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Increased expression of intercellular adhesion molecule-1 (ICAM-1) in a murine model of pulmonary eosinophilia and high IgE level

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SUMMARY

T lymphocytes and eosinophils are probably involved in the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA), a disease characterized by pulmonary eosinophilia and high serum and lavage IgE levels. We recently developed a murine model of ABPA. To investigate the mechanisms of T lymphocyte and eosinophil recruitment to the lung in this disease, we examined the expression of ICAM-1 in the lung tissue of mouse challenged with *Aspergillus fumigatus* (Af) antigen. C57Bl/6 mice were intranasally exposed to Af (Af group) or saline (control group) three times a week for 1, 2 or 3 weeks. On days 4, 7, 14 and 21, mice were killed and lung tissue was fixed in acetone and embedded in glycol methacrylate. Serial 2-μm sections were stained with chromotrope 2R and MoAbs against ICAM-1, CD11a/CD18 (LFA-1) and CD3. Af-challenged mice presented significant increases in eosinophil, T lymphocyte and LFA-1-positive cell count and up-regulated expression of ICAM-1 in the lung tissue at all the time points examined. ICAM-1 expression intensity correlated with the number of T lymphocytes ($r = 0{\cdot}59$, $P < 0{\cdot}01$), LFA-1-positive cells ($r = 0{\cdot}68$, $P < 0{\cdot}001$), but not of eosinophils ($r = -0{\cdot}24$, $P > 0{\cdot}05$). These findings suggest that up-regulation of ICAM-1 expression is involved in the inflammatory process of this murine model of ABPA, and that this up-regulation may be more relevant to the T lymphocyte accumulation in the lung.

Keywords ABPA immunohistochemistry T lymphocytes eosinophils intercellular adhesion molecule-1

INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disease caused mostly by the inhalation of *Aspergillus* species. Patients with ABPA show blood and pulmonary eosinophilia and elevated serum and bronchoalveolar lavage IgE levels. These two features may be T lymphocyte-dependent, as T cell-derived cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IL-3 and IL-4 can promote eosinophil maturation, survival and activation, and IgE production [1–3].

Eosinophil is probably an important immune effector cell in the development of ABPA. It can damage tissues by the release of granule-associated basic proteins, eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase and eosinophil-derived neurotoxin, and oxygen radicals, and can modulate the inflammatory process by producing mediators and cytokines, such as leukotriene C4, platelet-activating factor (PAF), IL-1, IL-3, IL-5, IL-6, GM-CSF, transforming growth factor-alpha (TGF-α), TGF-β and tumour necrosis factor (TNF) [2–5].

The mechanisms of T lymphocyte and eosinophil accumulation in inflammatory sites are only partially understood. Recent studies showed that adhesion of leucocytes to microvascular endothelium precedes their presence in the tissue. Adhesion of leucocytes to structural tissue components such as fibronectin, airway and alveolar epithelial cells, is also essential for their retention, survival and actions within the tissue [6,7]. The initiation of leucocyte adhesion to endothelial cells of inflamed tissue is realized by the binding of various adhesion molecules, which expression on the endothelial cells and leucocytes is up-regulated during the inflammatory process. ICAM-1 (CD54) is one of these adhesion molecules. It serves as a ligand for lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) on T cells, as well as other inflammatory cells such as macrophages, neutrophils and eosinophils [8].

Up-regulation of ICAM-1 expression has been found in many pathological processes [9–13]. An early study demonstrated that strong ICAM-1 expression on endothelial cells was associated with T cell migration in areas exhibiting delayed hypersensitivity in the skin [14]. Wegner *et al.* [15] showed that,

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in Ascaris-sensitized monkeys, repeated allergen provocation of the airways resulted in increased ICAM-1 expression on bronchial epithelia and submucosal vascular endothelia, and in an intense eosinophil influx into the tracheal mucosa which could be attenuated by i.v. infusion or inhalation of a blocking anti-ICAM-1 MoAb. These observations suggest that ICAM-1 may be relevant to the tissue inflammatory response involving significant eosinophil infiltration.

