

Nasal challenge with allergen upregulates the local expression of vascular endothelial adhesion molecules

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To understand the events involved in selective eosinophil migration into allergic inflammatory sites, we studied the expression of vascular endothelial adhesion molecules in the nasal mucosa. Ten subjects with asymptomatic seasonal allergic rhinitis and 13 nonallergic subjects underwent localized allergen challenge of one inferior turbinate. Twenty-four hours later, biopsy specimens were obtained from the inferior turbinates, bilaterally in the seasonally allergic subjects and unilaterally in the nonallergic control subjects. The specimens were divided, sectioned, and either stained for identification of eosinophils or analyzed immunohistochemically for intercellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and von Willebrand's factor. Intercellular adhesion molecule-1 expression was observed in all mucosal specimens, with no significant difference among groups. E-selectin showed minimal baseline expression, and low levels were significantly induced on the challenged mucosa of the allergic compared with nonallergic subjects ($p < 0.05$). VCAM-1 was expressed basally and was significantly upregulated by allergen challenge, compared with the nonchallenged side and nonallergic control subjects ($p < 0.05$). Submucosal eosinophils increased significantly in allergic subjects 24 hours after antigen challenge, compared with nonallergic control subjects and weakly correlated with VCAM-1 expression ($r_s = 0.33$, $p = 0.06$). Our results suggest that endothelial activation accompanies allergic inflammation. Furthermore, because the counterligand for VCAM-1, very late activation antigen-4, is present on eosinophils, VCAM-1 upregulation may contribute to the selective recruitment of these cells to the nasal mucosa. (J ALLERGY CLIN IMMUNOL 1994;94:1006-16.)

Key words: Adhesion molecules, intercellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1, allergic rhinitis, nasal biopsy, eosinophil

Tissue eosinophilia is a characteristic finding of allergic inflammation,¹⁻³ although the mechanisms for eosinophil migration are still poorly understood. After nasal allergen challenge of allergic individuals who were free of symptoms, eosinophil counts increased significantly in nasal lavages,

Abbreviations used

APAAP:	Alkaline phosphatase-anti-alkaline phosphatase
ICAM-1:	Intercellular adhesion molecule-1
IL:	Interleukin
mAb:	Monoclonal antibody
TBS:	Tris-buffered saline
TNF- α :	Tumor necrosis factor- α
VCAM-1:	Vascular cell adhesion molecule-1
VLA-4:	Very late activation antigen-4
vWF:	von Willebrand factor

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peaking at 8 hours and remaining significantly increased for 10 days.⁴ In contrast, nonallergic subjects challenged with allergen showed no increase. Nasal biopsy specimens of subjects with allergic rhinitis, obtained 24 hours after antigen challenge, also demonstrated significant increases

in the total number of eosinophils on the side of antigen provocation, compared with the unchallenged side or with the response of nonallergic subjects challenged with antigen.⁵ These findings are consistent with other reports on tissue eosinophilia in the nasal mucosa after challenge and during the allergy season.^{6, 7} The observation that the number of eosinophils in tissues and secretions exceeds the numbers in the peripheral blood suggests that mechanisms that favor their activation, recruitment, and/or survival exist at extravascular sites.

Accumulating evidence supports the importance of leukocyte and vascular endothelial cell adhesion molecules in the migration of inflammatory cells, including eosinophils, into tissue sites during allergic reactions.⁸ This involves a sequence of events, which includes margination of leukocytes along the walls of the microvasculature, adhesion to the endothelium, transmigration through the vessel walls, and migration along a chemotactic gradient within the extravascular compartment.⁸ These events are mediated by adhesion molecules such as integrins, selectins, and members of the immunoglobulin gene superfamily. For example, vascular endothelial cells are now widely perceived as active participants in this process by virtue of the adhesion molecules present on their surfaces.

Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin are three representative endothelial adhesion molecules. They can be upregulated on endothelial cells by interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and lipopolysaccharide.⁹⁻¹¹ ICAM-1, a ligand for the β_2 integrin molecules lymphocyte function-associated antigen-1 and Mac-1, which are present on the surface of leukocytes, mediates the attachment of all classes of leukocytes to endothelial cells. ICAM-1 is known to be constitutively expressed on vascular endothelial cells and can be induced by inflammatory mediators on other extravascular cells. Recently, ICAM-1 has also been identified as the major human rhinovirus receptor.¹²

E-selectin binds to sialyl Lewis X, which is expressed on the surface of leukocytes.¹³ It appears to be endothelial-specific in its expression, whereas ICAM-1 and VCAM-1 are also expressed on nonvascular structures.^{14, 15} VCAM-1, like ICAM-1, is a member of the immunoglobulin gene superfamily and supports the adhesion of leukocytes to endothelial cells through interactions with the integrin molecule very late activa-

tion antigen-4 (VLA-4) ($\alpha_4\beta_1$), which is present on the surface of lymphocytes, monocytes, eosinophils, and basophils, but not neutrophils.^{8, 15} VCAM-1 expression can be selectively induced on endothelium by treatment with IL-4.¹⁶

Recent studies have examined the role of vascular adhesion molecules in human allergic inflammation. Enhanced expression of E-selectin and ICAM-1 was observed in vivo on cutaneous vascular endothelium, along with a substantial infiltration of eosinophils, 2 to 8 hours after intradermal injection of allergen.^{17, 18} Montefort et al.¹⁹ observed increased expression of ICAM-1 and VCAM-1 in nasal mucosal biopsy specimens of subjects with perennial allergic rhinitis, compared with normal control subjects. Georas et al.²⁰ reported increased levels of a soluble form of E-selectin 19 hours after bronchoscopic allergen provocation in allergic subjects. Whether localized experimental allergen exposure of the nasal mucosa results in endothelial activation has not been determined.

