also significantly induced IL-4 secretion by basophils. As shown in Fig. 2*D*, time-course analyses showed a gradual increase of IL-4 in the supernatants of IL-33-stimulated basophils, reaching a plateau at 24 h. Furthermore, we assessed whether IL-33 affects IL-4 secretion from basophils stimulated by IL-3 or IgE-crosslinkage. As shown in Fig. 2*E*, IL-33 enhanced IL-4 secretion from basophils stimulated with IL-3. IL-4 production from basophils stimulated with CRA-1 mAb was also potently augmented by IL-33. We also tested for basophil LTC4 synthesis in response to IL-33, but only weak LTC4 secretion, below significant levels, was observed in IL-33-treated basophils (data not shown).

IL-33 enhances adhesiveness of human basophils

Next, we analyzed the adhesiveness of human basophils using Percoll-separated cell preparations. Plastic plates were coated with BSA and tested for basophil adhesion. As shown in Fig. 3*A*, in the presence of IL-33 at 100 ng/ml, a significantly increased number of basophils adhered to the plates compared with the baseline level of adhesion. Unexpectedly, the adhesion-inducing effect of IL-33 was much stronger than that of 300 pM IL-3. Similarly, IL-33 at 10–100 ng/ml significantly induced adhesion of human basophils to fibronectin-, ICAM-1- and VCAM-1-coated microplates, and, again, those effects were more potent than those of 300 pM IL-3 (Fig. 3, *B–D*). On the other hand, IL-1β and IL-18 failed to affect basophil adhesion to plates coated with fibronectin, ICAM-1 or VCAM-1.

IL-33 up-regulates CD11b expression on human basophils

Percoll-separated basophils were used to study the effect of IL-33 on basophil CD11b expression (Fig. 4, *A* and *B*). Consistent with previous reports (14), CD11b expression was markedly up-regulated by IL-3 at 300 pM (74 ± 8.7% above baseline, $p < 0.01$). IL-33 also significantly enhanced surface CD11b expression by basophils, although this enhancement was slightly weaker than that by 300 pM IL-3. This effect of IL-33 was dose-dependent, and the EC50 of IL-33 in terms of enhancement of basophil CD11b expression was approximately 1 ng/ml, which corresponds to 33 pM on a molar basis. The effect reached a plateau at 10–100 ng/ml of IL-33 (Fig. 4*C*). In the next experiments, we compared the effects of IL-33 incubation for 30 min and 18 h. The longer, 18-h incubation was not as effective at enhancing CD11b expression as the shorter, 30-min incubation (Fig. 4*C*). We next tested whether IL-33 affects the level of basophil CD11b expression induced by other well-known stimulants such as IL-3 and FcεRI-crosslinkage. As shown in Fig. 4*D*, IL-33 synergistically augmented surface CD11b expression on IL-3-treated basophils. IL-33 also showed slight enhancement of CD11b levels on anti-FcεRI mAb-treated basophils, but this effect was small and seemingly additive rather than synergistic. Neutralizing Ab for ST2 diminished the enhancement of basophil CD11b expression by IL-33, as shown in Fig. 4*E*. Pretreatment of basophils with anti-ST2 Ab at 10 μg/ml significantly suppressed the effect of 1 ng/ml IL-33 on CD11b expression, indicating that IL-33 regulates basophil CD11b expression by signaling through its receptor, ST2. However, IL-33 at 10 ng/ml or more seemed to be too high for anti-ST2 Ab to efficiently block the IL-33-induced up-regulation of CD11b (Fig. 4*E*).

IL-33 enhances basophil migration toward eotaxin

In vivo local administration of IL-33 was reported to attract inflammatory cells to inflammatory sites (18). Therefore, we investigated whether IL-33 regulates human basophil migration. IL-33 was added to the lower chamber of Chemotaxicell at 10–100 ng/ml, but no induction of basophil migration was observed (Fig. 5*A*). However, when added to the upper chamber with the cells, IL-33

