Anti-phospholipid antibodies (aPL) and apoptosis: prothrombin-dependent aPL as a paradigm for phospholipid-dependent interactions with apoptotic cells[★]

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Abstract

The natural targets of anti-phospholipid antibodies (aPL) and the stimuli that induce them remain unknown. Apoptotic cells have been proposed as both potential targets and immunogens for anti-phospholipid antibodies. Demonstration of selective recognition by anti-phospholipid antibodies provides support for apoptotic cells as antigenic targets. Here, we summarize data showing that prothrombin (PT) binds to apoptotic, but not viable, cells, and that apoptotic-cell bound prothrombin provides a target for human polyclonal and murine monoclonal lupus anticoagulant (LA) antibodies. We discuss findings for two monoclonal lupus anticoagulant antibodies that have high (antibody 29J3-62) or low (antibody 29I4-24) affinity, respectively, for soluble prothrombin. Despite their very different affinities for soluble prothrombin, both monoclonal antibodies reacted similarly with prothrombin bound to phospholipid or apoptotic cells. Furthermore, both antibodies enhanced the binding of prothrombin to apoptotic cells. We propose that the recognition of apoptotic cells by these prothrombin-dependent monoclonal antibodies provides a paradigm for other anti-phospholipid autoantibodies. 29I4-24 is prototypical of phospholipid-dependent anti-phospholipid antibodies, while 29J3-62 represents a prototype for phospholipid-independent anti-phospholipid antibodies. Proteins such as prothrombin and β 2-glycoprotein I (β 2GPI) bind to apoptotic cells, thereby enhancing the recognition of apoptotic cells by anti-phospholipid antibodies. Furthermore, anti-phospholipid antibodies potentiate the interaction of these proteins with apoptotic cells. While it is unclear whether apoptotic cells are the inducing stimuli in patients with anti-phospholipid antibodies or even whether anti-phospholipid antibodies interact with apoptotic cells in vivo, it is nonetheless clear that anti-phospholipid antibodies have the potential to affect both the procoagulant activity and the uptake and clearance of apoptotic cells.

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Keywords

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Introduction

Anti-phospholipid antibodies (aPL) are a family of autoantibodies that arise in a variety of autoimmune diseases, particularly systemic lupus erythematosus (SLE) and primary anti-phospholipid syndrome (APS) [1,2]. These auto-antibodies are heterogeneous, and encompass a broad range of target specificities and affinities, all recognizing various combinations of phospholipids and/or phospholipid-binding proteins [2,3]. While aPL were initially believed to target anionic phospholipids directly, we now appreciate that aPL recognize primarily phospholipid-binding proteins that interact with anionic phospholipids, such as β 2-glycoprotein I (β 2GPI), prothrombin (PT), and annexin V [4–8].

Certain aPL recognize phospholipid-binding proteins alone, whereas others require the presence of phospholipid for this recognition [9–11]. Lupus anticoagulant (LA) antibodies, a subset of aPL, are prototypical of the latter group of aPL. Although LA antibodies are defined functionally by their ability to prolong clotting times in in vitro coagulation assays, they are associated with thrombosis in vivo [12]. Immunochemically, LA antibodies react with a disparate array of antigens and fall into two major groups: antibodies that are β 2GPI-dependent [13] and antibodies that are PT-dependent [7,11,14]. Both groups of LA antibodies are thought to affect the interaction of their respective protein antigen with PL in vitro and, likely, also, in vivo. However, identification of the natural target(s) and/or immunogen(s) for LA antibodies and other aPL, as well as the sequence of events underlying their induction, remain unclear.