Our studies aimed to determine: (1) baseline expression of adhesion molecules in the nasal mucosa of both allergic individuals and normal control subjects, (2) whether nasal challenge with antigen upregulates the expression of vascular endothelial cell adhesion molecules, and (3) whether the infiltration of eosinophils into the nasal mucosa of subjects with allergic rhinitis, an established event after allergen challenge, is associated with endothelial activation.

To reach our goals, we obtained nasal biopsy specimens from subjects with allergic rhinitis and nonallergic control subjects 24 hours after nasal provocation with antigen. The timing of the biopsies relative to challenge was chosen on the basis of previous studies that showed that several inflammatory cells, including eosinophils, were significantly increased over baseline in the nasal submucosa 24 hours after allergen provocation.^{5, 21, 22}

METHODS

Reagents

Murine monoclonal antibodies (mAbs) recognizing E-selectin (H 18/7, IgG_{2a} subclass) and VCAM-1 (2G7, IgG₁ subclass) were generated as described previously and were generously donated by Dr. Michael Gimbrone, Jr., Harvard Medical School and Dr. Walter Newman, Otsuka America Pharmaceutical, Inc., respectively.^{23, 24} The following were purchased: mAb recognizing ICAM-1 (84H10, IgG₁ subclass) from AMAC Inc. (Westbrook, Maine), recombinant human TNF- α from Genzyme (Cambridge, Mass.), optimum

cutting temperature medium for embedding tissues from Tissue-Tek, Miles Inc. (Elkhart, Ind.), anti-human murine mAb against von Willebrand's factor (vWf), which specifically stains vascular endothelial cells (vWf, F8/86, IgG₁ subclass), mouse irrelevant IgG₁ and IgG_{2a} mAb used as negative controls, rabbit anti-mouse immunoglobulin and alkaline phosphatase-anti-alkaline phosphatase (APAAP) complex from Dako (Glostrup, Denmark), normal rabbit serum, poly-L-lysine solution, Mayer's hematoxylin solution, and levamisole from Sigma Diagnostics (St. Louis, Mo.).

Human subjects and ethical approval

Ten subjects with seasonal allergic rhinitis (8 men and 2 women) with an average age of 26 ± 1 years and 13 nonallergic control subjects (3 men and 10 women) with an average age of 29 ± 2 years participated in the study. All of the subjects with seasonal allergic rhinitis had a positive skin puncture test response to either ragweed (8 subjects) or grass extracts (2 subjects) and had seasonal allergic symptoms. Nonallergic control subjects gave no history of rhinitis symptoms and had negative skin puncture test responses to a range of common aeroallergens, including those used for challenge. The allergic subjects underwent biopsy outside their allergy season, and all subjects were receiving no medications, except acetaminophen, for at least 6 weeks before the study. The allergic subjects underwent biopsies of both inferior turbinates 24 hours after localized antigen challenge of one turbinate. The nonallergic subjects also underwent nasal biopsies of the inferior turbinate 24 hours after localized antigen challenge. The protocol was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions at The Francis Scott Key Medical Center, and each participant gave informed consent before enrollment.

Antigen challenge

Eight-millimeter filter paper disks, punched out from Shandon filter cards (Shandon Inc. Pittsburgh, Pa.), were saturated with 50 μ l of the relevant allergen extract (333 protein nitrogen units of grass or ragweed purchased from Greer Laboratories, Lenoir, N.C.) and placed on the anterior tip of the inferior turbinate for 1 minute. All nonallergic control subjects were challenged in a similar fashion with ragweed extract. All allergic subjects experienced rhinorrhea and sneezing within minutes after allergen challenge, whereas all nonallergic subjects reported no symptoms.

Nasal biopsy and tissue preparation

Twenty-four hours after allergen challenge, the nasal mucosa at the anterior tips of the inferior turbinates was decongested with oxymetazoline and anesthetized topically with tetracaine. Next, 0.5 to 1.0 ml of 1% lidocaine with 1:100,000 epinephrine (Elkins-Sinn, Inc., Cherry Hill, N.J.) was injected. Nasal biopsy

specimens averaging 4×3 mm in dimension, were then obtained from the anterior tips of the inferior turbinates with a punch forceps. Allergic subjects had biopsy specimens taken from the same site of challenge, as well as from the contralateral, unchallenged nasal cavity; nonallergic subjects underwent biopsy only at the site of challenge. Each specimen was bisected, and one part was fixed in formalin solution and later embedded in paraffin for hematoxylin-eosin and Orcein-Giemsa staining, and the other part was placed in optimum cutting temperature medium, snap-frozen with liquid nitrogen, and kept at -80° C until cryosectioning was performed.