In the present study, we examined the expression of ICAM-1 by immunohistochemistry in the lung tissue of mice with *Aspergillus fumigatus* (Af)-induced pulmonary eosinophilia and related it to tissue recruitment of eosinophils, T lymphocytes and LFA-1-positive cells.

MATERIALS AND METHODS

Animals

Thirty-six pathogen-free female inbred C57Bl/6 mice 6–8 weeks old were obtained from Charles River Inc. (St-Constant, Québec, Canada) and randomized before the sensitization.

Induction of ABPA

Induction of ABPA was performed as previously described [4]. Briefly, the mice were lightly anaesthetized with diethyl ether, and 50 μl of *A. fumigatus* antigen (100 μg in dry weight) (Af group) or 50 μl saline (Sa group) were administered to the tip of the nose and inhaled involuntarily. The inhalations were given three times a week during the first three successive days of each week for 1, 2 or 3 weeks. On days 4, 7, 14 and 21, mice were killed by exsanguination. There were five and four mice at each time point in Af and Sa groups, respectively.

Lung tissue processing

The whole lungs were removed from the thoracic cavity. They were immediately dissected into small pieces approximately $2 \times 2 \times 1$ mm. For each mouse, two to four pieces of tissue were randomly selected from the left and right lungs and processed as follows. Lung fragments were fixed in ice-cooled acetone containing the protease inhibitors, PMSF (2 mM) and idioacetomide (20 mM), and stored overnight at −20°C. The following day, the lung tissue was transferred into acetone and methybenzoyl at room temperature followed by immersion in glycol methacrylate (GMA) monomer (Polyscience Inc., Warrinton, PA) at 4°C for 7 h. Finally, the tissue was embedded in the resin prepared by mixing GMA monomer and benzoyl peroxide and polymerized overnight at 4°C. The blocks were stored in airtight containers at −20°C for later immunohistochemistry.

Histochemistry

For routine histologic evaluation, sections were stained with haematoxylin and eosin. Eosinophils in the tissue were identified by 1% chromotrope 2R staining.

Immunohistochemistry

Sequential 2-μm sections were cut by ultramicrotome (Reichert Ultracut S, Vienna, Austria), placed on glass slides coated with poly-L-lysine and dried at room temperature. To block endogenous peroxidase, the slides were placed in 0·1% sodium azide and 0·3% hydrogen peroxide solution for 30 min and rinsed in 0·05 M Tris-buffered saline (TBS, pH 7·6). Successive incubations in avidin for 20 min, and biotin for 20 min (Blocking Kit, Vector Labs, Burlingame, CA) were then performed to eliminate the endogenous biotin activity. To prevent non-specific protein binding, 10% normal goat or rabbit serum was applied to the sections for 30 min before the addition of primary antibodies.

Rat MoAbs against mouse ICAM-1, CD3 (PharMingen, San Diego, CA) and LFA-1 (Cedarlane Lab, Westbury, NY) were used at dilutions of 1 : 50, 1 : 50 and 1 : 1000, respectively. Sections were incubated with primary antibodies overnight at room temperature. Biotinylated secondary antibodies in appropriate dilutions in TBS containing 2% normal mouse serum were then applied for 2 h followed by the straptavidin-biotin-horseradish peroxidase complex (Sera Lab, Crawley Down, UK) for an additional 2 h. After rinsing the sections in TBS, 0·01% diaminobenzidine (DAB) in 0·02% hydrogen peroxide was used as substrate to develop a peroxide-dependent brown colour reaction. All the sections were counterstained with Harris haematoxylin, and some of the sections stained for ICAM-1 were also counterstained with chromotrope 2R to identify the eosinophils. Sections were finally dehydrated and coverslipped with Permount. Hamster IgG, rat IgG1 and mouse IgG2a were used to replace the primary antibodies as negative controls.

