Expression of priming-associated cellular markers on neutrophils during an exacerbation of COPD

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\textbf{Summary} Chronic inflammation of the airways is a hallmark of chronic obstructive pulmonary disease (COPD). We investigated the kinetics of priming of inflammatory cells in peripheral blood during exacerbations of COPD and during the resolution phase.

Modulation of the leukocyte compartment as a consequence of systemic activation by cytokines/chemokines was determined by measuring the expression of priming-associated epitopes by novel antibodies designated A17 and A27. Furthermore, $\text{H}_2\text{O}_2$ was determined in breath condensate as a read out for local inflammation. Leukocytes were obtained from COPD patients (GOLD II–IV) during and after an exacerbation of their disease.

During an exacerbation the expression of priming epitopes on leukocytes was increased. This priming phenotype disappeared upon treatment with intravenous corticosteroids. Similarly, $\text{H}_2\text{O}_2$ levels in breath condensate were also increased during an exacerbation and decreased upon treatment.

We conclude that the activation status of neutrophils in the systemic compartment can be used as a read-out for systemic innate immune signals involved in the pathogenesis of COPD. The correlation between $\text{H}_2\text{O}_2$ in exhaled air with A27 priming on neutrophils showed that local inflammation has systemic effects on cells of the innate immune system.

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Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a disease state characterized by an irreversible, usually progressive airflow limitation. Central in the pathogenesis of COPD is the inflammatory reaction found in the airways and the alveolar structures, which leads to persistent injury. The clinical course of COPD is often accompanied by recurrent exacerbations, which contribute to a more rapid decline in lung function and health status. Most prominent in the disease process is the ongoing inflammation with an increase of neutrophils, monocytes and T lymphocytes in the lung parenchyma.

An increase of cytokines and chemokines is often observed in sputum and bronchoalvolar lavage studies of COPD patients which are clear indicators for the inflammatory process. These cytokines/chemokines are involved in modulation of leukocyte responses in vivo. The unique mix of pro-and anti-inflammatory mediators will determine whether leukocytes are preactivated (primed) or inhibited functionally. Preactivation or priming is a prerequisite for homing to and optimal activation of granulocytes in the tissues and can be monitored by recently developed phage antibodies A17 and A27. Despite the fact that these antibodies recognize epitopes highly expressed on primed myeloid leukocytes the identity of these priming structures remains to be elucidated. A systemic increase in pro-inflammatory cytokines and chemokines as well as an increased expression of their receptors on the leukocytes are likely to lead to an increased number of systemically primed neutrophils. This will facilitate the margination, extravasation and accumulation of inflammatory cells into the tissues.

Therefore, only 'partially' primed cells can be found in the peripheral compartment. Leukocytes obtained from bronchoalveolar lavage (BAL) fluid have an increased expression of priming markers compared to the expression in the peripheral blood. Insight into the mechanisms of priming of peripheral blood leukocytes of patients with COPD will contribute to the understanding of the kinetics of the systemic inflammatory processes, which are associated with the disease. In addition, priming of leukocytes can be used as read-out for the in vivo action of pro- and anti-inflammatory cytokines.

We performed a study regarding the priming status of neutrophils during an exacerbation of COPD and during resolution of this clinical condition. To determine whether inflammation-associated priming of neutrophils in the peripheral blood was accompanied by a change in a tissue associated inflammatory marker, H$_2$O$_2$ in breath condensate was measured. We will show that systemic neutrophil preactivation is increased during an exacerbation of COPD and that local inflammation is inducing an activation of the systemic innate immune system.

Materials, methods and patients

Reagents

Recombinant human GM-CSF was from Genzyme (Boston, MA, USA). Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). RPMI 1640 medium with Glutamax was purchased from Life Technologies (Breda, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden).

