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

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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.

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Primary proliferative polycythaemia (PPP, polycythaemia vera) is a clonal myeloproliferative disorder originating in a neoplastic multilineage stem cell.¹ The principal clinical features of PPP are related to the increased red cell mass and abnormal platelet function.² Granulocytosis is also a frequent finding in PPP and, in most patients, all circulating neutrophils appear to be derived from the abnormal stem cell.³ Monocytes are also likely to be part of the abnormal clone as they and neutrophils are derived from a common antecedent, the colony forming unit granulocyte-macrophage.⁴

Previous studies demonstrate that unstimulated PPP neutrophils have a high glycogen content⁵ and exhibit increased alkaline phosphatase activity,⁶ glycogenolysis,⁷ glucose oxidation, oxygen consumption, and nitroblue tetrazolium reduction,⁸ suggesting that these cells are metabolically activated in vivo. However, there are conflicting reports concerning neutrophil migration,⁹⁻¹¹ adherence^{12,13} and phagocytic activity^{9,14} in PPP and the question of whether neutrophils are functionally activated in PPP remains controversial. Moreover, there have been no equivalent studies of monocytes in these patients.

Many of the functions of monocytes and neutrophils are mediated by surface receptors, the expression of which is upregulated upon activation. For example, the expression of CD11b/CD18 (Mac-1, CR3), which promotes adherence to endothelium and phagocytosis of iC3b coated particles, increases rapidly following phagocyte activation.¹⁵ CD14, a receptor for lipopolysaccharide (LPS)-LPS binding protein complexes, expressed strongly by monocytes but weakly by neutrophils,¹⁶ is upregulated on neutrophils stimulated with cytokines and chemoattractants.¹⁷ Similarly, CD64, the high affinity receptor for IgG (Fc γ RI), is strongly upregulated on monocytes¹⁸ and neutrophils¹⁹ by interferon- γ (IFN- γ). Consequently, CD64 is considered to be a marker of neutrophil activation: it promotes the recognition²⁰ and phagocytosis²¹ of IgG coated erythrocytes and activates the oxidative responses of neutrophils.¹⁹

In the present study we have therefore determined the expression of CD11b/CD18, CD14 and CD64 on monocytes and neutrophils from patients with PPP and have assessed the phagocytic activity of these cells in order to elicit evidence of in vivo activation. Blood concentrations of colony stimulating factors (CSFs) and IFN- γ were also measured to determine whether changes in surface pheno-

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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 $8.1\text{--}16.1 \times 10^9/\text{l}$) and one had a monocytosis ($1.1 \times 10^9/\text{l}$) 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 (Serotec, Oxford, UK) were used for immunofluorescence 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 $\leq 2\%$ 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 opsonised fluorescein isothiocyanate (FITC) labelled *Escherichia coli* (Orpegen, Heidelberg, Germany) were incubated with whole blood (2.5×10^7 *E. coli*/ml blood) at 37°C . Aliquots were removed at intervals up to 90 minutes and mixed with ice cold trypan 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 uningested 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 GNFS60 (G-CSF assay), TF1 (GM-CSF) or MNFS60 (M-CSF) factor dependent cells at 37°C in $5\% \text{CO}_2$ for 48 hours. Cultures were then pulsed with ^3H -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 (2M) 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 χ^2 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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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.

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 PPP²² at

presentation but has not required venesection for over 20 years and therefore is atypical. In contrast to those with PPP, monocytes and neutrophils from patients with secondary polycythaemia and apparent polycythaemia were

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

Surface receptor	Monocytes				Neutrophils			
	PPP (n = 18)	SP (n = 8)	AP (n = 13)	Normal (n = 10)	PPP (n = 18)	SP (n = 8)	AP (n = 13)	Normal (n = 10)
CD11b %	99 (0.3)	99 (0.2)	99 (0.3)	99 (0.3)	100 (0.1)	100 (0.2)	100 (0.2)	100 (0.2)
MFC	65* (9)	54 (9)	51 (9)	36 (6)	42 (6)	39 (8)	46 (9)	39 (13)
CD14 %	75 (2)	75 (2)	80 (2)	78 (3)	36*** (3)	14 (2)	24 (2)	18 (3)
MFC	91 (6)	68* (5)	92 (9)	91 (5)	8 (1)	13 (2)	8 (0.4)	10 (2)
CD18 %	82 (3)	72 (4)	86 (3)	80 (4)	94 (2)	83 (4)	94 (2)	93 (2)
MFC	77 (5)	73 (5)	69 (5)	66 (3)	35 (4)	39 (7)	36 (3)	37 (4)
CD64 %	52* (3)	40 (5)	37 (5)	36 (3)	42† (4)	18 (3)	13 (3)	11 (2)
MFC	47† (4)	29 (2)	24 (2)	26 (2)	8 (1)	10 (1)	8 (1)	10 (2)

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).

