

Role of Vascular Cell Adhesion Molecule 1/Very Late Activation Antigen 4 and Intercellular Adhesion Molecule 1/Lymphocyte Function-associated Antigen 1 Interactions in Antigen-induced Eosinophil and T Cell Recruitment into the Tissue

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Summary

To determine the role of vascular cell adhesion molecule 1 (VCAM-1)/very late activation antigen 4 (VLA-4) and intercellular adhesion molecule 1 (ICAM-1)/lymphocyte function-associated antigen 1 (LFA-1) interactions in causing antigen-induced eosinophil and T cell recruitment into the tissue, we studied the effect of the *in vivo* blocking of VCAM-1, ICAM-1, VLA-4, and LFA-1 by pretreatment with monoclonal antibodies (mAb) to these four adhesion molecules on the eosinophil and T cell infiltration of the trachea induced by antigen inhalation in mice. The *in vivo* blocking of VCAM-1 and VLA-4, but not of ICAM-1 and LFA-1, prevented antigen-induced eosinophil infiltration into the mouse trachea. On the contrary, the *in vivo* blocking of VCAM-1 and VLA-4, but not of ICAM-1 and LFA-1, increased blood eosinophil counts after antigen challenge, but did not affect blood eosinophil counts without antigen challenge in sensitized mice. Furthermore, the expression of VCAM-1 but not ICAM-1 was strongly induced on the endothelium of the trachea after antigen challenge. In addition, pretreatment with anti-IL-4 mAb decreased the antigen-induced VCAM-1 expression only by 27% and had no significant effect on antigen-induced eosinophil infiltration into the trachea. The *in vivo* blocking of VCAM-1 and VLA-4 inhibited antigen-induced CD4⁺ and CD8⁺ T cell infiltration into the trachea more potently than that of ICAM-1 and LFA-1. In contrast, regardless of antigen challenge, the *in vivo* blocking of LFA-1, but not of ICAM-1, increased blood lymphocyte counts more than that of VCAM-1 and VLA-4. These results indicate that VCAM-1/VLA-4 interaction plays a predominant role in controlling antigen-induced eosinophil and T cell recruitment into the tissue and that the induction of VCAM-1 expression on the endothelium at the site of allergic inflammation regulates this eosinophil and T cell recruitment.

Eosinophil and T cell infiltration into the tissue is a characteristic feature of allergic inflammation such as asthma. Increasing evidence suggests that the migration of leukocytes into the tissue is controlled by the interaction of cell-surface adhesion molecules between leukocytes and vascular endothelial cells (1). *In vitro* studies have shown that the adhesion of eosinophils to vascular endothelial cells and its transendothelial migration are mediated by the interactions of intercellular adhesion molecule 1 (ICAM-1)¹ and lymphocyte function-associated antigen 1 (LFA-1) (2–4) and of vascular cell adhesion

molecule 1 (VCAM-1) and very late activation antigen 4 (VLA-4) (5–9). The adherence of T cells to cultured endothelial cells and its transendothelial migration are also mediated by ICAM-1/LFA-1 (10–14) and VCAM-1/LFA-4 (15–17) interactions. In addition, it has been reported that the transendothelial migration of T cells *in vitro* is dependent on ICAM-1 but not VCAM-1, whereas the adhesion of T cells to cultured endothelial cells depends on both ICAM-1 and VCAM-1 (18, 19). Furthermore, it has been shown that inflammatory cytokines such as TNF and IL-1 induce ICAM-1 and VCAM-1 expressions on cultured endothelial cells (20–23) and that the adhesion of eosinophils and T cells to cultured endothelial cells is also increased by the stimulation of the endothelial cells with these cytokines (2, 5–17, 24–26). IL-4 has also recently been shown to selectively induce VCAM-1

¹ Abbreviations used in this paper: ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late activation antigen 4.

but not ICAM-1 expression on cultured endothelial cells (23) and increase the adherence of eosinophils and T cells to the endothelial cells in vitro (27–29). However, the in vivo functional importance of ICAM-1/LFA-1 and VCAM-1/VLA-4 interactions has not yet been clarified in causing antigen-induced eosinophil and T cell recruitment into the tissue. Therefore, to elucidate this issue, we studied the effect of the in vivo blocking of ICAM-1, VCAM-1, LFA-1, and VLA-4 using mAbs to these four adhesion molecules on antigen-induced eosinophil and T cell recruitment into the mouse airways. We also studied the expression of ICAM-1 and VCAM-1 after antigen inhalation in the airways of sensitized mice to determine the regulatory role of ICAM-1 and VCAM-1 expressions at the site of antigen challenge in causing the eosinophil and T cell recruitment into the tissue. Our results indicate that VCAM-1/VLA-4 interaction plays a predominant role in controlling antigen-induced eosinophil and T cell recruitment into the tissue and that the induction of VCAM-1 expression on the endothelium at the allergic inflammatory site regulates this eosinophil and T cell recruitment.

Materials and Methods

Mice and Immunization. Female BALB/c mice (age 8 wk) (Charles River Laboratories, Atsugi, Japan) were immunized intraperitoneally twice with 1 μ g of OVA (Sigma Chemical Co., St. Louis, MO) in 4 mg of aluminium hydroxide at a 2-wk interval. 10–14 d after the second immunization, the sensitized mice were challenged with aerosolized OVA as described below.

mAbs. Rat anti-murine VCAM-1 mAb M/K-1 (IgG2b) (30), rat anti-murine VLA-4 mAb PS/2 (IgG2b) (31), rat anti-murine ICAM-1 mAb YN1/1.7 (IgG2b) (32, 33), rat anti-murine LFA-1 mAb KBA (IgG2a) (34), and rat anti-murine IL-4 mAb 11B11 (IgG1) (35) were used in this study. The rat mAb were purified from ascites produced from the hybridomas with the use of a Protein G-Sepharose 4FF affinity column (Pharmacia LKB, Uppsala, Sweden), and all mAbs were used as purified IgG. The mAbs contained a negligible amount of endotoxin (<1 pg/mg) as determined by the Limulus amoebocyte lysate assay (Seikagaku Kogyo Co., Tokyo, Japan).

