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Ultrastructural evidence for extravascular platelet recruitment in the lung upon intravenous injection of platelet-activating factor (PAF-acether) to guinea-pigs

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Summary. Bronchoconstriction and degenerative lesions of the bronchial epithelium were observed microscopically 1 min after the i.v. injection of 100 ng/kg of platelet-activating factor (PAF-acether) to the anaesthesized guinea-pig. Constricted arterioles containing marginated polymorphonuclear neutrophils and platelet aggregates were seen, as well as alveolar capillaries obstructed by thrombi formed by partially or totally degranulated platelets. Three minutes after the injection of PAF-acether, platelet diapedesis to the alveolar septa and lumen was clearly observed. Bronchoconstriction was still present at 3 min, but subsided after 60 min, whereas oedema of the submucosa persisted accompanied by an infiltration of eosinophils and neutrophils. The infusion of prostacyclin prevented the formation of platelet aggregates and platelet diapedesis due to PAF-acether, but the morphological evidence of bronchial constriction was not modified. Aspirin failed completely to modify the effects of PAF-acether. Our results show that PAF-acether causes early formation and deposition of platelet aggregates, accompanied by the margination of polymorphonuclear neutrophil leucocytes in pulmonary vessels of the guinea-pig. Since bronchoconstriction persisted when platelet aggregation was inhibited with prostacyclin, aggregation by itself would not account for this effect. Early platelet diapedesis in the vicinity of bronchial smooth muscle corroborates previous evidence that platelets contain and release bronchoconstrictor substances, which operate by cyclo-oxygenase-independent mechanisms and are possibly involved with the physiopathology of lung inflammation during immediate hypersensitivity.

Keywords: bronchoconstriction, cyclo-oxygenase, inflammation, lungs, platelet-activating factor (PAF-acether) platelets

PAF-acether is a phospholipid endowed with a variety of biological effects, (Benveniste & Arnoux 1983) including the ability to induce bronchoconstriction when injected intravenously (i.v.) to guinea-pigs (Vargaftig *et al.* 1980). The analogy between the effects of PAF-acether and those accompanying various physiopathological conditions led to the hypothesis that PAF-acether plays a role as a trigger for platelet activation (Chignard *et al.*

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1979), as a mediator of acute inflammation and of anaphylaxis (Henson & Linch 1983). Bronchoconstriction induced by i.v. PAF-acether is suppressed by immune platelet depletion (Vargaftig *et al.* 1980), suggesting a role for platelets. Nevertheless, it has recently been shown that PAF-acether can trigger the release of thromboxane A2 activity from isolated platelet-free guinea-pig lungs. This release, as well as the accompanying bronchoconstriction are suppressed by aspirin (Lefort *et al.* 1984). We have now studied the ultrastructural changes in the lung following i.v. injection of PAF-acether to the guinea-pig, in an attempt to correlate the bronchopulmonary and the cellular effects. Since the anti-platelet drug prostacyclin, as well as a combination of aspirin with mepyramine (an anti-histamine) and methysergide (an anti-serotonin), prevent bronchoconstriction due to PAF-acether (Vargaftig *et al.* 1982), the latter was injected into treated animals, and the ultrastructural features were investigated.

Materials and methods

Female Hartley guinea-pigs (350–450 g) were anaesthesized with sodium pentobarbitone (i.p. 40 mg/kg), and prepared for the recording of bronchial resistance to inflation (here termed bronchoconstriction, BC), as described previously (Lefort & Vargaftig 1978). PAF-acether (a gift from Prof. J.J. Godfroid, Université de Paris VII; 100 ng/kg), prostacyclin (PG12, a gift from Dr S. Moncada, The Wellcome Research Laboratories; 10 μg/kg perfused for 1 min), aspirin (Aspégic, Laboratories Egic, Paris; 20 mg/kg), methysergide (Sandoz) and mepyramine (Rhône-Poulenc) (0.2 mg/kg of each), were all administered by injection into the cannulated jugular vein. PG12 was injected 1 min before PAF-acether, and the other potential inhibitors, alone or associated, were given 10 min before. In two experiments, ADP was used as a standard platelet-aggregating and bronchoconstrictor agent, at a dose of 300 μg/kg i.v. (Lefort & Vargaftig 1978). Lungs were removed 1, 3 and 60 min after the injection of PAF-acether, small pieces of around 1 mm^3 were dissected along the bronchovascular axis or peripherally, and immediately fixed for 1 h in ice-cold 2.5% glutaraldehyde diluted with sodium phosphate buffer at pH 7.3. The fragments were washed with the buffer, post-fixed in 2% osmium tetroxide, dehydrated and embedded in Epon. Semithin sections of 1 μm thickness were stained with toluidine blue and examined by light microscopy. Ultrathin sections were then prepared with a LKB ultramicrotome, stained with uranyl acetate and lead citrate according to Reynolds (1963), and examined with a Phillips EM 300 electronmicroscope.

