

Neutrophils released from the bone marrow by granulocyte colony-stimulating factor sequester in lung microvessels but are slow to migrate

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Neutrophils released from the bone marrow by granulocyte colony-stimulating factor sequester in lung microvessels but are slow to migrate. S.F. van Eeden, E. Lawrence, Y. Sato, Y. Kitagawa, J.C. Hogg. ©ERS Journals Ltd 2000.

ABSTRACT: Inflammatory mediators such as granulocyte colony-stimulating factor (G-CSF) release polymorphonuclear leukocytes (PMNL) from the bone marrow. This growth factor is used to promote the host response to infection but its effect on the behaviour of leukocytes at the inflammatory site is unclear. This study examined the sequestration and migration of PMNL released from the bone marrow by G-CSF in a model of streptococcal pneumonia.

Eight hours following the administration of either human G-CSF (n=6) or saline (n=3) in rabbits, a focal *Streptococcus pneumoniae* pneumonia was induced and the animals were followed for 2 h. The thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) was used to label PMNL (PMNL^{BrdU}) in the marrow and as a marker of PMNL newly released by the bone marrow. The PMNL^{BrdU} in the lung and blood were identified using immunohistochemistry.

G-CSF pretreatment elevated the circulating PMNL (3.6 ± 0.4 (mean \pm SEM) to $8.3 \pm 1 \times 10^9 \text{ L}^{-1}$, $p < 0.05$) and PMNL^{BrdU} (5.4 ± 2.1 to $12.5 \pm 3.1\%$, $p < 0.05$) counts at 8 h with little further increase caused by the subsequent 2 h pneumonia. These counts did not change in the control group. Morphometric studies of the lung showed that the total number of PMNL sequestered in lung capillaries were increased in the G-CSF group and the percentage of these PMNL that were BrdU-labelled, was higher than in circulating blood ($p < 0.05$). In the G-CSF group, only $11.2 \pm 2.6\%$ of the PMNL that migrated into the airspaces were PMNL^{BrdU} compared to $50.8 \pm 8\%$ PMNL^{BrdU} in the pulmonary capillaries. *In vitro* studies showed PMNL^{BrdU} released from the bone marrow by G-CSF are less deformable than unlabelled circulating PMNL ($p < 0.01$).

It is concluded that granulocyte colony-stimulating factor treatment causes the marrow to release polymorphonuclear leukocytes that preferentially sequester in lung microvessels but are slow to migrate out of the vascular space into the airspace at the pneumonic site.

Eur Respir J 2000; 15: 1079–1086.

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Keywords: Bone marrow release
5'-bromo-2'-deoxyuridine
polymorphonuclear leukocytes
migration
streptococcal pneumonia

Received: June 20 1999
Accepted after revision March 8 2000

This work was supported by the Medical Research Council of Canada (grant No. 4219) and the BC Lung Association. S. van Eeden was supported by a St Paul's Hospital Foundation Scholarship and E. Lawrence by a fellowship from the Medical Research Council of Canada and Genetech Inc.

Polymorphonuclear leukocytes (PMNL) are a critical component of the body's defence against acute bacterial infection [1]. Mature PMNL respond to microbial invasion by means of chemotaxis, phagocytosis and intracellular killing of the microbes *via* the release of hydrolytic enzymes and generation of oxygen free radicals [1, 2]. Since PMNL have a limited lifespan in the circulation (6–10 h), their supply must constantly be replenished by the bone marrow [3]. The bone marrow normally releases $\sim 1 \times 10^{11}$ PMNL per day [3–5] and the ability of the bone marrow to increase this output during an infection is a crucial factor in the host's ability to control and survive infections. An inability to mount this host response to infection is associated with a high morbidity and mortality particularly in patients with neutropenia [6, 7].

The use of the haematopoietic growth factor, granulocyte colony-stimulating factor (G-CSF), to increase the number of circulating PMNL and improve the outcome in neutropenic patients with infection is well established but its role in the treatment of non-neutropenic patients with infection needs to be determined [8]. G-CSF plays a pivotal

role in maintaining a normal circulating granulocyte count and mounting a neutrophilic response during infections. [9, 10]. G-CSF production is induced by bacterial products, by tumour necrosis factor- α and interleukin-1 from endothelial cells, fibroblasts and mononuclear phagocytes in the lung as well as stromal cells in the bone marrow [11]. G-CSF increases the circulating PMNL counts by stimulating the bone marrow where it accelerates proliferation of all stages of the PMNL development and releases maturing cells from the bone marrow storage pool into the circulation [10, 12]. In addition to its important role in the regulation of granulopoiesis, G-CSF also modulates numerous granulocyte functions such as increasing their chemotactic ability, enhancing cell adhesion to endothelium, and promoting phagocytosis and the respiratory burst [13, 14]. These beneficial effects of G-CSF on the development and functional capacity of mature PMNL as well as the proven efficacy in a variety of animal models of infection, provide the rationale for the use of this growth factor to promote host defence during local and systemic infections [15–17].