Antigen-induced Eosinophil Infiltration in Mouse Trachea. The eosinophil infiltration into the trachea was induced by the inhalation of antigen in sensitized mice, and the number of eosinophils infiltrating into the submucosal tissue of trachea was evaluated as described previously (36). Briefly, the sensitized mice were individually placed in 50-ml plastic tubes and inhaled with aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a nebulizer (model 646; DeVilbiss Co., Somerset, PA) for 20 min. As a control, 0.9% saline alone was administered by the nebulizer. At various intervals after the inhalation, the mice were killed by cervical dislocation and the tracheas were excised. After the tracheas were fixed in 10% formalin, the specimens were embedded in paraffin, sectioned in 3 μ m thick segments, and stained with Luna solution and hematoxylin-eosin solution. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per length of the basement membrane of trachea, which was measured with a digital curvimeter. The eosinophil infiltration into the trachea of sensitized mice began at 6 h after antigen inhalation and reached a peak at 24 h (36).

Antigen-induced T Cell Infiltration in the Trachea. T cell infiltra-

tion into the trachea was assessed by direct staining with streptavidin-biotinylated antibody technique as described previously (37). Briefly, the trachea was removed and frozen with OCT compound (Miles Laboratories, Naperville, IL) in a liquid nitrogen bath. After acetone-fixed cryostat sections (3 μ m thick) were treated with normal rabbit serum, the sections were incubated with biotinylated anti-Thy 1.2, anti-L3T4, or anti-Lyt-2 mAb (Becton Dickinson & Co., Mountain View, CA) at room temperature for 2 h. As a negative control, biotinylated normal rat IgG was used. Sections were then incubated with the streptavidin conjugated with horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 min, followed by the reaction with 3',3'-diaminobenzidine in Tris-HCl buffer containing H₂O₂ for 5 min. The immunostained cells were counted and expressed as described above. CD4⁺ and CD8⁺ T cell infiltration into the trachea occurred 2 h after inhalation and reach a peak at 12–24 h (37).

Effect of Anti-VCAM-1 and Anti-VLA-4 mAbs. To determine whether VCAM-1 on the endothelial cells is involved in antigen-induced eosinophil and T cell recruitment into the tissue, we examined the effect of the in vivo administration of anti-VCAM-1 mAb on antigen-induced eosinophil and T cell infiltration in the trachea of sensitized mice. OVA-sensitized mice were injected intraperitoneally with 1 mg of rat anti-murine VCAM-1 mAb (M/K-1) (30) 24 h before the inhaled OVA challenge. As a control, OVA-sensitized mice were injected intraperitoneally with purified rat IgG (1 mg) (Jackson ImmunoResearch Laboratories, West Grove, PA) 24 h before the inhaled OVA challenge. The eosinophil and T cell infiltration into the trachea was evaluated at 24 h after OVA inhalation.

Since VLA-4 is a counter-receptor for VCAM-1 (16) and is expressed on eosinophils and lymphocytes but not on neutrophils (5–8, 38), we also examined the effect of anti-VLA-4 mAb on antigen-induced eosinophil and T cell infiltration in the trachea of sensitized mice. Rat anti-murine VLA-4 mAb (PS/2) (1 mg) (31) or purified rat IgG (1 mg) was injected intraperitoneally 24 h before the inhaled OVA challenge. The eosinophil and T cell infiltration into the trachea was evaluated at 24 h after OVA inhalation.

Effect of Anti-ICAM-1 and Anti-LFA-1 mAbs. To determine whether ICAM-1 on the endothelial cells is involved in antigen-induced eosinophil and T cell recruitment into the tissue, we examined the effect of the in vivo administration of anti-ICAM-1 mAb on antigen-induced eosinophil and T cell infiltration in the trachea of sensitized mice. We also examined the effect of mAb to LFA-1, which is a ligand for ICAM-1 (39), on the antigen-induced eosinophil and T cell infiltration. OVA-sensitized mice were injected intraperitoneally with rat anti-murine ICAM-1 mAb (YN1/1.7) (1 mg) (32, 33), rat anti-murine LFA-1 mAb (KBA) (1 mg) (34), or purified rat IgG (1 mg) 24 h before the inhaled OVA challenge. The eosinophil and T cell infiltration into the trachea was evaluated at 24 h after OVA inhalation.

VCAM-1 and ICAM-1 Expression in the Trachea. The expression of VCAM-1 and ICAM-1 on the endothelium of the trachea was assessed at 24 h after OVA inhalation by the immunocytochemical staining using optimally diluted biotinylated anti-VCAM-1 mAb (M/K-1), anti-ICAM-1 mAb (YN1/1.7), and rat IgG (negative control) as described above. The intensity of staining was classified according to the criteria as described by Briscoe et al. (40). The VCAM-1 and ICAM-1 expression on the endothelium was evaluated as score 0 (absent staining or faint staining of an occasional vessel only), 1+ (faint staining of several vessels), 2+ (moderate intensity staining of most vessels), and 3+ (intense staining of most vessels). The sections were coded and then examined by two observers in a blind manner, and the average of the two determina-

tions for each section was used for subsequent calculations of VCAM-1 or ICAM-1 expression.

Because IL-4 has been shown to upregulate the expression of VCAM-1 but not ICAM-1 on cultured endothelial cells (23) and thereby enhance eosinophil migration *in vitro* (27, 28), we also examined the effect of anti-IL-4 mAb on antigen-induced VCAM-1 expression and eosinophil infiltration in the trachea of sensitized mice. OVA-sensitized mice were injected intraperitoneally with anti-murine IL-4 mAb (11B11) (5 mg) (35) or purified rat IgG (5 mg) 24 h before the inhaled OVA challenge. In preliminary experiments, greater than 2 mg of anti-IL-4 mAb completely prevented anti-OVA IgE production in BALB/c mice assessed by passive cutaneous anaphylaxis reaction. The VCAM-1 expression and eosinophil infiltration in the trachea was evaluated at 24 h after OVA inhalation. In addition, a significant amount of anti-IL-4 mAb was detectable (600–710 $\mu\text{g/ml}$), which was assessed by an ELISA using anti-rat IgG, in the mouse serum at the time of OVA inhalation.

