

The response of human bone marrow to chronic cigarette smoking

S.F. van Eeden, J.C. Hogg

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ABSTRACT: Chronic cigarette smoking in humans causes leukocytosis. Animal studies show that chronic smoking shortens the transit time of polymorphonuclear leukocytes (PMNLs) through the bone marrow. The present study examines the response of human bone marrow to chronic cigarette smoking.

Three characteristics of peripheral blood PMNLs that indicate active bone marrow release (band cell counts, surface L-selectin expression and myeloperoxidase (MPO) content), were measured in 38 healthy chronic smokers (23±5 pack-yrs) and 15 age- and sex-matched nonsmoking controls.

The total white cell ($6.8\pm0.3\times10^9$ versus $5.3\pm0.2\times10^9$ cells·L⁻¹, $p<0.0001$) and PMNL ($4.2\pm0.18\times10^9$ versus $3.2\pm0.1\times10^9$ cells·L⁻¹, $p<0.003$) counts were higher in smokers as were the percentage (4.8 ± 0.6 versus 1.1 ± 0.2 , $p<0.0001$) and total number ($0.21\pm0.04\times10^9$ versus $0.03\pm0.001\times10^9$ cells·L⁻¹, $p<0.01$) of band cells. Flow cytometry showed that the mean fluorescence intensity (MFI) of L-selectin (3.2 ± 0.2 versus 2.6 ± 0.1 , $p<0.05$) on PMNLs was higher in smokers. There was no difference in the baseline or N-formyl-methionyl-leucyl-phenylalanine-stimulated expression of CD63 or CD18/CD11b (surface markers of PMNL activation) between smokers and controls. The MPO content of PMNLs was higher in smokers (3.4 ± 0.3 versus 1.7 ± 0.2 MFI, $p<0.05$). Smokers with a low (<75% of the predicted value) diffusing capacity of the lung for carbon monoxide had higher PMNL MPO levels than smokers with a diffusing capacity of >75% pred ($p<0.05$).

In conclusion, chronic smoking causes phenotypic changes in circulating polymorphonuclear leukocytes that are characteristic of chronic stimulation of the bone marrow and it is speculated that the increased number of immature polymorphonuclear leukocytes contributes to the chronic lung inflammation associated with cigarette smoking.

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Chronic cigarette smoking produces a 20–25% increase in the peripheral blood leukocyte count compared with nonsmoking control subjects [1, 2]. This chronic increase in circulating leukocyte number correlates with a decrease in lung function over time [3, 4], and polymorphonuclear leukocytes (PMNLs) have been implicated in the pathogenesis of both chronic airways obstruction and emphysema associated with cigarette smoking [5]. The destruction of alveolar walls, the hallmark of emphysema, has been postulated to result from a functional proteolytic imbalance created by cigarette smoking [5]. This imbalance may be augmented by the sequestration of PMNLs in lung capillaries during smoking and the ability of the oxidative potential of PMNLs to inactivate the major antiprotease for neutrophil elastase, (α_1 -antitrypsin [6–10].

The number of circulating PMNLs is influenced by their rate of production in the bone marrow, their release into the circulation, the exchange between the circulating and the marginated pool of intravascular PMNLs and their permanent removal from the circulation. PMNLs are produced in the bone marrow, which is the site of proliferation,

terminal differentiation and maturation [11]. In previously reported animal studies, it was found that cigarette smoke exposure accelerated the release of PMNLs from the bone marrow, which was postulated to be mediated by soluble factors released from the lung [12]. Alveolar macrophages are an important source of pro-inflammatory mediators and, when stimulated by cigarette smoke, produce factors such as tumour necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, IL-8 and haematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) [13, 14]. When these factors circulate they are capable of stimulating haematopoietic precursors resulting in accelerated proliferation and release of PMNLs from the bone marrow into the circulation [15, 16].

The hallmark of an accelerated release of PMNLs from the bone marrow is the presence of an increase in the number of circulating nonsegmented PMNLs or band cells [17, 18]. The L-selectin level on PMNLs also serves as a marker of younger cells in the circulation [19, 20], and this molecule could contribute to PMNL sequestration in lung capillaries. Myeloperoxidase (MPO), an enzyme of

the primary granules of PMNLs, is produced at an early stage of PMNL proliferation in the bone marrow and is divided between progeny with subsequent cell divisions [11]. The authors postulate that an accelerated transit of PMNLs through the mitotic pool of the marrow could cause PMNLs to skip divisions resulting in the release of cells into the circulation with a higher MPO content [11, 12].

