

IN the allergic mucosa, there is a significant increase in numbers of CD25⁺ cells and activated eosinophils. To determine whether a link exists between the activated T-lymphocytes and tissue eosinophils in nasal allergy, we studied CD25⁺ cells in the nasal mucosa and compared the levels of soluble IL-2 receptor (sIL-2R) both in the serum and the nasal secretions, and further investigated expression of CD11b on eosinophils in the nasal lavage fluids and peripheral blood of patients with nasal allergy. We also examined the effects of the culture supernatant of Con A- and IL-2-activated T-lymphocytes on CD11b expression on eosinophils in the present study. The concentration of sIL-2R in the nasal secretions from patients with Japanese cedar pollinosis (JCP) was significantly higher than that from normal subjects ($p < 0.01$). The sIL-2R level was significantly higher in the nasal secretions than in the sera in patients ($p < 0.01$), and CD11b expression on eosinophils from nasal lavage fluid was significantly higher than that of eosinophils from peripheral blood of the same individuals ($p < 0.01$). The activated T-lymphocytes promoted eosinophil activation with up-regulation of CD11b *in vitro*, and eosinophils in the nasal secretions from patients significantly expressed more CD11b *in vivo*. These results indicate that activation of T-lymphocytes is linked to eosinophil activation in nasal allergy.

Key words: CD11b, Eosinophil, Nasal allergy, Soluble interleukin-2 receptor, T-lymphocyte

CD25⁺ T-lymphocytes induce CD11b on eosinophils in allergic nasal mucosa

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Introduction

Eosinophil infiltration into lesions is a hallmark of IgE-mediated allergic reactions in the lungs, nose and skin.¹ Eosinophils are activated in allergic lesions and release cytotoxic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and also leukotriene C₄ (LTC₄) and platelet-activating factor (PAF) which have been shown to lead to nonspecific hyper-responsiveness of the airway mucosa by causing mucosal epithelial dysfunction and/or damage.²⁻⁴ Eosinophils predominantly infiltrate into the tissue during allergen-induced late-phase responses suggesting the selective activation of eosinophils.⁵ However, the mechanisms of eosinophil activation in nasal allergy have not yet been elucidated fully.

To date, it has been clarified that the process of the tissue localization of eosinophils is mediated by families of cell adhesion molecules that are expressed on both eosinophils and vascular endothelial cells. The expression of adhesion molecules on eosinophils might be modulated by cytokines and/or chemical mediators released from some inflammatory cells. In the allergic mucosa, recent reports have demonstrated that there were significant increases in numbers of CD25⁺ cells

(interleukin-2 receptor-bearing cells, presumably activated T-lymphocytes) and activated eosinophils,^{6,7} while the dominant cells in the normal nasal mucosa have been reported to be CD3⁺, CD4⁺ and CD25⁻ lymphocytes. These reports suggest a relationship between these activated T-lymphocytes and activated eosinophils. Recent studies have suggested a possible relationship between IL-2 and IL-5 in the plasma of rodents and humans with eosinophilia.⁸ On the other hand, elevated levels of soluble form IL-2 receptor (sIL-2R) in the serum of patients with nasal allergy and bronchial asthma,⁹ and also nasal lavages¹⁰ were reported, suggesting the activation of T-lymphocytes in IgE-mediated allergic diseases.

Recently, CD11b was reported to be a marker for the degree of functional activation of eosinophils.¹¹ To determine whether a correlation exists between the activated T-lymphocytes and tissue eosinophils in nasal allergy, we studied CD25⁺ cells in the nasal mucosa and measured sIL-2R levels in both the serum and nasal secretions. We also examined the effects of the culture supernatant of ConA- and IL-2-activated T-lymphocytes (T-cell sup.) on CD11b expression on eosinophils in the present study and further investigated the expression of CD11b on eosinophils separated from the nasal lavage fluids and peripheral blood of nasal allergy patients.

Materials and Methods

Monoclonal antibodies: The monoclonal antibodies (mAbs) for immunocytometric analysis, Leu-15 (anti-CD11b), Leu-11a (anti-CD16), Leu-11c (anti-CD16), anti-CD25, Leu-4 (anti-CD3), mouse IgG1 and mouse IgG2a for isotype control were purchased from Becton Dickinson (Mountain View, CA, USA).

Cytokines and reagents: Recombinant human IL-2 (TGP-3) was kindly donated by Takeda Chemical Industries, Co., Ltd (Tokyo, Japan).

