

## ANTIBODIES TO PHOSPHOLIPID COFACTOR PROTEINS INHIBIT THEIR BINDING TO HIGH AFFINITY PLPS, BUT STRONGLY ENHANCE THEIR BINDING TO LOW AFFINITY PLPS.

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### INTRODUCTION

Auto-antibodies to phospholipid cofactor proteins (Annexin V, Prothrombin, Protein S,  $\beta$ 2-GPI, etc...) can be associated with disease states inducing thrombosis. The pathogenicity mechanisms are not completely understood, but they involve antibody binding to phospholipids and formation of auto-antigen/antibody complexes on these surfaces, targeting the immune response to cells or tissues. Diagnostic assays designed for measuring these auto-antibodies use conditions frequently far away from physio-pathological ones. Therefore, it is not always obvious to establish relationships between measurement of auto-antibodies and occurrence of disease. In this study, we evaluated the binding of phospholipid cofactor proteins to various phospholipid mixtures, with or without calcium, which can mimic the occurrence of cell membrane modifications in patho-physiological conditions. We also analysed how presence of specific antibodies (monoclonal, polyclonal, auto-antibodies) can affect protein binding to phospholipids, antibody binding, and immune-complexes formation on phospholipid surface.

### MATERIALS

- Purified human Prothrombin or  $\beta$ 2-GPI (prepared in non denaturing conditions).
- Recombinant Wild Type Annexin V (Dr Chris Reutelingsperger).
- Various phospholipids (Phosphatidyl Serine: PS; Phosphatidyl Ethanolamine: PE; Phosphatidyl Choline: PC; cholesterol: Chl; Phosphatidic Acid: PA, all from Sigma).
- Calcium chloride, BSA, PEG (8000).
- Polyclonal antibodies to Prothrombin,  $\beta$ 2-GPI, Annexin V.
- Monoclonal antibodies to Annexin V (Wac2A).
- Normal human citrated plasma.
- TMB substrate.
- Pathological plasmas with auto-antibodies to Prothrombin,  $\beta$ 2-GPI or Annexin V.
- Tris-NaCl physiological buffer at pH 7.50, with BSA, used as a diluent for antibodies and plasmas (Tris allows performing studies with calcium). This diluent does not contain phospholipid binding proteins.
- Micro-ELISA Plates, Nunc type I with certificate.
- Anti-phospholipid cofactor protein affinity purified rabbit antibodies, coupled with peroxidase.
- Anti-Ig (G or M) antibody coupled with peroxidase.

### METHODS

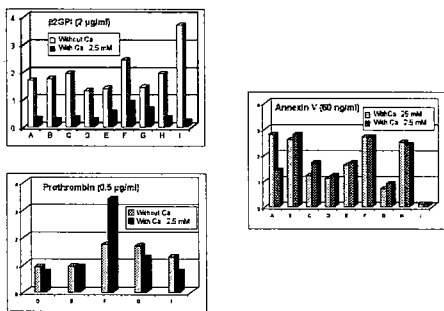
Phospholipids (PC, PS, PE, PA) or phospholipid mixtures were coated onto micro-ELISA plates (Nunc), then saturated with BSA and PEG: no phospholipid binding protein is present on the solid phase.

#### Phospholipid mixtures tested (%)

	PC	PE	PS	Chl	PA
A	100				
B	87.5	12.5			
C	50	37.5	12.5		
D	67				33
E	33				67
F		100			
G		100			
H	25	18	7	50	
I					100

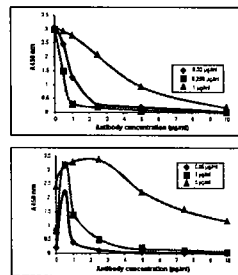
### RESULTS

#### Binding of $\beta$ 2GPI, Prothrombin or Annexin V to Phospholipids



#### Effect of antibodies on the Annexin V binding to phospholipids; the binding of antibodies to phospholipids via the antigen, follows a similar kinetics (data not shown)

shown only for Annexin V, but binding kinetics are similar with other systems



#### PROTOCOL 1

- Diluent with or without calcium (2.5 mM), supplemented with variable amounts of phospholipid binding protein (Prothrombin,  $\beta$ 2GPI, Annexin V), is incubated with phospholipids.
- Following washing, an anti-phospholipid cofactor protein coupled with peroxidase is added, and it allows measuring the amount of bound protein.
- TMB is used for colour development.

#### PROTOCOL 2

- Diluent, containing a constant phospholipid cofactor protein amount (with or without calcium) is supplemented with variable amounts of polyclonal (or monoclonal), antibodies to phospholipid cofactor proteins.
- The amount of protein bound is measured with an anti-protein-peroxidase conjugate, and the amount of antibody bound is evaluated with an anti-Ig (G or M)-peroxidase conjugate.

### CONCLUSIONS

- $\beta$ 2GPI binding to various phospholipids is much weaker in presence of physiological concentrations of calcium than in its absence. Little difference for Prothrombin. Annexin V binds only in presence of calcium.
- Phospholipid cofactor proteins and their antibodies binding to the various phospholipids is strongly affected by presence of calcium, presence of antibodies and affinity for phospholipids. Presence of antibodies stimulates binding to low affinity surfaces.
- These preliminary data demonstrate how antibodies can target important amounts of protein (phospholipid cofactor) antibody complexes to low affinity phospholipid surfaces, thus focusing the harmful effects of the immune system to these sites.
- This mechanism can be important for pathological complications in patients with phospholipid dependent antibodies.

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