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Eotaxin Stimulates Eosinophil Adhesion to Human Lung Microvascular Endothelial Cells

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Effects of the human C-C chemokines eotaxin, MIP-1α, and RANTES on human eosinophil or neutrophil adhesion to human lung microvascular endothelial cells (LMVEC) were investigated. Basal adhesion of unstimulated eosinophils to LMVEC was increased following pretreatment of LMVEC with TNFα (10ng/ml) for 6h. Stimulation of eosinophils with eotaxin (30 and 100ng/ml) resulted in increased adhesion to LMVEC pretreated with TNFα but not culture medium. Neutrophil adhesion was not increased by eotaxin under similar conditions. Neither MIP-1α (3-100 ng/ml) nor RANTES (3-100ng/ml) increased eosinophil or neutrophil adhesion to LMVEC pretreated for 6h with either TNFα (10ng/ml) or culture medium. Monoclonal antibodies (mAb) against eosinophil adhesion molecule VLA-4 (2B4; 30μg/ml) but not CD18 (6.5E; 10μg/ml) inhibited eotaxin-induced eosinophil adhesion to TNFα-activated LMVEC. 2B4 in combination with 6.5E reduced adhesion to basal levels. These data show that eotaxin, but not MIP-1α or RANTES, stimulates eosinophil adhesion to LMVEC and that this effect can be abolished by anti-VLA-4 and CD18 mAb in combination. These results suggest that eotaxin may facilitate eosinophil migration from blood vessels in the lung by increasing eosinophil adhesion to endothelial cells.

Eosinophils are considered to play a key role in perpetuating inflammatory changes and airway epithelial damage seen in asthma (1). Binding of eosinophils to endothelial cell adhesion molecules facilitates the migration of these cells from blood vessels into the respiratory tract (2, 3). A better understanding of how eosinophils are recruited from the microcirculation may have important implications for the development of new anti-asthma therapies (3). The chemokines are a family of secreted 8-10 KD proteins that attract leukocytes to inflammatory foci by: (i) activating leukocyte integrins thus promoting leukocyte-endothelial interaction; (ii) facilitating leukocyte trans-endothelial migration; (iii) establishing a chemotactic gradient (4, 5). Since leukocytes express multiple chemokine receptors and a given chemokine receptor binds multiple ligands, chemokines may attract several types of leukocytes (6). In contrast, the C-C chemokine eotaxin, is thought to be specific for eosinophils. Eotaxin was first identified as an eosinophil chemoattractant in a guinea pig model of asthma and is up-regulated in the lungs within 3h of antigen challenge (7, 8, 9). Purified eotaxin induces only eosinophil accumulation when administered to the skin or airways of naive guinea pigs (10). Recently the murine and human homologue of eotaxin have been identified and cloned (11-13) and human eotaxin has been shown to be directly chemotactic for eosinophils but not neutrophils or mononuclear cells (12, 13). Since eosinophil/endothelial interactions are an integral part of eosinophil migration from blood vessels, we questioned whether eotaxin might directly increase eosinophil adhesion to endothelial cells. In this study we have used an *in vitro* adhesion assay to investigate the effects of eotaxin on human eosinophil or neutrophil adhesion to human lung microvascular endothelial cells (LMVEC). We have also compared the effects on adhesion of other eosinophil chemoattractant C-C chemokines, macrophage inflammatory protein-1α (MIP-1α) or

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RANTES, and also C5a since these stimuli are known to induce migration and activation of human eosinophils (14).

MATERIALS AND METHODS

Materials. All reagents for culture of LMVEC were from TCS Biologicals Ltd (Buckingham, UK). Horse serum, foetal bovine serum (FBS), dimethyl sulphoxide (DMSO), Dulbecco's phosphate buffered saline (PBS) and gelatin were from Sigma (Poole, Dorset, UK). Percoll and dextran were from Pharmacia Biotech Ltd. (St. Albans, Herts. UK). Trisodium citrate and salts for Kreb's Ringer Phosphate Dextrose (KRPD) buffer were obtained from BDH Chemicals Ltd. (Poole, Dorset, UK). Human recombinant tumour necrosis factorα (TNFα) was obtained from Boehringer Mannheim UK (Lewes, East Sussex; specific activity $> 1 \times 10^8$ U/ml) and human eotaxin, RANTES and MIP-1α were from Peprotech EC Ltd. (London, UK). MACS CS separation columns and CD16 microbeads were purchased from Miltenyi Biotech (Camberely, Surrey, UK) and calcein-AM from Cambridge Bioscience (Cambridge, UK). 6.5E (anti-CD18) monoclonal antibodies (mAb) and the control antibody MOPC21 were gifts from Dr. M. Robinson, Celltech, Slough, UK. 2B4 (anti-VLA4) mAb was a gift from Dr. R. Pigott, British Biotechnology Ltd., Oxford, UK. C5a was a gift from Dr. J. J. van Oostrum, Ciba-Geigy, Summit, New Jersey, USA.

