Antibodies to Factor XII Associated with Lupus Anticoagulant

D. W. Jones, M. J. Gallimore, S. L. Harris, M. Winter

From the Kent Haemophilia Centre, Kent and Canterbury Hospitals NHS Trust, Canterbury, Kent, United Kingdom

Summary

Falsely low levels of factor XII (FXII) have been documented in patients who are lupus anticoagulant positive (LA+). In addition, we have previously noted a surprisingly high incidence (20.9%) of apparently true FXII deficiency in patients who were LA+. We have hypothesised that this may be partly due to the presence of antibodies to FXII.

The aim of the present study was to investigate whether LA+ patient plasmas contain antibodies directed either against FXII or FXII in association with phospholipids. Plasma samples from 60 blood donors, all LA negative, and 51 LA+ patients were tested using ELISA assays employing purified FXII, phosphatidylserine (PS) and phosphatidylethanolamine (PE). We have identified seven patients whose plasma contained either IgG or IgM that reacted with purified FXII in the absence of PS or PE. When PS was included in the assay system four additional patient plasmas were shown to contain either IgG or IgM that reacted with FXII. The plasma of one patient contained IgG that reacted with FXII both in the presence and absence of PS. There was no reactivity to FXII with either IgG or IgM when PE was included in the assay system. Affinity purified IgG from three patients whose plasma reacted with FXII in the ELISA assay in the absence of PS, gave a positive reaction in an immunoblot assay. These results suggest that FXII antibodies are present in a significant proportion of LA+ patients and may lead to an erroneous diagnosis of FXII deficiency.

Introduction

Patients with circulating phospholipid antibodies have a high incidence of thrombosis (1). Current evidence indicates that many of these phospholipid antibodies do not react with phospholipids alone but recognise phospholipid/protein complexes. These proteins include beta-2-glycoprotein I and/or prekallikrein associated with anionic phospholipids (2-4) and high molecular weight kininogen (HK), low molecular weight kininogen (LK) and/or proteins in complex with HK (factor XI or prekallikrein) associated with a zwitterionic phospholipid (phosphatidylethanolamine) (5, 6).

The contact system protein FXII has been shown to be a major component of the intrinsic pathway of fibrinolysis (7, 8) and is linked to the extrinsic fibrinolytic pathway through the generation of bradykinin from HK and subsequent release of tissue plasminogen activator (9, 10). These findings – together with reports of high incidences of FXII deficiency in thrombotic populations (11, 12) – have led to considerable debate as to whether FXII deficiency constitutes a thrombotic risk (13-15).

We recently reported that plasma samples from some LA+ patients exhibit “pseudo-FXII deficiency” (16). This state is characterised by the FXII level as measured by one-stage clotting assay (FXIIct) being below the lower limit of the normal range while the FXII level measured by either chromogenic peptide substrate (FXIIcs) or immunochemical (FXIIag) assay are within the limits of the normal range. We have also reported that 20.9% of a cohort of 67 LA+ patients appeared to have genuine FXII deficiency in that FXII levels as assessed by FXIIct, FXIIcs and FXIIag were all below the lower limit of the normal range (17). These studies were carried out on Caucasian populations. This figure of 20.9% differs significantly from the prevalence of FXII deficiency found in local blood donors (2.8%) or in patients with a history of venous thrombosis (7.8%) (18).

These findings have led us to surmise that antibodies to FXII might be present in some LA+ patients, in a situation analogous to the occasional reports of antibodies to prothrombin in such patients (4).

We have now tested plasma samples from a group of normal lupus anticoagulant negative (LA−) blood donors and a group of LA+ patients for the presence of antibodies to FXII, or to FXII in association with either an anionic (phosphatidylserine) or zwitterionic (phosphatidylethanolamine) phospholipid.

Subjects, Materials and Methods

Subjects

Samples from 60 LA– blood donors and 51 LA+ patients (referred for investigation of thrombosis or phospholipid syndrome) were collected and treated as previously described (17). All samples were assessed for lupus anticoagulant positivity by the dilute Russell’s viper venom time and platelet correction procedure, as detailed by the British Society of Haematology (19). All samples were also assayed for FXII levels using one-stage clotting (FXIIct), chromogenic peptide substrate (FXIIcs) and immunochemical (FXIIag) methods, as previously described (17).

