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Pulmonary Expression of ICAM-1 and LFA-1 in Experimental Goodpasture's Syndrome

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The functional importance of ICAM-1 and its ligands, the β_2-integrins, in leukocytic accumulation in pulmonary injury has been recently demonstrated in experimental models of lung disease. However, the exact location of these adhesion molecules remains unknown. In the current study we have used immunogold ultrastructural techniques to define the precise location of ICAM-1 in the lung and its interaction with β_2-integrin expressing leukocytes in the early stages of experimental Goodpasture's (GP) syndrome in the rat. In normal animals there is strong constitutive ICAM-1 expression on the luminal surface of the alveolar epithelium that is confined to type I cells and completely absent from type II cells. Constitutive expression of ICAM-1 on the pulmonary capillary endothelium is comparatively weak. In GP syndrome there is an increase in ICAM-1 expression, which is still confined to the alveolar type I epithelial cells and capillary endothelium. This is associated with an early (1.5 hours) influx of CD18 expressing polymorphonuclear leukocytes, which are seen migrating into alveoli and the pulmonary interstitium. There is a later (6–12 hours) influx of CD11a/CD18 expressing macrophages which are present in the interstitium and in large numbers in the alveolar spaces, where they are very closely apposed to and adherent to the alveolar epithelium. This is the first study to demonstrate the precise ultrastructural location of ICAM-1 in the normal rat lung and in disease. **In vivo** ***administered antibody to ICAM-1 gains access to the extravascular sites within the lung, in particular the surface of alveolar type I epithelial cells, and this raises the possibility that beneficial effects of such antibodies may extend beyond their ability to inhibit interactions between leukocytes and endothelial cells. (Am J Pathol 1994, 145:220–227)***

Goodpasture's (GP) syndrome is characterized by severe glomerulonephritis (GN) and hemorrhagic pneumonitis induced by autoantibodies to basement membrane antigens.[1] We have induced experimental GP syndrome in the rat by using a model of passive accelerated anti-glomerular basement membrane (anti-GBM) disease.[2,3] In the lung there is strong linear deposition of anti-GBM antibody on the alveolar BM associated with an early polymorphonuclear leukocytic (PMNL) accumulation and a more delayed macrophage infiltration with the development of granulomatous lesions.[3]

There is increasing evidence for the importance of the role of endothelial and leukocytic adhesion molecules in inflammatory injury.[4–8] Functional studies have demonstrated the important role of intercellular adhesion molecule (ICAM-1) and its ligands, the β_2-integrins (LFA-1, CD11a/CD18 and Mac-1, CD11b/CD18), in leukocytic recruitment in various models of pulmonary injury, including immune complex-mediated injury,[9,10] phorbol ester-induced inflammation,[11] pulmonary artery occlusion/reperfusion,[12] and lung injury after ischemia/reperfusion of rat hind limbs.[13]

Although a number of light microscopy studies have examined ICAM-1 expression in the lung in various disease states,[10,12,14–17] the exact location of the sites of adhesion molecule expression within the lung remains largely unknown. Therefore the aim of this study was to determine the precise ultrastructural location of ICAM-1 expression and ICAM-1/β_2-integrin interactions in the lung during the initiation of the pul-

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monary leukocytic infiltration in experimental GP syndrome in the rat to better understand the role of ICAM-1 in leukocytic movement and accumulation in this disease.

Materials and Methods

Animals

Inbred male Sprague-Dawley rats aged 2 to 3 months were obtained from the Monash Medical Centre Animal House.

Experimental GP Syndrome

Experimental GP syndrome was induced in Sprague-Dawley rats using the passive accelerated anti-GBM disease model. Rabbit nephrotoxic serum (NTS) was raised as previously described.[2] Experimental GP syndrome was induced in rats by subcutaneous immunization with 5 mg of normal rabbit IgG in Freund's incomplete adjuvant, followed 5 days later by intravenous administration of NTS (10 ml/kg body weight). Groups of two animals were sacrificed at 1.5, 6, 12, and 24 hours after administration of the NTS. A group of two normal rats was used as controls.

A further group of two animals with experimental GP syndrome were treated as follows: monoclonal anti-ICAM antibody (IA29) was administered, at a dose of 1 mg/kg body weight, by intraperitoneal injection daily over a period of 10 days beginning one day before the administration of the NTS; animals were sacrificed on day 11.