Cell culture. LMVEC, prepared by Clonetics (California, USA), were obtained as cryopreserved tertiary (3°) cultures from TCS Biologicals and were maintained in microvascular endothelial growth medium (EGM-MV), based on the MCDB 131 formulation and supplemented with 10ng/ml human recombinant epidermal growth factor, 1μg/ml hydrocortisone, 5% heat-inactivated FBS, 50μg/ml gentamicin, 50 ng/ml amphotericin-B and bovine brain extract containing 12μg/ml protein and 10μg/ml heparin. LMVEC were cultured for up to 12° without significant alteration in magnitude of TNFα-induced ICAM-1, VCAM-1 or E-selectin expression (Blease, Hellewell & Burke-Gaffney; unpublished observation). LMVEC used in this study were between 5°-8°. Confluent cells were washed with Hanks balanced salt solution (HBSS), trypsinised using 0.025% trypsin + 0.01% EDTA and collected into trypsin neutralizing solution. Cells were seeded at a density of 3.2×10^3 cells per well onto gelatin-coated Nunclon 96-well plates.

Isolation of human peripheral blood eosinophils or neutrophils. Granulocytes were isolated from peripheral blood of mildly atopic adult donors by the method of Haslett *et al.,* (15) as described by Burke-Gaffney & Hellewell (16). The granulocyte band was collected, washed in PPP and resuspended in an appropriate volume of KRPD (4.8mM KCl; 3.1mM NaH_2PO_4; 12.5mM Na_2HPO_4; 5% v/v glucose; 2% v/v FBS) to give a 1/10 dilution of anti-CD16 microbeads when 40μl of beads were added per 10^8 granulocytes, and incubated on ice for 45 min. A type-CS MACS separation column was set up in a magnetic separator and microbead-labelled granulocytes applied to the top of the column. Eosinophils (>97% pure) were eluted in 35ml KRPD and labelled (30 min, 37°C) with Calcein-AM (10μM dissolved in 1% DMSO in KRPD without FBS). Cells were washed twice in KRPD and resuspended at 1.25×10^6 cells per ml in KRPD containing 0.93mM $CaCl_2$ and 1.2 mM $MgSO_4$ for the adhesion assay. Neutrophils (>98% pure) were isolated from peripheral blood of normal adult donors (15, 16) and labelled with calcein-AM as described above for eosinophils.

Measurement of eosinophil or neutrophil adhesion to lung microvascular endothelial cells. LMVEC grown on 96-well plates were pre-treated with TNFα (10ng/ml) for 6h. LMVEC express basal ICAM-1 which is increased by TNFα; VCAM-1 and E-selectin which are not expressed under resting conditions were also induced (17). Cells were washed 3 $\times$ with PBS (containing Ca^{2+} and Mg^{2+}) to remove TNFα before carrying out the adhesion assay. One hundred microlitres of calcein-AM-labelled eosinophils or neutrophils and 50μl of KRPD or mAb (4$\times$ final concentration) and 50μl of chemokine, C5a or buffer were added per well and the plate was incubated at 37°C for 30 min. Fluorescence was measured using a Biolite F1 plate reader (excitation wavelength of 485±20nm and emission wavelength of 530±25nm) before and after washing the plate to remove non-adherent cells by two gentle washes with PBS containing 1% horse serum. Percent adhesion was expressed as fluorescence after washing the plate divided by fluorescence before washing plate $\times$ 100.