The recruitment of PMNL to an inflammatory site in the lung depends on the mechanical properties that influence their sequestration in pulmonary capillaries, their ability to adhere firmly to endothelium, and their response to chemo-attractant stimuli [18]. Recent studies from the authors' laboratory have shown that PMNL newly released from the bone marrow during streptococcal pneumonia preferentially sequester in the pulmonary capillaries but are slow to migrate into the airspaces [19]. PMNL taken from bone marrow are less deformable than their mature circulating counterparts [20], express higher levels of the adhesion molecule, L-selectin, [21], and show decreased mobility and chemotactic ability [22]. The authors' working hypothesis was that G-CSF would release PMNL from the bone marrow with this phenotype that results in increased sequestration in lung microvessels with less migration at the inflammatory site.

To address this hypothesis the sequestration and migration of PMNL released from the bone marrow by G-CSF in the lung was quantified. The migration of PMNL from the pulmonary capillaries into the alveolar space occurs early (1–2 h) after the deposition of *Streptococcus pneumoniae* into the rabbit lung [23] before the induction of a significant bone marrow release response by the infection [19, 24]. Therefore, PMNL release from the bone marrow by G-CSF was induced and the behaviour of these newly released PMNL in the early phase of pneumococcal pneumonia was determined. The thymidine analogue, 5'-bromo-2'-deoxyuridine (BrdU) was used to label the PMNL precursors (PMNL^{BrdU}) in the marrow and track the behaviour of newly released cells [19, 25, 26].

Materials and methods

Animals

Adult New Zealand white rabbits (n=12, weight 1.9–2.3 kg) were used in this study. The Animal Experimentation Committee of the University of British Columbia approved all experiments.

Effect of granulocyte colony-stimulating factor on the circulating polymorphonuclear leukocytes

To determine the optimum time to induce the pneumonia, three rabbits received G-CSF ($12.5 \mu\text{g}\cdot\text{kg}^{-1}$, s.c.) and blood samples were taken from the central ear artery at baseline, 15 min and 1, 2, 5, 8, 12, 24, 72, 144 and 220 h following G-CSF administration. PMNL and band cell counts as well as the expression of the adhesion molecules, L-selectin and CD18, on PMNL were measured as previously described [21].

Experimental protocol

Rabbits were pretreated with BrdU ($100 \text{ mg}\cdot\text{kg}^{-1}$; Sigma Chemical Co., St Louis, MO, USA) by infusion into the marginal ear vein at a dose of $40 \text{ mg}\cdot\text{mL}^{-1}$ in pyrogen-free saline over 15 min as previously described [19]. Twenty-four hours later, rabbits in the test group were given G-CSF ($12.5 \mu\text{g}\cdot\text{kg}^{-1}$ s.c., n=6) and rabbits in the control group were given an equivalent volume of saline (n=3).

Eight hours later, a focal pneumonia was induced in all animals by the instillation under fluoroscopic guidance of *Streptococcus pneumoniae* (5×10^8 organisms $\cdot\text{mL}^{-1}$) into one lung and an equal volume of the vehicle into the contralateral lung. Blood samples (3 mL each) were taken from the central ear artery before and 8 h after treatment with G-CSF, and 1 and 2 h after instillation of the bacteria. *S. pneumoniae* was instilled into the lungs following a method previously described [19]. Briefly, rabbits were anaesthetized with ketamine hydrochloride ($80\text{--}100 \text{ mg}\cdot\text{kg}^{-1}$ i.m.) and xylazine, ($10\text{--}15 \mu\text{g}\cdot\text{kg}^{-1}$ i.m.). *S. pneumoniae* (5×10^8 organisms) was instilled into the lower lobe of the anaesthetized rabbit under fluoroscopy. An equal volume of vehicle was instilled into the contralateral lower lobe of the same rabbit as a control. Animals were kept sedated in a prone position with additional anaesthetic as required throughout the experiment. Following the last blood sample, the animals were killed with an overdose of sodium pentobarbitone, the chest was opened rapidly and the base of the heart ligated to maintain the pulmonary blood volume. The trachea and both lungs were separated from other organs, weighed and inflated by intratracheal instillation of 10% formalin in phosphate buffer at 25 cmH₂O pressure, reweighed and then immersed in 10% formalin for 2 h. Following fixation, they were sliced horizontal to the plane of gravity and blocks of tissue were randomly selected from the pneumonic vehicle-treated sites and untreated areas. These blocks were processed in paraffin for histological evaluation using 3- μm -thick sections.

The blood samples, used to determine blood cell counts, 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 model SS80 Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and differential counts were made on Wright's-stained blood smears. Blood used for preparation of leukocyte-rich plasma (LRP) was collected in acid citrate dextrose, sedimented and cytopins prepared as previously described [19, 21].

Immunohistochemical detection of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes

A mouse monoclonal antibody to BrdU and the alkaline phosphatase anti-alkaline phosphatase method was used to stain for the presence of BrdU in PMNL both in cytopins of LRP and lung sections [27] as previously described in detail. Briefly, cells on cytopins were fixed in methanol and subjected to digestion in 0.04% pepsin. The lung sections were deparaffinized, rehydrated and digested for 10 min in 0.4% pepsin. Both the LRP and the lung sections were incubated in 2 N HCl to denature the DNA. The mouse monoclonal anti-BrdU antibody ($2 \mu\text{g}\cdot\text{mL}^{-1}$; DAKO Laboratories, Copenhagen, Denmark) was used to label leukocytes and nonimmune mouse immunoglobulin (IgG₁ ($2 \mu\text{g}\cdot\text{mL}^{-1}$)) was used as control. Following labelling, slides were counterstained with Gill's haematoxylin (BDH, Inc., Toronto, Canada), mounted and covered with a coverslip. Fixation and paraffin-embedding of lung tissue reduces BrdU labelling by 53% if compared to labelling of leukocytes in cytopins made with LRP obtained from whole blood [19]. The authors have used this factor in the comparisons of PMNL^{BrdU} in blood and lung tissue.

