Neutrophil Activation by Anti–Proteinase 3 Antibodies in Wegener's Granulomatosis: Role of Exogenous Arachidonic Acid and Leukotriene B₄ Generation

By Friedrich Grimminger,* Katja Hattar,* Charis Papavassilis,*
Bettina Temmesfeld,* Elena Csernok,‡ Wolfgang Ludwig Gross,‡
Werner Seeger,* and Ulf Sibelius*

From the *Department of Internal Medicine, Justus-Liebig-University, Giessen D-35392, Germany;
and ‡Department of Rheumatology of the Medical University of Lübeck, D-23538 Germany

Summary

Among the anti–neutrophil cytoplasmic antibodies (ANCA), those targeting proteinase 3 (PR3) have a high specificity for Wegener's granulomatosis (WG). It is known that a preceding priming of neutrophils with cytokines is a prerequisite for membrane surface expression of PR3, which is then accessible to autoantibody binding. Employing a monoclonal antibody directed against human PR3 and ANCA-positive serum from WG patients with specificity for PR3, we now investigated the role of free arachidonic acid (AA) in autoantibody-related human neutrophil activation. Priming of neutrophils with tumor necrosis factor (TNF-α) for 15 min or exposure to anti-PR3 antibodies or incubation with free AA (10 μM) as sole events did not provoke superoxide generation, elastase secretion or generation of 5-lipoxygenase products of AA. Similarly, the combination of TNF-α-priming and AA incubation was ineffective. When TNF-α-primed neutrophils were stimulated by anti-PR3 antibodies, superoxide and elastase secretion was provoked in the absence of lipid mediator generation. However, when free AA was additionally provided, a strong activation of the 5-lipoxygenase pathway was demasked, with the appearance of excessive quantities of leukotriene (LT)B₄, LTA₄, and 5-hydroxyeicosatetraenoic acid. Moreover, superoxide and elastase secretion were markedly amplified, and studies with 5-lipoxygenase inhibitors and a LTB₄-antagonist demonstrated this was due to an LTB₄-related autocrine loop of cell activation. In contrast, the increased synthesis of platelet-activating factor in response to TNF-α-priming and anti-PR3 stimulation did not contribute to the amplification loop of neutrophil activation under the given conditions. We conclude that anti-PR3 antibodies are potent inductors of the 5-lipoxygenase pathway in primed human neutrophils, and extracellular free AA, as provided at an inflammatory focus, synergizes with the autoantibodies to evoke full-blown lipid mediator generation, granule secretion and respiratory burst. Such events may be enrolled in the pathogenesis of focal necrotizing vascular injury in Wegener's granulomatosis.

Despite its relatively low incidence, Wegener's granulomatosis (WG) represents a major challenge for many disciplines in clinical medicine, chiefly because it is difficult to recognize and full-blown cases have a poor prognosis. Anti–neutrophil cytoplasmic antibodies (ANCA), directed against granule proteins of polymorphonuclear granulocytes (PMN) and monocytes, have been described in association with WG and systemic vasculitis (1, 2). At least two classes of ANCA are distinguished by characteristic immunofluorescence patterns: the cytoplasmic (classic) c-ANCA and the perinuclear p-ANCA. Proteinase 3 (PR3) contained in azurophilic granules has been identified as the target antigen of c-ANCA staining (3, 4), while p-ANCA staining is brought about by anti-myeloperoxidase antibod-

ies (5, 6); the identification of additional autoantigenic ANCA targets is under current research (7).

The c-ANCA fluorescence pattern has a specificity for WG that is close to 95%, and the titer correlates well with disease activity (8, 9), thus suggesting that the related autoantibodies are not only seromarkers of WG, but do possess pathogenic significance. However, the mode in which c-ANCA stimulates PMN and evokes vascular injury is still elusive. It was shown that priming of neutrophils with cytokines, as occurring during episodes of infection or inflammatory disease, induces membrane surface expression of PR3, which is then accessible to autoantibody binding (10–13) with subsequent provocation of respiratory burst and PMN degranulation to a limited extent (6, 12, 14, 15).

