

# A Novel C5a Receptor-Tissue Factor Cross-Talk in Neutrophils Links Innate Immunity to Coagulation Pathways<sup>1</sup>

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**Neutrophils and complement are key sentinels of innate immunity and mediators of acute inflammation. Recent studies have suggested that inflammatory processes modulate thrombogenic pathways. To date, the potential cross-talk between innate immunity and thrombosis and the precise molecular pathway by which complement and neutrophils trigger the coagulation process have remained elusive. In this study, we demonstrate that antiphospholipid Ab-induced complement activation and downstream signaling via C5a receptors in neutrophils leads to the induction of tissue factor (TF), a key initiating component of the blood coagulation cascade. TF expression by neutrophils was associated with an enhanced procoagulant activity, as verified by a modified prothrombin time assay inhibited by anti-TF mAb. Inhibition studies using the complement inhibitor compstatin revealed that complement activation is triggered by antiphospholipid syndrome (APS) IgG and leads to the induction of a TF-dependent coagulant activity. Blockade studies using a selective C5a receptor antagonist and stimulation of neutrophils with recombinant human C5a demonstrated that C5a, and its receptor C5aR, mediate the expression of TF in neutrophils and thereby significantly enhance the procoagulant activity of neutrophils exposed to APS serum. These results identify a novel cross-talk between the complement and coagulation cascades that can potentially be exploited therapeutically in the treatment of APS and other complement-associated thrombotic diseases. *The Journal of Immunology*, 2006, 177: 4794–4802.**

Inflammation and thrombosis are linked in many clinical disorders (1). Life-threatening diseases associated with acute or chronic inflammation, such as sepsis and systemic inflammatory response syndrome (2, 3), antiphospholipid syndrome (APS)<sup>3</sup> (4), and other autoimmune syndromes (5), show clinical manifestations of severe predisposition to thrombosis. However, it has remained a subject of intense debate whether persistent inflammation can actually trigger thrombosis or whether the ensuing procoagulant response is rather a bystander effect of inflammation, secondary to the dysregulated activation of the vascular endothelium by various inflammatory mediators.

Neutrophils and complement are crucial components of innate immunity. Both are considered among the earliest innate immune effectors that sense incoming inflammatory signals and are rapidly recruited to sites of injury and inflammation (3). Earlier studies

have indicated a role for complement in procoagulant pathways by showing that interception of C5a and its receptor C5aR results in distinct changes in pro-/antifibrinolytic proteins (3, 6) and in the induction of tissue factor (TF) in endothelial cells (7) and monocytes (8). Furthermore, studies in rodent models of APS-induced thrombophilia have indicated that activation of complement components C3 and C5 is required for the induction of thrombus formation (9–11). However, the mechanisms by which complement modulates thrombogenic responses and triggers the coagulation pathway, as well as the identities of the immune cells and specific receptors targeted by complement in this procoagulant response, have not been determined. Prompted by the fact that neutrophils are pivotal effectors of acute inflammation that are potently chemoattracted by C5a and rapidly extravasate to remote sites of injury or infection, we have investigated the potential interaction of complement and neutrophils in the induction of TF-dependent thrombosis.

TF, a 47-kDa transmembrane glycoprotein, enables cells to initiate the coagulation cascade. The extracellular N-terminal domain of TF contains functional sites for factor VIIa (FVIIa) binding (e.g., Lys<sup>20</sup>) (12); the coagulation cascade is triggered when TF binds to FVIIa (13). Traditionally, TF was believed to be expressed only in tissues, separated from blood cells and vascular endothelium (hemostatic envelope), exerting its thrombogenic effect after vascular damage (14). More recently, it has become evident that whole blood contains active TF; however, the exact source of this circulating or bloodborne TF is not fully understood (15). Recently, the existence of an alternatively spliced variant of human TF (asTF), which consists of 206 aa, has been described (16). This spliced form of TF is soluble, circulates in plasma, and seems to be biologically active. Although TF induction in activated endothelial cells and peripheral monocytes has been described (17, 18), the potential contribution of neutrophils to circulating or bloodborne TF has not yet been clarified, and the capacity of neutrophils to express and generate TF is currently under debate (15, 19–23).

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<sup>3</sup> Abbreviations used in this paper: APS, antiphospholipid syndrome; TF, tissue factor; asTF, alternatively spliced variant of human TF; cTF, common TF; rhC5a, recombinant human C5a; mPT, modified prothrombin time; FVII, factor VII.

Using human APS as a clinical model of thrombosis, we have now identified neutrophil-derived TF as a novel molecular target linking complement activation to the coagulation cascade. Our studies have shown that the complement anaphylatoxin C5a triggers the extrinsic coagulation pathway by inducing TF expression and TF-dependent procoagulant activity in neutrophils. Studies involving complement inhibitors and a specific C5aR antagonist have further confirmed our conclusion that APS autoantibodies trigger complement activation and that both C3 and C5aR stimulation are essential for TF-dependent procoagulant activity in neutrophils.

## Materials and Methods

### Preparation of APS serum

Serum samples were collected from 11 patients who displayed primary (six patients) or secondary (five patients) APS-associated pathology and who are being followed in the Academic University Hospital of Alexandroupolis. All harvest procedures were approved by the Institutional Review Board and were in compliance with institutional guidelines.

### Complement reagents

1) Compstatin, a 13-residue cyclic peptide (Ac-I[CVWQDWGAHRTC]T-NH<sub>2</sub>) that inhibits the cleavage of native C3 by the C3 convertase, was used as a specific inhibitor of complement activation at the C3 level (24). A linear inactive analog of compstatin (IAVWQDWGAHRTAT-NH<sub>2</sub>) was used as a control (25). 2) SB-290152, a selective nonpeptide antagonist of the complement anaphylatoxin receptor C3aR, was used in this study to dissect the involvement of C3aR signaling in the generation of neutrophil-derived TF (26). 3) To block C5aR stimulation on neutrophils, we used a small cyclic hexapeptide (AcF-[OPdChaWR]) that acts as a selective C5aR antagonist (27, 28). 4) Recombinant human C5a (rhC5a) containing a His-tag at the N terminus was expressed in *Escherichia coli* using the expression vector pQE30 (Qiagen). The expressed protein was purified as previously described (29). C3a (WWGKKYRASKLGLAR-COOH) (30) and C5a (YSFKPMPLaR) (31) agonists were synthesized, and both purity and identity were confirmed as previously described (24).

### Isolation of peripheral blood cells

Circulating leukocytes were immediately isolated from EDTA-treated peripheral blood. PBMCs and neutrophils were separated by Histopaque double-gradient density centrifugation according to the manufacturer's instructions (Histopaque; Sigma-Aldrich). The absolute number of neutrophils was adjusted to  $2\text{--}2.4 \times 10^7$  cells/ml in PBS. Cell purity after cytopsin (>98%), viability by trypan blue exclusion (>97%), and platelet contamination (<2 platelets/100 neutrophils) were assessed in all experiments. Moreover, May-Grünwald-Giemsa staining did not reveal adhering platelets to neutrophils.

