

Changes in neutrophil morphology and morphometry following exposure to cigarette smoke

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Summary. Acute cigarette smoking delays neutrophils within the pulmonary circulation in some smokers. Evidence from an in-vitro Micropore filter model of the pulmonary capillaries indicates that this may be due to a smoke induced decrease in cell deformability. In order to determine whether changes in cell shape are associated with the observed decrease in neutrophil deformability following smoke exposure, cell morphology, using scanning electron microscopy, and morphometric measurements, made using transmission electron microscopy, were performed on aliquots of neutrophils harvested from whole blood in non-smoking subjects before and after exposure *in vitro* to cigarette smoke. Smoke exposure increased the maximum diameter and circumference of neutrophils, without changing their area. There was also a change in the maximum to minimum cell diameter ratio, which indicated that the cells had become less spherical. Scanning electron microscopy showed that smoke exposed cells had developed blebbing of their surface membranes, suggestive of an oxidative injury to the cell membrane rather than the shape changes associated with cell activation. These changes in the morphology and morphometry of smoke exposed neutrophils may contribute to the reduction in cell deformability induced by cigarette smoke.

Keywords: cigarette smoke, neutrophil, morphology, morphometry

Cigarette smoking is a major risk factor in the development of pulmonary emphysema (Auerbach *et al.* 1972). Neutrophils are present in increased numbers in both the airspaces (Hunninghake & Crystal 1983) and alveolar walls (Ludwig *et al.* 1985) of smokers. Acute cigarette smoking delays the passage of polymorphonuclear leucocytes within the pulmonary circulation in some smokers (MacNee *et al.* 1989a). In order to

traverse the pulmonary microcirculation neutrophils must deform, as their average diameter of 7 μm (Schmid-Schonbein *et al.* 1980) is greater than that of the average pulmonary capillary segment diameter of 5 μm (Weibel 1963).

Studies using an in-vitro Micropore filter, whose dimensions mimic those of the average pulmonary capillary segment (MacNee *et al.* 1989b), suggest that neutrophils

exposed *in vitro* to either vapour or particulate phase cigarette smoke have decreased cell deformability. It is reasonable to expect that neutrophils which are less deformable will take longer to traverse the microcirculation and may consequently have more time to increase the proteolytic burden in the lungs.

A number of factors influence the deformability of a cell. These include the fluidity of the cell membrane, the turnover of the cytoskeleton, the viscosity of the cytoplasm, the size of the nucleus and the surface area: volume ratio of the cell (Chien *et al.* 1984). The neutrophil is a multi-nucleate cell with a cell membrane area calculated to be between 40 and 90% in excess of that necessary to contain its volume (Schmid-Schonbein *et al.* 1980; Bagge 1976). In its resting state the membrane of the neutrophil is ruffled, exhibiting folds. During deformation within the capillary segments a proportion of the excess surface area of the membrane is utilized to allow a passive change in cell shape. The aim of this study was to assess the smoke induced changes in neutrophil morphology and morphometry which may contribute to the reduced filterability of the cells *in vitro* following smoke exposure.

Methods

Polymorphonuclear leucocyte harvesting

Neutrophils were harvested from 30 ml of venous blood obtained from six healthy, normal non-smokers. Whole blood was mixed with 12 ml of dextran MW 70 000 (LOMODEX 70 Fisons P/C Pharmaceutical Division, Loughborough, UK) and allowed to sediment for one hour to separate the majority of the erythrocytes so as to obtain a leucocyte-rich plasma (LRP). This LRP (approx 20 ml) was washed twice with phosphate buffered saline (PBS, pH 7.4, 10 ml) at 100 *g* for 10 min. The resulting LRP cell pellet was resuspended in platelet-poor plasma (PPP) (4 ml), previously prepared by centrifuging 20 ml of blood for 10 min at