Immunohistochemistry

Specimens were cryosectioned into 6 μ m thickness (2800 Frigocut N; Reichert-Jung Co., Germany) and mounted on poly L-lysine-coated glass slides. Cryostat sections were air-dried and then fixed in acetone (4° C) for 10 minutes. Immunostaining was performed with the modified APAAP method, as previously described.^{1, 25, 26} Nonspecific binding of the second layer (link) antibody was prevented by an initial incubation with 5% normal rabbit serum in Tris-buffered saline (TBS) for 10 minutes.

Primary mouse antibody, suitably diluted in TBS (see below), was then applied to each specimen and incubated in a humid chamber for 45 minutes at room temperature. After washing in TBS, sections were incubated for 30 minutes with link antibody (rabbit anti-mouse Ig). After another wash in TBS, sections were incubated with APAAP complex for 30 minutes. Link antibody and APAAP complex were reapplied to potentiate staining. After a further wash in TBS, the specimens were stained with a substrate-chromogen solution, Fast Red-Naphthol AS-MX containing levamisole (Vector Laboratories, Burlingame, Calif.), to block endogenous alkaline phosphatase activity, which might be present in tissue specimens. The APAAP technique results in an intense red staining of the recognized antigens against a faint background. Finally, the specimens were counterstained with Mayer's hematoxylin solution and dried by brief consecutive dips in 95% and 100% ethyl alcohol, followed by xylene; and coverslips were mounted (mounting medium: Baxter Health Care Corporation, McGaw Park, Ill.). To minimize histologic differences from tissue to tissue, we always stained adjacent sections with vWf followed by VCAM-1, E-selectin, and ICAM-1. Eosinophils were counted on nonadjacent sections of the tissue samples from the same subjects.

In vitro activation of human nasal turbinate tissue

To confirm that endothelial adhesion molecules of the nasal mucosa could be induced by cytokines such as TNF- α and to determine the optimal dilution point of each mAb used in the staining of study subjects' biopsy

specimens for adhesion molecules, a preliminary experiment was performed with an inferior turbinate biopsy specimen obtained from a nonallergic subject. The specimen was cut into four pieces and processed as follows: the first piece was immediately fixed in formalin, and the second was snap-frozen and immunohistochemically stained as a control. The other two pieces were cultured in 1 ml of RPMI 1640 media (Gibco Laboratories, Grand Island, N.Y.) containing antibiotics (1% penicillin/streptomycin), L-glutamine (10 mmol/L), and polymyxin B (50 µg/ml) with or without TNF-α (10ng/ml) at 37° C for 5 hours. The cultured specimens were snap-frozen and processed for immunohistochemistry.

Tissue from this experiment was subsequently used to verify adequate dilutions of the mAbs that we used for the rest of the studies. These were determined on the basis of maximal staining of the vessels, along with minimal background staining. VCAM-1 and E-selectin were induced only on the specimens stimulated by TNF-α. ICAM-1 expression was present to a significant extent on all the specimens, including the ones that were snap-frozen immediately after biopsy (data not shown). Optimal concentrations were as follows: 10 µg/ml for 2G7 (VCAM-1), 1:5 for H18/7 (culture supernatant) (E-selectin), 1 µg/ml for 84H10 (ICAM-1), 30 ng/ml for F8/86 (vWf), 60µg/ml for rabbit anti-mouse secondary antibody, and 3 µg/ml for the APAAP complex. These specimens were also used as positive controls in the rest of the experiments. Negative controls consisted of tissue specimens stained with irrelevant antibodies (IgG₁ or IgG_{2a}).

Microscopic assessment of stained specimens

The stained sections were examined in blinded fashion by two observers independently. With the use of the eye reticule, the central 1 mm² of the lamina propria was examined under 100× magnification for endothelial adhesion molecules and under 400× magnification for eosinophils. Vessels were considered to be positively stained if the endothelial cells lining their lumen were stained red. Comparisons were always made between study specimens and positive and negative controls stained simultaneously. Qualitative evaluation of the intensity of staining was deemed to be too subjective and was not done. We counted only stained vessels with a distinct lumen to avoid counting nonvascular expression of adhesion molecules. This conservative approach eliminated capillaries without a distinct lumen. The counts of the two observers were closely correlated, and the average counts were used to report the results. Percent expression of endothelial adhesion molecules was obtained as follows:

$$\frac{\text{Number of vessels stained by mAb for a specific adhesion molecule}}{\text{Number of vessels stained by antibody for vWf}} \times 100$$

The numbers of vessels stained with a specific adhesion molecule and with vWf were obtained from consecutively cut sections. This percentage of expression of adhesion molecules was used to report and analyze the results.

Statistical analysis

Because the data were not normally distributed, we used nonparametric statistical tests to analyze the results. These were performed with the Statview II software package (Abacus Concepts, Berkeley, Calif.) and a Macintosh computer (Apple Computer, Cupertino, Calif.). Kruskal-Wallis analysis of variance was used to evaluate differences among three or more unpaired groups. If there was a statistically significant difference between the groups, post hoc analysis was performed with the Mann-Whitney U test. Paired data were compared with the Wilcoxon signed-rank test. To examine baseline expression of the adhesion molecules in the nonchallenged allergic mucosa and the challenged mucosa of the nonallergic subjects, we compared these values for each of the adhesion molecules to a set of the same number of zero values by using the Mann-Whitney U test. Correlations were determined with Spearman's rank correlation coefficient. Two tailed *p* values of less than 0.05 were considered significant.

