Pulmonary granulocyte kinetics in relation to endothelial and granulocyte activation

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A B S T R A C T

The aim of the study was to measure the peripheral blood levels of soluble E-selectin in patients with systemic inflammation and compare them with *in vivo* granulocyte activation, pulmonary intravascular granulocyte pooling, pulmonary extravascular granulocyte migration and ^{99m}Tc-diethylenetriaminepenta-acetic acid (DTPA) aerosol clearance, an index of lung injury. The level of soluble E-selectin was measured by capture ELISA. Granulocytes were labelled with ^{111}In and ^{99m}Tc for quantification of pulmonary granulocyte kinetics. The pulmonary vascular granulocyte pool (PGP) was expressed as a fraction of the total blood granulocyte pool. Pulmonary granulocyte migration was quantified on 24-h images using the ^{111}In signal. Granulocyte activation was quantified as the percentage of circulating cells showing shape change (' primed '). Lung injury was assessed from the clearance rate of inhaled ^{99m}Tc-DTPA aerosol. Eighteen patients with systemic inflammation were studied: five with inflammatory bowel disease, eight with systemic vasculitis, four with graft versus host disease and one with a recent renal transplant. The peripheral blood levels of soluble E-selectin were significantly elevated in patients with systemic inflammation. The level of soluble E-selectin showed a significant association with granulocyte migration (Spearman rank correlation coefficient, $Rs = 0.53$; $P < 0.05$) but not with PGP or with the percentage of cells showing shape change ($P > 0.05$ for both). Granulocyte migration was bimodal: patients were therefore subdivided into ' migrators ' and ' non-migrators '. Soluble E-selectin level, ^{99m}Tc-DTPA clearance and PGP, but not the percentage of cells showing shape change, were significantly higher in migrators than in non-migrators. We conclude that pulmonary intravascular granulocyte pooling is increased in the presence of increased numbers of circulating primed granulocytes but increased pooling does not by itself promote granulocyte migration into the lung interstitium. Insofar as an elevated level of E-selectin in peripheral blood reflects vascular endothelial activation, the data are consistent with the notion that pulmonary endothelial activation is required, in addition to granulocyte activation and an expanded PGP, for granulocyte migration into lung parenchyma and, therefore, for lung injury to occur.

INTRODUCTION

Granulocytes play a crucial role in acute lung injury [1,2], although the mechanisms involved remain controversial. Exposure of granulocytes to pro-inflammatory factors increases their cell stiffness through effects on the cytoskeleton and promotes a delay in pulmonary vascular transit [3,4]. However, increased pulmonary intra-

Key words: E-selectin, human granulocyte activation, labelled granulocytes, lung granulocyte kinetics.

Abbreviations: DTPA, diethylenetriaminepenta-acetic acid; IBD, inflammatory bowel disease; PGP, pulmonary vascular granulocyte pool; TBGP, total blood granulocyte pool.

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vascular pooling of granulocytes by itself may not be associated with lung damage, which also requires granulocyte migration into the pulmonary interstitium [5–7]. What makes granulocytes migrate or not migrate from an expanded pulmonary vascular granulocyte pool, and consequently injure the lung, is not clear.

The independence of granulocyte activation and migration in the lungs was recently emphasized in an imaging study using ^{111}In-labelled granulocytes and [^{18}F]fluorodeoxyglucose which showed that, in bronchiectasis, granulocytes migrate into the airways in large numbers but do not subsequently take up [^{18}F]fluorodeoxyglucose [8]. In this context, the relative importance of granulocyte and endothelial activation in granulocyte migration across the pulmonary endothelium is not fully understood. Circulating cytokines may be capable of inducing activation of both circulating granulocytes and pulmonary vascular endothelium, with simultaneous upregulation of several endothelial adhesion molecules [9–15]. The aim of this paper was to compare the soluble E-selectin level, as a marker of vascular activation, with pulmonary granulocyte kinetics in order to improve our understanding of the factors which promote granulocyte margination (pooling) in the pulmonary vasculature and migration into the lung interstitium.