Quantification of histochemical and immunohistochemical stainings

Sections were coded and counted blindly using a Leitz Wetzlar microscope at ×200 magnification field (0·62 mm^2). Because the distribution of CD3 and LFA-1-positive cells and eosinophils was not uniform, entire sections ($\geqslant 5 \times 200$ magnification fields per section) were counted. Count was expressed as number of cells (mean ± s.e.m.) per field. ICAM-1 expression was evaluated on each ×200 magnification field according to the staining intensity and the distribution pattern, and scored from 0 to 9, where 0 meant no, 3 for light, 6 for moderate and 9 for intense staining. Each mouse (two to four sections per mouse, $\geqslant 5$ fields per section) was given a score (mean ± s.e.m.) of ICAM-1 expression per field. Each section was counted or evaluated at least twice by the same observer. The coefficient of variation for repeated measurements was $\leqslant 7\%$.

Statistical analysis

Student's *t*-test was applied to the comparisons between Af and Sa groups. For the comparisons within the group at different time points, Fisher's test was used followed by Tukey's comparison. Correlations between various groups of data were carried out using Spearman's rank correlation coefficient. $P \leqslant 0{\cdot}05$ was regarded as significant.

RESULTS

Histological findings

Mice of the Sa group showed normal lung structure without any evidence of inflammatory lesions. In contrast, mice of the Af group at all time points examined demonstrated interstitial pneumonitis with various degrees of eosinophil infiltration (Table 1, Fig. 1). On day 4, an acute inflammatory cellular infiltrate appeared. Neutrophils, monocytes, eosinophils and increased numbers of alveolar macrophages were dispersed throughout the lung tissue. These inflammatory cells were

Table 1. Lung tissue immunohistochemical results and eosinophil count

	ICAM-1		LFA-1$^+$ cells		CD3$^+$ cells		Eosinophils	
Day	Af	Af/Sa	Af	Af/Sa	Af	Af/Sa	Af	Af/Sa
4	$6{\cdot}8 \pm 0{\cdot}3\dagger^{a}$	6·2	$41{\cdot}8 \pm 8{\cdot}7\dagger^{a}$	16·7	$52{\cdot}3 \pm 9{\cdot}0\dagger^{ab}$	4·9	$9{\cdot}6 \pm 1{\cdot}5\dagger^{b}$	32·0
7	$5{\cdot}3 \pm 0{\cdot}4*^{b}$	2·4	$20{\cdot}9 \pm 4{\cdot}3*^{b}$	3·9	$23{\cdot}9 \pm 7{\cdot}7^{c}$	2·1	$14{\cdot}7 \pm 4{\cdot}6\dagger^{b}$	29·4
14	$6{\cdot}4 \pm 0{\cdot}5\dagger^{ab}$	1·6	$44{\cdot}3 \pm 1{\cdot}9\dagger^{a}$	3·5	$69{\cdot}0 \pm 7{\cdot}1*^{a}$	3·2	$33{\cdot}3 \pm 3{\cdot}2\dagger^{a}$	57·3
21	$5{\cdot}3 \pm 0{\cdot}3\dagger^{b}$	3·8	$44{\cdot}9 \pm 5{\cdot}8\dagger^{a}$	17·3	$38{\cdot}4 \pm 4{\cdot}9\dagger^{bc}$	5·3	$47{\cdot}0 \pm 6{\cdot}8\dagger^{a}$	107·3

Values are expressed as mean ± s.e.m. ICAM-1 is expressed as staining score per ×200 magnification field. LFA-1 and CD3-positive cells as well as eosinophils were expressed as number of cells per ×200 magnification field. $*P < 0{\cdot}05$ and $\dagger P < 0{\cdot}01$ compared with control (not shown), Student's *t*-test. The values identified by a different letter are different ($P < 0{\cdot}05$), Fisher's test followed by Tukey's comparison. Af, Af group; Sa, saline group.

