Some Plasmin-Induced Antibodies Bind to Cardiolipin, Display Lupus Anticoagulant Activity and Induce Fetal Loss in Mice

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Antiphospholipid syndrome (APS) is a heterogeneous disease characterized by thrombosis, fetal loss, and anti-phospholipid Ab (aPL) (1–3). The characteristic aPL include anti-cardiolipin Ab (aCL) and lupus anticoagulants (LAC), as detected by their abilities to prolong certain in vitro phospholipid (PL)-restricted blood clotting tests. Both aCL and LAC serve as the diagnostic criteria for APS (1, 2). Accumulated studies show that, in addition to phospholipids (PL), aPL also recognize some plasma proteins, including β$_2$-glycoprotein 1, prothrombin, protein C, protein S, and tissue plasminogen activator (4, 5). Several mechanisms have been implicated in this disease pathogenesis, including coagulation abnormality and fibrinolytic dysfunction (1, 2).

Recently, our studies of seven monoclonal IgG aCL from two APS patients revealed that five aCL react with thrombin, activated protein C, and plasmin (6–8). All three proteins are trypsin-like serine proteases (SP), and are highly homologous in their catalytic domains. Therefore, common epitopes are likely to be shared by these SP autoantigens. Moreover, of the five cross-reactive aCL, the CL15 monoclonal IgG aCL can inhibit the anticoagulant activity of activated protein C and the fibrinolytic function of plasmin. Importantly, among the three SP autoantigens, mAb CL15 binds to plasmin with relatively high affinity (with a relative $K_d$ value of $1 \times 10^{-7}$) (8). In contrast, when plasma samples from 25 APS patients and 20 normal controls were analyzed for IgG anti-plasmin Ab and the mean plus 3 SD of the normal controls was used as the cutoff, IgG anti-plasmin Ab were positive in 7 (28%) of 25 APS patients (8).

Considering the relatively high affinity of CL15 for plasmin, we hypothesized that plasmin may be a key driving autoantigen for some IgG aCL in the APS patients who are positive for IgG anti-plasmin Ab. To test this hypothesis, we immunized mice with human plasmin and analyzed immune sera for aCL activity and reactivity with relevant SP. We found that some immune sera displayed aCL activity and/or bound to test SP. Subsequently, eight mAb were obtained and studied. The results revealed that one mAb displayed the aCL and the LAC activities and induced fetal loss when injected into pregnant mice. Immunohistological analyses of placentas revealed extensive deposits of activated C3 components. Combined, these data demonstrate that plasmin may serve as a driving Ag for some pathogenic aPL.

**Materials and Methods**

**Plasmin immunization and generation of mAb**

All experimental procedures involving mice were approved by the Animal Care and Use Committee of Shanghai Jiaotong University School of Medicine. Groups of 10 BALB/c mice (8 wk old) were each immunized s.c. with 50 μg human plasmin (BioDesign International) in CFA (Sigma-Aldrich) or CFA only (for the control group) on days 0, 14, and 28. On day 42, mice were boosted with 100 μg human plasmin. Two weeks later, blood samples were obtained and stored at −70°C, and spleen cells were isolated and fused with mouse myeloma cell SP2/0 lineage growing in selective medium described by standard procedure (9, 10). Hybridomas were screened for IgG anti-plasmin Ab by ELISA. Cells from confirmed positive wells were subcloned by limited dilution method. Eight IgG mAb were isolated and were used in following studies. The monoclonality of each mAb was determined according to the protocol of MABTEST kit (Adiatec).
IgG was purified by affinity chromatography using protein G-Sepharose chromatography columns (Amersham Pharmacia Biotech). All IgG samples were treated with Centriprep ultrafiltration devices (Millipore) to remove endotoxin for in vivo experiments. After treatment, endotoxin levels of mAb were determined by the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000; BioWhittaker); the endotoxin levels of all mAbs were determined by the quantitative chromogenic Limulus amebocyte lysate assay (QCL-1000; BioWhittaker); the endotoxin levels of all mAbs were<0.03 endotoxin units/μg IgG.