As described in an earlier paper [7], we used granulocytes double-labelled with ^{99m}Tc and ^{111}In to measure pulmonary intravascular granulocyte pooling and pulmonary extravascular migration, respectively, in patients with severe systemic inflammation. The much higher photon flux of ^{99m}Tc was exploited to acquire dynamic data after bolus injection on which measurement of pooling is based, whereas the longer physical half-life and greater intracellular stability of ^{111}In was utilized to measure migration from static images obtained 24 h after injection. The level of circulating soluble E-selectin was then correlated with the above-mentioned parameters of granulocyte kinetics, with *in vivo* granulocyte activation based on an *in vitro* shape-change assay, and with a sensitive index of lung damage, ^{99m}Tc-diethylenetriaminepenta-acetic acid (DTPA) aerosol clearance. Shape change parallels the state of the granulocyte termed 'priming', a condition in which granulocytes show an increased response to agents which stimulate superoxide release [16,17].

METHODS

Granulocyte kinetics in relation to soluble E-selectin in peripheral blood were studied in 18 non-smoking adults with systemic inflammation. Five of the 18 were referred for labelled granulocyte scintigraphy for diagnosis or follow-up of known inflammatory bowel disease (IBD) (two with ulcerative colitis, three with Crohn's disease; age range 22–50 years). All had abnormal scintigraphy, indicating active disease. Eight patients had clinically active systemic vasculitis (two with Behcet's syndrome, four with Wegener's granulomatosis and two with antineutrophil cytoplasmic antibody-positive systemic vasculitis; aged 26–73 years), four had graft versus host disease after bone marrow transplantation (performed for chronic myeloid leukaemia; aged 33–52 years), and one (aged 35 years) was the recipient of a recent renal transplant. Clinical management of the 18 patients was variable. Patients with vasculitis received prednisolone and azathioprine. All the bone marrow transplant recipients were on cyclosporin; they had also received total body irradiation and cyclophosphamide for conditioning before transplantation. Minor and variable abnormalities of lung function were documented in the vasculitic and marrow transplant patients but not in those with IBD.

Pulmonary granulocyte kinetics were studied with granulocytes double-labelled with ^{111}In and ^{99m}Tc (i.e. each cell labelled with both radionuclides), as described previously [18]. The injected doses of ^{99m}Tc and ^{111}In were 148 (S.D. 12) and 6.8 (0.21) MBq respectively. A dynamic first-pass study was acquired for 15 min on the ^{99m}Tc photopeak, utilizing the high-count density of this radionuclide. Regions of interest were placed over the right ventricle and right lung and time–activity curves generated for quantification of the pulmonary vascular granulocyte pool (PGP) as a fraction of the total blood granulocyte pool (TBGP), as described and validated previously [7,19]. As previously described [7,20], extravascular granulocyte migration was quantified from regions of interest drawn over the right lung and posterior iliac bone marrow (adjacent to the sacro-iliac joint) on static images recorded on the ^{111}In photopeak 24 h after injection, utilizing the high intracellular stability of this radionuclide. The contribution made by labelled granulocytes within ribs to the chest image was subtracted by quantifying the uptake in posterior iliac bone marrow (adjacent to the sacro-iliac joints) [20]. The basis of the measurement of extravascular granulocyte migration is that by 24 h there are no longer significant numbers of intravascular labelled cells [21]. Granulocyte shape change was measured by visual inspection after fixation by glutaraldehyde [16]. The granulocytes tested were from the cell pellet isolated for labelling, which itself has virtually no effect on this assay [18]. Pulmonary alveolar epithelial permeability was measured from the rate of clearance of an inhaled aerosol of ^{99m}Tc-DTPA 24 h after injection of labelled cells when background counts from ^{99m}Tc-labelled cells were negligible [7]. Soluble E-selectin was measured using two-antibody capture ELISA [22] in an aliquot of blood taken from the peripheral venous sample obtained for cell isolation and labelling. This sample was obtained at about 09.30 hours. Our lab-

oratory's normal range for soluble E-selectin is based on samples from normal subjects taken during the normal working day.

