

Neutrophil sequestration in rat lungs

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Abstract

Background – The transit of neutrophils through the pulmonary microvasculature is prolonged compared with red blood cells and is increased further during cigarette smoking and in exacerbations of chronic obstructive pulmonary disease. The increased residence time (sequestration) of neutrophils in the pulmonary capillaries in these conditions may be the first step leading to the accumulation of cells within the lung interstitium and in the bronchoalveolar space, so potentiating lung damage. A rat model has been developed to investigate the factors which may influence neutrophil transit through the lung microvasculature.

Methods – Intratracheal instillation of the heat killed organism *Corynebacterium parvum* was used to induce an acute neutrophil alveolitis. Neutrophils and red blood cells were isolated from donor rats, labelled with two distinct radioisotopes, and then reinjected into recipient rats to assess their transit through the pulmonary circulation. To ascertain whether peripheral blood neutrophils were minimally altered by the isolation procedure their functional status in vitro was compared with that of inflammatory neutrophils in a number of assays commonly used as descriptors of neutrophil activation. The influence of neutrophil activation on the accumulation of cells in the lungs was assessed by comparing the lung sequestration of control neutrophils, isolated from peripheral blood, with that of inflammatory neutrophils obtained from bronchoalveolar lavage of inflamed rat lungs. Lung sequestration of neutrophils was defined as the fold increase in the ratio of neutrophils labelled with chromium-51 to red blood cells labelled with technetium-99m in lung tissue compared with the same ratio in peripheral blood.

Results – Sequestration of peripheral blood neutrophils occurred in control rat lungs as shown by a 17.5 (2.1) fold increase in the ratio of neutrophils to red blood cells in the pulmonary circulation compared with the ratio of these cells in the peripheral circulation. When inflammatory neutrophils, obtained by bronchoalveolar lavage from *C parvum*-treated animals, were injected into control rats, the increase was 90.6 (11.0) fold. Induction of an inflammatory response in the lung tissue of the recipient rat also caused an increase in the sequestration of control neutrophils compared with the same cells in control rat lungs which was, however,

less marked than when inflammatory neutrophils were used (34.7 (4.7) fold). The mean (SE) pressure developed on filtration of inflammatory neutrophils in vitro through a millipore filter (7.53 (0.2) cm H₂O) was greater than that of peripheral blood neutrophils (1.18 (0.2) cm H₂O). Increased filtration pressure indicates a decrease in cell deformability and suggests that this may be a contributory factor to the increased sequestration of inflammatory neutrophils in the pulmonary vasculature.

Conclusions – This study shows that there is sequestration of neutrophils in the pulmonary vasculature in normal rat lungs which increases in acute lung inflammation and when inflammatory neutrophils are injected into control animals. In this model changes in the neutrophil, such as cell deformability, may have a more important role in inducing increased neutrophil sequestration than the inflammatory response in the lungs.

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The lungs contain a large so called “marginated” pool of neutrophils^{1,2} which are largely located in the capillary bed.^{3,4} While the average diameter of the pulmonary capillary segments is only 5 µm,⁵ that of the neutrophil is about 7 µm.⁶ This size differential is likely to influence neutrophil sequestration in the lungs since most neutrophils must therefore deform to pass through the smaller pulmonary capillaries. The effect of such size differential is that, in normal lungs, the transit of neutrophils through the pulmonary microvasculature is delayed compared with the passage of erythrocytes.^{7,8}

Delay in neutrophil transit within the pulmonary microcirculation allows interaction between capillary endothelial cells and the neutrophil and is likely to be a prerequisite for cell adhesion to the vascular endothelium and subsequent diapedesis and emigration. In inflamed lungs the slow transit of neutrophils through the pulmonary capillaries may potentiate their interaction with the capillary endothelium, so increasing their accumulation in the lung tissue.