predominantly localized around small blood vessels and bronchioles. Local oedema could be visualized in some specimens. The pathological changes present on day 7 were similar to those on day 4, but neutrophils and oedema were hardly seen. Peribronchiolar and perivascular inflammation was more significant on day 14: great numbers of eosinophils, macrophages and monocytes infiltrated the peribronchiolar and perivascular tissue, alveolar walls and air spaces. On day 21, the lung lesions were even more severe. Inflammatory infiltrates not only aggregated around small blood vessels and bronchioles, but also extended, sometimes massively, to the alveolar walls and distorted the lung parenchyma. In some mice, epithelioid granuloma with giant cells could be observed at this time.

Immunohistochemical findings

In comparison with the control mice, ICAM-1 expression, T cells and LFA-1$^+$ cells were significantly increased in Af-challenged mice (Table 1, Fig. 1). In both Af and Sa groups, immunostaining for ICAM-1 was localized at the luminal surface of the endothelia of arteries, arterioles, veins and venules, on alveolar macrophages, and in alveolar walls (Fig. 2a,b). Under light microscope, in the alveolar walls, it was difficult to determine whether ICAM-1 staining was on the surface of epithelial cells or endothelial cells. However, at ×1000 magnification, we could identify ICAM-1 staining on the alveolar epithelium and capillary endothelium in some thick portions of alveolar walls (Fig. 2c). In the lung tissue of Af-treated mice, more cells were positive for ICAM-1, and ICAM-

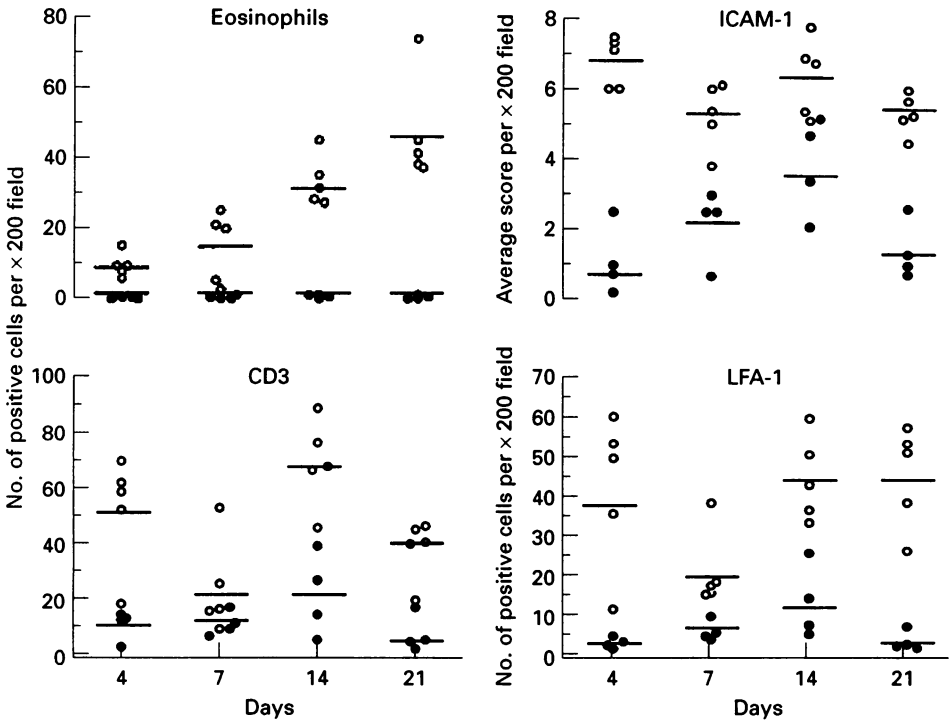

Fig. 1. Eosinophils, CD3 and LFA-1$^+$ cells, and ICAM-1 expression at the examined time points. Each dot (○, *Aspergillus fumigatus* (Af) group; ●, saline group) represents the result from an individual mouse lung tissue. The upper and lower cross bars represent the mean of Af and saline groups, respectively.