Results

Effects of PAF-acether

One minute after the i.v. injection of PAF-acether, intense changes were observed at both bronchial and vascular sites. Marked bronchoconstriction with total obstruction of the bronchial lumen by alveolar macrophages and cellular debris was seen by light microscopy (Fig. 1 inset). Degenerative lesions of the bronchial epithelium were seen by electron microscopy, with degranulated platelets associated with debris in the lumen (Fig. 1). The bronchial vessels were dilated and there was marked blood stasis (Fig. 2).

Arterioles were constricted, and marginated polymorph neutrophil leucocytes together with platelet aggregates were found inside many vessels (Fig. 3). Moreover thrombi, formed by platelets either partially or totally degranulated, obstructed the lumen of the alveolar capillaries (Fig. 4). In some cases membrane fusion was observed, together with gap junctions (Fig. 4 inset). When the aggregates were present, the alveolar septa were markedly thickened (Fig. 4). Circulatory stasis within the capillaries and the venules of the bronchial submucosa accompanied the accumulation of polymorphonuclear cells in the vascular lumen (Fig.

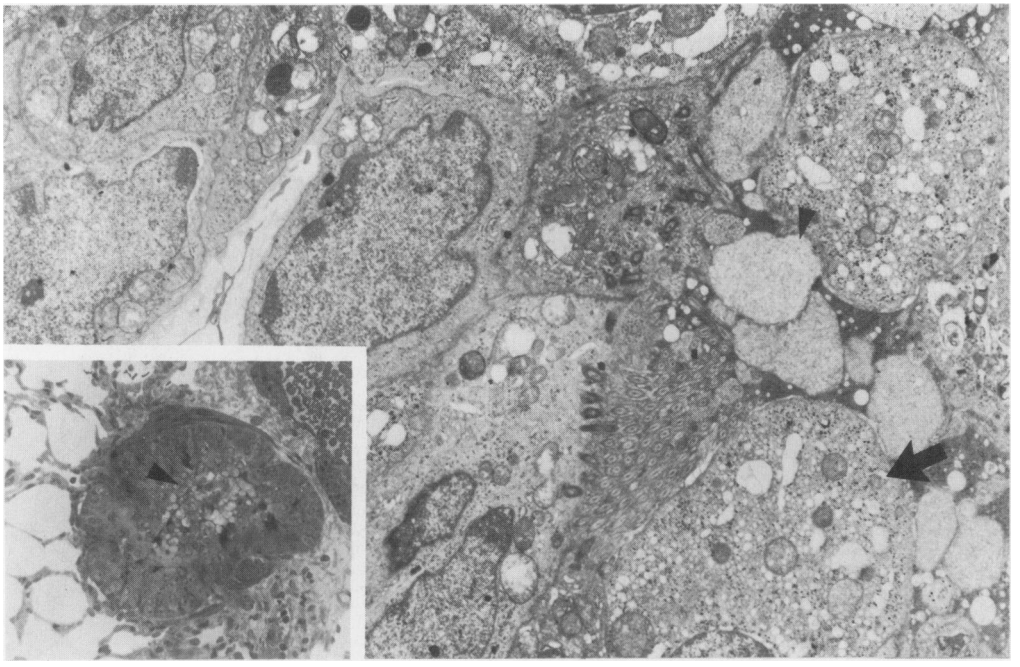

Fig. 1. Contracted bronchus, showing the accumulation in the lumen of degranulated platelets (arrowhead) and fragments of necrotic epithelial cells (arrow) 1.5 min after PAF-acether injection. ×4000. The inset shows a semithin section showing the intense bronchiolar constriction and the obstruction of the lumen by cellular debris and degranulated platelets (arrowhead). × 130.

