Effects of Polyclonal IgG Derived from Patients with Different Clinical Types of the Antiphospholipid Syndrome on Monocyte Signaling Pathways

Anastasia Lambrianides, Christopher J. Carroll, Silvia S. Pierangeli, Charis Pericleous, Ware Branch, Jurhee Rice, David S. Latchman, Paul Townsend, David A. Isenberg, Anisur Rahman and Ian P. Giles

*J Immunol* 2010; 184:6622-6628; Prepublished online 17 May 2010; doi: 10.4049/jimmunol.0902765
http://www.jimmunol.org/content/184/12/6622
Effects of Polyclonal IgG Derived from Patients with Different Clinical Types of the Antiphospholipid Syndrome on Monocyte Signaling Pathways

Anastasia Lambrianides,*† Christopher J. Carroll,* Silvia S. Pierangeli,‡ Charis Pericleous,*† Ware Branch,§ Jurhee Rice,§ David S. Latchman,* Paul Townsend,§ David A. Isenberg,† Anisur Rahman,† and Ian P. Giles*†

A major mechanism of hypercoagulability in the antiphospholipid syndrome (APS) is antiphospholipid Ab-mediated upregulation of tissue factor (TF) on monocytes via activation of TLRs, p38 MAPK, and NF-κB pathways. We examined whether monocyte signaling pathways are differentially activated by IgG from patients with vascular thrombosis (VT) alone compared with IgG from patients with pregnancy morbidity (PM) alone. We purified IgG from 49 subjects. A human monocyte cell line and ex vivo healthy monocytes were treated with 100 μg/ml IgG for 6 h, and cell extracts were examined by immunoblot using Abs to p38 MAPK and NF-κB. To further investigate intracellular signaling pathways induced by these IgGs, specific inhibitors of p38 MAPK, NF-κB, TLR4, and TLR2 were used to determine their effect on TF activity. Only IgG from patients with VT but no PM (VT+/PM−) caused phosphorylation of NF-κB and p38 MAPK and upregulation of TF activity in monocytes. These effects were not seen with IgG from patients with PM alone (VT−/PM+), anti-phospholipid Ab-positive patients without APS, or healthy controls. TF upregulation caused by the VT+/PM− samples was reduced by inhibitors of p38 MAPK, NF-κB, and TLR4. The effects of VT+/PM− IgG on signaling and TF upregulation were concentrated in the fraction that bound β2-glycoprotein I. Our findings demonstrate that IgGs from patients with diverse clinical manifestations of APS have differential effects upon phosphorylation of NF-κB and p38 MAPK and TF activity that may be mediated by differential activation of TLR4. The Journal of Immunology, 2010, 184: 6622–6628.

The antiphospholipid syndrome (APS) is diagnosed in patients who suffer vascular thrombosis (VT) and/or pregnancy morbidity (PM) in association with persistently positive blood tests for anti-phospholipid Abs (aPLs) (1, 2). APS is the most common cause of acquired venous and arterial thrombosis (3) and the most important treatable cause of recurrent miscarriage (4). Prospective clinical studies have shown a significant association between aPLs and arterial and venous thrombosis (5) as well as PM (6). Patients with APS develop a wide range of clinical manifestations (7), and pathogenic aPLs have been shown to exert their thrombotic effects through interactions with endothelial cells (ECs) (8), platelets (9), and monocytes (10).

aPLs are commonly identified by the anticardiolipin (aCL) ELISA, anti–β2–glycoprotein I (β2GPI) ELISA, and lupus anticoagulant (LA) assay (2). Some patients who test positive in these assays will develop VT, others will develop PM, some will have both, and some will develop neither despite the persistent presence of serum aPLs (7). Fewer than 4.2% of patients with PM due to APS go on to develop VT (11, 12). This study investigates the hypothesis that aPLs present in these VT−/PM− or VT+/PM+ patients with APS to increase the activity of tissue factor (TF), the major initiator of coagulation produced by monocytes.