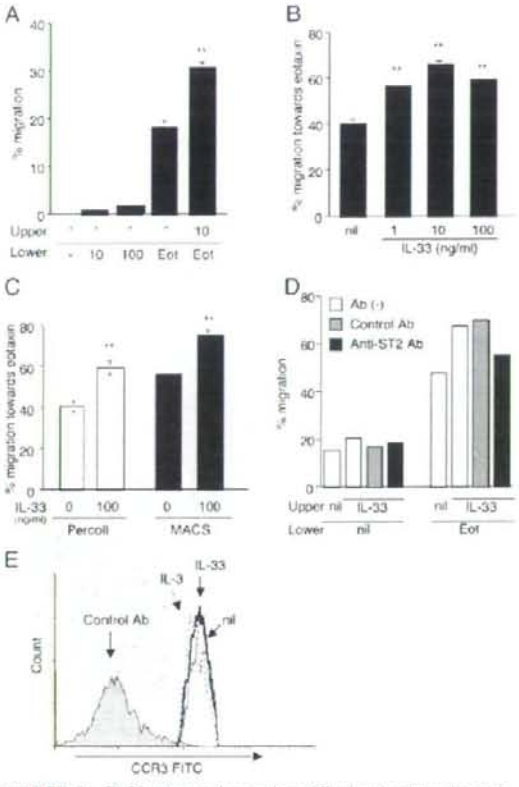

FIGURE 5. IL-33 enhances human basophil migration toward eotaxin. *A*, Two × 10^4 Percoll-separated basophils were added to the upper chamber. IL-33 at 10 or 100 ng/ml or eotaxin (Eot) at 10 nM was added to the lower chamber. Cells mixed with IL-33 at 10 ng/ml were also tested for migration toward eotaxin. The percentage of migrated cells was calculated by subtracting the spontaneous migration (9.6 ± 0.4% for medium only). Error bars represent the SEM ($n = 5$). **, $p < 0.01$ vs spontaneous migration in medium alone. *B*, Percoll-separated basophils were mixed with the indicated concentrations of IL-33 and then tested for migration toward eotaxin at 10 nM. The percentage of migrated cells was calculated by subtracting the spontaneous migration (16.8 ± 0.5% for medium only). Error bars represent the SEM ($n = 3$). **, $p < 0.01$ vs basophil migration toward eotaxin in the absence of IL-33. *C*, Both Percoll-separated (□) and MACS-separated (■) basophils were used for the migration assay. Basophil preparations with and without IL-33 at 100 ng/ml were placed in the upper chamber, and eotaxin at 50 nM was added to the lower chamber. The percentage of migrated cells was calculated by subtracting the spontaneous migration (16.8 ± 0.8% for Percoll-separated and 12.3 ± 1.2% for MACS-separated preparations). Error bars represent the SEM ($n = 3$). **, $p < 0.01$ vs migration of the corresponding basophils without IL-33. *D*, Effect of neutralizing Ab for ST2 on IL-33 enhancement of basophil migration. Percoll-separated basophils were mixed without Ab (□) or with control IgG at 20 μg/ml (▨) or anti-ST2 Ab at 20 μg/ml (■). IL-33 at 10 ng/ml was then added to the cells; eotaxin at 10 nM was added to the lower chamber. Data shown are mean values of an experiment performed in duplicate. Another experiment using basophils from a different donor yielded similar results. *E*, Effect of IL-33 on CCR3 expression by human basophils. Highly purified basophils were incubated with (bold line) and without (thin line) IL-33 at 10 ng/ml for 1 h at 37°C. The dotted line indicates basophils that were incubated with IL-3 at 300 pM. Cells stained with control Ab are shown as a shaded area. Data are representative of two separate experiments using cells from different donors and showing similar results.

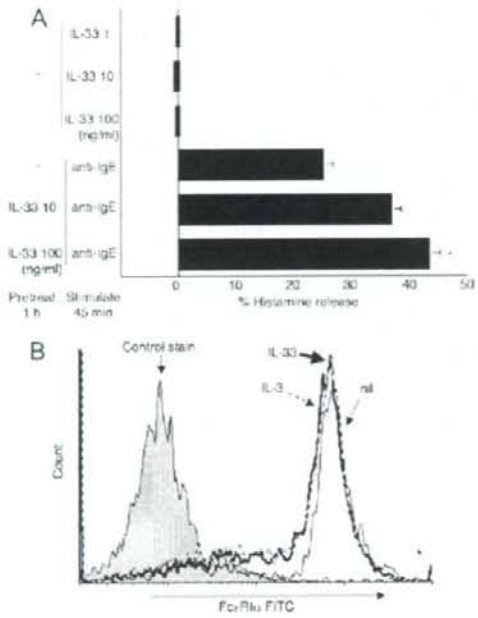

FIGURE 6. IL-33 enhances basophil degranulation following IgE cross-linkage. *A*, Percoll-separated basophils were incubated with and without IL-33 at the indicated concentrations for 1 h and then stimulated with either IL-33 at the indicated concentrations or anti-IgE Ab at 14 μg/ml. The percentage of release was calculated based on the total cellular histamine content. Error bars represent the SEM ($n = 3$). *, $p < 0.05$ vs corresponding release without IL-33 pretreatment. *B*, Effect of IL-33 on FcεRIα expression by basophils. Highly purified basophils were incubated with (bold line) and without (thin line) IL-33 at 10 ng/ml for 1 h at 37°C, and then the surface expression of FcεRIα was analyzed by flow cytometry. Basophils incubated with IL-3 at 300 pM are shown with a dotted line. Basophils stained with control Ab are shown as a shaded area. Data are representative of two separate experiments using cells from different donors and yielding similar results.

at 1–100 ng/ml enhanced basophil migration toward eotaxin (10 nM) (Fig. 5, *A* and *B*). Moreover, we found that IL-33 enhanced chemotaxis of highly purified basophils toward eotaxin (Fig. 5*C*), indicating that possible effects from contaminating cells can be ruled out. Furthermore, when neutralizing Ab for ST2 was added to the upper chamber, the effect of IL-33 on basophil migration toward eotaxin diminished, as shown in Fig. 5*D*, suggesting that IL-33 affects basophil locomotion via the ST2 receptor. As shown in Fig. 5*E*, treatment with IL-33 did not alter the surface level of CCR3, a receptor for eotaxin, on the human basophils, suggesting that IL-33 affects eotaxin-induced intracellular signal(s) downstream of CCR3.

IL-33 enhances degranulation of human basophils

Next, using Percoll-separated basophils, we studied the effect of IL-33 on basophil degranulation. As shown in Fig. 6*A*, freshly isolated basophils did not degranulate in response to IL-33. We next tested IL-33 for basophil priming. Importantly, pretreatment with IL-33 at 100 ng/ml for 15 min significantly enhanced degranulation of basophils stimulated with anti-IgE Ab. We confirmed that the expression level of surface FcεRI remained the same even after IL-33 pretreatment of basophils, as shown in Fig. 6*B*.

IL-33 does not alter survival of basophils

Finally, we analyzed the effect of IL-33 on the viability of highly purified basophils. Although IL-33 is known to enhance the survival of eosinophils (27), this cytokine induced no change in the number of viable or apoptotic basophils compared with basophils cultured in medium alone (data not shown). We next assessed whether IL-33 affects the viability of IL-3-cultured basophils, but it did not show any effect (data not shown).

Discussion

In this study, we demonstrated that human basophils express transcripts and protein for ST2, a receptor for IL-33, and neutralization studies showed that basophil ST2 is functional. IL-33 affected several arrays of basophil functions: this cytokine up-regulated CD11b expression on the cell surface of basophils, enhanced eotaxin-directed chemotaxis, induced Th2 cytokine IL-4 secretion, and augmented the IgE-mediated histamine release reaction. This is the first study to identify the roles of IL-33 and its ST2 receptor in the functional regulation of basophils. Importantly, basophil adhesion was potently enhanced by IL-33, and this action of IL-33 was greater than that of IL-3, a well-known basophil-active cytokine.