Apoptotic cells provide a potential natural target and/or immunogen for all aPL, and, in fact, for most autoantibodies. There is increasing evidence that apoptotic cells are involved in the initiation and/or maintenance of autoimmunity [15–17]. Apoptotic cells express autoantigens that are specifically targeted by autoantibodies found in SLE and in APS [18–21]. Of particular relevance to aPL, the external membrane leaflet of apoptotic cells contains anionic phospholipid, such as phosphatidylserine (PS) and possibly cardiolipin, not normally present on the surface of viable cells [22–24]. The apoptotic cell surface thus provides an appropriate microenvironment for the capture of phospholipid-binding proteins that interact with anionic and other phospholipids.

We have previously demonstrated that β 2GPI binds to the surface of apoptotic, but not viable, cells, and that the interaction of β 2GPI with the apoptotic cell surface generates epitopes that are both antigenic for aPL [25] and immunogenic in normal mice [26]. Recently, we have also shown that this paradigm for recognition of apoptotic cells by aPL can be extended to prothrombin (PT)-dependent aPL [27]. We review some of these findings here, and propose that this paradigm may apply to other aPL.

PT and PT-dependent SLE-derived LA antibodies bind to apoptotic cells

To address whether apoptotic cells are antigenic targets for PT-dependent aPL, it was necessary to first demonstrate that PT, itself, was capable of binding to the surface of apoptotic cells. PT is a phospholipid-binding protein known to interact with PS in the presence of calcium. We used as our model Jurkat T cells induced to undergo apoptosis in the presence of staurosporine [27]. Fluorescein-labeled PT (FITC-PT) was found to bind to the surface of apoptotic, but not viable, Jurkat cells in the presence, but not the absence, of calcium chloride (CaCl_2) (Fig. 1). The binding was relatively low, but specific, as shown by lack of binding of FITC-labeled human serum albumin to apoptotic cells, and approximately 2.6-fold higher than the binding to viable cells.

The next step was to evaluate whether PT-dependent aPL were capable of binding to apoptotic Jurkat cells in the presence of PT. Initially, we used IgG antibodies isolated from the sera of patients with SLE and known PT-dependent LA activity. IgG from four of the six patients showed greater binding to apoptotic (mean fluorescence intensity (MFI) range=23.87–145.21) than to viable (MFI range=10.66–42.02) cells in the presence of PT (Fig. 2). In contrast, IgG from two patients demonstrated slightly greater binding to viable than to apoptotic cells, irrespective of the presence of PT. PT-dependent binding to apoptotic cells correlated with binding of the same IgG fractions to phosphatidylethanolamine (PE)-bound PT [11], but not with the strength of LA activity or with clinical features of the patients [27].

PT-dependent monoclonal LA antibodies bind to apoptotic cells

The use of polyclonal PT-dependent LA antibodies from patients precludes identification of the epitopes on apoptotic cells that are recognized by these antibodies. To better understand and characterize the epitopes responsible for LA antibody binding to apoptotic cells, we used two IgG1 PT-dependent LA murine monoclonal antibodies (mAb), called 29J3-62 and 29I4-24. These mAb were derived from mice immunized with phospholipid/PT complexes, comprised of either dioleoylphosphatidylserine (DOPS) and PT, or DOPS/dioleoylphosphatidylcholine (DOPC) and PT, respectively. Both mAb exhibited similar and strong LA activity (Table 1) that was dose-dependent and specifically inhibited by hexagonal (II) phase PE [27]. Neither mAb reacted with cardiolipin, DOPS, DOPC, or $\beta_2\text{GPI}$ in enzyme-linked immunoassay (ELISA) (Table 1). Despite virtually identical LA activity, 29J3-62 reacted strongly with human PT alone (in the absence of PL), while 29I4-24 bound very weakly to PT alone (Fig. 3a). The relative affinities (K_d) of the two antibodies for soluble PT, defined as the concentration of soluble PT producing 50% inhibition, differed by roughly 9000-fold (0.01 nM for 29J3-62 compared to 90 nM for 29I4-24) (Fig. 3b). In contrast, the binding of the mAb to complexes of PT and phospholipid (DOPS/DOPC or DOPS/dipalmitoylphosphatidylcholine, hereafter referred to as PSPC) was equivalent (Fig. 3c and d).