There are many factors which can influence neutrophil sequestration in the lungs,⁸ amongst which are alterations in blood flow,⁹ adhesion molecule expression,¹⁰ and cell deformability.¹¹ We have shown in humans that the sequestration of neutrophils labelled with indium-111 10 minutes after their injection correlates with local blood velocity,¹² whereas

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the initial first pass sequestration in vivo correlates with neutrophil deformability in vitro.¹¹ Decreased neutrophil deformability is probably related to alterations in the cytoskeleton and can be oxidant-mediated or can result from cell activation.¹³ Thus, decreased deformability of neutrophils may contribute to the increased sequestration which occurs during cigarette smoking¹² and in exacerbations of chronic obstructive pulmonary disease (COPD).¹⁴

Sequestration of neutrophils in the lungs has been the subject of considerable research interest for many years,^{1-4, 8} with human studies being augmented by experimental studies in dogs and rabbits. We now report the development of a model of neutrophil sequestration in rat lungs in which we demonstrate increased sequestration in acute inflammation, which is influenced both by changes in neutrophil function and by the effects of lung inflammation.

Methods

CELL PREPARATIONS

Rat peripheral blood neutrophils were obtained as previously described by Williams and co-workers.¹⁵ Briefly, male HAN rats of around 250 g in weight were anaesthetised with intraperitoneal nembutal and transfused via the iliac vein with 40–50 ml of plasma expander (Hetastarch; Dupont, Stevenage, UK) to increase the total volume of blood retrieved from the animals. The blood/Hetastarch mixture was allowed to sediment for 40 minutes at room temperature and the leucocyte-rich plasma so formed was decanted and separated in one of two ways.

In the initial experiments a two-step separation was used in which 20 ml of leucocyte-rich plasma was layered over a double gradient of Percoll (top layer, 1.04 g/ml; bottom layer, 1.089 g/ml). The gradients were spun at 1050 rpm for 30 minutes at 10°C. A leucocyte-rich band was retrieved from the interface of the two Percoll layers and the cells were then washed with saline and resuspended in 10 ml ice-cold Hank's balanced salt solution (HBSS) (Gibco, Paisley, UK) containing 1% bovine serum albumin (BSA) (Sigma, Poole, Dorset, UK). One ml of the cell suspension was mixed with 3 ml of Sepracell (Sepratech Corp, Ohio, USA) and the neutrophils were isolated by centrifugation at 2600 rpm for 10 minutes, whereafter the neutrophils were located at the bottom of the centrifuge tube and the mononuclear cells at the top of the separation medium. This technique yielded a mean (SD) neutrophil population of $10.5 (5.0) \times 10^6$ /rat with a purity of 86 (12)%, the remaining cells being predominantly lymphocytes.

In later experiments a modification of the separation technique was used. In this procedure 4.5 ml leucocyte-rich plasma was mixed with 8 ml Percoll whose specific gravity had been adjusted to 1.1120 at 23°C. The leucocyte-rich plasma/Percoll mixture was centrifuged at 2600 rpm for 30 minutes at room temperature. The red blood cells (RBCs) sedimented to the bottom of the tube, with a buffy

coat of neutrophils on top; mononuclear cells migrated to the top of the separating medium. By removing the supernatant, Percoll, and mononuclear cells the neutrophils could be cleanly pipetted off the RBC layer. This technique gave a markedly improved cell yield of $34.0 (23.7) \times 10^6$ per rat with a greater purity of 94 (3)%, viability of >99% (as assessed by trypan blue exclusion), and no RBC contamination. In these experiments all in vitro procedures were carried out at room temperature.

Inflammatory neutrophils were obtained by bronchoalveolar lavage, as previously described,¹⁶ from rats which had been dosed intratracheally 16 hours previously with 1.4 mg in 0.2 ml of the heat-killed *Corynebacterium parvum* (Wellcome, London, UK). This procedure yielded $45.0 (9.4) \times 10^6$ neutrophils per rat, with a purity of 78 (9)%. The remaining cells in this population were alveolar macrophages and these were removed by differential centrifugation through Percoll as described above, giving a >95% pure neutrophil population.