ELISA

The ELISA for IgG anti-plasmin Ab was done as described previously (8). Briefly, high-binding microwell plates (Costar) were coated separately with 5 μg/ml human plasmin (BioDesign International) in ethanol was used to coat regular binding microwell plates. After incubated overnight at 4°C, plates were blocked with 10% FCS. Test mAb were diluted at 1/100 in PBS containing 0.1% gelatin, and purified mAb were diluted at 1 μg/ml in PBS containing 0.1% gelatin. Test mice sera were diluted at 1/100 in PBS, and test mAb were diluted overnight at 4°C, plates were blocked with 10% FCS. Test mice sera were diluted at 1/100 in PBS, and test mAb were diluted at 1 μg/ml in PBS containing 10% FCS.

The ELISA for IgG against thrombin and activated protein C were done as described previously (6, 7). They were similarly to the aforementioned assay for anti-plasmin Ab, except that the plates were coated with 5 μg/ml human thrombin (BioDesign) or activated protein C.

The LAC test

LAC activity of mAb was determined by a modified dRVVT according to Zhu et al. (13). Briefly, 20 μl of test IgG (final concentration, 160 μg/ml) were incubated with 80 μl of normal pooled human plasma, provided by Department of Clinical Laboratory at the Ruijin Hospital (Shanghai, China), for 3 min at 37°C. Then, 100 μl of the prewarmed dRVVT reagent (LAC-Screen; Gradipore) was added to initiate clotting, and the clotting time was determined in a semiautomated BBL fibrometer (BD Biosciences). If the ratio of the clotting time of a test mAb to that of a normal IgG exceeded 1.2 (14), its LAC activity was confirmed by a confirmatory test, in which the assay was repeated exactly as above except that the dRVVT-confirm reagent (LAC-Confirm; Gradipore) contained excess PL to neutralize the LAC effect. A LAC activity was confirmed positive if the clotting time ratio of dRVVT/dRVVT-confirm was also >1.2.

Function assay for the amidolytic activity of plasmin

Amidolytic activity assay was used to determine the effect of murine mAb on plasmin activity. Briefly, plasmin (20 nM) in 50 μl of HEPES buffer (20 mM HEPES, 150 mM NaCl, 3 mM CaCl2, pH 7.4) was incubated with 50 μl of mAb (200 μg/ml) for 1 h at room temperature in microtiter wells, then 100 μl of a chromogenic substrate S2251 (250 μg/ml final concentration) (4-nitroaniline dihydrochloride; Sigma-Aldrich) solution was added to each well, and the plate was incubated in a humidified environment for 1 h (15). OD value at 405 nm was measured with a microplate reader (Bio-Rad 550).

Effect of mAb on activated protein C activity

The effects of mAbs on amidolytic activity of activated protein C were determined using substrate S2366 (Chromogenix). Briefly, 50 μl of activated protein C (25 nM) was mixed separately with 50 μl of a test mAb or murine monoclonal control IgG (all at 200 μg/ml) in the HBSS buffer for 1 h at room temperature. One hundred microliters of S2366 (0.4 mM) was added to each reaction mixture. After 5 min, OD at 405 nM was measured.

Table I. Characteristics of sera from 10 mice immunized with plasmin

<table>
<thead>
<tr>
<th></th>
<th>Plasmin</th>
<th>CL/FCS</th>
<th>Activated Protein C</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutoff OD*</td>
<td>0.20</td>
<td>0.22</td>
<td>0.17</td>
<td>0.19</td>
</tr>
<tr>
<td>M1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The OD cutoff values were determined by means of 10 control mice immunized by CFA plus 3 SD. –, <3 SD; +, >3 SD; ++, >5 SD; ++++, >10 SD. Each value is the mean of three duplicates. Murine sera were diluted at 1/100.

FIGURE 1. Some anti-plasmin mAb display the aCL activity and react with thrombin and activated protein C. Test mAbs and the control murine IgG were used at 1 μg/ml, and the mean OD and SEM are given (n = 2). A, Reactivity of mAbs with plasmin; B, three anti-plasmin mAbs (B12, E9, and F10) display the aCL activity (a diagnostic criterion for disease-associated aPL); C, one anti-plasmin mAb (CS) binds to activated protein C; D, one anti-plasmin mAb (CS) bind to thrombin.
also same test sample. LAC activity of a test IgG was confirmed positive if this ratio was normal IgG.

The mean OD plus 3 SD of the 10 control murine sera were used as the cutoff, and the serum samples with OD values consistently higher than the cutoff in two separate experiments were considered positive. The one-way ANOVA was used to compare plasmin activity, the resorption rate, and fetal weights among groups. A value of $p < 0.05$ was considered to be significant.

