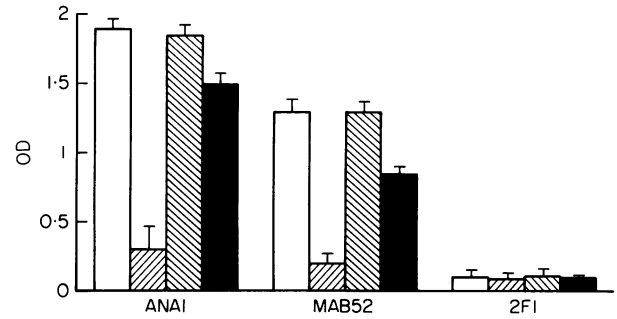

Fig. 2. Reactivity of antihistone MoAbs with histones bound to phospholipids. Histones (5 μg/ml) were added to wells coated with different phospholipids or dsDNA (■) as a positive control. After washing, bound histones were detected by ELISA using antihistone (ANA1 and MAB052) or control (2F1) monoclonal IgG. Results are the means of duplicates + s.d. CL (□), cardiolipin; PC (▨), phosphatidylcholine; PS (▧), phosphatidylserine. Reactivity of ANA1 and MAB052 was significant ($P < 0.001$) in all cases, except when histones were added to PC-coated wells ($P > 0.05$).

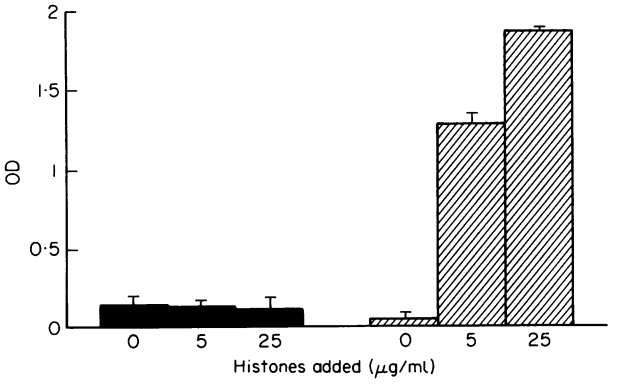

Fig. 4. 'Anticardiolipin' activity due to histone–antihistone complexes as detected by ELISA. Antihistone (ANA1) (▨) and control (2F1) (■) monoclonal IgG were preincubated with histones and then tested for anticardiolipin activity. Results are the mean of duplicates + s.d. Reactivity of ANA1 with cardiolipin was significant in the presence of histones ($P < 0.001$).

Table 1. Effect of exogenous histones on the anticardiolipin activity detected in sera containing antihistone antibodies (AHA)

		Histones added (μg/ml)						
		0	5			25		
Serum (Diagnosis)	AHA	ACA	ACA	%	*P*	ACA	%	*P*
1 (SLE)	501	55	81	48	0·04	165	200	0·001
2 (SLE)	158	10	12	20	NS	35	355	0·02
3 (SLE)	3000	8	295	>1000	0·001	473	>1000	0·001
4 (SLE)	1233	9	10	6	NS	10	3	NS
5 (SLE)	231	9	11	12	NS	18	87	NS
6 (SLE)	725	98	63	−35	0·03	31	−68	0·005
7 (APS)	<40	98	77	−21	0·01	36	−63	0·001
8 (APS)	<40	122	62	−49	0·007	29	−76	0·002
Controls ($n = 8$)	<40	9	11	25	NS	12	37	NS

AHA, Antihistone antibodies in arbitrary units/ml as detected by ELISA [9]. AHA were considered positive when greater than 40 U/ml. ACA, Anticardiolipin antibodies as detected by ELISA in a standard anticardiolipin assay [2]. ACA were considered positive when greater than 20 U/ml. Results are the mean of triplicates.

SLE, Systemic lupus erythematosus; APS, antiphospholipid syndrome.

concentrations (1·2 M or 2·4 M). This decrease was not due to cardiolipin desorption from the solid support, since reactivity of anticardiolipin antibodies was not impaired when control cardiolipin-coated wells, similarly treated, were used (Fig. 3).

Antihistone antibodies complexed with histones bind to cardiolipin

To test if histones complexed with IgG were still able to bind to cardiolipin, both histones and antihistone antibodies were preincubated together, added to cardiolipin-coated wells, and the IgG bound to the wells detected by ELISA. As shown in Fig. 4, antihistone MoAb (ANA1), but not a control MoAb (2F1), did bind to cardiolipin-coated wells if incubated with histones previously. Similar assays were carried out using, instead of MoAbs, sera from patients containing antihistone and/or anticardiolipin antibodies (Table 1). As can be seen, a significant increase in anticardiolipin activity was observed in three (sera 1, 2 and 3) out of six SLE antihistone-positive sera, but in none of the antihistone-negative sera (sera 7 and 8, and controls). Moreover, in these latter sera (sera 7 and 8), addition of histones greatly decreased the anticardiolipin activity, as also occurred in serum 6 with detectable antihistone antibodies (Table 1). Thus, there existed a significant association between the existence of antihistone antibodies and the increase of anticardiolipin activity by adding histones exogenously ($P = 0·03$; Fisher's exact test).

