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Novel monoclonal antibodies that recognize both rat and mouse phosphatidylserine/prothrombin complexes

Short title: Novel monoclonal anti-PS/PT antibodies

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To the Editor,

Anti-phospholipid syndrome (APS) is a prothrombotic disorder characterized by anti-phospholipid antibodies in the serum [1]. Anti-phospholipid antibodies recognize phospholipid-binding plasma proteins or phospholipid-protein complexes that include β2 glycoprotein I (β2GPI) and prothrombin [2-5]. Recent studies have suggested that anti-phosphatidylserine/prothrombin complex antibody (anti-PS/PT antibody) is one of the most relevant autoantibodies to APS [6-9]. However, the precise mechanism of *in vivo* thrombogenesis induced by anti-PS/PT antibody remains unrevealed due to the limited number of monoclonal antibodies that recognize rodent PS/PT complexes.

We had established transgenic rats carrying the *env-pX* gene of human T cell leukemia virus type I (HTLV-I) [10]. The pathogenic *env-pX* gene was introduced into the germline of WKAH rats under the control of the HTLV-I long terminal repeat promoter. Interestingly, these rats developed diverse collagen vascular diseases, including arthritis, sialo-/dacryoadenitis, thrombosis, myositis, and vasculitis, but not HTLV-I-associated lymphoid neoplasm. The onset of diseases was around 2 months after birth and the incidence at 6 months old was as follows approximately; arthritis: 80%, sialo-/dacryoadenitis: 40%, thrombosis: 30%, myositis: 30%, and vasculitis: 30%. Various autoantibodies, including anti-nuclear, anti-DNA, and anti-cardiolipin/β2GPI complex antibodies, were detected in the serum.

In this study, we determined the serum anti-PS/PT antibody levels in env-pX rats. Sera from thirteen 6-month-old morbid env-pX rats and twenty-five age-matched wild-type WKAH rats were subjected to a specific enzyme-linked immunosorbent assay. For this purpose, microtiter plates were first coated with phosphatidylserine (Sigma-Aldrich, St. Louis, MO) and then purified rat prothrombin (Oxford Biomedical Research, Rochester Hills, MI) was immobilized

on the phosphatidylserine-coated plates in the calcium-containing buffer. Phosphatidylserine-coated plates without immobilization of prothrombin were used as the control plates. Anti-PS/PT antibody levels (OD value at 450 nm) in the 1:101 diluted sera were significantly higher in env-pX rats than WKAH rats concerning both IgG (env-pX 0.094 ± 0.094, WKAH 0.027 ± 0.009, $p<0.0001$) and IgM (env-pX 1.770 ± 0.700, WKAH 0.457 ± 0.512, $p<0.0001$) subclasses. Since no reactivity was detected in the wells without prothrombin, the sera did not react directly with phosphatidylserine. These findings indicate that env-pX rats are suitable models of APS.

Therefore, we generated monoclonal antibodies toward rat PS/PT complex using env-pX rats. For this purpose, we employed the conventional hybridoma methodology using the splenocytes or lymph node cells of 6-month-old morbid env-pX rats and mouse myeloma cells, P3U1. Finally, five hybridoma clones that produced anti-PS/PT monoclonal antibodies (Sp7-1, Sp7-11, Sp7-12, Sp7-14, and Ly3-135) were established. Isotyping revealed that they were all IgM class.

In order to determine the specificity of these antibodies, culture supernatants of hybridoma clones were added in microtiter plates coated with rat, mouse, and human PS/PT complexes, respectively. Mouse PS/PT plates were prepared similarly to rat PS/PT plates using purified mouse prothrombin (Oxford Biomedical Research). Phosphatidylserine-coated plates without immobilization of prothrombin were used as the control plates. Cross-reactivity with human PS/PT complex was examined using commercially available human PS/PT plates (Medical & Biological Laboratories, Nagoya, Japan). As a result, all rat anti-PS/PT monoclonal antibodies showed high cross-reactivity with mouse PS/PT complex (Table 1). Since no reactivity was detected in the wells without prothrombin, the monoclonal antibodies did not react directly with phosphatidylserine. In contrast, these antibodies did not demonstrate cross-reactivity with human PS/PT complex. BLAST analysis revealed that the amino acid homology between rat

prothrombin and mouse prothrombin is 94%, whereas between rat prothrombin and human prothrombin, it is 80%. Our results are consistent with this evidence.

Among the five hybridoma clones, Sp7-11 had shown the most vigorous growth *in vitro*. Thus, we employed this clone for mass production of rat anti-PS/PT monoclonal antibody. By injection of Sp7-11 cells into the intraperitoneal cavity of three BALB/c nude mice ($1x10^7$/mouse), 7 ml ascites containing 5 mg/ml of rat IgM was obtained. For the following experiments, affinity-purified rat anti-PS/PT IgM monoclonal antibody (Sp7-11) was used.

Next, in order to determine the thrombogenic potential of the rat anti-PS/PT monoclonal antibodies *in vivo*, young pre-morbid env-pX rats (5-week-old, female, n=5) were given intravenous injection of the affinity-purified Sp7-11 (200µg/500µl/rat) at day 0 and day 7, and then killed for histological examinations at day 14. Thrombosis developed in 2 of 5 rats given intravenous injection of Sp7-11 at 7 weeks of age (Figures 1A-1D). The affected vessels included the pulmonary vein and artery, and a small arteriole in the subcutaneous connective tissue. In our experience, male rats are more susceptible than female rats in this model and spontaneous development of diseases, including thrombosis, has be seldom seen in female env-pX rats at this age (7 weeks old). In consistent with this, no thrombosis occurred in age- and sex-matched env-pX rats similarly given control rat IgM (eBioscience, Tokyo, Japan) (n=3, Figures 1E and 1F). These findings seem promising but are insufficient; hence, we are going to confirm through further investigations in order to submit as relevant results in the future.

