Activated phenotype in neutrophils and monocytes from patients with primary proliferative polycythaemia

N B Westwood, E R Copson, L A Page, A R Mire-Sluis, K A Brown, T C Pearson

Abstract

**Aim**—To investigate whether monocytes and neutrophils from patients with primary proliferative polycythaemia (PPP) exhibit increased expression of markers of cell activation and, if so, whether they are associated with the phagocytic activity of these cells and concentrations of circulating cytokines.

**Methods**—Expression of CD11b, CD14, CD18, and CD64 on monocytes and neutrophils was assessed by flow cytometry. Phagocytosis was analysed using immunoglobulin opsonised *Escherichia coli*. Serum concentrations of granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF) and macrophage CSF (M-CSF) were determined by bioassays, and interferon-γ (IFN-γ) by enzyme linked immunosorbent assay (ELISA).

**Results**—Patients with PPP (n = 18), when compared with normal subjects (n = 10), had increased percentages of CD64+ monocytes (52% v 36%) and neutrophils (42% v 11%) and of CD14+ neutrophils (36% v 18%). Monocytes from patients with PPP exhibited increased expression of CD64 (47 v 26) and of CD11b (65 v 36). These abnormalities were not found in patients with secondary (n = 8) or apparent (n = 13) polycythaemia. The percentage of neutrophils undergoing phagocytosis was higher in patients with PPP (mean 64%; n = 6) than in normal subjects (mean 42%; n = 5). G-CSF, GM-CSF and IFN-γ concentrations in patients’ serum samples were comparable with normal; M-CSF was not detected in any of the samples. There was no correlation between cytokine concentrations and the expression of CD11b, CD14, CD18, and CD64 on patients’ phagocytes.

**Conclusions**—Increased expression of CD11b and CD64 by monocytes, increased percentages of CD14+ and CD64+ neutrophils and the high phagocytic activity of neutrophils suggests that these cells are activated in vivo in patients with PPP. The phenotypic changes of PPP phagocytes were not associated with increased concentrations of circulating cytokines and probably reflect intrinsic abnormalities within the neoplastic PPP clone.

Keywords: CD antigen, primary polycythaemia, neutrophil activation, monocyte activation.
type or phagocytic activity, or both, were associated with increased concentrations of endogenous phagocyte activating cytokines.

Methods
The study population comprised 21 patients fulfilling the criteria for PPP;22 eight patients with secondary polycythaemia, caused by hypoxaemic chronic obstructive airways disease in five and by cyanotic congenital heart disease in the other three; 13 patients with a raised haematocrit but normal red cell mass (apparent polycythaemia); and 15 normal subjects. Patients were managed by venesection and in those with PPP busulphan was given intermittently to control elevated platelet counts. Of the PPP group, 12 had a granulocytosis (range 8·1–16·1 x 10⁹/l) and one had a monocytosis (1·1 x 10⁹/l) at the time of the study. Leucocyte counts of all other subjects were normal.

SURFACE RECEPTOR ANALYSIS
Monoclonal antibodies to CD11b (Becton Dickinson, Oxford, UK), CD14, CD18, (Dako, High Wycombe, UK), and CD64 (Sero tec, Oxford, UK) were used for immuno-fluorescence staining of whole blood followed by flow cytometry (Coulter, Luton, UK). Monocytes and neutrophils were identified by forward and side scatter characteristics and the percentage of labelled cells determined from 1024 channel fluorescence histograms set to ≤2% using isotype controls. The relative level of receptor expression was estimated using the mean fluorescence channel (MFC) of stained cells. Sterile endotoxin-free reagents and aseptic technique were used throughout to minimise the possibility of in vitro cell activation, and the optical alignment and fluorescence sensitivity of the instrument were checked daily.