### RNA extraction and semiquantitative RT-PCR analysis of TF mRNA synthesis

RNA isolation from peripheral blood neutrophils was performed using the TRIzol reagent (Invitrogen Life Technologies) according to the manufac-

turer's instructions. Semiquantitative analysis of TF mRNA expression was performed using the QuantumRNA classic II 18S kit (Ambion). The primers FCU and FCL (5'-CCGCAACTGGTAGAC-3' and 5'-TGGGGAGT TCTCATACAG-3', respectively) were used to amplify a common sequence of both TF mRNA isoforms (full-length TF and alternatively spliced TF), resulting in a 406-bp, common TF (cTF) PCR product (F3 accession sequence, NM\_001993). PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 50 s. All reactions were conducted according to the manufacturer's instructions (Invitrogen Life Technologies). The cycling parameters, linear range, and optimal 18S primer/competitor ratio (1:12) were optimized using PBMC cDNA from each volunteer, because it has been established that this population of cells expresses TF mRNA. The band intensities of cTF (406 bp) and 18S (318 bp) were analyzed using Kodak DS, 1D Image Analysis Software, version 3.0.1 (Eastman Kodak).

TF isoform-specific real-time PCR was also performed using neutrophils and PBMCs isolated from six healthy individuals before and after exposure to APS serum to quantify the relative expression levels of TF and asTF mRNA and distinguish possible differences between these cellular populations. In each sample, full-length TF (TF), alternatively spliced TF (asTF), and GAPDH mRNA sequence-specific primers and probes for detection were applied as previously described (32): To amplify the two TF isoforms separately, the primers and probe for the detection of TF were designed to hybridize to exon 5, which is missing in asTF, whereas the asTF primers were designed to hybridize to exons 4 and 6, with the probe covering the unique exon 4/6 boundary, which is present in asTF (32). Two microliters of each cDNA (prepared from 0.5  $\mu$ g of total RNA), was added to a final PCR mix volume of 25  $\mu$ l that contained 12.5  $\mu$ l TaqMan Universal Master Mix (Applied Biosystems), 0.5  $\mu$ l (10  $\mu$ M) of each primer (Biolegio), 0.5  $\mu$ l (10  $\mu$ M) of probe, and 11.5  $\mu$ l of H<sub>2</sub>O. Real-time PCR was performed using the MiniOpticon Real-Time Detection System (BioRad) under the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. Standards for each target were generated by RT-PCR, and serial dilutions of each standard were used to construct the respective standard curve.

The  $2^{-DDC_T}$  method (33) was used for quantification of the target genes (TF and asTF). In brief, we normalized the amount of target gene (TF or asTF) in the stimulated cells to that of the same type of unstimulated cells ( $2^{-DDC_T}$ , Table I). We also normalized the amount of target gene (TF or asTF) in the stimulated PMNs to that of the stimulated PBMCs ( $2^{-DDC_T}$ , Table II). The averages of the threshold values ( $C_T$ ), as well as  $DC_T$  and  $DDC_T$  values for the  $2^{-DDC_T}$  equation, are shown in Tables I and II. Values are means and SDs. Given the limited sample, a nonparametric test (the paired sign test) was selected to compare data within each group. The level of statistical significance was set to  $p < 0.05$ .

### Modified prothrombin time (mPT) assay to assess TF-mediated coagulation activity released by neutrophils

The supernatants of cells incubated with various agents were isolated by centrifugation at  $1000 \times g$  for 10 min; supernatants from neutrophil preparations were checked a second time to confirm the absence of cells and platelets. The coagulation activities (TF/FVIIa binding activity) of the cell supernatants were determined using a mPT assay. After performing the classic PT test (100  $\mu$ l of platelet-poor plasma plus 200  $\mu$ l of thromboplastin (Instrumentation Laboratory)), we conducted the modified PT analysis. Namely, 125  $\mu$ l of cell supernatant and 75  $\mu$ l of thromboplastin were

Table I.  $2^{-DDC_T}$  data analysis<sup>a</sup>

| Description             | Average $C_T$<br>(Target) | Average $C_T$<br>GAPDH | $DC_T$           | $DDC_T$          | $2^{-DDC_T}$      | Expression<br>Difference*   |
|-------------------------|---------------------------|------------------------|------------------|------------------|-------------------|-----------------------------|
| PBMCs unstimulated TF   | 31.75                     | 17.72                  | 14.02 $\pm$ 1.43 | 0 $\pm$ 0.93     | 1 $\pm$ 0.36      |                             |
| PBMCs stimulated TF     | 29.20                     | 18.13                  | 11.07 $\pm$ 1.80 | -2.95 $\pm$ 1.15 | 7.72 $\pm$ 0.28   | $\times 7.72$ ( $2 > 1$ )   |
| PMNs unstimulated TF    | 30.94                     | 20.71                  | 10.22 $\pm$ 2.29 | 0 $\pm$ 2.29     | 1 $\pm$ 0.20      |                             |
| PMNs stimulated TF      | 30.01                     | 20.85                  | 9.16 $\pm$ 1.99  | -1.06 $\pm$ 1.99 | 2.09 $\pm$ 0.25   | $\times 2.09$ ( $4 > 3$ )   |
| PBMCs unstimulated asTF | 35.42                     | 17.72                  | 17.69 $\pm$ 1.79 | 0 $\pm$ 2.45     | 1 $\pm$ 0.28      |                             |
| PBMCs stimulated asTF   | 32.39                     | 18.13                  | 14.26 $\pm$ 0.13 | -3.43 $\pm$ 1.06 | 10.79 $\pm$ 0.90  | $\times 10.79$ ( $6 > 5$ )  |
| PMNs unstimulated asTF  | 44.46                     | 19.82                  | 24.63 $\pm$ 1.74 | 0 $\pm$ 1.74     | 1 $\pm$ 0.29      |                             |
| PMNs stimulated asTF    | 33.48                     | 18.67                  | 14.81 $\pm$ 1.50 | -9.82 $\pm$ 1.50 | 905.97 $\pm$ 0.27 | $\times 905.97$ ( $8 > 7$ ) |

<sup>a</sup> Relative quantification of TF or asTF between unstimulated and stimulated cells. The comparison was performed in pairs using the same target gene and the same type of cells.

$C_T$ , Threshold value.  $DC_T$  = average target  $C_T$  - average GAPDH  $C_T$ .

$DDC_T$  (for the same target gene) = average  $DC_T$  of cells of interest - average  $DC_T$  of unstimulated cells.  $DDC_T = 0$  when the cells of interest are used as calibrator.

$2^{-DDC_T}$  = normalized target gene amount (TF or asTF) relative to unstimulated cells target gene amount.

\*,  $p < 0.05$ .

Table II.  $2^{-DDC_T}$  data analysis<sup>a</sup>

| Description          | Average $C_T$<br>TF | Average $C_T$<br>GAPDH | $DC_T$       | $DDC_T$      | $2^{-DDC_T}$ | Expression<br>Difference |
|----------------------|---------------------|------------------------|--------------|--------------|--------------|--------------------------|
| PBMC stimulated TF   | 29.21               | 18.13                  | 11.08 ± 1.15 | 0 ± 1.15     | 1 ± 0.45     | ×3.78 ( $I > 2$ )*       |
| PMNs stimulated TF   | 30.01               | 20.85                  | 9.16 ± 2.20  | 1.92 ± 2.20  | 0.26 ± 0.22  |                          |
| PBMC stimulated asTF | 32.39               | 18.13                  | 14.26 ± 1.06 | 0 ± 1.06     | 1 ± 0.48     |                          |
| PMNs stimulated asTF | 33.48               | 18.67                  | 14.81 ± 1.50 | -0.55 ± 1.50 | 1.46 ± 0.35  | ×1.46 ( $I > 3$ )**      |

<sup>a</sup> Relative quantification of TF or asTF between stimulated PBMCs and PMNs. The comparison was performed in pairs using the same target gene.