Role of histone-forming immune complexes in the anticardiolipin activity associated with antihistone MoAbs

We had observed that hybridoma supernatants derived from MRL-*lpr* mice were frequently found to react both with cardiolipin and histones. For example, the results of a single fusion were: out of 14 hybridomas cloned twice for being positive for any of these activities, six were scored positive to cardiolipin, of which four reacted, in addition, with histones by ELISA and with cell nuclei by immunofluorescence. Since the above results we were prompted to test whether the anticardiolipin activity detected in these particular supernatants could be explained by the presence of spontaneously formed immune complexes with histones. Figure 5 shows the kinetics of the antihistone and anticardiolipin activities as they appeared in the culture of 2F6, a representative hybridoma with both activities. As shown, antihistone activity was already detected in supernatants on day 1, while anticardiolipin activity appeared sharply later on day 3, without a parallel increase in the former. When a 5-day culture supernatant (2F6) was treated under dissociating conditions, the antihistone activity was relatively preserved, while decreasing more than 20 times the anticardiolipin activity (not shown). As these results suggested that the anticardiolipin activity associated with 2F6 was due to histone-binding immune complexes, IgG from these supernatants was absorbed to protein A-Sepharose beads and analysed by PAGE. As shown in a representative experiment (Fig. 6), core histones were seen associated with monoclonal IgG (heavy and light chains) obtained from 2F6, 2B2 or 2B3

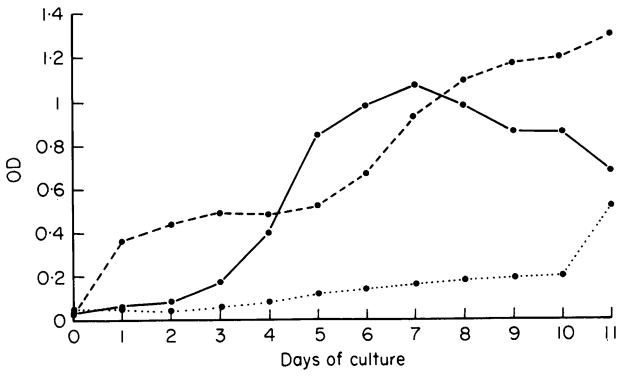

Fig. 5. Kinetics of antihistone (- - - -), anticardiolipin (——) and anti-dsDNA (· · · ·) activities detected in 2F6 hybridoma culture supernatant as measured by ELISA. Results are the mean of triplicates of a representative experiment.

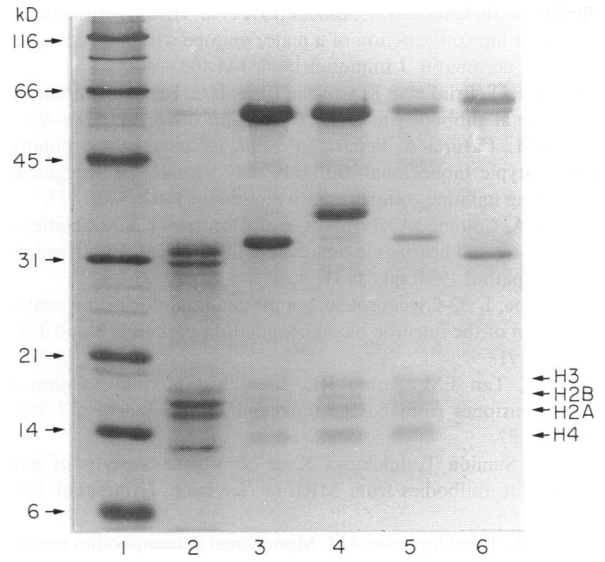

Fig. 6. SDS (20%)–PAGE analysis under reducing conditions of monoclonal IgG purified with protein A from 5-day culture hybridoma supernatants (coomassie blue staining). Lane 1, molecular weight standards; lane 2, H1 plus core histones (H2A, H2B, H3, H4); lanes 3, 4 and 5, IgG from 2B3, 2F6 and 2B2 hybridomas; lane 6, IgG purified in the same way from a hybridoma supernatant used as control.

hybridomas, all three of which had antihistone and anticardiolipin activities, but not with the IgG from another hybridoma supernatant that was scored negative for these activities and used as control. Uncoated Sepharose beads (protein A-free) were unable to retain any protein in control assays carried out in parallel (not shown).