PHAGOCYTOSIS ASSAY
Immunoglobulin oposiuned fluorescein isothiocyanate (FITC) labelled Escherichia coli (Orpogen, Heidelberg, Germany) were incubated with whole blood (2·5 x 10⁷ E coli/ml blood) at 37°C. Aliquots were removed at intervals up to 90 minutes with mixed with ice cold trypsin blue solution to halt phagocytosis and quench fluorescence from adherent E. coli. Monocytes and neutrophils were identified by flow cytometry as described above, in conjunction with propidium iodide staining to eliminate unengulfed bacteria and leucocyte aggregates from the analysis. The percentage of cells undergoing phagocytosis was then determined from histograms of FITC fluorescence. Plots of per cent phagocytosis against time, fitted using commercial software (Fig.P Biosoft, Cambridge, UK), approximated asymmetric sigmoidal curves with maxima between 20 and 60 minutes. These curves were used to interpolate the time taken to reach half-maximum values of phagocytosis as an estimate of the rate of phagocytosis.

CYTOKINE ASSAYS
Granulocyte CSF (G-CSF), granulocyte–macrophage CSF (GM-CSF) and macrophage CSF (M-CSF) were measured using cell proliferation bioassays.23 Briefly, serum samples were incubated with GFS60 (G-CSF assay), TF1 (GM-CSF) or MNFS60 (M-CSF) factor dependent cells at 37°C in 5% CO₂ for 48 hours. Cultures were then pulsed with 1H-thymidine (Amersham, Little Chalfont, UK), harvested onto filters and read in a scintillation counter. Assays were calibrated using the World Health Organisation International Standards (National Institute for Biological Standards and Control, NIBSC): G-CSF (NIBSC reference 88/502); GM-CSF (88/646); M-CSF (90/732). Serum samples stimulating TF1 cells were retested in the presence of a neutralising antibody to erythropoietin and the identity of any residual activity confirmed using anti-GM-CSF. Serum IFN-γ was measured by enzyme linked immunosorbent assay (ELISA)24 using microtitre plates (Falcon, Becton Dickinson) coated with murine monoclonal antihuman IFN-γ (NIBSC). Non-specific binding was blocked using 1% human albumin, followed by sequential incubations with serum samples, rabbit polyclonal antihuman IFN-γ (NIBSC), peroxidase conjugated goat antirabbit immunoglobulin (Biorad, Hemel Hempstead, UK) and substrate (tetra-methylbenzidine/hydrogen peroxide). Colour development was stopped with sulphuric acid (2 M) and absorbance read at 450 nm. The British Standard preparation of IFN-γ (NIBSC 82/587) was used for the calibration curve.

STATISTICS
Intergroup comparisons were made using the Student’s t test, Wilcoxon’s rank sum or the χ² test; p<0.05 was considered significant.

Results
SURFACE RECEPTOR ANALYSIS
The percentage of CD64+ monocytes was higher in patients with PPP (mean 52%) than in normal subjects (mean 36%; p<0.05; fig 1A) and the expression of CD64 on PPP monocytes was increased (mean MFC in PPP, 47%; normal mean MFC, 26; p<0.0001; fig 1B). The percentage of neutrophils expressing CD64 was also higher in patients with PPP (mean 42%) than in normal subjects (mean 11%; p<0.0001; fig 2A), although the level of CD64 expression on these cells was not increased (fig 2A). Table 1 shows that patients with PPP also had increased expression of CD11b on monocytes (PPP mean MFC, 65; normal mean MFC, 36; p<0.05) and a high percentage of CD14+ neutrophils (PPP mean 36%; normal mean, 18%; p<0.001). When monocyte CD11b (MFC), monocyte CD64 (% and MFC), neutrophil CD14 (%), and neutrophil CD64 (%) were assessed in each patient with PPP the

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In contrast to those with PPP, monocytes and neutrophils from patients with secondary polycythaemia and apparent polycythaemia were

majority (17/18) were found to have two or more results that were greater than the maximum values observed in normal subjects. The single patient who was entirely comparable with normal subjects met the criteria for PPP at

presentation but has not required venesection for over 20 years and therefore is atypical.

Figure 1 The percentage of CD64+ monocytes (panel A) and relative level of CD64 expression (panel B) on monocytes from patients with polycythaemia and normal subjects. Horizontal lines indicate mean values. SP, secondary polycythaemia; AP, apparent polycythaemia.