Subjects: Healthy blood donors ($n = 9$) in whom atopic dermatitis, asthma and allergic rhinitis were excluded by history, intracutaneous reaction and/or scratch tests, and IgE radioallergosorbent tests (RAST) volunteered to act as controls. Fourteen adult patients with Japanese cedar pollinosis (JCP) who had no other inflammatory diseases participated in the present study. All patients received no medication for at least 2 weeks prior to collection of blood and nasal lavage fluid. All volunteers and patients gave informed consent. The diagnosis of JCP was based on: (1) a history of sneezing attacks, watery rhinorrhoea and nasal obstruction during the cedar pollen season; (2) positive skin test with Japanese cedar pollen extract and negative with house dust or mite allergen; and (3) elevation of specific serum IgE shown by IgE RAST.

Measurement of sIL-2R in the sera: Blood samples were obtained from allergic patients with JCP during the pollen season. Soluble IL-2R levels were measured in both serum and nasal secretions by an enzyme-linked immunosorbent assay (ELISA) using a Cellfree Interleukin-2 Receptor Bead Assay Kit (T Cell Diagnostics, Cambridge, MA, USA). Briefly, sera were assayed for sIL-2R levels by a double epitope ELISA method according to the manufacturer's protocol.

Measurement of sIL-2R in the nasal secretions: Twenty ml of physiological saline with 1 μ M LiCl was used for the nasal lavage and the recovered fluids were stored at -80°C after separation of the cell components by centrifugation at $300 \times g$ for 10 min at 4°C . The concentration of lithium in nasal lavage fluid was used as an exogenous marker and measured by atomic emission spectrophotometry for calculation of sIL-2R concentration in the nasal secretions.¹² The following formula was used:

$$sIL-2R = sIL-2R_n \times Li_0 / (Li_0 - Li_n)$$

where $sIL-2R$ = concentration of sIL-2R in the nasal secretions; $sIL-2R_n$ = concentration of sIL-2R in the lavage fluid; Li_0 = Li concentration of the 0.9% NaCl solution used for washing; and Li_n = Li concentration in the lavage fluid.

Preparation of ConA- and IL-2-stimulated T lymphocyte conditioned medium: Normal human

peripheral blood lymphocytes were prepared by Ficoll-Hypaque (mono-poly resolving medium, ICN) density gradient centrifugation at $450 \times g$ for 30 min at 18°C . The lymphocytes were collected and washed three times with RPMI1640 (Cosmo Bio, Tokyo, Japan) and resuspended in RPMI1640 containing 10% FCS (Cosmo Bio, Tokyo, Japan) and 100 U/ml of penicillin and 100 mg/ml of streptomycin (RPMI1640 medium) for 3 h at 37°C . After removal of adherent cells, the cells were cultured at a concentration of 5×10^6 cells/ml in RPMI1640 medium containing Con A (2 μ g/ml) for 9 h, washed three times, and then the cells were cultured with 100 U/ml of IL-2 for 20 h. The culture supernatant (T-cell sup.) was collected and stored at -80°C until use.

Determination of eosinophil CD11b expression:

Cell culture. Peripheral blood granulocytes from normal subjects were separated by Ficoll-Hypaque centrifugation from 20 ml of heparinized blood. After three washes, granulocytes were transferred to tissue culture plates (Costar, Cambridge, MA) at a final concentration of 1×10^5 cells/ml. Cells were maintained in RPMI1640 medium or the T-cell sup. under defined conditions in a 100% humidified atmosphere containing 5% CO_2 at 37°C for 3 h. After incubation, the granulocytes were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4) containing 0.1% NaN_3 (PBS-azide). After the second centrifugation, the cell pellet was resuspended in 20 μ l of phycoerythrin (PE)-conjugated Leu-15 and 20 μ l of fluorescein isothiocyanate (FITC)-conjugated Leu-11a (anti-CD16) for 30 min at 4°C . A PE-conjugated mAb of the same IgG subclass as Leu-15 was used as a control for nonspecific fluorescence. After incubation with the mAb, the cells were washed twice with 1 ml of PBS-azide and were resuspended in 400 μ l of PBS-azide containing 1% paraformaldehyde for flow cytometric analysis.

Flow cytometry. The expression of CD11b on human neutrophils and eosinophils was determined by direct immunofluorescence with a PE-conjugated mAb (Leu-15) against CD11b and applied to flow cytometry (Epics Profile, Coulter Corp., Hialeah, FL) with laser excitation at 488 nm. Neutrophils were analysed as CD16-positive gated cells and eosinophils as CD16-negative cells. A minimum of 10 000 cells were analysed on the same day.