The present study compares the phenotypic characteristics of circulating PMNLs in smokers and nonsmokers to test the hypothesis that cigarette smoking in humans is associated with bone marrow stimulation and an accelerated release of PMNLs into the circulation.

Methods

Subjects

The study population consisted of 38 healthy adult smokers and 15 age- and sex-matched nonsmoking controls. The study was approved by the Human Experimentation Committee of St Paul's Hospital and the University of British Columbia.

Inclusion criteria for smokers were: age 25–50 yrs; a smoking history of ≥ 15 cigarettes·day⁻¹ for >3 yrs; and healthy with no symptoms or clinical signs of lung or other inflammatory disease and on no medication. Similarly, the controls were aged 25–50 yrs, lifetime nonsmokers and healthy with no underlying inflammatory disease. On enrolment, all subjects completed a health questionnaire and underwent physical examination, chest radiography, electrocardiography and lung function tests. These tests were done to exclude subjects with acute or chronic diseases that could influence circulating leukocyte counts.

Lung function tests were performed in the following manner: forced vital capacity and forced expiratory volume in one second (FEV1) were determined using spirometry; residual volume was determined with the helium-dilution method; total lung capacity was assessed with plethysmography; and diffusing capacity of the lung for carbon monoxide (DLCO) was obtained using a single-breath method and values corrected for alveolar volume and haemoglobin concentration. The predicted values for each subject based on sex, age and height were obtained from standard tables. Data are expressed as a percentage of the predicted value.

Smokers were requested not to change their smoking habits prior to venepuncture and blood was collected from all subjects between 8:00 and 10:00 h. The smoking status of subjects was assessed from carboxyhaemoglobin concentrations (IL-482 CO-Oximeter; System Instrumentation, Lexington, MA, USA).

Leukocyte counts

Blood samples were collected in standard tubes containing potassium ethylene diamine tetra-acetic acid (EDTA), (Vacutainer; Becton Dickinson, Rutherford, NJ, USA). White blood cell counts were performed using a Sysmex model E4000 (Sysmex Tao, Japan) and differential counts were made on Wright-stained blood smears by counting 100 leukocytes in randomly selected fields of view. Band cells were identified using the American Pathology Society

criteria [21] and the percentage of band cells obtained by counting 100 PMNLs in randomly selected fields of view. Two independent observers who were naive to the identity of the slides performed these counts and interobserver variability was small.

Flow cytometry

Expression of L-selectin, CD11b and CD63 on polymorphonuclear leukocytes. The surface expression of L-selectin, CD11b and CD63 [21] on PMNLs was quantified by means of immunofluorescence using flow cytometry. A whole blood method was used to label PMNLs to prevent artefactual changes in sensitive surface molecules [22]. Whole blood (100 μ L) was incubated with 200 μ L phosphate-buffered saline (PBS), pH 7.3 and 0.1 μ g fluorescein isothiocyanate (FITC)-conjugated antihuman L-selectin DREG 56 (Immunotech, Marseille, France), 0.1 μ g phycoerythrin (PE)-conjugated antihuman CD11b (Dakopatts, Copenhagen, Denmark) or 0.1 μ g FITC-conjugated antihuman CD63 (Immunotech, Marseille, France) for 10 min at room temperature (20°C) in the dark. Nonimmune mouse FITC-conjugated immunoglobulin G2a (Sigma Chemical Co, St Louis, MO, USA) in the same concentration served as a negative control. Cells were washed with PBS, the red blood cells in the samples lysed (Immunolyse; Coulter Electronics, Hialeah, FL, USA), leukocytes fixed with 1% paraformaldehyde and stored at 4°C. Cells were analysed using flow cytometry (Epics XL; Coulter Electronics) within 24 h. Analysis gates for PMNLs were established using distinctive forward- and side-scatter profiles and results expressed as the mean fluorescence intensity (MFI) of 5,000 cells.