Figure 2 The percentage of CD64+ neutrophils (panel A) and relative level of CD64 expression (panel B) on neutrophils from patients with polycythaemia and normal subjects. Horizontal lines indicate mean values. SP, secondary polycythaemia; AP, apparent polycythaemia.
Table 1 Expression of CD11b, CD18, CD14 and CD64 on monocytes and neutrophils from patients with polycythaemia

<table>
<thead>
<tr>
<th>Surface receptor</th>
<th>Monocytes</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPP (n=18)</td>
<td>SP (n=8)</td>
</tr>
<tr>
<td>CD11b %</td>
<td>99 (0.3)</td>
<td>99 (0.3)</td>
</tr>
<tr>
<td>MFC %</td>
<td>65* (9)</td>
<td>54 (9)</td>
</tr>
<tr>
<td>CD14 %</td>
<td>75 (2)</td>
<td>75 (2)</td>
</tr>
<tr>
<td>MFC %</td>
<td>91 (6)</td>
<td>68* (6)</td>
</tr>
<tr>
<td>CD18 %</td>
<td>82 (3)</td>
<td>72 (3)</td>
</tr>
<tr>
<td>MFC %</td>
<td>77 (5)</td>
<td>73 (5)</td>
</tr>
<tr>
<td>CD64 %</td>
<td>52* (3)</td>
<td>40 (3)</td>
</tr>
<tr>
<td>MFC %</td>
<td>47† (4)</td>
<td>29 (4)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SEM). Mean values significantly different from normal (Student's t test): *p<0.05, ***p<0.001, †p<0.0001.

SP, secondary polycythaemia; AP, apparent polycythaemia.

comparable with normal, except for lower expression of CD14 on secondary polycythaemia monocytes (MFC in secondary polycythaemia, 68; normal MFC, 91; p<0.01; table 1).

Table 2 Phagocytic activity of neutrophils and monocytes from patients with polycythaemia

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>PPP (n=6)</th>
<th>AP (n=5)</th>
<th>Normal (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undergoing phagocytosis (%)</td>
<td>64* (4)</td>
<td>36 (4)</td>
<td>42 (5)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>PPP (n=6)</td>
<td>AP (n=5)</td>
<td>Normal (n=5)</td>
</tr>
<tr>
<td>Undergoing phagocytosis (%)</td>
<td>31 (6)</td>
<td>18 (6)</td>
<td>24 (2)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SEM) of the maximum percentage of cells undergoing phagocytosis during a 90 minute incubation with immunoglobulin opsonised E.coli. *p<0.02 compared with normal group mean (Wilcoxon's rank sum test). AP, apparent polycythaemia.

Table 3 Cytokine concentrations in the serum samples of patients with polycythaemia

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PPP (n=14)</th>
<th>SP (n=7)</th>
<th>AP (n=12)</th>
<th>Normal (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF (pg/ml) No. of positive samples*</td>
<td>11 (3-0)</td>
<td>4 (3-0)</td>
<td>10 (3-0)</td>
<td>6 (3-0)</td>
</tr>
<tr>
<td>mean (SEM)*</td>
<td>25.1 (5-9)</td>
<td>22.5 (5-9)</td>
<td>16.6 (2-6)</td>
<td>19.2 (3-7)</td>
</tr>
<tr>
<td>range</td>
<td>8-679-98</td>
<td>11-438</td>
<td>7-233-2</td>
<td>6-128-4</td>
</tr>
<tr>
<td>GM-CSF (pg/ml) No. of positive samples*</td>
<td>1 (3)</td>
<td>15-7</td>
<td>1 (3)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>M-CSF (pg/ml) No. of positive samples*</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IL-6 (IU/ml) No. of positive samples*</td>
<td>2 (16-5)</td>
<td>0 (16-5)</td>
<td>3 (16-5)</td>
<td>4 (16-5)</td>
</tr>
<tr>
<td>mean (SEM)*</td>
<td>23.0 (16-5)</td>
<td>25.0 (16-5)</td>
<td>43.8 (32-2)</td>
<td>47.5 (32-2)</td>
</tr>
<tr>
<td>range</td>
<td>6-39-5</td>
<td>10-54-0</td>
<td>4-7-155-3</td>
<td></td>
</tr>
</tbody>
</table>

*Positive samples were those with measurable concentrations of cytokine in assays with the following sensitivities: 1-0 pg/ml (G-CSF and GM-CSF), 3-9 IU/ml (IFN-γ) and 15-2 pg/ml (M-CSF).