$C_T$ , Threshold value.  $DC_T$  = average target  $C_T$  - average GAPDH  $C_T$ .

$DDC_T$  (for the same target gene) = average  $DC_T$  of cells of interest - average  $DC_T$  of stimulated PBMCs.  $DDC_T = 0$  when stimulated PBMCs are used as the calibrator.

$2^{-DDC_T}$  = normalized target gene amount (TF or asTF) relative to stimulated PBMCs target gene amount.

\*,  $p < 0.05$ . \*\*,  $p = NS$ .

added to 100  $\mu$ l of platelet-poor plasma to measure the changes of PT. As control of modified PT, 125  $\mu$ l of PBS was used instead of cell supernatant and the in vitro clotting time was usually ranged from 31 to 34 s.

To verify that the thromboplastic activity was due to TF, supernatants were incubated for 30 min with a specific anti-TF mAb (no. 4509; American Diagnostica), 10  $\mu$ g/ml, at room temperature. PT was then measured by the mPT method. Controls involved incubation with the same subclass and concentration of mouse anti-human Abs (anti-CD11b/M0847, CD103/M0741, CD2/M0720, CD19/M0740, CD5/M0705, CD13/M0812, anti-MPO/M0748; DakoCytomation), as well as with different secondary Abs.

#### Complement inhibition and anaphylatoxin receptor studies

The mPT coagulation assay was optimized for APS serum (coagulation activity at saturation point, viability of neutrophils >97%), and all subsequent incubations were performed for 90–120 min at 37°C. Neutrophils from healthy donors were incubated with the following complement agonists or antagonists (in a total volume of 250  $\mu$ l): 1) serum from healthy individuals or APS patients (one of five); 2) APS IgG alone or APS IgG plus normal serum; 3) serum from APS patients or APS IgG plus normal serum preincubated for 30 min with compstatin (15  $\mu$ M final concentration), before incubation with neutrophils (28); 4) serum from APS patients preincubated for 30 min with a linear control peptide (15  $\mu$ M final concentration), before incubation with neutrophils; 5) serum from APS patients or APS IgG plus normal serum added to neutrophils that had been preincubated for 30 min with C5aRa (5–10  $\mu$ M final concentration) (28); 6) serum from APS patients added to neutrophils that had been preincubated for 30 min with C3aRa (5–10  $\mu$ M final concentration) (26); 7) rhC5a (2  $\mu$ M final concentration); 8) C5a agonist (200  $\mu$ M final concentration) (31); and 9) C3a agonist (200  $\mu$ M final concentration) (30). The effects of complement agonists and antagonists were found to be dose dependent, reaching peak activity at the doses chosen for this study.

#### Immunocytochemical staining

Cytospin films of incubated cells were stored at -20°C before use. Immunocytochemical staining for TF was performed using the alkaline phosphatase-anti-alkaline phosphatase method as previously described (34). An IgG1 mouse anti-human TF mAb directed against epitopes of aa 1–25 (no. 4509; American Diagnostica) was used in this study. The same procedure was followed for CD11b staining, using an anti-CD11b (Mac-1) mAb (M0741; DakoCytomation) and IgG1 anti-CD19 mAb (M0740; DakoCytomation) was used as negative control.

#### FACS analysis of isolated neutrophils

Isolated neutrophils were stimulated and then fixed and permeabilized for intracellular staining using the FIX & PERM System (Caltag Laboratories) according to the manufacturer's instructions. The cells ( $\sim 2.5 \times 10^4$ ) were stained with FITC-conjugated mouse mAb against human TF (4508CJ; American Diagnostica). FITC-labeled mouse IgG1 was used as an isotype control (BD Biosciences). The staining was performed in the dark for 1 h at 4°C. Flow cytometry was performed using a FACScan with CellQuest software (BD Biosciences), and neutrophils were identified by forward- and side-scatter characteristics.

#### Western blot analysis

Approximately  $3 \times 10^6$  cells were lysed at -20°C for 1 h in lysis buffer (1% Triton X-100 and 150 mM NaCl in 20 mM HEPES (pH 7.5) with protease inhibitors (Complete Protease Inhibitor Tablets; Roche)). After centrifugation, the supernatants were assayed for protein content using the BCA Protein Assay (Pierce). Proteins (90  $\mu$ g of protein per lane) were

diluted 1/1 in 2× SDS loading buffer, heated at 10°C for 5 min, resolved by SDS-PAGE, and electroblotted onto polyvinylidene difluoride membrane filters (Bio-Rad). Membranes were blocked for 2 h in 3% skim milk/TBT-T, followed by an overnight incubation at 4°C with anti-TF mAb (4509; American Diagnostica) or anti-Jak2 polyclonal Ab (SC7229; Santa Cruz Biotechnologies) at 1/500 and 1/1000 dilutions, respectively. After a thorough washing in TBS-T, membranes were incubated with HRP-linked whole anti-mouse (P0161; DakoCytomation) or anti-rabbit Ab (NA934; Amersham Biosciences), respectively, at 1/2000 for 1.5 h at room temperature. Immunoreactive proteins were detected using the ECL detection system (Supersignal West Pico Chemiluminescent Substrate; Pierce) and exposed to x-ray film (Fuji medical x-ray film; Fujifilm).

#### Antiphospholipid IgG purification

Antiphospholipid Abs (APS-IgG fraction) were affinity-purified from pooled sera of APS patients using protein G-agarose chromatography, as previously described (35, 36). The same procedure was used to purify IgG from healthy donors. The purity of the resulting APS IgG preparations was determined by SDS-PAGE, and protein concentrations were determined by measuring absorbance at 280 nm. Purified IgG was endotoxin-free, as determined by a *Limulus* ameocyte assay (Sigma-Aldrich).

#### Statistical analysis

Data are reported as means + SD. Statistical analyses were conducted using Student's *t* test to compare differences in means. Associations were considered to be statistically significant if the value of  $p < 0.05$ . Data were processed using the STATISTICA, version 5.0 (Statsoft), statistical program for Windows.

## Results

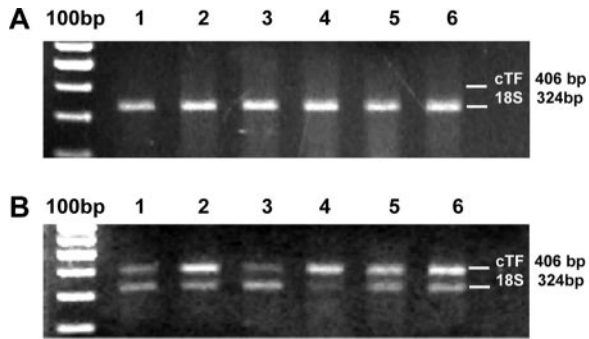
#### APS stimulates TF expression in neutrophils

To determine whether neutrophils constitutively produce TF in the absence of a priming inflammatory stimulus, we looked for the presence of TF mRNA by RT-PCR in freshly isolated neutrophils from six healthy individuals. TF mRNA was absent from unstimulated neutrophils (Fig. 1A, lanes 1–6).

