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Phenotypic characterization of alveolar capillary endothelial cells, alveolar epithelial cells and alveolar macrophages in patients with pulmonary fibrosis, with special reference to MHC class II antigens

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Summary. Major histocompatibility complex (MHC) class II antigens are essential in the presentation of antigens to T lymphocytes, and cells expressing MHC class II antigens are known to play a role as antigen presenting cells (APC). We investigated the distribution of MHC class II antigens and the reactivity of monoclonal antibodies OKM1 and OKM5 in normal and fibrotic lungs immunohistochemically. The results showed that alveolar capillary endothelial cells (ACEnd) expressed MHC class II antigens and were reactive with OKM5 in both normal lungs and the non-thickened parts of alveolar septa of pulmonary fibrosis. However, ACEnd did not express MHC class II antigens and were not reactive with OKM5 in thickened alveolar septa of pulmonary fibrosis. Type II alveolar epithelial cells (AEp) proliferating and replacing type I AEp in pulmonary fibrosis expressed MHC class II antigens strongly. Alveolar macrophages expressed MHC class II antigens strongly and reacted with OKM1 in pulmonary fibrosis, especially in alveolar spaces. These findings suggest that the phenotypic changes of ACEnd may be involved in the process of pulmonary fibrosis, and type II AEp and alveolar macrophages in the parts of thickened alveolar septa may play a role as APC.

Key words: MHC class II antigens – Antigen presenting cells – Pulmonary fibrosis – Immunohistochemistry

Introduction

Pulmonary fibrosis begins with alveolitis, which is often followed by fibrosis of alveolar septa (Crystal et al. 1981, 1984). Although the aetiology of idiopathic pulmonary fibrosis (IPF) has not been clarified, immunological responses or autoimmune mechanisms may be involved in IPF (Kravis et al. 1976; Crystal et al. 1984).

At the beginning of immune reactions, inhaled antigens in the alveolar spaces or septa have to be presented to lymphocytes by antigen presenting cells (APC), such as cells of a monocyte-macrophage lineage (Rosenthal 1980; Ettensohn and Roberts 1983). However, human alveolar macrophages obtained from bronchoalveolar lavage fluid (BALF) of healthy adults have a limited accessory function compared with peripheral blood monocytes (Wewers et al. 1984; Holt 1986; Rossi et al. 1986; Rich et al. 1987). However, vascular endothelial cells may serve immunological functions (Burger and Vetto 1982; Knowles et al. 1984; Jaffe 1984). Recently, we reported that alveolar capillary endothelial cells (ACEnd) share many phenotypic properties with APC, and speculated that they can replace monocytes or macrophages in the immune responses occuring in the lungs (Yamamoto et al. 1988). Furthermore, APC, which were thought initially to be restricted mainly to cells of the immune system, have a far wider distribution, and indeed various epithelial cells in a number of organs express Ia antigens and play a role as APC (Londei et al. 1984; Todd et al. 1985; von Willebrand et al. 1986). Alveolar epithelial cells (AEp) have also been suggested as a route for antigen presentation in patients with pulmonary fibrosis (Beaumont et al. 1986; Kallenberg et al. 1987a, b).

Major histocompatibility complex (MHC) class II antigens (HLA-DR, HLA-DP and HLA-DQ) are involved in a variety of immune responses, and cells expressing MHC class II antigens play a role as APC (Gonwa et al. 1983; Pober et al. 1983;

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Table 1. Clinical data of patients with pulmonary fibrosis

Case	Age/Sex	Diagnosis	Duration of symptoms (year)	Chest X-ray	Complications	Steroid	Smoking
1	54/M	IPF	2	reticular	−	−	−
2	62/M	IPF	2	reticular, honeycomb	−	−	+
3	59/M	IPF	1	reticular	LC[a]	−	+
4	56/F	PF	2	reticular	PSS[b]	−	−
5	49/F	PF	0.3	micronodular	PN[c]	−	+
6	55/F	PF	0.3	reticular	SS[d]	−	−
7	57/M	IPF	3	reticular, honeycomb	−	+	+
8	54/M	IPF	0.3	nodular, reticular	DM[e]	+	−

IPF: idiopathic pulmonary fibrosis; PF: pulmonary fibrosis

[a] lung cancer, [b] progressive systemic sclerosis, [c] periarteritis nodosa, [d] Sjögren's syndrome, [e] Diabetes mellitus, Cases 1–6: open lung biopsy, Cases 7, 8: autopsy

Austin et al. 1985). Although the distribution of HLA-DR, HLA-DP and HLA-DQ has been investigated in some organs (Mestecky et al. 1987; Sasaki et al. 1987; Teyton et al. 1987; Degener et al. 1988), little is known about them in the lung. In this study, we investigated the localization of a variety of antigens related to cells of a monocyte-macrophage lineage. Monoclonal antibodies (MoAb) OKM1 and OKM5 detect antigenic determinants distributed on functionally distinct human peripheral blood APC subsets (Shen et al. 1983). We also detected interleukin-1 (IL-1), one of the most important cytokines synthesized by cells of a monocyte-macrophage lineage (Bochner et al. 1987), which has been proven to proliferate fibroblasts in inflammatory processes (Schmidt et al. 1982). We also investigated the localization of factor VIII related antigen/von Willebrand factor antigen (FVIIIRAg), which is a coagulant factor generally used as a marker of endothelial cells (Hoyer et al. 1973; Takeuchi et al. 1988).

We expected that ACEnd, AEp and alveolar macrophages, all of which possibly have antigen presenting capability, were involved in the process of pulmonary fibrosis, and we investigated the differences in their phenotypic characterization.

Materials and methods

We used tissue specimens from 6 open lung biopsies and 2 autopsies within 3 h after death, obtained from patients with pulmonary fibrosis. Biopsy specimens were obtained from one or two lobes. Five of 8 cases were histologically and clinically proven to be IPF and 3 were pulmonary fibrosis associated with collagen diseaes (Table 1). Histologically normal tissues were also obtained from 10 patients undergoing lobectomy or pneumonectomy for bronchogenic carcinoma. A part of each specimen was immediately fixed in periodate-lysin 4% paraformaldehyde for 6 h, and washed in phosphate-buffered saline (PBS) containing increasing concentrations of sucrose. The fixed specimens were embedded in Tissue-Tec OCT compound (Miles Pharmaceutical, Naperville, IL, USA), frozen in dry ice ethanol, and sectioned at 6 μm on a cryostat microtome. The sections were placed on egg-albumin coated slides and dried in air.