The finding that both LA mAb, 29J3-62 and 29I4-24, recognized phospholipid-bound PT suggested that these mAb should be capable of binding to apoptotic cell-bound PT. Therefore, we evaluated the reactivity of the mAb with apoptotic and viable Jurkat cells by

flow cytometry. Both mAb bound strongly to apoptotic cells only in the presence of PT (Fig. 4). PT-dependent binding of both 29J3-62 and 29I4-24 was approximately 30-fold higher to apoptotic cells than to viable cells. Moreover, the binding of 29I4-24 to apoptotic cells was nearly equivalent to that of 29J3-62, despite the great difference in affinities of these two mAb for PT in the absence of phospholipid. These findings demonstrate that the interaction of PT with anionic phospholipid, whether in the form of PSPC or the surface of apoptotic cells, dramatically increases the affinity of 29I4-24 for PT. In contrast, the affinity of 29J3-62 for PT is minimally affected by the interaction of PT with anionic phospholipid.

Phospholipid-dependent binding of LA mAb to PT

We next investigated the mechanism responsible for the increase in binding of mAb 29I4-24 to PT in the presence of phospholipid (PSPC) or apoptotic cells. It is important to define the terms “affinity” and “avidity”, before discussing the possible mechanisms. Antibody “affinity” is defined as the strength of binding of a single antigen-binding site of an antibody to a monovalent antigen, while antibody “avidity” is defined as the total binding strength of an antibody having more than one antigen-binding site to a multivalent antigen [28]. Avidity, therefore, depends not only on the affinity of each individual antigen-binding site, but also on the number of antibody binding sites engaged by antigen.

We hypothesized that at least two independent processes may play a role in the enhanced binding of mAb 29I4-24 to complexes of phospholipid and PT. First, the interaction of PT with phospholipid (i.e., PSPC) may result in the expression of a novel epitope on PT. In this case, the strength of interaction of an individual antigen-binding site (“affinity”) of the mAb with that epitope would be increased. Second, the phospholipid may provide a surface upon which PT can attach at a sufficiently high density, or in a particular spatial orientation, such that both antigen-binding sites of the mAb, each with low affinity for soluble PT, would now interact with PT. In the latter situation, the overall increase in binding of the mAb would be due to increased “avidity”, which is therefore dependent on antibody bivalency. Increased avidity occurs when both antigen-binding sites interact simultaneously with antigen (i.e., PT that has been rendered effectively multivalent by its attachment to the phospholipid surface at a sufficiently high surface density).

We determined the relative contribution of these two mechanisms by evaluating the reactivity of bivalent mAb (IgG, IgG', and F(ab')₂) and monovalent mAb (Fab') with PT alone or with phospholipid/PT complexes. For ease of comparison, the data in these studies are expressed as a function of the molarity of antigen-binding sites (Fig. 5). Thus, the molarity of a given concentration of bivalent mAb will be twice that of the same concentration of monovalent mAb, since the bivalent mAb has twice as many antigen binding sites. The binding of monovalent (Fab') fragments of 29J3-62 to PT alone (Fig. 5, right panels) was strong and did not differ significantly from that of intact IgG, IgG', or F(ab')₂ fragments, whereas the binding of monovalent (Fab') fragments of 29I4-24 to PT was significantly lower than that of bivalent intact IgG, IgG', or F(ab')₂ fragments. These results demonstrate an important contribution of antibody bivalency (and therefore avidity) for binding of 29I4-24, but not 29J3-62, to PT alone. In contrast, the results with phospholipid/PT complexes (PSPC/PT) were very different, and are consistent with the