Data Analysis. Data are summarized as mean \pm SD. The statistical analysis of the results was performed by the analysis of variance using Fisher's least significant difference test for multiple comparisons. $p < 0.05$ was considered significant.

Results

Effect of In Vivo Blocking of VCAM-1 and VLA-4 on Antigen-induced Eosinophil Infiltration into the Mouse Trachea. The *in vivo* blocking of VCAM-1 by pretreatment with anti-VCAM-1 mAb decreased the eosinophil infiltration induced by antigen inhalation in the trachea of sensitized mice. The intraperitoneal preinjection with anti-VCAM-1 mAb (M/K-1; 1 mg) 24 h before the inhaled OVA challenge significantly decreased OVA-induced eosinophil infiltration into the trachea of OVA-sensitized mice at 24 h by 73% (control rat IgG 21.4 ± 3.2 vs. anti-VCAM-1 mAb 5.7 ± 1.2 eosinophils/mm, mean \pm SD, $n = 10$ mice in each group, $p < 0.001$) (Fig. 1). In addition, the eosinophil infiltration into the trachea of OVA-sensitized mice was negligible at 24 h after saline inhalation (0.21 ± 0.16 eosinophils/mm, $n = 10$).

The *in vivo* blocking of VLA-4, a ligand of VCAM-1, by pretreatment with anti-VLA-4 mAb also decreased antigen-induced eosinophil infiltration in the mouse trachea. The intraperitoneal preinjection with anti-VLA-4 mAb (PS/2; 1 mg) 24 h before the inhaled OVA challenge significantly decreased OVA-induced eosinophil infiltration into the trachea at 24 h by 74% ($n = 10$, $p < 0.001$) (Fig. 1). The preventive

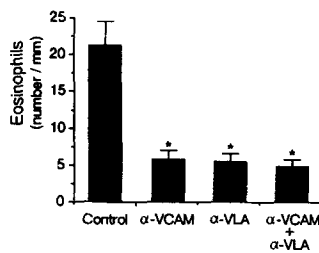


Figure 1. Effect of *in vivo* blocking of VCAM-1 and VLA-4 on antigen-induced eosinophil infiltration in mouse trachea. OVA-sensitized BALB/c mice were injected intraperitoneally with anti-murine VCAM-1 mAb (M/K-1), anti-murine VLA-4 mAb (PS/2), or rat IgG 24 h before inhaled OVA challenge, and the number of eosinophils infiltrating into the submucosal

tissue of trachea was then counted at 24 h after OVA inhalation. Data are means \pm SD for 10 mice in each group. *Significantly different from the mean value of the control response (rat IgG), $p < 0.001$.

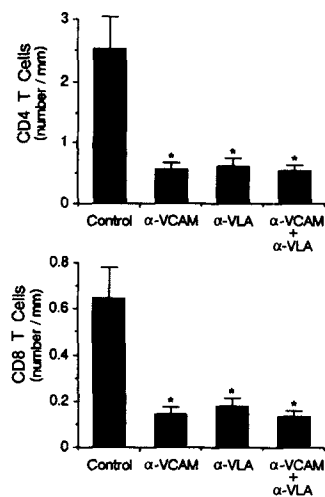


Figure 2. Effect of *in vivo* blocking of VCAM-1 and VLA-4 on antigen-induced T cell infiltration in mouse trachea. CD4⁺ (top) and CD8⁺ (bottom) T cell infiltration in the trachea was examined at 24 h. Experimental protocols are the same in Fig. 1. Data are means \pm SD for 10 mice in each group. *Significantly different from the mean value of the control response (rat IgG), $p < 0.001$.

effect of combination of anti-VCAM-1 mAb (M/K-1; 1 mg) and anti-VLA-4 mAb (PS/2; 1 mg) was at a similar degree of decrement when pretreated with anti-VCAM-1 mAb or anti-VLA-4 mAb alone (Fig. 1).

Effect of In Vivo Blocking of VCAM-1 and VLA-4 on Antigen-induced T Cell Infiltration into the Trachea. The *in vivo* blocking of VCAM-1 decreased antigen-induced T cell infiltration (both CD4⁺ T cells and CD8⁺ T cells) in the mouse trachea. The preinjection with anti-VCAM-1 mAb significantly decreased OVA-induced CD4⁺ T cell infiltration into the trachea at 24 h by 78% (control rat IgG 2.51 ± 0.53 vs. anti-VCAM-1 mAb 0.56 ± 0.11 CD4⁺ T cells/mm, $n = 10$, $p < 0.001$) (Fig. 2). The preinjection with anti-VCAM-1 mAb also significantly decreased OVA-induced CD8⁺ T cell infiltration into the trachea at 24 h by 78% (control rat IgG 0.65 ± 0.13 vs. anti-VCAM-1 mAb 0.15 ± 0.03 CD8⁺ T cells/mm, $n = 10$, $p < 0.001$) (Fig. 2).

The *in vivo* blocking of VLA-4 also decreased antigen-induced T cell infiltration in the trachea. The preinjection with anti-VLA-4 mAb significantly decreased OVA-induced CD4⁺ T cell infiltration into the trachea by 24 h by 75% ($n = 10$, $p < 0.001$) (Fig. 2). The preinjection with anti-VLA-4 mAb also significantly decreased OVA-induced CD8⁺ T cell infiltration into the trachea at 24 h by 72% ($n = 10$, $p < 0.001$) (Fig. 2). The combined effect of anti-VCAM-1 mAb and anti-VLA-4 mAb was at a similar degree of decrement when pretreated with anti-VCAM-1 mAb or anti-VLA-4 mAb alone (Fig. 2).