Study population and design

Ten patients with unstable moderate to very severe COPD were selected from our outpatient clinic population (Department of Pulmonary Diseases, Heart & Lung Center Utrecht, St. Antonius Hospital Nieuwegein) when they suffered from a severe exacerbation requiring hospitalization. The patients had to have a smoking history of at least 10 pack years and they fulfilled the criteria for the diagnosis of moderate to very severe COPD according to the GOLD guidelines. At the time of hospitalization they suffered from two or all three of the following symptoms: an increase from baseline of sputum production, sputum purulence or shortness of breath, and they were unresponsive to outpatient therapy. The exacerbations were not life-threatening and did not need intensive care unit management. Patients were allowed to use glucocorticosteroids (GCS) and bronchodilators at admission. Patients with uncontrolled severe diseases other than COPD contributing to the deterioration were excluded. At inclusion before the start of treatment, at the third or fourth day and at day 7 of the treatment period a lung function was performed, the Borg score was reported by the patient (0 = no dyspnea to 10 = maximum dyspnea), exhaled breath condensate was collected and blood samples were drawn. The subjects were treated according to the ERS guidelines on COPD. In short, GCS (Di-Adreson-F aquosum) was administered intravenously, 50 mg 24 h$^{-1}$ which was tapered to 25 mg 24 h$^{-1}$ after 3–4 days. Oxygen given through a nasal canula was titrated to reach a P$\mathrm{aO}_2$...
of 8.0 kPa or more, without the occurrence of hypercapnia. Physiotherapy was applied to improve clearance of secretions and to control breathing patterns. Current smokers refrained from smoking during admission. All subjects gave written informed consent to be included in the study, which was approved by the local Ethics Committee.

**Procedure for staining phagocytes with FITC-labeled MoPhabs A17, A27 and other activation markers**

Two human monoclonal phage antibodies were isolated from a synthetic bacteriophage antibody library. These phage antibodies recognize epitopes that are upregulated on neutrophils, eosinophils and monocytes in whole blood, and these epitopes are expressed on the cells at low priming concentrations of cytokines such as GM-CSF and TNF-α. Periperal leukocytes of normal donors do not exhibit a primed phenotype. An increase in expression of priming-associated cellular markers will be shortly named priming. Blood was collected from COPD patients and normal control and put on ice immediately after venapuncture and kept at 4°C. A volume of 100 µl of FITC-labeled MoPhab (A17 and A27) was added to 50 µl of whole blood before lysis of the red cells to minimize artificial activation by ex vivo manipulation of the samples. After incubation for 90 min on ice, red cells were lysed in ice-cold NH₄Cl. Hereafter, the cells were washed and taken up in PBS/1%HSA. For the measurement of expression of CD18 (DAKOcytomaion, Gostrup, Denmark), CD63 (Ancell, Bayport, USA), CD62L (BD Pharmingen, Franklin Lakes, USA) and CD11b (DAKOcytomaion, Gostrup, Denmark) cells were lysed after stimulation and incubated directly with FITC or PE-labeled antibody (1:100). An isotype control, FITC or PE-conjugated antibody was used. After incubation cells were washed and taken up in PBS/1%HSA. Cells were analyzed in a FACS vantage flow cytometer (Becton & Dickinson, Mountain View, CA, USA). The different phagocytes were identified according to their specific side-scatter and forward-scatter signals.

**Measurement of H₂O₂ in exhaled air condensate**

Exhaled air condensate was sampled in the morning after measurement of the lung function according to the guidelines of ATS/ERS Task Force as described before. Exhaled breath condensate samples were taken between 8.30 and 9.30 a.m. The patients were spontaneously breathing through a mask with a two-way valve (Downs CPAP Mask, Vital Signs Inc., USA) for a 20 min period. The expired breath condensate was collected by passing the exhaled air through a specially constructed collecting device which consisted of a 140 cm cold finger with an internal diameter of 15 mm. At the end of the cold finger, the condensate was collected into small glass container. This device was placed in a polystyrene foam container filled with ice, water and salt (−2 to −5°C). A second collecting device was coupled to the first device to be sure that all the exhaled breath did indeed condensate. After the collection the condensate was immediately transported to the laboratory in a light protected vial and stored at −70°C and processed within 7 days. The amount of H₂O₂ in the condensate was detected by a method described previously. Briefly, H₂O₂ causes the oxidation of the substrate 3,3′,5,5′-tetramethylbenzidine, a process that is catalyzed by horse-radish peroxidase. The reaction product was measured using a spectrophotometer at 450 nm using an automated microplate reader. The absorbency recorded is directly proportional to the concentration of H₂O₂.