TF expression is increased in monocytes from patients with APS (13, 14) and on healthy monocytes exposed to aPLs in vitro (15, 16). This aPL-mediated upregulation of TF in monocytes occurs via ERK-1, p38 MAPK, and NF-κB signaling pathways (10, 17). Similarly, aPL-mediated activation of p38 MAPK and NF-κB pathways has been shown in cultured ECs (18), and TLR4 has been implicated in this process by both in vitro (19) and in vivo (20) studies. aPLs react with the β2GPI–TLR4–Annexin A2 complex in human monocyte plasma membranes (21). TLR2 is implicated in the inflammatory activation of mouse fibroblasts by human aPLs, but there are no previous studies of the effects of aPLs on TLR2 in monocytes (22).

Previous studies (10, 17) of the effects of aPLs upon monocytes have tested samples of purified polyclonal aPLs from limited numbers of patients with mostly VT alone. Only one study, however, has clearly examined large numbers of patients with...
Isolation of healthy monocytes

Peripheral venous blood samples from a healthy donor were used to isolate mononuclear blood cells by Ficoll-Paque PLUS (GE Healthcare Life Science) density gradient centrifugation. Monocytes were purified using the immunomagnetic EasySep Human CD14 Positive Selection Protocol (StemCell Technology, U.K.). Cultures of monocytes from each individual sample, on 1 × 10^5 cells per milliliter (33 GPLU) were incubated with 10 μg/ml purified IgG, 3 μg/ml LPS, or 100 ng/ml TNF-α for time periods between 6 min and 24 h.

In some experiments, monocytes were pretreated with specific inhibitors for 30 min prior to exposure to IgG. Bay 11–7082 (Alexis Biochemicals, U.K.), a specific inhibitor of NF-κB activity, was used at a concentration of 50 μM. SB203580 (Calbiochem, U.K.), a specific p38 MAPK inhibitor, was used at a concentration of 1 μM. All of the inhibitors were dissolved in <1% DMSO in PBS. Anti-human TLR2 Ab (eBioscience, Hatfield, U.K.) and Escherichia coli lipopolysaccharide (LPS, 10 μg/ml) were used at concentrations of 1 μg/ml. To confirm specific effects of these inhibitors, we repeated experiments using 1% DMSO/PBS alone or SB202474 (Calbiochem), a nonfunctional analogue of SB203580 in DMSO.

Western blotting for the analysis of NF-κB and p38 MAPK signaling pathways

Cell extracts were prepared by addition of 100 ml lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 10 mM NaF, 1 mM Na3VO4, and complete mini protease inhibitor mixture tablets [Roche, U.K.]). Samples were resolved via 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, blocked, and incubated overnight at 4°C with a 1:1000 dilution of rabbit monoclonal anti-human phosphorylated (Ser52) NF-κB p65 and total NF-κB p65 phosphorylated (Ser276) and total NF-κB p65 and phosphorylated (Thr181/Tyr185), and total p38 MAPK, followed by 1 h of incubation in a 1:2000 dilution of HRP-conjugated goat anti-rabbit IgG. Equivalent protein loading was demonstrated using an anti-human actin polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by chemiluminescence (GE Healthcare Life Science), their intensities were quantified by densitometric analysis (QuantityOne software, Bio-Rad, Hercules, CA), and results were expressed as a ratio of relative expression.

Assay for monocyte TF activity

Monocyte TF activity was determined using a chromogenic assay (Actichrome TF, American Diagnostica, Stamford, CT) that measures factor Xa after activation by the TF–factor VII complex. U937 cells were treated for 6 h with IgG (100 μg/ml), and cell lysates then were tested for TF activity.

Statistical analysis

Nonparametric statistical analyses were performed. Mean values are shown, and Mann-Whitney-Wilcoxon scores were carried out to compare two groups of unpaired data using Prism software, version 4.0c (GraphPad, San Diego, CA).

