Differences in oxidative response of subpopulations of neutrophils from healthy subjects and patients with rheumatoid arthritis

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Abstract

Objectives—To determine whether blood neutrophils from healthy individuals and blood and synovial fluid neutrophils from patients with rheumatoid arthritis (RA) responded differently to priming agonists and stimuli of the oxidative burst and, if so, whether this was a property of a subpopulation of neutrophils.

Methods—Continuous flow electrophoresis was used to separate neutrophils into subpopulations based upon quantitative differences in net negative surface charge. The generation of superoxide anion \( \text{O}_2^- \) was used as a measure of oxidative activity using \( 10^{-7} \text{ mol/l} \) N-formylmethionyl-leucyl-phenylalanine (FMLP) as the stimulating agonist and \( 10^{-4} \text{ mol/l} \) platelet activating factor (PAF) as the priming agent.

Results—The production of \( \text{O}_2^- \) by blood and synovial fluid neutrophils from RA patients in response to FMLP was greater than that observed with control blood neutrophils \( (p < 0.001) \). Priming of normal blood neutrophils with PAF increased their FMLP induced oxidative burst \( (p < 0.001) \), but PAF treatment had no effect on rheumatoid neutrophils. Neutrophils from synovial fluid of RA patients were less electronegative than paired blood samples and exposure of blood neutrophils to FMLP but not PAF reduced their surface charge. Continuous flow electrophoresis isolated three neutrophil subpopulations: cells of least surface electronegativity were ascribed to pool P1 and cells of greatest surface electronegativity to P3. Normal blood neutrophils from P3, but not P1, showed increased oxidative activity after PAF priming (twofold increase; \( p < 0.01 \)), whereas the responsiveness of rheumatoid blood and synovial fluid neutrophils from P1 and P3 was not modified by PAF treatment under the same conditions.

Conclusion—It is suggested that most of the circulating neutrophils in RA are already in a state of readiness to generate \( \text{O}_2^- \) upon activation by an inflammatory stimulus. This is in contrast to normal blood neutrophils, which have both responsive and non-responsive subpopulations with respect to priming agonists.

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surface charge. The demonstration that exposure of neutrophils to agonists such as N-formyl-methionyl-leucyl-phenylalanine (FMLP), platelet activating factor (PAF), lactoferrin and C5a results in a decrease in the electrophoretic mobility of the cell supports the concept that a reduction in surface charge is associated with increased functional activity. In addition, studies by ourselves and others have shown that normal blood neutrophils respond in a heterogeneous manner to stimuli of the oxidative burst.

A major aim of this study was to investigate whether there were differences between neutrophils from control subjects and patients with RA in the production of superoxide anion (O2•-) and to examine the effect of priming on this response. We further sought to establish to what degree subpopulations of neutrophils from RA patients were in a primed or more activated state than cells from healthy individuals. To achieve this aspect of the work, subpopulations of neutrophils were prepared by CFE. The identification of primed and unprimed neutrophil subpopulations may prove to be of value in increasing the understanding of pathogenic mechanisms in RA.

Patients and methods

Patients and Controls

Blood was collected from 26 patients with definite or classical rheumatoid arthritis, who were attending the rheumatology clinics at The Royal London Hospital and Royal Free Hospital, and at the time of the investigation were either receiving non-steroidal anti-inflammatory drugs (NSAIDs) or were about to commence other anti-inflammatory treatment. Three patients provided both blood and synovial fluid during one visit to the clinic. Patients with severe disease had early morning stiffness lasting more than two hours and an articular index >20. Control blood was taken from healthy laboratory personnel who had previously given their informed consent.