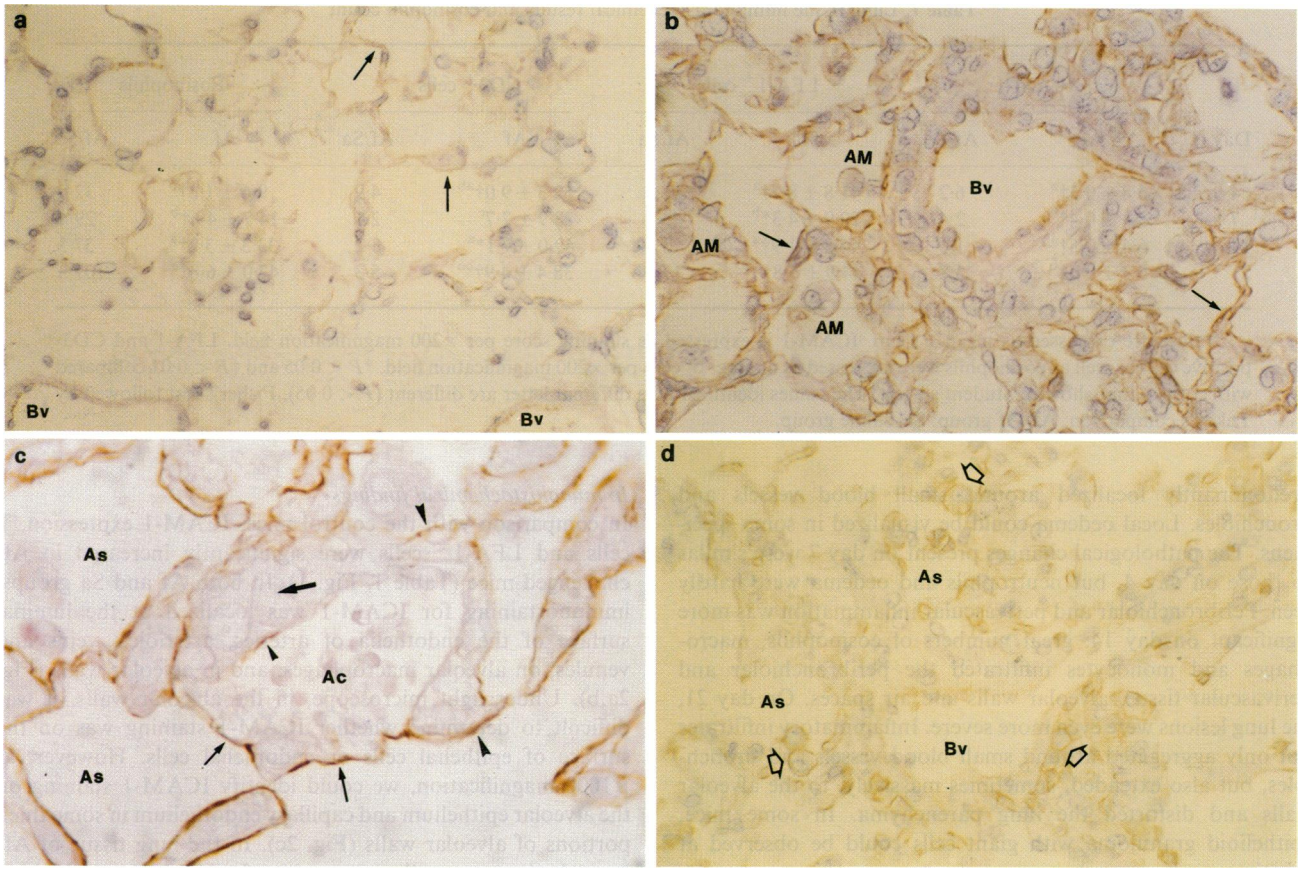

Fig. 2. Lung tissue preparations. (a) A normal mouse lung tissue stained with MoAb against ICAM-1 demonstrates weak staining on blood vessel (Bv) endothelia and in the alveolar walls (arrows). (b) At day 4, lung tissue of *Aspergillus fumigatus* (Af)-treated mouse shows a very strong ICAM-1 staining on alveolar macrophages (AM) and blood vessel (Bv) endothelia, and in alveolar walls (arrows). (c) In the thick portion of alveolar wall (thick arrow), ICAM-1 stainings were identified on the surface of epithelia (big arrowheads) and capillary endothelia (small arrowhead). However, localization of ICAM-1 staining between epithelia and endothelia was impossible in the thin portion of the alveolar wall (thin arrows) (Af group, day 4). Ac, Alveolar capillary; As, alveolar space. (d) At day 4, Af-treated mouse lung presents an increased number of LFA-1$^+$ cells (open arrows) around a small blood vessel (Bv) and in alveolar spaces (As). Original magnifications: c, ×1000; others, ×400.

1 staining intensity was significantly increased, especially in alveolar walls. In addition, ICAM-1 expression was found at the basolateral portion of some bronchial epithelia where some eosinophils aggregated. On day 4, there was a striking increase of ICAM-1 expression (staining score: $6{\cdot}8 \pm 0{\cdot}3$, Af/Sa = 6·2, $P < 0{\cdot}001$). ICAM-1 expression on day 7 and day 21 was weaker than on day 4 ($P < 0{\cdot}05$), but still higher than control level ($P < 0{\cdot}05$). The expression level of ICAM-1 did not show any significant difference between days 4 and 14 ($P < 0{\cdot}05$).