Effect of In Vivo Blocking of ICAM-1 and LFA-1 on Antigen-induced Eosinophil Infiltration into the Trachea. In contrast, the *in vivo* blocking of ICAM-1 by pretreatment with anti-ICAM-1 mAb (YN1/1.7; 1 mg) had no significant effect on antigen-induced eosinophil infiltration in the trachea of sensitized mice at 24 h ($n = 10$) (Fig. 3). The *in vivo* blocking of LFA-1, a ligand of ICAM-1, by pretreatment with anti-LFA-1 mAb (KBA; 1 mg) did not significantly decrease antigen-induced eosinophil infiltration into the trachea at 24 h, either ($n = 10$) (Fig. 3).

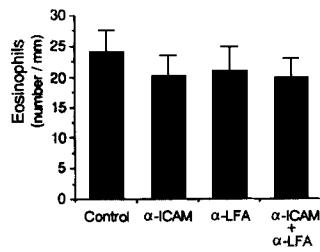


Figure 3. Effect of in vivo blocking of ICAM-1 and LFA-1 on antigen-induced eosinophil infiltration in mouse trachea. OVA-sensitized mice were injected intraperitoneally with anti-murine ICAM-1 mAb (YN1/1.7), anti-murine LFA-1 mAb (KBA), or rat IgG 24 h before OVA inhalation, and the eosinophil infiltration in the trachea was examined at 24 h. Data are means \pm SD for 10 mice in each group.

Effect of In Vivo Blocking of ICAM-1 and LFA-1 on Antigen-induced T Cell Infiltration into the Trachea. The in vivo blocking of ICAM-1 decreased antigen-induced T cell infiltration in the mouse trachea. The preinjection with anti-ICAM-1 mAb significantly decreased OVA-induced CD4⁺ T cell infiltration into the trachea at 24 h by 44% (control rat IgG 2.45 \pm 0.54 vs. anti-ICAM-1 mAb 1.38 \pm 0.25 CD4⁺ T cells/mm, $n = 10$, $p < 0.001$) (Fig. 4). The preinjection with anti-ICAM-1 mAb also significantly decreased OVA-induced CD8⁺ T cell infiltration into the trachea at 24 h by 51% (control rat IgG 0.66 \pm 0.19 vs. anti-ICAM-1 mAb 0.32 \pm 0.05 CD8⁺ T cells/mm, $n = 10$, $p < 0.001$) (Fig. 4).

The in vivo blocking of LFA-1 also decreased antigen-induced T cell infiltration in the trachea. The preinjection with anti-LFA-1 mAb significantly decreased OVA-induced CD4⁺ T cell infiltration into the trachea at 24 h by 50% ($n = 10$, $p < 0.001$) (Fig. 4). The preinjection with anti-LFA-1 mAb also significantly decreased OVA-induced CD8⁺ T cell infiltration into the trachea at 24 h by 50% ($n = 10$, $p < 0.001$) (Fig. 4). The combination of anti-ICAM-1 mAb and anti-LFA-1 mAb decreased OVA-induced CD4⁺ and CD8⁺ T cell infiltration into the trachea at 24 h by 62 and 65%, respectively, but these degrees of decrement were not significantly different from that of anti-ICAM-1 mAb or anti-LFA-1 mAb alone (Fig. 4).

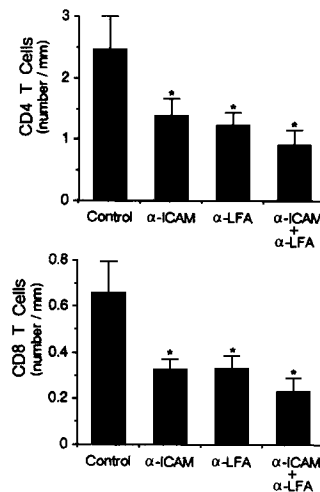


Figure 4. Effect of in vivo blocking of ICAM-1 and LFA-1 on antigen-induced T cell infiltration in mouse trachea. CD4⁺ (top) and CD8⁺ (bottom) T cell infiltration in the trachea was examined at 24 h. Experimental protocols are the same in Fig. 3. Data are means \pm SD for 10 mice in each group. *Significantly different from the mean value of the control response (rat IgG), $p < 0.001$.

Dose-response Curves of mAbs to VCAM-1, ICAM-1, VLA-4, and LFA-1 in Antigen-induced Eosinophil and T Cell Infiltration into the Trachea. To determine whether the amounts (1 mg, each) of mAb to the four adhesion molecules are sufficient to inhibit antigen-induced eosinophil infiltration in mouse trachea, we examined dose-response curves of these mAb (0.5–4 mg), which were administered intraperitoneally 24 h before the inhaled antigen challenge, in antigen-induced eosinophil infiltration in the trachea of sensitized mice. We also examined dose-response curves of mAbs to ICAM-1 and LFA-1 in antigen-induced T cell infiltration in the trachea. The inhibitory effects of these four mAb on the antigen-induced eosinophil and CD4⁺ T cell infiltration at 24 h were not significantly different at 0.5, 1, and 4 mg of mAbs tested ($n = 5$ mice in each group) (Fig. 5).

Effect of Combined Blocking of Both VCAM-1 and ICAM-1 on Antigen-induced Eosinophil and T Cell Infiltration into the Trachea. There was no significant additive effect of in vivo blocking of both VCAM-1 and ICAM-1 on antigen-induced eosinophil infiltration into the trachea. The decrement of antigen-induced eosinophil infiltration at 24 h by pretreatment with both anti-VCAM-1 mAb (M/K-1; 1 mg) and anti-ICAM-1 mAb (YN1/1.7; 1 mg) was similar to that of anti-VCAM-1 mAb alone ($n = 10$, each) (Fig. 6).

However, the combination of anti-VLA-4 mAb (PS/2; 1 mg) and anti-LFA-1 mAb (KBA; 1 mg) more potently prevented antigen-induced eosinophil infiltration at 24 h than anti-VLA-4 mAb alone (control rat IgG 20.1 \pm 3.2 vs. anti-VLA-4 mAb 5.3 \pm 1.1 vs. combination of anti-VLA-4 mAb and anti-LFA-1 mAb 0.7 \pm 0.3 eosinophils/mm, $n = 10$, $p < 0.001$) (Fig. 6), although anti-LFA-1 mAb had no significant effect (Figs. 3 and 6).