Preparation of Neutrophils from Blood and Synovial Fluid

Neutrophils were isolated from 40 ml of blood anticoagulated with potassium EDTA 1-5 mg/ml by a one step isolation procedure using polymorphoprep (Nycochrome, Inc). This was in accordance with the manufacturer's instructions. The neutrophils were then washed twice in ice cold Hanks' balanced salt solution (HBSS) and maintained at 4°C until required. Synovial fluid was collected by aspiration of knee joints in the course of treatment and incubated with 75 U/ml hyaluronidase (Type X; Sigma Chemical Co) for 30 minutes at 37°C. The cell suspension was added to an equal volume of ice cold HBSS during washing to help retard the effect of various inflammatory mediators present in the synovial fluid. The cells were gently washed twice at 400 g for five minutes. After each wash the supernatants were found to contain mainly mononuclear cells, which were discarded. The pelleted cells, enriched with neutrophils, were resuspended in ice cold isotonic ammonium chloride for 10 minutes to lyse contaminating erythrocytes and maintain osmolarity. This was followed by additional washes in ice cold HBSS; the cells were further maintained in HBSS at 1 x 10⁷ cells/ml. The purity of neutrophils isolated from blood and synovial fluid was approximately 94% and 90%, respectively.

Cell surface charge

Previous work in our group demonstrated that incubation of normal blood neutrophils with hyalase did not modify their surface charge. To determine whether exposure of neutrophils to priming and stimulating agents altered cell surface charge, neutrophils from healthy subjects were divided into three aliquots and either incubated with 10⁻⁴ mol/l PAF or 10⁻⁷ mol/l FMLP, or left untreated for 15 minutes at 37°C. The cells were then formaldehyde fixed to prevent further alteration of membrane constituents, by adding to the suspensions an equal volume of 0-8% v/v formaldehyde in HBSS (0-4% v/v final concentration). The suspension was then left to stand at room temperature for 30 minutes. The fixed cells were maintained in HBSS at 4°C until required for separation by CFE.

Separation of Subpopulations of Neutrophils by Continuous Flow Electrophoresis

Continuous flow electrophoresis was used to fractionate purified preparations of blood neutrophils from eight RA patients and eight healthy controls. A detailed description of the principles of CFE has been presented elsewhere. Figure 1A illustrates the apparatus used (Elphor Vap 22 CFE apparatus; Bender and Hobein, Munich, Germany) and figure 1B shows typical CFE separation profiles of formaldehyde fixed and unfixed neutrophils from a healthy donor. Briefly, neutrophils were resuspended in a chamber buffer (10 mmol/l triethanolamine, 280 mmol/l glycine and 30 mmol/l glucose) at a concentration of 2·5 x 10⁷ cell/ml and introduced by a continuous flowing film of buffer into the chamber of the apparatus. The cell suspension was injected into the chamber at a flow rate of 2·5 ml/h and the chamber temperature was maintained at 4·5 ml/h. The current was set at 100 mA, which gave a potential across the chamber of 900–1000 V. Triethanolamine, EDTA and all other running reagents were of analytical grade and purchased from BDH. The electrode buffer consisted of 100 mmol/l triethanolamine and the chamber was held at a constant temperature of 10°C. The mean peak fraction number was determined by turbidometric analysis in a spectrophotometer at an absorbance of 500 nm and confirmed by Coulter counting. Fractionated neutrophils were found in approximately 12 to 15 collecting tubes. Many of these tubes contained cells in concentration less than 1.
million and therefore several of them were pooled, on the basis of differences in net negative charge, into three major subpopulations of approximately equal cell number (P1, P2, and P3). The least and most electro-negative pools, P1 and P3 respectively, were washed in HBSS before being assayed at the same time as an aliquot of non-fractionated (total pool) cells for $O_2^-$ production during both stimulated and primed-stimulated conditions.