Monoclonal Antibodies

Mouse monoclonal antibodies (MAbs) used in this study were as follows: IA29,[18] an IgG1 to rat ICAM-1 (CD54); WT1,[19] an IgG2a to rat LFA-1α chain (CD11a); WT3,[19] an IgG1 to rat LFA-1β chain (CD18). All MAbs were supplied by Serotec (Australian Laboratory Services, Melbourne, Australia).

Transmission Electron Microscopy

One- to two-mm cubes of lung were immersion-fixed in 2.5% glutaraldehyde for 2 hours, post-fixed in 2% aqueous osmium for 2 hours, and then dehydrated through graded acetones and embedded in Epon-Araldite. 0.08-μ sections were stained with lead citrate and uranyl acetate and examined in a Philips CM12 electron microscope.

Immuno-Electron Microscopy

One-mm-thick slices of lung were immersion-fixed in PLP for 2 hours. The slices were then immersed in increasing concentrations of sucrose (5% to 20%) in 0.1 M phosphate-buffered saline (PBS) pH 7.4 over 36 hours and then placed in OCT and snap-frozen in liquid nitrogen. Subsequently, 30-μ cryostat sections were cut and incubated in 5% NGS in 0.01 M PBS, 0.8% bovine serum albumin (BSA), and 0.1% immunogold silver staining quality gelatin as a blocking step and then in the primary MAbs (IA29, WT1, and WT3) overnight at 4 C. MAbs were diluted 1 in 75 in 1% normal goat serum in 0.01 M PBS/0.8% BSA/0.1% gelatin (dilution buffer) to a final concentration of 13 μg/ml. For each animal control sections were incubated in dilution buffer only. Sections were then incubated in goat anti-mouse IgG labeled with 1-nm gold particles (Auroprobe One, Amersham) diluted 1 in 25 in dilution buffer. After extensive washing, sections were fixed in 2% glutaraldehyde in PBS for 15 minutes and then silver-enhanced using IntenSE M kit (Amersham, Melbourne). Post-fixation with 1% aqueous osmium for 30 minutes was followed by dehydration in graded acetones and flat-embedding of the sections in Epon-Araldite. 0.08-μ sections were cut straight from the surface of the block, mounted on copper grids, stained with lead citrate and uranyl acetate, and examined in a Philips CM12 electron microscope.

In the two animals administered *in vivo* anti-ICAM-1 MAb, the technique was identical to the above except for the omission of the primary MAb.

Results

Transmission Electron Microscopy

In the normal rat lung occasional intra-alveolar macrophages were seen. These large cells contained numerous lysosomal type dense bodies and were usually in contact at several points with the alveolar epithelium. The alveolar spaces were lined by flattened type I epithelial cells and cuboidal type II epithelial cells. By 1.5 hours after initiation of GP syndrome, erythrocytes and fibrin were apparent within alveolar spaces and alveolar capillaries contained numerous PMNL. PMNL were also seen migrating out from capillaries into the interstitial and alveolar spaces where their surface membranes were in close contact with or adherent to type I epithelial cells but not type II cells. Occasional lymphocytes and macrophages were present within alveolar capillaries as

early as 1.5 hours. At later stages (6–24 hours) macrophages, and to a lesser extent lymphocytes, were the more predominant inflammatory leukocytes within capillaries, the interstitium, and alveolar spaces. The numerous intra-alveolar macrophages often showed very close apposition of large areas of their surface membranes to the alveolar lining of type I cells but, as with PMNL, not type II cells.

Immuno-Electron Microscopy

ICAM-1 (CD54) and LFA-1 (CD11a/CD18) Expression in the Lung in Normal Animals

ICAM-1 was moderately strongly expressed on the luminal surface of the endothelium of arteries and veins but was not present on the bronchial epithelium. There was moderately strong expression of ICAM-1, in a even pattern of distribution, over the entire surface of the flattened type I epithelial cells lining the alveolar spaces (Fig. 1A). However, ICAM-1 expression was completely absent from the surface of type II epithelial cells (Fig. 1A). The type I cells often formed a collar around the type II cells and this was highlighted by the distribution of ICAM-1 staining (Fig. 1A). ICAM-1 was weakly, but evenly, expressed on the endothelial surface of alveolar capillaries (Fig. 1, A and B) and on the surface of occasional circulating monocytes (Fig. 1B) and intra-alveolar macrophages (Fig. 2A).