Consistent with the increased expression of ICAM-1, the number of LFA-1$^+$ cells was greater in the lung tissue from the Af group than from the Sa group. LFA-1$^+$ cells included macrophages, eosinophils, lymphocytes and neutrophils. Significant increases of CD3$^+$ cells were seen in the Af group on days 4, 14 and 21, but not on day 7. Most of the LFA-1 and CD3-positive cells were observed around bronchioles and small blood vessels (Fig. 2d).

When correlations between measured parameters were made in Af-treated mice at all the examined time points, we found that ICAM-1 expression significantly correlated with the number of T cells ($r = 0{\cdot}59$, $P < 0{\cdot}01$) (Fig. 3a), but neither with the intensity of eosinophil influx ($r = -0{\cdot}24$, $P > 0{\cdot}05$) nor with the number of LFA-1$^+$ cells ($r = 0{\cdot}30$, $P > 0{\cdot}05$). However, when the two groups were combined, there was a significant positive correlation between the expression of ICAM-1 and the LFA-1$^+$ cell count ($r = 0{\cdot}68$, $P < 0{\cdot}001$) (Fig. 3b).

DISCUSSION

T lymphocytes and eosinophils may interact closely in the pathogenesis of ABPA. T cells produce cytokines which recruit and activate eosinophils. Eosinophils may act as effector cells through the release of certain pro-inflammatory mediators, and as modulator cells through the production of cytokines such as IL-3, IL-5, TNF, GM-CSF, etc. [2–5]. In Af-challenged mouse lung tissue, the eosinophil and T lymphocyte count was significantly increased. This study shows that lung ICAM-1 expression was up-regulated, and that there was a significant positive correlation between the expression of ICAM-1 and the