ASSESSMENT OF IN VITRO FUNCTIONAL ACTIVITY OF CONTROL AND INFLAMMATORY NEUTROPHILS

Chemotaxis

Neutrophil chemotactic response was measured by assessing the migration of the cells towards zymosan-activated serum, through nucleopore filters of 3 µm pore size in Boyden chambers as previously described.¹⁷ 6.0×10^5 neutrophils in 400 µl were added to each chamber. Chemotaxis was assessed, following 30 minutes incubation at 30°C, by counting the number of migrated cells per high power field in 10 fields per filter.

Epithelial injury

Cells of the human type II alveolar epithelial cell line A549 were prepared at a concentration of 5×10^5 /ml in microtitre plates, allowed to form a monolayer, and radiolabelled with chromium-51 (⁵¹Cr); 5×10^4 A549 cells were added to each well. Peripheral blood and bronchoalveolar lavage neutrophils were incubated on the A549 monolayers at a ratio of 10 neutrophils to one A549 cell for four hours at 37°C without further stimulation. Epithelial injury was assessed as cell detachment from the monolayer by measuring the cell associated ⁵¹Cr counts released into the supernatant medium as previously described.¹⁷

Fibronectin proteolysis

The proteolytic activity of unstimulated neutrophils was tested by their ability to degrade fibronectin labelled with iodine-125 as previously described.¹⁶ Briefly, 1×10^5 neutrophils were cultured on a solid phase matrix of ¹²⁵I-labelled fibronectin in microtitre plates for four hours at 37°C. Proteolysis of fibronectin was assessed as the ¹²⁵I counts in the supernatant medium.

Superoxide anion production

Superoxide anion (O_2^-) production was measured in the presence of the phorbol ester, phorbol myristate acetate (PMA; 1 μ g/ml) using the cytochrome C reduction method. The release of O_2^- from 2.5×10^5 neutrophils was assessed spectrophotometrically as the absorbance at 550 nm as previously described.¹⁸

Neutrophil deformability

Neutrophils were diluted to a concentration of 1×10^5 /ml and filtered at constant flow (1.5 ml/min) through a nucleopore polycarbonate membrane in a filter chamber with a pore diameter of 5 μ m and length of 11 μ m. These dimensions mimic the average dimensions of human pulmonary capillary segments.⁵ Deformability was assessed by measuring the pressure which developed within the chamber over a period of six minutes as previously described.¹³

RADIOLABELLING OF CELLS

Red blood cells were labelled with technetium-99m (^{99m}Tc) using the BNL red blood cell labelling kit (Cadema Inc, New York, USA). Four ml of blood was withdrawn by cardiac puncture from a control rat and anticoagulated with heparin. The blood was then mixed with stannous tin reagent and rotated for four minutes at room temperature. One ml of 4.4% disodium EDTA was added to the tube and inverted five times. The tube was then spun at 2500 rpm for five minutes and the plasma and buffy coat removed and discarded. Technetium (12 MBq) was added to the cell pellet and the tube was rotated for 10 minutes at room temperature. The cell pellet was then diluted to the original blood volume and the labelling efficiency estimated by spinning an aliquot of the cells and measuring the counts remaining in the supernatant. Labelling efficiency of RBC using this system, measured as the uptake of label, was >95%.

Neutrophils were labelled with chromium-51 (^{51}Cr) by incubating 15×10^6 neutrophils with 15 MBq of ^{51}Cr in 0.2 ml saline. Following incubation for 20 minutes at 37°C the cells were washed twice with HBSS/0.2 rat plasma to removed unbound ^{51}Cr . After labelling the neutrophils were recounted and adjusted to a concentration of 10×10^6 /ml in HBSS/0.2% rat plasma. The labelling efficiency of neutrophils was >70%. Cell viability was always >99% as assessed by trypan blue exclusion.

The cell association of radiolabel and the ability of the cells to retain the radiolabel in vitro was assessed for both RBCs and neutrophils over a two hour period. Aliquots of each cell suspension were taken at 10 minute intervals, centrifuged, and counts per minute (cpm) in the supernatant were measured. There was no loss of label from the RBCs or neutrophils during this time (RBC T_0 6.5×10^5 , T_{120} 6.4×10^5 cpm; neutrophils T_0 9.2×10^4 , T_{120} 8.9×10^4 cpm).