Employing c-ANCA-positive serum from WG patients and a monoclonal antibody manufactured against human PR3 (MoAB-PR3), we now asked for the role of free arachidonic acid (AA) in ANCA-related neutrophil activation. Our interest in this precursor of eicosanoids stems from the fact that it is present in substantial quantities in the microenvironmental milieu of an inflammatory focus (16, 17), and may activate PMN by cell–cell contact with endothelial cells (18–20); moreover it was previously noted to amplify inflammatory ligand-evoked neutrophil activation (21–23). We report here, that the presence of AA demasks strong activation of the 5-lipoxygenase pathway in autoantibody-exposed human PMN, with generation of the potent chemoattractant leukotriene (LT)B$_4$ and, via triggering an autocrine loop of LTB$_4$-related cell activation, provocation of full-blown PMN mediator release. These findings provide new insights into the immunopathogenesis of the focal necrotizing vascular lesions characteristic for this enigmatic disease.

Materials and Methods

Preparation of Human PMN. Within a group of 30 healthy donors, 13 were noted to respond to ANCA-positive serum from WG patients in pilot experiments, and these were chosen as blood donors. EDTA-anticoagulated blood was centrifuged in a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient, erythrocytes were sedimented with polyvinyl alcohol (Merck-Schuchardt, Hohenbrunn, Germany) and residual erythrocytes were removed by hypotonic lysis as described previously (10). Cells were washed twice (150 g, 10 min, 4°C) and resuspended in phosphate-buffered saline (298 mM) with Ca^{2+} and Mg^{2+} (PBS) at a final concentration of 5 × 10^6/ml. Cell purity was >98% (Pappenheim staining) and cell viability >96% (trypan blue exclusion) throughout.

Analytical Procedures. LT and hydroxyeicosatetraenoic acids (HETE) were analyzed by HPLC techniques as previously described (24, 25). These include extraction from cell supernatants by octadecylsilyl solid phase extraction columns, RP-HPLC of methylated and non-methylated compounds and SP-HPLC of methylated compounds. LTA$_4$ was quantified by detection of its spontaneous degradation products (6-*trans*-diastereomeric pair of LTB$_4$ and 5,6-DiHETEs), summed as LTA$_4$ decay. Platelet-activating factor (PAF) release was determined by employing neutrophils prelabeled with 1-O-[^{3}H]alkyl-2-lyso-glycerophosphoryl-choline with subsequent SP-HPLC analysis of labeled PAF as described before (26). Superoxide anion generation was measured as superoxide dismutase inhibitable cytochrome c reduction, and elastase according to standard procedures (26).

Experimental Procedures. For priming, PMN were incubated with 0.5 ng/ml human recombinant TNF-α (Boehringer, Mannheim, Germany) for 15 min. Stimulation of the neutrophils was performed with 250 ng/ml MoAB-PR3 (monoclonal antibody to human proteinase 3, designated WGM2 (11), 250 ng/ml F(ab)'$_2$-MoAB (Fab fragment of the WGM2 monoclonal antibody; purity was checked by SDS-Page and was always higher than 95%) or 250 ng/ml murine control IgG1 (murine MoAB IgG, isotype control; Dranova, Hamburg, Germany), as well as 0.01, 0.1, and 1% vol/vol of IgG preparation three patients with PR3-ANCA-positive established Wegener's granulomatosis. Free AA was provided at a concentration of 10 μM AA, either as sole stimulus, or at different time intervals after antibody/serum admixture. Inhib-
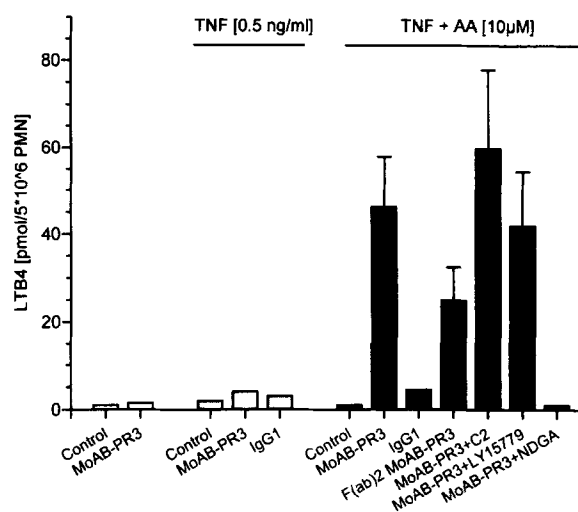