The IL-1 cytokine family is known to regulate various inflammatory reactions; among its members, IL-1β and IL-18 are especially potent proinflammatory substances. However, our knowledge regarding the effects of these cytokines on basophil functions is limited. To date, IL-18 has been demonstrated to induce cytokine production by basophils (34, 35), and IL-1α and IL-1β have been demonstrated to potentiate IgE-mediated histamine release from human basophils (36, 37).

IL-33 is a new member of the IL-1 family of cytokines. Schmitz et al. demonstrated that IL-33 has biological activities such as driving Th2-polarized cells to produce Th2 cytokines such as IL-5 and IL-13. In addition, in vivo studies revealed that administration of IL-33 induces histological changes in the mucosa, including eosinophilic infiltration, increased mucus production, and epithelial cell hyperplasia and hypertrophy (18). Thus, locally produced IL-33 may act as a potent inducer of Th2-dominant inflammation. IL-33 is produced by various cells, including epithelial cells and smooth muscle cells (38). Greater knowledge regarding the biological effects of IL-33 on basophils might shed light on the interplay between tissue structural cells and inflammatory granulocytes. In our present study, IL-33 potently enhanced basophil adhesiveness and surface CD11b expression, and these actions of IL-33 were by far the strongest among the tested IL-1 family members. Furthermore, the finding that IL-33 induced IL-4 secretion by basophils implies that this IL-1 family member may strengthen local Th2 dominance through effects not only on Th2 lymphocytes and mast cells but also on basophils, since IL-4 can exert multiple effects causing exacerbation of inflammation (39–41). In addition, our study indicates that transcripts for another Th2 cytokine, IL-13, are also increased by IL-33 in basophils. We further found that IL-4 secretion by basophils stimulated with IL-3 or FcεRI-crosslinkage was potently enhanced by IL-33. IL-33-stimulated basophils may thus be an important cellular source of Th2 cytokines in the pathogenesis of Th2-biased allergic inflammation.

IL-33 was recently identified as a biologically active ligand for ST2 (18), a Th2-associated receptor expressed on Th2 cells and mast cells. Before the ligand was identified, ST2 had been shown to function as an important effector molecule for Th2 responses in experimental models (20, 23). In addition, in the clinical setting, elevated ST2 protein expression was reported in the sera of patients suffering asthmatic exacerbation (42). Thus, ST2 is believed

to have strong relevance to the pathogenesis of Th2-associated diseases. Consistent with a recent study by others (29), we found that human basophils express ST2 protein. We also found that the surface ST2 levels on basophils are changeable, and that the ST2 receptor is functional in basophils. Real-time PCR revealed that the expression level of ST2 mRNA by basophils was lower than that by mast cells but significantly higher than that by eosinophils and neutrophils. We found that the levels of surface ST2 protein on freshly isolated basophils were very low, if any, but culture with IL-3 clearly increased the surface ST2 protein levels on those cells. Furthermore, neutralization experiments showed that ST2 plays a key role in many of IL-33's effects on basophils: anti-ST2 Ab inhibited up-regulation of CD11b expression on basophils and enhancement of basophil chemotaxis toward eotaxin. In our experiments analyzing cell adhesion (Fig. 3), we could not conduct ideal neutralization studies since the added IgG itself augmented basophil adhesion. Nevertheless, we think that ST2 may also be involved in regulation of basophil adhesion. Interestingly, the basophil expression level of ST2 mRNA was enhanced by IL-33 itself. Certain cytokines have previously been reported to regulate the expression of their respective receptors, and similar enhancement has been demonstrated in the case of IL-3 and its IL-3Rα receptor on eosinophils (28). The up-regulation of ST2 expression by its own ligand, IL-33, may contribute to long-term maintenance of IL-33's effects on basophils.

We have shown in this study that IL-33 augments basophil adhesion and CD11b expression. Basophils have previously been reported to express both β1 and β2 integrins on their surface (14), and in earlier studies we demonstrated that β2 integrin represents the first line of adhesion molecules that are involved in basophil transendothelial migration (16) and trans-basement membrane migration (17). Furthermore, basophil CD11b expression is up-regulated by IL-3, resulting in enhanced adhesion to the endothelium (14). Thus, the enhanced adhesion induced by IL-33 may be due at least in part to augmented expression of β2 integrin, and it will lead to increased accumulation of basophils at inflammatory sites.

It is increasingly recognized that basophils and eosinophils share important characteristics such as their growth factors, receptors, cellular functions, and secreted mediator profiles (43, 44). In our recent experiments analyzing the actions of IL-33, we found that this cytokine also activates human eosinophils. However, the precise action of IL-33 on eosinophils differs somewhat from that on basophils: IL-33 failed to enhance migration and degranulation of eosinophils but it suppressed eosinophil apoptosis (27), whereas basophil apoptosis was not affected by IL-33. The different spectrums of IL-33's effects on basophils and eosinophils may in part account for the different behaviors and fates of these effector cells in the pathogenic mechanisms of allergic inflammation. It will thus be important to analyze the extent to which IL-33 regulates the effector functions of basophils (and other cell types) in clinical settings. Further elucidation of the details of the involvement of IL-33 and its receptor, ST2, in the pathogenesis of allergies will enable us to evaluate their potential as useful targets in the therapeutic strategies for allergic diseases.

Acknowledgments

We thank Dr. Koichi Hirai for helpful discussion, and Yasuko Asada for excellent secretarial work. We also thank Drs. H. Kawasaki and C. Ra for providing mAbs for this work.

Disclosures

The authors have no financial conflict of interest.

