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Citation for published version (APA):

Regnault, V., Beguin, S., Wahl, D., de Maistre, E., Hemker, H. C., & Lecompte, T. (2003). Thrombinography shows acquired resistance to activated protein C in patients with lupus anticoagulants. *Thrombosis and Haemostasis*, 89(2), 208-212. <https://doi.org/10.1055/s-0037-1613433>

Document status and date:

Published: 01/01/2003

DOI:

[10.1055/s-0037-1613433](https://doi.org/10.1055/s-0037-1613433)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Rapid Communication

Thrombinography shows acquired resistance to activated protein C in patients with lupus anticoagulants

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Summary

In patients with lupus anticoagulants (LA), acquired resistance to activated protein C (APC) is difficult to demonstrate with clot-based assays due to the presence of the anticoagulant. Via the conversion of a fluorogenic substrate (thrombinography), we monitored the complete process of thrombin formation and decay and its delimitation by the protein C system in eight consecutive LA-patients without anticoagulant therapy and non-carriers of the V Leiden polymorphism. Thrombin generation was triggered in platelet-poor and platelet-rich plasma by recalcification in the presence of a low concentration of tissue factor.

In 7 out of 8 patients we observed a long lag-time before the thrombin burst (LA effect) together with a marked inability of

APC to diminish the thrombin activity. The lag-phase was however prolonged to some degree by APC. The effects were more outspoken in the presence of phospholipids from patients' platelets than with added phospholipids.

Thrombinography thus demonstrates APC resistance in LA-patients despite the occurrence of long lag-times (clotting times). The amount of thrombin activity generated in the presence of APC could be a better indicator of the thrombotic risk than the moment at which the thrombin burst starts.

Keywords

Lupus anticoagulant, acquired APC resistance, thrombin activity, platelets

Thromb Haemost 2003; 89: 208–12

Introduction

Lupus anticoagulants (LA) are the so-called antiphospholipid (auto)antibodies with *in vitro* anticoagulant activity. They delay the burst of thrombin formation and therefore the moment at which clotting occurs. Paradoxically, thrombosis [the antiphospholipid syndrome (APS) (1)], may develop in patients with such inhibitory antibodies (2, 3). Biochemical experiments have shown that these antibodies may interfere with the delimitation

of coagulation by APC (4) [also reviewed in (5)]. Significant association was reported between lupus-associated APS and a decreased ability of APC to prolong clotting times (6, 7). Plasma clots when ~5 nM of thrombin are formed, i.e. at the start of explosive thrombin generation. At that moment >95% of all thrombin is still to be formed (8). Such thrombin is presumably still thrombogenic. Heparins, for example, strongly diminish the amount of active thrombin formed without significantly affecting the lag-time of thrombin burst and clot formation (8).

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Received October 25, 2002

Accepted after revision November 22, 2002

We monitored the development of thrombin in clotting plasma via the conversion of a fluorogenic substrate (the thrombinogram) that allows us to observe both the lag-time (clotting time) and the burst and decay of thrombin that follows.

Thrombin generation and the formation and action of APC are critically dependent upon the composition of the phospholipid surface at which these reactions take place (5). In order to approach the *in vivo* conditions, we used the platelets of the patient as the source of phospholipid. *In vivo*, APC is formed by the activation of protein C by the thrombin-thrombomodulin (TM) complex. We therefore not only investigated the effect of added (exogenous) APC but also that of natural (endogenous) APC, the latter formed during thrombin generation in the presence of added recombinant TM.

Methods

Patients

We studied the 8 consecutive patients without anticoagulant therapy who were referred to our outpatient clinics between September 2001 and June 2002 and who were markedly LA-positive according to the criteria of the Subcommittee on LA of the International Society on Thrombosis and Haemostasis

(9); Rosner's indexes using the PTT-LA reagent from Diagnostica Stago (Asnières, France) ranged from 35 to 80 (cut-off 15). The clinical features of the patients are summarized in Table 1.