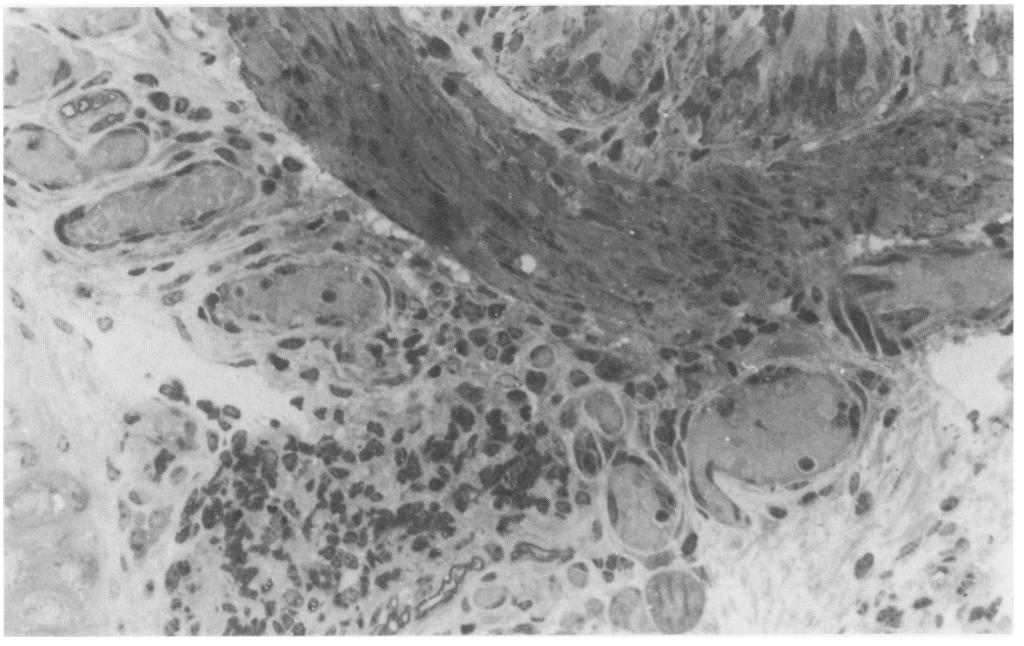

Fig. 2. Light micrograph of a section from the submucosa of a bronchus. Intense stasis, with capillary dilatation and infiltration by neutrophils, is observed 1.5 min after the injection of PAF-acether. × 350.

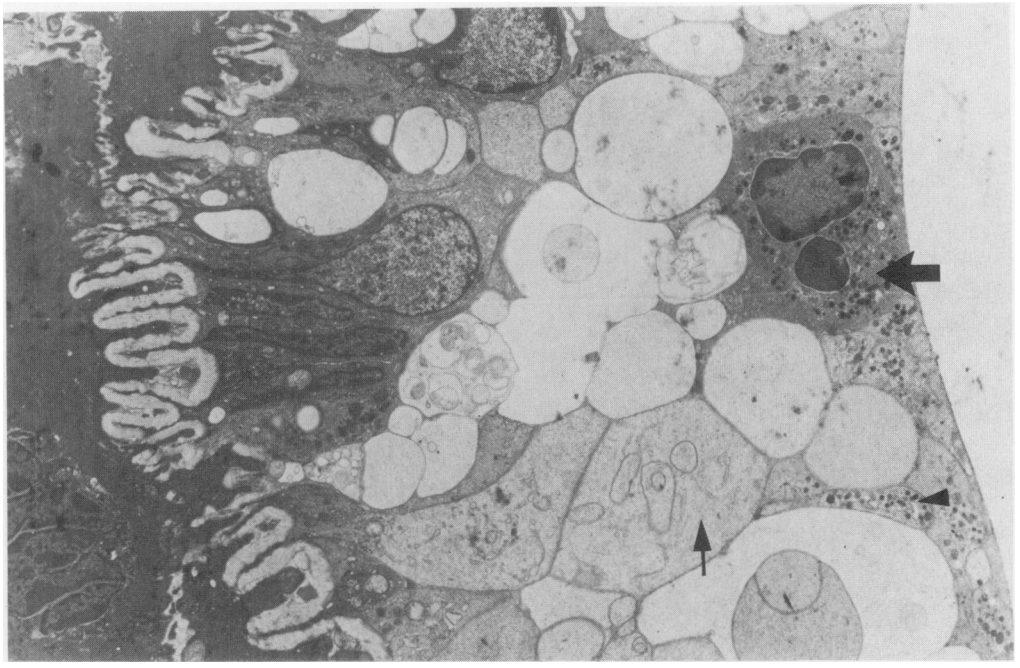

Fig. 3. Electron micrograph of the intima of a small pulmonary artery with a contracted elastica interna. Marginated neutrophil leucocytes (large arrow) platelets (arrowhead) and degenerated blood cells (small arrow) are seen 1.5 min after the injection of PAF-acether. × 2400.