**Results**

**Plasmin immunization induced IgG aCL and other relevant cross-reactive autoantibodies in some mice**

To test our hypothesis that plasmin may serve as a driving autoantigen for IgG aCL and other concerned aPL, sera from 10 immunized mice and 10 control mice were analyzed at 1/100 dilution for IgG anti-plasmin Ab, IgG aCL, IgG anti-thrombin Ab, and IgG anti-activated protein C Ab. The results showed that, as would be expected, plasmin immunization induced strong IgG anti-plasmin Ab, IgG aCL, IgG anti-thrombin Ab, and IgG anti-activated protein C Ab.

**Immunohistological analyses of Ab-induced fetal loss**

To investigate the role of complement activation in the Ab-induced fetal loss, the OCT slides were treated sequentially with 0.2% H2O2 in methanol (to quench endogenous peroxidase), normal rat serum (to block nonspecific binding), primary rat anti-mouse C3b/C3c poly-Ab (HyCult Biotechnology), and goat anti-rat IgG conjugated to HRP (Sigma-Aldrich). Bound HRP was detected with diaminobenzidine. Sections were counterstained with hematoxylin and covered with mounting medium (Cytoseal).

**Statistical analysis**

The mean OD plus 3 SD of the 10 control murine sera were used as the cutoff, and the serum samples with OD values consistently higher than the cutoff in two separate experiments were considered positive. The one-way ANOVA was used to compare plasmin activity, the resorption rate, and fetal weights among groups. A value of $p < 0.05$ was considered to be significant.

**Table II. dRVVT test of eight anti-plasmin mAb**

<table>
<thead>
<tr>
<th>mAb</th>
<th>dRVVT(s)</th>
<th>Ratio*</th>
<th>dRVVT-Confirm Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>B12</td>
<td>40.7</td>
<td>0.91</td>
<td>ND</td>
</tr>
<tr>
<td>C4</td>
<td>44.1</td>
<td>0.98</td>
<td>ND</td>
</tr>
<tr>
<td>C5</td>
<td>43.9</td>
<td>0.98</td>
<td>ND</td>
</tr>
<tr>
<td>E5</td>
<td>41.2</td>
<td>0.92</td>
<td>ND</td>
</tr>
<tr>
<td>E9</td>
<td>53.5</td>
<td>1.2</td>
<td>42.3</td>
</tr>
<tr>
<td>F5</td>
<td>43.5</td>
<td>0.98</td>
<td>ND</td>
</tr>
<tr>
<td>F10</td>
<td>43.4</td>
<td>0.97</td>
<td>ND</td>
</tr>
<tr>
<td>G11</td>
<td>42.5</td>
<td>0.95</td>
<td>ND</td>
</tr>
<tr>
<td>Control IgG (0.16 g/L)</td>
<td>44.6</td>
<td>—</td>
<td>43.8</td>
</tr>
</tbody>
</table>

* All of the mAb were tested at 160 mg/ml.
† Each value represents the clotting time ratio of dRVVT/dRVVT-confirm for the same test sample. LAC activity of a test IgG was confirmed positive if this ratio was also >1.2.

**Murine fetal loss model**

Ten-week-old female BALB/c mice were mated with male mice, and the presence of a vaginal plug was defined as day 0 of pregnancy (16–18). On days 8 and 12 of pregnancy, each mouse was either not treated (as the baseline control) or treated with i.p. injection of polyclonal anti-plasmin Ab or the control mouse IgG (500 μg each), or a test mAb or a corresponding isotype control IgG (50 μg each), or PBS. Mice were sacrificed on day 15, uteri were dissected, fetuses were weighed, and frequency of fetal resorption was determined (number of resorption divided by the total number of formed fetuses and resorption).

**FIGURE 2.** The effect of mAb on plasmin amidolytic activity using substrate S2251. A, Plasmin amidolytic activity (using substrate S2251) in the absence or presence of test mAb or normal murine IgG (all at 100 μg/ml) at the 60-min time point. The results are given in mean OD and SEM (n = 3). B, The mAb-mediated inhibition of plasmin activity was calculated from the above data and was expressed in mean percentage of inhibition and SEM. Significantly, mAb C5 and F10 could inhibit plasmin activity by −41.8% ($p = 0.001$) and 34.5% ($p = 0.016$), respectively.