IN VIVO FUNCTION OF ^{51}Cr -LABELLED NEUTROPHILS

Rats of approximately 250 g in weight were anaesthetised with 0.1 ml hypnorm (10 mg/ml) (Janssen, Lamberhurst, Kent, UK) intraperitoneally and 0.2 ml diazepam (10 mg/2 ml) (Roche, Welwyn Garden City, UK) was given intramuscularly as a muscle relaxant. The radiolabelled RBC and neutrophil suspensions were mixed gently in a ratio of 1:1 by volume immediately prior to reinjection. A 200 μ l bolus of this cell mixture was then injected into an anaesthetised rat via the iliac vein. Ten minutes later the thoracic cavity was opened and 200 μ l peripheral blood was obtained by cardiac puncture. The heart and major blood vessels were immediately ligated to prevent blood loss from the lungs which were then dissected free of the thoracic cavity. The lungs were inflated with 3% gluteraldehyde in saline and fixed overnight to prevent blood loss on dissection. The samples of blood and lung were counted the following day in a gamma counter to determine the amount of ^{51}Cr and ^{99m}Tc in each sample.

To check that the radiolabel remained associated with the cell, and to assess the persistence of radiolabelled cells in the circulation, 200 μ l samples of venous blood were taken from three rats at 5, 15, 30, 60, and 120 minutes after the injection of radiolabelled RBC/neutrophil via the iliac vein. These samples were anticoagulated with EDTA, the cells were pelleted by centrifugation, and the ^{99m}Tc and ^{51}Cr counts in the supernatant and pellet were then measured.

The ability of the radiolabelled neutrophils to function as native unlabelled cells was assessed in two experiments in which their ability to respond to an inflammatory stimulus was determined by measuring their accumulation in the bronchoalveolar space in control and inflamed rat lungs. In these experiments a 200 μ l bolus of RBC/neutrophil was injected into anaesthetised rats, followed immediately by an intratracheal injection of *C parvum*. Twenty four hours later the lungs were excised and lavaged and the cell-associated radioactivity in the bronchoalveolar lavage fluid was measured.

Measurement of neutrophil sequestration in the lungs

Sequestration of ^{51}Cr -labelled neutrophils (PMN) in the lungs was calculated by dividing the ratio of PMN:RBC counts in peripheral blood by the ratio of PMN:RBC counts in the lung tissue samples. This provides a measure of the fold excess of WBC:RBC in the pulmonary vasculature. The calculation is as follows:

$$\frac{\text{PMN (cpm in blood)}}{\text{RBC (cpm in blood)}} = X$$

$$\frac{\text{RBC (cpm in lung)}}{\text{PMN (cpm in lung)}} = Y$$

$$\text{Fold increase in PMN:RBC ratio} = \frac{X - Y}{X}$$

$$= \frac{Y}{X} - 1$$

STATISTICAL ANALYSIS

Significant differences between treatment groups were assessed by analysis of variance followed by a *t* test.

Results

IN VITRO FUNCTIONAL ACTIVITY OF CONTROL AND INFLAMMATORY NEUTROPHILS

There were no differences in the activity of control peripheral blood neutrophils harvested by the one-step or two-step techniques as measured by epithelial cell detachment (2410 (843) cpm *v* 2780 (515) cpm respectively, *n* = 4, *p* > 0.05).

The data on the functional status of control peripheral blood neutrophils are summarised in the table. These cells were quiescent compared with inflammatory neutrophils obtained by bronchoalveolar lavage from rat lungs exposed to *C parvum* as judged by significantly lower proteolytic activity, ability to detach epithelial cells, and chemotaxis. The exception to this was the ability of neutrophils to release superoxide anion (O₂⁻). Both cell populations released O₂⁻ in the presence of the phorbol ester, PMA. Although PMA-stimulated O₂⁻ production by control peripheral blood neutrophils was lower than that of neutrophils obtained by bronchoalveolar lavage after *C parvum* instillation, the difference was not statistically significant.