CD11a and CD18 were expressed on the surface and within the cytoplasm of intra-alveolar macrophages (Fig. 2B). The CD11a/CD18 was evenly dis-

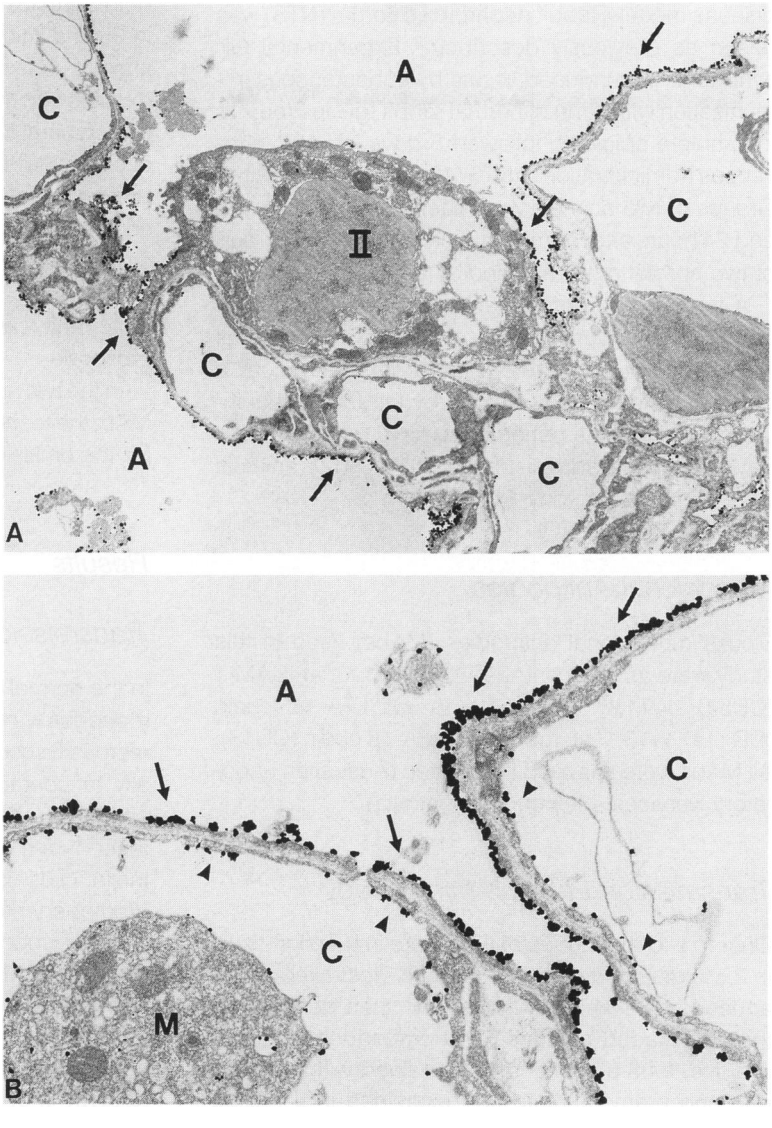

Figure 1. *Normal rat (immuno-electron microscopy).* **A**: *ICAM-1. There is moderately strong expression of ICAM-1 in an even distribution over the entire surface of the flattened type I epithelial cells lining the alveolar space* (**A, arrows**). *However, ICAM-1 is completely absent from the surface of type II cells (II).* **C**: *alveolar capillary ×7600.* **B**: *ICAM-1. At higher magnification, the moderately strong expression of ICAM-1 on the alveolar type I epithelium* (**arrows**) *contrasts with the weaker pattern of staining on the endothelium of the alveolar capillaries* (**arrowheads**). *An intracapillary monocyte (M) shows weak surface ICAM-1 expression. A, alveolar space; C, alveolar capillary (×20,000).*