Evaluation of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes in blood

PMNL on cytopins with any nuclear stain were counted as PMNL^{BrdU}. PMNL^{BrdU} were evaluated using a Zeiss Universal light microscope (Model IIR; Oberkochen, Germany) in random fields of view. The number of positive PMNL^{BrdU} per 100 PMNL was determined and results are expressed as the percentage or number of PMNL that are BrdU-labelled.

Morphometric evaluation of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes in lung sections

The number of PMNL^{BrdU} in tissue sections was determined using a sequential level stereological analysis as described by CRUZ-ORIVE and WEIBEL [28] and a modification of a method previously described [29]. Briefly, point counting was performed at 4× magnification and the volume fraction (V_v) of each component of lung estimated as follows:

$$V_v(\text{object}) = \frac{\text{Sum of the points on object}}{\text{Sum of the total points}}$$

Paraffin-embedded sections stained for BrdU were then point counted at 800× magnification using a Nikon Microphot-fx light microscope (Nikon, Tokyo, Japan) and using an image analysis system ("Bioview"; Infracan, Inc., Richmond, BC, Canada). Twenty computer-generated random fields were evaluated on each slide. At this level, the number of points falling on airspace and tissue PMNL^{BrdU} and unlabelled PMNL in the airspace or in tissue were counted and the V_v of each component was estimated. Tissue in this context consisted largely of alveolar walls and included capillaries and interstitial spaces. Results are expressed as the percentage of PMNL in airspace or tissue that are BrdU-labelled.

The number of PMNL^{BrdU} or total PMNL (PMNL^{total}) in the airspace or tissue from the pneumonic, vehicle-treated and untreated regions were also calculated. The calculation for the number of PMNL^{BrdU} in the airspace in the pneumonic region is shown as an example:

$$\frac{\text{Number of PMNL}^{\text{BrdU}} \text{ in pneumonic airspace} = \text{lung volume} \times V_v \text{ pneumonic region} \times V_v(\text{PMNL}^{\text{BrdU}})}{143 \text{ fL}}$$

where 143 fL is the assumed volume of a rabbit PMNL [29]. Results are expressed as the total number of PMNL^{BrdU} · mL⁻¹ of airspace or tissue in the pneumonic, vehicle-treated or untreated region.

Immunofluorescence flow cytometric analysis

Expression of PMNL surface L-selectin and CD18 were determined by flow cytometry on whole blood samples collected in EDTA using methods previously described [19, 21]. A mouse monoclonal CD18 (DAKO), DREG-200 (kindly donated by E.C. Butcher, Stanford University School of Medicine, Stanford, CA, USA) or nonimmune mouse IgG antibodies were used followed by fluorescein isothiocyanate-conjugated goat antimouse secondary antibody (Sigma). Flow cytometry was performed using an

Epics®-Profile II (Coulter Electronics) and results are expressed as mean fluorescence intensity of 3,000 cells.

In vitro deformability of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes

To test the deformability of BrdU-labelled PMNL relative to unlabelled PMNL, leukocytes were filtered through polycarbonate filters as previously described [30]. Blood samples were obtained from the rabbits 8 h after they were injected with either G-CSF or saline (before the pneumococci were instilled in the lung), and again at the end of the study period (2 h after the pneumococci were instilled into the lung). LRP was prepared as described above, and filtered *in vitro* according to the filtration method described by LENNIE *et al.* [31]. Briefly, a 20-mL polypropylene syringe (Sherwood Medical Co., St Louis, MO, USA) was filled with sample solution (LRP, 4 × 10⁵ cells·mL⁻¹) and filtered through polycarbonate filters (Poretics, Livermore, CA, USA) with defined pore size (pore diameter 5 µm; length 10 µm and pore density 4 × 10⁵ · cm⁻² (manufacturer's data)) using a syringe infusion pump (Pump 22; Harvard Apparatus, Millis, MA, USA) which provided a constant flow rate (3 mL·min⁻¹) of solution across the filter. Hydrostatic pressure was continuously monitored upstream from the filter using a pressure transducer (Validyne Engineering, Northridge, CA, USA) connected to a recording system. To reduce nonspecific adhesion of PMNL to filters, the membrane was coated with albumin (human albumin 5%; Sigma) before filtration, by first filtering phosphate-buffered saline/albumin alone for 8 min. The pressure sensing system was calibrated using a water manometer under conditions of no flow before each filtration. Samples were collected before (prefiltered) and after (postfiltered) filtration. Postfiltered samples were collected at the plateau phase (steady state) of the pressure curve. Leukocytes in the samples were fixed in 1% paraformaldehyde, spun on to precoated slides and stained for the presence of BrdU as described above. The retention of PMNL^{BrdU} in the filters was expressed as a ratio that was calculated as the fraction of BrdU-labelled PMNL in the postfiltered samples divided by the fraction of BrdU-labelled PMNL in the prefiltered samples. A ratio of 1.0 is compatible with BrdU-labelled cells passing through filters similar to nonlabelled cells; a low ratio represents retention of BrdU-labelled cells. Four measurements were made in the G-CSF-treated animals and three in the saline controls.

