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An immunohistochemical study on phenotypic heterogeneity of human pulmonary vascular endothelial cells

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Summary. The existence of a subpopulation of human vascular endothelial cells (EC) has been demonstrated in the liver with the aid of immunohistochemical techniques. In this study, we investigated the antigenic and functional properties of the vascular EC in human lung. Alveolar capillary EC shared antigens with a peripheral blood monocyte/macrophage subset capable of presenting soluble antigens and triggering autologous mixed lymphocyte reactions. That is to say that the alveolar capillary EC were HLA-DR$^+$, OKM1$^-$, and OKM5$^+$. In addition, these EC frequently expressed interleukin-1. These facts suggest that alveolar capillary EC may play an important role in immunological responses in the lung. The antigens were, however, absent or only faintly visible on the vascular EC of medium and small vessels. In contrast, Factor VIII/von Willebrand factor antigen (FVIIIRAg), which is produced in vascular EC was heavily stained in the EC of medium and small vessels, but only weakly stained in the alveolar capillary EC. These immunohistochemical findings suggest that in different anatomical compartments in the lung vascular EC express phenotypic properties heterogeneously. They may play differing biological roles or serve different immunological functions in normal and pathological states in the lung.

Key words: Endothelial cells – Macrophage – Lung – Immunohistochemistry – Monoclonal antibodies

Introduction

Functions of vascular endothelial cells (EC) other than those related to coagulation were described in the 1920's (Henschen 1928). Thus factor VIII/von Willebrand factor antigen (FVIIIRAg) is synthesized in vascular EC (Hoyer et al. 1973) but vascular EC have also been shown to share many phenotypic and functional properties with antigenic presenting cells. They are major participants in the development of cell-mediated immune responses and may play an important role (Hirschberg et al. 1974, 1975; Moraes and Stastny 1977; Hirschberg et al. 1979, 1980; Ryan et al. 1981; Burger and Vetto 1982; Nunez et al. 1983; Wagner et al. 1984, 1985a, 1985b).

Recently, we have emphasized that vascular EC in different anatomical compartments in the liver expressed surface antigens heterogeneously (Fukuda et al. 1986; Nagura et al. 1986). Vascular EC in the portal areas and central veins carried FVIIIRAg, whereas only sinusoidal EC expressed antigens found on a subset of macrophages. However, comparable functional properties of vascular EC in the lung have not been studied extensively. Monoclonal antibodies (MoAb) OKM1 and OKM5 have been shown to detect unique antigenic determinants distributed on a functionally distinct monocyte/macrophage subset (Breard et al. 1980; Shen et al. 1983), and interleukin-1 (IL-1) is thought to be the most important product of monocyte/macrophages as an inflammatory and immune mediator (Wewers et al. 1984). In this study, we investigated the localization of FVIIIRAg and the variety of antigens related to the cells of monocyte/macrophage lineage, and discussed the distinct antigenic and functional properties of vascular EC in different anatomical compartments in the lung.

Materials and methods

Histologically normal tissues were obtained from 15 patiens undergoing lobectomy or pneumonectomy for bronchogenic carci-

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Table 1. Antibodies used in the present study

1st Antibody	Dilution	2nd Antibody	Dilution
Monoclonal OKM1	1:25	HRP[a] goat anti-mouse IgGF(ab′)$_2$	1:100
Monoclonal OKM5	1:25	HRP goat anti-mouse IgGF(ab′)$_2$	1:100
anti-HLA-DR antibody	1:100	HRP goat anti-mouse IgGF(ab′)$_2$	1:100
rabbit anti-IL-1[b] antibody	1:1500	HRP goat anti-rabbit IgGF(ab′)$_2$	1:100
HRP rabbit anti-FVIIIRAg[c]	1:25	–	–

[a] horseradish peroxidase
[b] interleukin-1
[c] Factor VIII/von Willebrand factor antigen

noma at Nagoya University Hospital, as previously described (Yamamoto et al. 1987). Briefly, the tissues were promptly fixed in periodate-lysine-4% paraformaldehyde (Mclean and Nakane 1974) for 6 h, washed in phosphate-buffered saline (PBS) containing increasing concentrations of sucrose, frozen in OCT compound (Miles Pharmaceutical, Naperville, IL) and sectioned at 6 μm on a cryostat. The sections were placed on egg-albumin coated slides and dried in air.