Non-parametric statistics were employed for the assessment of differences between variables (Wilcoxon rank sum test) and to test significance of association (Spearman rank correlation coefficient). The study was approved by the local Ethics Committee of the Hammersmith Hospital and Royal Postgraduate Medical School and by the Administration of Radioactive Substances Advisory Committee of the Department of Health of the United Kingdom.

RESULTS

Compared with our laboratory's normal value of 0.07 (range up to 0.15) μg/ml, soluble E-selectin was increased in systemic inflammation: 0.71 (range 0.28–1.9) μg/ml in patients with transplants ($n = 5$, $P < 0.05$), 0.5 (0.22–1.0) μg/ml in vasculitis ($n = 8$, $P < 0.05$) and 0.29 (0.14–1.0) μg/ml in IBD ($n = 5$, $P < 0.05$). As previously reported [7], values of migration, PGP, shape change and ^{99m}Tc-DTPA clearance are all elevated in patients with systemic inflammation. There is a clear trend for the inflammatory conditions to be ranked in the order of transplant recipients, vasculitis and IBD; soluble E-selectin levels rank in the same order (Table 1).

As previously reported [7], the distribution of granulocyte migration values was bimodal, ranging from 0.02 to 0.2 c.p.m. $\cdot$ pixel^{-1} $\cdot$ MBq^{-1} ($n = 7$) and from 0.55 to 2.66 c.p.m. $\cdot$ pixel^{-1} $\cdot$ MBq^{-1} ($n = 11$) (Figure 1). Values for soluble E-selectin, PGP, ^{99m}Tc-DTPA clearance and granulocyte shape change were therefore compared between these two patient groups, denoted 'non-migrators' and 'migrators' respectively. There was a significant difference between the two groups for soluble E-selectin level (median 0.60 μg/ml in migrators versus 0.29 μg/ml in non-migrators, $P < 0.05$), PGP (median 0.33 in migrators versus 0.23 in non-migrators, $P < 0.05$) and for ^{99m}Tc-DTPA clearance (median $t\frac{1}{2}$ in migrators 37 min versus 59 min in non-migrators, $P < 0.05$) (Figure 2). However, there was no significant difference for shape change (median 39% in migrators versus 29% in non-migrators, $P > 0.05$).

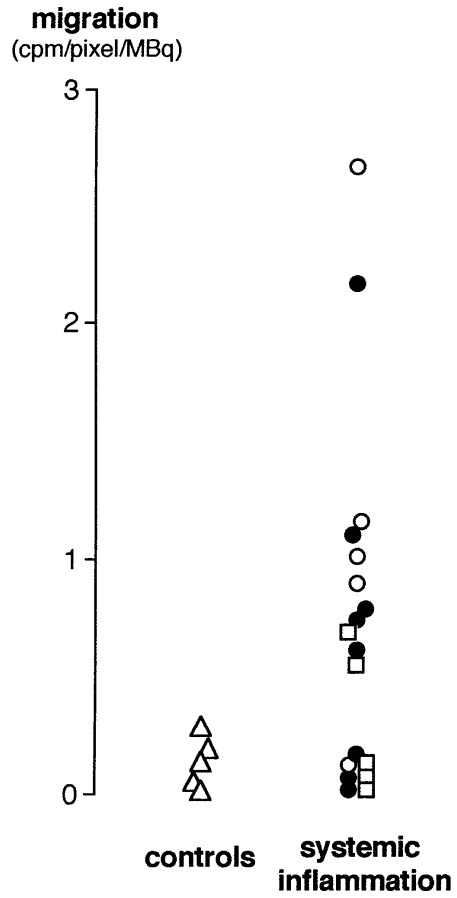

Figure 1 Values for the index of granulocyte migration in the 18 patients with systemic inflammation associated with recent transplantation (○), systemic vasculitis (●) and IBD (□), compared with five patient controls without evidence of inflammatory disease (△) [7]

The distribution of values appears to be bimodal.