RESULTS

VCAM expression

Biopsy specimens were obtained from both inferior turbinates of 10 allergic subjects and from one inferior turbinate of 13 nonallergic control subjects 24 hours after unilateral allergen challenge. The number of vessels counted in 1 mm² of tissue varied, depending on the adhesion molecule of interest. The range was from 17 to 76 vessels/mm² for vWf, 0 to 25 vessels/mm² for VCAM-1, 0 to 21 vessels/mm² for E-selectin, and 4 to 48 vessels/mm² for ICAM-1. Examples of positive vascular endothelial staining are shown in Fig. 1. The percentage of total vessels expressing each of the adhesion molecules (as detailed above) is reported. Most of the adhesion molecules, including VCAM-1 and ICAM-1, were expressed on the small vessels of the lamina propria rather than on the larger vessels of the submucosal vascular layer of the nasal mucosa.

To evaluate baseline expression of the adhesion molecules in the nasal mucosa of nonallergic control subjects (*n* = 13) and the unchallenged mucosa of subjects with allergic rhinitis (*n* = 10), we compared these numbers for each of the adhesion molecules to zero. Both VCAM-1 and ICAM-1 expression were significantly greater than zero in the unchallenged nasal mucosa of allergic subjects and nonallergic control subjects

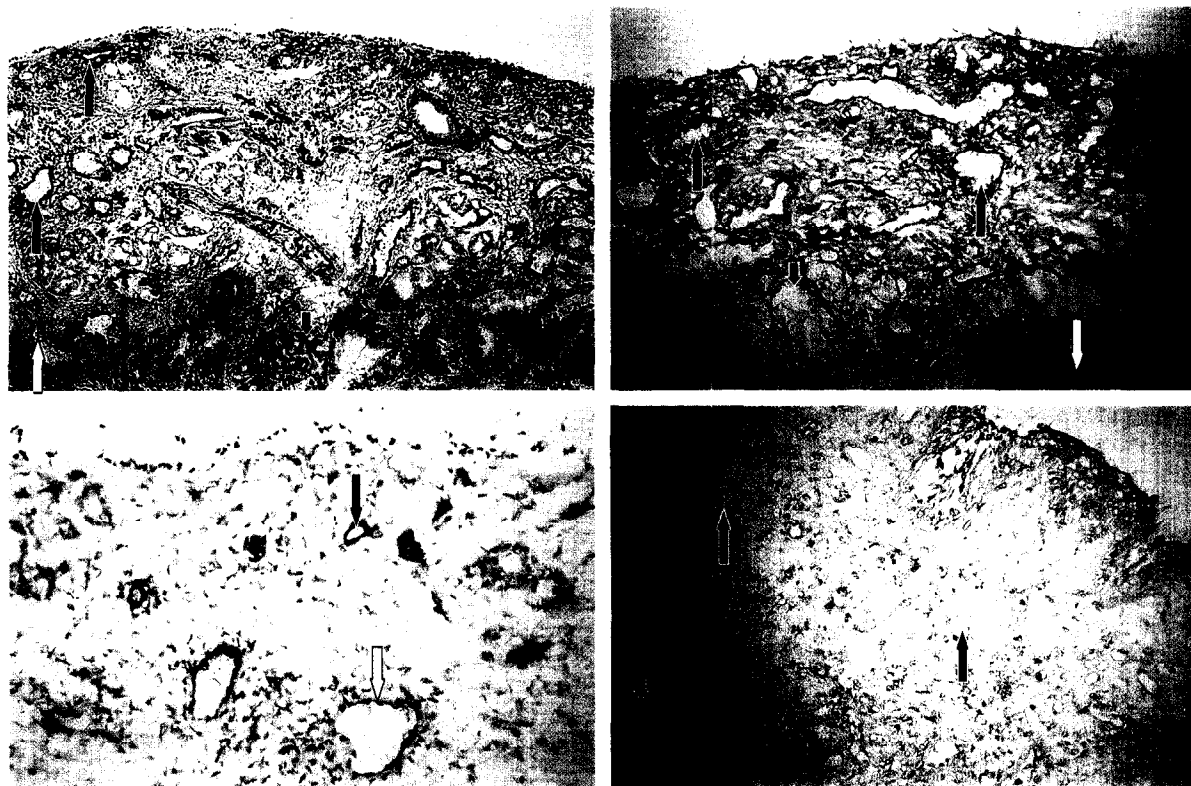


FIG. 1. Examples of adhesion molecule expression on vascular endothelium viewed at 100 $\times$ magnification. All specimens were obtained 24 hours after allergen provocation. Staining with antibodies against vWf (**top left panel**), VCAM-1 (**top right panel**), E-selectin (**bottom left panel**), and irrelevant IgG₁ antibody (**bottom right panel**) are shown. The basement membrane and lamina propria are at the top. The epithelium is not well visualized except in the **bottom right panel**, and this is due to the immediate freezing of the specimens after harvesting, a step known to damage the epithelium. The deeper layers of the nasal mucosa are rich in glands (*red arrows*) and large vessels (*open arrows*). Multiple vessels with a distinct lumen are identified by staining of endothelial cells with the different antibodies and appear dark red in color. *Solid arrows* identify representative vessels in the superficial portions of the tissue. Note the absence of any vascular endothelial staining of vessels with distinct lumens when the specimen is stained with irrelevant IgG₁ antibody (*solid arrows* in **bottom right panel**).

