Neutrophil extracellular traps formation in patients with eosinophilic granulomatosis with polyangiitis: association with eosinophilic inflammation

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ABSTRACT

Objective. Eosinophilic granulomatosis with polyangiitis (EGPA) is associated with an inflammation and the presence of antineutrophil cytoplasmic antibodies (ANCA). Thus, we investigated the impact of ANCAs and eosinophilic inflammation on neutrophil activation and extracellular traps (NETs) formation.

Methods. We recruited 29 patients in the remission of EGPA (17 ANCA-negative and 12 ANCA-positive, including 7 p-ANCA-positive and 5 c-ANCA-positive patients). Healthy donors’ neutrophils were stimulated with EGPA patients’ serum, NETs formation was assessed by immunofluorescence and scanning electron microscopy.

Results. EGPA patients presented enhanced ability to generate NETs compared to healthy subjects (20.3±8.2% vs. 2.7±1.5%, p=0.0036). However, there were no differences in NETs formation between ANCA-positive and ANCA-negative patients (23±11.2% vs. 17±6.1%, p=0.15). There was also no correlation between NETs generation and the amount of circulating DNA in EGPA patients. Among ANCA-positive patients, p-ANCA-positives showed the highest percentage of NETs as compared to cANCA-positive and ANCA-negative patients (27.3±10.3% vs. 17.8±10.5% and vs. 17±6.1%, both p<0.01, respectively). Eosinophil number correlated with the percentage of NETs in the whole EGPA group (r=0.53, p=0.039), but we failed to observe the correlation with an eosinophil cationic protein (r=0.49, p=0.058).

Conclusion. EGPA patients’ serum has the ability to induce NETosis with no regard to the ANCA status in contrast to other vasculitides, where p-ANCA were considered as the main factor. Interestingly, NETs formation in EGPA patients connected with the number of eosinophils might be of major relevance. Further studies are required to assess which eosinophil-derived factors might be responsible for the neutrophils activation in EGPA patients.

Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA), formerly named Churg-Strauss syndrome, is a rare systemic disease characterised by necrotic vasculitis involving small and medium blood vessels accompanied by eosinophilia in the peripheral blood, eosinophilic infiltration of the tissues and the presence of bronchial asthma (1, 2). The prevalence of EGPA ranges from 10-13 cases per 1 million individuals in the general population, depending on their geographical location and the classification criteria applied (3-6). Among patients with asthma the EGPA incidence is higher and is estimated from 34.6 (7) to 64.4 (4) cases per 1 million patient-years.

The current evidence indicates that EGPA develops following an inflammatory response directed at target antigens and a number of factors, including infectious agents (8), drugs (9), allergic hyposensitisations or vaccinations (10).

The most common laboratory abnormalities found in patients with EGPA are blood hypereosinophilia (8, 9) and high immunoglobulin E (IgE) titres that however, are non-specific and usually absent during therapy with corticosteroids (11). Another feature of EGPA is the presence of antineutrophil cytoplasmic antibodies (ANCA) among 30-40% of the patients (12, 13). ANCA-positive patients differ from those without circulating ANCA. In the former group mononeuritis multiplex is more frequent while glomerular nephritis and cardiomyopathy are less commonly observed.
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(9, 13). ANCA, when present, represent predominantly p-ANCA of anti-myeloperoxidase (MPO) specificity, but c-ANCA of anti-proteinase 3 (PR3) specificity can also be present (9). It is considered that pathogenic ANCA can bind to the cell surface MPO of the proinflammatory cytokine-primed neutrophils, which leads to excessive activation of neutrophils (14). It remains unclear why neutrophil-specific proteins are targeted in EGPA and other systemic autoimmune diseases. It has been suggested that this phenomenon is related to the neutrophil death at sites of inflammation where the exposure of normally sequestered antigens to the immune system may occur. There is a growing evidence that ANCA-activated neutrophils undergo degranulation and release neutrophil extracellular traps (NETs) (15-19). NET formation, which requires neutrophil death termed netosis, represents an unique, innate immune defence mechanism characterised by the destruction of the nucleus with active release of chromatin fibres decorated with histones and a variety of anti-microbial granular proteins, including MPO and PR3, (20) to bind, disarm and kill microbes. Although NETs are bactericidal, excessive NET formation as a result of non-infectious inflammatory processes, as well as a lack of DNAses to degrade them have been linked to the pathogenesis of inflammatory and autoimmune diseases such as lupus erythematosus (21, 22), rheumatoid arthritis (23) and microscopic polyangiitis (MPA) (16-19).