In this letter, we introduce the establishment of novel monoclonal antibodies toward both rat and mouse PS/PT complexes. Although further studies are required to ensure if the anti-PS/PT monoclonal antibodies would induce thrombosis in rats and mice, they could be useful tools for investigating the pathogenesis of APS, not only *in vitro* assay but also *in vivo* assay using rodent

models.

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Conflict of Interest

None.

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Table 1. Reactivity of anti-PS/PT monoclonal antibodies

Sample	OD (450 nm)		
	Rat PS/PT complex	Mouse PS/PT complex	Human PS/PT complex
Sp7-1	1.979	1.824	0.036
Sp7-11	1.937	1.836	0.033
Sp7-12	1.953	1.833	0.036
Sp7-14	1.966	1.847	0.035
Ly3-135	1.912	1.800	0.092
PBS	0.010	0.010	ND
Human standard serum*	0.192	ND	1.336

*Serum from a patient with APS (1:101 dilution)
ND: not done

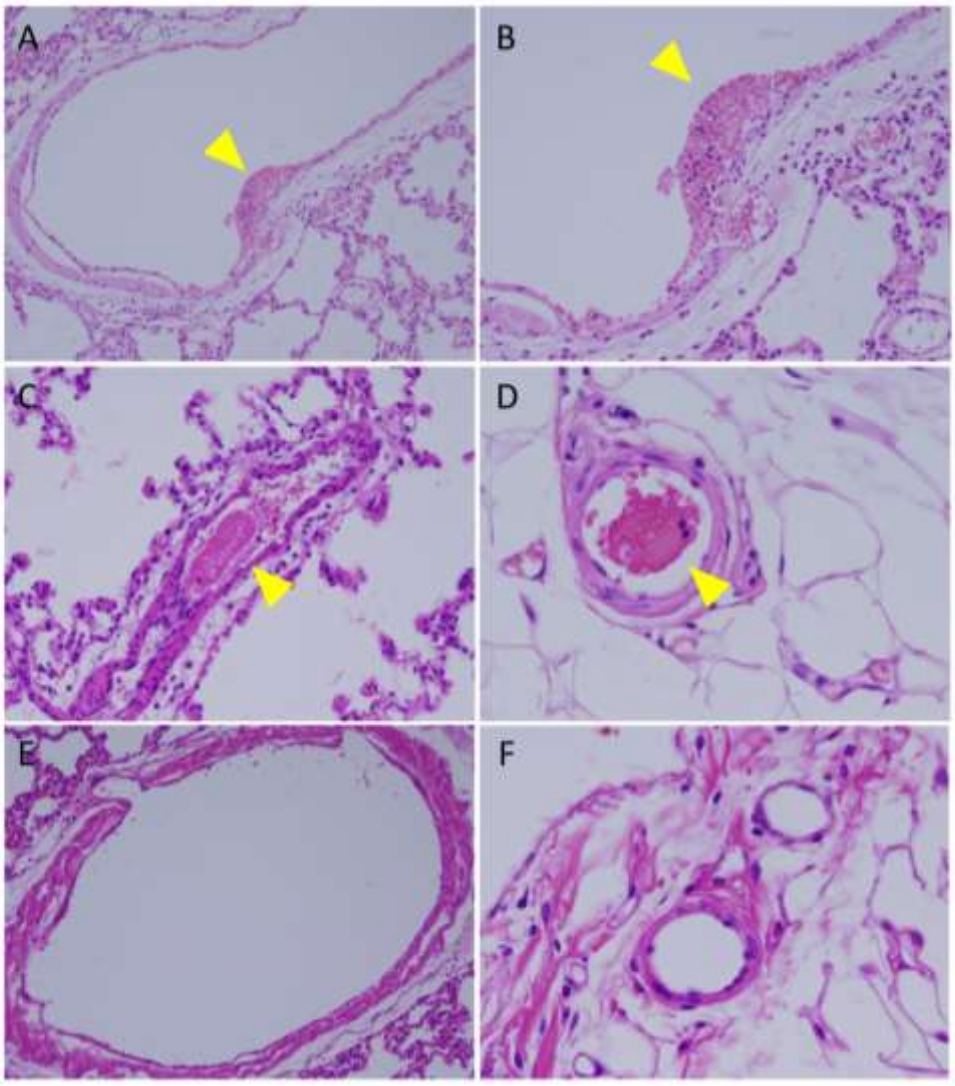


Figure 1. Thrombogenic potential of rat anti-PS/PT monoclonal antibodies

Young pre-morbid env-pX rats (5-week-old, female, n=5) were given intravenous injection of affinity-purified Sp7-11 (200µg/500µl/rat) at day 0 and day 7 and then killed for histological examinations at day 14. Thrombi observed in the pulmonary vein (A, original magnification: ×100; B, original magnification: ×200), pulmonary artery (C, original magnification: ×200), and subcutaneous arteriole (D, original magnification: ×400) (Yellow arrow heads). No thrombosis occurred in age- and sex-matched env-pX rats similarly given control IgM (n=3). (E) Pulmonary vein (original magnification: ×100). (F) Subcutaneous arteriole (original magnification: ×400).