There was a progressive increase in cell activity between peripheral blood neutrophils from control rats and those from *C parvum* treated animals. Neutrophils in bronchoalveolar lavage fluid from *C parvum* treated animals were the most active as measured by fibronectin degradation and epithelial cell detachment (table). This trend was not present for O₂⁻ release. The pressure developed after six minutes filtration of peripheral blood neutrophils through a millipore filter was significantly lower (0.94 (0.28) cm H₂O) than that produced by inflammatory neutrophils

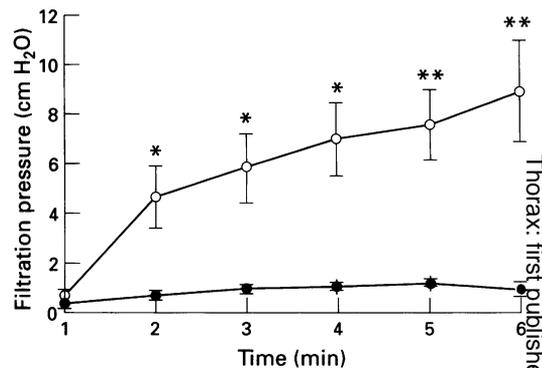


Figure 1 Mean filtration pressures of peripheral blood neutrophils from control animals (●) and inflammatory neutrophils obtained from bronchoalveolar lavage (BAL) of rats treated with *Corynebacterium parvum* (○) (*n* = 4). Data are given as the pressure developed during filtration of 1×10^5 cells/ml through a millipore filter of 8 μ m pore size. The bars represent the SE in each case. * *p* < 0.05; ** *p* < 0.01 compared with time zero.

obtained by bronchoalveolar lavage (8.89 (2.01) cm H₂O, *p* < 0.01) (fig 1), indicating the greater deformability of the peripheral blood neutrophils.

IN VIVO EXPERIMENTS WITH CONTROL AND INFLAMMATORY NEUTROPHILS

Persistence of neutrophils in the bloodstream

The intravenous retention of radiolabel on neutrophils and RBCs was assessed in three control rats in a single experiment over a time course which extended from five to 180 minutes. The proportion of ^{99m}Tc which remained associated with RBCs during this time was 98 (1)%, and the proportion of ⁵¹Cr associated with neutrophils was 88 (1)%.

Cell-associated counts due to ⁵¹Cr in 200 ml aliquots of peripheral blood were also measured over a period of 5–180 minutes. During this time there was a decrease in counts of ⁵¹Cr from 1272 (100)/blood sample to 683 (17)/blood sample (fig 2).

To determine if radiolabelled neutrophils functioned as native cells, the ability of the labelled neutrophils to migrate into a site of inflammation was assessed. In a single experiment the total neutrophil-associated counts in the bronchoalveolar lavage fluid were 51 cpm

Mean (SE) *in vitro* function of peripheral blood (PB) and bronchoalveolar lavage (BAL) neutrophils (PMN) from control and *C parvum* treated animals

	Control	<i>C parvum</i>		Number of experiments*	Comparison	<i>p</i>
	PB-PMN (Group 1)	PB-PMN (Group 2)	BAL-PMN (Group 3)			
Fibronectin proteolysis (cpm fibronectin degraded)	2452 (80)	3702 (154)	12367 (1129)	3 ^a	1 <i>v</i> 2 1 <i>v</i> 3 2 <i>v</i> 3	<0.05 <0.001 <0.01
A549 epithelial cell detachment (cpm detached cells)	2410 (487)	4150 (500)	7597 (1880)	4 ^a	1 <i>v</i> 2 1 <i>v</i> 3 2 <i>v</i> 3	<0.05 <0.01 >0.05
PMA-stimulated superoxide anion release (nmol)	8.16 (1.4)	19.73 (2.2)	14.4 (1.2)	3 ^a	1 <i>v</i> 2 1 <i>v</i> 3 2 <i>v</i> 3	>0.1 >0.1 >0.25
Chemotaxis (cells/high power field)	7.4 (1.8)	ND	19.2 (3.0)	6 ^b	1 <i>v</i> 3	<0.05

^aAnalysis of triplicate sample in each experiment.

