The effect of platelet activation on the hypercoagulability induced by murine monoclonal antiphospholipid antibodies

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Online Supplementary Design and Methods

Reagents

The anti-Fcγ-receptor RII (FcγRIIa) monoclonal antibody IV.3 was purchased from StemCell Technologies (Vancouver, Canada), abciximab from Centocor (Leiden, the Netherlands) and the anti-glycoprotein Ibα monoclonal antibody AK2 from Acris Antibodies (Hiddenhausen, Germany).

Human total IgG was purified on protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden).

Human ß$_2$GPI was purified according to Arvieux *et al.*[1] Purified human factors II, Va, Xa were from Synapse B.V. (Maastricht, The Netherlands) and S2238 (H-D-Phe-Pip-Arg-p-nitroanilide) from Chromogenix (Vienna, Austria). Recombinant human tissue factor, (Innovin®) was from Dade Behring (Marburg, Germany), rabbit lung thrombomodulin from American Diagnostica (Stamford, CT, USA), Z-Gly-Gly-Arg aminomethyl coumarin from Bachem (Bubendorf, Switzerland), human thrombin calibrator from Biodis (Signes, France). Bovine serum albumin, apyrase grade I, ionomycin, octyl-D-glucoside, annexin V, dipyridamole and milrinone were purchased from Sigma (St. Louis, MO, USA). Dioleylglycero-phospholipids were from Avanti-Polar lipids Inc. (Alabaster, AL, USA) and Horm-type collagen from Nycomed (Linz, Austria). Acetylsalicylic acid (Aspegic®) was from Sanofi Aventis (Paris, France) and hirudin from Pharmion (Windsor Berkshire, UK).

Washed platelet preparations for platelet procoagulant activity assays

Whole blood from a healthy subject was drawn into an acid-citrate dextrose solution (6v/1v). Platelet-rich plasma (PRP) was prepared by centrifugation at 170 g for 20 min at 20°C. Platelets were then sedimented by centrifugation at 1800 g for 10 min at 20°C and washed once at 1800 g for 10 min at 20°C using Tyrode buffer (5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, pH 6.2) without $CaCl_2$ and containing 50 µg/mL of apyrase. Platelets were finally resuspended in Tyrode buffer pH 7.4.

Thromboxane B_2 assay

Platelet mixtures used for the platelet procoagulant activity assay were centrifuged at 1750 g for 10 min at 20°C. The supernatants were centrifuged at 13000 g for 45 min at 4°C and stored at -80°C. Thromboxane B_2 concentrations were determined using a commercial enzyme immunoassay (Coger, Paris, France). The mean value for thromboxane B_2 in platelet suspensions alone was 0.9 ± 0.2 ng/mL (n = 3).

Supplementary Table S1

	assay	*control*	*7F6G*	*28F4*	*ALB6*
washed platelets at 750x10^9 platelets/L	TXB2	0.93 ± 0.19	22.02 ± 0.31	169.65 ± 4.88	154.62 ± 7.35
	PPA	0.22 ± 0.03	0.74 ± 0.30	7.04 ± 0.96	8.46 ± 1.13
washed platelets at 210x10^9 platelets/L	PPA	0.28 ± 0.05	0.39 ± 0.15	6.93 ± 1.15	7.80 ± 0.29
PRP at 300x10^9 platelets/L	PPA	0.22 ± 0.04	0.82 ± 0.24	1.95 ± 0.48	2.28 ± 0.60

Effect of platelet concentration and preparation on platelet-activating properties of murine monoclonal antibodies. Measurements of PPA or TXB$_2$ were performed in mixtures of washed platelets incubated with murine monoclonal antibodies at 200 µg/mL in the presence of their antigen at their plasma concentrations (200 µg/mL for β$_2$GPI and 100 µg/mL for FII) or ALB6 at 10 µg/mL and in normal PRP supplemented with murine monoclonal antibodies at 200 µg/mL or ALB6 at 10 µg/mL. Platelet concentration corresponds to the concentration during the activation step. Control corresponds to unstimulated washed platelets. Results are expressed in ng/mL for thromboxane B$_2$ (TXB$_2$) and µM phosphatidylserine equivalents for platelet procoagulant activity (PPA).

Surface plasmon resonance binding study

Surface plasmon resonance experiments were performed using a Biacore X (Biacore, Uppsala, Sweden) on a L1 sensor chip. Washed platelets in HBS containing 2 mM $CaCl_2$ were immobilized at a 2 µL/min flow rate. The reference flow cell was phosphatidylcholine liposomes. The average surface response was 4500 RU. Binding experiments were performed at 25°C at a 10 µL/min flow rate. The sensor chip was regenerated with 25 mM NaOH.

Flow cytometry analysis

Washed platelet suspensions were analyzed on a FACScalibur fluorescence cytometer (Becton Dickinson, San Jose, CA, USA) using Cell Quest software. Double-labeling was used to determine either platelet selectin or CD63 (granulophysin) expression in the platelet population (CD42b positive). Exposure of phosphatidylserine at the surface of platelets was measured as the percentage of ^{FITC}annexin V-positive cells.

Preparation of platelet microvesicles

Washed human platelets were prepared according to Mustard *et al.*[2] with some modifications.[3] The platelet count was adjusted to $600x10^9$ platelets/L and platelets were incubated with ionomycin (final concentration of 10 µM) for 10 min at 37 °C. Platelets were centrifuged at 1500 g for 15 min and platelet microvesicles were isolated from the supernatant by centrifugation at 13000 g for 45 min according to Barry *et al.*[4] The platelet microvesicles were washed at least five times in HBS buffer without bovine serum albumin, resuspended in HBS buffer and stored at - 80°C.

Preparation of plasma for thrombography studies

Blood was drawn into Monovette® (Sarstedt) syringes containing 1/10 volume of 0.106 M sodium citrate. Platelet-rich plasma was prepared by centrifugation at 190 g for 10 min at 20°C. The platelet count was adjusted to $150x10^9$ platelets/L by addition of autologous platelet-poor plasma obtained by centrifugation at 1750 g for 10 min at 20°C. For experiments with platelet-poor plasma, centrifugation at 13000 g for 30 min at 4°C was performed in order to discard endogenous microvesicles (PPP 13000 g). PPP 13000 g was stored at –80°C. Platelet-rich plasma was incubated with 20 µg/mL collagen for 15 min at 37°C and centrifuged at 1500 g for 15 min to obtain platelet-poor plasma enriched in platelet microvesicles.

Additional References

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