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Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils

Maho Suzukawa[1], Rikiya Koketsu[1], Motoyasu Iikura[2], Susumu Nakae[3], Kenji Matsumoto[3], Hiroyuki Nagase[4], Hirohisa Saito[3], Kouji Matsushima[5], Ken Ohta[4], Kazuhiko Yamamoto[1] and Masao Yamaguchi[1]

Eosinophils are important effector cells in allergic diseases, but the mechanisms regulating their biological functions remain obscure. Interleukin-33 (IL-33) is a recently identified cytokine of the IL-1 family, and it reportedly accelerates the production of Th2-associated cytokines and promotes tissue inflammation. However, the action of IL-33 on effector cells such as eosinophils has remained unclear. In this study, we investigated the effects of IL-33 on eosinophil activation, assessed in terms of the cells' adhesiveness, expression of CD11b and apoptosis. Adhesiveness was quantified by measuring eosinophil peroxidase content of adherent eosinophils, and expression of CD11b was measured by flow cytometry. Apoptosis was determined by flow cytometry based on the ability of cells to bind annexin V. Real-time PCR analysis showed that eosinophils expressed mRNA for ST2, a putative receptor for IL-33. IL-33 at 1–100 ng/ml enhanced the adhesiveness and CD11b expression of eosinophils even more potently than IL-5. IL-33 maintained the viability of eosinophils. Treatment with neutralizing antibodies to ST2 eliminated the effects of IL-33 on eosinophil CD11b expression and cell survival. However, IL-33 did not elicit degranulation or leukotriene C4 synthesis in eosinophils. These findings indicate that IL-33 potently induces eosinophil adhesion and CD11b expression and enhances eosinophil survival. The IL-33-ST2 pathway might be an important regulator of eosinophil biology in the pathogenesis of Th2-biased allergic diseases.

Laboratory Investigation (2008) **88,** 1245–1253; doi:10.1038/labinvest.2008.82; published online 1 September 2008

KEYWORDS: eosinophil; human; ST2; IL-1; IL-33; IL-18

Blood and local tissue eosinophilia is an outstanding feature of allergic diseases and other disorders such as helminthic parasitic infections and various neoplasms.[1] In the allergic inflammation observed locally in bronchial asthma and atopic dermatitis, eosinophils constitute the major line of effector cells. They possess the capacity to generate and release a wide array of preformed mediators such as major basic protein and eosinophil peroxidase (EPO), as well as newly synthesized mediators including leukotriene (LT) C4 and platelet-activating factor.[2,3] These mediators can cause tissue destruction, modify the smooth muscle tone and vascular permeability and also attract and activate other inflammatory cells. Eosinophils are thus thought to be an important source of proinflammatory mediators. However, only little has been known about which factors are primarily responsible for the accumulation and activation of eosinophils *in vivo*.

ST2, also called DER4, Fit-1 or T1, is a member of the IL-1 receptor family originally identified as a serum-inducible secreted protein in murine fibroblasts.[4] ST2 cDNAs have been also cloned from humans[5] and rats.[6] This receptor is expressed in both soluble and membrane-bound forms as a result of differential splicing,[7] although the expression pattern of ST2 protein differs between humans and mice. ST2 is stably expressed on mouse Th2 cells, but not on mouse Th1 cells.[8] For this reason, this molecule is considered to be a stable cell marker on murine Th2 effector cells. On the other hand, ST2 is inducible in human Th2 cells, and human Th2 cells express ST2 on their cell surface and secrete ST2 following activation.[9] Several lines of evidence suggest that

[1]Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [2]Department of Respiratory Medicine, National Disaster Medical Center of Japan, Tokyo, Japan; [3]Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan; [4]Department of Respiratory Medicine, University of Teikyo School of Medicine, Tokyo, Japan and [5]Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Correspondence: Dr M Suzukawa, MD, PhD, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.

E-mail: fueta-tky@umin.ac.jp

Received 1 February 2008; revised 7 July 2008; accepted 15 July 2008

the ST2 expressed on Th2 cells[8] and mast cells[10] is linked to important Th2 effector functions.[8] Exogenous administration of soluble ST2 has been demonstrated to effectively neutralize the putative ligand, resulting in alleviation of inflammation by abrogating Th2 cytokine production and induction of the eosinophilic inflammatory response.[11] Moreover, mice deficient in ST2 did not develop a Th2 response to *Schistosoma* egg antigen.[12] In addition, although the ligand for ST2 had not been known for years, elevated levels of the soluble form of ST2 were reported to be present in the circulation of patients with various inflammatory diseases.[13] These results have collectively suggested that ST2 may be an important receptor mediating various inflammatory reactions.

Schmitz *et al*[14] recently identified a new cytokine, interleukin-33 (IL-33), which mediates its biological effects through ST2 and accelerates production of Th2-associated cytokines by *in vitro* polarized Th2 cells. Furthermore, *in vivo* experiments have revealed that exogenous administration of IL-33 markedly increased expression of IgE, IgA, IL-4, IL-5 and IL-13 in the serum and led to obvious pathological changes including eosinophilic and mononuclear infiltration of arterial walls, lungs and intestinal tissues, increased mucus production and epithelial cell hyperplasia and hypertrophy. A very recent study showed that IL-33 induces IL-8 secretion and autocrine production of IL-13 in human cultured mast cells.[15]

Although these novel findings seem to imply that IL-33 potentiates the effector functions of Th2 cells and mast cells, there have been no report showing whether IL-33 acts directly on allergic inflammatory granulocytes such as eosinophils, or whether these inflammatory cells possess functional ST2. Therefore, we for the first time conducted a series of analyses designed to detect IL-33-induced eosinophil activation *in vitro*. In this report, we demonstrate that ST2 is expressed on eosinophils, and that IL-33 affects the viability, increases the adhesiveness and upregulates CD11b expression of human eosinophils.