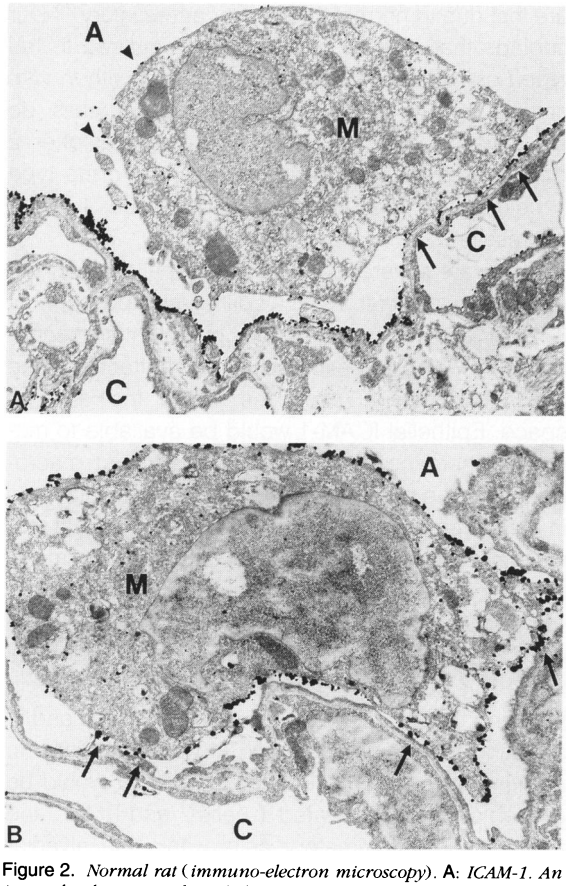

Figure 2. *Normal rat (immuno-electron microscopy).* **A:** *ICAM-1. An intra-alveolar macrophage (M) expressing sparse surface ICAM-1* (**arrowheads**) *is in close contact with and adherent to type I epithelium* (**arrows**), *which shows strong surface expression of ICAM-1. A, alveolar space; C, alveolar capillary (×10,000).* **B:** *CD11a. CD11a is evenly distributed over the surface of this intra-alveolar macrophage (M) and is present at the sites of close contact or adherence to the alveolar type I epithelium* (**arrows**). *There is also weaker staining for CD11a within the macrophage cytoplasm. A, alveolar space; C, alveolar capillary (×10,500).*

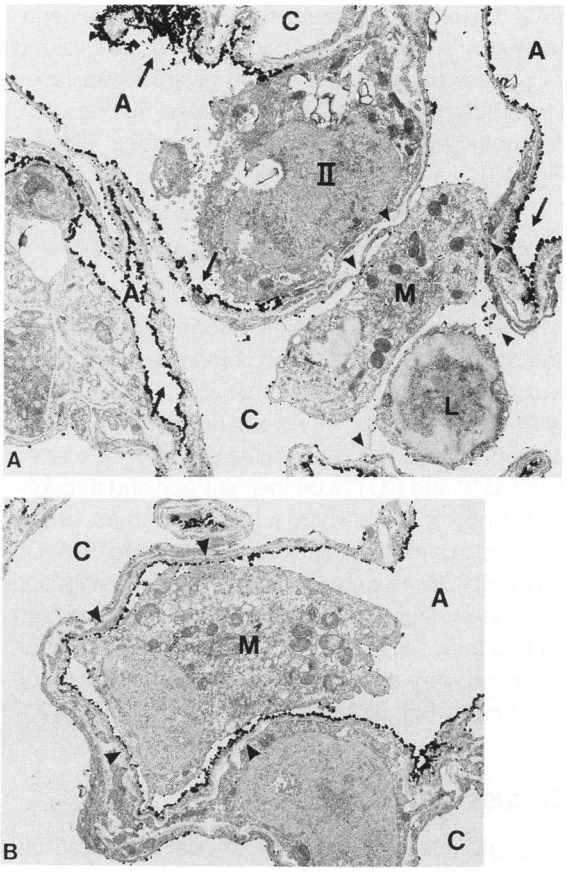

Figure 3. *GP syndrome (immuno-electron microscopy); in vitro anti-ICAM-1 MAb, 12 hours.* **A:** *ICAM-1 is strongly expressed on the entire surface of the type I epithelial cells lining alveolar spaces* (**A, arrows**) *but is absent from the type II cells (II). Alveolar capillary (C) endothelial ICAM-1 expression is comparatively weak. Intracapillary monocytes (M) and lymphocytes (L) show weak surface ICAM-1 expression. ICAM-1 staining is present at the sites of close contact or adherence of leukocytes to the endothelial surface* (**arrowheads**), *which often appears tethered at these points (×10,000).* **B:** *An intra-alveolar macrophage (M), which shows sparse surface ICAM-1 expression in close contact with or adherent to the alveolar type I epithelium at several points* (**arrows**). *The type I epithelium shows strong ICAM-1 expression, which is evenly distributed over its entire surface, including sites of macrophage adherence to the epithelium. A, alveolar space; C, alveolar capillary (×7600).*

tributed over the entire surface of the macrophages, including the sites of close contact/adherence to the alveolar epithelium (Fig. 2B).