PHAGOCYTOSIS

As shown in table 2, the percentage of neutrophils undergoing phagocytosis was higher in patients with PPP (mean 64%) than in normal subjects (mean 42%; *p*<0.02). The time taken to reach half-maximum phagocytosis was lower, but not significantly, in those with PPP

(mean 6.8 minutes) than in normal subjects (mean 13.0 minutes). The patients with PPP in these assays had higher neutrophil counts (mean $7.5 \times 10^9/l$) than the normal subjects ($3.8 \times 10^9/l$) and, therefore, *E. coli*:neutrophil ratios were lower (3.2 *v* 9.1). When data from all subjects were taken into account, the percentage of neutrophils undergoing phagocytosis correlated with the percentage of CD64+ neutrophils (*r*=0.801, *p*<0.01). The phagocytic activity of monocytes from patients with PPP and monocytes and neutrophils from those with apparent polycythaemia were comparable with those from normal subjects.

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

	PPP (n = 6)	AP (n = 5)	Normal (n = 5)
Neutrophils undergoing phagocytosis (%)	64* (4)	36 (5)	42 (5)
Monocytes undergoing phagocytosis (%)	31 (6)	18 (4)	24 (2)

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

Cytokine	PPP (n = 14)	SP (n = 7)	AP (n = 12)	Normal (n = 10)
G-CSF (pg/ml)				
No. of positive samples ^a	11	4	10	6
mean (SEM) ^b	25.1 (3.0)	22.5 (5.8)	16.6 (2.6)	19.2 (3.7)
range	8.7–49.8	11.4–38.7	7.2–33.2	6.1–28.4
GM-CSF (pg/ml)				
No. of positive samples ^a	1	1	0	1
value	11.3	15.7	—	17.8
IFN-γ (IU/ml)				
No. of positive samples ^a	2	0	3	4
mean (SEM) ^b	23.0 (16.5)	—	25.9 (11.5)	43.8 (32.2)
range	6.5–39.5	—	10.0–54.0	4.7–155.3
M-CSF (pg/ml)				
No. of positive samples ^a	0	0	0	0

^aPositive 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).

^bMean (SEM) of positive samples.

SP, secondary polycythaemia; AP, apparent polycythaemia.

CYTOKINES

G-CSF was detected in the serum samples of most subjects (table 3) and there was no significant difference in the frequency of G-CSF positive serum samples between patient groups and normal subjects (χ^2 test). The mean G-CSF concentration in each patient group was comparable with normal and there was no correlation between G-CSF concentration and receptor expression (percentage positive or MFC) for any of the markers tested. IFN-γ was detected in nine of 43 samples and all patient values were within the range observed in normal subjects. GM-CSF could be measured in three serum samples (one PPP, one secondary polycythaemia, one normal) with similar values obtained in each. M-CSF was not detected in any of the samples tested.

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).

Increased expression of the high affinity receptor for IgG, CD64 (FcγRI), is considered to be a marker of monocyte¹⁸ and neutrophil¹⁹ activation. In addition to promoting phagocytosis,^{20,21} CD64 mediates cytotoxicity²⁵ and activates the oxidative burst.¹⁹ IFN-γ up-regulates CD64 on monocytes¹⁸ and neutrophils¹⁹ in vitro and on neutrophils in vivo²⁶ whilst administration of G-CSF stimulates the de novo production of neutrophils expressing CD64²⁷ via its action on myeloid progenitor cells.²⁸ Phagocytes from patients with streptococcal pharyngitis exhibit increased expression of CD64,²⁹ which has been attributed to the endogenous production of IFN-γ²⁶ or G-CSF²⁷ in such infections. CD14, a receptor for LPS-LPS binding protein complexes, mediates phagocyte activation¹⁶ and enhances the adhesive activity of neutrophil CD11b.¹⁷ Expression of CD14 increases during differentiation of myelomonocytic cells¹⁶ and can be further upregulated on mature neutrophils by G-CSF and GM-CSF in vitro¹⁷ and by G-CSF in vivo.³⁰ Thus, the increased percentages of CD64+ monocytes and neutrophils and of CD14+ neutrophils in patients with PPP suggests that these cells were activated in vivo. However, activation of monocytes and neutrophils by inflammatory stimuli results in the rapid upregulation of CD11b/CD18,¹⁵ 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,³¹ 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.³² 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 neutrophils³³ and platelets³⁴ 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.²⁸ 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.² However, the ability of activated normal neutrophils to stimulate platelet aggregation³⁵ and the expression of procoagulant activity by activated normal macrophages,³⁶ suggests that monocytes and neutrophils could contribute to the pathogenesis of thrombotic complications in PPP.

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