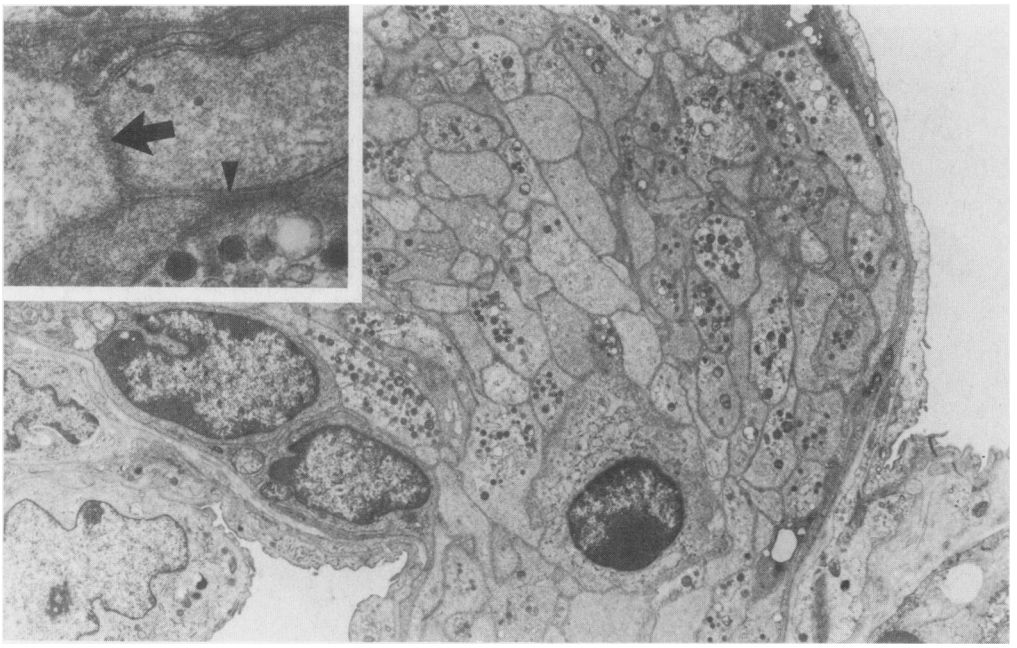

Fig. 4. Electron micrograph of a large aggregate formed by intact and degranulated platelets in an alveolar capillary 1.5 min after the injection of PAF-acether. Note the extent of the alveolar septum. × 5000). The inset shows platelet fusion (arrow) and the presence of inter-platelet junctions (arrowhead) × 40 000.

5). Finally, a marked stasis was observed in the pulmonary veins.

Three minutes after the injection of PAF-acether, the bronchi were still contracted but the number of platelet aggregates was reduced. Images of platelet diapedesis in the septa and in the alveolar lumen were observed (Fig. 6). Degranulated platelets were found in the bronchial wall, in close contact with the smooth muscle (Fig.7). Marked lesions of the vascular walls were also seen, accompanied by alterations of the endothelial cells which showed oedema and in some instances zones of disruption, with cell dissociation and a discontinuous appearance of the basement membrane (Fig. 8). In some instances, foci of haemorrhagic and oedematous alveolitis were seen (Fig. 6).

Sixty minutes after the injections of PAF-acether, bronchoconstriction disappeared. Submucosal oedema persisted, and was accompanied by eosinophilic infiltration in the mucosa and in the submucosa (Fig. 9). Occasional platelet aggregates were still seen within the alveolar capillaries, whereas neutrophils were present in the vessels and in the septa. The latter were infiltrated by inflammatory cells, i.e., monocytes, lymphocytes, neutrophils and eosinophils. Finally, macrophagic alveolitis was seen (Fig. 10).

Effects of potential antagonists on PAF-acether-induced pulmonary changes

Infusion of PG12 to the control animals inhibited 80–90% of the recorded bronchoconstriction Nevertheless, when, examined microscopically, the bronchi were found to be markedly constricted, but with rare platelet aggregates. Aspirin also failed to modify the bronchial constriction produced by PAF-acether. Furthermore, in the aspirin-treated animals, septal and venular platelet aggregates formed by a mixture of intact and