expression of a novel epitope formed by the interaction of PT with phospholipid (Fig. 5, left panels). Monovalent Fab' fragments of 29J3-62 and 29I4-24 bound with similarly high affinity to PSPC/PT, despite their very different binding affinities for PT alone. These data suggest that the epitope recognized by 29I4-24 is dependent upon interaction of PT with phospholipid, and that the affinity of 29I4-24 for this phospholipid-dependent epitope on PT is far greater than its affinity for the epitope that it recognizes on PT alone. While these findings demonstrate that PL likely plays a crucial role in the antigenicity of PT for mAb 29I4-24, the precise epitope recognized by this antibody remains unclear. There are a number of possible ways in which phospholipid may be involved in creating the epitope that this LA mAb recognizes. First, as PT has been shown to undergo a conformational change upon binding to PS-containing membranes [29], it is possible that 29I4-24 is directed against a conformationally induced neoepitope exposed on PT when it interacts with phospholipid. Second, 29I4-24 may recognize an epitope formed by the interaction of phospholipid and PT, with no conformational change in PT. Third, 29I4-24 may bind to an epitope on PT created by chemical (e.g., oxidative) modifications that occur when PT interacts with phospholipid. The finding that high concentrations of PT alone can inhibit the binding of 29I4-24 to plate-bound PT (Fig. 3) suggests that the epitope may be found on PT alone, but is minimally expressed in the absence of phospholipid.

To determine whether these mechanisms of antibody interaction also apply to apoptotic cells, we evaluated the reactivity of the mAb fragments with apoptotic cells by flow cytometry. As in the ELISA on PSPC-bound PT, bivalent F(ab')₂ fragments of both mAb exhibited elevated binding to apoptotic cells, which was similar to the binding of their respective IgG and IgG' controls (MFI: 345.99 and 239.62 for 29J3-62 and 29I4-24, respectively, at 20 µg/ml PT) (Fig. 6a). Importantly, and as in the ELISA, monovalent Fab' fragments of these two mAb had virtually identical reactivity with PT in association with apoptotic cells (MFI: 19.15 and 17.61 for 29J3-62 and 29I4-24, respectively, compared to 8.26 for the negative control mAb). However, in contrast to reactivity with phospholipid/PT complexes, the binding of monovalent Fab' fragments of both mAb to apoptotic cell-bound PT was low and dramatically decreased, compared to that of the F(ab')₂ fragments. These data indicate that the contribution of antibody bivalency is relatively minor for binding of both mAb to PSPC-bound PT in ELISA, but is more important for binding of the mAb to apoptotic cell-bound PT. This difference is likely due to the fact that PSPC/PT complexes are presumably irreversibly bound to an ELISA plate, while the interaction of PT with apoptotic cells is an equilibrium state. Thus, in the case of PSPC/PT complexes, the sole way that mAb can detach from the ELISA plate surface is for the mAb to dissociate from PSPC-bound PT. In contrast, in the case of apoptotic cells, the mAb can dissociate from the apoptotic cell surface in one of two ways: (1) leaving the PT behind still attached to the apoptotic cell or (2) taking the PT with it (i.e., bound to its antigen-binding site). A comparison of the reactivity of the two mAb to PT bound to different surfaces, including apoptotic cells, is summarized in Table 2.

LA antibodies enhance the binding of PT to apoptotic cells

The data shown thus far have evaluated the ability of LA antibodies (polyclonal or monoclonal) to bind to apoptotic cells in the presence of PT. This is interesting and