Genotyping for FV R506Q was performed as previously described (10). ELISA Asserachrom APA® (Diagnostica Stago), which is known to detect antibodies to β_2 -glycoprotein I, was used to detect "antiphospholipid" antibodies. A first-generation assay for sensitivity to APC was performed as originally described (11) with an automated APTT measurement (Akzo-Nobel). The sensitivity ratio of a normal pooled plasma was used to derive a normalized ratio (n-APC-SR) for patients.

Course of thrombin activity during clotting

Thrombinography was carried out according to the principles described by Hemker et al. (12). Venous blood was collected on 1/10 volume 0.129 M trisodium citrate and centrifuged to obtain platelet-rich and platelet-poor plasmas (PRP/PPP). Thrombinograms were recorded in citrated plasma to which trace amounts (0.5 pM f.c.) of recombinant human tissue factor (Dade Behring, Marburg, Germany) were added. We used the fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) (417 μ M f.c.), a plate reading fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) and

Table 1: Clinical and laboratory features of patients. All patients were non-carriers of the FV Leiden polymorphism. Patient LA1 was studied at two occasions 10 months apart. SLE: systemic lupus erythematosus; PAPS: primary APS; DVT: deep venous thrombosis; APL: antiphospholipid antibodies. Platelet count was measured in whole blood. The value of APTT without APC obtained with the normal pooled plasma was 34 s. N/D: not done; N/A: n-APC-SR expression of the result deemed not applicable.

	underlying disease	platelet count ($10^9/L$)	thrombotic event	anti-thrombotic treatment	Rosner's index	APL	APC sensitivity		
						ELISA (units/mL)	APTT -APC (s)	APTT +APC (s)	n-APC-SR
LA1a	SLE	430	none	none	80	<5	N/D		
LA1b		439			58	14	51.5	>140	N/A
LA2	B lymphoproliferative disease	175	none	none	35	<5	45.1	111.1	0.89
LA3	B lymphoproliferative disease	144	none	none	48	758	29.5	54.1	0.66
LA4	B lymphoproliferative disease	231	none	none	63	4625	N/D		
LA5	PAPS	238	DVT	aspirin	40	8	37.0	60.4	0.59
LA6	SLE	204	DVT	none	58	10	47.7	>140	N/A
LA7	SLE	209	DVT	none	46	261	>60	>140	N/A
LA8	SLE	78	none	aspirin	64	<5	52.4	>140	N/A

appropriate software (Synapse, Maastricht, The Netherlands). The total amount of thrombin activity was assessed as the area under the curve (i.e. the thrombin potential).

Recombinant human TM was a kind gift of Asahi (Japan) and APC was prepared in-house (13). TM was added at 10 nM and APC at 6.7 and 13.9 nM (f.c.). Thrombin generation was triggered by adding Ca^{2+} (16.7 mM f.c.). The two normals who had been previously established to be at the upper and lower limits of the interindividual variation of thrombin activity (N = 9) were run in parallel.

Sources of phospholipids

PRP (obtained by centrifugation at $190 \times g$) was adjusted to 150×10^9 platelets/L in all patients but the thrombocytopenic one (LA8, platelet count in PRP of only $117 \times 10^9/L$). Platelet procoagulant phospholipid activity was provoked in three ways: 1: by activation during the thrombin generating process, 2: by freezing and thawing, or 3: by inducing microvesicle formation with collagen (Horm, Argene, Varilhes, France) (20 $\mu\text{g}/\text{mL}$, 15 min, 37°C) and centrifuging at $1500 \times g$ to obtain PPP enriched in platelet microvesicles (PPP/PMV).

To PPP centrifuged at $13,000 \times g$ in order to discard microvesicles, synthetic procoagulant phospholipids were added as a mixture of phosphatidyl-serine (20 mole%), phosphatidyl-ethanolamine (20 mole%) and phosphatidyl-choline (60 mole%); (dioleilylglycerol)phospholipids from Avanti Polar Lipids Inc. (Coger, Paris, France).