Recent findings have demonstrated a marked ability of serum to induce NETs in patients with MPO-ANCA associated MPA (19, 24). In the present study we sought to investigate the ability of serum derived from the EGPA patients, both ANCA-positive and ANCA-negative, to induce the NETs formation, reflecting its potential engagement in the disease.

Materials and methods

Patients

We enrolled 29 consecutive patients with EGPA on remission. EGPA was diagnosed according to the 1990 American College of Rheumatology (ACR) classification criteria (25). The patients were eligible if they met the following criteria: age over 18 years, and clinically stable disease according to the Birmingham Vasculitis Activity Score version 3 (BVAS v. 3) (25, 26). The exclusion criteria were: any signs of acute illness, known cancer, hepatic or renal dysfunction, and current anticoagulant therapy. The most frequent therapy used was oral glucocorticosteroids (n=27), while 18 subjects received immunosuppressive drugs, including cyclophosphamide (n=7, 20.5%), methotrexate (n=7, 20.5%), or azathioprine (n=4, 12%), all three in monotherapy. Peripheral blood neutrophils were obtained from 10 young healthy volunteers recruited from hospital personnel and acquaintances. The Bioethics Committee of the Jagiellonian University approved the study protocol and informed written consent was obtained from all the participants.

Laboratory investigations

After an overnight fast, venous blood was collected to determine blood cell count, glucose, and creatinine using routine laboratory assays. Blood was drawn from the antecubital vein into serum tubes (Sarstedt, Numbrecht, Germany). To determine absolute peripheral blood eosinophil counts, a peripheral blood eosinophil count, glucose, and creatinine using routine laboratory assays. Blood was drawn from the antecubital vein into serum tubes (Sarstedt, Numbrecht, Germany). To determine absolute peripheral blood eosinophil counts, a manual method and a Bürker’s chamber were used.

ANCA were determined using an indirect immunofluorescence method (Euroimmun, Lübeck, Germany) according to manufacturer’s protocol. Briefly, reagent slides were incubated with EGPA patients’ serum diluted at 1:10 for 30 minutes, washed, and followed by an incubation with secondary fluorescein-labeled IgG. All specimens were assessed independently by 2 technicians. The end-point titre was defined as the highest dilution at which the ANCA staining pattern was visible. Commercially available immunoenzymatic assay was used to determine plasma interleukin 6 (IL-6) (R&D Systems, Indianapolis, USA). Serum eosinophil cationic protein (ECP) was measured using a commercial fluoroimmunoassay kit (PharmaciaECP UniCap System FEIA; Pharmacia Diagnostics, Uppsala, Sweden), with a detection limit of 2 µg/L. Technicians unaware of the sample status performed the tests. Intra- and interassay coefficients of variation ranged from 5% to 8%.

Isolation of human neutrophils

Neutrophils were isolated from peripheral blood drawn from healthy donors by Polymorphprep gradient centrifugation and dextran sedimentation, as described previously (27). Briefly, 5 ml of whole blood containing ethylenediamine tetraacetic acid (EDTA) was layered on 5 ml of Polymorphprep (Gentaur, Brussels, Belgium). After 35 minutes centrifugation at 500 xg, the neutrophils were separated from the polymorphonuclear neutrophils-rich pellet. The residual erythrocytes were then eliminated via red blood cell lysis. Neutrophils were washed with physiological saline, spun down (10 minutes, 400 xg) and resuspended in 2 ml of RPMI medium (phenol red-free) supplemented with 10 mM Hepes. Post cell counts were performed using May-Grunwald Giemsa (Sigma-Aldrich, MO, United States) counterstaining and trypan blue (Sigma-Aldrich, MO, United States) white blood cells viability analysis was performed. Neutrophil purity after isolation was at least 94% and the mean cell viability was 99%.