To determine whether PMNLs are primed, 100 μ L of whole blood was incubated for 10 min in a waterbath at 37°C with 10 nM cytochalasin B in 0.02% dimethyl sulphoxide (Sigma Chemical Co), the receptor stimulus N-formyl-methionyl-leucyl-phenylalanine (fMLP; 10 nM final concentration; Sigma Chemical Co) was added and the blood was incubated for 10 min. The reaction was terminated by washing the sample twice with a large volume of PBS (15 mL). Cells were labelled for L-selectin, CD11b and CD63 and analysed as described above. CD63 is a primary granule membrane protein and surface expression is a marker of degranulation of primary granules containing MPO [23].

Leukocyte myeloperoxidase levels

The MPO content of PMNLs was determined using the FITC-conjugated monoclonal antibody ((Dakopatts, Copenhagen, Denmark). Briefly, 100 μ L of whole blood and 100 μ L of fixative (reagent A of "Fix and Perm®"; Caltag, Austria) were incubated for 10 min at room temperature, washed twice in PBS, and the cell pellet suspended in 100 μ L of reagent B (Fix and Perm®) with 0.2 μ g of FITC-conjugated MPO. Cells were incubated for 15 min, washed in PBS, fixed with 1% paraformaldehyde and stored at 4°C. Cells were analysed using flow cytometry as described above.

Double labelling

To evaluate the relationship between PMNL MPO levels and surface markers, leukocytes were labelled for MPO

(FITC) as well as either L-selectin or CD11b. Whole blood (100 µL) was incubated with 200 µL PBS, pH 7.3 and 0.1 µg PE-conjugated antihuman L-selectin (immunotech); or 0.1 µg of PE-conjugated antihuman CD11b for 10 min at room temperature in the dark. Nonimmune mouse PE-conjugated IgG2a (Sigma Chemical Co) in the same concentration served as negative control. Cells were prepared as described for MPO labelling. Cells were analysed using flow cytometry and either L-selectin or CD11b were analysed on FITC-positive (MPO label) PMNLs that had low (30% of PMNLs with the lowest levels of MPO) and high (30% of PMNLs with the highest levels of MPO) levels of MPO. A total of 5,000 cells were analysed in each population.

Statistical analysis

All values are expressed as mean±SEM. Analysis of variance was carried out to compare leukocyte counts, cell surface markers and PMNL myeloperoxidase content. Bonferroni corrections for multiple comparisons were performed where appropriate and a p-value of <0.05 was accepted as significant.

Results

Subject characteristics

Table 1 shows the demographic data, carboxyhaemoglobin levels and lung function of the two groups. The groups were comparable with regard to age and sex. Smokers smoked 15–40 cigarettes·day⁻¹ at the time of the study with a mean 23±1 pack-yrs (range 15–41) history. This was reflected in higher carboxyhaemoglobin levels (5±0.4%) than controls (2.2±0.2%). Both FEV₁ (p<0.05) and DL_{CO} (p<0.001) (% pred) were lower in the smokers but values were still within the accepted normal range.

Peripheral blood cell counts

Table 2 summarizes the blood cell counts in the two groups. Peripheral white blood cell counts were higher in the smokers, mainly due to an increase in PMNL counts

Table 1. – Demographic parameters

	Smokers	Controls	p-value
Subjects n	38	15	
Age	37±1.2	34±2.9	NS
Sex ratio male/female	0.48	0.58	NS
Smoking			
Cigarettes·day ⁻¹ *	23±5	0	-
Pack-yrs**	23±1	0	-
FEV ₁ % pred	105±2.5	114±3.2	0.05
DL _{CO} % pred	83±2.6	103±3.1	0.001
TLC % pred	94±5	103±6	NS
HbCO (%)	5.1±0.4	2.2±0.2	0.01

Data are presented as mean±SEM. *: in the preceding 3 months; **: 20 cigarettes·day⁻¹ for 1 yr. FEV₁: forced expiratory volume in one second; DL_{CO}: diffusing capacity of the lung for carbon monoxide (corrected for alveolar volume and haemoglobin concentration); TLC: total lung capacity; HbCO: carboxyhaemoglobin; % pred: percentage of the predicted value (for age, sex and height).