Rabbit anti-human FVIIIRAg was purchased from Dako (Kyowa Medics, Tokyo, Japan). The antibody specificity of the anti FVIIIRAg was documented by immunoelectrophoresis and immunodiffusion. The Fab' fragments of the γ-globulin fraction of the antisera were conjugated with horseradish peroxidase (HRP) (Wilson and Nakane 1978). For use in control experiments, the Fab' fragments of non immune γ-globulin were also labeled with HRP.

OKM1 and OKM5 monoclonal antibodies (MoAb) were obtained from Ortho Pharmaceutical Co. (Ortho Japan, Tokyo, Japan). Anti-HLA-DR MoAb were purchased from Becton-Dickenson (Fujisawa Pharmaceutical Co., Tokyo, Japan) and rabbit anti IL-1 antibody was from Genzyme Co (Cosmo Bio Co, Tokyo, Japan). Goat anti-mouse F (ab′)$_2$ fragments of IgG labeled with HRP (Tago Inc., Cosmo Bio Co, Tokyo, Japan) and Goat anti-rabbit F(ab′)$_2$ fragments of IgG labeled with HRP (presented by Prof. Watanabe, Tokai University, Japan) were the second antibody. Antibodies used in the present study are listed with dilution values in Table 1.

The direct peroxidase-labeled antibody method was used for the immunohistochemical staining for FVIIIRAg and the indirect peroxidase-labeled antibody method was applied for the staining for IL-1, HLA-DR and antigens detected by MoAb OKM1 and OKM5 (Shioda et al. 1984, Nagura et al. 1986).

For light microscopy, cryostat sections were treated with 100% methyl alcohol and 0.03% hydrogen peroxide to inactivate endogenous peroxidase (Streefkerk 1972). Then, either the direct or indirect horseradish peroxidase-labeled antibody method was applied for the immunological reaction. This involves successive incubations for 12 h at 4° C. The sections for the indirect method were incubated with the second antibody for 6 h at 4° C, the sections were then reacted with 0.25% diaminobenzidine (DAB) solution containing 0.01 M sodium azide and 0.01 M hydrogen peroxide, and counter-stained with methyl green.

For electron microscopy, cryostat sections adjacent to those taken for light microscopy were treated similarly through the antibody incubation steps and sections were postfixed in 0.5% glutaraldehyde in PBS for 5 min at 4° C. The postfixed sections were washed and incubated with 0.25% DAB solution without hydrogen peroxide for 15 min, and then with 0.25% DAB solution containing 0.01 M hydrogen peroxide for 5 min. The sections were then washed, reacted with 2% osmium tetroxide in PBS for one hour at room temperature, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections, either unstained or stained with lead citrate, were viewed with a Hitachi H-300 electron microscope.

The specificity of histochemical staining was confirmed by use of HRP-labeled nonimmune rabbit γ-globulin fragments in the direct peroxidase-labeled antibody technique, and by use of ascitic fluid from mice injected with nonsecreting hybridoma cells or non-immune rabbit serum instead of primary antiserum in the indirect method.

Results

The results of the immunohistochemical characterization of the vascular EC in the lung are summarized in Table 2. In this study, we classified the EC of vessels as follows: (1) alveolar capillary, (2) medium and small vessels, which include arteries from muscular pulmonary arteries to arterioles and veins from veins of medium caliber, to venules. We did not examine the large vessels, such as elastic pulmonary arteries and veins of large caliber.

MoAb OKM1 did not react with any EC of the pulmonary vascular system, although it detected a large proportion of alveolar macrophages. In contrast, MoAb OKM5 was reactive with the majority of alveolar capillary EC (Fig. 1 A). Alveolar macrophages and the vascular EC of medium and small vessels, were however, unreactive with the MoAb OKM5 (Fig. 1 A, 3 A). Electron microscopy revealed reaction products of the MoAb

Table 2. Immunohistochemical characterization of endothelial cells (EC) in the lung

	FVIIIRAg	HLA-DR	OKM1	OKM5	IL-1
Alveolar capillary EC	− ~ ±	+	−	+	+
Vascular EC of medium and small vessels	+	− ~ ±	−	−	− ~ ±
Capillary EC in the wall of medium and small vessels	− ~ ±	+	−	−	− ~ ±