Figure 1. Influence of TNF priming, antibody stimulation and supply with free AA on neutrophil LTB$_4$ generation. 5 × 10^6 PMN were primed with TNF-α or sham-incubated for 15 min. Next, anti-proteinase 3 monoclonal antibody (MoAB), its F(ab)'$_2$ fragment, IgG1 or vehicle only (*Control*) was added. When indicated, free AA was additionally admixed 30 s later. Inhibitors and botulinum C2 toxin (C2) were co-applied with antibodies. Reactions were stopped after 15 min, and LTB$_4$ was quantified in the supernatant. Mean ± SEM of five independent experiments each are given. Experiments with MoAB-PR3, F(ab)'$_2$MoAB-PR3 and MoAB-PR3 + C2 significantly differed from control.

itors, applied simultaneously with the immunoglobulins when indicated, included the cyclooxygenase inhibitor acetylsalicylic acid (ASA, 100 μM; Bayer AG, Leverkusen, Germany), the LTB$_4$-antagonist LY15779 (10 μM, Eli Lilly & Company, Indianapolis, IN), the 5-lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 10 μM; Sigma Chem. Co., St. Louis, MO), the 5-lipoxygenase inhibitor AA-886 (10 μM; Dr. M. Nishikawa, Takeda Chemical Industries, Osaka, Japan) and the PAF-antagonist WEB 2086 (10 μM; Boehringer, Ingelheim, Germany). Botulinum C2 toxin, which is composed of a membrane translocation component (C$_{2II}$) and a component (C$_{2I}$) effecting ADP-ribosylation of non-muscle G-actin, thereby acting as a barbed end-capping protein and effecting selective loss of the non-muscle F-actin content (30), was graciously provided by K. Aktories (Department of Pharmacology, University of Freiburg, Freiburg, Germany). It was co-applied with antibodies at a concentration of 400 ng/ml C$_{2I}$ and 800 ng/ml C$_{2II}$. Reactions were stopped by centrifugation at 4°C for 5 min at 1200 g.

Statistics. For statistical comparison, one-way analysis of variance was performed. A level of P <0.05 was considered as to be significant.