In contrast to antigen-induced eosinophil infiltration, the combination of anti-VCAM-1 mAb and anti-ICAM-1 mAb inhibited antigen-induced CD4⁺ and CD8⁺ T cell infiltration at 24 h more potently than anti-VCAM-1 mAb alone ($n = 8$, each, $p < 0.01$) (Fig. 7). The combination of anti-VLA-4 mAb and anti-LFA-1 mAb also decreased antigen-induced CD4⁺ and CD8⁺ T cell infiltration at 24 h more

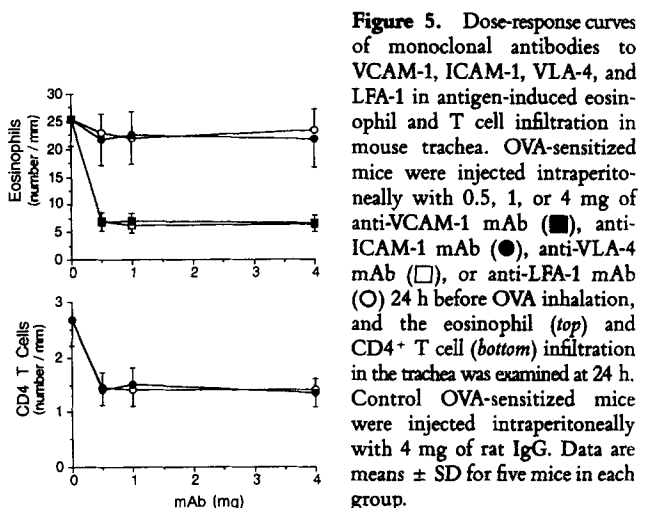


Figure 5. Dose-response curves of monoclonal antibodies to VCAM-1, ICAM-1, VLA-4, and LFA-1 in antigen-induced eosinophil and T cell infiltration in mouse trachea. OVA-sensitized mice were injected intraperitoneally with 0.5, 1, or 4 mg of anti-VCAM-1 mAb (■), anti-ICAM-1 mAb (●), anti-VLA-4 mAb (□), or anti-LFA-1 mAb (○) 24 h before OVA inhalation, and the eosinophil (top) and CD4⁺ T cell (bottom) infiltration in the trachea was examined at 24 h. Control OVA-sensitized mice were injected intraperitoneally with 4 mg of rat IgG. Data are means \pm SD for five mice in each group.

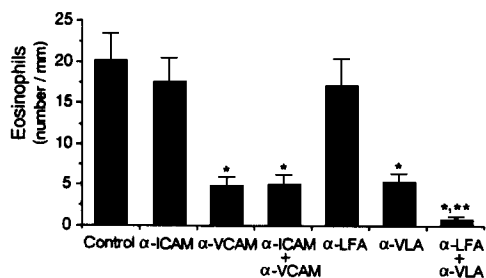


Figure 6. Effect of combined blocking of both VCAM-1 and ICAM-1 on antigen-induced eosinophil infiltration in mouse trachea. OVA-sensitized mice were injected intraperitoneally with anti-VCAM-1 mAb, anti-ICAM-1 mAb, the combination of anti-VCAM-1 mAb and anti-ICAM-1 mAb, anti-VLA-4 mAb, anti-LFA-1 mAb, the combination of anti-VLA-4 mAb and anti-LFA-1 mAb, or rat IgG 24 h before OVA inhalation, and the eosinophil infiltration in the trachea was examined at 24 h. Data are means \pm SD for 10 mice in each group. *Significantly different from the mean value of the control response (rat IgG), $p < 0.001$. **Significantly different from the mean value of the response by anti-VLA-4 mAb pretreatment, $p < 0.001$.

potently than anti-VLA-4 mAb alone ($n = 8$, each, $p < 0.01$) (Fig. 7).

Effect of In Vivo Blocking of VCAM-1/VLA-4 and ICAM-1/LFA-1 on Blood Leukocyte Counts. The in vivo blocking of VCAM-1 and VLA-4, but not of ICAM-1 and LFA-1, increased blood eosinophil counts after antigen challenge in sensitized mice. The preinjection with anti-VCAM-1 mAb and anti-VLA-4 mAb significantly increased blood eosinophil counts at 24 h after OVA inhalation by 211 and 186%, respectively, (control rat IgG 94 ± 26 vs. anti-VCAM-1 mAb 292 ± 63 vs. anti-VLA-4 mAb 269 ± 76 eosinophils/mm³,

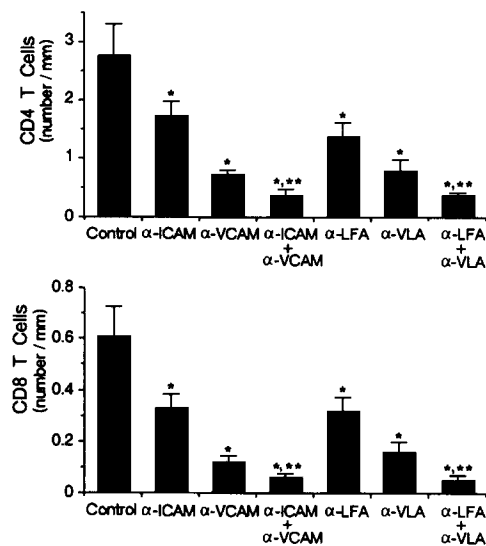


Figure 7. Effect of combined blocking of both VCAM-1 and ICAM-1 on antigen-induced T cell infiltration in mouse trachea. CD4⁺ (top) and CD8⁺ (bottom) T cell infiltration in the trachea was examined at 24 h. Experimental protocols are the same in Fig. 6. Data are means \pm SD for eight mice in each group. *Significantly different from the mean value of the control response (rat IgG), $p < 0.001$. **Significantly different from the mean value of the response by anti-VCAM-1 mAb or anti-VLA-4 mAb pretreatment, $p < 0.01$.