+ : positive staining; ± : weak staining; − : negative staining

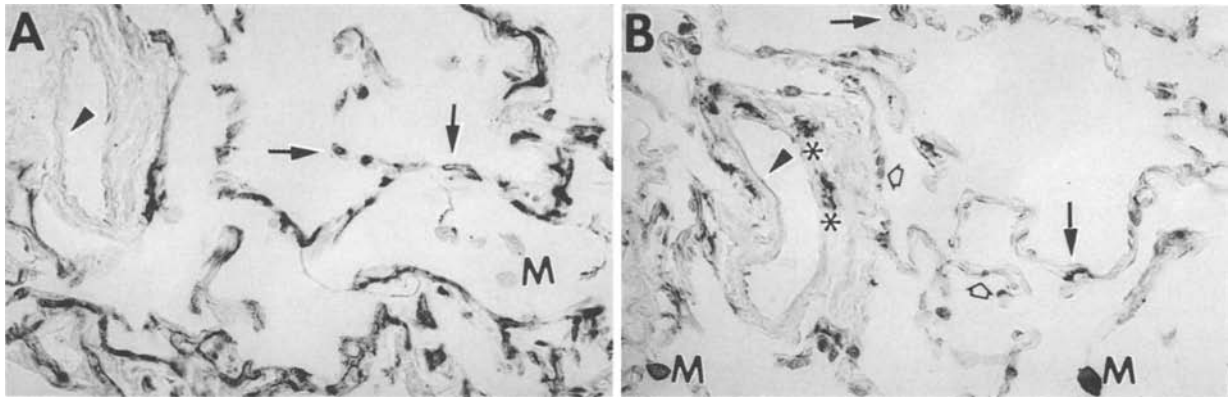

Fig. 1 A, B. Immunohistochemical localization of OKM5 (**A**) and HLA-DR (**B**); (A) OKM5 is reactive with alveolar capillary endothelial cells (EC) (solid arrow). Vascular EC of small vessels (*arrow head*) and alveolar macrophages (*M*) are unstained. (**B**) Anti-HLA-DR monoclonal antibody (MoAb) is reactive with alveolar macrophages (*M*), alveolar capillary EC (*solid arrow*), capillary EC (*) in the wall of small vessel, and the portions (*open arrow*) which seem to be compatible with type II alveolar epithelial cells, whereas it is only faintly reactive with the vascular EC (*arrow head*) of the small vessel. Magnification: **A**, ×280. **B**, ×280

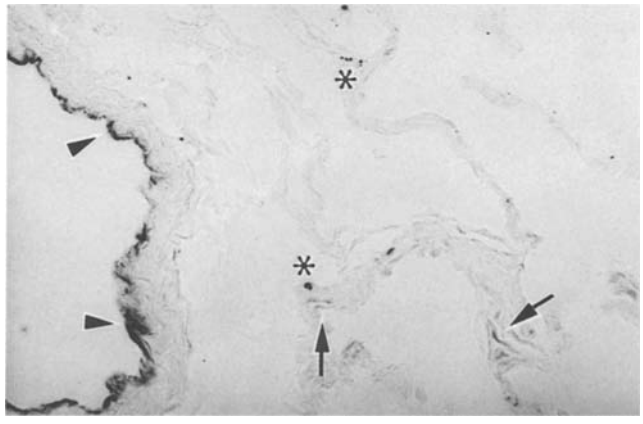

Fig. 2. Immunohistochemical localization of Factor VIII/von Willebrand factor antigen (FVIIIRAg); FVIIIRAg is clearly identified in vascular EC of small vessel (arrow head), but only faintly identified in a few alveolar capillary EC (*arrow*). (*) indicates the deposition of dust particles. Magnification: ×280

OKM5 along the plasma membranes of the alveolar capillary EC and in endocytic-like invaginations of the plasma membranes (Fig. 6). Both type I and type II alveolar epithelial cells were unreactive with the MoAb OKM5 (Fig. 6). HLA-DR was strongly expressed on the majority of alveolar capillary EC and capillary EC in the wall of medium and small vessels, whereas it was absent or only faintly expressed on the vascular EC of the medium and small vessels (Fig. 1 B, 3 B). The majority of alveolar macrophages and type II alveolar epithelial cells carried the HLA-DR, but type I alveolar epithelial cells did not (Fig. 1 B). These findings were also confirmed by immunoelectron microscopic examinations (Fig. 7). IL-1 was frequently expressed on alveolar capillary EC, whereas the vascular EC of medium and small vessels and capillary EC in the wall of those vessels carried little or no IL-1 (Fig. 4). Both type I and type II alveolar epithelial cells were unreactive with the anti-IL-1 antibody. In addition, alveolar macrophages faintly expressed the IL-1 (Fig. 4). Electron microscopically, the reaction products of the anti-IL-1 antibody were present along the plasma membranes of the alveolar capillary EC and in endocytic-like invaginations of the membranes (Fig. 8), similar in pattern to those of MoAb OKM5. In contrast, FVIIIRAg was strongly stained in the vascular EC of all of medium and small vessels (Fig. 2, 3 C), but absent or only faintly identified in the capillary EC in the wall of these vessels and alveolar capillary EC (Fig. 2). The control sections were uniformly negative (Fig. 5).