Results

Characteristics of subjects and IgG samples

Table I gives the relevant clinical and laboratory features of the 49 subjects. Forty-six (94%) were women. aCL and anti-b2GPI levels and LA positivity were similar in the three APS groups (VT+/PM−, VT−/PM+, and VT+/PM+) but considerably lower in the aPL+/APS− group as expected because higher titers of IgG aCL and IgG anti-b2GPI are markers of increased risk of developing VT or PM (2). The comparable levels of aPL detected by all three assays in the VT+/PM− and VT−/PM−
groups indicate that differences in the functional effects of IgG from these two groups are not likely to be due to differences in levels of aPL.

Pooled IgG from VT+/PM− patients but not VT−/PM+ patients promoted phosphorylation of NF-κB and p38 MAPK in U937 cells

To establish the effects of exposing monocytes to IgG, initially we used pooled IgG samples from the four clinical groups VT+/PM−, VT−/PM+, aPL+/APS−, and aPL-Negative. U937 cells were treated with pooled IgG for 0, 5, 10, and 15 min and 1, 6, and 24 h. Maximal differences in phosphorylation of NF-κB and p38 MAPK were observed between the different groups after 6 h of exposure to IgG (as shown in Fig. 1). U937 cells exposed to these IgGs or to untreated cells and positive control), whereas cells cultured for 24 h under any conditions (i.e., exposure to IgG from all of the groups, TNF-α, or medium alone) showed equal levels of nonspecific phosphorylation of both NF-κB p65 and p38MAPK. After 6 h of incubation, VT+/PM− IgG caused a ~4-fold increase in phosphorylation of NF-κB compared with that caused by IgGs from the other three groups (p < 0.05) (as shown in Fig. 1A). Similarly, a ~6-fold increase in phosphorylation of p38 MAPK was seen with the VT+/PM− sample compared with those of the other groups (p < 0.05) (as shown in Fig. 1B). In contrast, IgG from VT−/PM+ patients had no greater effect on NF-κB and p38 MAPK phosphorylation than IgG from the control (aPL+/APS− and aPL-negative) groups or medium alone.

These experiments with pooled samples enabled us to establish the ideal incubation time for further experiments as 6 h of exposure of monocytes to IgG. This finding was consistent with the previous work of López-Pedrera et al. (10, 23). To investigate the biological relevance of our initial findings using pooled samples and U937 cells, we addressed the following questions: Are similar differences between VT+/PM− and VT−/PM+ samples seen in monocyte signaling when IgG from individual subjects is tested? Are similar effects seen when ex vivo monocytes rather than U937 cells are used? What is the functional consequence of the 6 h of exposure to IgG?

Similar profiles of NF-κB and p38 MAPK phosphorylation occurred in U937 cells and ex vivo monocytes exposed to IgG from individual subjects

We examined the effects of IgG from individual patients chosen at random from the four groups previously tested (VT+/PM−, VT−/PM+, aPL+/APS−, and aPL-negative). In general, the same pattern of phosphorylation found with pooled IgG was observed with individual IgG samples. NF-κB p65 (Fig. 2A) and p38 MAPK (Fig. 2B) phosphorylation in U937 cells exposed to IgG from VT+/PM− patients were ~3-fold higher (p < 0.05) than those from the other groups. To investigate the possibility that differences in NF-κB and p38 MAPK phosphorylation between groups were due to differences in titer of aPL, we plotted aPL level against each of these outcome measures for all 28 samples in the VT+/PM−, VT−/PM+, VT+/PM+, and aPL+/APS− groups (Fig. 3A, 3B). There was no correlation between aPL titer and either phosphorylation of NF-κB (r² = 0.003117) or p38 MAPK (r² = 0.03016).

We then studied the effects of IgGs from four individual patients chosen at random upon ex vivo monocytes isolated from a healthy donor (Fig. 4A). We found a similar profile of IgG-mediated NF-κB p65 and p38 MAPK phosphorylation in ex vivo monocytes compared with what we previously found in U937 cells (Figs. 1, 2). Therefore, IgG from patients with VT+/PM− increased NF-κB p65 and p38 MAPK phosphorylation compared with IgG from patients with VT−/PM+, although it only reached statistical significance (p < 0.05) for NF-κB p65.