ICAM-1 (CD54) Expression in the Lung in GP Syndrome

The distribution of ICAM-1 was similar to that seen in normal animals, being expressed strongly on alveolar type I epithelial cells and comparatively weakly on the surface of the alveolar capillary endothelium (Fig. 3, A and B). As in normal animals, ICAM-1 was not present on type II epithelial cells (Fig. 3A). There appeared to be an increase in intensity of ICAM-1 staining at all stages of GP syndrome as compared with normal, but this was only a subjective observation, as quantitation of this staining technique is not feasible. The ICAM-1 was evenly expressed on the endothelial and epithelial surfaces and was present at sites of close contact/adherence of PMNL, macrophages, and lymphocytes to both these surfaces (Fig. 3, A and B). The endothelium often appeared to be tethered at these sites of ICAM-1 expression associated with leukocyte adhesion (Fig. 3A). ICAM-1 was also weakly expressed on the surface of macrophages and lymphocytes (Fig. 3, A and B).

In the animals in which anti-ICAM-1 MAb was administered *in vivo*, although the intensity of staining was overall weaker, the pattern of ICAM-1 expression was very similar to that described above. Alveolar type I epithelial cells showed moderately strong

ICAM-1 expression, whereas type II cells were negative (Fig. 4, A and B). The endothelium of arteries and veins was strongly positive and alveolar capillaries demonstrated weak ICAM-1 expression (Fig. 4B). Macrophages expressed sparse surface ICAM-1 (Fig. 4B).

LFA-1 (CD11a/CD18) in the Lung in GP Syndrome

PMNL showed moderately strong CD18 expression both on their surface membranes and within the cytoplasm (Fig. 5A). In contrast, CD11a expression by PMNL was either very weak or absent (Fig. 5B). Macrophages and lymphocytes strongly expressed both CD18 and CD11a on their surface and throughout their cytoplasm, where it appeared to be largely localized to small cytoplasmic vacuoles (Fig. 5, A–C). CD18 and CD11a were present in an even distribution over the surface membranes of the leukocytes, including their sites of close contact/adherence to alveolar capillary endothelium and type I alveolar epithelial cells (Fig. 5, A–C).

Discussion

Although functional studies have demonstrated the importance of ICAM-1 and the β_2-integrins in experimental models of pulmonary injury,[9–13] this is the first study to identify the precise ultrastructural location of ICAM-1 in both the normal and inflamed lung. Pulmonary alveolar epithelial ICAM-1 expression was localized to the surface of type I cells in both normal and GP syndrome animals and was completely absent from type II cells. Type II cells, in addition to producing pulmonary surfactant, are the progenitor cells of the alveolar space and differentiate into type I cells to reconstitute the epithelial surface when type I cells are lost due to normal turnover or acute injury.[20] Our findings that ICAM-1 expression is confined to the type I epithelial cell *in vivo* is consistent with *in vitro* studies which demonstrate that type II cells do not initially express ICAM-1 in culture but after 2 days undergo a morphological change to the type I cell phenotype and acquire surface ICAM-1 expression.[16]

There are several potential functional roles for the pulmonary alveolar type I epithelial expression of ICAM-1 and hence a number of potential mechanisms by which this expression might affect immune and inflammatory responses within the alveolar space. Epithelial ICAM-1 would be available to mediate adherence of β_2 expressing alveolar macrophages to the surface of alveolar type I epithelial cells in normal animals and in disease. It is of interest that we observed very close contact of the surface membranes of macrophages to the surface of alveolar type I cells but, in sharp contrast, no evidence of adherence to type II cells. This adherence of macrophages to the alveolar epithelium could be important in both retaining these cells within the alveolar space and in assisting their migration along the alveolar walls. In addition, alveolar type I epithelial ICAM-1 would be available to bind activated T cells[21] and PMNL and retain these inflammatory cells within the alveolar space. The tight adherence of leukocytes to alveolar epithelial cells *in vitro* has been shown to be a very important component of the cytotoxic process[22,23] and hence by mediating leukocyte adherence ICAM-1 could be important in enhancing the cellular damage caused by inflammatory leukocytes in the lung.