Discussion

Using immunohistochemical techniques, we observed a distinct distribution of FVIIIRAg and a panel of antigens related to the cells of monocyte/macrophage lineage in different anatomical compartments of the human lung.

Although FVIIIRAg is considered to be produced in vascular EC generally as a part of the coagulation system (Hoyer et al. 1973), we found in the present study that it was strongly stained in the vascular EC of medium and small vessels, whereas it reacted very weakly with the capillary EC in the walls of these vessels and in alveolar capillary EC. Thus, the vascular EC of medium

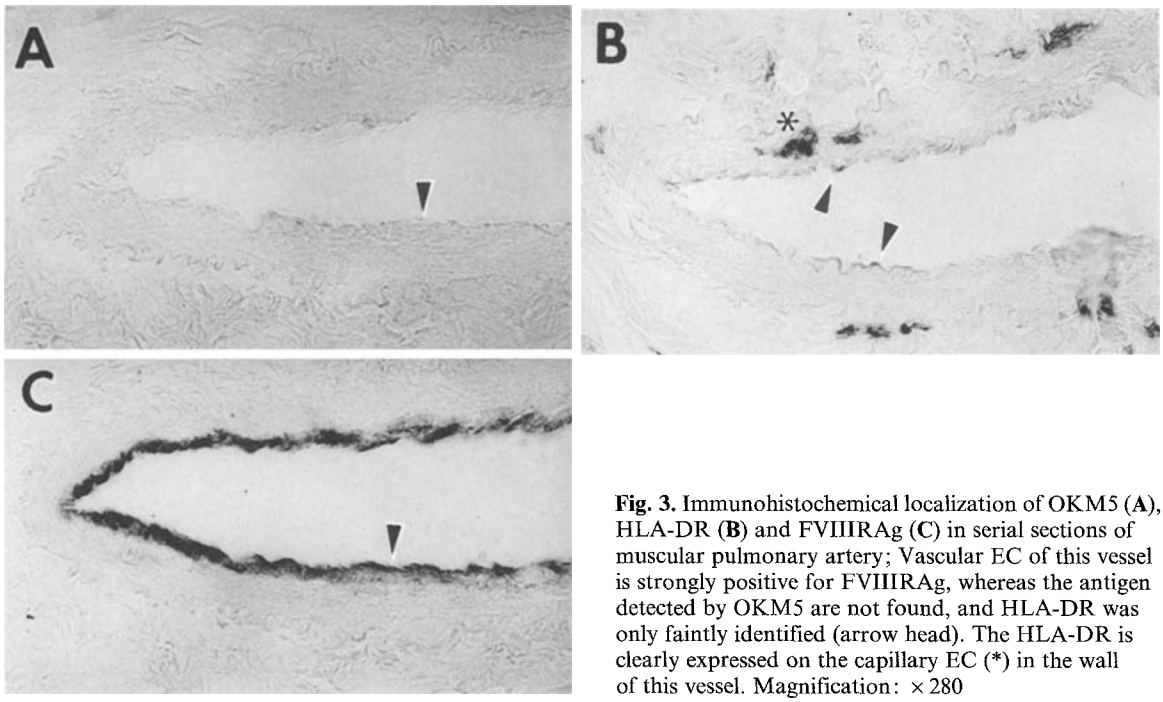

Fig. 3. Immunohistochemical localization of OKM5 (**A**), HLA-DR (**B**) and FVIIIRAg (**C**) in serial sections of muscular pulmonary artery; Vascular EC of this vessel is strongly positive for FVIIIRAg, whereas the antigen detected by OKM5 are not found, and HLA-DR was only faintly identified (arrow head). The HLA-DR is clearly expressed on the capillary EC (*) in the wall of this vessel. Magnification: × 280