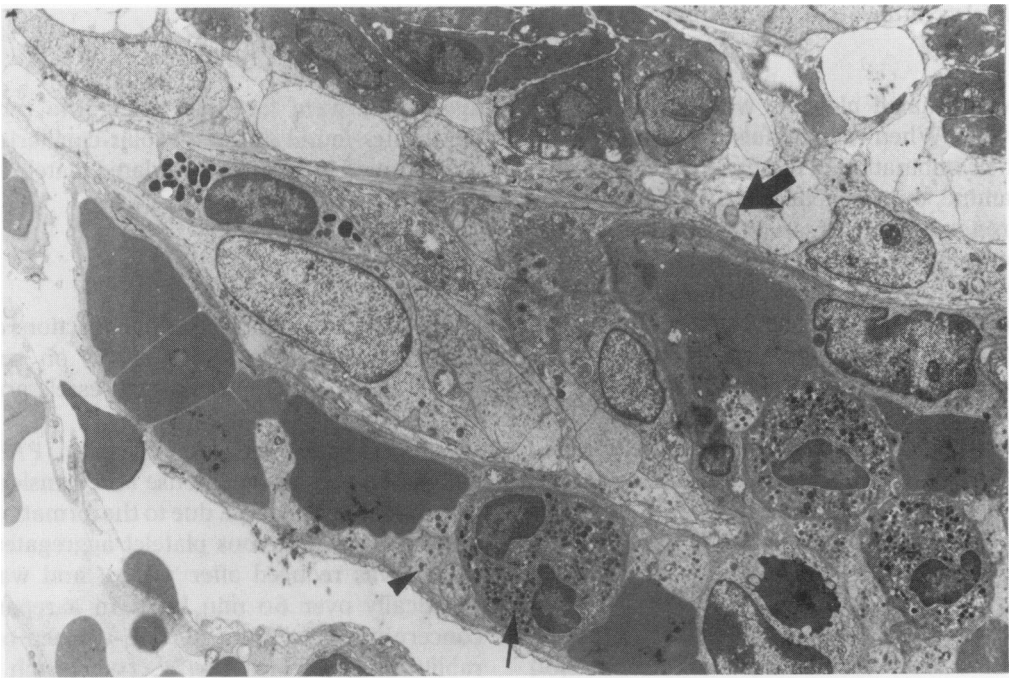

Fig. 5. Electron micrograph of the bronchial sub-mucosa showing marked vascular dilatation and stasis, erythrocyte accumulation, platelets (arrowhead) and neutrophil margination (small arrow), 1.5 min after the injection of PAF-acether. The interstitial tissue contains cell ghosts of degranulated platelets (large arrow). × 2000.

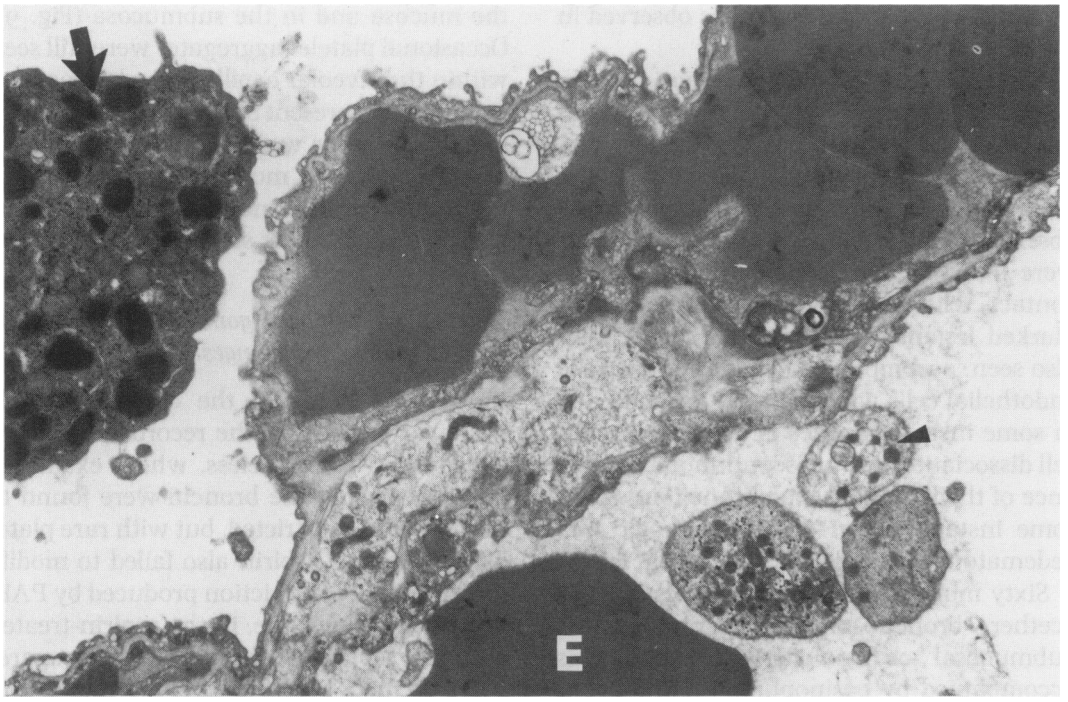

Fig. 6. Electron micrograph of an alveolar septum enlarged by capillary stasis 3 min after the injection of PAF-acether. Haemorrhagic and oedematous alveolitis is observed, with the presence of intra-alveolar platelets (arrowhead), one erythrocyte (E) and one eosinophil (arrow) ×6000.

degranulated platelets were large and frequent. When the animals were treated with the combination of methysergide and mepyramine, only a moderate constriction of the bronchi was observed. Many partially degranulated platelet aggregates were seen in the pulmonary artery, the peribronchial veins and the alveolar capillaries and pulmonary artery constriction was present, despite the treatment. Finally, when aspirin was added to the anti-amine drugs, bronchial constriction was completely suppressed whereas the other morphological modifications induced by PAF-acether, including the platelet effects, persisted.

Effects of ADP

One minute after the injection of ADP (300 μg/kg, i.v.) no constriction of the bronchi was observed microscopically, even though bronchoconstriction was recorded. Platelet aggregates were few and small; the rare aggregates found at the alveolar capillaries were formed by non-degranulated platelets.

Discussion

Our results demonstrate that i.v. injections of PAF-acether induce major effects on cell recruitment and diapedesis, on vessels and on bronchi.