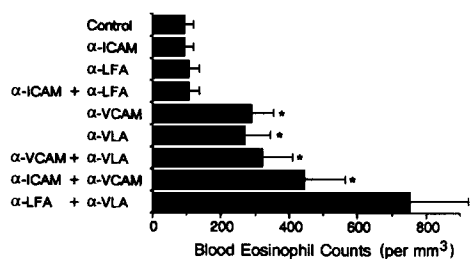


Figure 8. Effect of in vivo blocking of VCAM-1/VLA-4 and ICAM-1/LFA-1 on blood eosinophil counts in mice. OVA-sensitized mice were injected intraperitoneally with anti-VCAM-1 mAb, anti-VLA-4 mAb, anti-ICAM-1 mAb, anti-LFA-1 mAb, the combinations of these mAb, or rat IgG 24 h before OVA inhalation, and blood eosinophil counts were examined at 24 h after OVA inhalation. Data are means \pm SD for 10 mice in each group. *Significantly different from the mean value of the control response (rat IgG), $p < 0.001$.

($n = 10$, $p < 0.001$), whereas the in vivo blocking of ICAM-1 or LFA-1 did not significantly affect blood eosinophil counts ($n = 10$, each) (Fig. 8). Furthermore, the combined blocking of VCAM-1 and ICAM-1 increased blood eosinophil counts more potently than that of VCAM-1 alone (combination of anti-VCAM-1 and anti-ICAM-1 444 ± 122 eosinophils/mm³, $n = 10$, $p < 0.01$) (Fig. 8). The combined blocking of VLA-4 and LFA-1 also increased blood eosinophil counts more potently than that of VLA-4 alone (combination of anti-VLA-4 and anti-LFA-1 752 ± 171 eosinophils/mm³, $n = 10$, $p < 0.001$) (Fig. 8). In contrast, the in vivo blocking of VCAM-1 and VLA-4 did not significantly increase blood eosinophil counts after the inhalation of saline in sensitized mice (data not shown). The combined blocking of VCAM-1 and ICAM-1 and of VLA-4 and LFA-1 had no effect on blood eosinophil counts after saline inhalation in sensitized mice, either (data not shown).

The in vivo blocking of LFA-1 increased blood lymphocyte counts at 24 h after antigen challenge in sensitized mice by 99% (control rat IgG 3303 ± 481 vs. anti-LFA-1 mAb 6559 ± 664 lymphocytes/mm³, $n = 10$, $p < 0.001$), whereas the in vivo blocking of ICAM-1 did not significantly increase blood lymphocyte counts (Fig. 9). The in vivo blocking of

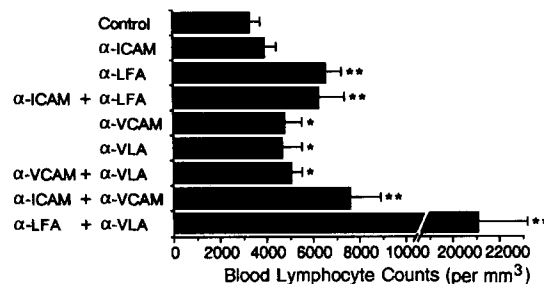


Figure 9. Effect of in vivo blocking of VCAM-1/VLA-4 and ICAM-1/LFA-1 on blood lymphocyte counts in mice. Experimental protocols are the same in Fig. 8, and blood lymphocyte counts were examined at 24 h after OVA inhalation. Data are means \pm SD for 10 mice in each group. *, **Significantly different from the mean value of the control response (rat IgG), * $p < 0.01$, ** $p < 0.001$.

VCAM-1 and VLA-4 also increased blood lymphocyte counts by 45 and 41%, respectively ($n = 10$, each, $p < 0.01$) (Fig. 9). The combined blocking of VCAM-1 and ICAM-1 and of VLA-4 and LFA-1 also increased blood lymphocyte counts by 129 and 538%, respectively ($n = 10$, each, $p < 0.001$) (Fig. 9). In addition, these increases in blood lymphocyte counts were similarly observed after the inhalation of saline in sensitized mice (data not shown).

Antigen-induced VCAM-1 and ICAM-1 Expression on the Endothelium of the Trachea. ICAM-1 was expressed moderately and universally on the endothelium in the trachea of sensitized mice before the inhaled OVA challenge, and ICAM-1 expression on the endothelium was not significantly increased at 24 h after the inhaled OVA challenge (ICAM-1 score; saline inhalation 2.26 ± 0.28 vs. inhaled OVA challenge 2.66 ± 0.39 , $n = 5$) (Fig. 10). In contrast, VCAM-1 was not significantly expressed on the endothelium in the trachea before antigen challenge. However, VCAM-1 expression on the endothelium was strongly increased at 24 h after antigen challenge (VCAM-1 score; saline inhalation 0.20 ± 0.18 vs. inhaled OVA challenge 1.73 ± 0.27 , $n = 5$, $p < 0.001$) (Fig. 10).

Pretreatment with anti-IL-4 mAb (11B11; 5 mg) decreased antigen-induced VCAM-1 expression on the endothelium only by 27% ($n = 5$, $p < 0.02$) (Fig. 10). In contrast, anti-IL-4 mAb did not significantly affect ICAM-1 expression on the endothelium (Fig. 10). The pretreatment with anti-IL-4 mAb did not significantly affect antigen-induced eosinophil infiltration in the trachea, either ($n = 10$, data not shown).