potentially physiologically relevant, as PT is present in the circulation at reasonably high concentrations. However, the potential effect of a LA autoantibody on the interaction of PT, itself, to an apoptotic cell could be of even greater physiological relevance. PT, in combination with factors Va and Xa, calcium, and phospholipid, forms the prothrombinase complex. This complex converts PT to thrombin, a key player in coagulation with both procoagulant and anticoagulant functions. Thus, potentiation or inhibition of the binding of PT to a cellular surface could have a major effect on coagulation. We determined whether the two LA mAb could augment the binding of PT to apoptotic cells by comparing the binding of FITC-PT to apoptotic cells in the presence or absence of 29J3-62 and 29I4-24 by flow cytometry. Binding of FITC-PT to apoptotic cells was enhanced by 5-fold or more in the presence of either mAb (29J3-62, MFI=51.16; 29I4-24, MFI=38.03), as compared to binding of FITC-PT in the presence of an isotype-matched control antibody (MFI=7.85) (Fig. 6b). This effect was restricted to apoptotic cells, as the mAb did not significantly affect the binding of FITC-PT to viable cells (data not shown). We evaluated whether antibody bivalency was necessary to observe the enhanced binding of PT. IgG, IgG', and F(ab')₂ fragments of both LA mAb enhanced binding of FITC-PT to apoptotic cells (maximal MFI of F(ab')₂=36.27 and 14.70 for 29J3-62 and 29I4-24, respectively), while monovalent Fab' fragments of the mAb had no effect on FITC-PT binding (maximal MFI=6.97) [27]. These data show that antibody bivalency is essential for the enhancement of PT binding to apoptotic cells by LA mAb.

A paradigm for the interaction of aPL with apoptotic cells

The findings for the PT-dependent LA mAb 29J3-62 and 29I4-24 provide an interesting view of two antibodies with similar, and yet, very different characteristics. Notably, the binding characteristics of these mAb for two physiologically relevant surfaces (phospholipid in the in vitro assay to detect LA, or apoptotic cells) were similar, while those for the protein (PT) alone were very different. This leads one to speculate about the antigens that are used for aPL screening (both for PT-dependent aPL and for other aPL), and whether the detection and understanding of aPL could be improved by screening for aPL with more physiologically relevant antigens (such as phospholipid-bound or cell-bound protein). Phospholipid-bound β 2GPI is already used for screening for anti-cardiolipin antibodies, and phospholipid-bound PT has been proposed as a more clinically useful assay for PT-dependent aPL [30]. LA antibodies, which are detected by their interaction with phospholipid-bound proteins in in vitro coagulation assays, are considered to be highly specific for APS [31]. Thus, it is well recognized that a considerable subset of aPL require the presence of phospholipid for their binding to phospholipid-binding proteins.

The two LA mAb described here, however, provide a clear picture of the degree to which phospholipid may alter recognition of a phospholipid-binding protein such as PT. 29I4-24, which had an extremely low affinity for soluble PT, reacted with phospholipid-bound or apoptotic cell-bound PT with the same affinity as 29J3-62, which had a high affinity for soluble PT. Importantly, we have demonstrated that this increased reactivity of mAb 29I4-24 for phospholipid-bound PT is not simply due to an increased density of PT on the phospholipid surface, but rather to an increased affinity of the individual antigen-binding sites of the antibody for PT presented in the context of phospholipid. This was shown by

comparing the reactivity of monovalent Fab' fragments of the mAb with that of bivalent fragments (F(ab')₂) or complete antibody (IgG' or IgG). We found that, for mAb 29I4-24, the reactivity of monovalent Fab' fragments with soluble PT was extremely low, whereas reactivity of the same monovalent fragments with phospholipid-bound PT was markedly higher and similar to that of 29J3-62. Thus, the "affinity" of 29I4-24 for soluble PT is very low, but its "affinity" for phospholipid-bound PT is high (Table 2). This finding is consistent with recognition of an epitope on PT that requires interaction of the protein with phospholipid. Interestingly, bivalency was found to contribute somewhat to the high binding of both mAb to phospholipid-bound PT, as bivalent fragments of both mAb bound slightly better than monovalent fragments. In contrast, when soluble PT was used as an antigen, bivalency dramatically affected the binding of only 29I4-24 to soluble PT, but had absolutely no effect for 29J3-62. Together, these findings expand our understanding of the potential interactions of aPL with phospholipid-binding proteins, both in the presence and absence of phospholipid.