(VCAM-1, medians: 9% and 6%, respectively, $p < 0.002$), (ICAM-1, medians: 66% and 52%, respectively, $p < 0.0001$). In contrast, baseline expression of E-selectin was not significantly greater than zero in either the unchallenged nasal mucosa of allergic subjects or nonallergic control subjects (medians: 0% and 0%, respectively).

ICAM-1 was expressed on endothelial cells lining the vascular lumen in the unchallenged mucosa of subjects with allergic rhinitis and the challenged mucosa of nonallergic control subjects (median values: 66% and 52%, respectively). ICAM-1 was also expressed on the challenged mucosa of the subjects with allergic rhinitis who underwent biopsy 24 hours after antigen challenge (median = 50%). Furthermore, ICAM-1

expression was found on various nonvascular cells, which could not be definitively identified. There were no significant differences among groups in the expression of ICAM-1 (analysis of variance: $p = 0.84$) (Figs. 2 and 3).

E-selectin was rarely observed at baseline in the unchallenged mucosa of subjects with allergic rhinitis and challenged nonallergic control subjects (median = 0% and 0%, respectively). In the challenged mucosa of subjects with allergic rhinitis, the expression was minimally upregulated (median = 5%). This increase was statistically significant compared with the nonallergic control subjects ($p < 0.05$) (Figs. 2 and 3) but was not different from contralateral, unchallenged sites of the allergic subjects.

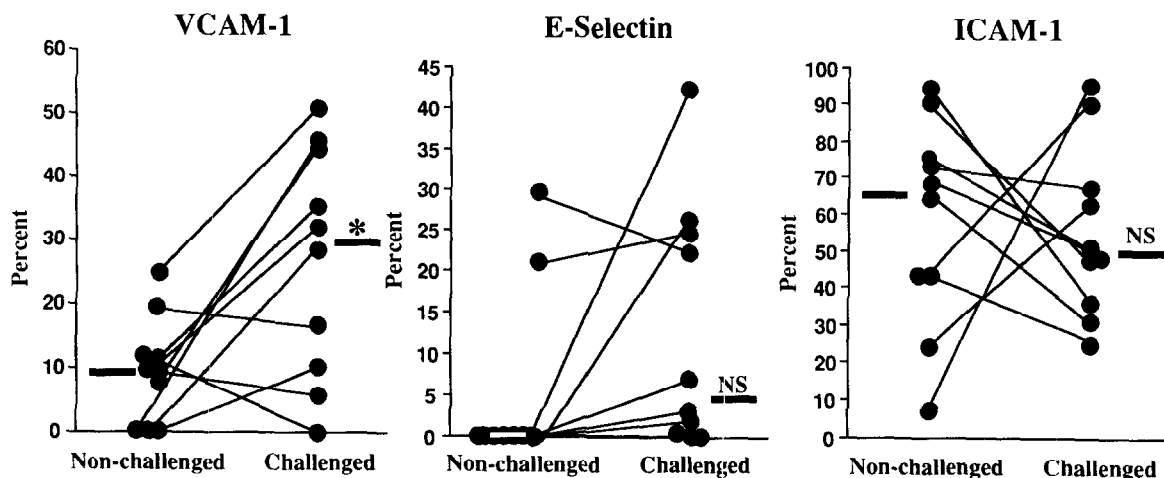


FIG. 2. Expression of vascular endothelial cell adhesion molecules in the nasal mucosa 24 hours after allergen challenge of subjects with allergic rhinitis. Data are presented as the percentage of vessels stained by mAb against each adhesion molecule of the total vessels (as identified by vWf positive stain) in 1 mm² of tissue. Individual data are shown with the solid bars representing median values for $n = 10$. *Challenged* represents counts from the inferior turbinate biopsy specimen obtained on the side of antigen challenge. *Nonchallenged* represents counts from the inferior turbinate biopsy specimen obtained from the contralateral nostril of the same individuals. * $p < 0.05$ challenged versus nonchallenged. NS, Nonsignificant.

