Enhanced Lipid Peroxidation in Patients Positive for Antiphospholipid Antibodies

By Luigi Iuliano, Domenico Praticò, Domenico Ferro, Valerio Pittoni, Guido Valesini, John Lawson, Garret A. FitzGerald, and Francesco Violi

The mechanism leading to the formation of antiphospholipid antibodies (aPL) is still unknown. Because an in vitro study suggested that aPL may derive from pro-oxidant conditions, we sought a relationship between aPL and isoprostanes, indices of lipid peroxidation in vivo. Thirty patients with systemic lupus erythematosus have been studied. Seventeen (56.6%) were positive for aPL because they had lupus anticoagulant and/or high titer of anticardiolipin antibodies (aCL). Plasma levels of tumor necrosis factor (TNF) and urinary excretion of two isoprostanes, 8-epi-PGF2α and IPF2α-I, free radical catalyzed oxidation products of arachidonic acid, were measured. Patients with systemic lupus erythematosus had higher urinary excretion of 8-epi-PGF2α and IPF2α-I than controls; urinary excretion of the two isoprostanes was highly correlated (Rho = 0.74, P < .0001). Urinary 8-epi-PGF2α was highly correlated with both aCL titer (Rho = 0.70, P < .0001) and TNF (Rho = 0.84, P < .0001), a measure of disease severity. Excretion of this isoprostane was also higher in those patients who exhibited aPL (P < .0001). Comparable correlations were observed with the isoprostane IPF2α-I. No difference of 8-epi-PGF2α was observed between patients with and without previous history of thrombosis. This study, showing the existence of a close association between aPL and increased in vivo lipid peroxidation, supports the hypothesis that these antibodies may result from pro-oxidative conditions and suggests that inflammation may play an important role.

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MATERIALS AND METHODS

Study population. Between September 1995 and July 1996 we studied 30 consecutive patients (28 women and 2 men, ages 17 to 58) diagnosed as having SLE in accordance with the criteria of the American College of Rheumatology, formerly the American Rheumatism Association, and 20 healthy subjects selected from the hospital personnel (19 women and 1 man, ages 18 to 58) as controls. The duration of disease averaged 8 ± 3 years (range 2 to 16) in the patients. Twenty-two patients were being treated with corticosteroids (prednisone 5 to 25 mg/d or methylprednisolone 4 to 24 mg/d) and/or methotrexate (0.25 to 0.30 mg/kg intravenously once a week). Nine patients were considered hypertensive having values of blood pressure >140/90 mm Hg in at least two different occasions; 8 were treated with diuretics, angiotensin-converting enzyme inhibitors, or calcium antagonists. Four patients had diabetes mellitus, 2 of whom were treated with insulin.

Eleven (36%) patients (10 women and 1 man, ages 31 to 58) had a history of thrombosis and/or fetal loss in the previous 8 to 32 months; 7 had deep venous thrombosis, 2 had deep venous thrombosis and thromboembolic stroke, 1 had retinal thrombosis, and 1 had recurrent fetal loss. Deep venous thrombosis was confirmed by venous Doppler ultrasound, and thromboembolic stroke was confirmed by computed tomographic scan.

Eight (73%) out of 11 patients with thrombosis were positive for aPL. At the time, all patients with previous thrombotic events were being treated with anticoagulant therapy (International Normalized Ratio 2.5 to 3.5). Four patients without a history of thrombosis were...
on treatment with aspirin (325 mg/d). Neither patients nor controls took vitamin supplements before 1 month of the study.

Among laboratory indexes, we measured serum levels of some proteins that were known to change during the acute phase of disease, namely, C3 and C4, C-reactive protein, and clottable fibrinogen, as previously described. No patient had had active infections, trauma, surgery, liver diseases, or other factors known to influence isoprostane levels, such as alcohol and acetaminophen abuse, during the previous 3 months. Among the healthy subjects, none had cardiovascular risk factors, but three were smokers.

**Laboratory tests.** Blood samples were taken into tubes containing 3.8% trisodium citrate and centrifuged at 500g after overnight fasting and supine rest for at least 10 minutes. The plasma was used immediately for measurement of fibrinogen and lupus anticoagulant (LA). Blood samples were also taken to measure serum aCL, C-reactive protein, the complement components C3 and C4, and tumor necrosis factor (TNF).