MATERIALS AND METHODS

Reagents

The following reagents were purchased as indicated: human recombinant IL-33 (Adipogen Inc., Seoul, South Korea); human recombinant IL-18 (MBL, Nagoya, Japan); human IL-1β (Wako, Osaka, Japan); human IL-5 (Peprotech, London, UK); human recombinant VCAM-1 and ICAM-1 (R&D, Minneapolis, MN, USA); Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); PBS and RPMI 1640 medium (GIBCO, Grand Island, NY, USA); FCS and fibronectin (0.1% solution) (Sigma, St Louis, MO, USA).

The following antibodies were purchased as indicated: mouse anti-human ST2 neutralizing mAb (IgG1, clone 97203), mouse anti-IL-4 neutralizing mAb (IgG2b, clone 34019.111), mouse anti-IL-5 neutralizing mAb (IgG1, clone 14611) and mouse anti-GM-CSF neutralizing mAb (IgG1, clone 3209) (R&D); mouse anti-CD18 neutralizing mAb (IgG1, clone L130) (BD Pharmingen, San Diego, CA, USA); control mouse IgG1 (MOPC21) and mouse IgG2a (UPC10) (Sigma); mouse anti-ST2 mAb (IgG1, clone HB12) (MBL); FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA); mouse anti-CD29 neutralizing mAb (IgG1, clone 4B4), FITC-conjugated mouse anti-CD16 mAb (IgG1, clone 3G8), PE-conjugated mouse anti-CD11b mAb (IgG1, clone Bear 1) and PE-conjugated mouse IgG1 (clone 679.1Mc7) (Coulter Immunotech, Marseille, France).

Isolation of Eosinophils, Neutrophils and Culture of Mast Cells

Leukocytes were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases.

Eosinophils were purified by density gradient centrifugation. In some experiments, eosinophils were further purified by negative selection using anti-CD16-bound beads (Miltenyi BioTech, Belgisch-Gladbach, Germany) as previously described.[16] After this negative selection, the eosinophil purity was >99%.

Human neutrophils were separated by density gradient centrifugation followed by positive selection using anti-CD14-bound micromagnetic beads (Miltenyi BioTech).[16] The purity of neutrophils was approximately 96–99% after the selection.

Human cord blood-derived mast cells were obtained by culturing cord blood CD34-postive cells in the presence of stem cell factor (100 ng/ml) and IL-6 (50 ng/ml) for more than 10 weeks.[17] Purity of mast cells assessed using Toluidine blue stain was >99%.

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR analysis was performed as previously described.[16] In brief, total RNA was extracted from MACS-separated eosinophils, neutrophils and mast cells from separate donors using RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time PCR was performed using the 7500 Real Time PCR System (PE Applied Biosystems, Foster City, CA, USA). The primers and the probes for ST2 were designed by PE Applied Biosystems. A standard curve was constructed with serial dilutions of specific PCR products, which were obtained by amplifying peripheral leukocyte cDNA as previously described.[18]

ST2 Protein Expression

Highly purified eosinophils were used for flow cytometric analysis of ST2 expression. Eosinophils were incubated for 30 min at 4°C with 10 μg/ml of either anti-ST2 mAb or control antibody and then stained with PE-conjugated goat anti-mouse IgG at 10 μg/ml for 60 min at 4°C. For intracellular staining, the cells were fixed with PBS containing 4% paraformaldehyde at 4°C for 30 min followed by permeabilization in PBS containing 0.1% Tween 20 at 4°C for 30 min. The cells were then stained and analyzed using an Experimental

Physics and Industrial Control System, XL System II (Coulter, Miami, FL, USA).

Survival Assay

Highly purified eosinophils were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Differential analysis of apoptotic and live cells was performed using a MEBCYTO apoptosis kit (MBL) and flow cytometry as previously described.[19] Early apoptotic cells were quantitatively determined by their ability to bind annexin V and exclude propidium iodide (PI). Cells stained with PI were considered to be necrotic cells. Cells without binding to annexin V or PI were judged to be alive.

Adhesion Assay

A 96-well culture plate (IWAKI, Tokyo, Japan) was coated with 100 μl of BSA (20 mg/ml), fibronectin (20 μg/ml), ICAM-1 (100 ng/ml) or VCAM-1 (100 ng/ml) dissolved in PBS overnight at 4°C. The coated wells were washed twice with blocking buffer (2% BSA in PBS) and incubated with 100 μl of this buffer for 1 h at 37°C. The wells were washed twice with RPMI 1640 medium containing 0.3% HSA before adding eosinophils.

Approximately 3×10^4 highly purified eosinophils were added to each well containing the stimulating reagent dissolved in RPMI 1640 medium including 0.3% HSA and incubated at 37°C in 5% CO_2 for 45 min. In some experiments, either anti-CD18 mAb, anti-CD29 mAb or control antibody at 10 μg/ml was added to each well. After incubation, the wells were gently washed twice with RPMI 1640 medium to remove nonadherent cells. Eosinophil adhesion was monitored by quantification of the EPO activity released from the adherent eosinophils as previously reported.[16,20] In brief, 200 μl of 50 mM Tris-HCl, pH 8.0, containing 0.1% (v/v) Triton X-100, 0.1 mM O-phenylenediamine dihydrochloride (Sigma) and 50 mM hydrogen peroxide was added to each well. The plate was left at room temperature until reaching the desired extinction, and the reaction was terminated with 50 μl of 1 M sulfuric acid solution. The optimal density was read at 490/570 nm using an ELISA plate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed with the Microplate Manager III program (Bio-Rad Laboratories), and the numbers of the adherent cells were calculated from a calibration curve established with varying known numbers of eosinophils. The adherent cells were expressed as a percentage of the total eosinophils added to each well.