Statistics. All values are means ± S.E.M. of n experiments. Analysis was by one-way ANOVA followed by Dunnett's multiple comparison test which compares all test values to control, unless otherwise stated, and $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of C-C Chemokines on Eosinophil Adhesion to LMVEC

Basal adhesion of eosinophils to untreated LMVEC was 9.2±1.4% (n=7) and was not significantly increased in the presence of eotaxin (3-100ng/ml; Fig 1a). Activation of LMVEC with TNFα (10ng/ml, 6h) increased adhesion of unstimulated eosinophils (31.0±3.2%, n=7; Fig 1a). Eosinophil adhesion to TNFα-activated LMVEC was significantly increased following exposure of eosinophils, during the adhesion assay (30 min), to eotaxin at concentrations of 30ng/ml (46.7±3.9%, n=7; $P<0.05$) or 100ng/ml (51.4±2.6%, n=7; $P<0.01$; Fig 1a). C5a

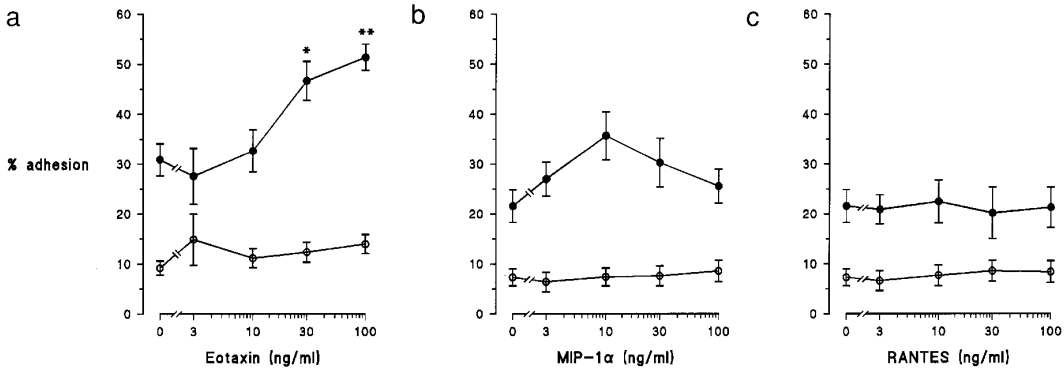

FIG. 1. Eotaxin but not MIP-1α or RANTES stimulate eosinophil adhesion to cultured lung microvascular endothelial cells. Adhesion of unstimulated or chemokine-stimulated eosinophils to LMVEC exposed to culture medium (open circle) or TNFα (10ng/ml; solid circle) for 6h. Data are expressed as the mean ± SEM of 5-7 separate experiments. *, **, $P<0.05$ or 0.01 compared to eosinophil adhesion to TNFα-activated LMVEC in the absence of chemokine.

(10^{-7}M) significantly increased eosinophil adhesion to unactivated (17.0±2.8, n=5; $P<0.05$) or TNFα-activated LMVEC (46.3±3.8%, n=5; $P<0.05$). Neither MIP-1α or RANTES (3-100ng/ml) significantly increased eosinophil adhesion to untreated or TNFα-activated LMVEC (Fig 1b, c).

Effects of C-C Chemokines on Neutrophil Adhesion to LMVEC

Basal adhesion of neutrophils to untreated LMVEC was 6.9±1.8% (n=5) and was not significantly increased by eotaxin (3-100ng/ml; Fig 2a). Activation of LMVEC with TNFα (10ng/ml, 6h) increased adhesion of unstimulated neutrophils (27.9±5.4%, n=5) however, eotaxin (3-100ng/ml) did not further increase neutrophil adhesion (Fig 2a). In contrast, C5a (10^{-7}M) significantly increased neutrophil adhesion to LMVEC from a basal value of 5.0±0.7% (n=5) to 12.0±2.5% and significantly increased adhesion to TNFα-activated LMVEC from 18.9±9.0 to 33.5±8.7% (n=5). In addition, neither MIP-1α nor RANTES (3-100ng/ml) significantly increased neutrophil adhesion to LMVEC (unactivated or pretreated with TNFα; Fig 2b, c).