Early (1 min) after its injection, PAF-acether caused a very intense and transient circulatory obstruction, due to the formation of large and numerous platelet aggregates, which was reduced after 3 min, and was practically over 60 min later. In a report concerning the effects of PAF-acether on rabbit lungs (McManus *et al.* 1983) such a platelet-dependent obstruction was not noted. Despite the intensity of aggregation *in situ*, no fibrinous material was observed,

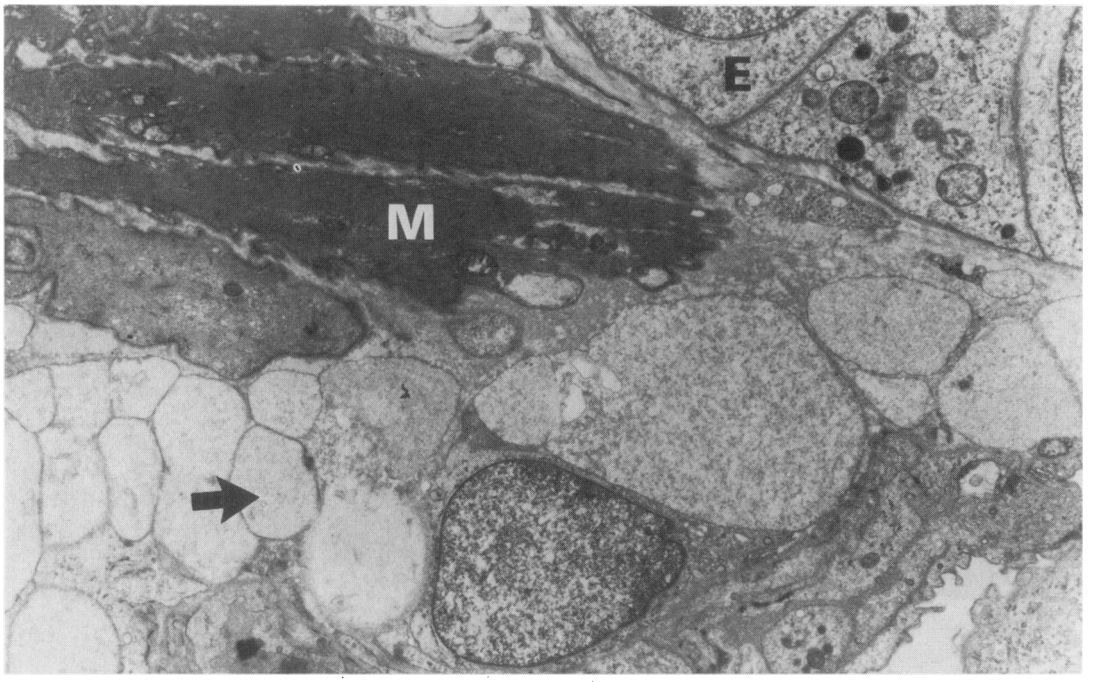

Fig. 7. Electron micrograph of the bronchiolar mucosa 3 min after the injection of PAF-acether. Note the accumulation of cellular debris (arrow), containing an aggregate formed by degranulated platelets, in close contact with the epithelium (E) and with the smooth muscle (M). × 8000.