**MEASUREMENT OF SUPEROXIDE PRODUCTION FROM PRIMED AND STIMULATED NEUTROPHILS**

Aliquots of isolated neutrophils ($1 \times 10^6/100\mu l$) maintained in Ca$^{2+}$ and Mg$^{2+}$ containing HBSS were suspended in 900 $\mu l$ of prewarmed HBSS containing 0.1 mmol/l cytochrome c, 5 mmol/l glucose with and without 50 $\mu g/ml$ superoxide dismutase (SOD) (178 U/ml) and placed in temperature controlled cuvettes of a spectrophotometer (Pye Unicam SP8-400). Cells were stimulated to generate superoxide by addition of various concentrations of FMLP ($10^{-9}$-10$^{-10}$ mol/l).

Platelet activating factor was used as the priming agent of choice, at a concentration (10$^{-8}$ mol/l) that was previously demonstrated to be optimal for neutrophil priming. Blood neutrophils from six control subjects and six RA patients were exposed to PAF by the addition of PAF 10$^{-8}$ mol/l to the prewarmed cells for two minutes before the addition of FMLP 10$^{-7}$ mol/l. During this period, baseline activity was monitored at 550 nm to ensure PAF did not directly stimulate production of superoxide. In control experiments, cells were stimulated without priming.

On addition of FMLP, superoxide activity was measured over a period of five minutes and expressed as nmol/min/10$^7$ cells calculated from an extinction coefficient for reduced cytochrome $c$ of 21.2 $\times$ $10^3$ (mol/l)$^{-1}$ cm$^{-1}$.

Cytochrome $c$ (horse heart, type VI), FMLP, PAF, and SOD (from bovine erythrocytes) were purchased from Sigma Chemical Co.

**STATISTICS**

The data were analysed using either paired Student's $t$ test (subpopulation comparisons) or Wilcoxon test (healthy subjects vs RA patients), using Statsworks 1.3 Cricket Software Inc, PA, USA. All values are

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**Figure 1** A: Schematic diagram of the CFE chamber and the isolation of neutrophil subpopulations. Neutrophils flow through the CFE chamber between two electrodes and collect in sample tubes. Tubes on the left contain neutrophils which displayed the least deflection in the electrical field (the lowest net negative surface charge). B: Typical CFE separation profile of non-fixed (●) and formaldehyde fixed (○) neutrophils from a healthy donor. The cells in each fraction are counted and pooled into three fractions of approximately equal cell numbers—P1 (least), P2, and P3 (most) electronegative.
expressed as mean (SEM); the threshold for statistical significance is $p < 0.05$.

### Results

#### Cell Surface Charge

Neutrophils fixed in formaldehyde showed an electrophoretic profile similar to that of fresh neutrophils isolated from the same individual (fig 1B).

CFE fractionated blood neutrophils from both control subjects and RA patients exhibited comparable isolation profiles, which ranged from broad to slightly skewed to the left. Interestingly, irrespective of the profile, the peak mean number of cells were generally of a lower electrophoretic mobility in the RA samples (table 1). Within each experiment, the isolated cells did not always appear in the same numbered collecting tubes because individual blood samples were separated on different days, when small variations occurred in buffer composition, pH, conductivity, osmolarity, and electrical conditions. However, on the basis of differences in electronegative charge, the neutrophils always separated into approximately 12 to 15 fractions (collecting tubes).

Neutrophils derived from the blood and synovial fluid of three patients with RA and fractionated on the same day under identical conditions showed a substantial cathodal shift in the separation profiles of those from synovial fluid compared with autologous blood (fig 2). Priming alone did not alter the cell surface charge properties of blood neutrophils from healthy subjects, but FMLP stimulation led to a cathodal shift in their charge properties (fig 3). The electrophoretic profiles of these cells resembled those of synovial fluid neutrophils (fig 2), in that they appeared to have an overall decrease in net surface charge characteristics when compared with non-treated autologous blood.