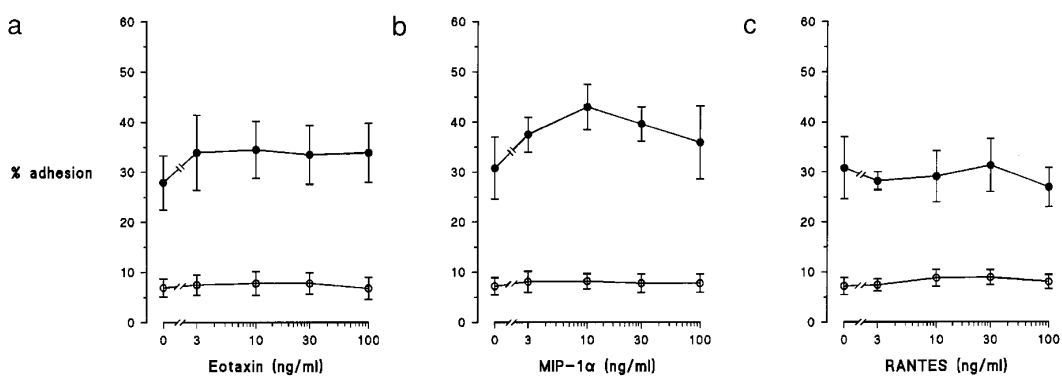

FIG. 2. Eotaxin, MIP-1α, or RANTES do not stimulate neutrophil adhesion to cultured lung microvascular endothelial cells. Adhesion of unstimulated or chemokine-stimulated neutrophils to LMVEC exposed to culture medium (open circle) or TNFα (10ng/ml; solid circle) for 6h. Data are expressed as the mean ± SEM of 5 separate experiments.

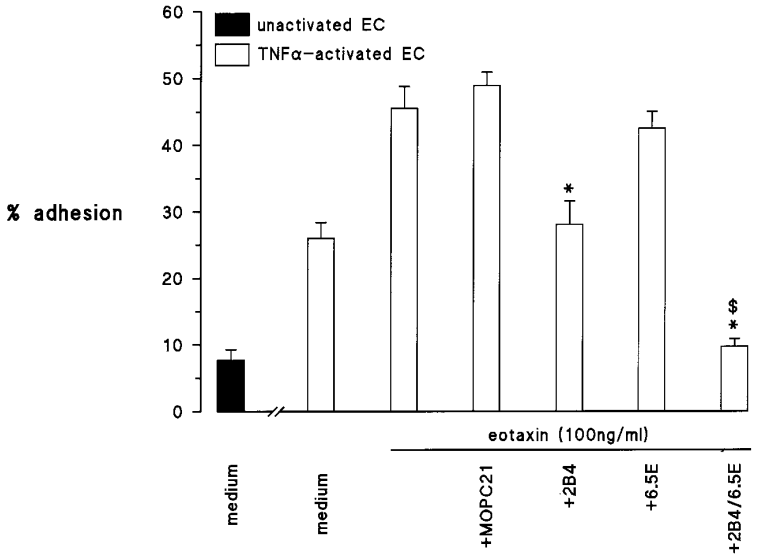

FIG. 3. Effect of anti-VLA-4 and CD18 monoclonal antibodies on eotaxin-stimulated eosinophil adhesion to TNFα-activated LMVEC. Adhesion of eotaxin (100ng/ml)-stimulated eosinophils to TNFα-activated LMVEC (10ng/ml, 6h) in the presence of a control antibody MOPC21 (40μg/ml), anti-VLA-4 (2B4; 30μg/ml), anti-CD18 mAb (6.5E, 10μg/ml), or 2B4 in combination with 6.5E). Data are expressed as the mean $\pm$ SEM of 5 separate experiments. * $P<0.001$ compared to MOPC21 control antibody and $ $P<0.001$ compared to 2B4 antibody alone.

Effect of anti-CD18 and anti-VLA-4 Monoclonal Antibodies on Eotaxin-Mediated Eosinophil Adhesion

Eotaxin (100ng/ml)-induced eosinophil adhesion to TNFα-activated LMVEC (45.5$\pm$3.3%, n=5) was significantly reduced, compared to a control antibody (MOPC21, 40μg/ml), by anti-VLA4 mAb (2B4, 30μg/ml; $P<0.001$; n=5) to levels observed with unstimulated eosinophils adhering to TNFα-activated LMVEC (Fig 3). An anti-CD18 mAb (6.5E; 10μg/ml) had no significant effect on eotaxin-mediated eosinophil adhesion (Fig 3). A higher concentration of 6.5E (30μg/ml) also had no significant effect (data not shown). A combination of 2B4 and 6.5E reduced adhesion to 9.7$\pm$1.2% which was not significantly different from adhesion of unstimulated eosinophils to unactivated LMVEC (Fig 3).