**TF activity in U937 cells was stimulated by VT+/PM− IgG but not by VT−/PM+ IgG and this stimulation is blocked by inhibiting NF-κB, p38 MAPK, or TLR4 signaling pathways**

Exposure to pooled VT+/PM− IgG caused a ~4-fold increase in TF activity (p < 0.05) compared with those caused by VT−/PM+, aPL+/APS−, or aPL-negative IgGs (Fig. 5A). In contrast, VT−/PM+ IgG did not significantly increase TF activity compared with aPL+/APS− or aPL-negative IgG.
Fig. 5B shows the effects of inhibitors of p38 MAPK (SB203580), NF-κB (Bay 11-7082), and TLR4 (E. coli K12 msbB LPS) and of anti-TLR2 Ab on TF activity induced by the IgG samples. The low levels of TF activity in cells exposed to IgGs from the VT2+/PM+, aPL+/APS2, and aPL-negative groups were not affected by any of the inhibitors. The increase in TF activity stimulated by VT+/PM2 IgG was significantly reduced by inhibitors of p38 MAPK (p = 0.01), NF-κB (p = 0.01), and TLR4 (p = 0.01) but not anti-TLR2 Ab (p = 0.06). Addition of either DMSO alone or a nonfunctional analogue of SB203580 did not have any appreciable effects on TF activity (data not shown).

Stimulatory effects of VT+/PM− IgG on NF-κB phosphorylation are concentrated in the IgG fraction that binds β2GPI

Affinity purification of anti-β2GPI Abs from a total IgG pooled sample of four VT+/PM− patients confirmed that the anti-β2GPI subfraction is responsible for increased phosphorylation of NF-κB p65 (Fig. 6). In contrast, the anti-β2GPI−depleted IgG fraction showed an appreciable reduction in ability to promote NF-κB p65 phosphorylation compared with those of both the anti-β2GPI-enriched and whole IgG fractions. Anti-β2GPI activity was calculated as mean percentage binding to a concentration of 100 μg/ml HCAL and was found to be 131 for the anti-β2GPI affinity-purified IgG sample and 65 for the anti-β2GPI−depleted IgG.

What are the effects on monocytes of IgG samples from APS patients who have both VT and PM (VT+/PM+)?

The results of testing IgG from seven VT+/PM+ individuals are included in Fig. 2. Three of these seven VT+/PM+ IgG samples showed both the greatest ability to cause p38 MAPK phosphorylation and the greatest NF-κB phosphorylation. These samples resemble VT+/PM− samples. The other four VT+/PM+ samples had far less effect on either signaling pathway and resemble VT−/PM+ samples. There was no correlation with the most recent clinical event. In four cases, the most recent event was PM, in one it was VT, and in two VT and PM had occurred during the same clinical episode. We found a similar spread of results when we tested the effect of seven individual VT+/PM+ samples on TF activity in monocytes. The results for these seven samples were 1, 3.3, 6.8, 11.8, 18.5, 32.5, and 54 (TF activity in pM, results are mean of two separate experiments).

Discussion

We have shown that IgG from VT+/PM− patients promotes upregulation of monocyte TF activity that is prevented by specific inhibitors of p38 MAPK and NF-κB. In contrast, IgG from VT−/PM+ patients could not promote phosphorylation of these signaling...
molecules or stimulate upregulation of TF activity. These functional differences were seen despite the fact that the VT−/PM+ and VT+/PM− samples had similarly high levels of CL binding and β2GPI binding.