**Some murine IgG anti-plasmin mAb display aCL activity and bind to concerned serine proteases**

Subsequently, we used splenocytes from mice M1 and M5 to generate IgG anti-plasmin mAb. Eight mAb were obtained; all from the M5 mouse (B12, C4, C5, E5, E9, F5, F10, and G11). Of these mAb, E9 and F10 were IgG1; B12, C4, E5, and G11 were IgG2a; and C5 and F5 were IgG2b. Fig. 1A shows the binding activity of these mAb to plasmin.

Thereafter, these mAb were analyzed for aCL activity, as well as binding to thrombin and activated protein C. The results showed that three displayed aCL activity (i.e., B12, E9, and F10, Fig. 1B); of these, mAb E9 bound strongly to thrombin (Fig. 1C). In addition to the three CL-reactive anti-plasmin mAb, mAb C5 reacted with activated protein C (Fig. 1D). Combined, these data indicated that plasmin could serve as a driving Ag for some IgG aCL, IgG anti-thrombin, and IgG anti-activated protein C Ab.

**A plasmin-induced IgG aCL mAb display LAC activity**

As noted earlier, aPL include aCL and LAC. Therefore, it was of interest to investigate whether some anti-plasmin mAb display LAC activity. To this end, all mAb were analyzed for their LAC activity by the dRVVT test and the dRVVT-Confirm test. Considering that the human plasma IgG concentration is 8–16 g/L in
To test our hypothesis that plasmin is a driving autoantigen for fetal loss, we studied the pathogenic potentials of two anti-plasmin mAb: mAb E9 and C5. These two mAb were chosen because mAb E9 is the only mAb that displays both diagnostic aCL and LAC activity, and mAb C5 is the only mAb that binds to activated protein C, suggesting that it may mimic the CL15 mAb in reducing activated protein C activity and promoting thrombosis (19). As can be seen in Fig. 3A, the fetal resorption rates in mice treated with E9 and C5 were 25.4 and 18.5%, respectively, as compared with 11.3 and 10.6% in the corresponding isotype control mice. The fetal resorption rate in mice treated with polyclonal anti-plasmin Ab did not increase significantly as compared with the corresponding control mice treated with mouse IgG (17.8% vs 11.2%). Considering that only one of eight anti-plasmin mAb displayed significantly the diagnostic aCL activity and that another one of eight anti-plasmin mAb bound to activated protein C, the negative data probably reflected that only part of polyclonal anti-plasmin Ab was pathogenic. In addition, both mAb also significantly reduced the fetal weights from treated mice as compared with those from the corresponding control mice (Fig. 3B).

**Immunohistological analysis of anti-plasmin/aCL mAb-induced fetal loss**

Placentas were examined immunohistologically to ascertain the role of the E9 anti-plasmin/aCL mAb in observed fetal loss. Under confocal microscope, IgG depositions could be seen around the sinus of the placentas of the E9-injected mice. C. There was no IgG deposits in the kidney from control mice. D. Some IgG deposit was seen in the glomeruli of kidneys from the mAb E9-injected mice.

**FIGURE 4.** IgG deposition in the placentas and kidney of the mAb E9-injected mice. A. There was no IgG deposit in the placentas from IgG1 control mice when examined with a confocal microscope. B. Much IgG deposit was seen around the sinus and small vessel of trophoblasts from the mAb E9-injected mice. C. There was no IgG deposits in the kidney from control mice. D. Some IgG deposit was seen in the glomeruli of kidneys from the mAb E9-injected mice.
The main goal of our present study was to demonstrate that plasmin may drive generation of a pathogenic aPL that displays the aCL and/or the LAC activity and could promote fetal loss (and/or thrombosis, the two main clinical manifestations of APS). Therefore, we did not try various immunization protocols and use different mouse strains (particularly those autoimmune-prone strains) to optimize induction of IgG aCL. Consequently, the serum data in Table I and mAb data in Fig. 1 and Table II may underestimate the likelihood of plasmin-induced pathogenic aPL in APS patients, as well as the characteristics of the observed plasmin-induced aCL in mice. In the future, it will be warranted to perform such studies to obtain better understanding regarding the real characteristics of plasmin-induced pathogenic aPL in APS patients.