Results

As a rule in the plasma of the patients the burst of thrombin generation started later (Table 2) and reached a lower peak than in normal plasma. The area under the curve (thrombin potential) was not decreased however (Fig. 1, right panel).

In normal plasma preparations a decrease in thrombin peak and thrombin potential was seen upon addition of TM and APC

(Fig. 1, left panel, Table 2). In all patients but one (LA5; Fig. 1 and Table 2) TM or APC hardly diminished these parameters. The lag-phase of the thrombin burst was prolonged to some degree by APC indicating that it was less insensitive to the APC effect than the other parameters (Fig. 1). Resistance to TM or APC was observed in the presence of phospholipids from the patients' platelets but not necessarily with added phospholipids (PPP) (Table 2). This stresses the importance of studying these reactions in the presence of the natural phospholipids of the patient as brought by their platelets. Patient 1 showed an identical pattern of thrombin generation when measured at a 10-month interval (Table 2).

The traditional test for APC-sensitivity failed to give interpretable results due to a prolonged APTT in 4 of our patients (Table 1). The n-APC-SR of patients 3 and 5 fell within the range of values obtained with patients heterozygous for V Leiden polymorphism (Fig. 2) and appeared to be misleading since the thrombin potentials in the presence of APC of these 2 patients were affected to a lesser extent (Table 2). Conversely the n-APC-SR of patient LA2 fell close to the median for non LA, non V-Leiden patients (Fig. 2), but thrombinography clearly showed APC resistance (Table 2).

Discussion

Our results show that in LA-patients abnormally high thrombin activity can be detected in an experimental setup approaching the conditions prevailing *in vivo*, i.e. minimally diluted plasma, natural (platelet) procoagulant phospholipids and the presence of an active protein C system. In contrast to traditional clotting times, in our test the course of thrombin-concentration after clotting are recorded. In PRP of both normals and patients the presence of platelets induces a resistance to APC that is seen to a lesser extent with frozen-thawed platelets or platelet microvesicles. The effect of TM seems much less subject to this phenomenon (results with normals, Table 2). At present we have no explanation for this observation.