Statistical analysis

All results are expressed as mean ± SEM. A two-way analysis of variance was used where appropriate to compare the number of PMNL and the percentage of PMNL^{BrdU} in peripheral blood samples. One-way analysis of variance was used to compare the distribution of PMNL^{BrdU} in the pneumonic region with the vehicle-treated and untreated region in the contralateral lung and also when comparing the airspaces with the lung tissue. To analyse the deformability of PMNL, each curve was fitted to an equation to determine an estimate for the plateau. The mean plateau for each subject (taken over two to four trials per subject) in

both groups was calculated and the difference in mean plateau between the two groups compared. An overall mean difference was computed and a t-statistic was calculated to determine significance. Bonferroni's correction was used for multiple comparisons. A p-value of $p < 0.05$ was accepted as statistically significant.

Results

Effect of granulocyte colony-stimulating factor on circulating polymorphonuclear leukocytes

Administration of G-CSF caused a transient drop in the circulating PMNL counts followed by an increase that peaked at 12 h after treatment (fig. 1). No changes in PMNL CD18 and L-selectin expression, signifying intravascular cell activation, occurred over this time-period (data not shown). Therefore, the 8 h time point was selected to instill the *S. pneumoniae* as this point was on the steep part of the release of PMNL from the marrow into the circulation.

Release of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes into the circulation

Administration of G-CSF stimulated a significant rise in the number of circulating PMNL (fig. 2) 8 h after treatment with no further rise during the 2-h pneumonia. The 2-h pneumonia also did not significantly increase the circulating PMNL counts in the control group. The percentage of PMNL^{BrdU} in the circulation (fig. 3) increased significantly from $5.4 \pm 2.1\%$ to $12.5 \pm 3.1\%$ with the G-CSF treatment before the pneumonia was induced with little further increase during the 2 h *S. pneumoniae* pneumonia. In the saline controls, a small increase in PMNL^{BrdU} ($2.6 \pm 0.9\%$ to $5.6 \pm 2.3\%$) occurred during the 10-h study period (fig. 3). The values in the G-CSF group were significantly higher than values in the control group of animals given saline instead of G-CSF ($p < 0.01$).

Expression of CD18 and L-selectin

The expression of CD 18 and L-selectin on all circulating PMNL at baseline ($11.4 \pm 3.5\%$ and $10.2 \pm 0.3\%$), 8 h

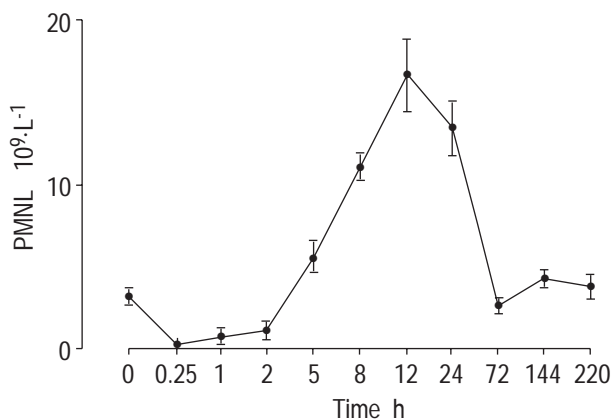


Fig. 1. – The effect of granulocyte colony-stimulating factor ($12.5 \mu\text{g}\cdot\text{kg}^{-1}$) on the circulating polymorphonuclear leukocyte (PMNL) counts of rabbits ($n=3$). An initial transient drop (within 1 h) in PMNL counts was followed by a steep rise that peaked at 12 h, at which point the counts were six times the baseline levels. Values are mean \pm SEM.

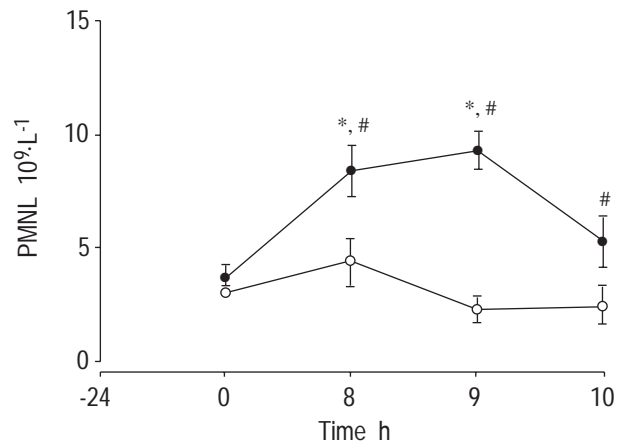


Fig. 2. – The effect of granulocyte colony-stimulating factor ($12.5 \mu\text{g}\cdot\text{kg}^{-1}$) followed by pneumonia on the circulating polymorphonuclear leukocyte (PMNL) counts ($n=6$). There was an increase in PMNL counts induced by G-CSF (8 h) and no further increase caused by the 2-h pneumonia. In the control group rabbits ($n=3$) that received saline instead of G-CSF, the PMNL counts did not change over the study period. At the 8- and 9-h time period, in the G-CSF treated animals, PMNL counts were significantly different from the controls. Values are mean \pm SEM. 5'-bromo-2'-deoxyuridine was applied at -24 h, and G-CSF at 0 h, and pneumonia was induced at 8 h. ●: G-CSF; ○: control. *: $p < 0.05$; #: $p < 0.01$, compared to control.