#### Comparison of Blood Neutrophils from Healthy Subjects and RA Patients

**FMLP Stimulated Generation of Superoxide**

Table 2 shows the production of $O_2^-$ by blood neutrophils from six control subjects and six patients with severe RA, in response to stimulation by $10^{-6}$ to $10^{-4}$ mol/l FMLP. At $10^{-7}$ mol/l FMLP, the rate of $O_2^-$ production for RA patients was significantly greater than that for controls ($p > 0.001$). When $10^{-6}$ or $10^{-4}$ mol/l FMLP was used as the stimulus there was no significant difference between the two groups. Accordingly, a concentration of $10^{-5}$ mol/l FMLP was considered optimal for distinguishing differences between the two sources of neutrophils and was therefore used throughout the study. FMLP concentrations of $10^{-6}$ to $10^{-8}$ mol/l were not studied further as it was reasoned that, if these concentrations of FMLP could not distinguish differences between whole populations of neutrophils from control and RA subjects, they would be unlikely to identify differences between the P1 and P3 subpopulations in response to FMLP.
superoxide production

Table 2  Dose response effects of FMLP on the rate of superoxide production by neutrophils isolated from six healthy controls (C) and six patients with severe rheumatoid arthritis (RA)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Superoxide (nmol/min/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FMLP 10⁻⁴ mol/l</td>
</tr>
<tr>
<td></td>
<td>C  RA  C  RA  C  RA  C  RA</td>
</tr>
<tr>
<td>1</td>
<td>44  45  28  44  5  10 5  18</td>
</tr>
<tr>
<td>2</td>
<td>46  55  20  41  5  18 5  18</td>
</tr>
<tr>
<td>3</td>
<td>48  55  38  52  2  10 2  10</td>
</tr>
<tr>
<td>4</td>
<td>40  51  26  57  4  13 4  13</td>
</tr>
<tr>
<td>5</td>
<td>51  44  25  55  5  13 5  13</td>
</tr>
<tr>
<td>6</td>
<td>35  53  20  49  14  9 9  12</td>
</tr>
<tr>
<td>Mean</td>
<td>41  51  26  50  6  12 6  12</td>
</tr>
<tr>
<td>SEM</td>
<td>2 4  2 2  2 6  2 5  1 7  1 5</td>
</tr>
</tbody>
</table>

Values are mean of duplicate measurements.

*Significant difference between neutrophils from RA patients and control neutrophils with 10⁻³ mol/l FMLP (p < 0.001; unpaired Student’s t test) (no difference with 10⁻⁴ or 10⁻² mol/l FMLP).

stimulation. Analysis of neutrophils from a further 17 healthy subjects and 14 RA patients with mild or moderate disease activity again revealed the patients’ cells to be more efficient than control cells at producing O₂⁻ (mean 39·2 (SEM 4·7) nmol/min/10⁷ cells in RA patients compared with 22·5 (1·7) nmol/min/10⁷; p < 0·001, Wilcoxon test) (fig 4). There was a considerable variation in the range of O₂⁻ production in response to 10⁻² mol/l FMLP by blood neutrophils from control subjects (12·38 nmol/min/10⁷ cells) and from patients with RA (25·94 nmol/min/10⁷ cells).

Effect of priming with PAF

O₂⁻ production by normal neutrophils was significantly enhanced by PAF, increasing from a mean O₂⁻ production of 27·6 (SEM 2·4) nmol/min/10⁷ cells without PAF to 41·2 (2·3) nmol/min/10⁷ cells (p < 0·001) after priming with PAF (fig 5A). In contrast, priming had no significant effect on O₂⁻ production by rheumatoid neutrophils (fig 5B).

Effect of priming with PAF

With control neutrophils, the O₂⁻ production by the P3 subpopulation was significantly increased by PAF treatment (table 3, p < 0·01) whereas priming had no effect on the P1 subpopulation. Pretreatment of the P1, P3, and unfractionated cells from RA blood and synovial fluid with PAF did not enhance their responsiveness to FMLP stimulation. However, the production of O₂⁻ by rheumatoid blood and synovial fluid neutrophils in P1 and P3 was greater than that of P1 and P3 control samples (p < 0·01).