In our model of GP syndrome, there is a strong linear deposition of anti-GBM antibody along the alveolar basement membranes within 30 minutes of injection.[3] Such antibody deposition has been shown to

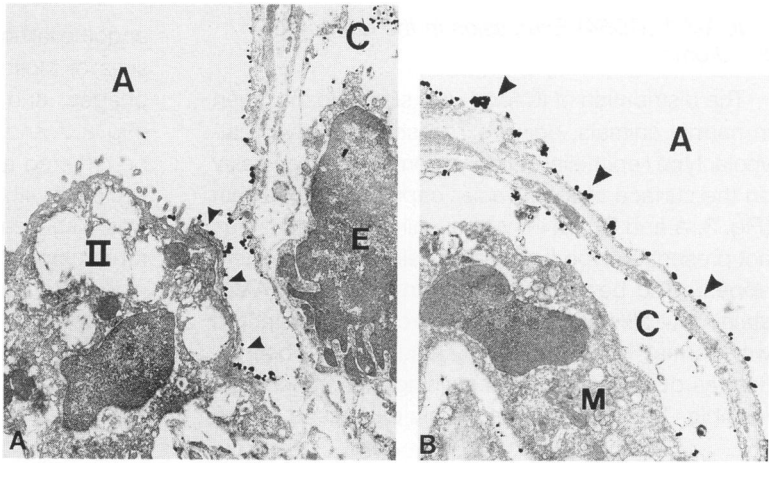

Figure 4. *GP syndrome (immuno-electron microscopy);* in vivo *anti-ICAM MAb.* **A**: *ICAM-1 is moderately strongly expressed on the surface of an alveolar type I epithelial cell which extends a thin finger-like process* (**arrowheads**) *over the adjacent type II cell (II) which is negative for ICAM-1. ICAM-1 is also present on the endothelial surface of an alveolar capillary (C). E, endothelial cell; A, alveolar space (×10,000).* **B**: *At higher magnification ICAM-1 is seen to be evenly distributed, in a moderately strong staining pattern, on type I alveolar epithelium* (**arrowheads**) *and more weakly on the endothelium lining alveolar capillaries (C). The intracapillary monocyte (M) also demonstrates weak surface ICAM-1 expression. A, alveolar space (×16,000).*