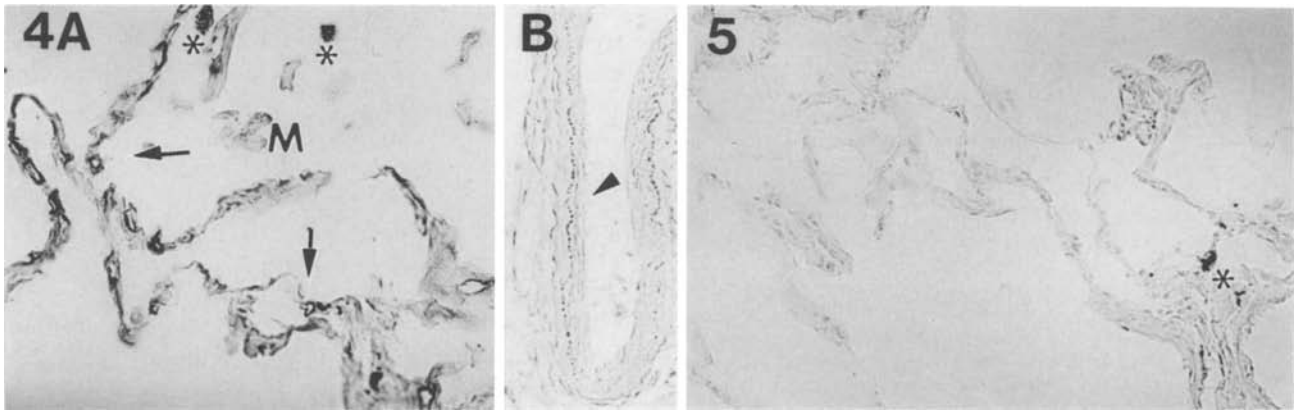

Fig. 4. Immunohistochemical localization of interleukin-1 (IL-1); **A** IL-1 is expressed on alveolar capillary EC (*arrow*), but faintly expressed on alveolar macrophages (*M*). Dust particles (*) are phagocytized by alveolar macrophages. **B** IL-1 is not expressed on vascular EC (*arrow head*) of small vessels. Magnification: **A**, × 340; **B**, × 250

Fig. 5. Control staining; No reaction products are observed. (*) indicates the deposition of dust particles. Magnification: × 230

and small vessels may be that mainly involved in blood coagulation.

In contrast, HLA-DR was identified in these capillary EC, whereas it was only faintly stained in the vascular EC of medium and small vessels. It has been generally accepted, however, that human vascular EC carry the HLA-DR antigen (Moreas and Stastny 1977; Hirschberg et al. 1979). Interestingly, the HLA-DR was also identified in type II alveolar epithelial cells, in addition to the vascular EC above mentioned. Furthermore, OKM5 was only reactive with alveolar capillary EC. Thus, alveolar capillary EC were HLA-DR$^+$, OKM1$^-$ and OKM5$^+$, sharing antigen with a peripheral blood monocyte/macrophage subset which possessed the capacity to present soluble antigens and induce autologous mixed lymphocyte reactions (AMLR) (Shen et al. 1983). Knowles