Stimulation of NETs formation

The resuspended neutrophils were seeded into 24-well culture plates with glass cover slips pretreated with 0.001% polylysine in a final concentration of 2×10⁵ per well (28). Incubations were performed at 37°C in the presence of 5% CO₂ for 30 minutes to adhere and followed by 180 minutes incubation with EGPA serum in a final ratio 2:1 (29) or with phosphor 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) in a final concentration 600 nM (28) to serve as a positive control. Neutrophils treated with serum derived from healthy donors served as a negative control.
Detection of NETs

• Immunofluorescence assays

After appropriate incubation neutrophils were fixed with 4% paraformaldehyde (28), endogenous peroxidase activity was blocked with 3% H2O2 in methanol (15 min) at room temperature (RT) and unspecific background was blocked with 3% bovine albumin (Sigma Co, St. Louis, MO, US) for 30 min at RT. Then, slides were incubated with anti-2H2AB-DNA complex antibodies (HIST2H2AB, US Biological, Salem, MA, USA) conjugated with fluorescein isothiocyanate (FITC) diluted in blocking serum at final concentration 1:500 for 60 min at RT. DNA was stained with DAPI (Invitrogen, Life Technologies, Grand Island, NY, USA).

NETs quantification was performed as previously described (30). Briefly, the percentage of neutrophils releasing NETs was quantified in blinded samples by evaluating neutrophils displaying expanded nuclei and releasing DNA fibres in at least 10 random microscope fields. The NET percentage was calculated as 100x number of netting neutrophils/total number of neutrophils. The images were analysed with Olympus BX43 (Olympus America Inc., Center Valley, PA, USA) microscope and the ImageJ software v. 1.48.

• MPO ELISA

MPO was measured in supernatants derived from the in vitro stimulated-neutrophiles. Ten microliters of the supernatant was tested for MPO presence in each culture well. Using anti-MPO IgG ELISA (Euroimmun, Lübeck, Germany) according to the producer’s instruction. Results were calculated as a ratio = optical density (OD) of the sample / OD of the calibrator.

• Scanning electron microscopy (SEM)

After appropriate incubation cells were fixed in 2.5% glutaraldehyde for 2 h at 4°C, then slides were washed three times with PBS, dehydrated using a graded ethanol followed by a tert-butyl alcohol series, and then dried in a critical point dryer. The specimen surface was coated with a gold layer using a thin layer evaporator and analysed with JEOL JCM-6000 (JEOL Ltd., Tokyo, Japan) microscope.

Circulating DNA measurement

Free DNA circulating in EGPA patients and healthy controls (n=12) was assessed in serum samples as previously described (31) using the Quant-iT™ Pico Green Assay (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instruction. The fluorescence of the samples was read on the fluorescence plate reader Fluoroscan Ascent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were excited at 485 nm and fluorescence intensity was measured at 520 nm with a detection limit of 25 pg/ml. Intra- and interassay coefficients of variation ranged from 4% to 7%.

Statistical analysis

Data are expressed as numbers (percentage) for categorical variables and mean ± SD or median (lower-upper quartile) for continuous variables (normally or non-normally distributed, respectively). The Shapiro-Wilk test was used to assess normality. Categorical variables were compared using the Pearson’s chi-squared test or Fischer’s exact test as appropriate. The two groups were compared using t-test for independent samples or the Mann-Whitney test, as appropriate. The Pearson coefficient was computed to study simple correlations. All tests were two-sided and p-values of <0.05 were considered statistically significant. Statistical analyses were performed using Statssoft 10.0 PL package (StatSoft, Inc.).

Results

A total of 29 patients with EGPA were included in the final analysis in which 17 (59%) patients were ANCA-negative and 12 (41%) were ANCA-positive (among them 7 patients represented p-ANCA and 5 patients cANCA-positive specifically). The median disease duration was 3 years (IQR, 5 months to 12 years). Clinical data are summarised in Table I. Additionally, p-ANCA-positive patients were characterised by higher eosinophil count and IgE levels when compared with c-ANCA-positive patients (437 [6–3304] vs. 378 [0–872], \(p=0.045\) and 40 [19–352] vs. 23 [7–47] IU/ml, \(p=0.042\), respectively).