LA was measured in platelet poor plasma centrifuged twice at 5,000g using four different coagulation tests, as previously described. Patients were considered positive for LA if they had at least two abnormal (prolonged) clotting tests, which returned to normal values after adding 0.05 mmol/L phosphatidylcholine-phosphatidylserine liposomes (confirmatory test). An enzyme-linked immunosorbent assay, validated in an international workshop, was used for measurement of aCL. IgG or IgM aCL were considered positive when the activity was greater than 10 GPL or 10 MPL units, respectively. Patients were considered positive for aPL if LA and/or aCL were detected in two separate occasions at least 2 months apart.

Serum TNF was assayed in duplicate by an enzyme immunoassay (Biokine tumor necrosis factor alpha test kit, T Cell Diagnostics Inc, Cambridge, MA). The detection limit was calculated to be 10 pg/mL. Intra-assay and interassay coefficients of variation were 8% and 9%, respectively. Among 20 healthy subjects, 2 showed detectable TNF-α serum levels (median <10 pg/mL; range <10 to 34 pg/mL).

The same day, 12-hour urine specimens were collected from each patient. Urinary 8-epi-PGF sub alpha, and IPF sub alpha, were assayed by GC/MS, as previously described. The internal standards used were [18O]8-epi-PGF sub alpha, and [1H]IPF sub alpha. The intra-assay and interassay variabilty in urine obtained from healthy volunteers was ±3% and ±4% for 8-epi-PGF sub alpha, and ±4% and ±5% for IPF sub alpha, respectively.

**Statistical analysis.** Statistical analysis was performed by χ² statistic or Fisher’s exact test (if n < 5) for independence and by appropriate t-test. When necessary, appropriate nonparametric tests were used. Correlation analysis was carried out by Spearman test. Data were presented as median ± standard deviation (SD). Median and range are given for TNF, 8-epi-PGF sub alpha, and IPF sub alpha because they show appreciably skewed distribution. Only P values lower than .05 were regarded as statistically significant. All calculations were made with the computer program STAT-View II (Abacus Concepts, Berkeley, CA).

## RESULTS

Patients with SLE had a higher urinary excretion of 8-epi-PGF sub alpha than controls (median, 166.5; range, 60 to 405 v median, 87.5; range, 26 to 161 pg/mg creatinine; P < .0001; Fig 1). Seventeen patients (57%) had 8-epi-PGF sub alpha values higher than the cut-off point of 154 pg/mg creatinine (mean ± 2 SD of controls). Among SLE patients, 17 were considered aPL positive because they had LA and/or high titer of aCL; 8 had positivity for LA, 16 were positive for aCL with a titer ranging from 20 to 110 GPL, and 7 were positive for both LA and aCL. TNF was significantly higher in SLE patients than in the control population (median, 101.6; range, 26.8 to 290.4 v median <10; range <10 to 34 pg/mL; P < .0001).