To test whether exposure of neutrophils to a priming factor derived from APS serum stimulates TF expression, neutrophils from six healthy individuals were incubated individually with serum samples from six APS patients. In contrast to unstimulated cells, neutrophils that had been exposed to APS serum were able to express TF isoforms, as verified both by RT-PCR (Fig. 1B, lanes 1–6, and Table I) and immunocytochemical staining with a specific anti-TF mAb (Fig. 2AIIa).

#### Neutrophil-derived TF has procoagulant activity and triggers the extrinsic coagulation pathway

Prompted by our finding that APS serum induces TF expression in neutrophils, we decided to assay APS-exposed neutrophils for TF-dependent procoagulant activity, performing an mPT assay as described in *Materials and Methods*. Normal-donor neutrophils were incubated separately with 11 APS sera (6 primary APS, 5 secondary) and also with 22 sera from healthy volunteers. The mPT assay enabled us to obtain accurate and easily reproducible coagulant



**FIGURE 1.** Patterns of cTF mRNA expression by neutrophils. APS induces TF expression in neutrophils. *A*, Representative pattern of cTF mRNA expression (see *Materials and Methods*) in unstimulated neutrophils of healthy individuals; no TF expression is observed in the neutrophils of six healthy individuals (*lanes 1–6*). *B*, mRNA expression pattern of cTF by neutrophils for six healthy individuals (*lanes 1–6*) after exposure to APS serum for 2 h; after exposure to APS, the neutrophils express cTF mRNA.

measurements of supernatants originating from cells incubated with serum from healthy individuals, because these supernatants lack TF activity.

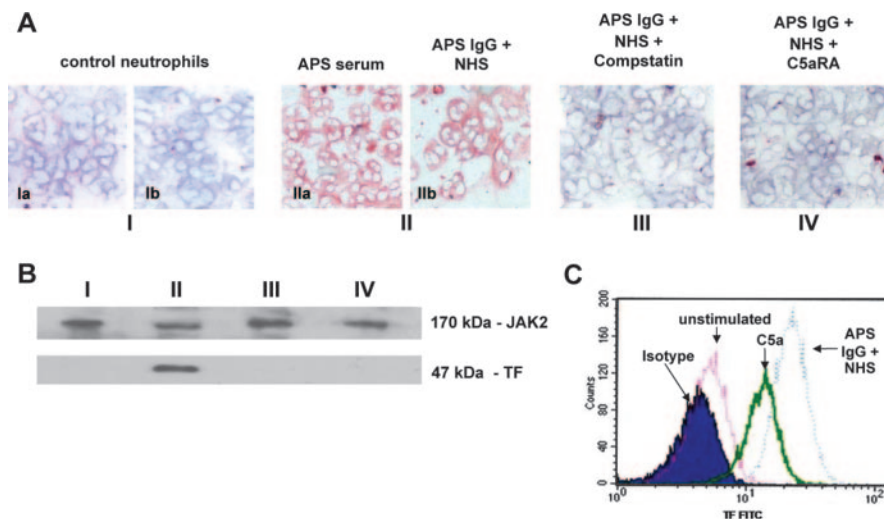
The mean classic PT was  $12.57 \pm 0.16$  s at baseline, whereas the mean control mPT at baseline was  $33.11 \pm 0.75$  s (Fig. 3*A*, *bar 1*). Supernatant from unstimulated neutrophils or cells incubated with the serum of healthy individuals exhibited no procoagulant activity (mPT,  $33.27 \pm 0.73$  and  $32.03 \pm 0.71$  s; Fig. 3*A*, *bars 2* and *3*, respectively); in contrast, supernatants from cells incubated with APS serum showed markedly increased procoagulant activity, because the mPT decreased to  $21.15 \pm 1.94$  s (a 64% difference between the control modified and classic PT values;  $p < 0.0001$ ; Fig. 3*A*, *bar 4*). This coagulation activity was found to be entirely dependent on TF, because the addition of the highly specific anti-TF mAb at a dilution of 1/100 resulted in the return of the mPT to baseline values ( $32.54 \pm 0.89$  s; Fig. 3*A*, *bar 5*). Incubation with

various concentrations of other monoclonal or polyclonal Abs (see *Materials and Methods*) had no effect on the mPT values. Likewise, no effect on mPT values was observed with any of the reagents used for the experiments, or with APS patients' or healthy individuals' serum alone. The specific anti-TF mAb at high concentration (1/5, w/v) as well as at 1/50 or 1/100 dilution, did not inhibit the exogenous thromboplastin, and the mPT remained at baseline level (data not shown).

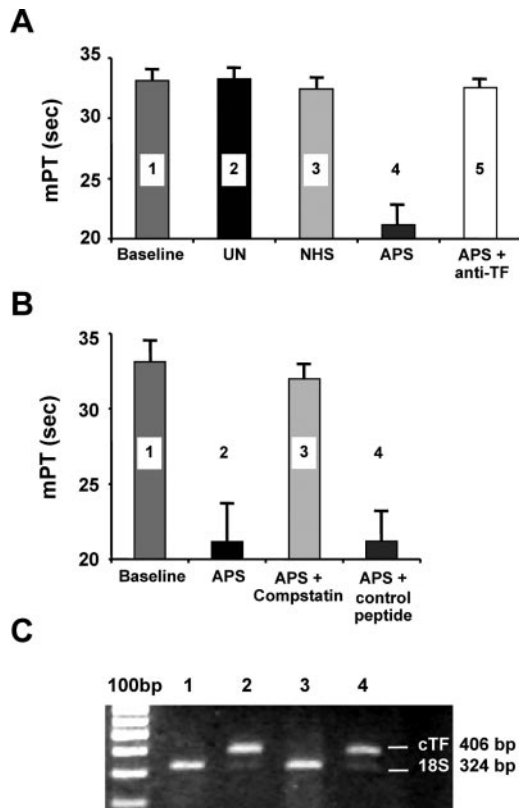
*TF-dependent coagulation activity of neutrophils (mPT) is not affected by trace amounts of contaminating PBMCs or platelets*

It is known that the PBMC layer (mononuclear cells and platelets) can generate high levels of TF after incubation with certain agents and APS serum (37, 38). We similarly observed that this TF coagulation activity could be induced by stimulation of the PBMC layer with APS serum (Fig. 4, *A* and *B*). To determine whether trace contamination of the enriched neutrophil preparations with PBMCs could interfere with the coagulation activity assay, we measured the mPT in three healthy individuals using supernatants from neutrophils and PBMCs in different dilutions (incubated with a 20% pool of APS serum). We found that the mPT was cell number dependent and that it returned to baseline when  $0.5\text{--}1 \times 10^5$  neutrophils or  $0.5\text{--}1 \times 10^5$  PBMCs with  $0.8\text{--}1 \times 10^6$  platelets were stimulated with APS serum (Fig. 4, *C* and *D*). Analysis of the enriched neutrophil preparations used in the functional assays demonstrated that the contamination never exceeded  $0.6\text{--}1 \times 10^5$  PBMCs and  $0.5\text{--}0.8 \times 10^5$  platelets per  $0.5 \times 10^7$  neutrophils. Thus, the APS serum-activated neutrophils are usually contaminated with the same or lower number of PBMCs and platelets than the number that has been shown to be ineffective in shortening the mPT ( $p = \text{NS}$ ; Fig. 4).