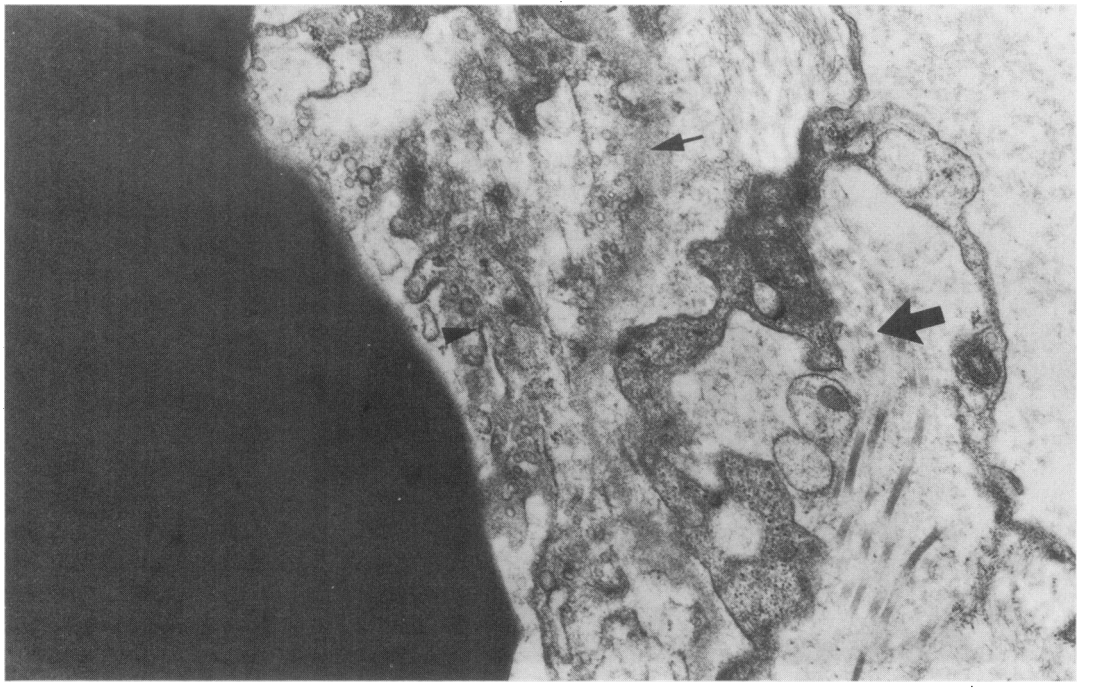

Fig. 8. Electron micrograph of an alveolar capillary showing oedematous degeneration of endothelial cells (arrowhead), dissociation of the basement membrane(small arrow), and interstitial oedema of the septum (large arrow), 3 min after the injection of PAF-acether. × 50 000.

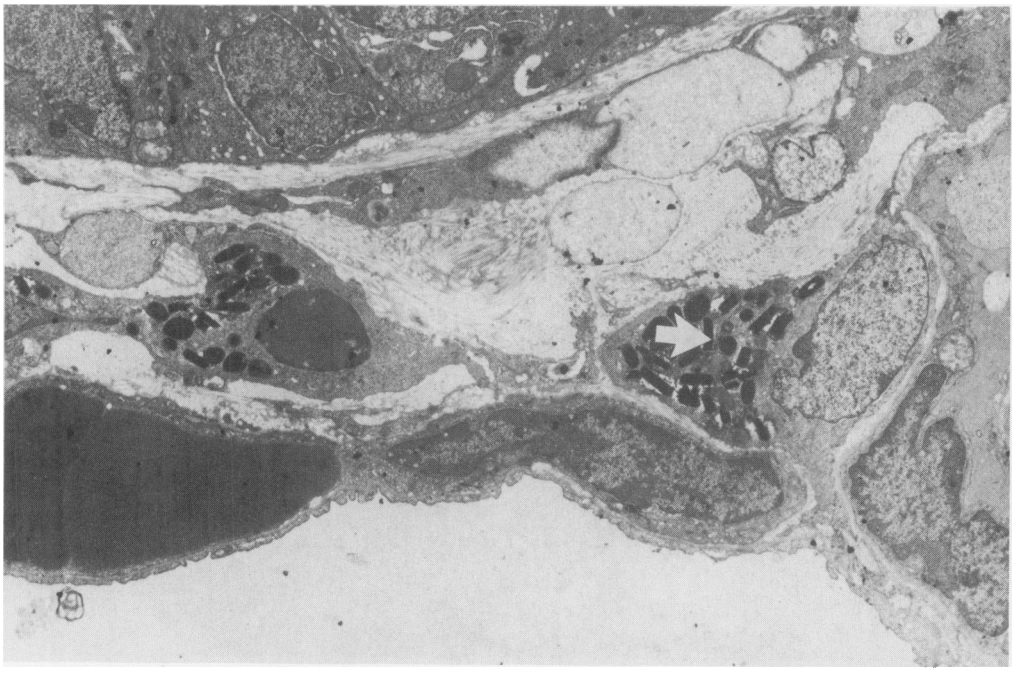

Fig. 9. Electron micrograph of the bronchial submucosa, showing an eosinophilic infiltrate in the process of degranulation (arrow), associated with oedematous lesions 1 h after the injection of PAF-acether. × 4000.