Analysis of data: Specific fluorescence associated with binding of antibody to the CD11b was calculated by subtracting the mean fluorescence intensity (MFI) of the cells stained with the subclass control mAbs from the MFI of cells stained with specific mAb. After conversion of the MFI from a logarithmic to a linear value, the increase in the level of eosinophil CD11b expression was calculated according to the following formula:

Increase in CD11b expression index (ΔMC) = MFI of stimulated eosinophils – MFI of control

Statistical analysis: Statistical analysis was performed by using Student's *t*-test for paired and unpaired data and by using Wilcoxon's rank-sum test. A *p* value of < 0.05 was considered statistically significant.

Results

Levels of sIL-2R in the sera and nasal secretions: sIL-2R concentrations of the normal sera from healthy subjects and allergic patients were 334.1 ± 142.6 U/ml (mean \pm S.D., $n = 9$) and 432.4 ± 110.2 ($n = 14$), respectively. The difference was not statistically significant. The mean values of sIL-2R concentrations in the nasal secretions of allergy patients and control subjects were 1367.1 ± 951.4 U/ml ($n = 14$) and 371.1 ± 182.1 U/ml ($n = 9$), respectively (Fig. 1). The sIL-2R concentration in the nasal secretions of the patients was significantly higher than that of control subjects ($p < 0.01$).

Effects of CD25-positive cells on CD11b expression on eosinophils: Flow cytometric analysis revealed that only 2% of T-lymphocytes in peripheral blood expressed CD25. Con A- and IL-2-supplemented medium led to an increase in CD25-expressing T lymphocytes up to 54.3% (Fig. 2a). T-cell sup. significantly enhanced CD11b expression on eosinophils ($n = 4$, Fig. 2b).

Levels of CD11b expression on eosinophils in the peripheral blood and in nasal lavage fluids: Four allergic patients participated in this study. CD11b expression on eosinophils from the allergic nasal lavage fluid was significantly increased compared with that of eosinophils from the peripheral blood ($p < 0.01$) (Fig. 3a). The mean values of MFI of eosinophils from the peripheral blood and the nasal

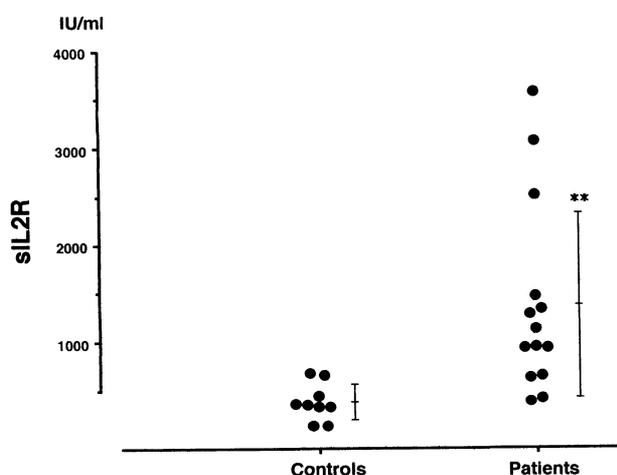


FIG. 1. Levels of sIL-2R in nasal secretions from patients with Japanese cedar pollinosis. The concentration of sIL-2R in nasal secretions from JCP patients was significantly higher than that from normal subjects (** $p < 0.01$). The sIL-2R level was significantly higher in the nasal secretion than in the sera.

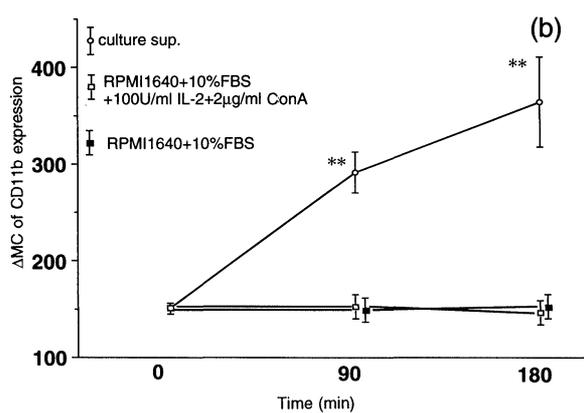
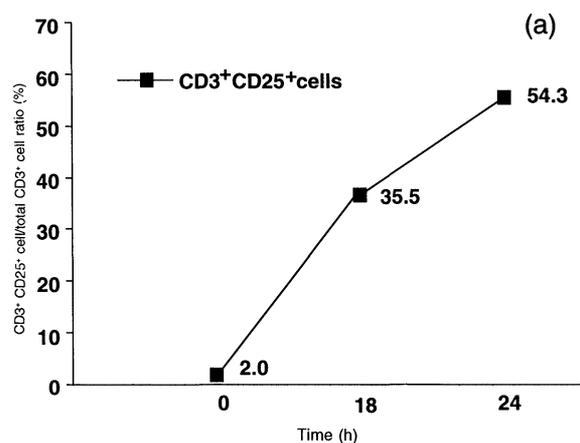