following G-CSF ($8.9 \pm 2.4\%$ and $9.9 \pm 0.4\%$) and 2 h following the instillation of the pneumonia ($10.4 \pm 3.2\%$ and $11.3 \pm 0.6\%$) respectively did not change.

Morphometry of lung

The volume of pneumonia in the G-CSF group (15.5 ± 2.3 mL) was similar to that in the control group (12.5 ± 3.3 mL). There were 11 ± 1.7 mL of airspace and 4.2 ± 0.5 mL of alveolar wall in the pneumonic area in the G-CSF group

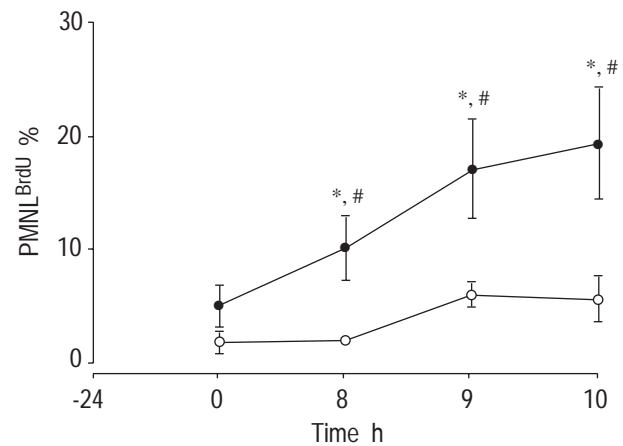


Fig. 3. – Changes in the percentage of circulating 5'-bromo-2'-deoxyuridine (BrdU)-labelled polymorphonuclear leukocytes (PMNL^{BrdU}) in rabbits treated with either granulocyte colony-stimulating factor (G-CSF) ($n=6$) or saline ($n=3$). G-CSF increased the percentage of PMNL^{BrdU} from $5.4 \pm 2.1\%$ to $12.5 \pm 3.1\%$ and the 2-h pneumonia further increased it to $19.3 \pm 4.9\%$. In the saline-treated group the percentage of PMNL^{BrdU} did not change with saline treatment and increased from $1.6 \pm 0.3\%$ to $5.6 \pm 2.3\%$ with the 2-h pneumonia. Values are mean \pm SEM. BrdU was applied at -24 h, and G-CSF at 0 h, and pneumonia was induced at 8 h. ●: G-CSF; ○: control. *: $p < 0.05$; #: $p < 0.01$, G-CSF versus saline.

that was similar to the control group (7.9±1.3 mL of airspace and 4.7±1.5 mL of alveolar wall). The volume of airspace and alveolar wall in the contralateral lungs was similar between groups (data not shown).

Polymorphonuclear leukocyte in the lung

In the G-CSF group, the PMNL total (labelled and unlabelled) in the lung tissues (alveolar walls) were 13.2±1.2×10⁷·mL⁻¹ of tissue in the untreated lung, 17.6±3×10⁷·mL⁻¹ in the vehicle-treated (colloidal carbon) lung tissue and 25.6±4.3×10⁷·mL⁻¹ in the pneumonic region (table 1). These numbers represent a ~25.3, 33.8, and 68.4 times enrichment of PMNL in the untreated, vehicle-treated and pneumonic region of the lungs respectively compared to circulating counts (0.52±0.9 ×10⁷·mL⁻¹ when rabbits were sacrificed). In the saline-treated control group, this enrichment of PMNL in pulmonary capillaries was significantly less (table 1). The enrichment of PMNL in pulmonary capillaries in the controls was ~13.2, 26.6 and 43.4 times that in the untreated, vehicle-treated and pneumonic regions respectively. In the pneumonic area of the G-CSF group, the PMNL total (labelled and unlabelled) in the airspaces was 39.5±7×10⁷·mL⁻¹ of airspace showing significant migration of PMNL. That was not different from values in control animals (27.2±5 ×10⁷·mL⁻¹ of airspace). Very few PMNL migrated into the alveolar space in the vehicle-treated regions (table 1). The number of PMNL^{BrdU} in circulating blood and lung are shown in table 2. Significantly more PMNL^{BrdU} (p<0.01) were present in all lung regions in the G-CSF-treated animals.

Figure 4a shows, that in the G-CSF group, the percentage of PMNL^{BrdU} in the circulating blood was significantly lower than the percentage of PMNL^{BrdU} in lung microvessels at the pneumonic site, as well as the vehicle-treated or untreated lung regions (p<0.05). The control group showed similar differences (data not shown). These findings are compatible with preferential sequestration of PMNL^{BrdU} in the infected and noninfected regions of the lung in both groups.