Many studies have shown that passive transfer of aPL (from APS patients) into pregnant mice induce fetal loss, as indicated by increased embryo resorption and lower weights of pups and placentas (22–27). These data indicate a pathogenic role for aPL in pregnancy loss. Moreover, immunohistological analysis of placentas from aPL-injected mice revealed decidual necrosis and intravascular IgG aPL and fibrin deposition. Recently, in vivo studies in mice showed that complement activation is an important and necessary intermediary step for the aPL-mediated fetal loss and thrombosis (20). The activated fragments of complements (i.e., C3b and C5a) can initiate a proinflammatory amplification loop, resulting in tissue damage and fetal death (20, 28).

In this context, to determine whether excessive complement activation is associated with fetal loss and the placental damage in pregnant mice treated with the E9 anti-plasmin/aCL mAb, we performed immunohistological analysis of placentas to search for IgG deposition and activated C3 in placentas. The results revealed IgG deposits around the sinus of the placentas (Fig. 4B) and C3b/C3c deposits in the small vessel and the basal membrane of the villi in the placentas (Fig. 5, B and C). These data indicated that anti-plasmin Ab might induce fetal loss and placenta damage via the activation of the complement pathway. Of note, several studies suggested that aCL interacted with trophoblasts (26, 29, 30). Therefore, the E9 anti-plasmin/aCL may deposit in placenta via interacting with trophoblasts. Further studies are warranted to examine this hypothesis.

In addition to anti-plasmin/aCL IgG deposits in placenta, IgG deposit was also found in kidney. Recently, an expanding spectrum of renal diseases was reported to be associated with APS (31–33); Therefore, in the future, it will be of interest to explore the possibility of using the E9 IgG anti-plasmin/aCL mAb to develop a mouse model for APS nephropathy (34).

How does plasmin serve as a driving autoantigen in patients? One possibility may be that plasmin is presented as the plasmin-bacterium complexes. For example, during an infection by Streptococcus pyogenes (also known as the group A streptococcus (GAS)), bacterial plasminogen receptors pick up plasminogen from host blood. At the same time, streptococci secrete streptokinase, which binds to plasminogen to form a complex that converts plasminogen to plasmin on bacterial surface (35, 36). Consequently, plasmin is presented to the host with streptococci, clearly a danger signal, and thus may induce anti-plasmin Ab response according to the “danger signal” hypothesis (37, 38). However, because plasmin is an autoantigen, anti-plasmin B cells are supposed to be tolerized by immune regulation in normal individuals, but may escape tolerance regulation and persist in genetically susceptible individuals with abnormal immune regulation. In addition to GAS, several other human pathogens also express plasminogen activators, including staphylokinase of Staphylococcus aureus and the P4 surface protease of Yersinia pestis (35). Of note, GAS is the most common cause of clinically significant bacterial infection of the pharynx (such as pharyngitis and tonsillitis) and skin (such as...
impetigo, pyoderma, and erysipelas). Consequently, repeated infections may lead to repeated stimulation of the host with plasminogen-GAS complexes, resulting in high-affinity anti-plasmin Ab and the mutated anti-plasmin Ab that display aCL and LAC activity. We plan to test this hypothesis in our future study.

What is the quantitative importance of anti-plasmin Ab and/or plasmin-driven aCL in APS patients? To address this issue, we have been studying the frequency of anti-plasmin Ab and/or plasmin-driven aCL in APS patients, using at least two positive IgG anti-plasmin Ab from blood samples of a patient that are at least 6 wk apart (following the 1999 criteria for aCL, since the study was initiated in 2003) (39). In addition, we are continuing to search and characterize additional unidentified aPL (in terms of their binding specificity, pathogenic potential, and functional activity) in patients. Once we identify all possible aPL, we will study the quantitative importance of each aPL in the order of the strength of pathogenic potential and functional activity of each aPL.

In summary, the present study shows that plasmin immunization in mice may induce IgG anti-plasmin Ab that display the aCL and the LAC activity and induce fetal loss in pregnant mice. Moreover, the Ab, as well as C3b/C3c, was found to be deposited in placentas in treated mice, suggesting that the aCL-positive IgG anti-plasmin Ab may induce fetal loss via complement activation. Taken together, our data indicate that plasmin may serve as a driving autotnigen for some pathogenic aCL in certain APS patients who are positive for IgG anti-plasmin Ab.

Acknowledgment

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Disclosures

The authors have no financial conflict of interest.

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