Table 2. Antibodies used in the present study

1st antibody	Dilution	2nd antibody	Dilution
anti-HLA-DR	1:100	HRP[a] goat anti-mouse IgG $F(ab')_2$	1:80
anti-HLA-DP	1:50		1:80
anti-HLA-DQ	1:25		1:80
OKM1	1:25		1:80
OKM5	1:25		1:80
rabbit anti-IL-1[b]	1:1000	HRP goat anti-rabbit IgG $F(ab')_2$	1:80
rabbit anti-FVIIIRAg[c]	1:200		1:80

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

Anti-HLA-DR, -DP and -DQ MoAb were purchased from Becton Dickenson (Fujisawa Pharmaceutical Co., Tokyo, Japan), and OKM1 and OKM5 MoAb were obtained from Ortho Pharmaceutical Co. (Ortho Japan, Tokyo, Japan). Rabbit anti-IL-1 antibody was from Genzyme Co. (Cosmo Bio Co, Tokyo, Japan) and rabbit anti-human FVIIIRAg was purchased from Dako (Kyowa Medex, Tokyo, Japan). Goat anti-mouse $F(ab')_2$ fragments of IgG labeled with horseradish peroxidase (HRP) (Tago Inc., Cosmo Bio Co., Tokyo, Japan) and goat anti-rabbit $F(ab')_2$ fragments of IgG labeled with HRP (donated by Prof. Watanabe, Tokai University, Japan) were the second antibody. Antibodies used in the present study are listed with dilution values in Table 2.

Cryostat sections to be observed by light microscopy were treated with 100% methanol containing 0.03% hydrogen peroxidase to inactivate endogenous peroxidase. Then, indirect HRP-labeled antibody method was applied for the immunological reaction as previously described (Nagura et al. 1986; Yamamoto et al. 1988). Briefly, the procedure involves successive incubations with the first antibodies in optimal dilutions for 12 h at 4° C, and the second antibodies for 6 h at 4° C. They were

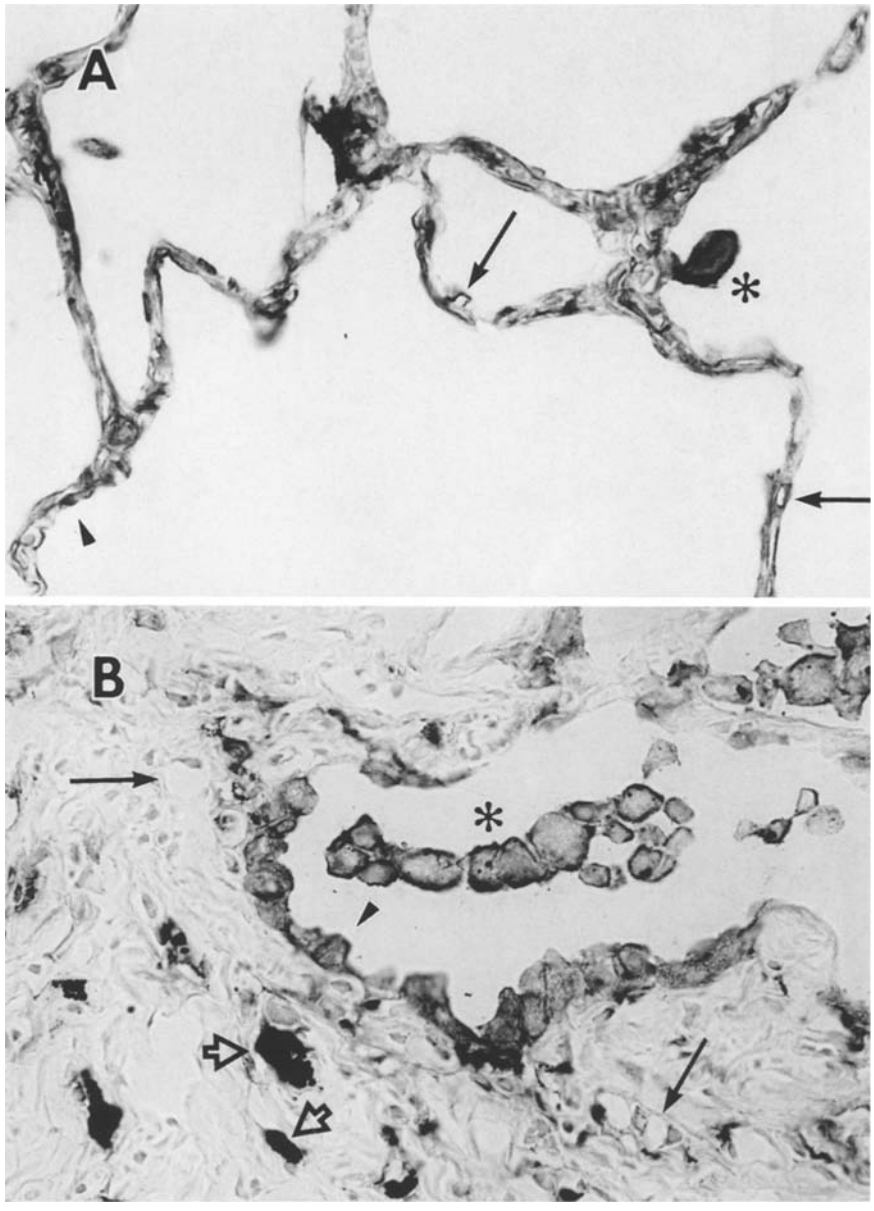

Fig. 1 A, B. Immunohistochemical localization of HLA-DR (case 1). **A** A non-thickened part near the thickened alveolar septa of pulmonary fibrosis. Anti-HLA-DR MoAb is reactive with ACEnd (*arrow*), alveolar macrophage (*) and the portions (*arrowhead*) which are compatible with type II AEp in the non-thickened alveolar septa. **B** Anti-HLA-DR MoAb is reactive with AEp lining over the surface of the thickened alveolar septa (*arrowhead*) and the clusters of alveolar macrophages in the alveolar spaces (*). However, ACEnd are negative (*arrow*) in the thickened alveolar septa. Open *arrowhead* indicates the deposition of dust particles. Magnification: (**A**) ×450; (**B**) ×450

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.

The specificity of histochemical staining was confirmed by use of either non-immune rabbit serum or ascitic fluid from mice injected with nonsecreting hybridoma cells instead of primary antiserum.

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.