The level of soluble E-selectin showed a significant association with migration ($Rs = 0.53$, $P < 0.05$), weak associations with shape change ($Rs = 0.42$, $0.05 < P < 0.1$) and ^{99m}Tc-DTPA clearance ($Rs = 0.23$), but no association with PGP ($Rs = 0.08$) (Figure 3).

Table 1 Values of ^{99m}Tc-DTPA clearance, migration, PGP, shape change and soluble E-selectin in patient groups

Medians shown; ranges not given for clarity. *Data from [7].

	DTPA (min)	Migration (c.p.m. $\cdot$ pixel^{-1} $\cdot$ MBq^{-1})	PGP (%)	Shape change (%)	E-selectin (μg/ml)
Transplant recipients	37	101	31	41	0.71
Vasculitis	42	68	30	36	0.5
IBD	57	34	24	23	0.29
Controls*	75	14	14	17	0.07

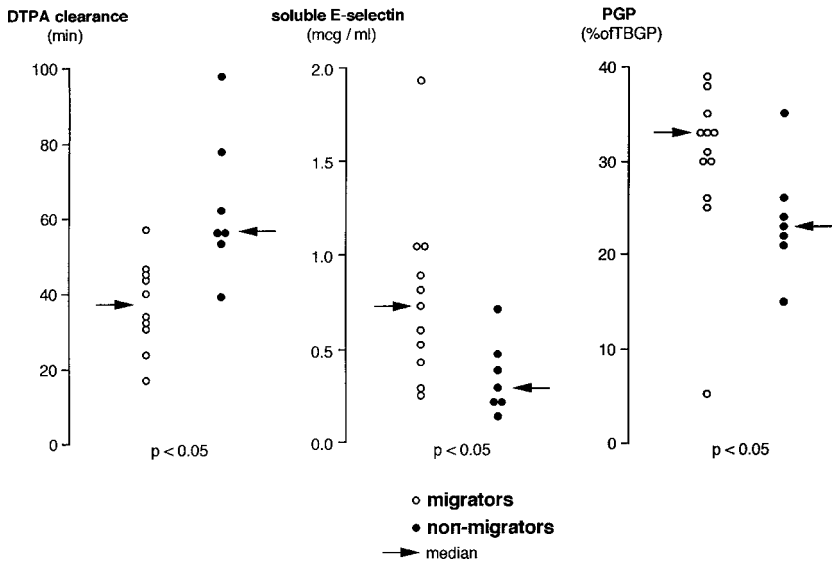

Figure 2 Values for ^{99m}Tc-DTPA clearance, soluble E-selectin and PGP in patients in whom the migration index was not more than 0.2 c.p.m. · pixel^{-1} · MBq^{-1} ('non-migrators') compared with those in whom the index was not less than 0.55 c.p.m. · pixel^{-1} · MBq^{-1} ('migrators')

All three variables are significantly higher in migrators compared with non-migrators.

DISCUSSION

The conclusions from this study depend on the validity of the interpretation of the kinetic measurements that were made. First, it is reasonable to assume that the early lung signal from injected granulocytes reflects granulocyte pooling, an interpretation that has been the basis for numerous previous studies of granulocyte traffic through the lungs [23–25]. Secondly, since ^{111}In-labelled granulocytes circulate in the blood with a $t_{\frac{1}{2}}$ of only about 6 h [21], it is also reasonable to assume that the residual ^{111}In signal in the lung after 24 h represents cell migration. Although some authors, directly observing pulmonary capillaries through skin windows, have described the presence of granulocytes sequestered in pulmonary capillaries for periods of many minutes to hours [26–28], these must represent a dwindling minority of the pulmonary granulocyte population, even in patients with an expanded PGP, otherwise a very large fraction of the whole-body TBGP would eventually accumulate in the pulmonary vasculature. Thirdly, shape change is a simple, rapid, robust and well-validated method of assessing the effect of activating stimuli on polarization of cytoskeletal elements and is therefore relevant for assessing whether re-infused granulocytes are likely to be retarded during passage through the pulmonary capillaries as a result of reduced deformability [29]. Shape-changed cells are likely to be primed [16,17]. Fourthly, E-selectin is a cytokine-inducible adhesion molecule which is expressed by endothelial cells in inflammation. Although it is largely internalized after expression, some is shed into the circulation and its blood level therefore reflects generalized vascular activation. A circadian rhythm for soluble E-selectin has recently been demonstrated in normal subjects with a maximum value at noon and a minimum value at midnight, about 12% less than the maximum [30]. The blood samples for measurement of soluble E-selectin in the patients with systemic inflammation were obtained at about 09.30 hours. The values from which our normal range is derived are based on samples taken during the working day, so any variation resulting from this circadian rhythm and its impact on the assessment of the levels in the patients should be minimal.