ELISA Assays for FXII Antibodies

Antibodies to FXII

Purified human FXII (Enzyme Research, Swansea, UK), was reconstituted to give a concentration of 500 µg/ml in distilled water and then further diluted in 0.05 M carbonate buffer pH 9.6 to give a final concentration of 5 µg/ml. Nunc Maxisorb microtitre plates (Life Technologies, Paisley, UK) were coated with 50 µl of the diluted FXII and left for 1 h at room temperature
and then washed 3 times using Tris buffered saline (TBS) pH 7.3. Seventy-five µl of blocking/diluting buffer (TBS containing 1% bovine serum albumin (Sigma Aldrich, Poole, UK)) was then added to each well, left to incubate for 1 h and then washed 3 times using TBS. Fifty µl of patient or control plasma, diluted 1:50 in blocking/diluting buffer was incubated in duplicate on the microtitre plates for 1.5 h at room temperature. The plates were washed 3 times using TBS.

Fifty µl of either peroxidase conjugated rabbit anti human IgG or peroxidase conjugated rabbit anti human IgM (diluted 1:5000 or 1:1000 in blocking/diluting buffer respectively) was added to each well of the microtitre plate. The plate was incubated for 1 h at room temperature and then washed 3 times with TBS. Colour development was produced using 1,2 ortho-phenylenediamine substrate (Sigma Aldrich). Exactly 20 min after the substrate was added the reaction was stopped using 50 µl of 1 M sulphuric acid. The optical density readings were measured at 492 nm using a Spectramax 340 (Molecular Devices, Crawley, UK).

**Antibodies to FXII in Combination with PS or PE**

Nunc Maxisorb microtitre plates were coated with 30 µl of either 50 µg/ml phosphatidylserine (PS) or phosphatidylethanolamine (PE) (Sigma Aldrich) and placed on a shaker to evaporate overnight at 4°C. The plates were washed 3 times using TBS. Seventy-five µl of blocking/diluting buffer was then added to each well of the microtitre plate which were left to incubate for 1 h at room temperature and then washed 3 times using TBS. Fifty µl of the diluted FXII was then added to each well of the microtitre plate, the plates were left to incubate for 1 h at room temperature and then washed 3 times using TBS. From this point the assay was identical to that of the assay for determining antibodies to FXII. Reactivity of patients’ plasma with either PS or PE alone was measured using the above assay conditions but with the omission of FXII. Optical density readings were then subtracted from those of the FXII in association with PS or PE before calculating antibody positivity.

The cut-off point for positivity was established as 2 SD above the mean on log transformed data of the anti FXII levels (as 492 nm OD values) in the 60 LA– blood donors.

**Affinity Purification of Plasma IgG**

Affinity purified IgG was prepared with HiTrap protein G columns using the supplied methodology (Amersham Pharmacia Biotech, St Albans, UK).

**Immunoblot**

A nitrocellulose membrane (Amersham Pharmacia) had 10 × 1 µl volumes of purified FXII (50 µg/100 µl) pipetted onto it. Excess protein binding sites were then blocked using 10% non-fat milk (NFM) in TBS for 2 h. Affinity purified IgG fractions from 4 patients (W, X, Y and Z) found positive by ELISA against FXII in the absence of phospholipids were diluted 1:50 in 10% NFM in TBS. Affinity purified fractions from three blood donor samples were also diluted 1:50 in 10% NFM in TBS. Using a DECA blot system (Amersham Pharmacia) the diluted affinity-purified IgG along with a positive control [affinity-purified goat anti-human FXII IgG (Nordic, Tilburg, The Netherlands) diluted 1:10,000 in 10% NFM in TBS] was incubated with the FXII dot blots overnight. Peroxidase conjugated rabbit anti-human IgG (Dako, Cambridge, UK) diluted 1:1000 in 10% NFM in TBS was used as a detector antibody for the patient and blood donor samples. Peroxidase conjugated rabbit anti-goat IgG (Dako) diluted 1:1000 in 10% NFM in Tris buffered saline was used as the detector antibody for the positive control. The immunoblot was developed using 1,2-ortho-phenylenediamine dihydrochloride (Sigma Aldrich) containing 0.02% hydrogen peroxide.

**Results**

**ELISA Assays**

Of the 51 LA+ patients investigated for the presence of antibodies to FXII we have found:

(i). In the absence of phospholipid:

The plasma of 3 patients contained IgG antibodies that reacted with FXII.