Results

In pulmonary fibrosis, the results differed between the non-thickened alveolar septa and the thickened parts. The non-thickened alveolar septa consisted of the parts without fibrosis and showed almost normal appearance, while the thickened alveolar septa consisted of the parts with fibrosis on haematoxylin and eosin stained sections. ACEnd showed conspicuous differences in the immunostaining between the non-thickened parts and thickened al-

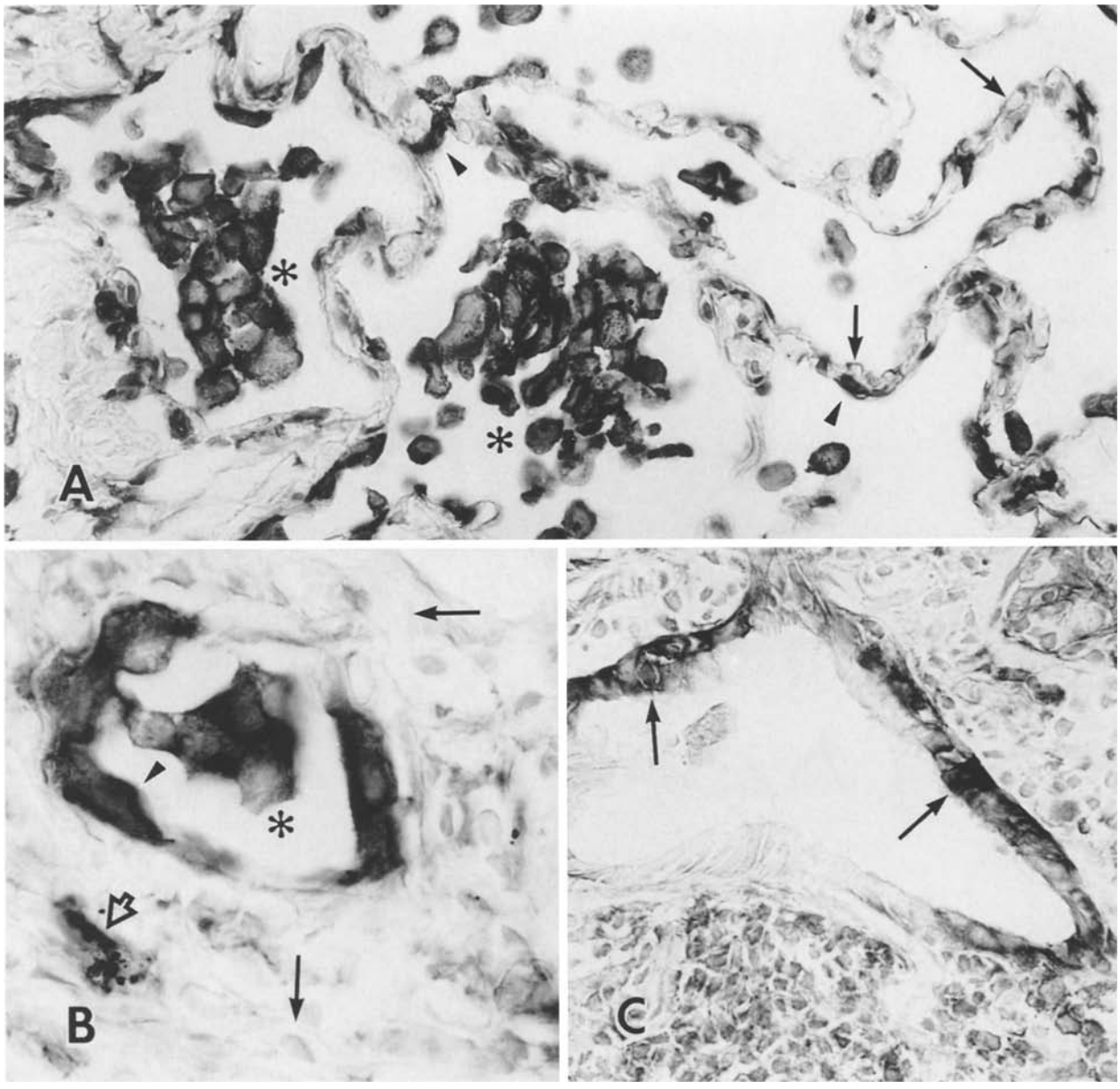

Fig. 2A–C. Immunohistochemical localization of HLA-DP (case 5). **A** Anti-HLA-DP MoAb is reactive with ACEnd (*arrow*), the portions (*arrowhead*) which are compatible with type II AEp and the clusters of alveolar macrophages (*) in alveolar spaces of the non-thickened alveolar septa. **B** Anti-HLA-DP MoAb is reactive with AEp in th thickened alveolar septa (*arrowhead*) and the cluster of alveolar macrophages in the alveolar space (*), but is not reactive with ACEnd (*arrow*). Open arrowhead indicates dust particles. **C** Bronchiolar epithelial cells in pulmonary fibrosis are positive for HLA-DP (*arrow*). Magnification: (**A**) ×600; (**B**) ×900; (**C**) ×450

veolar septa. In the non-thickened alveolar septa, the pattern of immunostaining of ACEnd was similar to that of normal lungs. That is, HLA-DR (Fig. 1A) and HLA-DP (Fig. 2A) were expressed on almost all AECnd, and HLA-DQ (Fig. 3A) were positive on more than three quarters of ACEnd. OKM5 was also reactive with a majority of ACEnd (Fig. 4) although OKM1 was not. IL-1 was expressed on almost all ACEnd (Fig. 6A). Although FVIIIRAg was clearly identified in vascular endothelial cells of small blood vessels, it was absent or weakly positive in ACEnd (Fig. 7A). However, in the thickened alveolar septa, alveolar capillaries were decreased in number, and HLA-DR (Fig. 1B), HLA-DP (Fig. 2B) and HLA-DQ (Fig. 3B) were not expressed on ACEnd. They were not reactive with OKM5 (Fig. 4), and IL-1 was scarcely expressed on them (Fig. 6B).