Results

TNF-α priming of neutrophils per se did not provoke any significant lipid mediator generation, superoxide release or elastase secretion (Figs. 1–4). This was also true for the exposure of non-primed PMN to MoAB-PR3 and pooled ANCA-positive human serum. Incubation of TNF-primed neutrophils with the monoclonal antibody provoked the liberation of some very minor quantities of LTB$_4$, 5-HETE and LTA$_4$, accompanied by superoxide
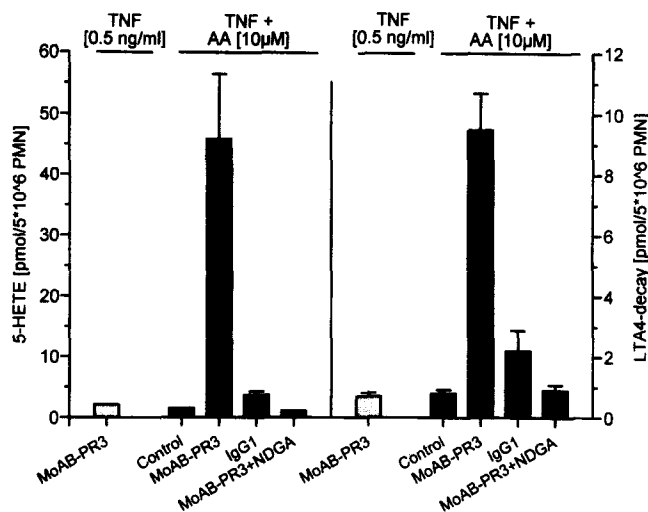
Figure 2. Influence of antibody stimulation and supply with free AA on neutrophil 5-HETE and LTA$_4$ release. 5 × 10^6 PMN were primed with TNF-α for 15 min, followed by the admixture of anti-proteinase 3 monoclonal antibody (MoAB), IgG1 or vehicle only (*Control*). When indicated, free AA was additionally admixed 30 s later. NDGA was co-applied with antibodies when indicated. Reactions were stopped after 15 min, and the 5 lipoxygenase products were quantified in the supernatant. Mean ± SEM of five independent experiments each are given. Experiments with MoAB-PR3 significantly differed from control.

Figure 3. Influence of TNF priming, antibody stimulation and supply with free AA on neutrophil superoxide generation. 5 ×10^6 PMN were primed with TNF-α or sham-incubated for 15 min. Next, anti-proteinase 3 monoclonal antibody (MoAB), its F(ab)'$_2$ fragment, IgG1 or vehicle only (*Control*) was added. When indicated, free AA was additionally admixed 30 s later. Inhibitors were co-applied with antibodies. Reactions were stopped after 15 min. Mean ± SEM of five independent experiments each are given. Experiments with MoAB-PR3, its F(ab)'$_2$ fragment and with MoAB-PR3 + LY15779 significantly differed from control.

generation and elastase secretion. In contrast, control IgG1 was totally ineffective.

Exposure of primed PMN to free AA did not enhance the liberation of leukotrienes, superoxide or elastase over baseline levels. However, when primed neutrophils were exposed to both MoAB-PR3 and free AA, a dramatic amplification of 5-lipoxygenase product synthesis ensued: LTB$_4$, LTA$_4$ and 5-HETE were all increased to >10-fold levels (10 µM AA) or even >25-fold levels (30 µM AA) as compared to the various control groups. This burst of lipid mediator release was not reproduced by control IgG1 and was fully suppressed by NDGA. As displayed for LTB$_4$ (Fig. 1), the F(ab)'2 fragment of MoAB-PR3 was approximately half as effective as the complete antibody, and co-exposure of the cells to botulinum C2 toxin and the LTB$_4$ antagonist LY15779 did not affect the LT release. In the presence of 1% homologous serum to provide complement factors, the TNF plus MoAB-PR3 plus AA-elicited 5-lipoxygenase product formation was not changed (data not given).

Supply with free AA also markedly increased the superoxide and elastase release from TNF-primed PMN in response to MoAB-PR3, which was not reproduced by control IgG1. Again, the F(ab)'$_2$ fragment of MoAB-PR3 was approximately half as effective as the complete antibody. 5-lipoxygenase inhibition with NDGA and AA861 (used in cases of superoxide detection, since NDGA interferes with this assay), and the presence of the LTB$_4$-antagonist LY15779 suppressed the AA-related increase in these secretory responses. ASA, in contrast, was ineffective.