Discussion

In this study, we show that VCAM-1/VLA-4 interaction plays a predominant role in controlling antigen-induced eosinophil recruitment into the tissue. We found that the *in vivo* blocking of VCAM-1 and VLA-4, but not of ICAM-1 and LFA-1, prevented antigen-induced eosinophil infiltration into the mouse trachea (Figs. 1 and 3). We also found that, on the contrary, the *in vivo* blocking of VCAM-1 and VLA-4, but not of ICAM-1 and LFA-1, increased blood eosinophil

counts at 24 h after antigen challenge (Fig. 8). In addition, the *in vivo* blocking of VCAM-1 and VLA-4, however, did not increase blood eosinophil counts without inhaled antigen challenge in sensitized mice. Furthermore, the expression of VCAM-1 but not ICAM-1 was found to be strongly induced on the endothelium of the trachea after inhaled antigen challenge (Fig. 10). Taken together, these results indicate that VCAM-1/VLA-4 interaction mediates antigen-induced eosinophil recruitment into the tissue and that the induction of VCAM-1 expression on the endothelium at the allergic inflammatory site regulates this eosinophil recruitment.

In vitro studies previously showed that both ICAM-1/LFA-1 (2–4) and VCAM-1/VLA-4 (5–9) interactions were involved in the adhesion of eosinophils to vascular endothelial cells and its transendothelial migration. It was also shown that there was an additive effect of CD11/CD18-dependent pathway and VCAM-1-dependent pathway on eosinophil adherence to cultured endothelial cells (5, 9). However, the *in vivo* importance of these adhesion molecules has not been evaluated in antigen-induced eosinophil recruitment into the tissue. Our present study shows that ICAM-1/LFA-1 interaction is not significantly involved in causing antigen-induced eosinophil recruitment into the tissue (Fig. 3). Wegner et al. (41) demonstrated that pretreatment with anti-ICAM-1 mAb prevented eosinophil migration into the bronchoalveolar lavage fluid in monkeys. Their finding is in disagreement with our present observation and might be due to the interference of epithelial ICAM-1 and subsequent inhibition of eosinophil migration from the mucosal side of airways to the luminal side because ICAM-1 is expressed on airway epithelium as well as endothelium (41). However, our findings that the combined blocking of VCAM-1 and ICAM-1 and of VLA-4 and LFA-1 had greater effects on blood eosinophil counts than that of VCAM-1 or VLA-4 alone (Fig. 8) suggest that ICAM-1/LFA-1 interaction might exert a minor role in antigen-induced eosinophil recruitment into the tissue when VCAM-1/VLA-4 interaction is blocked. Furthermore, the finding that the combined blocking of VLA-4 and LFA-1 increased blood eosinophil counts greater than that of VCAM-1 and ICAM-1 (Fig. 8) also suggests a possible involvement of LFA-1/ICAM-2 interaction in the antigen-induced eosinophil recruitment because ICAM-2 has been shown to be another ligand for LFA-1 (42, 43).

We demonstrate that antigen inhalation induces VCAM-1 expression on the endothelium at a site of antigen challenge in sensitized mice (Fig. 10), indicating that antigen-induced VCAM-1 expression on endothelial cells is an important regulatory step of antigen-induced eosinophil migration into the tissue. It has been shown that inflammatory cytokines such as TNF and IL-1 induce VCAM-1 expression on cultured endothelial cells *in vitro* (22, 23) and on the endothelium in the skin *in vivo* (40) and that the adhesion of eosinophils to cultured endothelial cells is also increased by the stimulation of the endothelial cells with these cytokines (5–9). Furthermore, IL-4 has recently been shown to selectively induce VCAM-1 but not ICAM-1 expression on cultured endothelial cells (23) and increase eosinophil adherence to the endothelial

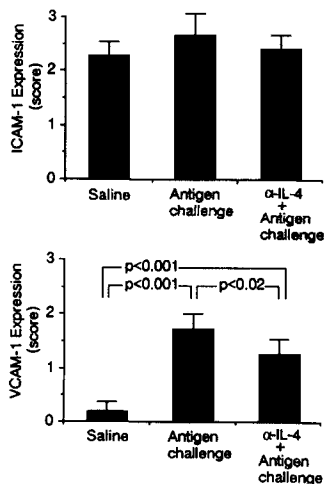


Figure 10. Antigen-induced VCAM-1 and ICAM-1 expression on the endothelium of mouse trachea. The expression of VCAM-1 and ICAM-1 on the endothelium of the trachea was assessed at 24 h after OVA or saline inhalation by the immunocytochemical staining using biotinylated anti-VCAM-1 mAb and anti-ICAM-1 mAb. The intensity of staining was then scored according to the criteria as described in Materials and Methods. A group of sensitized mice was also injected intraperitoneally with anti-murine IL-4 mAb (11B11) 24 h before the inhaled OVA challenge. Data are means \pm SD for five mice in each group.

cells *in vitro* (27, 28). We therefore investigated the role of IL-4 in antigen-induced VCAM-1 expression in the trachea. We found that pretreatment with anti-IL-4 mAb decreased the antigen-induced VCAM-1 expression only by 27% (Fig. 10). In addition, pretreatment of anti-IL-4 mAb had no significant effect on antigen-induced eosinophil infiltration into the trachea, which is shown to be dependent on VCAM-1/VLA-4 interaction (Fig. 1). These results suggest that other cytokines such as IL-1 and TNF- α as well as IL-4 might be important in causing antigen-induced VCAM-1 expression on the endothelium of the airways. Briscoe et al. (40) reported that the intradermal injection of IL-4 induced little or no VCAM-1 expression on the endothelium but enhanced TNF-induced VCAM-1 expression, which is consistent with our present observation.