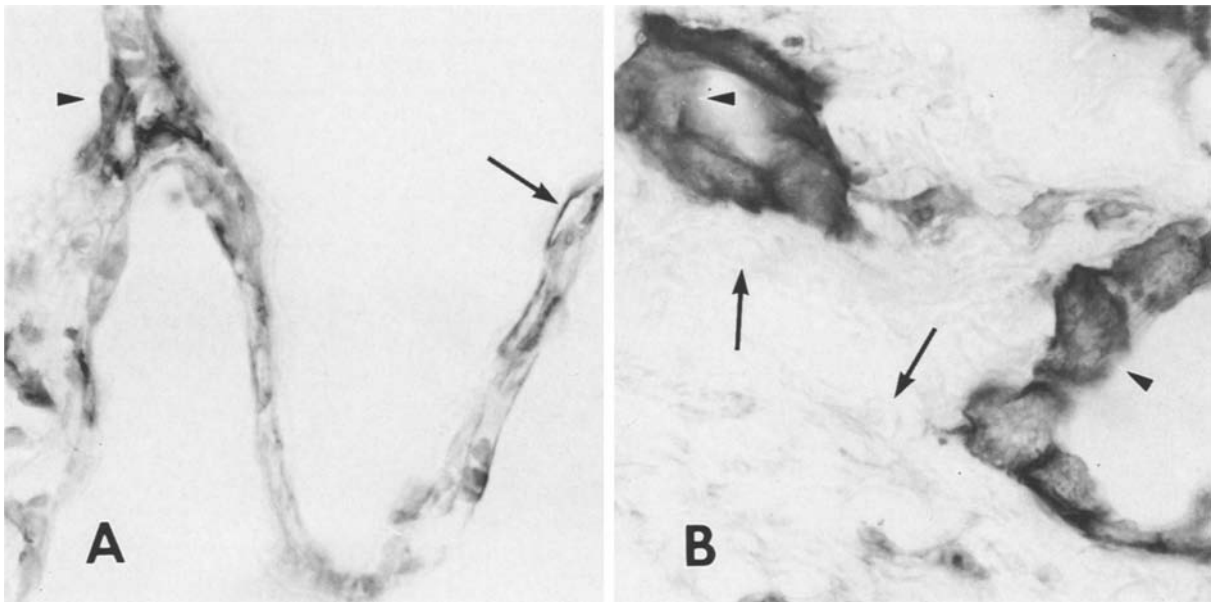

Fig. 3A, B. Immunohistochemical localization of HLA-DQ (case 6). **A** Anti-HLA-DQ MoAb is reactive with ACEnd (*arrow*) and the portions (*arrowhead*) which are compatible with type II AEp in the non-thickened alveolar septa. **B** HLA-DQ is strongly expressed on AEp lining over the surface of the thickened alveolar septa (*arrowhead*), but is not reactive with ACEnd (*arrow*). Magnification: (**A**) ×900; (**B**) ×900

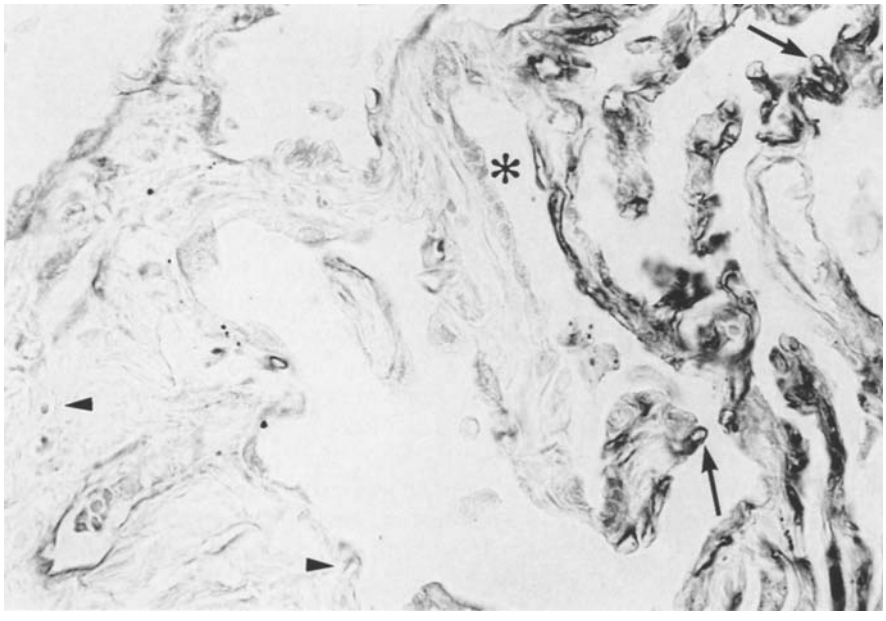

Fig. 4. Immunohistochemical localization of OKM5 (case 1); OKM5 is reactive with ACEnd in the non-thickened alveolar septa (*arrow*), while ACEnd in the thickened alveolar septa are decreased in number and almost not reactive with OKM5 (*arrowhead*). Type II AEp are not reactive with OKM5 (*). Magnification: ×450

FVIIIRAg was present in ACEnd (Fig. 7B). The immunohistochemical characterization of ACEnd in normal and fibrotic lungs is summarized in Table 3.

Type II AEp of the non-thickened alveolar septa in pulmonary fibrosis showed similar immunohistochemical characteristics to those of normal lungs. That is, HLA-DR (Fig. 1A), HLA-DP (Fig. 2A) and HLA-DQ (Fig. 3A) were positive. AEp lining over the surface of the thickened alveolar septa strongly expressed HLA-DR (Fig. 1B), HLA-DP (Fig. 2B) and HLA-DQ (Fig. 3B). These cells were classified as type II AEp, because lamella bodies were observed in their cytoplasm by electron microscopy (Fig. 10). OKM1 and OKM5 (Fig. 4) were not reactive and IL-1 was not identified on them. Type I AEp were not reactive with any of the antibodies used. Some of bronchiolar

Table 3. Immunohistochemical characterization of alveolar capillary endothelial cells

	HLA-DR	HLA-DP	HLA-DQ	OKM1	OKM5	IL-1	FVIIIRAg
Fibrotic lungs							
non-thickened	+	+	−/+	−	+	+	−/±
thickened	−	−	−	−	−	−	+
Normal lungs	+	+	−/+	−	+	+	−/±