Discussion

The present study demonstrates that patients with PPP have increased percentages of circulating CD64+ neutrophils, CD64+ monocytes and CD14+ neutrophils. Monocytes from these patients also displayed increased expression of CD11b and CD64. These surface molecules were not upregulated on phagocytes from polycythaemic patients with polyclonal haemopoiesis (secondary and apparent polycythaemia).
Activated phenotype in neutrophils and monocytes in PPP

Increased expression of the high affinity receptor for IgG, CD64 (FcγRI), is considered to be a marker of monocyte and neutrophil activation. In addition to promoting phagocytosis, CD64 mediates cytotoxicity and activates the oxidative burst. IFN-γ upregulates CD64 on monocytes and neutrophils in vitro and on neutrophils in vivo whilst administration of G-CSF stimulates the de novo production of neutrophils expressing CD64 via its action on myeloid progenitor cells. Phagocytes from patients with streptococcal pharyngitis exhibit increased expression of CD64, which has been attributed to the endogenous production of IFN-γ or G-CSF in such infections. CD14, a receptor for LPS-LPS binding protein complexes, mediates phagocyte activation and enhances the adhesive activity of neutrophil CD11b.

Expression of CD14 increases during differentiation of myelomonocytic cells and can be further upregulated on mature neutrophils by G-CSF and GM-CSF in vitro and by G-CSF in vivo. Thus, the increased percentages of CD64+ monocytes and neutrophils and of CD14+ neutrophils in patients with PPP suggest that these cells were activated in vivo. However, activation of monocytes and neutrophils by inflammatory stimuli results in the rapid upregulation of CD11b/CD18, but in the present study the expression of this molecule by PPP neutrophils was comparable with that in normal subjects. That CD14 and CD64, but not CD11b, were upregulated on PPP neutrophils may be explained by the augmented expression of surface adhesion molecules being dependent upon the nature of the stimulus and the local environment. For example, the response of neutrophils to activating factors in the bone marrow could be distinct from those operating in the circulation or at sites of inflammation/infection. Moreover, this responsiveness may also be dependent upon the cell type, as illustrated by the finding of enhanced CD11b on PPP monocytes in our study.

In the present study the concentrations of G-CSF, GM-CSF and IFN-γ in the serum samples of patients with PPP were not increased and there was no correlation between concentrations of these cytokines and the expression of surface receptors on phagocytes. Although patients with PPP have been previously reported to have increased serum immunoreactive M-CSF, we were unable to detect biologically active M-CSF in any of our samples despite using a sensitive cell proliferation bioassay. The lack of concordance between receptor expression on circulating phagocytes and cytokine concentrations in serum may reflect the paracrine nature of the haemopoietic microenvironment. Measurements of cytokines in serum may not reflect their concentrations in the bone marrow as cytokines, such as the CSFs produced in the bone marrow stroma, are immobilised by the local extracellular matrix. Alternatively, the abnormal phenotype of PPP phagocytes may be an inherent characteristic transmitted by the neoplastic stem cell rather than the result of cytokine mediated activation. Such a model has been previously proposed to account for increased expression of Fc receptors by neutrophils and platelets in PPP and could involve maturation defects within the abnormal clone. For example, CD64 is expressed by normal granulocyte precursors up to the metamyelocyte but is lost during terminal differentiation. However, the present finding of increased phagocytic activity of PPP neutrophils, despite a lower E coli:neutrophil ratio than for normal subjects in the assay system used, together with abnormally high percentages of CD14+ and CD64+ neutrophils, suggests activation rather than persistence of an immature phenotype.

The clinical complications of PPP, which include manifestations of increased blood viscosity, thrombosis and haemorrhage, are largely attributed to elevated red cell mass and abnormal platelet function. However, the ability of activated normal neutrophils to stimulate platelet aggregation and the expression of procoagulant activity by activated normal macrophages suggests that monocytes and neutrophils could contribute to the pathogenesis of thrombotic complications in PPP.


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