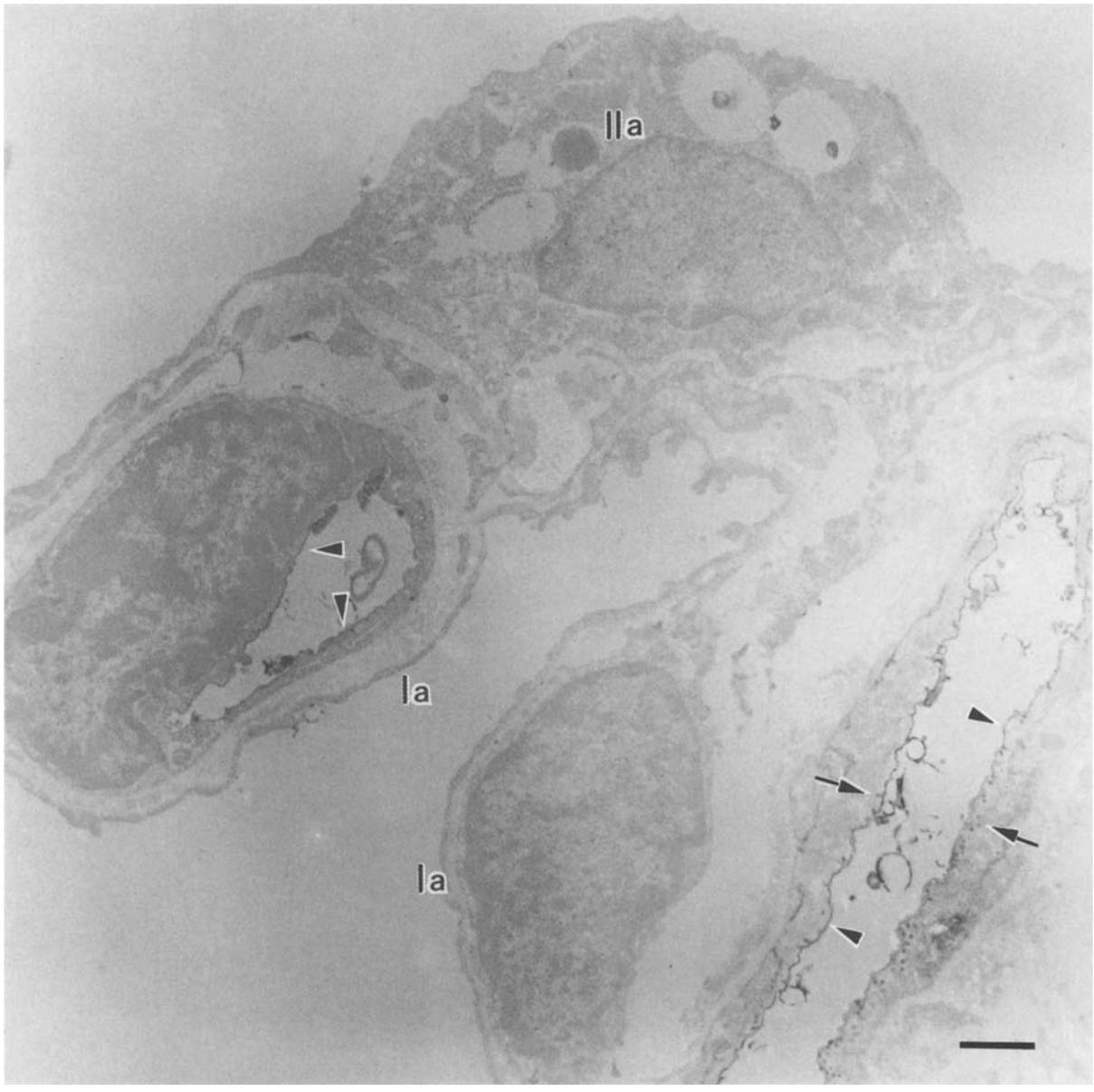

Fig. 6. Ultrastructural localization of OKM5; The reaction products are present along the plasma membranes (*arrow head*) of alveolar capillary EC and in endocytic-like invaginations (*arrow*). Type I alveolar epithelial cell (*Ia*) and type II alveolar epithelial cell (*IIa*) are unreactive with the MoAb OKM5. Magnification: ×11000

et al. (1984) have also shown that the alveolar capillary EC exhibited intense reactivity with OKM5 at light microscopic level. In addition, we found that these alveolar capillary EC frequently expressed IL-1. This is not surprising, because it has been reported that some human vascular EC also express IL-1 (Wagner et al. 1985a, 1985b), which is thought to be an important product of monocyte/macrophages (Wewers et al. 1984). Thus, alveolar capillary EC may be able to replace monocytes or macrophages in the immune responses occurring in the lung. However, it is necessary to determine whether similar phenomena will be demonstrated in isolated alveolar capillary EC.

It has been well documented that various stimuli from antigens in inhaled air result in an inflammatory process, in and around the alveoli, in capillaries and small airways of the lung, and that this inflammatory process mediates injury to lung parenchymal cells and interstitial fibrosis (Hunninghake and Mosely 1984). Alveolar macrophages are involved in defending the lung from such stimuli