+ : positive staining, ± : weakly positive staining, − : negative staining

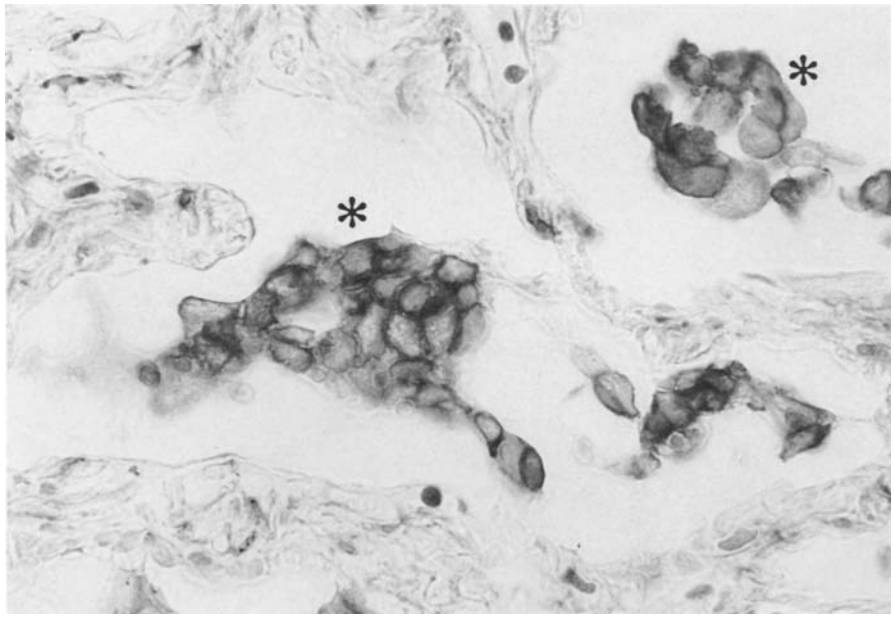

Fig. 5. Immunohistochemical localization of OKM1 (case 5); OKM1 is reactive with the clusters of alveolar macrophages in the alveolar spaces (*). Magnification: ×450

epithelial cells in pulmonary fibrosis were positive for HLA-DR, HLA-DP (Fig. 2C) and HLA-DQ. These MHC class II antigens were almost negative on bronchiolar epithelial cells of normal lungs.

More than two thirds of alveolar macrophages in normal lungs expressed HLA-DR, HLA-DP and HLA-DQ, and over one half of them were reactive with OKM1, but few were reactive with OKM5. In pulmonary fibrosis, more than three quarters of alveolar macrophages expressed HLA-DR, HLA-DP and HLA-DQ, and were also reactive with OKM1. IL-1 was expressed in alveolar macrophages in pulmonary fibrosis, but only faintly in normal lungs. Interestingly, alveolar macrophages in the alveolar spaces of pulmonary fibrosis with the non-thickened and thickened alveolar septa were increased in number and often gathered in clusters, and the expression of HLA-DR (Fig. 1B), HLA-DP (Fig. 2A, B) and HLA-DQ was especially strong on these clusters. They were also strongly reactive with OKM1 (Fig. 5), and expressed IL-1 (Fig. 6B). These clusters, however, were absent in normal lungs. The results of AEp in normal and fibrotic lungs are summarized in Table 4. The control sections were uniformly negative (Fig. 8).

These findings were confirmed by immunoelectron microscopy. That is, MHC class II antigens were present along the plasma membranes of ACEnd in the non-thickened alveolar septa (Fig. 9), but not expressed along the plasma membranes of ACEnd in thickened alveolar septa (Fig. 10). MHC class II antigens were present along the plasma membranes of AEp lining over the surface of thickened alveolar septa (Fig. 10).

All 8 cases represented the same phenotypic characterization, regardless of the complication of collagen diseases, steroid therapy and smoking.

Discussion

The present investigation demonstrated different expression of antigens found on a peripheral blood monocyte-macrophage subset by ACEnd in normal and fibrotic lungs. ACEnd in the non-thick-