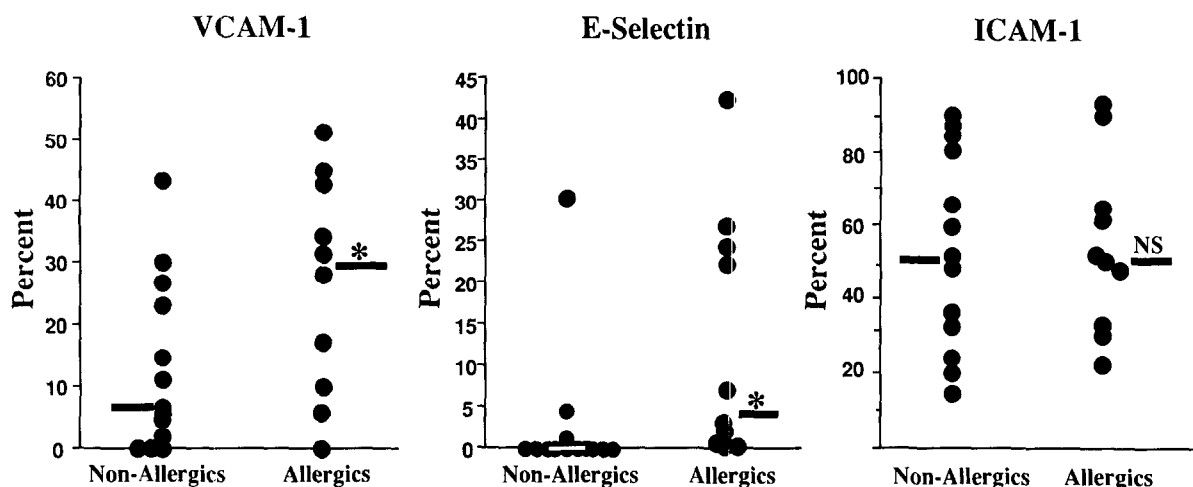


FIG. 3. Expression of vascular endothelial cell adhesion molecules in the nasal mucosa 24 hours after allergen challenge of subjects with allergic rhinitis ($n = 10$) and nonallergic control subjects ($n = 13$). Data are presented as in Fig. 2. * $p < 0.05$ allergic subjects versus nonallergic subjects. NS, Nonsignificant.

VCAM-1 appeared to be expressed modestly at baseline in the unchallenged mucosa of the allergic subjects and nonallergic control subjects, with no significant differences between these two groups (median = 9% and 6%, respectively; $p = 0.75$). It was significantly upregulated at the site of allergen challenge 24 hours later in the subjects with allergic rhinitis (median = 26%), compared with the other two groups ($p < 0.05$)

(Figs. 2 and 3). Extravascular expression of VCAM-1 was also found on unidentifiable cells in the lamina propria of the challenged mucosa of subjects with allergic rhinitis and infrequently in the other two groups.

Eosinophils

Eosinophils were present in all layers of the nasal mucosa but were chiefly located in the

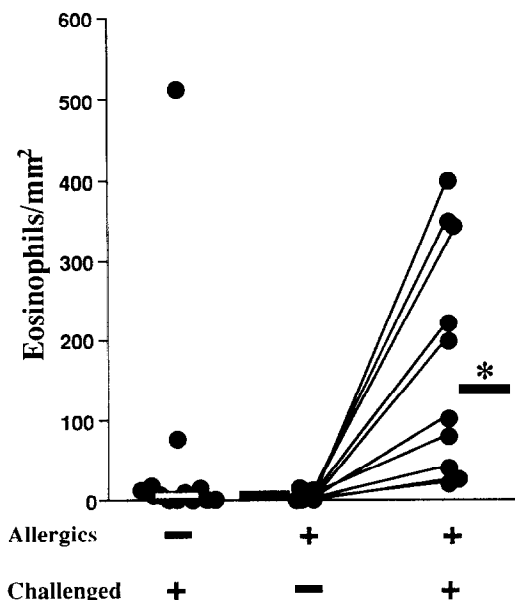


FIG. 4. Number of eosinophils per square millimeter in nasal mucosal tissue as identified by Orcein-Giemsa staining. Counts from the inferior turbinate biopsy specimens obtained at the site of antigen challenge in nonallergic subjects ($n = 13$) and in nonchallenged and challenged sites of subjects with allergic rhinitis ($n = 10$) are shown. Individual data are shown, with the solid bars representing median values. * $p < 0.01$ versus unchallenged allergic subjects and challenged nonallergic subjects.

lamina propria. They were rarely observed in the unchallenged mucosa of subjects with allergic rhinitis and nonallergic control subjects (median = 2.5 cells/mm² and 2.0 cells/mm², respectively). In contrast, eosinophils were significantly increased at the site of allergen challenge 24 hours later in the subjects with allergic rhinitis compared with the other two groups (median = 144 cells/mm², $p < 0.01$) (Fig. 4).

Correlation between endothelial adhesion molecules and eosinophils

The correlation between the expression of endothelial adhesion molecules and infiltrating eosinophils was evaluated in 33 tissue specimens (10 obtained from the site of challenge in allergic subjects, 10 obtained from the site contralateral to the challenge site in allergic subjects, and 13 obtained from nonallergic control subjects 24 hours after allergen challenge). There was a significant positive correlation between the expression of VCAM-1 and E-selectin ($r_s = 0.36$, $p < 0.05$). The correlation between VCAM-1 expression and eosinophil counts closely approached statistical significance ($r_s = 0.33$, $p = 0.06$) (Fig.

5). The expression of E-selectin also showed a significant correlation with eosinophil counts ($r_s = 0.53$, $p = 0.003$) (Fig. 5). The expression of ICAM-1 did not correlate with expression of any other adhesion molecule or inflammatory cell.