400 *g*, aspirating the plasma, followed by centrifugation at 1000 *g* for 30 min. This was overlaid on a discontinuous plasma-Percoll (Pharmacia, Uppsala, Sweden) 42 and 65% density gradient and centrifuged at 200 *g* for 10 min. The resultant neutrophil band which settled at the interface between the 65 and 42% plasma-Percoll gradient was aspirated and washed with PBS at 200 *g* for 10 min. Any contaminating red blood cells were lysed with 0.2% saline for 20 s. The tonicity of the solution was re-established immediately with an equal volume of 1.6% saline. The neutrophils were washed and then resuspended in PBS to give a final concentration of 1×10^6 cells/ml.

Smoke exposure

Two-millilitre aliquots of whole blood or harvested neutrophils at a concentration of 1×10^6 cells/ml, were placed in the siliconized flask of a tonometer and exposed to four puffs (37 ml/puff) of vapour phase cigarette smoke over 4 min. Vapour phase cigarette smoke was produced by drawing particulate cigarette smoke, generated by a smoking machine, through a Cambridge filter. Control blood and cell samples were agitated in the tonometer for the same length of time but were not exposed to smoke. Carboxyhaemoglobin (CoHb) was measured in the whole blood samples using a co-oximeter (Co-oximeter 282 II, Instrumentation Laboratory) as an indication of smoke exposure. The neutrophils were washed after exposure in PBS at 200 *g* for 10 min, then fixed in freshly prepared 3% glutaraldehyde and processed for electron microscopy.

Morphometry

Samples of neutrophils post fixation were placed in 1% osmium tetroxide in sodium cacodylate buffer for 1 hour, dehydrated through to absolute alcohol (10, 50 and 100%, 15 min per change), then transferred to propylene oxide (10 min, twice) and impregnated overnight in Araldite resin.

This preparation was finally embedded in Araldite resin and polymerized at 50°C. The sections were cut on a LKB Ultratome Nova to a thickness of 60 nm and stained with uranyl acetate and lead citrate. Eight random fields were then photographed on a Jeol 100 S electron microscope at a magnification of 2.1×10^3 . Morphometric analysis was done using a Tektronix 4050 series computer linked to a digitizing tablet (GIS Blairgowrie, Scotland), on a total of 200 cells for each condition, taken from 6 paired experiments. The following parameters were measured; minimum diameter, maximum diameter (perpendicular to the minimum diameter), circumference and area. Two other parameters were then calculated from these measurements:

$$\text{diameter ratio} = \frac{\text{minimum diameter}}{\text{maximum diameter}}$$

$$\text{form factor} = \frac{\text{area}}{\text{circumference}^2} \times 4 \pi$$

The form factor of a perfect circle is 1 representing the maximum area bound by a given circumference. This tends towards zero where the area bound by the circumference is not maximal, due to increases in ellipticity and/or undulations of the circumference. It differs from the diameter ratio in that it is little affected by minor undulations of the circumference.

Morphology

Neutrophil morphology was assessed before and after smoke exposure in 30 cells randomly selected from two subjects using scanning electron microscopy. The changes in cell shape were categorized as:

- (1) Neutrophils with a normal surface membrane ruffling and a spherical shape.
- (2) Neutrophils producing lamella podia.
- (3) Cells with membrane blebbing.
- (4) Cells which have lamella podia and surface blebbing.

An aliquot of neutrophils activated with 1

$\mu\text{g/ml}$ of phorbol myristate acetate (PMA) was processed for scanning electron microscopy for comparison with the smoke exposed cells. Cell viability was assessed before and after smoke exposure by trypan blue exclusion.

Statistical analysis

Results were analysed using Kruskal-Wallis one-way ANOVA.

Results

Carboxyhaemoglobin in whole blood increased from $1.1\% \pm 0.3\%$ to $4.5\% \pm 2.2\%$ following smoke exposure ($P < 0.05$). Cell viability remained $> 98\%$ after smoke exposure, as assessed by trypan blue exclusion.