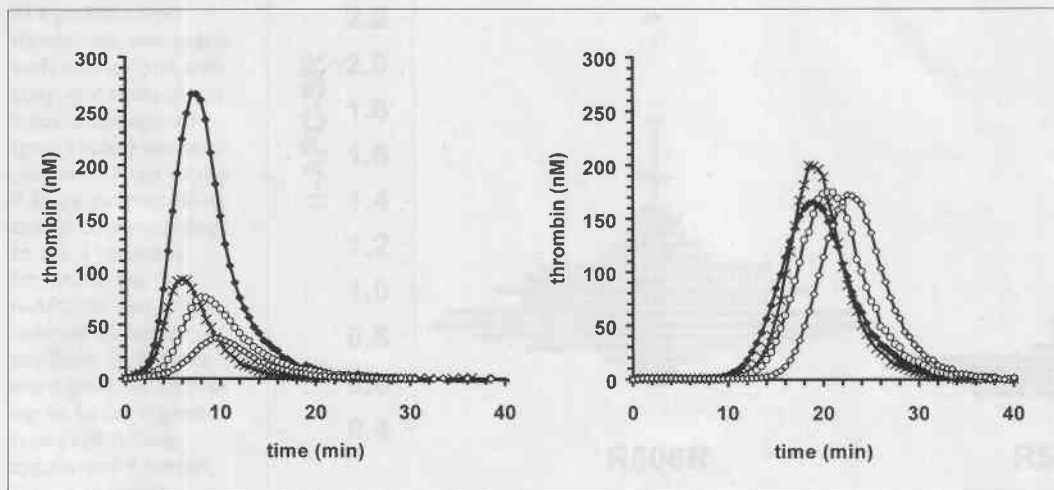
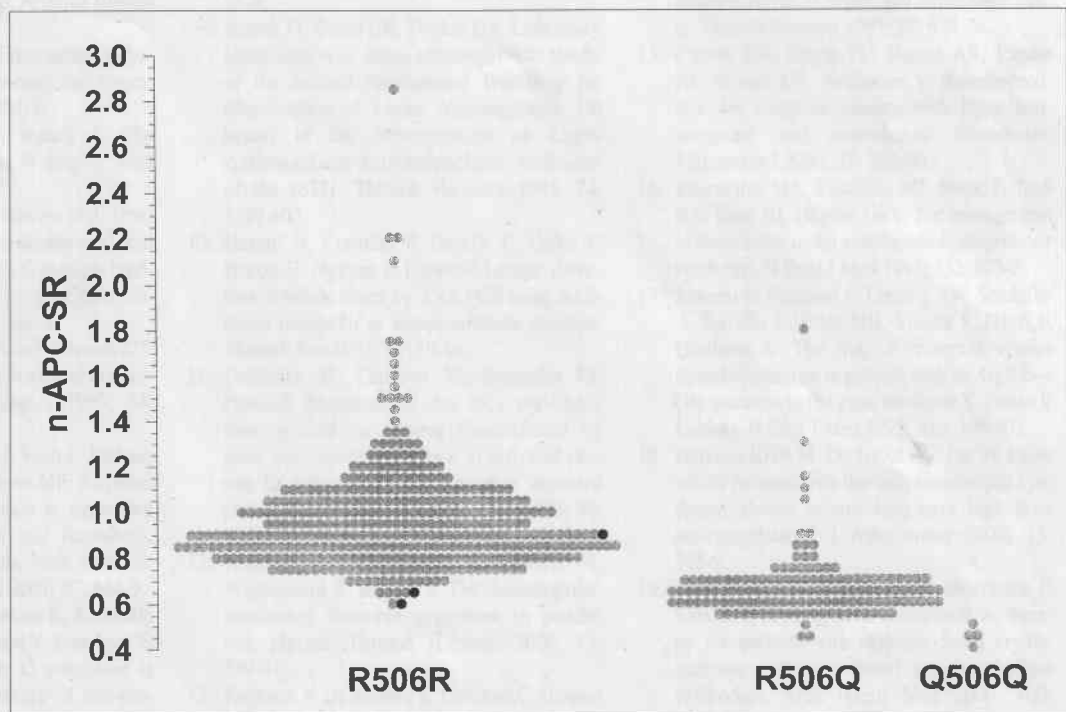


Figure 1: Representative thrombinograms of patient LA1. Thrombinograms with frozen-thawed normal (left panel) or LA-positive (right panel) platelet-rich plasma triggered with 0.5 pM tissue factor and 16.7 mM CaCl_2 in the absence (closed symbols) or presence of 10 nM TM (crosses), 6.7 nM (open circles) or 13.9 nM (open diamonds) APC.

Table 2: Thrombinography. Thrombin activity was recorded in the absence or presence of an active protein C system. Lag-time to burst of thrombin generation is only given in its absence. As expected it is short when procoagulant phospholipids are made available as soon as the reaction starts (frozen-thawed PRP and PPP/PMV). Inhibition of thrombin activity in the presence of TM or APC is expressed as percentage of the uninhibited area under the curve. The standard deviation in the uninhibited plasma being 13%, inhibitions $\leq 13\%$ were considered not significant (—). The figures in italics are out of the normal range. PRP = fresh platelet-rich plasma; PPP/PMV = platelet microvesicles in platelet-poor plasma; PPP = platelet-poor plasma centrifuged at $13,000 \times g$. N/D: not done.