Table I. Characteristics of EGPA subjects regarding ANCA status.

<table>
<thead>
<tr>
<th>Variable</th>
<th>ANCA-negative (n=17)</th>
<th>ANCA-positive (n=12)</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>47.83 ± 12.02</td>
<td>45.91 ± 8.43</td>
<td>0.9</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>11 (64.7)</td>
<td>9 (75)</td>
<td>0.7</td>
</tr>
<tr>
<td>Laboratory parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.79 ± 1.35</td>
<td>4.35 ± 1.18</td>
<td>0.9</td>
</tr>
<tr>
<td>Creatinine, μmol/l</td>
<td>61.27 ± 20.76</td>
<td>69 ± 22.72</td>
<td>0.9</td>
</tr>
<tr>
<td>WBC, x10⁹/μl</td>
<td>7.68 ± 2.73</td>
<td>7.74 ± 3.90</td>
<td>0.8</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>75.7 ± 13.6</td>
<td>62.7 ± 12.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Eosinophils, μL⁻¹</td>
<td>0.205 (0.151)</td>
<td>0.378 (0.3304)</td>
<td>0.045</td>
</tr>
<tr>
<td>IgE, IU/ml</td>
<td>19.9 (18.7-269)</td>
<td>35.9 (18.7-352)</td>
<td>0.043</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.06 (0.15-6.51)</td>
<td>2.5 (0.25-8.67)</td>
<td>0.052</td>
</tr>
<tr>
<td>ECP, μg/L</td>
<td>70.9 ± 23.5</td>
<td>80.3 ± 31.3</td>
<td>0.7</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>1.27 (0.86–2.04)</td>
<td>1.41 (073–2.35)</td>
<td>0.6</td>
</tr>
<tr>
<td>pANCA, n (%)</td>
<td>-</td>
<td>7 (38.33)</td>
<td>-</td>
</tr>
<tr>
<td>cANCA, n (%)</td>
<td>-</td>
<td>5 (41.6)</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are given as number (%), mean ± SD or median (lower-upper quartile).

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NETosis formation did not correlate with the titre of p-ANCA or c-ANCA antibodies, however the highest value of netosis (47%) was observed in one individual patient with the highest p-ANCA titre (1:160). However netosis intensity was positively correlated with the eosinophil numbers ($r=0.53$, $p=0.039$). The amounts of circulating DNA, measured in serum of EGPA patients, were higher in p-ANCA-positive subjects as compared to c-ANCA-positive as well as ANCA-negative patients ($2.84±0.2$ $\mu$g/ml vs. $2.01±0.29$ $\mu$g/ml, $p=0.04$ and $2.84±0.2$ $\mu$g/ml vs. $1.98±0.13$ $\mu$g/ml, $p=0.038$, respectively; Fig. 1C). Of note, healthy subjects showed the lowest concentrations of circulating DNA, even if DNA levels were compared with ANCA-negative patients (0.143±0.018 vs. $1.98±0.13$ $\mu$g/ml, $p<0.0001$). However, the percentage of in vitro NET releasing cells did not correlate with the amount of in vivo circulating DNA in any of examined subgroups. Similar order was observed between the percentage of NETs releasing neutrophils and IgE level, which correlated only in p-ANCA-positive patients ($r=0.41$, $p=0.018$) but not in c-ANCA-positive or ANCA-negative patients ($r=0.39$, $p=0.083$; $r=0.45$, $p=0.103$, respectively). Serum CRP level correlated with the percentage of NET releasing cells in the whole EGPA group ($r=0.57$, $p=0.046$). There were no significant correlations between the percentage of netting neutrophils and either IL-6 or ECP levels and other laboratory parameters or medication used (data not shown).