Grouping patients according to the positivity for aPL, we found that positive patients had higher 8-epi-PGF sub alpha than negative ones (median, 225; range, 72 to 405 v median, 130; range, 60 to 175 pg/mg creatinine; P < .001; Fig 1). Fourteen (82%) aPL-positive patients had 8-epi-PGF sub alpha values higher than 154 pg/mg creatinine. Table 1 reports on clinical and laboratory characteristics of aPL-positive and -negative patients. No significant differences in urinary 8-epi-PGF sub alpha were noticed as a function of sex, age, or cardiovascular risk factors, such as hypertension, dyslipidemia, or smoking. Also, they did not show differences in renal function and acute phase reactant proteins, such as C-reactive protein, C3 and C4, and fibrinogen (not shown). Conversely, aPL-positive patients had higher values of TNF than aPL-negative patients; median values of TNF were 170.5 pg/mL (range, 26.8 to 294.4) for aPL-positive patients and 74.1 pg/mL (range, 27.8 to 138.2) for aPL-negative patients (P < .003). Among patients with 8-epi-PGF sub alpha values higher than 154 pg/mg creatinine, 14 were aPL positive and 3 were negative. A significant correlation was observed between aCL titer and 8-epi-PGF sub alpha (Rho = 0.70, P < .0001; Fig 2). Patients taking aspirin (3 with normal and 1 with 20 GPL aCL titer) had 8-epi-PGF sub alpha values similar to those of the remaining SLE population (median, 175; range, 90 to 168 v median, 172.5; range, 60 to 405 pg/mg creatinine; P > .05). This lack of difference persisted when patients taking aspirin were matched for sex, age, aCL titer, and disease activity (median, 133; range, 105 to 180 v median, 143; range, 90 to 168 pg/mg creatinine) with SLE ones not taking aspirin. Patients with 8-epi-PGF sub alpha >154 pg/mg creatinine had higher TNF values than patients with 8-epi-PGF sub alpha <154 pg/mg creatinine (median, 170.5; range, 72.5 to 290.4 v median, 48.5; range, 27.8 to 138.2 pg/mL; P < .001; Fig 3). A strong significant correlation was found between 8-epi-PGF sub alpha and TNF (Rho = 0.84, P < .0001). aPL-positive patients with previous thrombosis had 8-epi-PGF sub alpha values (median, 220; range, 150 to 335 pg/mg creatinine) similar to those of aPL-
positive patients without thrombosis (median, 240; range, 75 to 405 pg/mg creatinine; \( P > .05 \)).

To further confirm that in vivo lipid peroxidation is enhanced in SLE patients, we decided to measure another member of the \( F_2 \)-isoprostane family, \( IPF_{2a} \). Similar to our observations with \( 8\text{-ePi-PGF}_{2a} \), SLE patients had urinary levels of \( IPF_{2a} \), higher than controls (median, 1,252; range, 449 to 2,400 pg/mg creatinine \( v \) median, 470; range, 225 to 710 pg/mg creatinine; \( P < .0001 \)). Excretion of the isoprostanes in patients with SLE was highly correlated (\( Rho = 0.74; \ P < .0001; \) Fig 4). \( IPF_{2a} \) exhibited the same pattern of the other isoprostane in the respect of aCL, aPL, and TNF (data not shown).

**DISCUSSION**

SLE is an autoimmune disease of unknown cause.\(^2\) Coincidence of this condition with the detection of aPL in the circulation confers a striking risk of venous as well as arterial thrombotic events and fetal wastage.\(^1,3\) It is unknown whether this represents a direct causative effect of aPL or its association with an unknown risk factor. There is some evidence to suggest that aPL may modify procoagulant proteins and/or interfere with the anticoagulant function of endothelium.\(^5\) The nature of aPL is currently being explored with the objective of addressing its functional importance in thrombogenesis.

Hörkko et al have recently provided evidence that aPL are directed against epitopes of oxidized phospholipids and suggested that aPL may result from phospholipid oxidation.\(^7\)

To test this hypothesis in vivo, we measured the urinary excretion of \( 8\text{-ePi-PGF}_{2a} \) in SLE patients with or without aPL positivity with the aim of assessing if there was a relationship between \( 8\text{-ePi-PGF}_{2a} \) and aPL. \( 8\text{-ePi-PGF}_{2a} \) was used as a marker of lipid peroxidation because it is elevated in clinical settings associated with oxidant stress\(^11,13\) and is generated during low density lipoproteins oxidation in vitro in temporal correlation with formation of lipid peroxides.\(^18,24\)

We found that urinary \( 8\text{-ePi-PGF}_{2a} \) excretion was higher in patients with SLE than in age- and gender-matched controls. However, within the patients, those positive for aPL had higher levels of the isoprostane. Indeed, whereas 82% of the SLE patients who were aPL positive had levels of urinary \( 8\text{-ePi-PGF}_{2a} \) above the upper bound of 95% confidence interval for its excretion in healthy individuals (154 pg/mg creatinine), only 16% of the aPL-negative SLE patients fell into

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**Table 1. Clinical and Laboratory Characteristics of SLE Patients**