Table 2. – Peripheral blood leukocyte counts in smokers and nonsmoking controls

	Smokers	Controls	p-value
Subjects n	38	15	
Total leuc 10 ⁹ cells·L ⁻¹	6.8±0.3	5.3±0.2	0.001
PMNLs 10 ⁹ cells·L ⁻¹	4.2±0.18	3.2±0.19	0.003
Band cells 10 ⁹ cells·L ⁻¹	0.18±0.04	0.031±0.006	0.01
%	4.8±0.6	1±0.2	0.0001
MCs 10 ⁹ cells·L ⁻¹	2.6±0.16	2.1±0.18	NS

Data are presented as mean±SEM. leuc: leukocyte; PMNL: polymorphonuclear leukocyte; MCs: mononuclear cells (including lymphocytes and monocytes).

(p<0.003). The total number (p<0.01) as well as the percentage (p<0.0001) of nonsegmented PMNLs or band forms was higher (p<0.01) in the smokers. The numbers of other leukocytes (lymphocytes, monocytes and eosinophils) were not different between the groups.

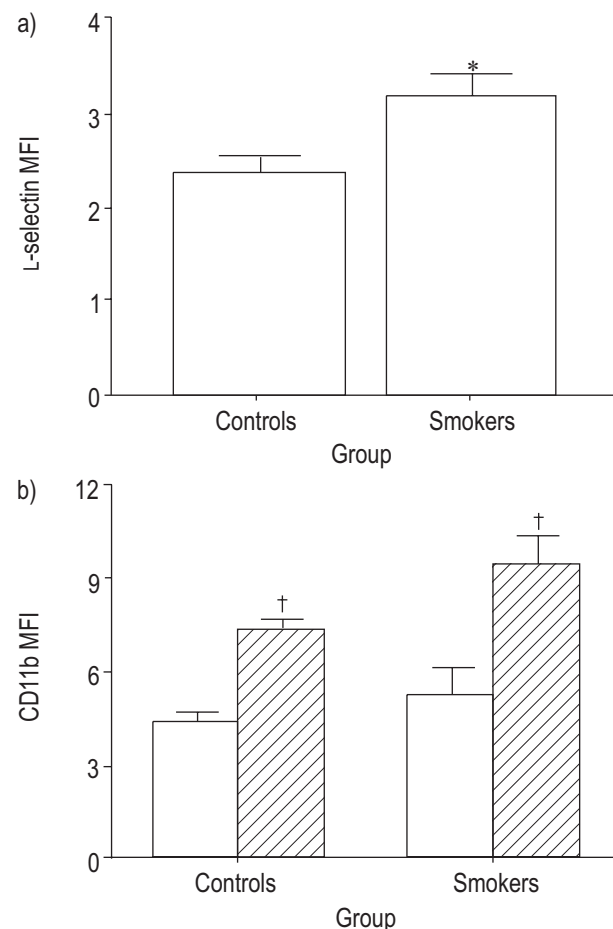


Fig. 1. – a) L-selectin expression on circulating polymorphonuclear leukocytes (PMNLs) as measured by flow cytometry using the monoclonal antibody DREG 56 (see *Flow cytometry* section). L-selectin levels on PMNLs in smokers were higher than in control subjects. b) CD11b expression on circulating PMNLs as measured by flow cytometry using a phycoerythrin-conjugated monoclonal antibody (see *Flow cytometry* section). Baseline expression (□) between groups was similar. A similar increase in expression was observed following stimulation with 10 nM N-formyl-methionyl-leucyl-phenylalanine (▨). Data are presented as mean±SEM (38 smokers and 15 controls). MFI: mean fluorescence intensity. *: p<0.05 versus controls; †: p<0.05 versus baseline.

Surface L-selectin, CD11b and CD63 expression on circulating polymorphonuclear leukocytes

The expression of L-selectin on circulating PMNLs was higher in smokers (fig. 1a) and decreased to a similar extent in smokers and controls following fMLP stimulation (data not shown). There was no difference in the baseline CD11b levels between smokers and controls (fig. 1b) and stimulation of PMNLs with fMLP caused a more than two-fold increase in CD11b expression in smokers that was similar to changes in the controls (fig. 1b). Baseline CD63 expression was no different from background readings in both groups and increased to the same level with fMLP stimulation (1.8 ± 0.2 in controls *versus* 1.84 ± 0.1 in smokers).