29I4-24 and 29J3-62 are mAb that were derived from immunized mice, and therefore do not directly reflect the specificities of aPL found in patients with SLE or APS. Nevertheless, these mAb may serve as a paradigm for human aPL in terms of both the heterogeneity of patient-derived aPL and the potential interactions of aPL with apoptotic cells. First, LA mAb and polyclonal LA from patients with SLE behaved similarly, in that both reacted with apoptotic cells in a PT-dependent manner. In each case, the binding to apoptotic cells correlated with binding to phospholipid-bound PT. Second, monoclonal and polyclonal LA antibodies overlapped in their recognition of some epitopes, as demonstrated by competitive inhibition assays on PSPC-bound PT [27]. Third, like polyclonal aPL found in patients with SLE and APS, the monoclonal LA antibodies encompassed both phospholipid-dependent and phospholipid-independent aPL. 29I4-24 is prototypical of a phospholipid-dependent aPL, while 29J3-62 presents a prototype for phospholipid-independent aPL.

While our analysis was limited to two PT-dependent LA, one phospholipid-dependent and the other phospholipid-independent, we believe our findings have broader implications relating to the heterogeneity of epitopes recognized by aPL; the role of phospholipid in creating neoepitopes and in enhancing antibody affinity; and the importance of bivalency in some, but not all, aPL interactions. Moreover, our finding that PT-dependent aPL, whether of low or high affinity for soluble PT, can recognize PT on the surface of apoptotic cells with a comparable high affinity most likely extends to β 2GPI-dependent and other aPL.

Extending these findings to other subsets of aPL, particularly those that recognize phospholipid-binding proteins, makes perfect sense, if one envisions the apoptotic cell as a potential immunogen or target, or both. An initial immune response to a single protein present on the apoptotic cell surface could, in the right immunologic and/or genetic context, conceivably give rise to antibody responses to other proteins and phospholipids derived from the same cell (i.e., epitope spreading). Similarly, the apoptotic cell provides an array of potential autoantigens targeted not only by aPL of different specificities (e.g., PTor β 2GPI-dependent), but also by other autoantibodies [18–21].

Our data provide evidence that PT-dependent LA antibodies not only bind directly to apoptotic cell-bound PT, but also enhance the binding of PT to the apoptotic cell. This is consistent with other data in the literature for both PT and β 2GPI. Several recent reports have shown that LA antibodies can enhance the binding of PT to phospholipid [32–34] and to endothelial cells [33,35] in vitro. In this way, LA antibodies may increase the affinity of PT for the phospholipid or cell surface, so that PT competes with other coagulation factors for the available catalytic surface [34]. In fact, enhanced PT binding to endothelial cells has been shown to increase thrombin generation and shorten the clotting time in human umbilical vein endothelial cell-based coagulation assays [35].

We further demonstrate that bivalency is important both for enhanced binding of PT to apoptotic cells and for the indirect binding of LA antibodies to apoptotic cells in the presence of PT. Similar observations have been made by Field et al. [32] using phospholipid vesicles. Bivalent, but not monovalent, fragments of patient-derived LA-positive IgG enhanced binding of PT to PSPC vesicles. The model proposed is one in which bivalent antibody binds two molecules of PT that are interacting with the phospholipid surface, thus stabilizing the binding of PT to phospholipid [32]. In contrast, binding of monovalent IgG to only one molecule of PT does not have the same stabilizing effect. A similar model can explain the enhanced binding of PT to apoptotic cells in the presence of bivalent, but not monovalent, mAb, as well as the need for bivalency for direct binding of mAb to apoptotic cells. It is of interest to note that bivalent β 2GPI-dependent aPL have an analogous stabilizing effect on the interaction of β 2GPI with phospholipid [36–38]. Furthermore, native β 2GPI binds to phospholipid membranes of physiologically relevant composition with relatively weak affinity [36,39], while dimeric forms of β 2GPI bind to these surfaces strongly [40], are recognized by aPL with increased avidity [41], and demonstrate functional LA activity that is enhanced by aPL [40].