FIG. 2. (a) CD3⁺CD25⁺ cells/whole CD3⁺ cell ratio. T-lymphocytes were collected from peripheral blood of normal subjects and cultured with Con A and IL-2. After 18 h incubation, 35.5% of T-lymphocytes expressed CD25. After 24 h incubation, 54.3% of T-lymphocytes expressed CD25. We used this culture supernatant to stimulate eosinophils. (b) ΔMC of eosinophils stimulated with ConA activated T-lymphocyte supernatant. The Con A activated T-lymphocyte culture supernatant significantly enhanced CD11b expression on eosinophils. However, IL-2 only could not induce CD11b expression. (** $p < 0.01$.)

lavage fluid were 168.6 ± 18.2 ($n = 4$) and 343 ± 109.2 ($n = 4$), respectively. Typical fluorescence intensities are shown in Fig. 3b. Eosinophils were collected from the same individuals.

Discussion

Nonspecific hyper-responsiveness of the nasal mucosa along with eosinophil infiltration is a distinct clinical feature of nasal allergy,¹⁻³ resulting in persistent nasal symptoms which consist of recurrent sneezing attacks, watery nasal discharge and nasal obstruction. Eosinophils are involved in the mechanisms of nonspecific hyper-responsiveness of the nasal mucosa releasing cytotoxic granule cationic proteins. PAF and LTC₄ presumably cause mucosal epithelial dysfunction and/or damage.

Recently, it was demonstrated immunohistochemically that the number of CD3⁺ and/or CD25⁺ cells increased in allergic nasal mucosa.⁶ On the other hand, in non-allergic nasal mucosa, the