Previous studies on monocytes (10, 13, 17, 21) tested polyclonal aPL samples from limited numbers of patients with VT alone. Two groups (10, 26) purified total IgG from seven patients with VT+/PM−, whereas another study (21) used polyclonal IgG anti-β2GPI Abs from three patients with VT+/PM−. We investigated IgG from larger numbers of subjects and are the first to compare effects of purified IgGs from VT+/PM− and VT−/PM+ patients with APS. The only previous studies to compare VT+/PM− and VT−/PM+ groups looked at ex vivo monocytes (10, 23) and showed that monocytes extracted from APS patients with VT (including both VT+/PM− and VT+/PM+) had higher TF mRNA, higher surface expression of TF, and altered signaling compared with those of monocytes from VT−/PM+ APS patients, aPL-negative patients with VT, and healthy controls (10). Furthermore, monocytes from patients with APS and thrombosis have higher levels of vascular endothelial growth factor and tyrosine kinase vascular endothelial growth factor receptor 1 than those from patients with APS and no thrombosis or healthy controls (26). Proteomic analysis and mass spectrometry identified six proteins whose expression was significantly different in monocytes from the APS/VT group than those in the three other groups. They showed the same results of TF expression, signaling, and proteomics in monocytes from healthy people exposed to purified IgG from patients with APS, but these IgG samples were derived only from patients with VT and were pooled before addition to the monocytes. Our results agree with and complement those of López-Pedrera et al. (10). We also found increased TF and increased p38 MAPK and NF-κB signaling in monocytes exposed to VT+/PM− IgG but have further demonstrated that purified VT−/PM+ IgG does not cause similar effects. This finding is consistent with the fact that those effects have not been seen on monocytes derived from VT−/PM+ patients (9). López-Pedrera et al. studied TF expression, whereas we studied activity. Expression and activity of TF are not linked directly because cell surface TF may be inactive due to encryption or complex formation with inhibitors. In future studies, we will measure both activity and expression because it is now recognized that TF may have pathogenic proinflammatory effects in APS separate from the effects on thrombosis that are measured in the activity assay (27, 28).

The distinct differences in IgG-mediated phosphorylation of monocyte signaling molecules that we observed at 6 h support the findings of López-Pedrera et al. and other groups who have examined the time course of aPL-mediated signaling activation in platelets and HUVECs (18). We then confirmed the biological significance of this 6 h exposure to IgG by correlating these differences in signaling with our findings from the TF activity assay.

**FIGURE 3.** Effects of IgG on monocyte activation are not related to the aPL titers. aPL titers of all 28 patients in the VT+/PM−, VT−/PM+, VT+/PM+ and aPL+/APS− groups (not healthy controls, who have no aPL) against both (A) p38 MAPK ($r^2 = 0.03016$) and (B) NF-κB p65 ($r^2 = 0.0003117$) phosphorylation show no correlation between aPL titer and either outcome.

**FIGURE 4.** IgGs from individual patients display similar patterns of activation of p38 MAPK and NF-κB on ex vivo monocytes. Western blot analysis of cell lysates from healthy monocytes treated with IgG (100 μg/ml) or LPS (3 μg/ml) for 6 h. A. Representative blot with Abs specific for human phosphorylated and total proteins against NF-κB p65 and p38 MAPK. The positive control (LPS) in lane 1 shows stimulation of p38 MAPK and NF-κB p65. Four individual patients from the VT+/PM− group are shown in lanes 2−5, four individual patients from the VT−/PM+ group are shown in lanes 6−9, four individual patients from the aPL+/APS− group are shown in lanes 10−13, and a pooled IgG sample from four healthy controls (aPL-negative) is shown in lane 14. B. Quantitative densitometric analysis of the blots shown in A displaying ratio of phosphorylated to total protein against NF-κB. C. Quantitative densitometric analysis of the blots shown in A displaying ratio of phosphorylated to total p38 MAPK. Statistically significant differences are shown (∗p < 0.05).
A possible confounder of our results would be misallocation of samples between clinical groups. A patient who had only ever suffered PM might nevertheless possess aPLs capable of causing VT and might subsequently develop VT. Therefore, they would be allocated to the VT+/PM group, whereas their true allocation should be VT+/PM+. Such misallocations would reduce our ability to detect true differences between the VT+/PM+ and VT−/PM+ samples. It is unlikely that these effects are due to non-aPL autoantibodies, because affinity-purified anti-β2GPI Abs from our VT+/PM+ samples had minimal effect on monocyte signaling. Use of pooled samples was necessary to carry out the multiple experiments to establish the optimal time of incubation with IgG. To reduce the chance of erroneous findings arising from atypical individual samples within pools, we tested two different pools for each clinical group and repeated the experiments using individual samples drawn at random from those used to create each pool. These experiments confirmed the differences between effects of VT+/PM+ and VT−/PM+ samples. It is unlikely that these effects are due to non-aPL autoantibodies, because affinity-purified anti-β2GPI Abs from our VT+/PM+ samples had a particularly strong effect. IgG from the aPL+/APS− group had minimal effect on monocytes in any assay, despite the fact that 9 of 12 patients from this group had SLE and a range of other serum autoantibodies (e.g., anti-dsDNA and anti-Ro).