CD11b Expression

CD11b expression experiments were performed as previously described.[21] Briefly, following stimulation, purified cells were incubated with 10 μg/ml of either PE-conjugated anti-CD11b mAb or PE-conjugated control mouse IgG1 and then stained with FITC-conjugated anti-CD16 antibody at 10 μg/ml. Stained cells were analyzed by flow cytometry. Cells that stained negative for CD16 were identified as eosinophils. The median values of fluorescence intensity of eosinophils were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as previously described.[21] Surface expression levels were semi-quantified using the following formula: ΔMESF = (MESF of cells stained with anti-CD11b mAb) − (MESF of cells stained with control IgG).

Enzyme Immunoassay for LTC4 and Eosinophil-Derived Neurotoxin

For enzyme immunoassay (EIA), 5×10^5 cells per ml of highly purified eosinophils were cultured with stimulating reagents in RPMI medium containing 0.3% HSA for 18 h at 37°C, and the supernatant was collected after centrifugation. Cell lysates were obtained by addition of 0.5% nonidet P-40 (Sigma) to the cell pellets. Samples were stored at −80°C until assay.

Immunoreactive LTC4 was measured using an EIA kit for LTC4 (Cayman Chemicals, Ann Arbor, MI, USA; detection range: 10–1000 pg/ml) by following the manufacturer's instructions.

The concentration of eosinophil-derived neurotoxin (EDN) was measured using an EDN ELISA kit (MBL) by following the manufacturer's instructions. The detection limit was 0.62 ng/ml.

Statistics

All data are expressed as the mean ± s.e.m. Differences between values were analyzed by the one-way ANOVA test. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

RESULTS

Eosinophils Express ST2

First, eosinophil expression of mRNA for ST2 was quantified by real-time PCR in comparison with neutrophils and mast cells. As shown in Figure 1a, ST2 mRNA expression was observed in eosinophils as well as neutrophils and mast cells. Consistent with previous reports,[10] mast cells expressed high levels of mRNA for ST2. The expression level of ST2 by eosinophils was lower than that by mast cells (about 10-fold lower) but much higher than that by neutrophils (about 10-fold higher). Certain cytokines have been previously reported to regulate their respective receptors;[22] thus, we tested the effect of IL-33 on ST2 mRNA expression. However, the ST2 mRNA expression level by eosinophils did not change as a result of 4 h incubation with IL-33 at 100 ng/ml (Figure 1b). By flow cytometric analysis, we found that hardly any ST2 was expressed on the surface of eosinophils (data not shown), but intracellular staining of eosinophils showed a low but detectable level of ST2 (Figure 1c).

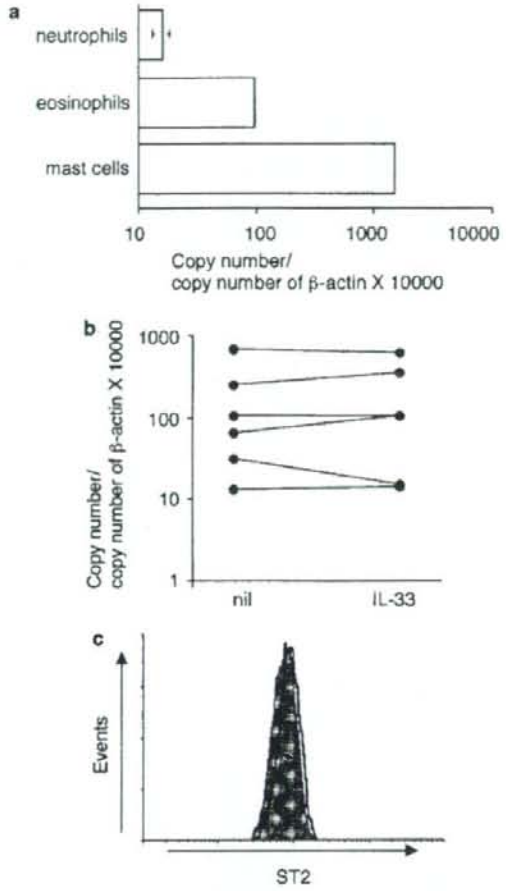

Figure 1 Real-time quantitative PCR analysis and flow cytometric detection for ST2, a receptor for IL-33. (**a**) Quantitative PCR was performed using cDNAs from highly purified eosinophils ($n=10$), neutrophils ($n=9$) and mast cells ($n=4$). The data are calculated as follows: copy number of ST2 gene/copy number of β-actin gene $\times$ 10 000. (**b**) Highly purified eosinophils ($n=6$) were incubated with and without IL-33 at 100 ng/ml for 4 h before extraction of the RNA. The expression levels for the same donor are connected with a solid line in the graph. (**c**) Fixed eosinophils were analyzed for ST2 protein expression by flow cytometry. The cells stained with the control antibody are indicated with a thin line, and the cells stained with anti-ST2 antibody are indicated with a thick line. The data are representative of three separate experiments using cells from different donors and showing similar results.

IL-33 Upregulates Adhesiveness of Eosinophils

As *in vivo* administration of exogenous IL-33 in murine models resulted in local accumulation of eosinophils, we performed a migration assay on eosinophils with IL-33. However, IL-33 added to the lower chambers failed to attract eosinophils. In addition, IL-33 added to the upper chambers did not show enhancement of eosinophil migration toward eotaxin (data not shown). Therefore, next, eosinophil adhesion was quantified by measuring EPO released from lysed adherent cells after 45-min incubation. As shown in Figure 2a, eosinophil adhesion to albumin-, fibronectin-, ICAM-1- and VCAM-1-coated wells was significantly upregulated by IL-33. This effect was apparent at a concentration of 1 ng/ml and increased up to 100 ng/ml. Notably, the effect of IL-33 at 100 ng/ml on eosinophil adhesion was significantly greater than that of IL-5 at 300 pM ($P<0.01$), the most potent known eosinophil-activating cytokine. Two other cytokines of the IL-1 family, ie, IL-1β and IL-18 at 100 ng/ml, did not show any effect on eosinophil adhesion. As shown in Figure 2b, adhesion of eosinophils to albumin-, fibronectin- and ICAM-1-coated wells in the presence of IL-33 was almost completely blocked by anti-CD18 neutralizing antibody, indicating that mainly $\beta 2$ integrin on IL-33-treated eosinophils is involved in the adhesion process to albumin, fibronectin and ICAM-1. On the other hand, adhesion to VCAM-1-coated wells was strongly diminished by the combination of anti-CD18 plus anti-CD29 antibodies, suggesting that eosinophil $\beta 1$ integrin is also involved in adhesion to VCAM-1.