These observations provide a clearer understanding of the mechanisms by which LA antibodies promote thrombosis in vivo. As apoptotic cells have been shown to exhibit procoagulant properties in vitro [42], it is possible that they contribute to the thrombotic state associated with APS. Our findings suggest that interactions between LA antibodies and apoptotic cells in the presence of PT may potentiate this procoagulant effect. They also indicate that apoptotic cell-bound PT represents an important target of LA antibodies, whether they have low or high affinity for PT. Finally, our data provide support for the concept that apoptotic cells play a central role in the induction and/or perpetuation of APS.

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Abbreviations

aPL

anti-phospholipid antibodies

APS	anti-phospholipid syndrome
β2GPI	β2-glycoprotein I
CaCl₂	calcium chloride
DOPC	dioleoylphosphatidylcholine
DOPS	dioleoylphosphatidylserine
PSPC	DOPS/dipalmitoylphosphatidylcholine or DOPS/DOPC
ELISA	enzyme-linked immunoassay
FITC-PT	fluorescein-labelled prothrombin
LA	lupus anticoagulant
MFI	mean fluorescence intensity
mAb	murine monoclonal antibodies
PE	phosphatidylethanolamine
PS	phosphatidylserine
PT	prothrombin
PT-dependent	prothrombin-dependent
PSPC/PT	PSPC and PT complexes
SLE	systemic lupus erythematosus

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Figure 1.

Selective binding of PT to the surface of apoptotic cells. Binding of FITC-PT (100 $\mu\text{g/ml}$) (filled histograms) to apoptotic cells required the presence of calcium (2.5 mM). FITC-HSA (100 $\mu\text{g/ml}$) (open histograms) was used as a negative control. Copyright 2003. The American Association of Immunologists [27].

Figure 2.

Binding of IgG from SLE patients to apoptotic cells in the presence of PT. Purified IgG (250 $\mu\text{g/ml}$) from the plasma of 6 SLE patients (1–6) was evaluated for binding to apoptotic cells in the presence (solid bars), or absence (cross-hatched bars), of PT (50 $\mu\text{g/ml}$); or viable cells in the presence (dotted bars), or absence (hatched bars), of PT by flow cytometry. SLE patient 7 had known high-titer anti-PT antibodies and LA antibodies, and was included as a positive control. Purified IgG from pooled normal human plasma was used as a negative control (normal). Reactivity of IgG fractions with phospholipid (PE)-bound PT in ELISA is indicated by a plus (+) sign for positive reactivity or a minus (–) sign for no reactivity, based on previous data [11]. Copyright 2003. The American Association of Immunologists [27].

Figure 3.

Anti-PT reactivity of LA murine mAb. (a) Binding of purified mAb to PT by ELISA. Purified 29J3-62 (solid line) and 29I4-24 (dashed line) were titrated and tested for binding to PT-coated ELISA plates at high (20 μg/ml, filled circles) and low (5 μg/ml, open circles) concentrations. Negative control mAb 27D2-83 showed no significant binding (data not shown). (b) Competitive inhibition of binding of purified mAb to PT by soluble PT. Binding of purified 29J3-62 (filled circles) or 29I4-24 (open circles) to PT-coated ELISA plates (20 μg/ml) was inhibited by soluble PT at increasing concentrations. (c and d) Effect of phospholipid on the reactivity of LA mAb with PT. Binding of purified 29J3-62 (c) and 29I4-24 (d) to PSC/PT (filled circles), PC/PT (open circles), or PT alone (filled triangles) coated directly onto ELISA plates (final concentration of CaCl₂ in coating solution was 5 mM) was measured by ELISA. Values represent the means of duplicate samples, and the data are representative of two independent experiments. Copyright 2003. The American Association of Immunologists [27].

Figure 4.