Myeloperoxidase levels in circulating polymorphonuclear leukocytes

Myeloperoxidase levels in PMNLs from smokers were higher than those from controls (fig. 2a). On subgroup

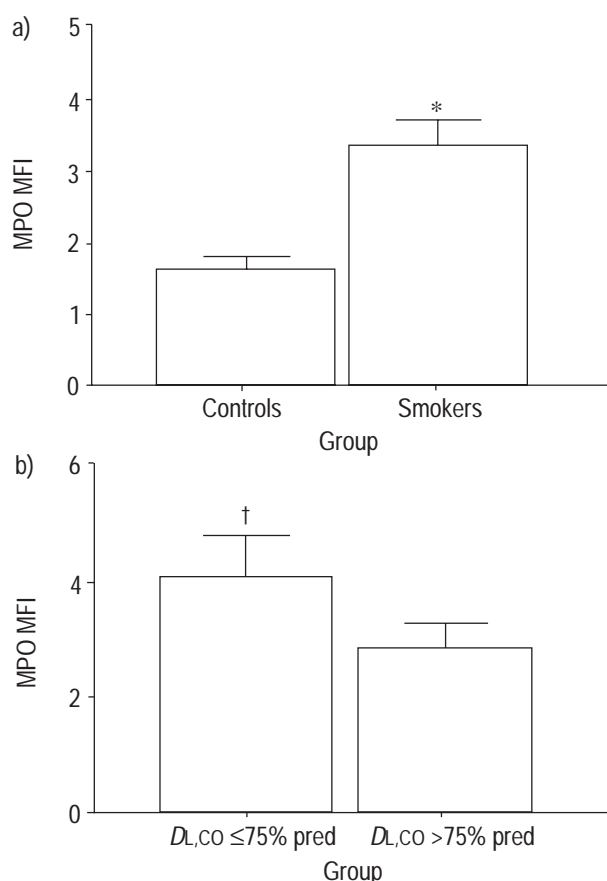


Fig. 2. – Myeloperoxidase (MPO) content of circulating polymorphonuclear leukocytes (PMNLs) as measured by flow cytometry using a fluorescein isothiocyanate-conjugated monoclonal antibody (see *Double labelling* section): a) in 38 smokers and 15 controls; and b) after dividing the subjects into those with a diffusing capacity of the lung for carbon monoxide (DL_{CO}) of $\geq 75\%$ of the predicted value ($n=21$) and those with a DL_{CO} of $>75\%$ pred ($n=17$) (both corrected for alveolar volume and haemoglobin concentration). Data are presented as mean \pm SEM. MFI: mean fluorescence intensity. *: $p < 0.05$ versus controls; †: $p < 0.05$ versus $DL_{CO} > 75\%$ pred.

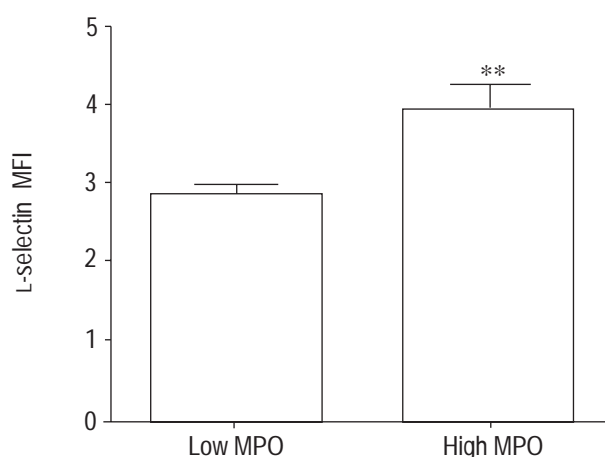


Fig. 3. – L-selectin expression on polymorphonuclear leukocytes (PMNLs) with high and low myeloperoxidase (MPO) levels. PMNLs were double labelled with fluorescein isothiocyanate-conjugated anti-MPO monoclonal antibodies and phycoerythrin-conjugated anti-L-selectin antibodies. L-selectin was measured on the 30% of PMNLs with the highest and lowest levels of MPO. Data are presented as mean \pm SEM. MFI: mean fluorescence intensity. **: $p < 0.01$ versus low MPO.

analysis, smokers were divided into those with a DL_{CO} of $\leq 75\%$ pred ($n=21$) and those with a DL_{CO} of $>75\%$ of pred ($n=17$) (both corrected for alveolar volume). The current and past smoking history of these two groups was similar and there were no differences in their total white blood cell, PMNL and band cell counts (data not shown). However, the PMNL MPO content (fig. 2b) was significantly higher in the smokers with DL_{CO} of $<75\%$ pred ($p < 0.05$).