**Data analysis**

Data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed with repeated measurement ANOVA, using the Student’s t-test for paired observations when appropriate. Correlation coefficients were calculated by using Pearson’s method.

**Results**

**Characteristics of the patient population with COPD**

Ten COPD patients with a severe, non-life-threatening exacerbation were included. In the days prior to admission, all subjects developed a deterioration of their lung disease. The most important clinical features at the time of admission are presented in Table 1. Mean age of the patients was 64.6 ± 2.4 yr (male/female: 6/4). Patients had a mean FEV₁ of 38% of predicted at admission (see Fig. 3B), whereas they had a mean FEV₁ of 54% of predicted (range 35–69%) under stable conditions.
They all used inhaled bronchodilators. No clinical or radiological signs of pneumonia were present in any of the patients. Hospital management included standard treatment with bronchodilator nebulisation, oxygen administration and physiotherapy, along with systemic theophylline and high-dose GCS. Antibiotics were prescribed when patients with worsening dyspnea and cough also had increased sputum purulence and were adjusted when appropriate. All patients completed the study and could be discharged after 13.6 ± 1.4 days.

**Comparison of different activation epitopes on granulocytes-induced activation of whole blood with GM-CSF**

Whole blood was stimulated for 15 min with GM-CSF. After stimulation expression of several priming markers was measured by flow cytometry (see materials and methods). In Fig. 1A the fold increase in expression of priming epitope A17 on GM-CSF stimulated neutrophils is compared to non-stimulated neutrophils. Stimulation with GM-CSF 10⁻⁹ and 10⁻¹⁰ M significantly increased the expression of priming epitope (P < 0.05). The expression of A27 is significantly induced by GM-CSF stimulation in the concentration range 10⁻⁹, 10⁻¹⁰ and 10⁻¹¹ M (P < 0.05). In panel C, the expression of different priming markers on neutrophils stimulated with GM-CSF 10⁻⁹ M are compared and shown in Fig. 1.

**Modulation of systemic neutrophil compartment in COPD**

Patients who where admitted to the hospital with an exacerbation of COPD showed an increase in the expression of moPhab A17 and A27 on peripheral blood neutrophils (A17 52.6 ± 14.0 vs. A27 265.3 ± 34.1) (Fig. 2A and B). No subpopulations of neutrophils with discrete expression patterns were found. After 3 days of treatment of the exacerbation the priming of neutrophils was decreased. At 7 days of treatment the priming of A17 was decreased to 20.2 ± 4.2 AU (P < 0.005), and the expression of A27 was decreased to 102.4 ± 34.9 AU (P < 0.05). Tapering of the systemic GCS after 3 days of treatment did not induce a rebound effect on the expression of moPhab A17 or A27 expression on neutrophils.

**Table 1** Patient characteristics at inclusion.

<table>
<thead>
<tr>
<th>Sex, male/female (n/n)</th>
<th>(6/4)</th>
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<tr>
<td>Age, yr</td>
<td>64.6 ± 2.4</td>
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<tr>
<td>Pack-years smoking</td>
<td>28.9 ± 3.6</td>
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<tr>
<td>FEV₁, l</td>
<td>1.10 ± 0.17</td>
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<tr>
<td>FEV₁, % predicted</td>
<td>37.8 ± 4.5</td>
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<tr>
<td>FVC, l</td>
<td>2.58 ± 0.38</td>
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<tr>
<td>FVC, % predicted</td>
<td>66.9 ± 7.9</td>
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<tr>
<td>moPhage A17 (AU) neutrophils</td>
<td>52.6 ± 14.0</td>
</tr>
<tr>
<td>moPhage A27 (AU) neutrophils</td>
<td>265.3 ± 34.1</td>
</tr>
<tr>
<td>H₂O₂, nmol l⁻¹</td>
<td>452.5 ± 33.9</td>
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</table>

**Figure 1** GM-CSF (1–0.001 nM)-induced expression of priming epitopes on neutrophils of normal donors recognized by FITC-labeled MoPhaps A17 and A27 is shown in panel (A) and (B), respectively. In panel (C), a comparative analysis of expression of activation epitopes recognized CD11b, CD18, CD 62L and CD 63 antibodies on neutrophils stimulated with 1 nM GM-CSF are shown. The antibodies were directly labeled with FITC or PE. The expression of priming markers is shown as the mean-fold increase of the GM-CSF stimulated sample compared to the unstimulated sample (error bars ± SEM, n = 7, *P < 0.05).
Local oxidative stress in unstable COPD