<table>
<thead>
<tr>
<th></th>
<th>aPL-Negative Patients</th>
<th>aPL-Positive Patients</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>36 ± 10</td>
<td>38 ± 13</td>
<td>ns</td>
</tr>
<tr>
<td>range</td>
<td>(24-49)</td>
<td>(17-58)</td>
<td></td>
</tr>
<tr>
<td>Male sex n (%)</td>
<td>1 (8)</td>
<td>1 (6)</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes mellitus n (%)</td>
<td>2 (15)</td>
<td>2 (12)</td>
<td>ns</td>
</tr>
<tr>
<td>Hypertension n (%)</td>
<td>4 (31)</td>
<td>5 (29)</td>
<td>ns</td>
</tr>
<tr>
<td>Total cholesterol (mg %)</td>
<td>Mean ± SD 210 ± 40</td>
<td>213 ± 45</td>
<td>ns</td>
</tr>
<tr>
<td>Smoking n (%)</td>
<td>1 (8)</td>
<td>3 (18)</td>
<td>ns</td>
</tr>
<tr>
<td>TNF (pg/mL) Median</td>
<td>74.1</td>
<td>170.5</td>
<td>( P &lt; .003 )</td>
</tr>
<tr>
<td>Range</td>
<td>(27.8-138.2)</td>
<td>(26.8-294.4)</td>
<td></td>
</tr>
<tr>
<td>8-epi &gt;154 (pg/mg creatinine) n (%)</td>
<td>3 (23)</td>
<td>14 (82)</td>
<td>( P &lt; .01 )</td>
</tr>
</tbody>
</table>

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![Fig 2. Linear regression analysis between urinary excretion of 8-ePi-PGF\(_{2a}\) and serum aCL titer in patients with systemic lupus erythematosus (Rho = 0.70; \( P < .0001 \)).](image)

![Fig 3. Scattergram showing TNF value distribution in SLE patients with 8-ePi-PGF\(_{2a}\) values ≤ or >154 pg/mg creatinine (mean ± SD of controls).](image)
this category. Urinary excretion of the compound also corre-
lated closely with the absolute levels of aCL. This observa-
tion, given the mechanism of formation of isoprostanes,8,9,25
is consistent with the hypothesis that aPL is directed against
oxidized epitopes in phospholipids. Oxidant stress may char-
acterize inflammatory episodes in autoimmune diseases such
as SLE.26 Furthermore, we have shown that monocytes may gen-
erate 8-epi-PGF$_{2\alpha}$ in response to inflammatory stimuli in
vitro and have immunolocalized the compound to these cells
in situ in human atherosclerotic plaque.18,27 Thus, it is of
interest that the levels of 8-epi-PGF$_{2\alpha}$ excretion correlated
with circulating TNF, which is generated by activated mono-
cytes and is elevated in the active phase of the disease.28,29

We have previously shown that COX enzymes exhibit a
minor capacity to generate 8-epi-PGF$_{2\alpha}$ but no other iso-
prostanes.15,16 However, this pathway appears to make a tri-
ivial contribution of overall biosynthesis of the compound as
reflected by its excretion in urine even in syndromes of
COX activation.11 In the present study we showed increased
urinary excretion of IPF$_{2\alpha}$-I, a second isoprostane that is
formed solely in a free radical dependent manner, and the
excretion of 8-epi-PGF$_{2\alpha}$ was highly correlated with that of
IPF$_{2\alpha}$-I. Finally, 22 patients were also taking steroids and
methotrexate. The possibility that these drugs can influence
the level of the isoprostanes measured cannot be excluded,
but in a preliminary study of patients with rheumatoid arthri-
tis who were receiving steroids and methotrexate, F$_2$-iso-
prostanes excretion did not differ significantly from that of
healthy controls.30

We also analyzed whether there was a relationship be-
tween lipid peroxidation and thrombosis. We did not find
any difference in isoprostane levels in patients with and
without previous thrombosis. However, the small cohort in-
vestigated did not allow us to reach definitive conclusion.
Therefore, further study is necessary to analyze this issue.

Thus, elevated levels of both isoprostanes in the aPL-
positive patients are consistent with the hypothesis that lipid
peroxidation may underlie the antiphospholipid syndrome.
However, further prospective study is necessary to clear-cut
establish whether a cause-effect relationship exists between
lipid peroxidation and aPL in vivo.

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