Morphometry

The maximum diameter and circumference of smoke exposed neutrophils increased following smoke exposure, and consequently the diameter ratio and form factor decreased, compared to control cells (Table 1). However, there was no significant change in either the minimum diameter or area following smoke exposure. The maximum diameter of the smoke exposed neutrophils was approximately 10% larger than that of the control neutrophils, with a shift in the frequency of distribution of these cells towards the higher values (Fig. 1). Neither the skewness nor the kurtosis of these frequency distributions changed after smoke exposure ($P > 0.05$). This suggests an increase in maximum diameter in the majority of cells, as opposed to an increase in the size of only the smaller cells. The mean circumference of the neutrophils also increased by approximately 14% whilst the shape of the frequency distribution of the circumference remained the same (Fig. 2).

Morphology

Scanning electron microscopy indicated that

Table 1. Comparison of the morphometry of control and in-vitro smoke exposed neutrophils

Measurement	Control cells	Smoke exposed cells
Minimum diameter (μm)	6.02 ± 1.18	6.08 ± 1.25
Maximum diameter (μm)	7.33 ± 1.88	$8.09 \pm 1.79^*$
Diameter ratio	0.84 ± 0.12	$0.76 \pm 0.12^*$
Circumference (μm)	24.14 ± 5.58	$27.43 \pm 5.19^*$
Area (μm^2)	32.44 ± 11.72	34.08 ± 10.84
Form factor	0.71 ± 0.15	$0.58 \pm 0.14^*$

* $P < 0.001$ compared with control cells. Results represent mean \pm s.d. of 200 cells.

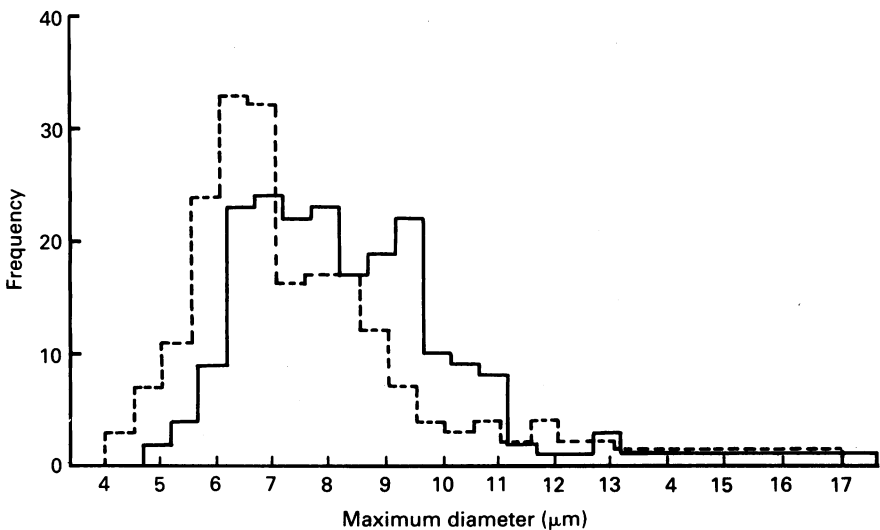


Fig. 1. The effect of in-vitro smoke exposure on the maximum diameter of human neutrophils. ---, Control cells; —, smoke exposed cells.

a greater proportion of smoke exposed cells had developed surface blebbing after exposure to smoke (Table 2). Examples of control, smoke exposed and PMA stimulated neutrophils are shown in Figs 3-5.

Discussion

The acute effect of cigarette smoke on neutrophils has not been previously studied. This study has shown that exposure to vapour phase cigarette smoke *in vitro* causes

changes in cell shape. These result in increased cell circumference and maximum diameter with accordingly decreased diameter ratio and form factor so that the cells become less spherical. The presence of surface blebbing indicates that smoke exposure produces a change in cell shape which is dissimilar to that which occurs when cells are triggered with PMA.