DISCUSSION

Our results show that the human C-C chemokine eotaxin, which is a selective chemoattractant for human eosinophils (12, 13), also induced eosinophil but not neutrophil adhesion to TNFα-activated LMVEC monolayers in culture. Eosinophil adhesion to endothelial cells plays a key role in eosinophil migration from blood vessels (2, 3) and eotaxin may therefore contribute to this process by increasing eosinophil adhesion in addition to inducing chemotaxis and transendothelial migration (12, 13). A selective increase in eosinophil adhesion may play a key role in triggering eosinophil accumulation *in vivo* reported following administration of eotaxin to the skin or airways of naive guinea-pigs (10) or to the skin of rhesus monkeys (13). C5a-induced adhesion to TNFα-activated LMVEC was similar to that induced by eotaxin. C5a but not eotaxin, also increased adhesion to unactivated LMVEC. This suggests that eotaxin may not induce changes in eosinophil adhesion molecule expression or activation that would facilitate binding to basal ICAM-1 expressed on LMVEC. In contrast, C5a has been reported to increase expression of CD11b (18) and this may contribute to C5a-stimulated eosinophil adhesion to unactivated LMVEC.

Eotaxin-induced eosinophil adhesion was reduced by an anti-VLA4 mAb (2B4) to levels of unstimulated eosinophil adhesion to TNFα-activated LMVEC. Adhesion was reduced to basal adhesion by 2B4 in combination with the anti-CD18 mAb (6.5E), although 6.5E alone had no effect on adhesion. The lack of effect of anti-CD18 mAb alone on eosinophil adhesion suggests adhesion may become CD18 independent when the CD18 pathway is blocked. A similar compensatory effect has been shown for chemokine-induced monocyte migration across IL-1-activated endothelium (19). These results may also explain, in part, the observation that neutrophil but not eosinophil migration is inhibited in patients suffering from leukocyte adhesion deficiency disease in whom leukocyte CD18 is absent or dysfunctional (20). It is not clear whether eotaxin alters VLA-4-mediated eosinophil adhesion. We have shown that eotaxin does not increase VLA-4 expression (unpublished observation, ABG & PGH) but whether eotaxin increases activation by changing VLA-4 from a low to a high affinity state, in a manner similar to that described for manganese (21), remains to be demonstrated.

Other C-C chemokines, RANTES and MIP-1α, which are known to induce monocyte and lymphocyte in addition to eosinophil chemotaxis and/or transendothelial migration (12-14, 19, 22, 23), did not increase eosinophil or neutrophil adhesion to TNFα-activated LMVEC in our study. Previous reports have shown that RANTES causes an increase in eosinophil adhesion to plastic coated with soluble ICAM-1 without an increase in CD11b or CD18 expression (24). Another study showed a small increase in CD11b or CD18 expression following stimulation with RANTES, but a functional effect on adhesion was not investigated (22). The extent of the functional significance of eotaxin- but not RANTES- or MIP-1α-induced eosinophil adhesion is not clear since these chemokines have all been shown to induce eosinophil recruitment *in vivo* (13, 25, 26). It is possible that eotaxin, but not other C-C chemokines, may induce eosinophil adhesion to airway epithelial cells, in addition to endothelial cells, and in this manner exacerbate eosinophil-mediated airway epithelial damage seen in asthma (1) and this is subject to further investigation. In conclusion, eotaxin increased eosinophil but not neutrophil adhesion to TNFα-activated LMVEC and this may play an important role in triggering selective eosinophil recruitment into the lungs. Inhibition of eotaxin-induced eosinophil adhesion may provide a selective therapeutic approach to reducing eosinophil recruitment in diseases such as asthma.