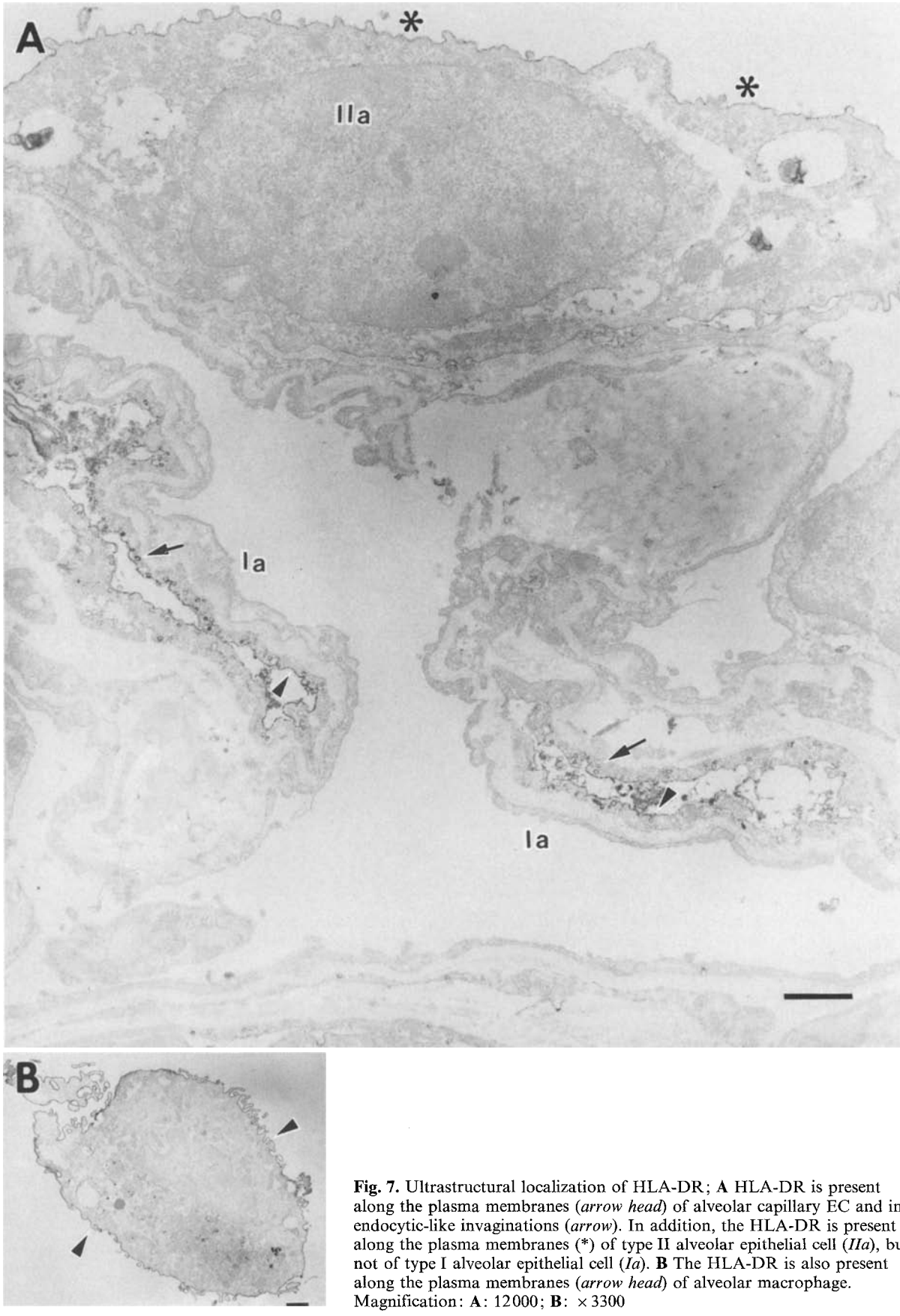

Fig. 7. Ultrastructural localization of HLA-DR; **A** HLA-DR is present along the plasma membranes (*arrow head*) of alveolar capillary EC and in endocytic-like invaginations (*arrow*). In addition, the HLA-DR is present along the plasma membranes (*) of type II alveolar epithelial cell (*IIa*), but not of type I alveolar epithelial cell (*Ia*). **B** The HLA-DR is also present along the plasma membranes (*arrow head*) of alveolar macrophage. Magnification: **A**: 12000; **B**: ×3300