^bDuplicate samples in six separate experiments.

ND = not done; PMA = phorbol myristate acetate.

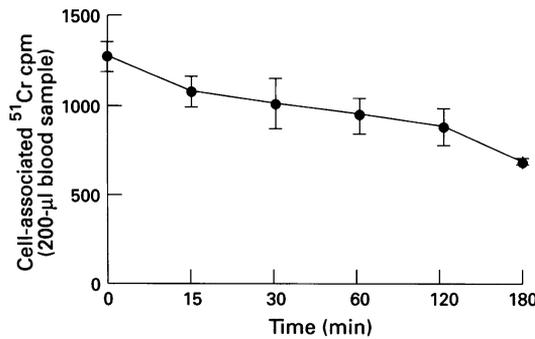


Figure 2 Mean (SE) cell-associated counts of ⁵¹Cr in 200 µl blood samples over a three hour period.

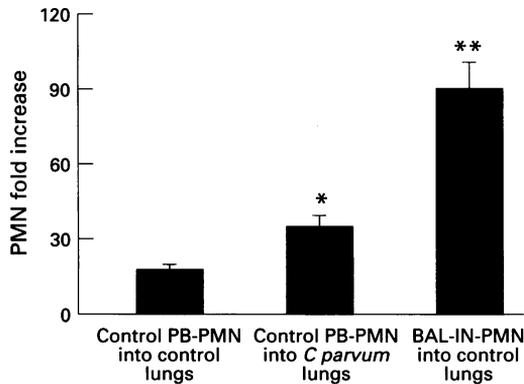


Figure 3 Sequestration of ⁵¹Cr-labelled neutrophils in the lungs 10 minutes after reinjection of peripheral blood (PB) neutrophils from control rats (PB-PMN) ($n=12$), inflammatory neutrophils from bronchoalveolar lavage fluid (BAL-IN-PMN) ($n=11$) into control rat lungs, and control PB-PMN into *C parvum* treated animals ($n=10$). The histograms represent the mean and the bars the SE of the fold increase of the PMN:RBC ratio in the lungs over that in peripheral blood. * $p<0.05$; ** $p<0.01$.

and 42 cpm for two control rats, and 4680 cpm and 2415 cpm for two rats with lung inflammation induced by *C parvum*.

NEUTROPHIL SEQUESTRATION

Effect of neutrophil activation

The role of neutrophil activation in modulating the sequestration of neutrophils in the lung was assessed by comparing the pulmonary sequestration of untreated peripheral blood neutrophils obtained from control animals and inflammatory bronchoalveolar neutrophils when injected into control rats. The increased sequestration of inflammatory neutrophils in the lungs of control rats was significantly greater than for control peripheral blood neutrophils (fig 3).

Effect of lung inflammation

To investigate the contribution which lung inflammation might have on neutrophil sequestration in this model we compared the lung sequestration of control peripheral blood neutrophils when reinjected into control rats with that of control neutrophils injected into rats in which an acute alveolitis had been elicited by the intratracheal instillation of *C parvum* 16 hours previously. An increased sequestration of control neutrophils in the inflamed lungs compared with control lungs was seen (fig 3).

Discussion

The role of intravascular neutrophil sequestration in the pathogenesis of pulmonary disease has been of interest for a number of years.^{1,2} Much of the work in previous experimental studies of neutrophil sequestration was carried out using rabbits^{3,10} and dogs.^{9,19} In view of the large database of information on rat models of lung inflammation^{20,21} we have developed a rat model to investigate the mechanisms which govern neutrophil sequestration in the lungs. The rat has previously been considered unsuitable for studies of neutrophil kinetics because of the large numbers of neutrophils which need to be harvested in such experiments, and the difficulty of obtaining sufficient numbers and purity of cells which maintain their functional integrity from such a small animal. In addition, rat neutrophils sediment with RBCs in dextran which further complicates standard isolation procedures. Rats have been used in previous studies of neutrophil sequestration but, in order to obtain sufficient numbers of cells, the influx of neutrophils into the peritoneal cavity was induced by an intraperitoneal injection of thioglycollate medium or into the bloodstream following an intravenous injection of endotoxin.^{22,23} However, the nature of the methods to elicit these cells will induce cell activation producing a population of cells with different properties from normal circulating neutrophils.²³