**Discussion**

Although the ability of p-ANCA to induce the NETs formation in vasculitis has been reported previously (19, 24), to our knowledge, this study is the first to show that the intensity of NETosis in EGPA patients is related to eosinophil number. Interestingly, we demonstrated that in patients with EGPA the ability to generate NETs is present not only in subjects positive for c-ANCA but also for p-ANCA patients. So far, only PR3 and PR3-ANCA induced enhancement of inflammation has been demonstrated in an animal model (32, 33). Surprisingly, we observed the formation of NETs in ANCA-negative patients as well. In our opinion, the ability of the EGPA serum to induce NETs formation regardless of the ANCA status might be of major importance, suggesting that in EGPA patients netosis is related rather to eosinophilic inflammation than to the presence of ANCA like in other vasculitides (19, 24). Especially, the correlation between eosinophil number and NETosis intensity was observed. As the pathology of EGPA includes not only ANCA-related vasculitis, but mainly eosinophil-rich granulomatous inflammation. It could be speculated that eosinophil-derived factors such as ECP, major basic protein (MBP), eosinophil peroxidase (EPO), and lysophospholipase, which determine a pro-inflammatory environment and are implicated in the vascular damage (34) could be responsible for activation of NETs formation in EGPA patients. We failed to show that ECP correlates with netosis intensity. However, the other proteins, especially MBP, which induces release of interleukin-8, the main neutrophils activator (35) and NET formation (20), might potentially be implicated in the NET generation. Moreover, it is known that MBP contributes to the pathophysiology of acute asthma (35). In EGPA patients the intensity of NETs formation is correlated with inflammation reflected by the level of circulating CRP. In our opinion CRP itself does not contribute to the process of NET formation, as we observed using high level CRP serum from patients without EGPA (data not shown). The correlation of CRP with netosis rate suggests the important role of the inflammatory status in EGPA patients. As shown by Tadie et al., the level of HMGB-1 (high mobility group box 1), which is closely correlated with circulating CRP (36) increases the NET formation in vitro in ANCA associated vasculitis patients (36, 37). However, it seems that in EGPA patients, factors other than ANCA are able to induce NETs as well. In our opinion the lack of the correlation between DNA concentration and NETosis intensity is not surprising since the stimulation of netosis was performed in vitro, whereas
the level of free DNA was measured in the EGPA patients' serum where the free DNA might be not only neutrophils-derived but also could come from eosinophil traps (ETosis) (38), mast cells or monocytes and macrophages which were also shown to release extracellular traps, but to a lesser extent when compared to neutrophils (39, 40). Moreover, in EGPA patients resulting eosinophils activation epithelial damage-derived DNA should also be included to the pool of free DNA. Further studies with EGPA serums depleted of ANCA IgG are needed to validate this hypothesis.

Study limitations
This study has several limitations. First, the number of subjects is low, especially in the subgroups regarding the ANCA status, however EGPA is a rare disease. Second, the stimulation of neutrophils was performed with serum. On one hand this “global model” does not allow to estimate precisely which single factor is involved in NET generation. On the other hand, this model enabled to assess the role of the other factors present in the patients’ sera, including proinflammatory cytokines, and eosinophilic proteins that might contribute to NET formation. It would be also of major importance to examine neutrophils stimulation regarding the G-protein coupled receptors CXCR1, CXCR2. In generally, studies of NET formation are difficult to design. Third, serum contained DNases, which might diminish the amount of NETs. To our knowledge, there have been no reports showing differences in this enzyme activity in EGPA compared with other vasculitides. The issue of a potential impact of DNases was beyond the scope of the current study.

Conclusions
In conclusion, we showed that serum obtained from the EGPA patients has the ability to induce NETs formation regardless of the ANCA status, which might be associated with the unique eosinophil activation in vivo in contrast to other vasculitides, where it seems that the main factor determining NETs formation is the presence of p-ANCA. In our opinion eosinophilic-derived proteins can be involved in NETosis induction or eosinophils itselfs can undergo so called ETosis triggering NETosis, which can enhance inflammation in a vicious circle and might contribute to thromboembolic complications (41). The important question remains to be answered of which eosinophil-derived factors might be responsible for the neutrophils activation.

Acknowledgement
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References
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