Second, we also show that VCAM-1/VLA-4 interaction is functionally predominant over ICAM-1/LFA-1 interaction in controlling antigen-induced T cell recruitment into the tissue, as indicated by the finding that the *in vivo* blocking of VCAM-1 and VLA-4 inhibited antigen-induced CD4⁺ and CD8⁺ T cell infiltration into the mouse trachea more potently than that of ICAM-1 and LFA-1 (Figs. 2, 4, and 7). Previous studies showed that ICAM-1/LFA-1 interaction (10–14) and VCAM-1/VLA-4 interaction (15–17) were involved in the adherence of T cells to endothelial cells and its transendothelial migration *in vitro*. Our finding of inhibition of antigen-induced T cell recruitment by *in vivo* blocking of VCAM-1 and VLA-4 (Fig. 2) is consistent with the *in vitro* observation by Dustin and Springer (13) that the adhesion of T cells to cytokine-stimulated endothelial cells is mostly mediated through an LFA-1-independent pathway. Our finding of the involvement of ICAM-1 and LFA-1 in antigen-induced T cell recruitment (Fig. 4) is also consistent with their finding that T cell adherence to cytokine-stimulated endothelial cells is partially mediated by ICAM-1/LFA-1 interaction (13). The interference of ICAM-1/LFA-1 interaction *in vivo* might also inhibit initial T cell activation during the antigen presentation because ICAM-1 has been shown to contribute to antigen-independent adhesion between T cells and antigen-presenting cells (44). In contrast to our findings, it has recently been reported that ICAM-1 plays a prominent role in the transendothelial migration of T cells *in vitro*, whereas VCAM-1 does not significantly mediate the migration (18, 19). The reason for this discordance in the role of VCAM-1 and ICAM-1 in T cell migration is unclear and might be explained by a possible contribution of the interaction between VLA-4 and fibronectin in the matrix *in vivo*.

There has been a recent observation that memory T cells selectively traffic from blood to peripheral tissues to lymph nodes via afferent lymph vessels, whereas naive T cells selectively traffic from blood to lymph nodes via high endothelial venules (45). It has also been shown that memory T cells have increased levels of LFA-1 and VLA-4 expressions compared with naive T cells (46, 47). Therefore, in addition to the induction of VCAM-1 and ICAM-1 expressions on the endothelium, it is possible that the increased expression of LFA-1 and VLA-1 on memory T cells might also contribute to the T cell infiltration into the airways. Indeed, memory

T cells show stronger adhesion to cytokine-stimulated cultured endothelial cells than naive T cells (48).

We have also found that the *in vivo* blocking of LFA-1, but not of ICAM-1, increases blood lymphocyte counts (Fig. 9), suggesting that an LFA-1-dependent, ICAM-1-independent basal adhesion, which was not significantly affected by cytokines (13), operates basal lymphocyte recirculation *in vivo*. In contrast, as already mentioned, our results indicate that the extravasation of lymphocytes to the tissue is operated through an LFA-1- and ICAM-1-dependent pathway (Fig. 4), which was previously shown to be strongly upregulated by cytokines (13), as well as VCAM-1/VLA-4 interaction (Fig. 2). It has been shown that *in vitro* adherence of lymphocytes to high endothelial venules of peripheral lymph nodes is substantially inhibited by anti-LFA-1 mAb (49, 50) and that the administration of anti-LFA-1 mAb decreases the migration of normal lymphocytes into lymph nodes and Peyer's patches by 40–60% (49). The difference in the effects of *in vivo* blocking of LFA-1 and ICAM-1 on blood lymphocyte counts suggests a possible involvement of LFA-1/ICAM-2 interaction in basal lymphocyte recirculation *in vivo* because ICAM-2 is another ligand for LFA-1 (42, 43) and LFA-1-dependent binding of lymphocytes to endothelium has been shown to be totally accounted for by ICAM-1 and ICAM-2 (51). Consistent with this possibility, it has been shown that anti-ICAM-1 mAb does not inhibit the binding of lymphocytes to high endothelial venules (52). Our finding that the combined blocking of VLA-4 and LFA-1 increased blood lymphocyte counts greater than that of VCAM-1 and ICAM-1 (Fig. 9) might also be explained by a possible involvement of LFA-1/ICAM-2 interaction when VCAM-1/VLA-4 interaction is blocked.

In addition, our finding that the *in vivo* blocking of VCAM-1 and VLA-4 increased blood lymphocyte counts less than that of LFA-1 (Fig. 9) suggests that the contribution of VCAM-1/VLA-4 interaction to lymphocyte recirculation *in vivo* is relatively small. *In vitro* adherence of lymphocytes to high endothelial venules of Peyer's patches, but not of peripheral lymph nodes, has also been shown to be strongly inhibited by anti-VLA-4 mAb (53). The increase in blood lymphocyte counts by *in vivo* blocking of VCAM-1 and VLA-4 might also be due to the release of lymphocytes from the bone marrow by the interference of attachment of lymphocytes to stroma cells because VCAM-1/VLA-4 interaction has been shown to be important in binding of lymphocytes to bone marrow stroma cells (30).

Our results show that the most prominent difference between antigen-induced T cell and eosinophil recruitments is the dependence of ICAM-1/LFA-1 interaction in T cell recruitment and the independence of this interaction in eosinophil recruitment (Fig. 3 and 4). There might be a difference in the ratio of cell surface expression of LFA-1 and VLA-4 between T cells and eosinophils. Another possible explanation is that the blocking of ICAM-1/LFA-1 interaction might inhibit subsequent T cell activation by antigen-presenting cells (44) at the same time that it inhibits T cell migration through vascular endothelial cells.

Eosinophil infiltrate is a characteristic feature of allergic

inflammation such as asthma. Increasing evidence suggests that the infiltrating eosinophils cause the tissue damage of the airways and airway hyperreactivity by releasing the cytotoxic granules and lipid mediators (54). Therefore, our finding of an important role of VCAM-1/VLA-4 interaction in antigen-induced eosinophil recruitment into the airways suggests that the interference with VCAM-1/VLA-4 interaction by a specific antagonist for the adhesion molecules and by a specific antisense oligonucleotide for the mRNA would be a potential therapeutic approach to control airway inflammation of asthma. Indeed, it has been reported that a synthetic peptide can specifically interfere with the binding of VLA-4 to its ligand (55). It has also been shown that

the expression of a cell surface adhesion molecule on the endothelium can be inhibited by an antisense oligonucleotide (56).

In summary, we have shown that VCAM-1/VLA-4 interaction plays a predominant role in controlling antigen-induced eosinophil and T cell recruitment into the tissue and that the induction of VCAM-1 expression on the endothelium at the allergic inflammatory site regulates this eosinophil and T cell recruitment. These results suggest that antagonism of VCAM-1/VLA-4 interaction would be a rational therapeutic approach to allergic airway inflammation such as asthma.

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