Figure 4b shows the percentage of PMNL^{BrdU} in the alveolar walls and alveolar airspace in the G-CSF group. There were approximately 5 times more PMNL^{BrdU} in alveolar walls than in alveolar spaces in the pneumonia

Table 1. – Total number of polymorphonuclear leukocytes (PMNL) in the lungs

Regions	G-CSF group		Control group	
	Alv-walls	Alv-space	Alv-walls	Alv-space
Lung				
Pneumonic	25.6±4.3	39.5±7	11.3±2.2 [#]	27.2±5
Vehicle-treated	17.6±3	3.5±0.6	6.9±1.4 [#]	1.1±0.5
Untreated	13.2±1.2 ⁺		3.4±1.7 ^{#,+}	
Circulating blood	0.52±0.09		0.24±0.02*	

The numbers are PMNL×10⁷·mL⁻¹ of alveolar wall (Alv-wall) or alveolar space (Alv-space). Granulocyte colony-stimulating factor (G-CSF) group: n=6, control group: n=3. *: circulating PMNL counts were lower in the control group (p<0.05); [#]: PMNL counts in all three lung-regions in the alveolar walls were lower in the control group (p<0.05); ⁺: PMNL counts in the untreated lung region were lower than in the pneumonic region (p<0.04).

Table 2. – Total number of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes (PMNL) in the lungs

Regions	G-CSF group		Control group	
	Alv-walls	Alv-space	Alv-walls	Alv-space
Lung				
Pneumonic	13±2.08	4.4±1	2.8±0.5 [#]	2.6±0.9
Vehicle-treated	6.8±1.2 ⁺	0.08±0.01	1.2±0.3 ^{#,+}	0.009±0.001
Untreated	4.9±1.5 ⁺		0.49±0.04 ^{#,+}	
Circulating blood	0.11±0.002		0.012±0.003*	

The numbers are PMNL^{BrdU} ×10⁷·mL⁻¹ of alveolar wall (Alv-wall) or alveolar space (Alv-space). Granulocyte colony-stimulating factor (G-CSF) group: n=6, control group: n=3. *: circulating PMNL^{BrdU} counts were lower in the control group (p<0.05); [#]: PMNL^{BrdU} counts in all three lung-regions in the alveolar walls were lower in the control group (p<0.05); ⁺: PMNL^{BrdU} counts in both the vehicle-treated and the untreated lung regions were lower than in the pneumonic region (p<0.04).

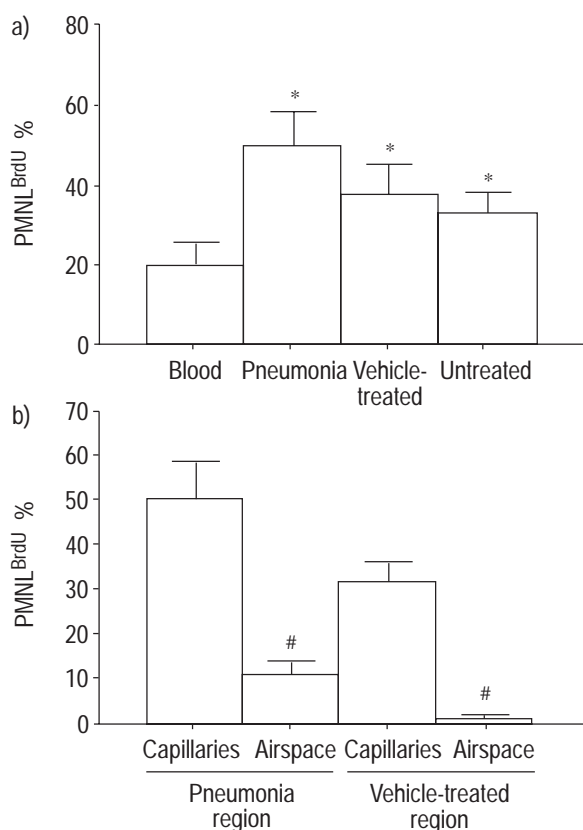


Fig. 4. – a) The percentage of 5'-bromo-2'-deoxyuridine (BrdU)-labelled polymorphonuclear leukocytes (PMNL^{BrdU}) in the circulating blood and pulmonary capillaries in rabbits treated with granulocyte colony-stimulating factor (G-CSF) (n=6) for 8 h followed by a 2-h pneumonia. The percentage of PMNL^{BrdU} in the pneumonia, vehicle-treated and untreated regions of the lungs was higher than in the circulating blood. Differences between PMNL^{BrdU} percentages in the pneumonia, vehicle-treated and untreated regions were not significant. b) Migration of PMNL^{BrdU} into the airspaces in rabbits treated with G-CSF (n=6) for 8 h followed by a 2-h pneumonia. The percentage of PMNL^{BrdU} was lower in the airspaces compared to the alveolar walls in the pneumonic region as well as the vehicle-treated region. Values are mean±SEM. *: p<0.05; #: p<0.01.

region ($50.4 \pm 7.8\%$ versus $11.2 \pm 1.3\%$, $p < 0.01$) and just a small number of PMNL^{BrdU} migrated into the airspace in the vehicle-treated region. In the control group similar differences were seen (data not shown) that are consistent with pre-ious published work [27].