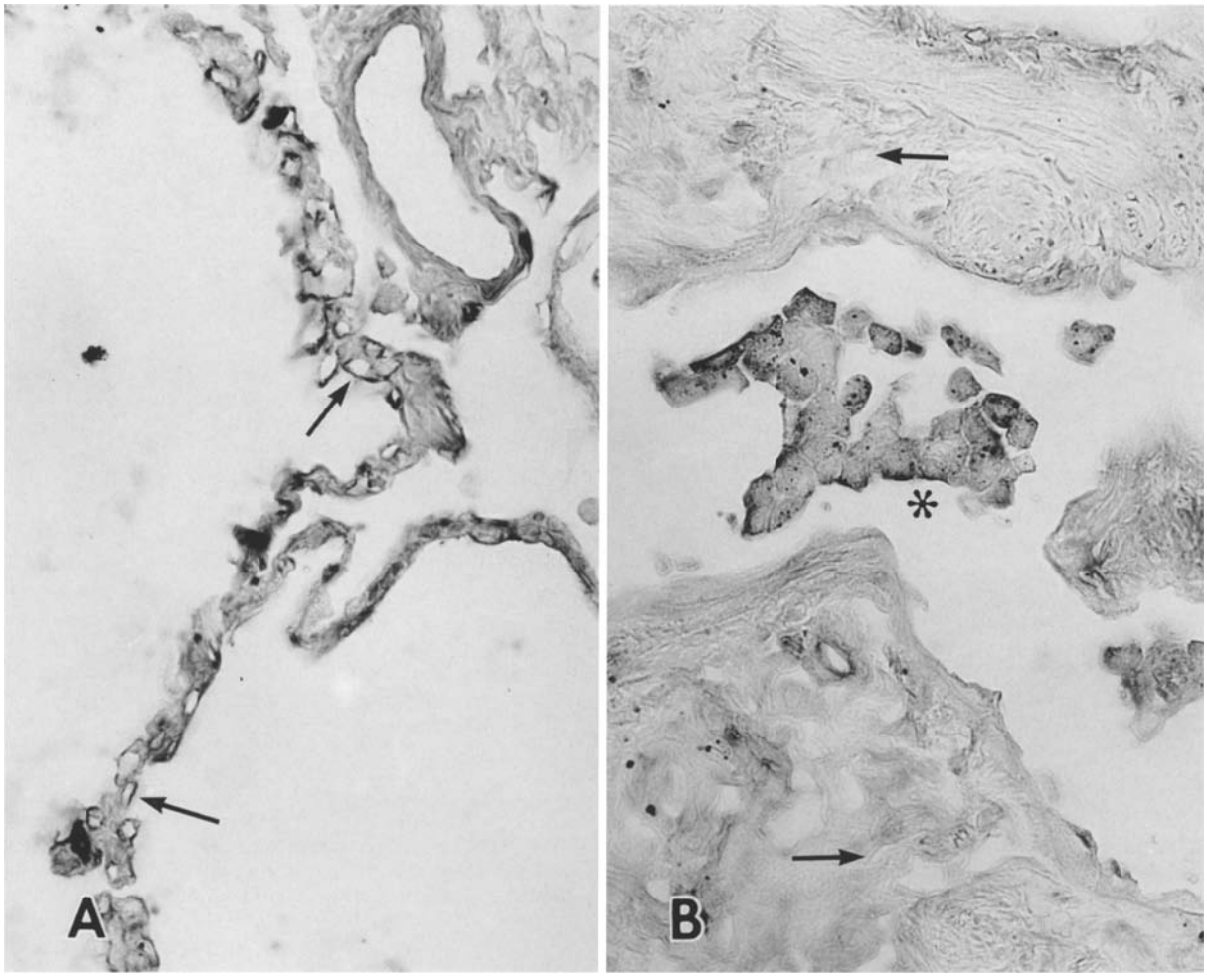

Fig. 6A. B. Immunohistochemical localization of IL-1 (case 1). **A** IL-1 is expressed on ACEnd in the non-thickened alveolar septa (*arrow*). **B** IL-1 is expressed on the cluster of alveolar macrophages (*), but little expressed on ACEnd (*arrow*) in the thickened alveolar septa. Magnification: (**A**) ×450; (**B**) ×450

Table 4. Immunohistochemical characterization of alveolar epithelial cells and alveolar macrophages

	HLA-DR	HLA-DP	HLA-DQ	OKM1	OKM5	IL-1
A) Alveolar epithelial cells						
Fibrotic lungs						
non-thickened						
type I[a]	−	−	−	−	−	−
type II[b]	+	+	+	−	−	−
thickened (alveolar lining cells)	++	++	+~++	−	−	−
Normal lungs						
type I	−	−	−	−	−	−
type II	+	+	+	−	−	−
B) Alveolar macrophage						
Fibrotic lungs	++	++	+~++	++	−	+
Normal lungs	+	+	+	+	−	±

++ : strongly positive staining, + : positive staining, ± : weakly positive staining, − : negative staining

[a] Type I alveolar epithelial cells
[b] Type II alveolar epithelial cells

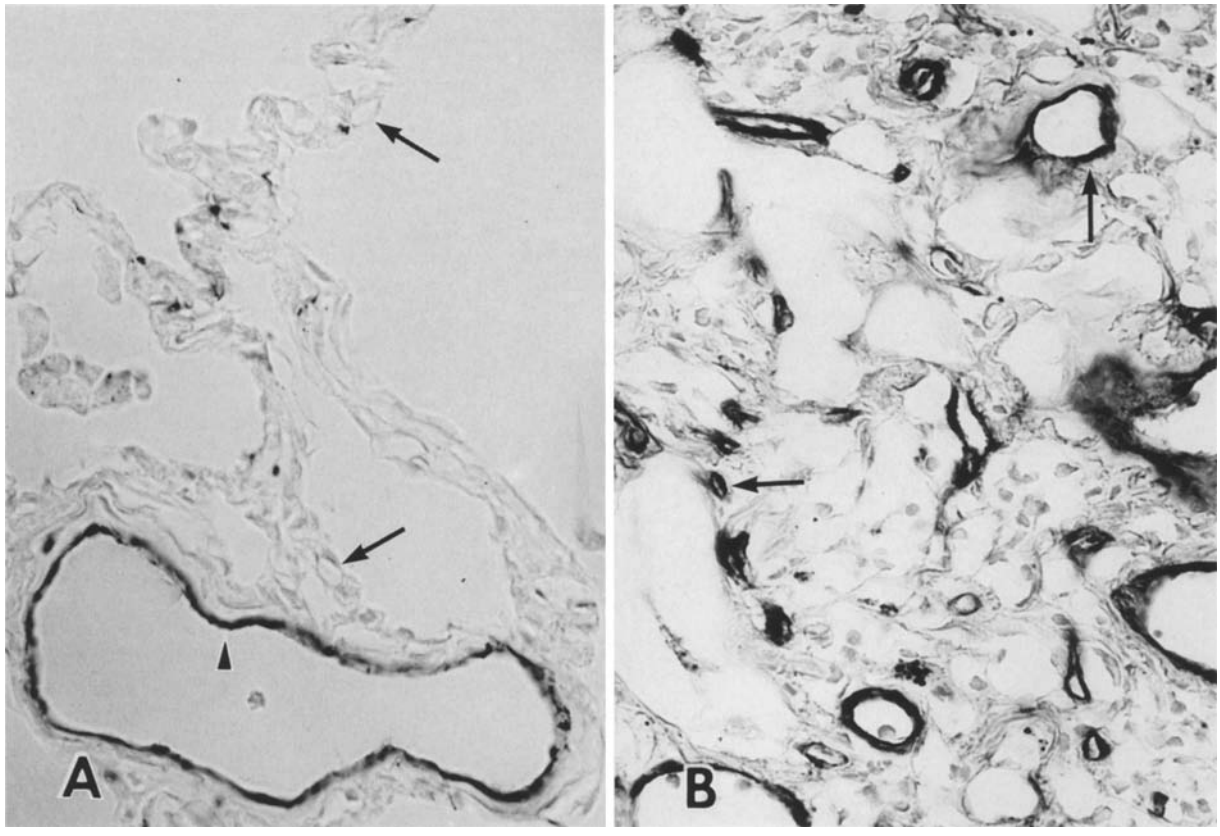

Fig. 7A, B. Immunohistochemical localization of FVIIIRAg (case 6). **A** FVIIIRAg is clearly identified in vascular endothelial cells of small vessel (*arrowhead*), but only faintly identified in ACEnd (*arrow*) in the non-thickened alveolar septa. **B** FVIIIRAg is identified both in ACEnd and vascular endothelial cells in the thickened alveolar septa (*arrow*). Magnification: (**A**) ×450; (**B**) ×450