The method of cell isolation which we have utilised in the present study permitted us to obtain large numbers of non-activated peripheral blood neutrophils from a single rat. The most important aspect of the separation technique is the use of Hetastarch (hydroxyethyl starch) exchange transfusion.¹⁵ This has several benefits. Firstly, by transfusing the animal the largest possible yield of plasma was obtained. Secondly, the increased time that is taken to bleed the animal permits recovery of the marginated pool of neutrophils from organs such as the lungs and liver. Finally, while rat RBCs sediment in Hetastarch, neutrophils remain in suspension so Hetastarch is a suitable alternative separating medium to dextran. The use of Hetastarch exchange to obtain peripheral blood neutrophils from rats was originally described and validated by Williams and co-workers.¹⁵ The functional activity in vitro of the isolated neutrophils in the experiments reported by Williams *et al* was not significantly different from that of neutrophils obtained by simple phlebotomy; in addition, radiolabelled neutrophils isolated with Hetastarch retained normal transit and adherence capacities in vivo.¹⁵

In our experiments we have extended the work of Williams and colleagues by comparing the in vitro chemotactic activity, fibronectin proteolysis, superoxide anion production, and the injurious effects on epithelial cells of peripheral blood neutrophils prepared by the Hetastarch method with that of exudated neutrophils obtained by bronchoalveolar lavage from inflamed rat lungs. In three of the parameters tested – fibronectin proteolysis, chemotaxis, and epithelial detachment – the control

peripheral blood neutrophils were significantly less active than exudated neutrophils from bronchoalveolar lavage fluid obtained from *C parvum* treated animals. The results of these experiments suggest that the peripheral blood neutrophils were not activated by the isolation procedure and are in agreement with a previous study in which we demonstrated increased proteolytic activity and injurious effects on epithelial cells of inflammatory bronchoalveolar neutrophils.²⁴ Furthermore, peripheral blood neutrophils in the rat could be activated in vitro by PMA.²⁵ Increased chemotaxis of inflammatory neutrophils has also been shown previously in rabbits.²⁶

These results were not due to the suppressive effect of the Hetastarch on the peripheral blood neutrophils since in vitro exposure of the exudated neutrophils to Hetastarch did not alter their functional activity (data not shown). In addition, exposure to hydroxyethyl starch in vitro has previously been shown to have no adverse effect on human peripheral blood neutrophils.¹⁵ Furthermore, in vivo transfusion with Hetastarch has no adverse effects in humans, rats, or several other laboratory animals.^{27,28}

The superoxide anion released by inflammatory neutrophils in bronchoalveolar lavage fluid was greater (although not significantly greater) than that of the control peripheral blood neutrophils. This may be due to down-regulation of the oxidant activity of the inflammatory neutrophils, which has been reported previously in neutrophils lavaged from lungs of rats exposed to asbestos²⁹ and in human bronchoalveolar neutrophils obtained from a patient with acute respiratory failure.³⁰ It has been proposed that such down-regulation of oxidant responses may be due to negative feedback by hydrogen peroxide.³¹

Interestingly, the activity of peripheral blood neutrophils obtained from *C parvum* treated animals was also higher than peripheral blood neutrophils obtained from control animals, but not as high as the activity from cells obtained by bronchoalveolar lavage from inflamed lungs. This result was significant for fibronectin proteolysis, although a similar but non-significant trend was present for epithelial cell detachment. These data suggest that neutrophils become more activated in migrating from the blood to the air spaces, possibly due to the action of cytokines.