In vitro deformability of 5'-bromo-2'-deoxyuridine-labelled polymorphonuclear leukocytes

In the control group, using leukocytes obtained before the pneumonia was instilled, the baseline pressure was 2.5 ± 0.3 cmH₂O, reaching a plateau (after 6–8 min) at 3.3 ± 0.4 cmH₂O. These values did not change significantly when leukocytes obtained after the 2-h pneumonia were filtered. In the G-CSF group using leukocytes obtained before the pneumonia was instilled, the baseline pressure was 2.9 ± 0.5 cmH₂O and reached a plateau (after 5–7 min) at 6.7 ± 1.3 cmH₂O. The 2-h pneumonia did not change these values significantly. Plateau pressures in the G-CSF group were higher than those in the control group ($p < 0.05$). PMNL^{BrdU} from the G-CSF-treated rabbits were preferentially retained in the filters (fig. 5, $p < 0.01$). The ratio of PMNL^{BrdU} between pre- and postfiltered samples was 0.99 ± 0.04 (prepneumonia) and $0.96.5 \pm 0.03$ (postpneumonia) in the control group and 0.62 ± 0.06 (prepneumonia) and 0.73 ± 0.03 (postpneumonia) in the G-CSF group (fig. 5). Filtration of PMNL did not change the CD18 or L-selectin expression of PMNL (data not shown).

Discussion

The potential beneficial effects of G-CSF on PMNL function suggest that G-CSF could be useful in increasing the host response to infection. However, most studies describing these beneficial effects, focus on the effect of G-CSF on mature circulating PMNL [13, 14, 16]. In this study the authors describe the effects of G-CSF on PMNL

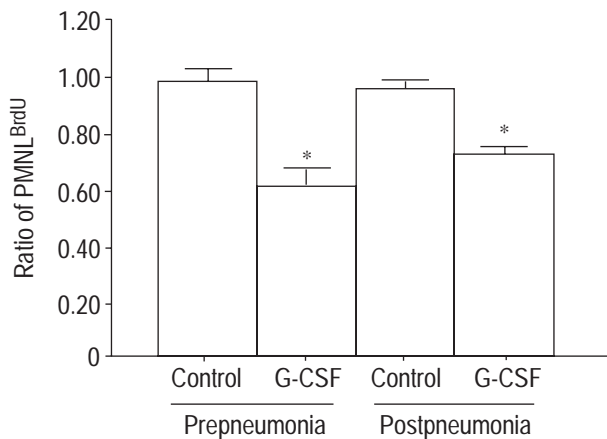


Fig. 5. – Deformability of 5'-bromo-2'-deoxyuridine (BrdU)-labelled polymorphonuclear leukocytes (PMNL^{BrdU}) released from the bone marrow during treatment with granulocyte colony-stimulating factor (G-CSF). Leukocyte-rich plasma from rabbits, 8 h after they were injected with either $12.5 \mu\text{g}\cdot\text{kg}^{-1}$ G-CSF or saline (prepneumonia) and following a 2-h pneumonia (postpneumonia), was filtered through 5-mm filters *in vitro*. Values are from four rabbits in the G-CSF group and three in the control group and expressed as the mean \pm SEM of the ratio of PMNL^{BrdU} postfiltered to prefiltered (see text). G-CSF caused the PMNL^{BrdU} to be retained in filters, while no retention was observed in saline-treated controls. *: $p < 0.01$ compared to control.

newly released from the bone marrow, specifically the ability of PMNL to sequester in lung microvessels and migrate into the alveolus during pneumococcal pneumonia. The results show that G-CSF rapidly release PMNL from the bone marrow and increase the total number of PMNL present in the lung compared to controls. The new PMNL released from the bone marrow preferentially sequester in microvessels in both the infected and noninfected regions of the lung. These newly released PMNL were also slow to migrate into the alveolus. The authors speculate that these PMNL are structurally and functionally still immature, resulting in preferential sequestration in lung microvessels and an inability to migrate into an infectious site.

To test the hypothesis that G-CSF changes the sequestration and migration of PMNL released from the bone marrow, the authors decided to study lung sequestration and migration of these cells 10 h after G-CSF administration and 2 h following an inflammatory stimulus instilled in the lung. The reasoning for this approach was that migration of PMNL from the pulmonary capillaries into the alveolar space occurs early (1–2 h) following *S. pneumoniae* instillation in rabbits [23]. This was supported by the study (migration $39.5 \pm 7 \times 10^7 \cdot \text{mL}^{-1}$ in G-CSF $27.2 \pm 5 \times 10^7 \cdot \text{mL}^{-1}$ in control group at the 2-h time point). This occurred before a significant bone marrow release response was induced by the infection [19, 24]. At this time point it was assumed that G-CSF is responsible for the majority of the marrow response (figs. 1 and 3), which allowed the authors to study the behaviour of PMNL released from the bone marrow by G-CSF at a site of inflammation.