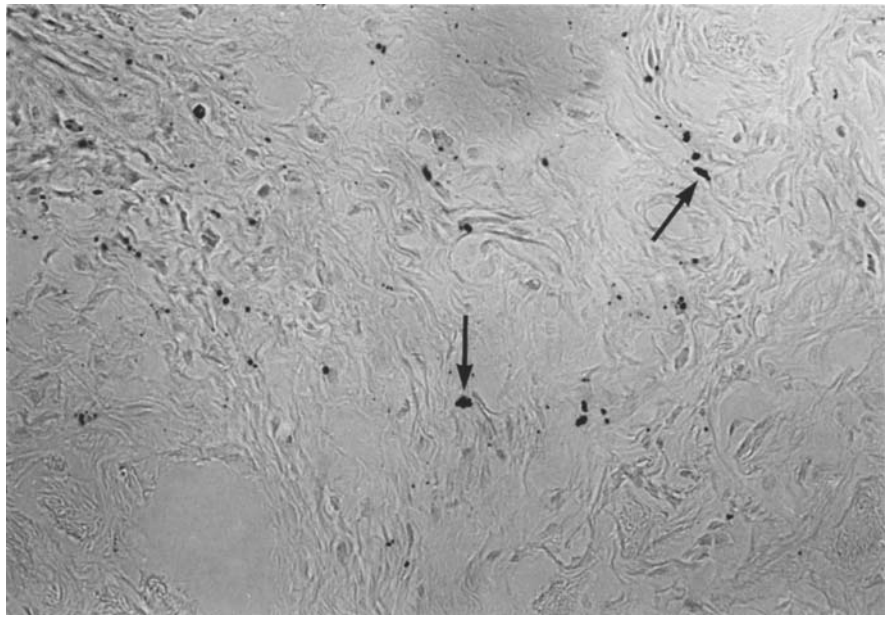

Fig. 8. Control staining (case 1); No reaction products are observed. *Arrow* indicates the deposition of dust particles. Magnification: ×450

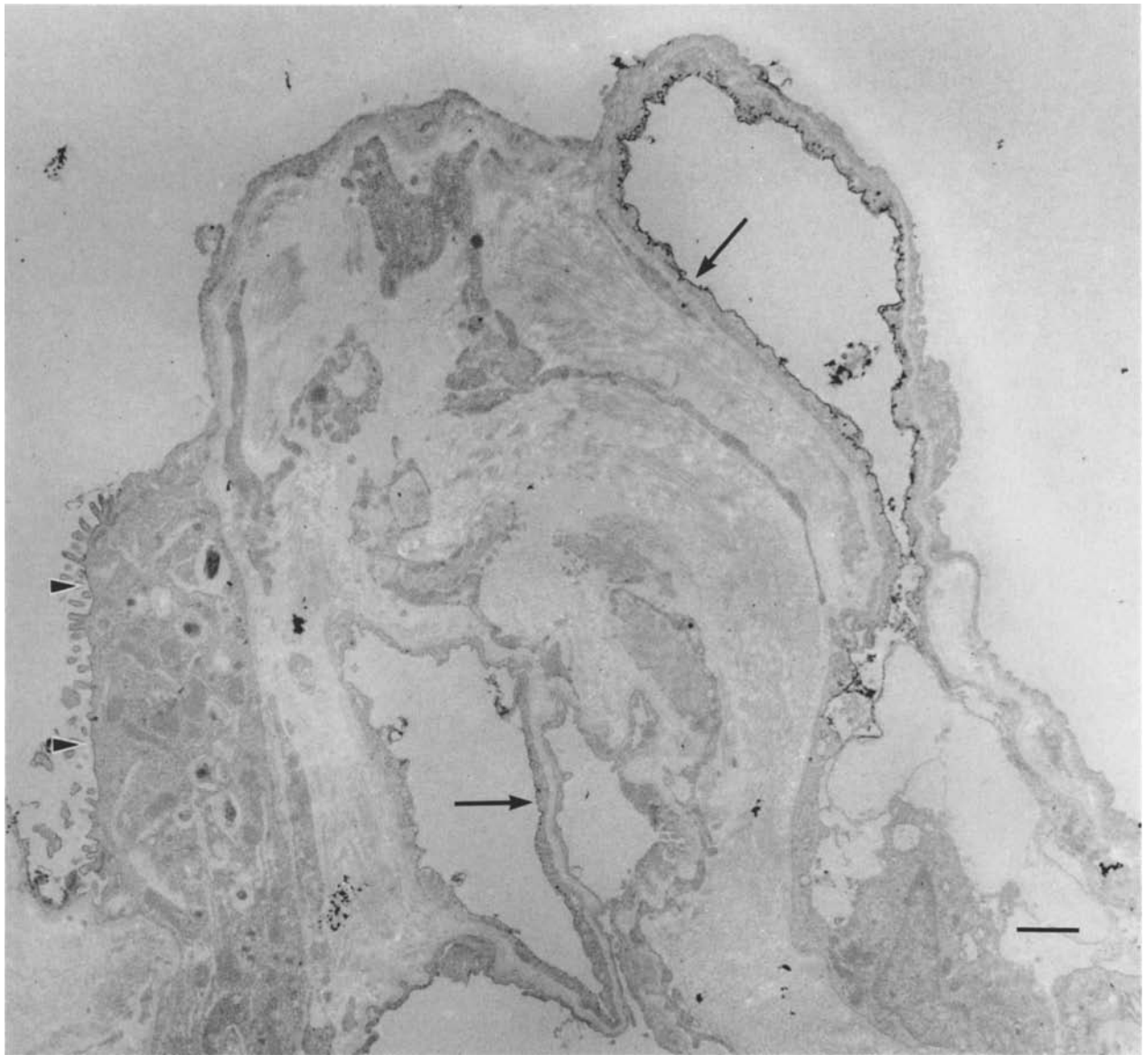

Fig. 9. Ultrastructural localization of HLA-DR (case 1); HLA-DR is present along the plasma membranes of ACEnd in the non-thickened or slightly fibrotic alveolar septa (*arrow*). Type II alveolar epithelial cell is positive (*arrowhead*). Magnification: ×8400

ened alveolar septa of pulmonary fibrosis expressed the same phenotypes as ACEnd in normal lungs, and may participate in immune reactions as APC (Yamamoto et al. 1988). In fact, Burger and Vetto (1982) suggested that Ia-positive vascular endothelial cells play a central role in the development of immune respones. In the thickened alveolar septa of pulmonary fibrosis, alveolar capillaries were decreased in number, as described by Matsubara et al. (1986) and they lost MHC class II antigens and were not reactive with OKM5. IL-1 was hardly detected. Evidently, the phenotypic changes of ACEnd occur during the development of pulmonary fibrosis. With regard to the origin of ACEnd in the thickened alveolar septa, Coalson (1982) reported that ACEnd in fibrosing alveolitis represented regenerating endothelial cells. It is reasonable to speculate that ACEnd are first injured by some antigenic stimulation, and that endothelial cells with different phenotypic properties are regenerated. These regenerated endothelial cells may be involved in coagulation instead of immunological functions, because FVIIIRAg is synthesized and antigens found on a monocyte-macrophage subset are lost in those cells in the thickened alveolar septa.