The H$_2$O$_2$ samples of exhaled air displayed a high level of this reactive oxygen intermediate, indicating increased oxidative stress in the airways at admission (Fig. 3A). By the time of discharge, the exhaled H$_2$O$_2$ content had fallen from 452.5 ± 33.9 to 236.3 ± 27.0 nmol l$^{-1}$, i.e. 35.7 ± 6.3% of the admission day 3-4 day 7

![Figure 2](image)

**Figure 2** The effect of 1-week treatment of patients with an exacerbation of COPD on expression of priming epitopes on neutrophils. (A) MoPhab A17 expression on circulating neutrophils, (B), MoPhab A27 expression on circulating neutrophils. Data are presented as individual values (median fluorescence intensity, $n$ = 10) and as means of the 10 individual experiments. The expressions of MoPhab A17 and A27 were significantly increased ad admission compared to the follow-up days.

![Figure 3](image)

**Figure 3** The effect of 1-week treatment of patients with an exacerbation of COPD on (A) exhaled H$_2$O$_2$, (B) lung function (FEV$_1$) and (C) (Borg) dyspnea score. Data are shown as individual values ($n$ = 10) and as means.
initial exhaled concentrations \((P<0.0005)\). No significant change in the lung function (FEV\(_1\)) was observed during the first week of treatment (Fig. 3B). On the contrary, the perception of dyspnea, determined by each patient with the help of the Borg dyspnea scale decreased significantly during treatment \((P<0.05)\) (Fig. 3C).

A moderate but significant correlation was found between \(H_2O_2\) values and the expression of the priming epitope A27 on circulating neutrophils \((r = 0.41, P<0.05)\) (Fig. 4A). A17 was not significantly related with \(H_2O_2\) \((r = 0.04, P = 0.83)\) (Fig. 4B). In addition, the Borg score correlated with the \(H_2O_2\) \((r = 0.5, P<0.01, \text{Fig. 4C})\) as well as with the A27 expression \((r = 0.65, P<0.0001, \text{Fig. 4D})\).

**Discussion**

The present study was undertaken to investigate aspects of systemic inflammation in COPD patients.

![Figure 4](image-url) **Figure 4** Correlations between markers of systemic inflammation and local inflammatory markers. \(H_2O_2\) concentration in exhaled air, a marker for local inflammation correlates with moPhab A27 expression on peripheral blood neutrophils (A) and not with moPhab A17 (B). In addition, the Borg score correlated with \(H_2O_2\) concentration in exhaled air (C) and with moPhab A27 expression on peripheral blood neutrophils (D). Every point represents the values corresponding to an individual subject during the study period and fluorescence is expressed as median fluorescent intensity. Correlation coefficients (Pearson) and \(P\) values are shown in each figure.
Systemic inflammation during an exacerbation of COPD

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during an exacerbation of their disease and in the following recovery phase in a unique context. Analysis of modulation of the leukocyte compartment in peripheral blood by determination of priming epitope expression on peripheral leukocytes is an elegant method to study the kinetics of systemic innate immune responses.8,16 Previously, we showed that the local inflammatory response in COPD patients is reflected by a systemic activation of leukocytes.16 In another study, we demonstrated a changed gene expression profile of pro- and anti-inflammatory genes in peripheral blood neutrophils obtained from patients with COPD.23 Expression of CD83, MIP-1α, IL-1RA, IL-1β, IL1-R2 in peripheral neutrophils correlated with the severity of COPD as measured by FEV1.23 This observation confirms the hypothesis that the inflammatory process in COPD is not restricted to the lungs but communicates with the systemic immune response.