Although, as we have previously suggested [25,31], there is normally only modest granulocyte pooling in human lungs, PGP is usually increased in patients with severe systemic inflammatory conditions, including active IBD, vasculitis and graft versus host disease [7,19,32,33], or increased briefly in normal subjects by inhalation of inflammatory mediators such as platelet activating factor [34,35]. A form of cell activation also occurs *in vitro* during labelling itself and this may contribute to a delay in granulocyte transit through the lung vasculature [36]. Nevertheless, these 'artefactual' kinetics are different, with relatively rapid clearance of the pulmonary activity, a very low recovery of labelled cells in circulating blood and, ultimately, prominent uptake by the liver [36], and were not seen in the current study. Although systemic inflammation is usually associated with an expanded PGP, these patients do not display evidence of overt lung damage nor is an increased PGP necessarily associated with granulocyte migration into the pulmonary interstitium [6–8]. Nevertheless, granulocyte migration as described here correlates with a

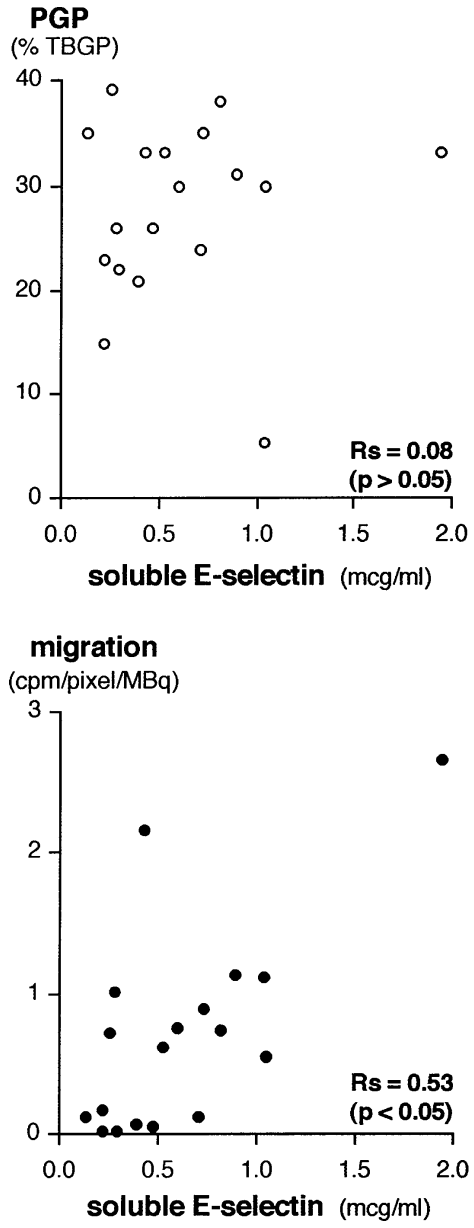

Figure 3 Associations of soluble E-selectin with PGP and migration index

The association is significant for migration but not for PGP.

sensitive index of lung damage, ^{99m}Tc-DTPA clearance, suggesting that injury, even subclinical injury, requires granulocyte migration to occur. Granulocytes are larger than pulmonary capillaries [29] and are normally trapped for variable periods during transit through the pulmonary microvessels [26,27]. Transit time is delayed after granulocytes have been exposed to inflammatory stimulation *in vivo* [37] or to pro-inflammatory mediators *in vitro* before injection [4], in part due to a reduction in their deformability [3,4,28].