The effects of cigarette smoke on the morphology of alveolar macrophages have been more widely studied than the effects on

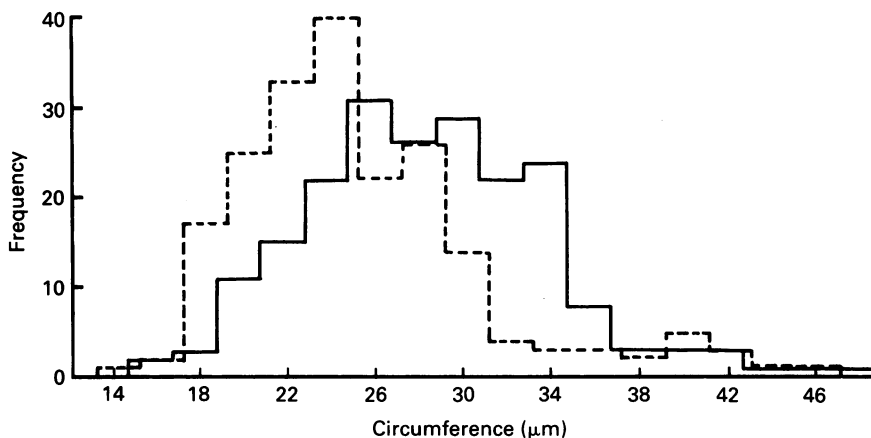


Fig. 2. The effect of in-vitro smoke exposure on the circumference of human neutrophils. ---, Control cells; —, smoke exposed cells.

Table 2. Analysis of the morphology of neutrophils from two subjects before and after smoke exposure in-vitro by scanning electron microscopy

Morphological category	Number of neutrophils	
	Control	Smoked
Normal	49	6
Lamella podia (1)	11	0
Surface blebbing (2)	0	49
Combined (1 + 2)	0	5

neutrophils but have produced conflicting results. Some studies indicate that macrophages from smokers are larger than those from non-smokers (Warr & Martin 1978; Finch *et al.* 1980) whereas others found no differences (Harris *et al.* 1970). The use of differing culture systems with or without the presence of serum and a lack of standardization of the smoking history immediately prior to the bronchoalveolar lavage may explain some of these contradictory results. Since we were interested in assessing the acute effect of components of cigarette smoke which might affect cells in transit in the pulmonary capillaries, we studied vapour

phase cigarette smoke. Guarnari (1978) found that alveolar macrophages recovered from lavage and then exposed to in-vitro smoke did not become less viable, which is in agreement with our observations using neutrophils.

Smith *et al.* (1986) found that alveolar macrophages from healthy cigarette smokers generated a greater pressure during filtration than cells obtained from non-smokers, associated with an increase in cell size and loss of the ruffles on the surface membrane. They concluded that the loss of the excess surface membrane available for deformation, due to cell shape change, may be responsible for the impaired filtration. The changes in neutrophil morphometry and morphology following smoke exposure in this study may also act to reduce the ability of cells to passively deform in a Micropore membrane (MacNee *et al.* 1989b). These shape changes are dissimilar to those induced by stimulation with PMA (Fig. 5) and thus do not support the hypothesis that smoke exposed neutrophils are activated.

Studies of the effects of smoking on alveolar macrophages may also help to explain the mechanism of the changes induced in neutrophils by smoke. Rasp *et al.* (1978) found that up to 60% of alveolar macrophages from