Comparison of electrophoretically separated subpopulations of neutrophils

The amount of O₂⁻ generated by the P1 fraction pool of normal neutrophils in response to 10⁻³ mol/l FMLP was significantly greater than (up to twice) the amount generated by the P3 fraction which contained the most electronegative cells (p < 0·001) (fig 6). The P1 and P3 subpopulations of RA neutrophils did not differ significantly in their FMLP stimulated responsiveness, though both pools produced more O₂⁻ than the corresponding control subpopulations (p < 0·01). A similar finding occurred with the P1 and P3 subpopulations of neutrophils from rheumatoid synovial fluid (table 3).

Figure 4  Production of superoxide by unfractionated rheumatoid and healthy control blood neutrophils stimulated by 10⁻³ mol/l FMLP. Horizontal bars represent mean value for each group. Significant difference between group means (p < 0·001).

Figure 5  Effect of priming with platelet activating factor (PAF) on superoxide anion production by unfractionated six healthy control (A) and six rheumatoid (B) samples of blood neutrophils primed (■) or not primed (□) with PAF before stimulation with 10⁻³ mol/l FMLP. Significant effect of priming between normal and rheumatoid neutrophils (p < 0·001).
Discussion

Superoxide anion production by neutrophils, which is believed to contribute to both defence against microbial invasion and tissue damage, is regulated by priming of the cells with physiological agonists such as leukotriene B4, interleukin, granulocyte macrophage colony stimulating factor, tumour necrosis factor, and PAF. 2,3 In the present study, blood neutrophils from rheumatoid patients in various stages of disease activity had higher rates of \(O_2^-\) production than cells from healthy individuals in response to stimulation by \(10^{-7}\) mol/l FMLP, but not with higher (\(10^{-6}\) mol/l) or lower (\(10^{-8}\) mol/l) concentrations of FMLP. It therefore appears that the concentration of stimulant is important in detecting differences in \(O_2^-\) production by neutrophils from these two subject groups; this probably explains why previous studies using \(10^{-6}\) mol/l FMLP failed to show a difference between oxidative responsiveness of neutrophils from RA and control subjects. 2,3

Primming of the rheumatoid cells with PAF did not augment their responsiveness, in contrast to the enhancing effect of PAF on control neutrophils. With greater concentrations of FMLP, pretreatment of control cells with PAF did not increase their oxidative burst (data not shown), presumably because the rate of \(O_2^-\) generation induced by the stimulant alone was at its optimum level. At present it is unclear why the primed and stimulated control neutrophils did not reach the high rates of production of \(O_2^-\) seen with RA neutrophils challenged only with \(10^{-7}\) mol/l FMLP. Rheumatoid blood may contain many inflammatory mediators and it is conceivable that, in this disease, circulating neutrophils are primed in vivo by agents that are more potent than those used in in vitro studies. Alternatively, there may be synergistic effects which have not yet been explored.

A particular feature of this study was the identification of subpopulations of normal blood neutrophils which differed in their respiratory burst in response to priming agonists and stimulation. The subpopulation containing the least electronegative neutrophils was the most responsive to a ‘stimulant’ only challenge. Priming of these cells with PAF did not enhance their generation of \(O_2^-\) production by FMLP, which was in contrast to the neutrophils with a high net negative surface charge, which exhibited significant increases in responsiveness following PAF priming. These findings add support to the concept that there is functional and metabolic heterogeneity within neutrophils, 4,12 and that the magnitude of these parameters relates inversely to surface membrane electronegativity. In addition, our studies agree with the recent proposal that in healthy individuals there are at least two populations of circulating neutrophils which differ from one another in their response to priming agents. 18