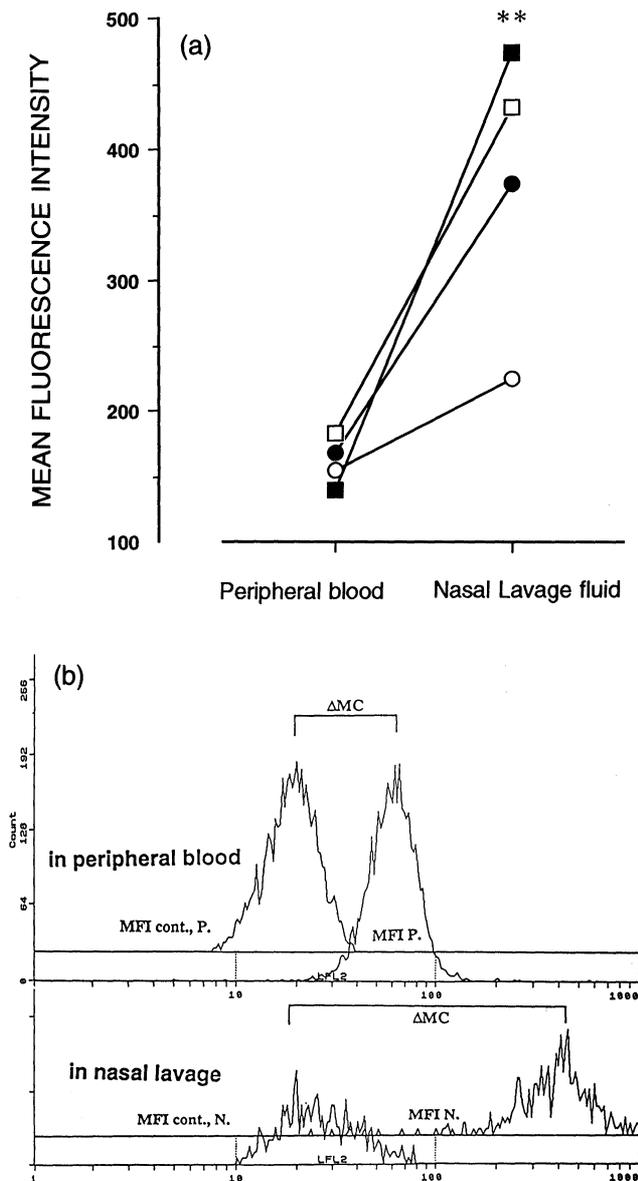


FIG. 3. (a) The ΔMC of eosinophils from peripheral blood and nasal lavage fluid. The ΔMC of eosinophils from the nasal lavage fluid was significantly higher than that of peripheral blood eosinophils from the same individuals (** $p < 0.01$). (b) Typical fluorescence intensity histogram of eosinophils from peripheral blood and from nasal lavage fluid. The CD11b expression on eosinophils from nasal lavage fluid was significantly higher than that of eosinophils from peripheral blood of the same individuals. ΔMC denoted the increase in the mean fluorescence intensity (MFI). MFI cont., P. and MFI cont., N. denote MFI of peripheral blood eosinophils and eosinophils from the nasal lavage fluids which were stained with mouse IgG2a, respectively. MFI P. and MFI N. denote MFI of the above eosinophils which were stained with mAb against CD11b respectively. MFI cont., P = MFI of peripheral eosinophils stained with isotype control. MFI cont., N = MFI of tissue eosinophils stained with isotype control mAb. MFI P = MFI of peripheral eosinophils stained anti-CD11b mAb. MFI N = MFI of tissue eosinophils stained anti-CD11b mAb.

dominant cells are CD3⁺CD25⁻ lymphocytes, and moreover, CD3⁺CD25⁺ lymphocytes were very rare in the population of CD3⁺ cells.¹³ It was also reported that a significant increase in the ratio of CD4⁺/CD8⁺ lymphocytes was observed in nasal biopsy specimens but not in peripheral blood after challenge with allergen.⁶ Immunohistochemical studies have also shown a significant increase in the number of CD25⁺ and CD4⁺ cells, and a significant correlation between

CD4⁺ cells and CD25⁺ cells in the allergic nasal mucosa after allergen exposure.¹⁴ Consequently, these reports suggest activation of helper T-lymphocytes in the allergic nasal mucosa.

sIL-2R is the shed extracytoplasmic component of the α chain subunit of the IL-2 receptor, and possibly an indicator of activation of T-lymphocytes in nasal allergy.¹⁰ We demonstrated a significant elevation of sIL-2R level in the nasal secretions from patients with JCP compared with that of the sera which was not significantly different from the value in sera obtained from normal subjects. In other words, the activation of T-lymphocytes possibly localized only in the nasal mucosa could be quantitatively demonstrated in JCP.

Recent reports have shown that the administration of recombinant IL-2 to human subjects caused systemic eosinophilia and significant elevation of the serum sIL-2R level,¹⁵⁻¹⁷ and induction of IL-5 was also reported.¹⁸⁻²¹ However, T-lymphocyte-induced eosinophil activation has not been clearly elucidated in IgE-mediated allergy. In the present study, some cytokines such as IL-5, IL-3 and GM-CSF might be released from the activated T-lymphocytes, which could take part in the eosinophil activation with CD11b upregulation.^{11,21} This is the potential mechanism of the *in vitro* CD11b up-regulation by activated T-lymphocytes and the *in vivo* CD11b up-regulation observed in allergic patients.

To date, studies of eosinophil activation have mainly used the following indicators: (1) eosinophil chemotaxis; (2) determination of EG-2 antigen-positive cells; (3) transmigration or adherent cell count; and (4) cell survival. It was recently shown that the determination of CD11b on eosinophils may be a useful marker for the degree of functional activation. Studies examining purified leukocyte subtypes binding to cultured human umbilical vein endothelial cells (HuVEC) *in vitro* have suggested that CD11b up-regulation on eosinophils is a marker of activation rather than enhancement of adhesion capacity.^{11,22} If CD11b is used at the time of transmigration, eosinophils must shed this molecule. However, transmigration through the HuVEC layer caused up-regulation of CD11b on eosinophils. Up-regulation of CD11b expression causes increased capacity to generate an oxidative burst in response to opsonized zymosan. Thus, CD11b on eosinophils may be a useful marker for flow cytometric analysis of the degree of functional activation. CD11b is rapidly mobilized from cytoplasmic stores, and up-regulation does not require *de novo* protein synthesis.^{23,24} In the present study, we measured enhancement of CD11b levels to clarify whether a correlation exists between the activation of T-lymphocytes and tissue eosinophils. The conditioned media from CD25⁺ cell-rich cultures showed an augmenting effect on CD11b expression on eosinophils. In JCP patients,

we showed that eosinophils from nasal lavage fluid expressed more CD11b than those from the peripheral blood.

In conclusion, we demonstrated the presence of activated T-lymphocytes in the nasal mucosa of nasal allergy patients with JCP along with the elevation of sIL-2R level of the nasal secretions but not in peripheral blood. The activated T-lymphocytes promoted eosinophil activation with up-regulation of CD11b *in vitro*, and eosinophils in the nasal secretions expressed significantly higher levels of CD11b. These results indicate that the activation of T-lymphocytes is linked to eosinophil activation and accumulation in allergic nasal mucosa.

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