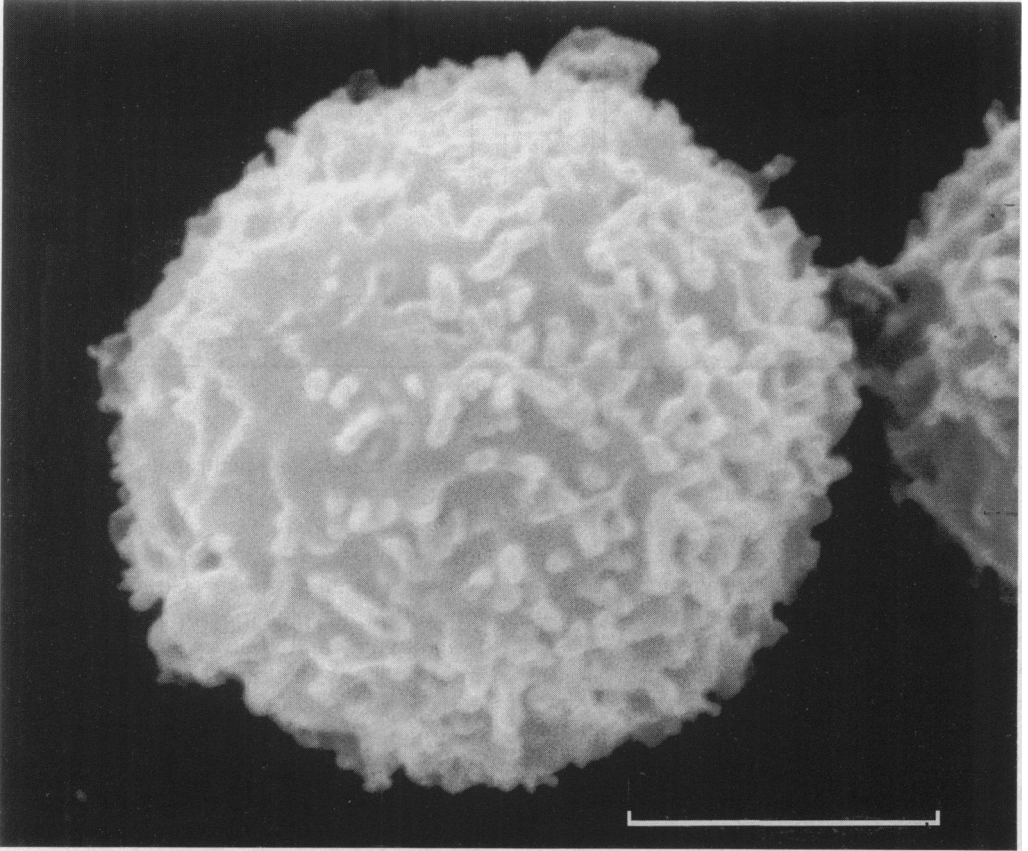


Fig. 3. Scanning electron micrograph of an untreated human neutrophil showing a spherical shape and normal membrane ruffling. The line represents 2.31 μm .

smokers had a blebbed surface, which was not the case in non-smokers. Cell membrane blebbing or zeinosis has been observed in Type I pneumocytes after oxidative injury (Hayashi *et al.* 1987). This may result from oxidation of the sulphhydryl groups in actin (Mirabelli *et al.* 1988) producing an increase in F-actin. Oxidative stress produced by quinones, a component of cigarette smoke, has been shown to deplete intracellular glutathione, followed by oxidation of protein sulphhydryl groups which in turn leads to dissociation of the cytoskeleton from the plasma membrane (Billamo & Mirabelli 1987–88). This results in disruption of the active binding sites of the actin binding proteins which attach the cytoskeleton to the

plasma membrane, resulting in surface blebbing. Tulenko *et al.* (1988) also suggested that chronic cigarette smoking produces reorganization of the phospholipid bilayer in smooth muscle cell membrane. Cigarette smoke has an immense oxidant potential since it is estimated to contain 10^{17} oxidant molecules per puff (Church & Pryor 1985). Thus oxidant induced changes in both the cytoskeleton and the cell membrane are likely to have resulted in the shape changes which we noted in neutrophils after exposure to cigarette smoke.