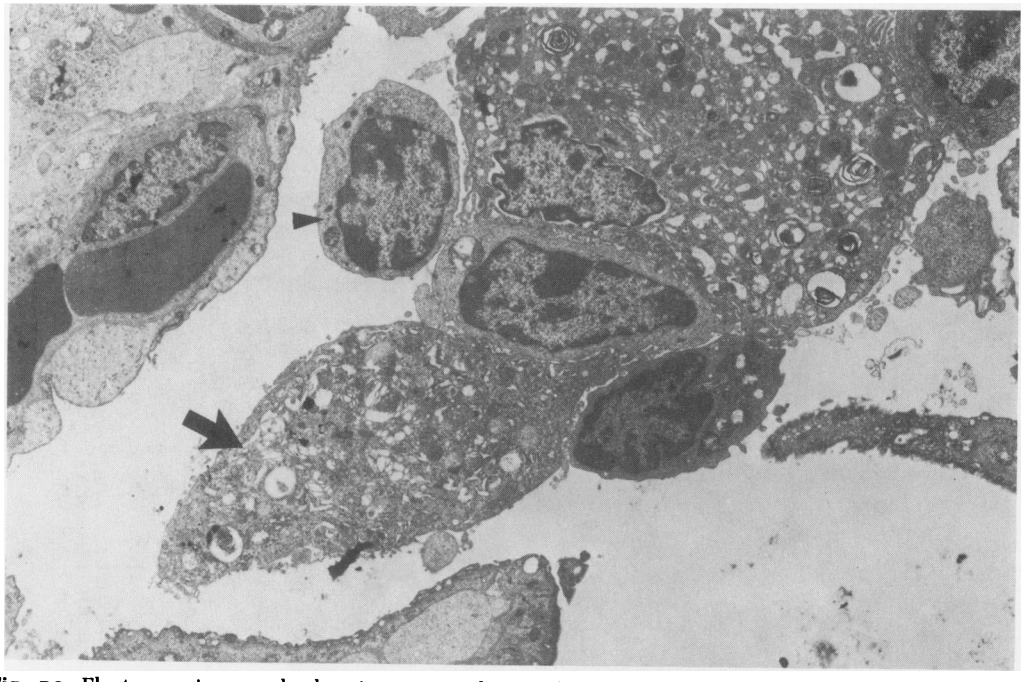

Fig. 10. Electron micrograph showing macrophagic alveolitis (arrow), and lymphocytic infiltration (arrowhead). × 2700.

which is probably related to the inability of PAF-acether to induce clot retraction, as shown by Bottecchia *et al.* (1984) for human blood.

PAF-acether is a recognized stimulator of polymorphonuclear neutrophil leucocytes (Camussi *et al.* 1981; O'Flaherty *et al.* 1981), and induces acute leukopenia when injected i.v. to the guinea-pig (Lefort *et al.* manuscript in preparation). Our present results indicate that these leucocytes accumulate in the pulmonary vessels and, most importantly, that they migrate across the capillaries as early as 1 min after the administration of PAF-acether. The leucocytes observed were mostly polymorphonuclear neutrophils, as described in rabbit skin by Humphrey *et al.* (1983) and in rat skin by Pfister *et al.* (1983) and Pirotzky *et al.* (1984). Moreover, activated (degranulated) eosinophils, located in the bronchial submucosa, appeared 3 min after injection of PAF-acether and persisted for 1 h. To the best of our knowledge, this is the first report of such an effect. The involvement of eosinophils is associated with immuno-allergic reactions, and that of neutrophils with non-specific inflammation.

Platelets, accompanied by neutrophils, were also observed outside the capillaries, as described in rat skin after the injection of PAF-acether (Pirotzky *et al.* 1984). The participation of platelets is a new and potentially important finding, particularly since many of them were degranulated, and in close contact with the bronchial smooth muscle, (as seen in Figs 7 and 9). This finding is in accordance with our previous pharmacological study that bronchoconstriction following i.v. administration of PAF-acether to guinea-pigs is platelet-dependent (Vargaftig *et al.* 1980), and that platelets transport bronchoconstrictor substances, which are released by PAF-acether (Vargaftig *et al.* 1982), and possibly by other stimuli. At present platelets are considered to be likely candidates for active participants in allergic and/or non-allergic inflammatory reactions (Braunstein *et al.* 1980; Knauer *et al.* 1981).

Vasoconstriction, involving arteries of all sizes and, to a lesser extent, the venules as well, was also observed after PAF-acether. This is in accordance with its recognized constricting effects on other vessels such as the coronaries (Benveniste *et al.* 1983), the arterioles of the retina (Braquet *et al.* 1983), those of the hamster cheek pouch (Bjork & Smedegard 1983), and of the guinea-pig mesentery (R.H. Bourgain, personal communication). Vasoconstriction was accompanied by oedema, dissociation and rupture of the endothelial cells and of the basement membrane (Fig. 8). All these effects characterize the acute inflammatory response.

Camussi *et al.* (1983), who instilled PAF-acether intratracheally to rabbits, also noted an acute inflammatory response with accumulation of neutrophils and platelets in the capillary lumina. Cell migration was limited to neutrophils and, later, to macrophages, which were not observed by us in lungs collected up to 1 h after PAF-acether administration. When the latter was instilled by Camussi *et al.* (1983), the alveolar epithelium was primarily affected, in contrast to our observations with PAF-acether given by the i.v. route. Camussi *et al.* (1983) did not observe eosinophil activation nor the massive obstruction of the pulmonary capillaries with platelet aggregates that we report here. This discrepancy may reflect a late tissue collection in their studies, and/or an inappropriate route of PAF-acether administration.

In conclusion, we demonstrated that i.v. injected PAF-acether induces in the guinea-pig a very early inflammatory response, involving polymorphonuclear neutrophil leucocytes, eosinophils and platelets. The last are degranulated and found in the vicinity of the constricted bronchial smooth muscle, suggesting strongly that PAF-acether induces bronchoconstriction via the release of as yet uncharacterized platelet mediators. Since PAF-acether itself may be involved in the dual response to allergen in asthmatic patients (Basran *et al.* 1984; Page *et al.* 1984), the present findings of its

stimulating effect on the early migration of platelets and of other inflammatory cells to the vicinity of the bronchi, strongly suggests a mediator role for this phospholipid in lung allergy.

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