Binding of LA mAb to apoptotic cells in the presence of PT. Purified 29J3-62, 29I4-24, or a subclass-matched control (27D2-83) (20 $\mu\text{g}/\text{ml}$) were evaluated for binding to apoptotic (filled histograms) or viable (open histograms) cells in the presence (+PT) or absence (–PT) of human PT (20 $\mu\text{g}/\text{ml}$) by flow cytometry. Data are representative of two independent experiments. Copyright 2003. The American Association of Immunologists [27].

Figure 5.

Effect of phospholipid on the reactivity of bivalent and monovalent fragments of LA mAb with PT. Binding of purified intact IgG (filled circles), reduced/alkylated IgG (IgG') (open circles), and F(ab')₂ (filled triangles) and Fab' (open triangles) fragments of 29J3-62 (top) and 29I4-24 (bottom) to PSPC/PT (left) or PT alone (right) coated directly onto ELISA plates. IgG and fragments were used at concentrations that provided equivalent molarities of antigen-binding sites. Values represent the means of duplicate samples, and the data are representative of two independent experiments. Copyright 2003. The American Association of Immunologists [27].

Figure 6.

(a) Binding of bivalent and monovalent fragments of LA mAb to apoptotic cells in the presence of PT. Purified IgG (filled circles), IgG' (open circles), and F(ab')₂ (filled triangles) and Fab' (open triangles) fragments of 29J3-62 or 29I4-24 were evaluated for binding to apoptotic cells in the presence of PT (20 μg/ml) by flow cytometry. IgG and mAb fragments were used at concentrations that provided the equivalent molarity of antigen-binding sites for all samples. A subclass-matched negative control (27D2-83) was included in each experiment, and data are representative of two independent experiments. (b) Enhanced binding of PT to apoptotic cells in the presence of LA mAb. Binding of FITC-PT (20 μg/ml) to apoptotic cells in the presence (filled histograms), or absence (open histograms), of 29J3-62, 29I4-24, or a subclass-matched control (27D2-83) (20 μg/ml) by flow cytometry. Copyright 2003. The American Association of Immunologists [27].

Characteristics of murine monoclonal antibodies

Table 1

Antibody	Immunogen	Subclass	Reactivity in ELISA (OD ₄₀₅) ^a				APTT (s) ^b	
			CL	DOPS	DOPC	β2GPI	PT	
29J3-62	PT+DOPS	IgG1 (κ)	0.070	0.211	0.060	0.038	>2.65	>180
29I4-24	PT+DOPS/DOPC	IgG1 (κ)	0.045	0.012	0.018	0.013	0.781	>180
27D2-83	PT+DOPS/DOPC	IgG1 (κ)	0.025	0.088	0.033	0.012	0.001	50.1

Positive reactivity was defined by an OD₄₀₅ reading >0.4.

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^aReactivity of purified mAb (5 µg/ml) in ELISA. Phospholipid (CL, DOPS, or DOPC) was coated at 90 µg/ml, human β2-glycoprotein I (β2GPI) at 15 µg/ml, and human prothrombin (PT) at 20 µg/ml.

^bLupus anticoagulant activity of purified mAb 29J3-62, 29I4-24, and negative control 27D2-83 (200 µg/ml).

Table 2

Affinity and avidity of mAb 29I4-24 and 29J3-62 for prothrombin (PT) bound to different surfaces

Surface to which PT was bound	Antibody valency	<u>Antibody reactivity</u>	
		29I4-24	29J3-62
Polystyrene plates			
Affinity ^a	Monovalent	Negligible	High
Avidity ^a	Bivalent	Low	High
Phospholipid ^b			
Affinity	Monovalent	High	High
Avidity	Bivalent	High	High
Apoptotic Jurkat cells			
Affinity	Monovalent	Low	Low
Avidity	Bivalent	High	High

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^a Affinity was determined using monovalent Fab' fragments, while avidity was determined using intact IgG, IgG', and bivalent F(ab')₂ fragments.

^b Phospholipid was incubated with PT, and then coated to polystyrene plates.