Double labelling experiments showed that PMNLs with high levels of MPO also have high levels of surface L-selectin (fig. 3). CD11b expression was similar in the PMNLs with high and low levels of MPO (data not shown).

Discussion

This study confirms previous findings that chronic cigarette smoke exposure caused leukocytosis and a neutrophilia [1–4]. In addition, the neutrophilia was associated with an increase in numbers of circulating band cells, a hallmark of early bone marrow release of PMNLs and an increase in L-selectin expression on PMNLs, indicating a larger number of PMNLs recently released from the bone marrow [11, 16]. These two findings support the hypothesis that cigarette smoking stimulates the bone marrow. Based on previous animal studies [12, 24], it is suspected that this stimulation causes an accelerated transit time of PMNLs through the bone marrow, resulting in the release of younger less mature PMNLs into the circulation. These PMNLs that sequester preferentially in lung microvessels have a higher level of MPO (fig. 2a) [24] and could play an important role in the pathogenesis of smoke-induced lung disease.

Chronic smokers show a 20–30% increase in their circulating total white blood cell counts, primarily due to an increase in granulocytes, suggesting stimulation of the bone marrow with a higher turnover of these leukocytes

[1–4]. An increase in circulating PMNL counts is a hallmark of the systemic response to stress [17, 18, 25]. Mild stress such as exercise increases the circulating PMNL counts without an increase in band cell counts [26], in contrast to more severe stress, such as infection, which causes an increase in circulating PMNL and band cell counts [17, 18]. The neutrophilia in the smokers reported here was associated with a four-fold increase in the percentage of circulating band cells (table 2), which provides definitive evidence of accelerated bone marrow release of granulocytes.

The expression of the cell adhesion molecule, L-selectin, on circulating PMNLs was higher in the smokers compared to the control subjects. L-selectin is constitutively expressed on circulating PMNLs [27] and it has been shown that PMNLs released from the bone marrow as a result of stimuli such as intravascular complement activation [19] or pneumococcal pneumonia [20] express high levels of L-selectin. Studies from the authors' own [28, 29] and other [30] laboratories have shown that PMNLs in the maturation pool of the bone marrow express high levels of L-selectin and that active bone marrow release enriches the circulating pool with PMNLs expressing high levels of L-selectin [19, 20]. The present study shows that the circulating PMNLs of chronic smokers express higher levels of L-selectin than controls (fig. 1), suggesting that active bone marrow release of PMNLs into the circulation is an important mechanism for the granulocytosis induced by cigarette smoking.

MPO, present in the primary granules of PMNLs, is formed at an early stage (promyelocyte) in the bone marrow and the number of these granules per cell is reduced by mitoses as the cells divide in the mitotic pool [11]. BRIDGES *et al.* [31] reported that PMNLs from smokers have greater MPO activity than those from nonsmokers, whereas other workers found no difference [32]. The present study supports the findings of BRIDGES *et al.* [31] and DASH *et al.* [33] showing high MPO levels in the PMNLs of chronic smokers. The authors' previous animal studies show that chronic cigarette smoking shortens the transit time of myeloid cells through the mitotic pool in the marrow, and the authors suspect that PMNLs skip divisions in the marrow and are released into the circulation with high MPO levels [12]. The double labelling experiments also showed that the PMNLs with the highest levels of MPO have the highest levels of surface L-selectin (fig. 3). This finding suggests that smoking causes the release of a population of younger cells with high MPO levels due to a rapid transit through the bone marrow [28, 34].