The concept of enhanced homing of primed neutrophils to the tissues is corroborated by recent findings. Ariga et al. demonstrated an increased neutrophil recruitment in the lungs of mice challenged with LPS.24 In vitro, an increase in adhesion receptor expression and chemotaxis was observed after stimulation with inflammatory mediators like TNFα and fMLP.25 Furthermore, neutrophils isolated from COPD patients with a fast decline in lung function were shown to express higher levels of the integrin Mac-1.26,27 In addition, previous experiments showed that the expression of priming epitopes is markedly increased on eosinophils obtained from the BAL fluid of allergic patients locally challenged with allergen.8 Therefore, we hypothesized that the increase in peripheral primed neutrophils observed during an exacerbation of COPD16 should be normalized upon optimal treatment.

To compare and validate our method of priming of systemic neutrophils we compared the expression of several well-known priming/activation markers such as α subunit of MAC-1 (CD11b), β1 subunit of MAC-1 (CD18), L-selectin (CD62L) and azurophil granula marker (CD63) with our newly developed priming markers, A17 and A27. The markers we used in this study for the measurement of the expression of priming epitopes on leukocytes were more sensitive to priming stimuli compared to the other markers (see Fig. 1 and Luijk et al.8 and Koenderman et al.16). Therefore we used the markers A17 and A27 to investigate the priming/activation status of neutrophils in this study.

Increased expression of priming-associated markers on neutrophils was observed during a severe exacerbation of COPD. Already after 3–4 days of treatment of the exacerbation the expression of priming epitopes on the neutrophils was normalizing (see Fig. 2). In addition to the measurement of priming, we determined the concentration of H2O2 in exhaled air to investigate if the local inflammation was related to the systemic inflammation (Fig. 3). Previously it was shown by Dekhuijzen et al. that the concentration of H2O2 in expired air was increased during an exacerbation of COPD.28 We show that upon treatment the increased H2O2 levels decreased, indicating that the inflammation due to the exacerbation was decreasing.29 The lung function as measured by FEV1 did not respond on treatment in contrast to the inflammation markers and the dyspnea score.30 This discrepancy suggests that the lung function is a weak tool for monitoring an exacerbation of COPD.

The increase in expression of priming epitopes on peripheral blood neutrophils during an exacerbation of COPD can be explained by an increase in the local inflammatory processes. In a similar study performed by Gerritsen et al.22 the serum concentrations of IL-8, ICAM-1 and E-selectin were measured. During treatment of the exacerbation the concentrations of IL-8 and ICAM-1 declined significantly.22 This is in line with our observation that the priming of neutrophils decreased upon treatment.

These data are consistent with studies that show an aggravated inflammatory process in COPD during an exacerbation of the disease and an increase in the number of neutrophils and lymphocytes in the sputum or BAL fluid.30–33 Interestingly, high baseline levels of IL-6 and IL-8 in the sputum showed that patients had a higher exacerbation frequency, indicating that an activated inflammatory process is related to recurrent exacerbations.34 The local increase of inflammation in the lungs results in an increased production of inflammatory mediators and low doses of peripheral cytokines/chemokines are already capable of priming the leukocytes.16 Therefore the increase of primed granulocytes in the peripheral blood in patients with COPD can be associated with a worsening of their clinical condition due to an activation of the inflammatory process.

The concentration of H2O2 in exhaled air can be used as a marker for local inflammation, because an increase in the number and activation of inflammatory cells in the lungs will be reflected by an increase in H2O2 production.35 This has been shown in several studies, e.g. patients with community acquired pneumonia had elevated levels of H2O2 in their exhaled air condensate.30 In addition, a significant correlation was observed between serum CRP concentration and the concentration of H2O2 in exhaled air.36 The results of this latter study
are in line with our observation that the peripheral blood neutrophil A27 expression and H2O2 concentration in exhaled air were correlated. To our surprise, systemic inflammation reflected in priming of peripheral blood neutrophils as well as local inflammation as determined by an increase in H2O2 concentration was correlated with the patients’ sensation of dyspnea (Borg score). This observation indicates that the systemic immune response, the local inflammation and patients' well being are in close relation with each other. Unexpected was the concentration was correlated with the patients’ inflammation as determined by an increase in H2O2 in the peripheral blood neutrophils as well as local inflammation reflected in priming-associated markers in peripheral blood during an exacerbation of COPD. In conclusion, primed neutrophils can be found in peripheral blood by antibodies isolated from a synthetic phage antibody library. J Leukoc Biol 2000;68:58–64.

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