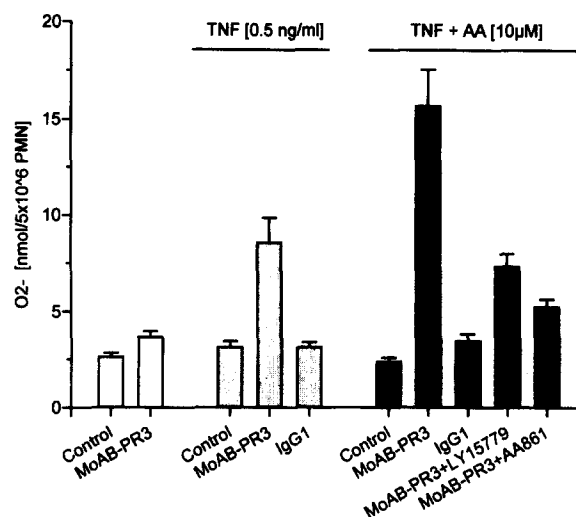The enhancing effect of free AA on MoAB-PR3-elicited secretory responses in TNF-primed PMN displayed

distinct time dependency, as shown in Fig. 5. The highest efficacy was noted for a lag time of 30 s between antibody admixture and AA supply, which was therefore used in the routine protocol.
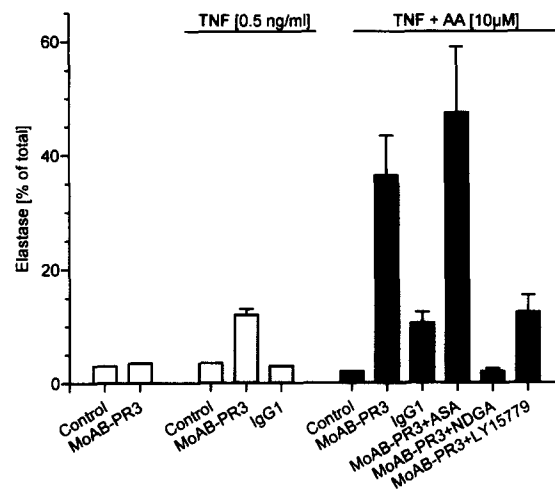

Figure 4. Influence of TNF priming, antibody stimulation and supply with free AA on neutrophil elastase secretion. 5 × 10^6 PMN were primed with TNF-α or sham-incubated for 15 min. Next, anti-proteinase 3 monoclonal antibody (MoAB), its F(ab)'$_2$ fragment, IgG1 or vehicle only (*Control*) was added. When indicated, free AA was additionally admixed 30 s later. Inhibitors were co-applied with antibodies. Reactions were stopped after 15 min, and elastase was quantified in the supernatant. Mean ± SEM of five independent experiments each are given. Experiments with MoAB-PR3, its F(ab)'$_2$ fragment, MoAB-PR3 + ASA and MoAB-PR3 + LY15779 significantly differed from control.
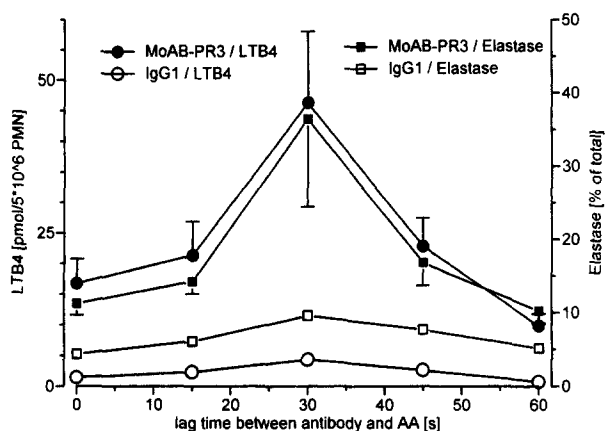
Figure 5. Influence of the lag time between antibody stimulation and AA supply on neutrophil LTB_4 and elastase secretion. 5×10^6 PMN were primed with TNF-α for 15 min. Next, anti-proteinase 3 monoclonal antibody (MoAB) or IgG1 was added. Free AA was co-applied with antibody or admixed at different time intervals up to 60 s. Reactions were stopped after 15 min, and the secretion products were analyzed in the supernatant. Mean ± SEM of five independent experiments each are given. Experiments with MoAB-PR3 significantly differed from control.