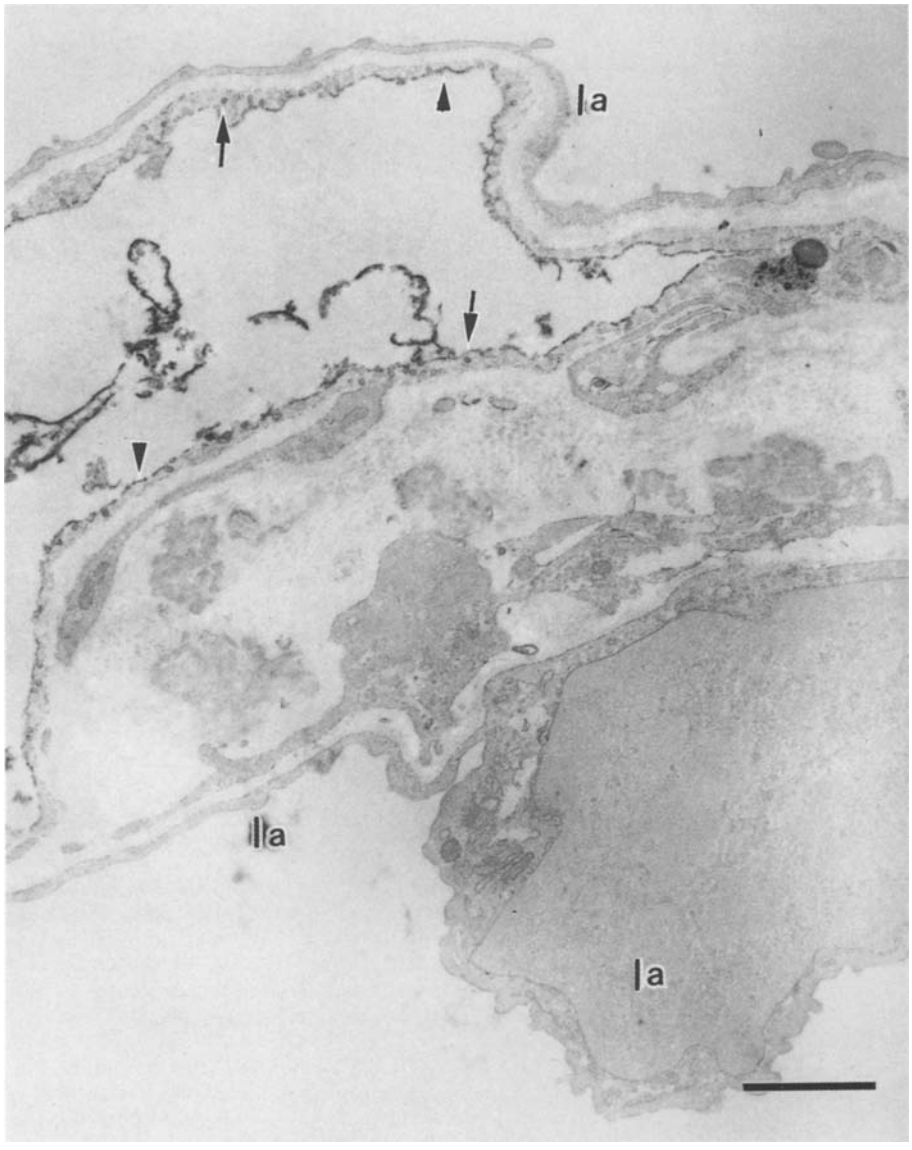

Fig. 8. Ultrastructural localization of IL-1; Anti-IL-1 antibody is expressed along the plasma membranes (*arrow head*) of alveolar capillary EC and in endocytic-like invaginations (*arrow*). Type I alveolar epithelial cells (*Ia*) are unreactive. Magnification: ×17000

(Green et al. 1977). Alveolar macrophages are also important cells for the initiation and regulation of immune responses the lung (Laughter et al. 1977; Yeager et al. 1982; Ettensohn and Roberts 1983; Toews et al. 1984). However, in the present study alveolar macrophages were only faintly stained with anti-IL-1 antibody, and it has been reported that normal human alveolar macrophages obtained by bronchoalveolar lavage have a limited capacity to release IL-1 (Wewers et al. 1984; Weissler et al. 1986; Monick et al. 1987). Alveolar macrophages have also been reported to function poorly as accessory cells for the stimulation of antigen-induced lymphoproliferation and autologous mixed lymphocyte reactions, when compared with peripheral blood monocytes (Ettensohn and Roberts 1983; Toews et al. 1984; Rossi et al. 1986; Weissler et al. 1986). In addition, from the antigenic properties defined by the present immunohistochemical observation, we speculate that alveolar capillary EC participate in various immune processes in the lung, replacing monocytes and macrophages. In fact, Burger and Vetto (1982) suggested that vascular EC play a central role in the development of cell-mediated immune responses.

A major implication of the present study is that the vascular EC in different anatomical compartments in the lung express phenotypic properties heterogeneously and that they may play different roles or functions in normal and disease states. It may also be important to investigate the regulation of immune response and coagulation mecha-

nism by vascular EC in the lung for a greater understanding of the pathogenesis of lung diseases.

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