Use of pooled samples was necessary to carry out the multiple experiments to establish the optimal time of incubation with IgG. To reduce the chance of erroneous findings arising from atypical individual samples within pools, we tested two different pools for each clinical group and repeated the experiments using individual samples drawn at random from those used to create each pool. These experiments confirmed the differences between effects of VT+/PM+ and VT−/PM+ samples. It is unlikely that these effects are due to non-aPL autoantibodies, because affinity-purified anti-β2GPI Abs from our VT+/PM+ samples had a particularly strong effect. IgG from the aPL+/APS− group had minimal effect on monocytes in any assay, despite the fact that 9 of 12 patients from this group had SLE and a range of other serum autoantibodies (e.g., anti-dsDNA and anti-Ro).

What is the mechanism of the effects exerted on monocytes by our VT+/PM− IgG samples? We believe that IgG anti-β2GPI Abs within those samples bind to β2GPI (derived from FCS used in cell culture of U937 cells or attached to the monocytes extracted from human serum) and that these complexes interact with TLR4 on the monocyte surfaces. This hypothesis would be consistent with our results showing that the effect of APS IgG upon monocytes is concentrated within the anti-β2GPI fraction of VT+/PM− IgG and inhibited by the TLR4 antagonist E. coli K12 msbB LPS. This antagonist is known to be specific for the TLR4 pathway (from experiments in ECs) and acts upstream of the MyD88 adaptor (29–31). However, aPLs can also act by other mechanisms...
involving other receptors (32). Our results did show some effect of TLR2 blocking Ab, though this was not clear enough to convince us that TLR2 is involved in the effect of our VT+/PM+ samples on monocytes. For example, aPLs could be acting by a complement-dependent mechanism, as suggested in other studies (33, 34) because complement is present in the FCS used in our U937 culture medium. Furthermore, we have previously detected β2GPI in IgG purified monoclonal aPLs (24) and have confirmed (data not shown) that copurified β2GPI is also present in our purified polyclonal IgG aPL.

It would be of interest to know whether these differences in effects of IgG samples from patients with different clinical manifestations of APS are mirrored in their effects when tested in vivo models. Though several such models have been described for both thrombosis (35, 36) and pregnancy loss (37), careful analysis of the methods sections of these papers (both ours and others) shows that very few samples from patients with PM alone have ever been tested in in vivo models. Thus, it is currently not possible to reach a conclusion as to whether VT+/PM→VT−/PM+ IgG aPL samples have different effects in these models.

In conclusion, our data support our hypothesis that aPLs from VT+/PM+ and VT−/PM+ patients with APS differ in their effects on p38 MAPK and NF-κB signaling pathways and TF activity. These differences may be mediated by preferential activation of TLR4 by IgG aPL from VT+/PM+ patients.

Disclosures
S.S.P. is a co-owner and founder of Louisville APL Diagnostics, Inc. The authors have no other conflicting financial interests.

References