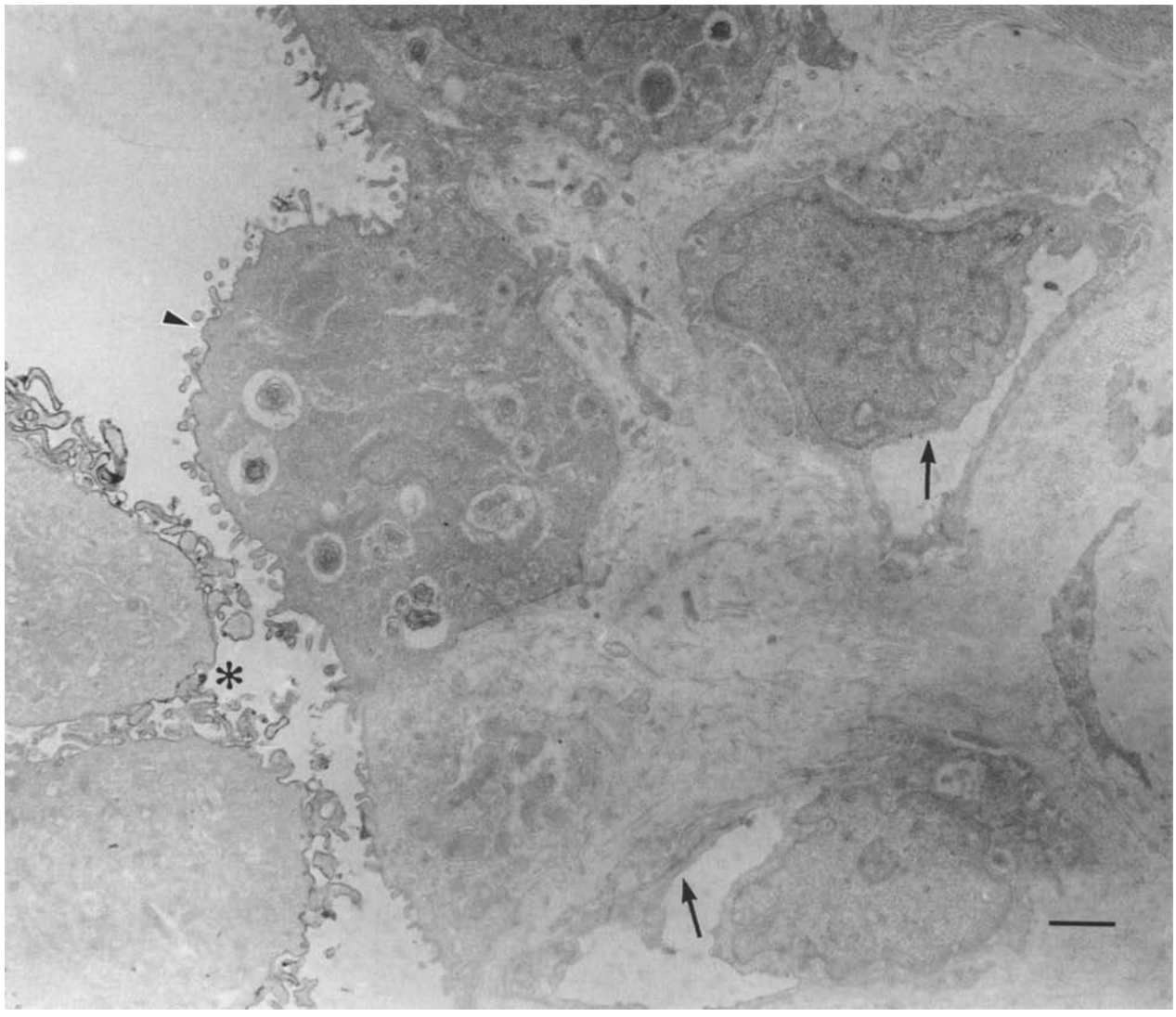

Fig. 10. Ultrastructural localization of HLA-DR (case 1); HLA-DR is present along the plasma membranes of type II AEp lining over the surface of the thickened alveolar septa (*arrowhead*), and the cluster of alveolar macrophages (*) in the alveolar space. But HLA-DR is not present along the plasma membranes of ACEnd in the thickened alveolar septa (*arrow*), and the capillaries were decreased in size and ACEnd were plump. Magnification: ×8400

We observed that proliferative alveolar lining cells on the thickened alveolar septa of pulmonary fibrosis expressed MHC class II antigens, such as HLA-DR, HLA-DP and HLA-DQ, much more strongly than type II AEp in normal lungs. The strong expression of MHC class II antigens in the alveolar lining cells in pulmonary fibrosis suggests that they play an important role in various immune reactions at alveolar epithelial proliferative period (Beaumont et al. 1986; Kallenberg et al. 1987a, b) because ACEnd in the thickened alveolar septa did not express MHC class II antigens. Alveolar lining cells on the thickened alveolar septa were classified as type II AEp in previous ultrastructural reports (Stachura et al. 1980; Corrin et al. 1985). After type I AEp are damaged in pulmonary fibrosis, type II AEp proliferate on the alveolar surface to cover the alveolar septa (Stachura et al. 1980).

Normal human alveolar macrophages reportedly function poorly as accessory cells and have limited capacity to secrete IL-1 (Wewer et al. 1984; Holt 1986; Rossi et al. 1986). However, alveolar macrophages obtained from BALF in active pulmonary fibrosis have increased HLA-DR antigen expression, higher reactivity with OKM1, and greater capacity to secrete IL-1 than those of normal subjects (Razma et al. 1984; Izumi et al. 1986; Eden and Turino 1986). In addition, IL-1 stimu-

lates the proliferation of fibroblasts (Schmidt et al. 1982). In our present study, alveolar macrophages in the alveolar spaces of pulmonary fibrosis with both non-thickened and thickened alveolar septa were strongly positive for HLA-DR, HLA-DP, HLA-DQ and IL-1, and highly reactive with OKM1, especially on the clusters. Such clusters are mainly composed of alveolar macrophages (Stachura et al. 1980; Corrin et al. 1985). Thus, alveolar macrophages in alveolar spaces may also be important in presenting antigens, initiating immune reactions, and developing fibrosis.

Based on these results, we suggest that the phenotypic changes of ACEnd are involved in the process of IPF and pulmonary fibrosis with collagen diseases, and that type II AEp on the thickened alveolar septa, and alveolar macrophages gathering in clusters in alveolar spaces, play a role as APC and are involved in the development of pulmonary fibrosis.

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