DISCUSSION

In nasal biopsy tissues, ICAM-1 was the most predominantly expressed endothelial cell adhesion molecule and was observed in all nasal mucosal specimens, including those of nonallergic subjects. There was no increased expression of ICAM-1 24 hours after allergen challenge in allergic subjects. Our observations are consistent with previous reports that ICAM-1 is constitutively expressed on vascular endothelial cells in skin, nasal mucosal tissues, synovial tissue, and lymph nodes.^{17, 19, 27, 28} ICAM-1 was reported to be upregulated in the nasal mucosa by in vitro stimulation with interferon- γ or TNF²⁹ and was also significantly increased in the nasal biopsy specimens of subjects with perennial allergic rhinitis, compared with normal control subjects.¹⁹ We did not find similar changes 24 hours after antigen challenge. In our study ICAM-1 was also found to be expressed on extravascular cells, which could not be identified. ICAM-1 is known to be expressed on several types of cells, including macrophages, fibroblasts, epithelial cells, lymphocytes, and follicular dendritic reticulum cells.^{27, 30}

E-selectin expression was negligible in the nasal mucosa of nonallergic control subjects and on the unchallenged side of the subjects with allergic rhinitis. In the mucosal specimens obtained 24 hours after antigen challenge, the expression of E-selectin was slightly elevated compared with biopsy specimens of the unchallenged mucosa of subjects with allergic rhinitis, but this did not reach statistical significance. E-selectin expression, however, increased when the challenge sites of allergic subjects were compared with those of nonallergic control subjects. E-selectin expression was not as high as that of ICAM-1 or VCAM-1. This is compatible with the knowledge that E-selectin is not normally present at baseline and must be induced by cytokines such as TNF- α and IL-1 on endothelial cells^{14, 31} or after exposure to antigen.^{18, 19}

E-selectin is involved in the initial interaction of granulocytes and a subpopulation of lymphocytes with endothelial cells in the early phase of inflammatory reactions.¹⁴ Our observation of low expression of E-selectin, even after allergen challenge, might be due to the 24-hour lag between antigen

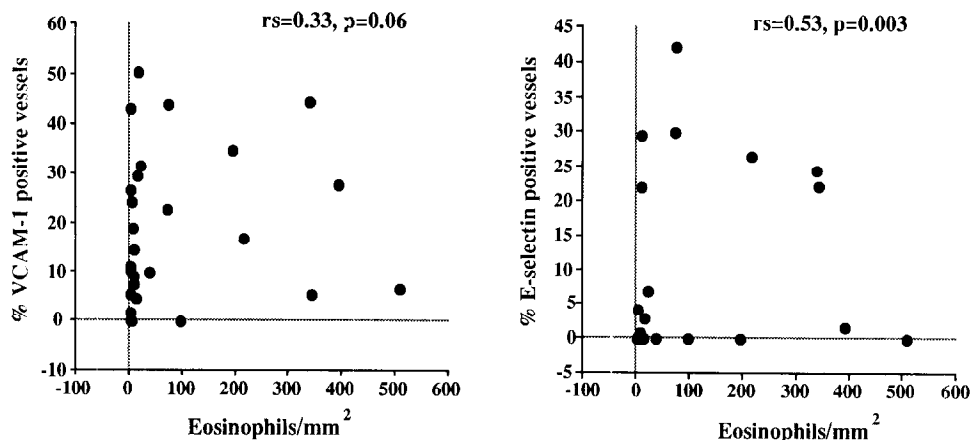


FIG. 5. Scattergrams of the number of eosinophils per square millimeter and percent of VCAM-1-positive vessels (**left panel**) and the percent of E-selectin-positive vessels (**right panel**) in the nasal mucosa. The correlations were evaluated in 33 tissue specimens (10 obtained from the site of challenge in allergic subjects, 10 obtained from the site contralateral to the challenge in allergic subjects, and 13 obtained from nonallergic control subjects 24 hours after allergen challenge).

challenge and biopsy. Had we obtained the biopsy specimens earlier after allergen challenge, E-selectin expression might have been more prominent. This is supported by *in vitro* studies with human umbilical vein endothelial cells, which showed that E-selectin expression in response to TNF- α and IL-1 started by 1 to 2 hours after exposure, was maximal at 4 to 6 hours, and decreased markedly by 24 hours.^{23, 31} This is also supported by studies of Montefort et al.,³² who demonstrated upregulation of E-selectin but not VCAM-1 in bronchial biopsy specimens obtained 6 hours after endobronchial segmental allergen challenge. Another study demonstrated that the expression of E-selectin in skin biopsy specimens of allergic subjects occurred concurrently with the development of inflammatory cellular infiltration between 2 and 4 hours after intradermal injection of allergen, peaked at 6 hours, and was significantly decreased at 24 hours.¹⁸ Like E-selectin, each of the inducible endothelial adhesion molecules showed distinct time-dependent patterns of expression in response to TNF- α , *in vitro*. ICAM-1, present basally in human umbilical vein endothelial cells, was increased starting 4 hours after exposure to TNF- α and IL-1, was maximal at 12 to 24 hours, and remained elevated for several days. VCAM-1 was induced after 4 hours of exposure to the cytokines and remained maximal for 24 to 48 hours.³³ Our studies support these observations on the kinetics of adhesion molecule expression in tissues after challenge. Twenty-four hours after provocation, VCAM-1 is the most prominently upregulated adhesion mol-

ecule, with E-selectin expression being only modestly increased. The lack of upregulation of ICAM-1 and the low levels of E-selectin in our studies suggest that IL-1, TNF- α , and interferon- γ are not continuously produced up to 24 hours after exposure to antigen.