These functional results are further strengthened by our analysis of the levels of TF and asTF mRNA in APS serum-stimulated neutrophils and PBMCs by isoform-specific real-time analysis (Tables I and II). Although both populations expressed TF- and asTF-specific mRNAs, PBMCs produced approximately four times more TF than did neutrophils, and the neutrophils produced



**FIGURE 2.** TF expression at the protein level by neutrophils (PMNs). *A*, *I*, Control neutrophils; *Ia*, staining of stimulated cells with anti-CD19 (irrelevant Ab); *Ib*, staining of unstimulated neutrophils with anti-TF. *II*, Immunostaining of neutrophils for TF after stimulation; *IIa*, immunostaining after APS serum treatment for 2 h; *IIb*, immunostaining after treatment with APS IgG plus normal serum from healthy individuals (NHS). *III* and *IV* show immunostaining of neutrophils exposed to APS IgG plus NHS pretreated with compstatin for 30 min (*III*), and neutrophils pretreated for 30 min with C5a receptor antagonist before their exposure to APS IgG plus NHS (*IV*). *B*, TF Western blot analysis of neutrophils before and after stimulation for 2 h with APS IgG plus NHS (*lanes I* and *II*, respectively). *Lanes III* and *IV* show the effects of compstatin and C5a receptor antagonist, respectively, in stimulated neutrophils. *C*, FACS analysis of TF expression on purified neutrophils, before and after stimulation with APS IgG plus NHS or rhC5a, as determined by forward- and side-scatter characteristics.



**FIGURE 3.** Promotion of neutrophil TF-dependent coagulation by APS, and complement dependence of the procoagulant response by APS-treated neutrophils. *A*, Accelerated blood coagulation activity (mPT) in neutrophil supernatants treated for 2 h with APS serum ( $p < 0.0001$ ; bar 4), compared with supernatants incubated with normal serum from healthy individuals (NHS; bar 3), unstimulated neutrophil supernatants (UN; bar 2), and baseline values (bar 1). mPT returned to baseline value after incubation of APS-treated neutrophils with anti-TF-specific mAb (bar 5), indicating that the procoagulant activity of neutrophils is TF dependent. *B*, Complete blockade of TF-dependent coagulation activity in neutrophils by complement inhibition: Incubation of neutrophils for 2 h with APS serum (bar 2) significantly reduces the increase in mPT above baseline (bar 1). Preincubation of APS serum with compstatin ( $15 \mu\text{M}$  for 30 min) completely inhibits the procoagulant activity of neutrophils, and the mPT remains at baseline (bar 3), whereas the nonfunctional linear compstatin control fails to inhibit the procoagulant effect (bar 4). *C*, Inhibition of complement activation blunts TF mRNA expression in neutrophils: cTF mRNA expression by neutrophils after exposure to APS serum (lane 2), compared with unstimulated neutrophils (lane 1). Preincubation of APS serum with the complement inhibitor compstatin for 30 min abrogates cTF expression, as demonstrated by RT-PCR (lane 3). cTF induction in stimulated neutrophils remains unchanged in the presence of a linear compstatin analog (control peptide) (lane 4).

slightly more asTF than did the PBMCs (Fig. 5). Because the level of contamination of the neutrophil layer by PBMCs was  $\sim 1\text{--}2\%$  (and neutrophils express more asTF than do PBMCs (see Fig. 5, Table II)), it seems reasonable to assume that the observed procoagulant activity was derived from neutrophils rather than PBMCs.

Furthermore, pure platelets ( $5 \times 10^8$ ) did not show any TF activity after APS serum stimulation (data not shown), a finding that is in accordance with recent data reporting the transfer of TF from CD15-positive leukocytes to platelets (39). Finally, FACS analysis with neutrophil and platelet markers (CD15 and CD42a, respectively) after activation using APS failed to reveal any contaminating platelets adhering to the surface of activated neutrophils (data not shown).

Collectively, the apparent differences in the expression profiles of TF and asTF in the PBMC and neutrophil populations analyzed (Fig. 5, Table II), the failure of purified platelets to produce TF activity, the apparent absence of significant sequestration of platelets on the surface of the isolated neutrophils (data not shown), and the cytofluorometric detection of TF only in permeabilized neutrophils (Fig. 2) argue strongly that the production of TF and asTF mRNA and protein is an intrinsic property of neutrophils and cannot be attributed to PBMCs or platelets contaminating the neutrophil preparations.

#### *Complement activation is required for the TF-mediated procoagulant activity of neutrophils*

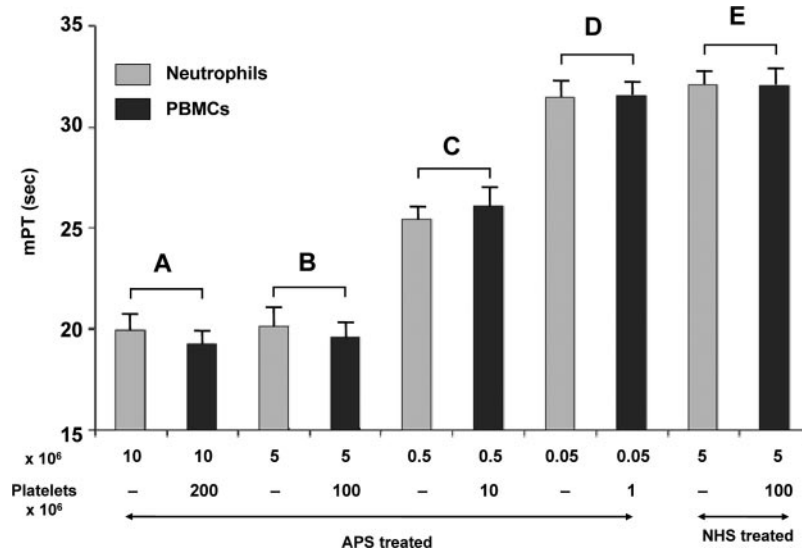
In preliminary experiments designed to clarify the nature of the factor(s) mediating TF production, we observed that this effect was dependent on heat inactivation. Heat inactivation of APS serum at  $56^\circ\text{C}$  for 30 min resulted in the loss of its ability to induce TF expression by neutrophils and also caused the mPT to return to baseline values (baseline mPT,  $33.11 \pm 0.75$  s; APS serum mPT,  $21.15 \pm 1.94$  s ( $p < 0.0001$  compared with baseline); and heat-inactivated APS serum mPT,  $32.13 \pm 0.72$  s ( $p < 0.0001$  compared with APS serum only;  $p = \text{NS}$  compared with baseline)).