The increase in circulating band cell counts, the high levels of L-selectin on circulating PMNLs and the increased MPO content of circulating PMNLs in smokers all support the hypothesis that cigarette smoking causes bone marrow stimulation. There is a growing body of evidence that PMNLs released from activated bone marrow are more harmful to the host [20, 24, 28, 35, 36]. L-selectin initiates the adherence of PMNLs to endothelium under conditions of flow and has been shown to be important for the recruitment of PMNLs to inflamed tissue [37, 38]. DOYLE *et al.* [39] have shown that L-selectin contributes to the prolonged sequestration of PMNLs in lung capillaries. The high levels of L-selectin on PMNLs (fig. 1) may contribute to prolonged retention of PMNLs in lung capillaries and also signifies younger cells that are less de-

formable, which could further enhance the retention of these cells in lung capillaries. The finding of high levels of L-selectin on PMNLs from smokers is in contrast to that of PITZER *et al.* [40] of no changes in levels of CD18 and L-selectin on circulating PMNLs from smokers. They postulate that PMNLs with enhanced adhesion properties due to activation by cigarette smoke are all sequestered in lung microvessels.

There is a marked increase in the mobility, deformability and chemotactic responsiveness of PMNLs during their maturation in the postmitotic pool in the bone marrow [41–43]. PMNLs in the marrow are larger and less deformable than those in peripheral blood [41], and when prematurely released are predisposed to sequestration in lung capillaries [20, 28]. MACNEE *et al.* [44] have shown that PMNLs are retained in the human lung by cigarette smoking, and recent studies by TERASHIMA *et al.* [24] have shown that immature PMNLs are preferentially sequestered in lung microvessels. Assuming a cardiac output of $5 \text{ L} \cdot \text{min}^{-1}$ and using circulating band cells as representative of younger immature PMNLs, the calculated burden of these immature PMNLs in the lung is ~ 5.8 times higher in smokers than in controls (1.296×10^9 versus 2.23×10^{11} band cells $\cdot 24 \text{ h}^{-1}$). Activation of these PMNLs sequestered in the pulmonary capillaries could contribute to the destruction of alveolar walls and the development of emphysema.

Activated PMNLs release an array of toxic products that include proteolytic enzymes, reactive oxygen species and cationic proteins that have been implicated in the damage to alveolar walls leading to emphysema [5–10]. The increased levels of MPO in the PMNLs of smokers (fig. 2a) would show increased redox potential when activated by cigarette smoke. Similar to MPO, other proteolytic enzymes such as elastase, cathepsin G and proteinase 3 are also present in the primary granules and preformed early in cell division in the marrow. The authors postulate that PMNLs loaded with these enzymes are retained in lung capillaries and, when activated by cigarette smoking, could release these enzymes (including MPO) that are damaging to alveolar walls. As only 15–20% of smokers develop emphysema [45], for reasons that are not clear, it is possible that the bone marrow response to cigarette smoking is an important determinant for the development of emphysema. The higher MPO levels in healthy smokers with a low DL_{CO} (fig. 2b) support the hypothesis that the response of the bone marrow to cigarette smoking could be an important determinant for the development of lung destruction and emphysema.

The mechanisms that control the release of PMNLs from the bone marrow into the circulation are poorly understood. Releasing factors such as glucocorticoids, endotoxins, complement fragments and haematopoietic growth factors such as GM-CSF and G-CSF have all been implicated in the initiation of PMNL release [46, 47]. Stimulation of the bone marrow by cigarette smoke is probably mediated by soluble factors released from the lung. Alveolar macrophages are an important source of pro-inflammatory soluble factors and when stimulated by cigarette smoke produce factors such as $\text{TNF-}\alpha$, IL-1, IL-8 and GM-CSF [13, 14]. These factors are capable of stimulating haematopoietic precursors in the marrow and cause the release of granulocytes from the bone marrow into the circulation [46, 47].

In conclusion circulating polymorphonuclear leukocytes from smokers have phenotypic characteristics that indicate that they are released from a stimulated bone marrow. These phenotypic characteristics contribute to the preferential sequestration of polymorphonuclear leukocytes in the lung (cell immaturity and high L-selectin expression), and could enhance their ability to damage alveolar walls (high myeloperoxidase content). The authors postulate that the response of bone marrow to cigarette smoking plays an important role in the pathogenesis of chronic obstructive lung disease.

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