DISCUSSION

In this study we show that histones are able to bind to cardiolipin and phosphatidylserine. This binding seems to be electrostatic in nature, as has been suggested for apolipoprotein H [16], since it does not take place with phosphatidylcholine, an electrically neutral (zwitterionic) phospholipid. An electrostatic histone–anionic phospholipid interaction is also supported because of its ionic strength dependence, since it is inhibited by increasing the medium salt concentration. In this regard, the relatively high molarity required to prevent it suggests that histones bind to cardiolipin with a rather high avidity. The results also show that once histones are bound to cardiolipin, they are still recognized by antihistone antibodies. Moreover, histones could bind to cardiolipin in the presence of both monoclonal and polyclonal antihistone antibodies, probably as histone-forming immune complexes, therefore being detected as 'anticardiolipin' activity.

Binding of histones to anionic phospholipids would be relevant when considering cross-reactivities of antihistone antibodies, as histones are widely distributed proteins that may be found in cell culture supernatants, serum or other organic fluids [17]. In fact, here we have shown that this may be the case when testing hybridoma supernatants of fusions derived from MRL-*lpr* mice, a lupus-prone mouse strain used to obtain antinuclear and anticardiolipin antibodies [18] by the observations that: (i) a significant number of antihistone-positive hybridomas also showed anticardiolipin activity; (ii) both antibody reactivities appeared in culture supernatants with different kinetics, suggesting the involvement of immune complexes [19]; (iii) anticardiolipin, but not antihistone, reactivity disappeared if supernatants were treated under dissociating conditions; (iv) core histones were found associated with IgG derived from these culture supernatants. Moreover, as histones spontaneously released in cell cultures are complexed with DNA, probably in the form of nucleosomes [19,20], the results suggest that DNA/histone-binding immune complexes may react with cardiolipin as well. This is of note, since these complexes, rather than free histones, are more likely to be expected in a physiological situation. Interestingly, these hybridoma supernatants showed very poor binding to DNA (Fig. 5), while showing a good reactivity with heparan sulphate (not shown), in addition to cardiolipin. This may indicate that this type of immune complex, which is able to interact with various anionic substances, does not necessarily bind to DNA, suggesting a higher avidity to cardiolipin than to DNA. This is not surprising, since it has already been described that histones may complex with other anionic substances (heparan sulphate) more avidly than with DNA [10].

Cross-reactivities due to immune complexes have been found not only using hybridoma supernatants but also sera of SLE patients [9,21], especially when testing for antibody activities against charged antigens [14]. Although the present results do not make clear whether histone–antibody complexes are involved in the anticardiolipin activity detected in sera containing antihistone antibodies, it should be noted that anticardiolipin antibodies are frequently detected in autoimmune diseases like SLE, drug-induced lupus or rheumatoid arthritis (RA) [1], where the presence of antihistone antibodies is well described [7]. Both antibody activities may also be found in patients with infectious diseases [1,22] and other conditions [1,23]. Moreover, some striking features such as enhancement of antibody activity by the heat treatment of sera, have been described just with anticardiolipin [24] and antihistone [25] antibodies, and not with any other antibody activity tested so far. In any case, the results do show that the presence of histones may greatly influence the anticardiolipin activity detected in sera containing antihistone and/or anticardiolipin antibodies. Thus, when histones were added to antihistone-positive sera, an increase in anticardiolipin activity was seen in a significant number of them, while histones were highly inhibitory for most sera showing anticardiolipin activity (Table 1). This inhibition could be explained by competition of histones with other cardiolipin-binding proteins required in the anticardiolipin assay (e.g. apolipoprotein H) or just by blocking of epitopes recognized by true anticardiolipin antibodies. In this regard, a similar inhibitory effect has been described in cardiolipin-positive sera from syphilis patients by adding exogenous ACA-cofactor (apolipoprotein H) during the anticardiolipin assay [3]. Taken together, these findings would indicate that the anticardiolipin activity detected in a given serum can be enhanced or suppressed by cardiolipin-binding proteins, depending on the presence or absence, respectively, of antibodies reacting with them.

Histone (or DNA/histone) interaction with phospholipids might be relevant when considering the pathogenic mechanisms

of antihistone, and even anti-DNA, antibodies. It has long been suggested that the pathogenic effect of autoantibodies reacting with nuclear components, like DNA/histones, may require cell destruction, release of antigen and formation of phlogistic immune complexes. Once in the circulation these immune complexes may deposit in different structures (e.g. glomerular basement membrane) by means of charge-based intermolecular associations, mainly through the histone moeity [11,26,27]. The results presented here suggest that anionic phospholipids might be another target structure for these immune complexes. Although these phospholipids are known to be present in the inner part of the cell membrane, they may be exposed under certain circumstances, e.g. upon cell activation [28]. Indeed, in some conditions anticardiolipin antibodies have been described to bind to erythrocytes [29], to macrophages [30] or to platelets [31]. Whether this may also be the case for histone-binding complexes needs to be elucidated, although a recent report showing that nucleosomes bind to activated monocytes [32] may point in that direction.

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