Because complement components are known to be inactivated by heat treatment, and complement activation has been implicated in fetal injury associated with the APS syndrome (9), we decided to investigate the effect of complement inhibition in our experimental setting. To ascertain whether complement activation and cleavage of C3 are required for the induction of TF expression and the subsequent enhancement of coagulation by neutrophils, we used the specific C3 inhibitor compstatin. APS serum was preincubated with either compstatin or a nonfunctional linear analog (as control peptide), and the mixture was then used to stimulate neutrophils. Pretreatment of APS serum with compstatin abolished TF production, as determined by mPT functional tests, whereas the linear analog had no effect (compstatin plus APS serum mPT,  $31.99 \pm 0.37$  s ( $p < 0.0001$  compared with APS serum only); linear control plus APS serum mPT,  $21.18 \pm 1.87$  s ( $p = \text{NS}$  compared with APS serum only)) (Fig. 3*B*, bars 3 and 4). The above functional studies have been also confirmed by RT-PCR (Fig. 3*C*). Taken together, these studies clearly indicate that complement activation is essential for TF production by neutrophils, thus identifying a novel molecular pathway by which complement can mediate the procoagulant response of neutrophils.

#### *APS IgG triggers complement activation and is required for TF-dependent coagulation*

To determine whether TF induction and subsequent neutrophil-dependent coagulation are initiated by a specific component of the APS serum (e.g., whether they are IgG dependent), we affinity-fractionated the APS-specific autoantibodies and tested their ability to induce TF-dependent coagulation by neutrophils. When neutrophils were incubated separately with a purified IgG pool from sera of two primary APS patients (APS IgG), no TF mRNA (Fig. 6*B*, lane 1) or protein expression assessed by immunostaining (data not shown) was observed, and the mPT in supernatants remained at baseline level when compared with that of total APS serum (Fig. 6*A*, bar 3); similarly, neither the IgG pool from healthy individuals (Fig. 6*A*, bar 4) nor the IgG-depleted fraction of APS serum or normal serum was able to activate neutrophil TF (data not shown).

In contrast, when neutrophils were exposed to purified APS IgG that had been pretreated with 5% normal serum or IgG-depleted APS serum, they were able to express TF both at mRNA (Fig. 6*B*, lane 3) and protein level, as shown by immunostaining (Fig. 2,



**FIGURE 4.** Coagulation activity (mPT) of the PBMC layer and neutrophils contaminated with trace amounts of PBMCs and platelets. Coagulation activity of cell supernatants incubated with APS serum is cell number dependent. *A*, mPT performed after incubation of  $1 \times 10^7$  neutrophils (contaminated with  $1 \times 10^5$  PBMCs and  $1 \times 10^5$  platelets) or  $1 \times 10^7$  PBMCs and  $2 \times 10^8$  platelets with APS serum. *B*, mPT performed after incubation of  $0.5 \times 10^7$  neutrophils (contaminated with  $0.5 \times 10^5$  PBMCs and  $0.7 \times 10^5$  platelets) or  $0.5 \times 10^7$  PBMCs and  $1 \times 10^8$  platelets with APS serum. *C*, mPT performed after incubation of  $0.5 \times 10^6$  neutrophils or  $0.5 \times 10^6$  PBMCs and  $1 \times 10^7$  platelets with APS serum. *D*, mPT performed after incubation of  $0.5 \times 10^5$  neutrophils or  $0.5 \times 10^5$  PBMCs, the usual number of contaminating neutrophils in our experimental setting) and  $1 \times 10^6$  platelets with APS serum. *E*, mPT performed after incubation of  $0.5 \times 10^7$  neutrophils (contaminated with  $0.5 \times 10^5$  PBMCs and  $0.7 \times 10^5$  platelets) or  $0.5 \times 10^7$  PBMCs and  $1 \times 10^8$  platelets with serum from a healthy individual.

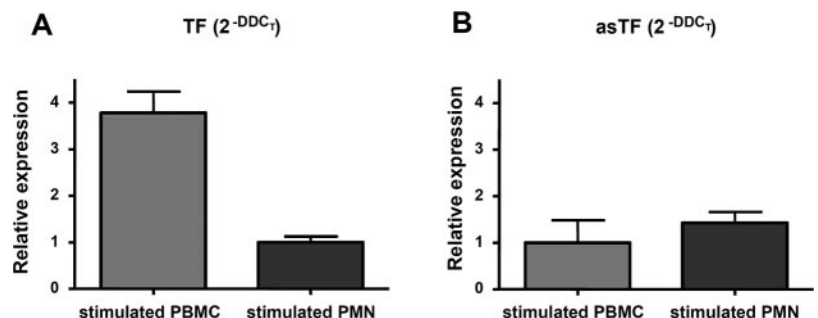
*Ailb* and *C*) and Western blot analysis (Fig. 2*B*, lane 2) and their supernatant showed marked coagulation activity that was inhibited by the anti-TF mAb (Fig. 6*A*, bar 5). In contrast, purified IgG from healthy individuals had no effect on neutrophils in a similar experiment (data not shown). Interestingly, the TF mRNA expression (Fig. 6*B*, lane 4), alkaline phosphatase-anti-alkaline phosphatase anti-TF immunostaining (Fig. 2*AIII*), Western blot analysis (Fig. 2*BIII*), and procoagulant capacity of normal serum containing APS IgG (Fig. 6*A*, bar 6) were completely blocked by compstatin (APS IgG plus normal serum plus compstatin mPT,  $32.10 \pm 0.63$  s;  $p < 0.0001$  compared with APS IgG plus normal serum), indicating that APS autoantibodies trigger complement activation and that convertase-mediated cleavage of C3 is required for the TF-dependent coagulation process initiated by neutrophils.

*TF production in neutrophils is regulated by C5aR*

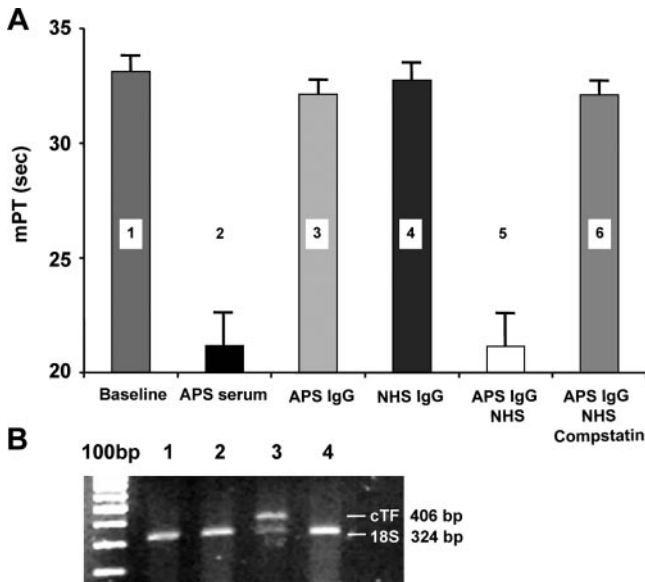
To further elucidate the mechanism by which complement mediates TF production by neutrophils, we examined the effect of C3aR and C5aR blockade on the TF-dependent coagulation activity of neutrophils. Both anaphylatoxin receptors are present in neutrophils, and their anaphylatoxin-mediated effects are exerted via activation of these receptors (40). To inhibit anaphylatoxin signaling, we used two highly selective C3aR and C5aR antagonists (C3aRa