In several previous studies based on microscopy or flow cytometry, granulocytes have been shown to undergo shape change in vasculitis [38], IBD [39], renal transplant recipients [40] and vascular graft recipients [41], probably as a result of exposure to locally generated cytokines in inflamed tissue. Shape-changed granulocytes have reduced deformability and, as a result, take longer to transit the lungs. This accounts for the association between shape change and PGP. E-selectin, while promoting granulocyte margination in systemic capillaries, is less likely to play a significant role in delaying the transit of shape-changed granulocytes through the lung since this is adequately explained by reduced deformability. The lack of an association, therefore, between soluble E-selectin and PGP may not be surprising and is consistent with the notion that E-selectin is not involved in leucocyte interactions with lung capillaries. On the other hand, if soluble E-selectin originates predominantly from peripheral tissues in systemic inflammation, then insofar as shape change is a marker of inflammatory activity, an association between soluble E-selectin and PGP would be anticipated. There was a weak association between soluble E-selectin and shape change but the association between soluble E-selectin and pulmonary granulocyte migration was stronger, hinting that soluble E-selectin may partly reflect other elements of pulmonary endothelial activation which promote migration into the pulmonary interstitium.

In accordance with the above argument, the role of E-selectin in experimental pulmonary inflammation is poorly defined. In acute pulmonary injury, E-selectin has been shown to be up-regulated in proportion to leucocyte accumulation and to markers of lung injury [42–44]. Furthermore, monoclonal antibodies to E-selectin or its ligand have been shown to prevent leucocyte accumulation and protect against lung injury [44–47]. There are several reports, however, showing that neutrophil accumulation in the lung and subsequent lung injury are E-selectin-independent [48–52]. Given that even quiescent granulocytes transit the lung slowly, it is not obvious why the lung needs to express E-selectin, the function of which at least systemically is to retard passing granulocytes. The E-selectin expressed in these models may be located more in large vessels than capillaries [53], through which, it is claimed, granulocytes migrate to the pulmonary interstitium [6,54]. The situation is further complicated by the fact that in many models of experimental lung inflammation it is not explicit whether sequestered leucocytes are intravascular or interstitial. Since separate measurements of granulocyte numbers respectively located in the vascular and interstitial spaces have seldom been made, exactly how the pulmonary capillary endothelium orchestrates migration into the interstitium has not previously been adequately addressed. Furthermore, many of these models have been produced by direct insults to the lung via intratracheal instillation of noxious agents. This route of adminis-

tration produces effects that are different compared with intravenous administration [55], although the latter is a closer paradigm of the human lung injury secondary to severe systemic inflammation.

The precise circumstances which trigger pulmonary granulocyte migration are incompletely understood but are likely to include up-regulation of pulmonary endothelial ICAM-1 (intercellular adhesion molecule 1) by peripherally generated inflammatory cytokines [13,15] with associated up-regulation of β_2 integrins [12,13,56]. Even though an expanded PGP is frequently observed in the absence of migration, PGP and soluble E-selectin were both significantly higher in migrators compared with non-migrators, which is consistent with the following sequence: systemic vascular activation (partly reflected by soluble E-selectin), granulocyte priming (manifesting as decreased deformability), delayed lung transit (manifesting as an expanded PGP), pulmonary endothelial activation, granulocyte migration and lung injury. If the endothelium is not activated, migration fails to occur.

In conclusion, we suggest that decreased granulocyte deformability leads to increased granulocyte pooling in the lung as a result of intravascular delay but does not necessarily lead to migration. The evidence here supports the notion that granulocytes pool only modestly in the normal lung, increase their pooling in systemic inflammation as a result of being primed but only migrate into the pulmonary interstitium when the pulmonary endothelium is also activated.

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