The later stages of the separation procedure involved differential separation of the leucocyte-rich plasma using Percoll, a colloidal silica preparation. This type of separation medium is preferable to the usual alternative, Ficoll Hypaque, which may activate the cells during the isolation procedure.³² The rapid one-stage separation technique which we used in all of the later experiments enabled us to obtain larger numbers of cells, of high purity, in about half the time required for our original method. No differences in the activity of neutrophils prepared by either techniques were observed in vitro or in vivo. This novel technique will permit the use of rats in research programmes where peripheral blood neu-

trophils are required and where larger animals would previously have been used to provide sufficient numbers of neutrophils. The techniques which we utilised to radiolabel the erythrocytes and neutrophils were standard procedures which have been used previously and which do not alter cell function or viability.^{19,23,25} In the present study we have shown that the radiolabel remains associated with the cells both in vitro and in vivo. In this study 54% of neutrophils injected into untreated rats remained in the circulation for up to three hours after the injection. This suggests that the cells were in a condition which was similar to that of neutrophils in previous studies where the half life of radiolabelled neutrophils in the circulation was calculated to be seven hours in humans³³ and six hours in dogs.³⁴ The number of neutrophils remaining in the circulation in the present study compares favourably with one previous study using peripheral blood neutrophils in dogs where only 17% of radiolabelled neutrophils remained in circulation at four hours.⁹ Moreover, in the present study the neutrophils migrated into the air spaces in response to the inflammogen *C parvum*, which is further evidence of their ability to respond like native circulating cells.

These studies of radiolabelled neutrophils confirm in the rat, as has been shown in animal studies in dogs,¹⁹ rabbits,¹⁰ and in humans,¹¹ that there is sequestration of neutrophils in the normal pulmonary vasculature. We calculated neutrophil sequestration as the fold increase of intravascular neutrophils in excess of that predicted by the local blood volume (estimated by the RBC counts). This calculation produced a 17.4 (2.1) fold excess of neutrophils in the normal pulmonary circulation, which compares with the 15 fold increase obtained using a similar technique when radiolabelled cells were injected into patients undergoing lung resection.³⁵ These data therefore support the use of this model in the rat as a surrogate for human studies.

Several mechanisms may contribute to the delayed passage of neutrophils within the capillary bed. In this study we have investigated the relative roles of neutrophil activation and acute lung inflammation on the transit of neutrophils through the pulmonary vasculature. We have also investigated, in a preliminary way, the effect of neutrophil deformability on neutrophil sequestration in the lungs. The most marked neutrophil sequestration was observed in experiments where cells obtained by bronchoalveolar lavage from rat lungs inflamed with *C parvum* were reinjected into control animals. The sequestration of these cells in control lungs was six times greater than that of the sequestration of control peripheral blood neutrophils. We have previously shown that these inflammatory neutrophils are activated as defined by a number of criteria.^{24,36} In this and previous studies we have shown that neutrophil activation is associated with altered deformability of the cell^{8,14} which is probably due to polymerisation of F-actin.¹³ The association between the reduced deformability of the inflammatory neutrophils in the present study and their increased intravascular retention

strongly suggests that cell deformability may be a major factor contributing to neutrophil sequestration in the normal lung microvasculature, and is supported by studies in humans where a correlation has been shown between neutrophil sequestration in the lungs of healthy subjects in vivo and neutrophil deformability in vivo.¹¹ However, the increased sequestration of control peripheral blood neutrophils in inflamed rat lungs in the absence of any difference in cell deformability implicates other mechanisms which enhance the sequestration of neutrophils during lung inflammation.⁸ Neutrophil sequestration in the lungs has been shown previously to be variously dependent on blood flow.^{9,12} Expression of adhesion molecules on the surface of the neutrophil and the capillary endothelial cells can alter cell adhesion and hence neutrophil sequestration.¹⁰ Such integrin-mediated neutrophil sequestration in the lungs is thought to be important in conditions of neutrophil or lung endothelial cell activation,³⁷ whereas cell deformability alone may account for the sequestration in normal lungs.^{8,11} The differences in lung sequestration of neutrophils under the various conditions in this study demonstrate the multifactorial nature of the signals which can elicit increased neutrophil sequestration in the lungs. The use of this rat model of neutrophil sequestration will enable further studies to be carried out to elucidate the role of the various factors which can influence neutrophil sequestration in lung inflammation and injury.

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