and C5aRa) (26–28, 38–41). C3aRa treatment had no effect on TF production by neutrophils incubated with APS IgG plus normal serum or total APS serum (data not shown); in contrast, C5aRa treatment completely abolished TF expression by neutrophils at mRNA level (data not shown), at protein level as shown by immunochemical staining (Fig. 2*AIV*) and Western blot analysis (Fig. 2*BIV*), and the procoagulant effect (mPT functional test) (Fig. 7, bar 3) after incubation with APS IgG plus normal serum (baseline mPT,  $33.11 \pm 0.75$  s; APS IgG plus normal serum mPT,  $21.15 \pm 1.94$  s; C3aRa+APS IgG plus normal serum mPT,  $21.25 \pm 1.7$  s ( $p = \text{NS}$  compared with APS IgG plus normal serum); C5aRa+APS IgG plus normal serum mPT,  $31.79 \pm 0.33$  s (Fig. 7, bar 3 ( $p < 0.0001$  compared with APS IgG plus normal serum)). The same inhibition of TF expression (at the mRNA and protein levels) and TF-dependent procoagulant activity (mPT) by C5aRa was also observed in neutrophils incubated separately with total APS serum from six (three primary and three secondary APS) patients (data not shown).

To substantiate the finding that C5aR inhibition attenuates the procoagulant activity of APS-treated neutrophils and to confirm the role of C5a in TF induction, we assessed the ability of neutrophils to produce TF when incubated with rhC5a, as well as C3a and C5a agonists. We performed experiments with serial dilutions



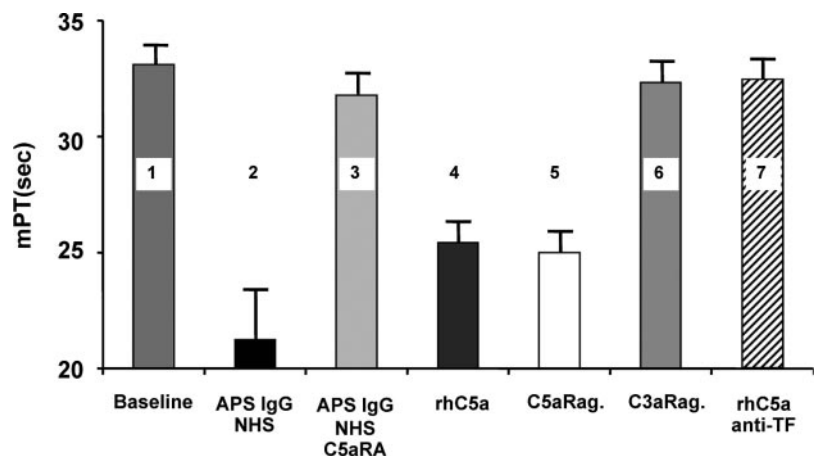
**FIGURE 5.** Real-time relative quantification of TF isoforms. Schematic presentation of the expression difference ( $2^{-DDC_T}$ ) of TF (*A*) and asTF mRNA (*B*) by the stimulated PMNs and PBMC layer. The  $2^{-DDC_T}$  has been estimated according to average  $C_T$  of target genes, average  $C_T$  of GAPDH, and  $DC_T$  values (Tables I and II).



**FIGURE 6.** Triggering of complement activation in serum by APS-IgG and its mediation of the TF-dependent procoagulant response of neutrophils. **A**, Incubation of normal neutrophils for 2 h with purified IgG from APS patients (bar 3) or healthy individuals (bar 4) does not result in procoagulant activity by the neutrophil supernatant (mPT remains similar to baseline, bar 1). In contrast, when neutrophils are coincubated with APS IgG plus serum from healthy individuals, a marked shortening of the mPT was observed (bar 5), similar to that seen for incubation with unfractionated APS serum (bar 2). Neutrophil TF-dependent activity is complement associated, because it is completely abolished by compstatin (15  $\mu$ M) (bar 6). **B**, Representative pattern of cTF mRNA expression by neutrophils after incubation (for 2 h) with APS IgG (lane 1), normal IgG from serum of healthy individuals (NHS) (lane 2), APS IgG plus NHS (lane 3), or APS IgG plus NHS after treatment with compstatin for 30 min (lane 4).

of rhC5a (10 nM, 100 nM, 500 nM, 2  $\mu$ M, and 4  $\mu$ M) and C3a and C5a agonists and found that both rhC5a and a C5a agonist induced TF production in a linear fashion, whereas C3a agonist treatment had no effect on TF-dependent coagulation by neutrophils (data not shown). Neutrophil activation reached a peak at higher concentrations of rhC5a (2  $\mu$ M) and C5a agonist (200  $\mu$ M), resulting in significant shortening of the mPT values (baseline mPT, 33.11 + 0.75 s; rhC5a mPT, 25.41 + 0.51 s ( $p < 0.0001$  compared with baseline); C3a agonist mPT, 32.33 + 0.55 s ( $p = \text{NS}$  compared with baseline); C5a agonist mPT, 25.01 + 0.61 s ( $p < 0.0001$  compared with baseline)) (Fig. 7). This TF-dependent procoagulant activity of neutrophils elicited after rhC5a (2  $\mu$ M) treatment

**FIGURE 7.** Mediation of the TF-dependent procoagulant activity of neutrophils by C5a and requirement downstream C5aR stimulation. When neutrophils from healthy individuals are incubated (for 2 h) with rhC5a (2  $\mu$ M, rhC5a, bar 4), the mPT is significantly shortened when compared with baseline (bar 1,  $p < 0.0001$ ). The same effect is observed after incubation of neutrophils with a C5a agonist (200  $\mu$ M) (bar 5), whereas incubation with a C3a agonist (200  $\mu$ M) has no effect on the coagulation activity of neutrophils (bar 6). Preincubation of neutrophils (30 min) with C5aRa (10  $\mu$ M) completely inhibits the procoagulant activity of neutrophils treated with APS IgG and normal serum from healthy individuals (bar 3). The procoagulant activity of neutrophil supernatants after rhC5a incubation is completely abolished by anti-TF mAb (bar 7).



was in accordance with FACS analysis (Fig. 2C) and TF mRNA expression studies (data not shown).

## Discussion

In recent years, the potential interplay between the inflammatory response and the coagulation process has been a subject of intense debate and has yielded several discordant findings. Our study provides evidence for a critical role for neutrophils and complement, two integral components of the innate immune system, in the triggering of the extrinsic coagulation cascade in a well-defined human thrombotic model, APS, which is characterized by a potent autoantibody-driven inflammatory response and a predisposition to thrombosis. Specifically, our findings indicate that 1) normal neutrophils are able to express TF and generate TF-dependent coagulation activity after stimulation with APS serum; 2) the TF procoagulant property of APS serum is complement dependent, and APS autoantibodies can directly trigger complement activation that results in TF generation by neutrophils and TF-dependent coagulation; and 3) C5a and its receptor C5aR/CD88 mediate the expression of TF in neutrophils.