Several studies have shown that PMNL released from the bone marrow by inflammatory stimuli such as pneumococcal pneumonia [19], bacteraemic pneumonia [24] and endotoxaemia [26] preferentially sequester in lung microvessels. The principle factors that regulate the sequestration of PMNL in the lung are the discrepancy between their size and the size of pulmonary capillary segments and the ability of PMNL to deform [29, 32]. Pneumococcal pneumonia prolongs the transit time of PMNL in the lung and causes sequestration of PMNL in both the infected and noninfected regions of the lung [19, 32]. Previous studies have shown enrichment of newly released PMNL at 5 and 8 h following a focal pneumococcal pneumonia [19]. The present study shows that pretreatment of animals with G-CSF did not change this pattern (fig. 4), suggesting that G-CSF does not change the phenotypic or functional characteristics responsible for this increase in sequestration of newly released PMNL in lung microvessels.

G-CSF caused a shortening of the transit time of PMNL through the maturation pool in the bone marrow and an accelerated release of these PMNL into the circulation. Studies have shown that PMNL in the post mitotic pool in the bone marrow are less deformable, less motile and have a reduced chemotactic ability compared to peripheral blood PMNL [20, 22]. The *in vitro* deformability of newly released PMNL was measured using 5- μm filters because the mean size of rabbit pulmonary capillaries is 5–6 μm [29]. These studies show that these younger PMNL^{BrdU} released from the bone marrow by G-CSF are indeed less deformable (fig. 5) and this depressed deformability was maintained during the period of pneumonia. These results are consistent with the hypothesis that PMNL released from the bone marrow by G-CSF have similar

deformability characteristics to PMNL harvested from the bone marrow. No changes in the expression of CD18 or L-selectin on PMNL were observed, suggesting that these PMNL adhesion properties were not responsible for the retention in the filters or sequestration in lung microvessels. Taken together, these findings support the notion that PMNL deformability is one of the major determinants for their sequestration in pulmonary capillaries [4, 29] and that G-CSF does not change the deformability of PMNL newly released from the bone marrow.

Several *in vitro* and *in vivo* studies have demonstrated that G-CSF can augment host defence in response to invading pathogens such as bacteria and fungi by enhancing PMNL functional capabilities [13, 14, 16]. In low concentrations, G-CSF enhances PMNL mobility and chemotaxis *in vitro*, with an inhibitory effect at higher concentrations [33]. Normal volunteers receiving G-CSF for 5 days have a reduced migration of PMNL into a skin window compared to pretreatment values [34]. The current study shows that PMNL released from the bone marrow by a single dose of G-CSF are slow to migrate toward an inflammatory stimulus in the alveolus compared to mature PMNL. This is consistent with previous observations that younger PMNL released from the bone marrow during a focal pneumococcal pneumonia are slow to migrate into the alveolus [19, 24]. The slow migration of PMNL in this model shows that pre-exposure to G-CSF does not alter the defect in migration in younger cells.

Several animal studies have shown the beneficial effect of G-CSF pretreatment in models of wound sepsis, intra-abdominal sepsis, pneumonia and lipopolysaccharide-induced acute lung injury [17, 35–37]. However, TERASHIMA *et al.* [38] have shown that G-CSF pretreatment of guinea-pigs increases sequestration of PMNL in the lung and exacerbates acute lung injury induced by intratracheal endotoxin, and KING *et al.* [15] showed that pretreatment with G-CSF enhances lung damage in two models of non-infectious acute lung injury. These findings are supported by clinical observations that G-CSF treatment could precipitate and aggravate acute lung injury [39]. G-CSF causes an increased burden of PMNL in pulmonary capillaries (table 1) and it is speculated that the systemic release of inflammatory mediators from the focus of infection, could activate these younger PMNL trapped in the microvascular bed and contribute to the development of acute lung injury observed by others [15, 33, 38, 39].

The use of granulocyte colony-stimulating factor to increase the number of circulating polymorphonuclear leukocytes and the outcome in neutropenic patients with infection is well established [10, 12, 16] and studies are ongoing to determine the efficacy of granulocyte colony-stimulating factor treatment to non-neutropenic patients [8, 40]. These beneficial effects are thought to result from the influence of granulocyte colony-stimulating factor on the production of polymorphonuclear leukocytes in the bone marrow and the enhancement of the functional activity of circulating polymorphonuclear leukocytes. Whether these beneficial effects hold true for patients when sepsis occurs with a normal functioning bone marrow remains to be determined. The inability of granulocyte colony-stimulating factor to increase migration of younger polymorphonuclear leukocytes into the infectious site results in large numbers of less mature polymorphonuclear leukocytes in the circulation. These younger polymorpho-

nuclear leukocytes preferentially sequester in the lung and possibly other organs and subsequent activation of the polymorphonuclear leukocytes within the vascular space could potentially be harmful for the host. This balance between the beneficial effect of granulocyte colony-stimulating factor on the functional capacity of mature circulating polymorphonuclear leukocytes and the potentially harmful effects of large numbers of less mature polymorphonuclear leukocytes in the circulation may determine the overall beneficial or harmful effects of granulocyte colony-stimulating factor administration in infectious diseases.

Acknowledgements. The authors want to thank S. Greene for photography, L. Verbrugg for assistance with the statistical analysis and J. Hards for immunocytochemistry.

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