VCAM-1 is the endothelial adhesion molecule that showed the highest significant increases in the allergic subjects after allergen challenge, compared with the unchallenged side of the same individuals and challenged mucosa of nonallergic subjects. It also showed a close relationship with eosinophils ($p = 0.06$). Montefort et al.¹⁹ reported that both ICAM-1 and VCAM-1 expression were increased in the nasal mucosa of subjects with perennial allergic rhinitis, compared with nonallergic control subjects, whereas we report significant increases in VCAM-1 and modest elevations in E-selectin after antigen provocation. Although the identified VCAM-1 expression in their studies was localized only to the vascular endothelium, we observed many cells positively stained by the mAb for VCAM-1, chiefly in the challenged mucosa of allergic subjects. The distribution and number of these cells were closely comparable to those of eosinophils observed on consecutive sections stained by a modified Wright's stain (Diff-Quick; Baxter Healthcare Corporation), although expression of VCAM-1 on eosinophils has never been observed.³⁴ Other nonvascular cells reported to express VCAM-1 include macrophages, follicular dendritic cells, and neural cells.^{35, 36}

Our observation that there was significant baseline expression of VCAM-1 and minimal to absent baseline expression of E-selectin in the unchallenged allergic or nonallergic nasal mucosa is also different from that of Montefort et al.,¹⁹ who reported expression of E-selectin and no baseline VCAM-1. On the other hand, both studies confirm the baseline, constitutive expression of ICAM-1 in the nasal mucosa. We also failed to observe differences in the expression of these molecules between nonallergic subjects and allergic subjects who were free of symptoms out of season. The reasons for these discrepancies are unclear but might include differences in subject populations and in staining techniques.

The mechanism by which eosinophils selectively migrate from the peripheral circulation to sites of allergic inflammation is still under investigation. The VLA-4/VCAM-1 adhesion pathway has been suggested as a mechanism for specific eosinophil, as opposed to neutrophil, migration into these sites.^{8, 16, 34} Several laboratories have demonstrated that blocking VCAM-1 with a specific antibody was extremely effective in inhibiting eosinophil adherence *in vitro* but had no effect on neutrophil adherence, supporting the importance of VCAM-1 in the migration of eosinophils but not neutrophils.^{34, 37-39} The importance of VCAM-1 in eosinophil recruitment is implicated further by our results, which showed a significant increase in the expression of this molecule on nasal mucosal vascular endothelial cells 24 hours after antigen provocation and also its upregulation in the nasal mucosa of subjects with perennial allergic rhinitis, as observed by Montefort et al.¹⁹ The initial step may involve adherence of unstimulated eosinophils to activated endothelial cells via the VLA-4/VCAM-1 pathway. Previous studies suggested that IL-4 was capable of selectively inducing VCAM-1, but not ICAM-1 and E-selectin expression, on endothelial cells.^{16, 33} The expression of adhesion molecules in our *in vivo* studies is consistent with these observations, showing an increase in VCAM-1 expression, very modest increases in E-selectin, and no significant increase in ICAM-1 expression 24 hours after allergen challenge. This is consistent with a possible role for IL-4 in antigen-induced upregulation of VCAM-1 expression in the nasal mucosa.

Evidence supporting the presence and probable contribution of cytokines in regulating allergic inflammation was recently generated by *in situ* hybridization studies performed by Durham et al.⁴⁰ on nasal mucosal biopsy specimens. They

found significant increases in mRNA⁺ cells for IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor in nasal biopsy specimens obtained after antigen challenge, compared with specimens from normal control subjects. The number of these cells correlated positively with the number of activated eosinophils (EG2⁺). Activated T lymphocytes, among other cell types, are potential sources of these cytokines and can be identified by the expression of the IL-2 receptor (CD25). Human mast cells have also been reported to be a source of IL-4.⁴¹ Whether these or other cells are producing IL-4 after local intranasal antigen challenge remains to be determined.

In conclusion, our results confirm the previously reported constitutive expression of ICAM-1 on the vascular endothelium in nasal biopsy specimens. The percentage of vessels expressing VCAM-1 is upregulated 24 hours after allergen challenge, and E-selectin is modestly upregulated, but ICAM-1 is not. This is consistent with a role for IL-4 in the induction of these changes and suggests that these events are mediated through an IgE-dependent mechanism, because they were observed in allergic, but not in nonallergic, subjects after antigen challenge. The minimal expression of E-selectin in this study might be due to minimal induction of expression or to its known rapid induction and decline within hours, compared with the other two adhesion molecules. Because the counterligand for VCAM-1, VLA-4, is present on eosinophils, but not on neutrophils, we speculate that the upregulated VCAM-1 contributes, at least in part, to the selective recruitment of eosinophils into the nasal mucosa after antigen provocation and may therefore play an important role in the pathogenesis of allergic rhinitis.

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