Using two physically distinct cell separation techniques (continuous flow electrophoresis
and partitioning in two phase aqueous polymer systems), our earlier studies showed that blood neutrophils are heterogeneous with respect to cell surface membrane electrophoretic properties and functional responsiveness.\textsuperscript{13, 14} Our previous work also demonstrated that neutrophils of a low electrophoretic mobility were increased in the blood and particularly in the synovial fluid of RA patients,\textsuperscript{9, 11} and that immune complexes reduced the surface charge of normal blood neutrophils.\textsuperscript{10} In the present study, rheumatoid synovial fluid neutrophils separated by CFE were found to be of a consistently lower electrophoretic mobility than autologous blood neutrophils. Neutrophils within rheumatoid joints are reported to be activated as judged by their O\textsubscript{2}\textsuperscript{-} production\textsuperscript{20} and receptor expression.\textsuperscript{31} The current demonstration that normal blood neutrophils stimulated with FMLP, but not those primed with PAF, possessed a low electrophoretic mobility similar to that expressed by synovial fluid neutrophils, strengthens the association of charge heterogeneity with cell surface charge. The cathodal shift in profile observed with 10\textsuperscript{-7} mol/l FMLP appears to be a concentration dependent. When 5 \times 10\textsuperscript{-7} mol/l FMLP was used instead (data not shown), there was no observable change in the shape or peak position of the CFE profile.

Why electrophoretic subpopulations of normal neutrophils differ in their production of O\textsubscript{2}\textsuperscript{-} remains to be resolved. Little attention has focused on the relationship between the functional status of cells and their surface charge, even though a reduction in the electrophoretic mobility of neutrophils by foreign or natural polycations leads to an increased responsiveness to stimulants,\textsuperscript{30} and neuraminidase increases phagocytic activity.\textsuperscript{30} Surface charge heterogeneity may arise from subpopulations of different stem cell origin, from late maturation differences emerging before and after entry into the circulation,\textsuperscript{15} or as a result of cytokines, primers, stimulants, and other factors acting on the cell surface to initiate membrane reorganisation. Examination of electrophoretic subpopulations of neutrophils in the present study revealed that priming with PAF enhanced the respiratory burst activity of only the P3 subpopulation (most electronegative) from control blood neutrophils. The inability of PAF to increase superoxide production of the other subpopulations of control and RA neutrophils in response to FMLP suggests that these cells were at their maximum threshold of responsiveness under the present experimental conditions. Furthermore, upon activation, the P1 and P3 subpopulations of RA blood and synovial fluid neutrophils produced higher levels of superoxide anion than control cells, implying that these cells had been influenced by one or several in vivo inflammatory factors. This in part would explain why the heterogeneity seen in normal controls is not detectable in RA cells, as exposure of these latter cells to a number of inflammatory agents before isolation may have enhanced the functional responsiveness of these cells above control levels. In addition to surface charge, it is likely that rheumatoid neutrophils differ from control cells in receptor expression and that their surface and functional properties are modified by the effect of in vivo factors such as immune complexes and, in relation to synovial fluid neutrophils, apoptosis. Future studies are planned to examine these possibilities. Although NSAIDs are known to suppress a number of neutrophil functions, there is no information to suggest that cell surface properties are modified by these drugs. The implications of neutrophil heterogeneity are considerable in the context of infection and tissue damage, where the rapid extravasation of a primed subpopulation of cells would benefit the acute inflammation but, if continued, would exacerbate the chronic inflammatory response. Unravelling the mechanisms by which neutrophils become activated and their recruitment into the joint cavities of patients with rheumatoid arthritis, by the combined application of CFE with other biochemical and functional techniques, will be a useful approach, once changes in electrophoreticity are more clearly defined and understood.

We are grateful to the Arthritis and Rheumatism Council of Great Britain for financial support. We also acknowledge the help and advice of Dr L Poulter and his clinical colleagues in the Department of Rheumatology, Royal Free Hospital School of Medicine and Dr J D Perry, Department of Rheumatology, The Royal London Hospital.

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