Supply with free AA also markedly enhanced the secretory responses provoked in TNF-primed neutrophils by ANCA-positive pooled serum. For the highest titer currently used (0.1% serum, vol/vol), the release of lipid mediators and elastase approached 60% of the maximum secretory responses elicited by MoAB-PR3 under these conditions.

TNF-priming of neutrophils did not augment the baseline PAF release by these cells. Subsequent exposure to MoAB-PR3 enhanced the liberation of this lipid mediator to 35 ± 5 ng/5 $\times$ 10^6 PMN (10 min incubation time), whereas control IgG1 was ineffective (3.4 ± 1.3 ng/5 $\times$ 10^6 PMN). Co-supply with free AA did not further augment the MoAb-PR3-related PAF release. The provocation of LT generation as well as superoxide and elastase release by TNF plus MoAB-PR3 plus AA were not suppressed in the presence of the PAF antagonist WEB 2086 (data not shown).
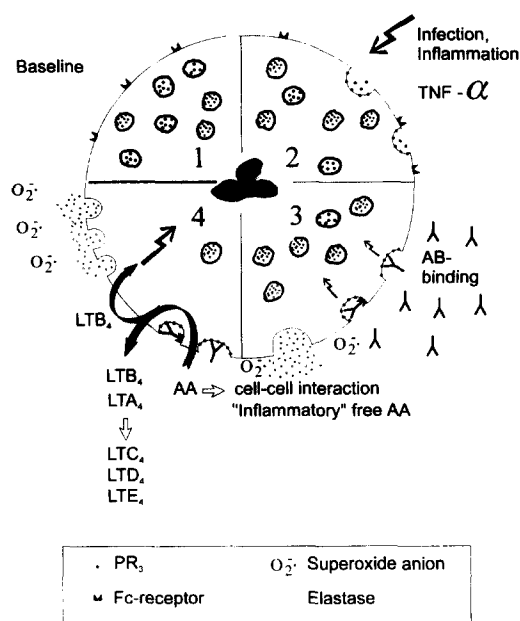

Figure 6. Postulated sequence of ANCA-induced PMN activation-role of cytokine priming and exogenous free AA supply. (1) Quiescent PMN without PR3-membrane expression. (2) Cytokine-induced membrane expression of PR3. (3) ANCA-binding to PR3 target antigen with possible antigen-Fc-receptor bridging. (4) Activation of intercellular leukotriene synthesis in the presence of free AA (liberated from inflammatory tissue). LTB_4-mediated autocrine amplification of inflammatory PMN-responses. Shift of the PMN-derived leukotriene intermediate LTA_4 to adjacent endothelial cells with transcellular synthesis of vasoactive cysteinyl LTs (LTC_4, LTD_4, LTE_4).

Discussion

It has previously been shown that the priming of human neutrophils with cytokines such as TNF-α induces a translocation of PR3 from the intragranular loci to the cell surface (10–13), notwithstanding the question whether this proceeds only via membrane fusion or requires additional receptor binding of PR3 on the neutrophil plasma membrane. Binding of the autoantibodies to these targets then evidently triggers cell signaling events, and the presently

Table 1. Neutrophil Stimulation by ANCA-positive Pooled Human Serum-impact of Free AA*

ANCA-Serum		LTB_4	LTA_4	5-HETE	Elastase
		pmol/5* 10^6 PMN	pmol/5* 10^6 PMN	pmol/5* 10^6 PMN	% of total
0.01%	+AA	12.37 ± 0.79	7.71 ± 0.76	23.87 ± 1.91	16.37 ± 1.49
	−AA	1.13 ± 0.09	0.83 ± 0.06	1.37 ± 0.29	5.73 ± 0.57
0.1%	+AA	18.45 ± 0.37	10.95 ± 1.29	32.43 ± 2.45	23.58 ± 0.29
	−AA	1.43 ± 0.09	0.93 ± 0.06	1.57 ± 0.09	9.03 ± 0.96