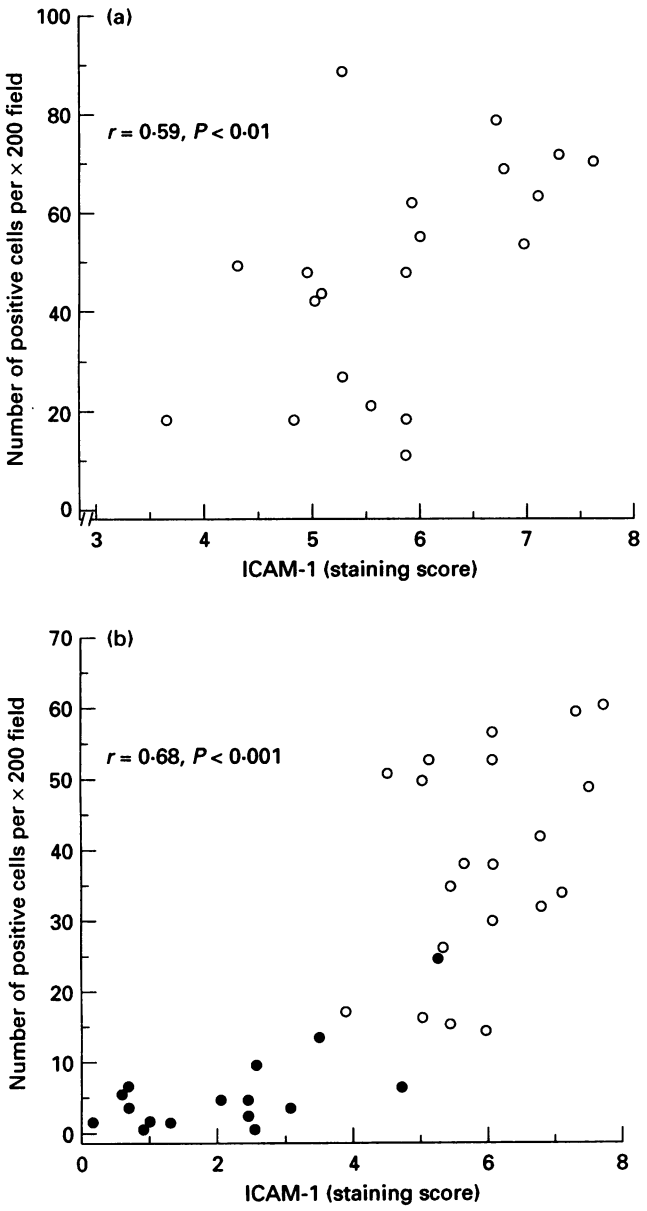

Fig. 3. Correlations between ICAM-1 expression and CD3 (a) or LFA-1$^+$ cell (b) count. ○, *Aspergillus fumigatus* (Af) group; ●, saline group.

number of T cells but not of eosinophils. To the best of our knowledge, this is the first demonstration of increased ICAM-1 expression in the animal model of ABPA. That ICAM-1 was significantly up-regulated as early as on day 4, and this up-regulation persisted throughout the whole period of the experiment, indicates that ICAM-1 was involved in the acute and subacute inflammatory processes.

ICAM-1 is constitutively expressed to varying degrees in different tissue sites and cell types such as endothelial cells, epithelial cells and macrophages. A study of asthmatic subjects showed that sputum but not blood eosinophils could express ICAM-1 [16]. ICAM-1 expression levels and the number of ICAM-1-expressing cell types could be increased by many stimuli such as IL-1, TNF-α, interferon-gamma (IFN-γ), endotoxins and even eosinophil's own products MBP and ECP [7,17,18]. In our experiments, ICAM-1 was weakly expressed by some endothelial cells, alveolar epithelial cells and alveolar macrophages in the lung of control mice. In Af-challenged mice, ICAM-1 expression increased significantly, not only on vascular endothelia and alveolar epithelia, but also on bronchial epithelia where aggregated eosinophils, lymphocytes and macrophages which were usually LFA-1$^+$. This finding may partly explain the histological profiles seen in the Af-challenged lung: clusters of inflammatory cells at the locations of increased ICAM-1 expression. We did not find any eosinophil positive for ICAM-1 in both Af and Sa groups. This may be due to the true absence of ICAM-1 on eosinophils, or to a low expression which was not demonstrated by immunohistochemistry.