These findings identify novel molecular targets and pathways that link innate immunity and inflammation to coagulation processes. Furthermore, they raise several intriguing questions as to 1) what extent neutrophils contribute to the extrinsic pathway of coagulation in various human thrombotic models via TF induction, and 2) how this complex interplay between C5a, neutrophils, and TF is involved in the regulation of procoagulant responses in different clinical models.

It is well known that activated monocytes are able to produce TF after stimulation by several agonists or APS serum (37, 38); we have now functionally confirmed the results of these previous studies and shown that PBMC preparations (that include monocytes and platelets) produce active TF after incubation with APS serum, resulting in TF-dependent coagulation (Fig. 4). However, opposite results have also been obtained regarding the capacity of neutrophils to express/produce TF and concerning their contribution to circulating TF. Therefore, we have focused on neutrophils for two main reasons: 1) in an effort to contribute to the elucidation of this phenomenon; and 2) to confirm the participation of neutrophils in TF synthesis, because these cells represent a very interesting component of innate immunity, because they rapidly accumulate in inflamed tissues, infiltrate the injured sites, and are found in large numbers when compared with monocytes. Although neutrophils have been shown to be positive for TF by immunohistochemical staining, their ability to produce active TF or engulf and transport it to the site of thrombus production has not been established (15).

Our findings are in agreement with a recent report demonstrating the expression of TF by neutrophils upon stimulation with P-selectin or the chemotactic peptide fMLP peptide (42).

Our findings indicate that peripheral neutrophils are able to express active TF after stimulation with APS serum. In addition to RT-PCR findings showing marked TF mRNA expression after APS stimulation and immunostaining combined with Western blot analysis indicating the existence of mature TF protein, we have demonstrated that the supernatant from neutrophils has TF-dependent activity. Moreover, although previous studies have suggested that human neutrophils may acquire functional TF from monocytes or platelets under pathophysiological conditions (20, 43, 44), we have shown in our experimental setting that neutrophils can generate TF activity independent of that produced by contaminating monocytes and platelets. Furthermore, the TF-dependent activity of neutrophil and PBMC supernatants was similar (Fig. 4), suggesting a substantial participation of activated neutrophils in TF regulation.

Previous studies have shown that residue 20 (blocked by the Ab) is essential for the binding of TF to FVII and the subsequent activation of coagulation (12); in addition, mutation of residue 20 results in a strong prolongation of clotting time, indicating an effect on coagulation (12). Because the anti-TF mAb we used recognizes an epitope within a portion (residues 1–25) of the N-terminal domain that is common to full-length TF and asTF, future studies (using Abs specific for the unique asTF sequence) are needed to demonstrate the precise percentage of asTF in the active soluble factor of the neutrophil supernatants.

Prompted by our observation that neutrophils express functionally active TF, we focused our studies on identifying which inflammatory factors mediate this effect and on characterizing the mechanism by which inflammatory circuits associated with APS pathology intercept the coagulation pathway at the neutrophil-TF interface. Based on recent findings that have implicated complement C5aR and neutrophils in APS-induced fetal loss (9), we decided to test the hypothesis that complement acts on neutrophils through specific receptors to modulate TF biosynthesis and thus promote the TF-dependent procoagulant response of these cells. In addition, recent studies have implicated complement components C3 and C5 in APS-induced thrombophilia, and interception of complement activation has been shown to attenuate endothelial cell activation and prevent thrombus formation in a mouse APS-induced thrombophilia model (10). To date, however, the mechanism by which complement mediates its effect on APS-induced thrombosis and the identities of its potential target cells and receptors remain ill-defined. Furthermore, different mechanisms have been proposed to explain the hypercoagulability in APS, including up-regulation of TF in monocytes and endothelial cells (38, 45) or suppression of TF pathway inhibitor-dependent inhibition through antiphospholipid Abs (46, 47). Our *in vitro* results bridge previous findings and indicate for first time that APS Abs activate the complement system, leading to a complex interplay between C5a, C5aR, neutrophils, and TF.

It is our conviction that this newly identified cross-talk between complement and thrombosis may have important implications in the context of several inflammatory diseases that present a well-documented predisposition to thrombosis. This molecular cascade may also apply to other inflammatory syndromes that are associated with remote organ injury, such as sepsis and systemic inflammatory response syndrome. It is well known that during the course of sepsis, neutrophils become activated by C5a that is overproduced through complement activation in the periphery (3). It is therefore intriguing to speculate that a similar C5aR-TF cross-talk may affect the outcome of sepsis and predispose septic patients to

TF-dependent coagulation and disseminated thrombosis. Support for such an alternative comes from recent studies that have documented an increased expression of C5aR in a number of organs during the course of experimental sepsis (48). This increase in remote organ C5aR expression might critically affect the balance between pro- and anticoagulant proteins in a TF-dependent manner. Furthermore, cross-talk between the C5a/C5aR axis and the fibrinolytic system has been suggested by recent studies (49–51).

There are several lines of evidence supporting a role for C5a and its receptor, C5aR, in the coagulation process: Recent studies have implicated C5a in the procoagulant response associated with severe sepsis and remote organ injury in a rat cecal ligation and puncture model of sepsis (6). *In vivo* blockade using anti-C5a IgG results in amelioration of several fibrinolytic/coagulation protein changes occurring in sepsis. Furthermore, it has recently been shown that C5a induces the expression of plasminogen activator-inhibitor-1 in human mast cells and basophils, thereby affecting the balance between pro- and antifibrinolytic proteins (49). Similarly, cross-talk between the inflammatory and fibrinolytic systems has been suggested by recent studies in which urokinase-type plasminogen activator receptor activation has been shown to up-regulate C5aR expression in human mesangial cells (50). Taken together, these findings support a role for innate immunity, and particularly the C5a/C5aR signaling axis, in the regulation of the early molecular events leading to a disseminated procoagulant response during inflammation.

Thus, complement may modulate coagulation pathways in a TF-dependent or -independent manner by critically affecting the balance between activators and inhibitors of the coagulation cascade (e.g., TF pathway inhibitor or plasminogen activator inhibitors).

Our results can be summarized in the following model: Neutrophils are among the first effector cells to be rapidly mobilized to sites of inflammatory insult or tissue injury during the course of inflammation. They are thought to be a pivotal effector population in this process, given that they largely outnumber monocytes in the peripheral circulation. Neutrophils migrate to remote tissues via C5aR receptors expressed on their surfaces and in response to the potent chemotactic factor C5a that is generated upon complement activation. C5a plays a dual role in this process by 1) attracting neutrophils through chemotactic forces and 2) stimulating the expression of TF in these cells. C5a stimulation primes neutrophils and other cells (e.g., monocytes, endothelial cells) to initiate the extrinsic coagulation pathway and thereby promote TF-dependent thrombosis in remote, inflamed tissues. Pharmacological agents that inhibit TF production through complement inhibition at different levels may therefore offer a novel and attractive therapeutic approach to treating complement-associated thrombotic disorders.

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## Disclosures

J. D. Lambris, along with the University of Pennsylvania, have one issued and two pending patents on compstatin. These patents have been licensed for ophthalmic indications to Potentia Pharmaceuticals, Inc. J. D. Lambris is also a non-equity-holding member of the Scientific Advisory Board of Potentia Pharmaceuticals.

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