*5 $\times$ 10^6 PMN were primed with TNF-α for 15 min, different concentrations of ANCA-positive pooled human serum were admixed, and free AA was additionally added 30 s later when indicated. Metabolite release after 15 min reaction time is given (mean ± SEM of five independent experiments each).

observed superoxide generation and elastase release support previous studies in this field (6, 12, 14, 15). There is controversy whether the signaling is directly related to the binding of the F(ab)' domain, or additionally or even exclusively proceeds via antibody binding to co-expressed Fc-receptors (14, 15, 28, 29); the present data with F(ab)'$_2$ fragments support the view that both antibody domains may be involved. The subsequent signal transduction pathway is largely unsettled; phenomena of antibody and receptor internalization are apparently not enrolled, as disrupture of the neutrophil F-actin content with botulinum C2-toxin (30) did not suppress the PMN mediator generation. The current findings do, however, strongly support the notion that activation of the PMN 5-lipoxygenase pathway is an important feature in the cascade of events. As similarly noted for neutrophil stimulation by inflammatory ligands (21–23), this may occur without major stimulation of neutrophil phospholipases, thus explaining the very low quantities of Lts and HETEs in cytokine plus antibody-exposed PMN in the absence of exogenous AA. Extracellular supply with this fatty acid may then compensate for the restricted availability of endogenous precursor to be readily metabolized to 5-lipoxygenase products. The finding of an optimal interval between antibody admixture and exogenous AA supply of ~30 s may be explained by the fact that even quiescent neutrophils display rapid uptake of exogenously offered free fatty acids (31, 32) with subsequent incorporation into different phospholipid species, which results in rapidly decreasing precursor availability for the 5-lipoxygenase pathway if the exogenous AA is offered "too early" (33, 34).

The total amounts of 5-lipoxygenase products elicited by the monoclonal antibodies to PR3 and the ANCA-positive serum in the presence of free AA approached the quantities elicited by the calcium ionophore A23187, the most potent stimulus for PMN lipoxygenase metabolism hitherto described (22, 23). LTB$_4$ and 5-HETE are well-known chemoattractants, capable of recruiting additional neutro-

phils to an inflammatory focus (35). The PMN-derived LTA$_4$ has been shown to be shifted to adjacent endothelial cells, with transcellular synthesis of the pro-inflammatory cysteinyl Lts by use of the enzymatic equipment of this acceptor cell type (18, 24, 36, 37). Moreover, the present data demonstrate that the arising LTB$_4$ serves as potent autocrine amplification loop for full-blown activation of autoantibody-exposed neutrophils: both the superoxide release and the elastase secretion were markedly increased in the presence of AA, and this increase was completely blocked by different 5-lipoxygenase inhibitors and a specific LTB$_4$ receptor antagonist. Such an autocrine function of LTB$_4$ is well in line with the previously characterized efficacy profile of this autacoid in human neutrophils (35). In contrast, the second autoantibody-evoked autacoid, PAF, did evidently not substantially contribute to the autocrine loop of neutrophil activation under the present experimental conditions, as deduced from the inefficacy of the PAF antagonist WEB 2086. We did not address the question, whether some suppression of PAF-binding to ANCA-exposed neutrophils, as previously suggested (29), might contribute to this finding.

We conclude that, in addition to their direct prosecretory effect in PMN, anti-PR3 antibodies are potent inductors of human neutrophil 5-lipoxygenase metabolism, with the arising LTB$_4$ serving as autocrine amplification loop. These observations suggest a "three hit" scenario: when the presence of autoantibodies is supplemented with cytokine priming of circulating neutrophils and the presence of free AA at an inflammatory focus, a vicious cycle of focal accumulation of neutrophils, full-blown activation of these cells and further PMN recruitment may ensue, which could then cause the severe necrotizing vascular injury known to be the hallmark of WG. The current findings thus further support and substantially extend the concept that c-ANCAs represent not only the best seromarker for WG so far available, but are centrally involved in the pathogenetic sequelae of this disease.

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