			(nM)	N1	N2	LA1a	LA1b	LA2	LA3	LA4	LA5	LA6	LA7	LA8
PRP	time to burst (min)	-	-	6	5	25	20	13	10	8	7	14	13	28
	inhibition of thrombin activity (%)	TM	10	59	56	37	29	30	42	65	78	37	41	25
		APC	6.7	—	—	—	—	—	—	—	23	N/D	—	—
		APC	13.9	27	23	—	—	—	—	—	44	—	—	—
frozen-thawed PRP	time to burst (min)	-	-	1	1	10	10	6	1	3	2	4	5	6
	inhibition of thrombin activity (%)	TM	10	69	72	—	—	—	29	15	97	—	—	—
		APC	6.7	66	79	—	—	—	33	—	73	—	—	—
		APC	13.9	83	91	—	—	—	63	—	89	—	—	—
PPP/PMV	time to burst (min)	-	-	3	5	12	N/D	11	3	9	3	9	12	17
	inhibition of thrombin activity (%)	TM	10	65	70	—	N/D	—	30	32	98	22	18	30
		APC	6.7	42	51	—	N/D	—	—	—	36	—	—	—
		APC	13.9	64	72	—	N/D	—	27	—	61	—	—	—
PPP + synthetic PL	time to burst (min)	-	-	4	2	18	17	16	12	23	6	12	23	42
	inhibition of thrombin activity (%)	TM	10	96	95	16	37	41	97	88	100	70	73	80
		APC	6.7	99	94	34	23	31	97	47	100	47	58	100
		APC	13.9	98	100	24	35	37	97	50	100	67	100	100

Figure 2: Normalized APC sensitivity ratios. The original APC resistance clot-based test was performed with plasma of 514 patients with thrombotic events but with neither oral anti-coagulant therapy nor lupus anticoagulant (grey circles) and with plasma of 7 out of the 8 study patients (black circles corresponding to the 3 patients for whom the n-APC-SR was deemed applicable – see Table 1). Patients were grouped according to factor V genotype (138 heterozygous and 4 homozygous patients).



The apparent paradox of prolonged clotting times accompanying thrombophilia disappears if one assumes that not the lag-time to thrombin burst but rather the amount of thrombin formed in the presence of the APC-system is the parameter that indicates thrombotic risk. This is in line with the observations that no shortening of clotting times is usually seen in most prothrombotic states and that antithrombotic effect of heparin is not necessarily accompanied by lengthening of TF-induced clotting times (14).

The combination of APC resistance and delay of thrombin burst could be explained by assuming that LA inhibits the activation of factors V and VIII by (meizo)thrombin as well as their inactivation by APC. Both processes occur at the phospholipid surface and thus may be thought to be influenced by phospholipid-dependent antibodies.

An inhibitory effect on thrombin-induced, TM-dependent protein C activation has been reported in LA-patients (15). This seems less likely in the present patients because then thrombin generation would have been resistant to TM but not to APC.

The degree of inhibition of the APC-effect in our patients is on the whole much higher than that seen in subjects homozygous for the factor V Leiden polymorphism, where about half

of the activity of the APC-system remains (results to be published). This is in keeping with the risk of recurrent thrombosis over 6 years reported in clinical settings: LA: 69% (16), factor V Leiden 39% (17).

Our results suggest that thrombin activity might be a better indicator of thrombotic risk than clotting times are. Our study was not designed, however, to establish correlations with clinical or laboratory data. Because the current method has been completely automated and allows the parallel measurement of up to 48 samples (see www.thrombin.com) it is suited for the larger clinical studies that are required for this purpose. It might also serve to choose and dose antithrombotic therapy in LA patients, at the present time a very challenging issue (18, 19).

Acknowledgments

We thank L. Clément, C. Hulin, P. Kaminsky, M. Morisset and B. Witz for providing us the opportunity to study their patients. The routine blood analyses were performed in the haematology laboratory of the Nancy University Hospital (CHU), and the genotyping of factor V was performed in the haematology laboratory of the Reims University Hospital, France (P. Nguyen and coworkers).

Financial support: INSERM and the Conseil Régional de Lorraine

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