IL-33 Augments CD11b Expression on Eosinophils

Eosinophils have been reported to express β_2 (CD11a, CD11b and CD18) integrins on their surface, and the levels of CD11b on eosinophils are enhanced by eosinophil-activating cytokines such as IL-5. In this study, expression of CD11b on eosinophils was analyzed by flow cytometry. As shown in Figure 3, IL-33 at 1–100 ng/ml significantly upregulated the expression of CD11b on eosinophils dose-dependently, and the effect of IL-33 at 100 ng/ml was stronger than that of IL-5 at 300 pM ($P<0.001$). On the other hand, two other IL-1 family cytokines, IL-1β and IL-18, did not show any effect on eosinophil CD11b expression.

To elucidate the role of ST2, neutralizing antibody for ST2 was added together with IL-33. When eosinophils were pretreated with anti-ST2 neutralizing antibody, the effect of IL-33 on CD11b expression was diminished, as shown in Figure 4a and b, indicating that IL-33 affected eosinophils by binding to and signaling through ST2. When IL-33 at 1 or 10 ng/ml was used to stimulate eosinophils, the effect declined significantly upon addition of anti-ST2 antibody. These results suggest that eosinophil CD11b expression is regulated by IL-33 and its receptor, ST2.

IL-33 Enhances Survival of Eosinophils

Next, we used highly purified eosinophils and analyzed the effect of IL-33 on their viability. As shown in Figure 5a and b, IL-33 at 10–100 ng/ml significantly enhanced the survival of eosinophils dose-dependently although the effect was weaker than that of IL-5 at 300 pM. IL-33 at 100 ng/ml increased the number of live eosinophils by approximately

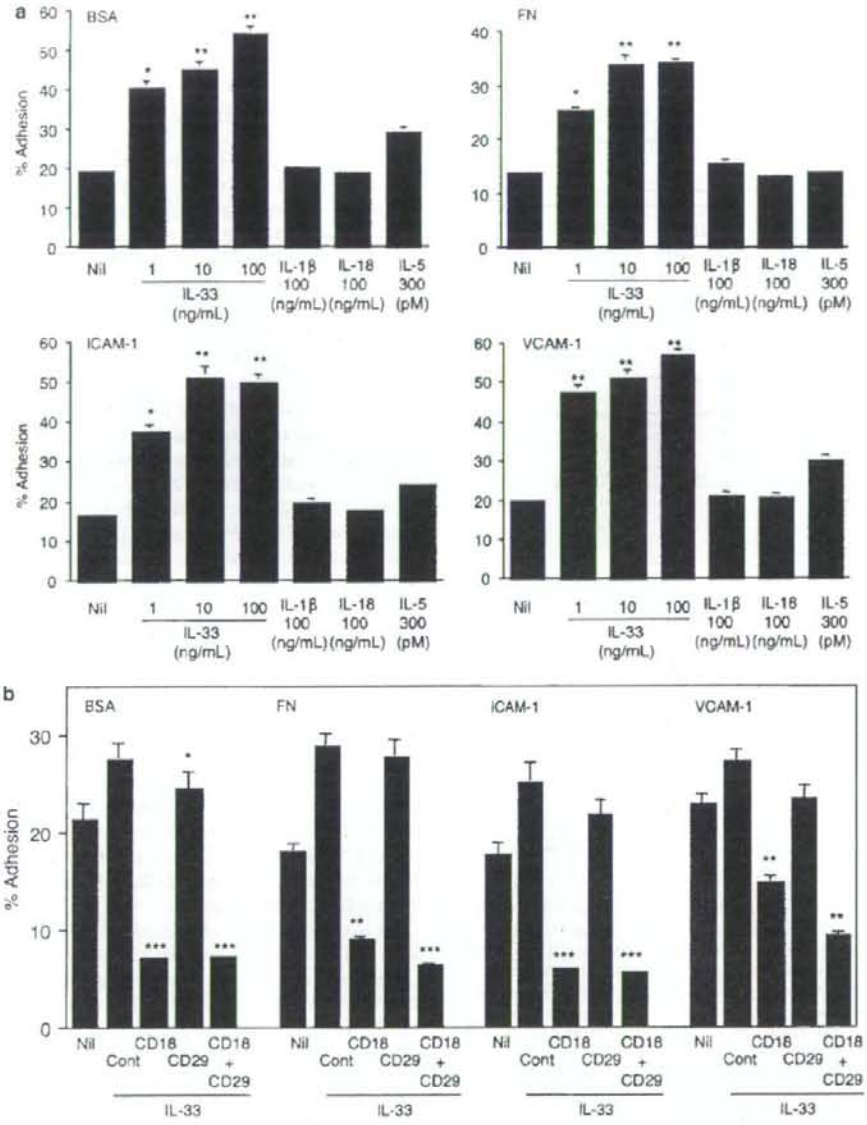

Figure 2 IL-33 enhances adhesion of eosinophils. (**a**) Human eosinophils were used to analyze adhesion to BSA-, fibronectin-, ICAM-1- or VCAM-1-coated culture plates. The cells were incubated with the indicated concentrations of IL-33 or IL-1β at 100 ng/ml, IL-18 at 100 ng/ml or IL-5 at 300 pM for 45 min. The number of adherent cells is expressed as a percentage of the total number of cells placed in each well. Bars represent the s.e.m. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* medium alone (nil). (**b**) Human eosinophils were preincubated with either neutralizing antibody or control antibody at 10 μg/ml plus IL-33 at 100 ng/ml, for 45 min. The number of adherent cells is expressed as the percentage of the total number of cells placed in each well. Bars represent the s.e.m. ($n = 4$–5). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* control antibody.