The plasma of 3 patients contained IgM antibodies that reacted with FXII.

The plasma of 1 patient contained IgG and IgM antibodies that reacted with FXII.

(ii). In the presence of PS:

The plasma of 3 additional patients contained IgG antibodies that reacted with FXII.

The plasma of 1 additional patient contained IgM antibodies that reacted with FXII.

The plasma of 1 additional patient contained IgM antibodies that reacted with FXII.

**Table 1** Results from ELISA, immunoblot and FXII assays together with a summary of clinical manifestations in 11 LA+ patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Contains antibody to</th>
<th>Antibody type</th>
<th>ELISA OD v FXII-IgG Normal range 0.02-0.751</th>
<th>ELISA OD v FXII-IgM Normal range 0.06-0.721</th>
<th>ELISA OD v FXII/PS-IgG Normal range 0.035-0.141</th>
<th>ELISA OD v FXII/PS-IgM Normal range 0.029-0.290</th>
<th>Immunoblot</th>
<th>FXII clotting Normal range 48-149 aU/ml</th>
<th>FXII IgG clotting Normal range 51-151 aU/ml</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>FXII</td>
<td>IgG</td>
<td>0.320*</td>
<td>0.164</td>
<td>0.037 (0.055- [blank] 0.006)</td>
<td>0.031 (0.099- [blank] 0.066)</td>
<td>Positive</td>
<td>30</td>
<td>34</td>
<td>40</td>
</tr>
<tr>
<td>X</td>
<td>FXII</td>
<td>IgG</td>
<td>0.318*</td>
<td>0.100</td>
<td>0.077 (0.087- [blank] 0.010)</td>
<td>0.011 (0.011- [blank] 0)</td>
<td>Positive</td>
<td>143</td>
<td>136</td>
<td>129</td>
</tr>
<tr>
<td>Z</td>
<td>FXII &amp; FXII/PS</td>
<td>IgG</td>
<td>0.273*</td>
<td>0.187</td>
<td>0.327 (0.386- [blank] 0.058)</td>
<td>0.039 (0.025- [blank] 0.066)</td>
<td>Negative</td>
<td>105</td>
<td>101</td>
<td>106</td>
</tr>
<tr>
<td>1</td>
<td>FXII</td>
<td>IgM</td>
<td>0.162</td>
<td>0.630*</td>
<td>0.072 (0.112- [blank] 0.038)</td>
<td>0.243 (0.438- [blank] 0.195)</td>
<td>ND</td>
<td>8</td>
<td>79</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>FXII</td>
<td>IgM</td>
<td>0.222</td>
<td>0.365*</td>
<td>0.027 (0.086- [blank] 0.059)</td>
<td>0.039 (0.051- [blank] 0.012)</td>
<td>ND</td>
<td>99</td>
<td>96</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>FXII</td>
<td>IgM</td>
<td>0.169</td>
<td>0.353*</td>
<td>0.063 (0.079- [blank] 0.016)</td>
<td>0.081 (0.099- [blank] 0.014)</td>
<td>ND</td>
<td>92</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>Y</td>
<td>FXII</td>
<td>IgG &amp; IgM</td>
<td>0.343*</td>
<td>0.366*</td>
<td>0.082 (0.315- [blank] 0.233)</td>
<td>0.046 (0.072- [blank] 0.050)</td>
<td>Positive</td>
<td>102</td>
<td>102</td>
<td>114</td>
</tr>
<tr>
<td>4</td>
<td>FXII/PS</td>
<td>IgG</td>
<td>0.237</td>
<td>0.005</td>
<td>0.378 (0.323- [blank] 0.145)</td>
<td>0.07 (0.096- [blank] 0.019)</td>
<td>ND</td>
<td>46</td>
<td>120</td>
<td>118</td>
</tr>
<tr>
<td>5</td>
<td>FXII/PS</td>
<td>IgG</td>
<td>0.106</td>
<td>0.143</td>
<td>0.356 (0.511- [blank] 0.165)</td>
<td>0.108 (0.193- [blank] 0.083)</td>
<td>ND</td>
<td>79</td>
<td>86</td>
<td>81</td>
</tr>
<tr>
<td>6</td>
<td>FXII/PS</td>
<td>IgG</td>
<td>0.175</td>
<td>0.010</td>
<td>0.241 (0.371- [blank] 0.033)</td>
<td>0.058 (0.068- [blank] 0.010)</td>
<td>ND</td>
<td>116</td>
<td>113</td>
<td>110</td>
</tr>
<tr>
<td>7</td>
<td>FXII/PS</td>
<td>IgM</td>
<td>0.247</td>
<td>0.165</td>
<td>0.018 (0.018- [blank] 0)</td>
<td>0.324 (0.527- [blank] 0.203)</td>
<td>ND</td>
<td>38</td>
<td>66</td>
<td>62</td>
</tr>
</tbody>
</table>