If cigarette smoke does lead to a change in the neutrophil membrane with the lipid bilayer becoming less fluid, such cells will take longer to change shape and may be

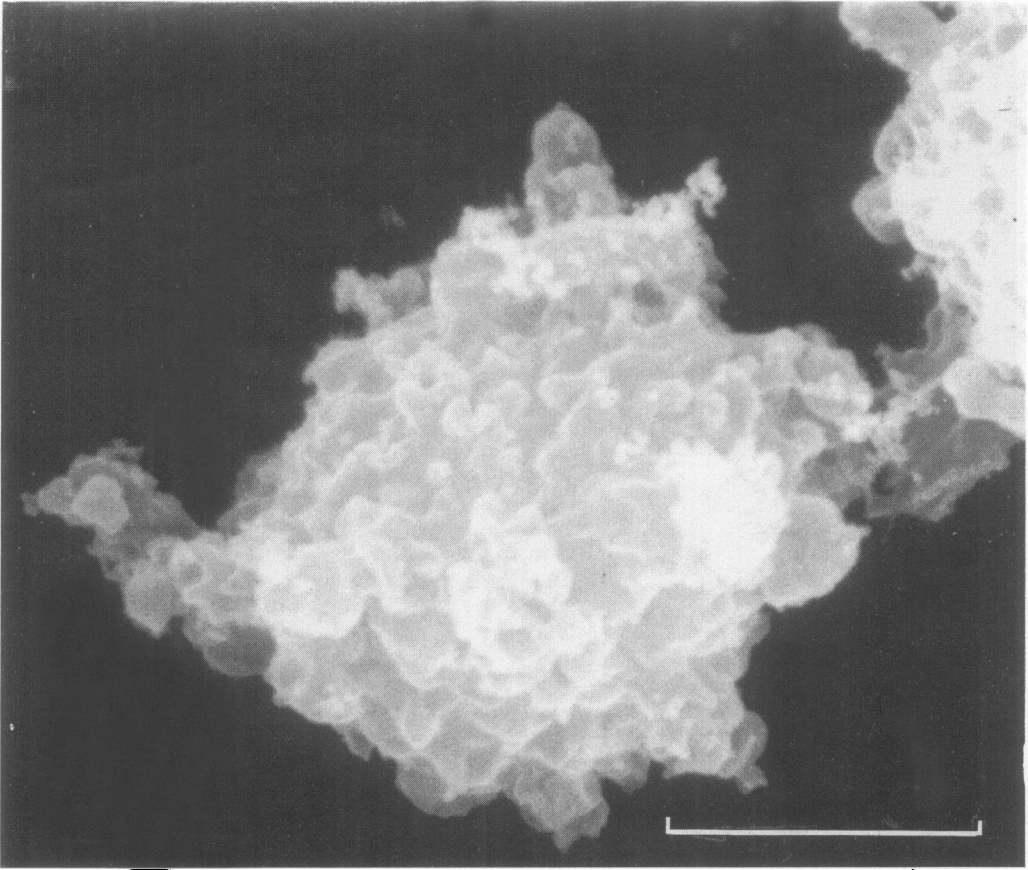


Fig. 4. Scanning electron micrograph of a human neutrophil exposed to cigarette smoke for 4 min. The cell has undergone zeinosis shown by membrane 'blebbing'. The line represents 2.7 μm .

slowed when attempting to deform in order to negotiate the smaller pulmonary capillary segments. This change in cell deformability will be compounded by the changes in the cytoskeleton, induced by smoke, due to alteration in the configuration of actin and destruction of the active sites of the membrane binding proteins.

A prerequisite for the hypothesis that neutrophils are delayed in the pulmonary microcirculation by a direct effect of cigarette smoke on the cell is that oxidative products of inhaled cigarette smoke must reach the intravascular space in the pulmonary capillaries. The fact that inhalation of ozone, a component of cigarette smoke, causes intra-

vascular oxidative stress as shown by a decrease in erythrocyte glutathione and an increase in erythrocyte fragility (Buckley *et al.* 1975) is at least supporting evidence that cigarette smoking could produce a similar effect *in vivo*.