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REFERENCES

1. Busse, W. W., and Sedwick, J. B. (1992) *Ann. Allergy* **68,** 286–290.
2. Resnick, M. B., and Weller, P. F. (1993) *Am. J. Respir. Cell Mol. Biol.* **8,** 349–355.
3. Teixeira, M., Williams, T. J., and Hellewell, P. G. *Trends Pharmacol. Sci.* **16,** 418–423.
4. Baggiolini, M., Dewald, B., and Moser, B. (1994) *Adv. Immunol.* **55,** 97–179.
5. Furie, M. B., and Randolph, G. J. (1995) *Am. J. Pathol.* **146,** 1287–1301.
6. Wells, T. N., Power, C. A., Lusti-Narasimhan, M., Hoogewerf, A. J., Cooke, R. M., Chung, C. W., Peitsch, M. C., and Proudfoot, A. E. (1996) *J. Leuk. Biol.* **59,** 53–60.
7. Jose, P. J., Griffith-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Totty, N. F., Truong, O., Hsuan, J. J., and Williams, T. J. (1994) *J. Exp. Med.* **179,** 881–887.
8. Jose, P. J., Adcock, I. M., Griffiths-Johnson, D. A., Berkman, N., Wells, T. N., Williams, T. J., and Power, C. A. (1994) *Biochem. Biophys. Res. Comm.* **205,** 788–794.
9. Rothenberg, M. E., Luster, A. D., Lilly, C. M., Drazin, J. M., and Leder, P. (1994) *J. Exp. Med.* **181,** 1211–1216.
10. Griffiths-Johnson, D. A., Collins, P. D., Rossi, A. G., Jose, P. J., and Williams, T. J. (1993) *Biochem. Biophys. Res. Commun.* **197,** 1167–1172.
11. Rothenberg, M. E., Luster, A. D., and Leder, P. (1995) *Proc. Natl. Acad. Sci. USA.* **92,** 8960–8964.

12. Garcia-Zepeda, E. A., Rothenberg, M. E., Ownbey, R. T., Celestin, J., Leder, P., and Luster, A. D. (1996) *Nature Med.* **2,** 449–456.
13. Ponath, P. D., Qin, S., Ringler, D. J., Clark-Lewis, I., Wang, J., Kassam, N., Smith, H., Shi, X., Gonzalo, J-A., Newman, W., Gutierrez-Ramos, and Mackay, C. R. (1996) *J. Clin. Invest.* **97,** 604–612.
14. Rot, A., Krieger, M., Brunner, T., Bischoff, S. C., Schall, T. J., and Dahinden, C. A. (1992) *J. Exp. Med.* **176,** 1489–1495.
15. Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnson, R. B. Jr., and Henson, P. M. (1985) *Am. J. Pathol.* **164,** 714–721.
16. Burke-Gaffney, A., and Hellewell, P. G. (1996) *Am. J. Physiol.* **270,** C552–C561.
17. Shen, J., Ham, R. G., and Karmiol, S. (1995) *Microvascular Res.* **50,** 360–372.
18. Smith, J. B., Kunjummen, R. D., and Raghavender, B. H. (1991) *Pediatric. Res.* **30,** 355–361.
19. Chuluyan, H. E., Schall, T. J., Yoshimura, T., and Issekutz, A. C. (1995) *J. Leuk. Biol.* **58,** 71–79.
20. Anderson, D. C., and Springer, T. A. (1987) *Ann. Rev. Med.* **38,** 175–194.
21. Werfel, S. J., Yednock, T. A., Matsumoto, K., Sterbinsky, S. A., Schleimer, R. P., and Bochner, B. S. (1996) *Am. J. Respir. Cell Mol. Biol.* **14,** 44–52.
22. Alam, R., Stafford, S., Forsythe, P., Harrison, R., Faubion, D., Lett-Brown, M. A., and Grant, J. A. (1993) *J. Immunol.* **150,** 3442–3447.
23. Ebisawa, M., Yamad, T., Bickel, C., Klunk, D., and Schleimer, R. P. (1994) *J. Immunol.* **153,** 2153–2160.
24. Kakazu, T., Chihara, J., Saito, A., and Nakakajima, S. (1995) *Int. Arch. Allergy Immunol.* **108,** 9–11.
25. Lukas, N. W., Strieter, R. M., Shaklee, C. L., Chensue, S. W., and Kunkel, S. L. (1995) *Eur. J. Immunol.* **25,** 245–251.
26. Meurer, R., van Riper, G., Feeney, W., Cunningham, P., Hora, D., Springer, M. S., MacIntyre, D. E., and Rosen, H. (1993) *J. Exp. Med.* **178,** 1913–1921.