In the lung, there are two blood circulation systems: pulmonary and bronchial circulations. In pulmonary circulation, inflammatory cells enter the lung only in the capillary bed. In bronchial circulation, inflammatory cell passage into the tissue occurs in the postcapillary venules [19]. We know little about the relative importance of these two systems in the inflammatory cell emigration of our murine model. Morphologically on a lung section, it is hard to determine if an ICAM-1-positive blood vessel belongs to the pulmonary or the bronchial circulation. We found that some inflammatory cells were distributed around ICAM-1-positive venules adjacent to bronchioles, which may suggest these cells emigrated from the venules and indirectly support the bronchial circulation origin of these venules. Increased ICAM-1 expression appeared more important in the alveolar walls than in venules; it probably indicates that in inflamed lung, alveolar capillaries are the main site where inflammatory cells enter the interstitium and air spaces.

LFA-1, the ligand of ICAM-1, belongs to the family of β-2 integrins. It is expressed to a certain extent by most types of leucocyte [18]. A significant increase of LFA-1$^+$ cells was found in Af-treated mouse lung. The association of ICAM-1 and LFA-1 was emphasized by the significant correlation we found between the expression of these two adhesion molecules in the mice of both Af and Sa groups analysed together, though such a correlation could not be observed in the Af group analysed alone. The fact that in Af-treated mice ICAM-1 expression correlated well with lung tissue T cell count suggests that, in our murine model, this adhesion molecule is involved more specifically in T cell emigration from the microvessels and migration or retention within the lung. Increased T cell infiltration could play an important role in the development of ABPA, as cytokines IL-5 and IL-4, derived from the Th2 subset of CD4$^+$ cells, have been shown to be directly responsible for eosinophilia and IgE production [20–23].

Montefort *et al.* [11] showed that, in patients with perennial allergic rhinitis, correlation did not exist between ICAM-1 expression and eosinophil count. We also failed to demonstrate a significant correlation between the number of eosinophils and ICAM-1 expression in this study. This finding suggests that, in our murine model, ICAM-1 does not play a direct role in the accumulation of eosinophils. Instead, it may be an important modulator for the recruitment of T cells or other types of leucocytes such as macrophages and neutrophils.

There are several mechanisms contributing to the

recruitment of eosinophils to the inflammatory sites. Besides ICAM-1, eosinophils can also adhere to vascular endothelial cells by binding to vascular cell adhesion molecule-1 (VCAM-1), which is found mainly on vascular endothelial cells. It is inducible upon exposure to IL-1 and TNF-α, but not to IFN-γ. Its receptor on leucocytes is the β-1 integrin, very late antigen-4 (VLA-4). Lymphocytes, monocytes and eosinophils have been shown to bind to VCAM-1 [4]. Eosinophils can express VLA-4, and a MoAb against this integrin has been shown to specifically inhibit eosinophil but not neutrophil adhesion to IL-1-stimulated human umbilical vein endothelial cells [24]. We may speculate that VLA-4–VCAM-1 interactions are more important than ICAM-1–LFA-1 ones in the recruitment of eosinophils into allergic inflammatory lesions.

Furthermore, the migration of eosinophils depends on their responses to a number of chemoattractants, which comprise humoral mediators such as C5a, PAF, and cytokines IL-2, IL-3, IL-5, GM-CSF, Rantes, and so on [4]. Although many investigators seek explanations for eosinophil predominance at the sites of allergic inflammation, no single agent, at present, is known to be fully responsible for the preferential accumulation of eosinophils, and an interplay of multiple mechanisms or pathways seems to be essential to the preferential mobilization of any cell type, including eosinophils, into tissue sites.

In conclusion, our findings indicate up-regulation of ICAM-1 in the murine ABPA model, and this up-regulation may be involved in the recruitment of T lymphocytes rather than eosinophils in the pathogenesis of this disease.

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