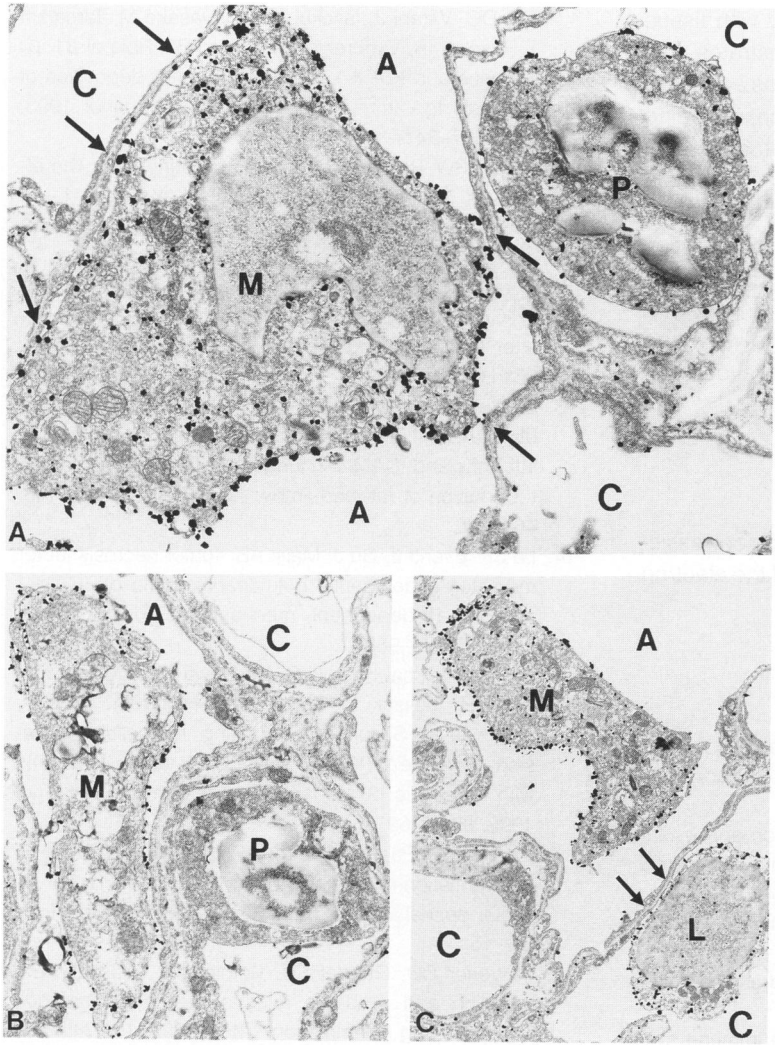

Figure 5. *GP syndrome (immuno-electron microscopy).* **A:** *12 hours; CD18. The intra-alveolar macrophage (M) shows strong expression of CD18 in an even distribution over its entire surface and within the cytoplasm. Much of the cytoplasmic staining appears to be located within small cytoplasmic vacuoles. The macrophage is closely apposed and adherent to the alveolar type I epithelium with CD18 being expressed along these adherent surfaces of the macrophage* (arrows). *An intracapillary PMNL (P) shows a comparatively weaker surface and cytoplasmic expression of CD18 which is present at sites of close contact/adherence to the capillary endothelium. A, alveolar space; C, alveolar capillary* (×*13,000*). **B:** *6 hours; CD11a. CD11a is moderately strongly expressed on the surface of an intra-alveolar macrophage (M). The intracapillary PMNL (P), however, shows an absence of CD11a expression. This contrasts with the moderate CD18 expression by PMNL in* **A**. *A, alveolar space; C, alveolar capillary* (×*12,500*). **C:** *12 hours; CD18. Both an intra-alveolar macrophage (M) and an intracapillary lymphocyte (L) express CD18 moderately strongly on the surface membrane and more weakly within the cytoplasm. The lymphocyte appears to be adherent to the alveolar capillary endothelium at several points* (arrows). *A, alveolar space; C, alveolar capillary* (×*5700*).

be accompanied by complement activation, chemotactic attraction of leukocytes, and activation of the coagulation system with endothelial damage.[24] There is an associated release of cytokines (interleukin-1 and tumor necrosis factor-α), which are known to be important regulators of pulmonary ICAM-1 expression.[14,25,26] The early (1.5 hours) inflammatory infiltrate is characterized by PMNL adherent to and migrating out from alveolar capillaries into the interstitium and alveolar spaces. PMNL adhesion and migration are dependent on both ICAM-1 expression by the endothelium[11] and the expression of β_2-integrins (both LFA-1 and Mac-1) on the PMNL surface.[27] In this study we have demonstrated that infiltrating PMNL express surface CD18 but very little or no CD11a, which suggests that Mac-1 (CD11b/CD18) is the more important PMNL counter-receptor for ICAM-1 in the lung in this disease. Macrophages and lymphocytes, which we see accumulating at a later stage (6 to 24 hours) in GP syndrome, show strong staining for CD18 and CD11a, representing LFA-1 expression, both on their surface membranes and within the cytoplasm. Both ICAM-1 and LFA-1 expression are important in mononuclear leukocyte adhesion to and migration through the endothelium.[28] In previous studies we have shown that it is the inflammatory macrophages that are the major cellular participants in progressive pulmonary injury in GP syndrome.[3] Hence, although the expression of ICAM-1 by alveolar capillary endothelium in GP syndrome is relatively weak compared with the strong ICAM-1 expression by alveolar type I epithelial cells, our results suggest that endothelial ICAM-1 interactions with leukocytic β_2-integrins play a role in the pulmonary leukocytic accumulation in the early stages of this disease.

Our demonstration that *in vivo* administration of anti-ICAM-1 antibody results in binding of the antibody to all sites of ICAM-1 expression within the lung in a pattern very similar to the staining seen with *in*

vitro incubation of anti-ICAM-1 antibody with tissue sections is important in that it demonstrates that blocking antibody administered *in vivo* has access to all sites of pulmonary ICAM-1 expression, including the extravascular sites. The beneficial effects of anti-ICAM-1 antibodies in ameliorating pulmonary injury[9–13] are likely to be due not only to blocking of endothelial ICAM-1 and the adherence and migration of leukocytes out from capillaries into the interstitium and alveolar spaces but, perhaps more importantly, to blocking of the leukocytic interactions with, and subsequent damage to, the type I epithelial cells lining the alveolar space.

Acknowledgments

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