* denotes positive result

ND = not done
The plasma of 1 patient whose plasma contained IgG antibodies that reacted with FXII in the absence of phospholipid also reacted with FXII in the presence of PS. (iii). In the presence of PE:

None of the plasmas were found to react with FXII in association with PE.

Clinical manifestations, FXII levels and the results of the ELISA and immunoblot assays for the patients with antibodies to FXII are summarised in Table 1.

Immunoblot

Results for the immunoblot assay using affinity-purified IgG from 4 patients whose plasma reacted with the FXII in the absence of phospholipid and from 3 blood donors are shown in Fig. 1. The affinity-purified IgG from the 3 blood donors showed no reactivity with the FXII present on the immunoblot while 3 of the 4 patient plasmas showed reactivity with FXII.

Discussion

Lupus anticoagulants are known to interfere with aPTT based coagulation factor assays and have been shown to give falsely low levels of FXII. We have termed this effect “pseudo-FXII deficiency” which describes patients whose plasmas give results that are below the lower limit of normal in the FXIIct assay while giving normal results in the FXIICs and FXIIag assays (16). This effect on FXII assays has been demonstrated to be more pronounced as well as more variable than that seen in assays for factors VIII, IX or XI (17).

We also reported a high incidence (20.9%) of apparent true FXII deficiency (low FXIIct, FXIICs and FXIIag) in patients who were LA+ and suggested that this may in part be due to the presence of antibodies to FXII, leading to removal of FXII from the circulation (17).

We now demonstrate, in the present study, the presence of antibodies against FXII and to FXII in association with an anionic phospholipid (PS) in patients who are LA+. In 51 LA+ patients so far investigated we have found 7 whose plasma contained antibodies (3 IgG, 3 IgM and 1 IgG + IgM) that reacted with FXII in the absence of phospholipids. Four additional patient plasmas were found to contain antibodies that reacted with FXII in association with PS (3 IgG and 1 IgM) and one patient plasma that reacted with FXII both in the presence and absence of PS (IgG). Thus antibodies against FXII alone or in association with PS could be demonstrated in 11 of 51 (21.6%) lupus anticoagulant positive patients.

No plasma showed reactivity with FXII in association with PE. These results for FXII are therefore unlike those reported for high and low molecular weight kininogens where antibodies against these proteins could only be demonstrated in the presence of PE a zwitter-ionic phospholipid (6, 7).

Immunoblot analysis of the affinity-purified IgG fractions confirmed the presence of antibodies to FXII, in the absence of phospholipids, in 3 patients whose plasma reacted with FXII by ELISA (Fig. 1). This positivity could only be demonstrated at a dilution of 1/50 or below.

There have been a number of anecdotal reports of circulating anticoagulants against FXII (20-22) most of which have been associated with lupus anticoagulants. The anticoagulant activity against FXII in these studies was based on coagulation assays alone which are known to be sensitive to interference in the presence of phospholipid antibodies (17).

We have now clearly demonstrated the presence of FXII antibodies in the plasma of some LA+ patients. These antibodies would appear to react with either FXII alone or with FXII associated with an anionic phospholipid (phosphatidylserine), suggesting that more than one population of antibodies may be present (Table 1). The effect of these antibodies on FXII levels appears to be variable, leading in some patients to a pattern of pseudo-FXII deficiency, in others to a reduction in FXII levels regardless of assay type, presumably through removal of FXII from the circulation and in others to no obvious effect. Similar patterns of FXII levels are seen in LA+ patients with no demonstrable antibodies to FXII.

The variable effect and significance of these antibodies on FXII levels as measured by FXIIct, FXIICs and FXIIag, as well as functional activities of FXII, are as yet unexplained but it is of interest to note that of the eleven LA+ patients so far identified as having antibodies to FXII, nine have suffered vascular events while one has suffered recurrent foetal loss (Table 1).

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