We conclude that in-vitro exposure of PMN to vapour phase cigarette smoke leads to zeinosis, a cell surface change which is regarded as a response to oxidative injury. Smoke probably also produces concurrent changes in the cytoskeleton and associated proteins which, taken together, result in a change in both cell shape and cell size. These changes could be major contributory factors in the increased pressures observed during

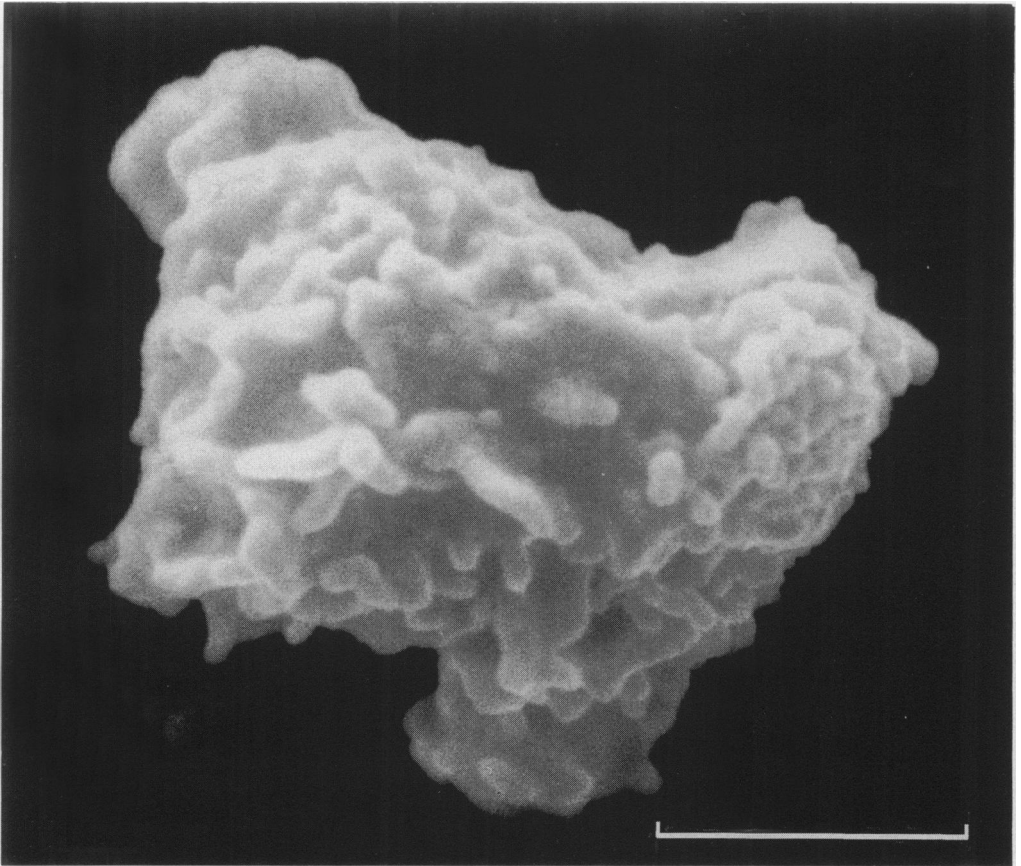


Fig. 5. Scanning electron micrograph of a human neutrophil exposed to PMA for 4 min. The cell has lost its spherical configuration and the surface has developed folds. The line represents $2.31 \mu\text{m}$.

filtration of smoke exposed cells *in vitro* in a model of the pulmonary capillary bed (MacNee *et al.* 1989b) and help to explain the increase in the normal delay of neutrophils in transit in the pulmonary microcirculation which occurs during smoking *in vivo* (MacNee *et al.* 1989a).

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