20% after 24 h. Apoptotic cells, ie, positive for annexin V staining and negative for PI staining, were significantly decreased by addition of IL-33 (Figure 5c). IL-1β and IL-18, at 100 ng/ml, did not enhance the survival of eosinophils, indicating that this effect is specific for IL-33 among these cytokines.

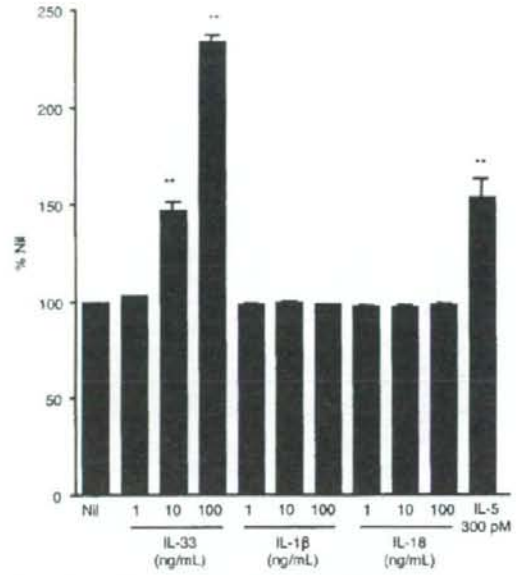

Figure 3 The effect of IL-33 on eosinophil CD11b expression. Human eosinophils were incubated with the indicated reagent for 30 min, and then the cell-surface expression of CD11b was analyzed by flow cytometry. The data are expressed as the percentage of the calculated MESF values of eosinophils cultured without stimulus (% nil). Bars represent the s.e.m. ($n=3$). $^{**}P<0.01$ *vs* nil.

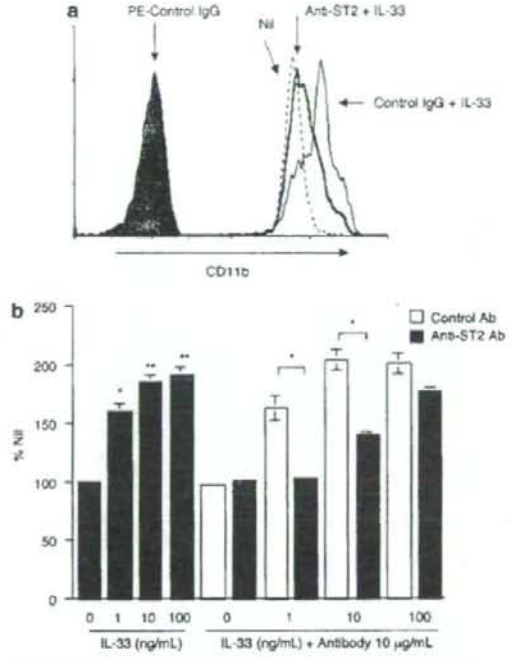

Figure 4 The effect of anti-ST2 neutralizing antibody on CD11b expression by eosinophils. (**a**) Human eosinophils were preincubated with anti-ST2 antibody at 10 μg/ml for 60 min and then with IL-33 at 10 ng/ml for 30 min. The surface CD11b expression level was assessed by flow cytometry. Eosinophils stained with PE-control mouse IgG1 are shown as a shaded area. Data are representative of four separate experiments showing similar results. (**b**) Human eosinophils were preincubated with or without anti-ST2 antibody or control antibody at 10 μg/ml for 60 min and then with IL-33 at the indicated concentrations for 30 min. The data are expressed as the percentage of the calculated MESF values of eosinophils cultured without antibodies or stimulus (% nil). Bars represent the s.e.m. ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$ *vs* nil.

To elucidate the role of ST2 in eosinophil survival, neutralizing antibody for ST2 was added together with IL-33. As a result, anti-ST2 antibody at 20 μg/ml significantly downmodulated the effect of IL-33, as shown by a decreased number of live cells and an increased number of apoptotic cells (Figure 6a and b), indicating that IL-33 enhances eosinophil survival by signaling through the ST2 receptor.

It has been reported that eosinophils can produce IL-5 upon stimulation. We used neutralizing antibody for IL-5 to test the possibility that IL-33 enhancement of eosinophil survival was mediated by IL-5. However, anti-IL-5 antibody did not affect the number of viable eosinophils cultured with IL-33 (data not shown). Similar tests using anti-IL-4 and anti-GM-CSF neutralizing antibody gave the same result. These results indicate that the effect of IL-33 on eosinophil survival is not mediated by autocrine activation involving IL-5, IL-4 or GM-CSF.

Analysis of Degranulation and Lipid Mediator Synthesis

We conducted experiments to see whether IL-33 induces degranulation and lipid mediator synthesis in human eosinophils. Eosinophil degranulation was analyzed by measuring EDN, but IL-33 was negative for this activity (data not shown). LTC4 synthesis was analyzed by ELISA, but no apparent release of LTC4 was induced by IL-33 (data not shown).

DISCUSSION

In the present study, we assessed the potential role of IL-33 in regulation of eosinophil functions. We demonstrated that IL-33 is a potent activator of human eosinophils, enhancing their surface CD11b expression and adhesion and prolonging their life span. Surprisingly, the maximal enhancing effects of IL-33 on adhesion and CD11b expression were comparable to, or even greater than, the effects of IL-5, a potent eosinophil-activating cytokine. This is the first report showing direct effects of IL-33 on the biological functions of eosinophils. Importantly, the effects of IL-33 on eosinophils were limited to cell adhesion and survival, in clear contrast to

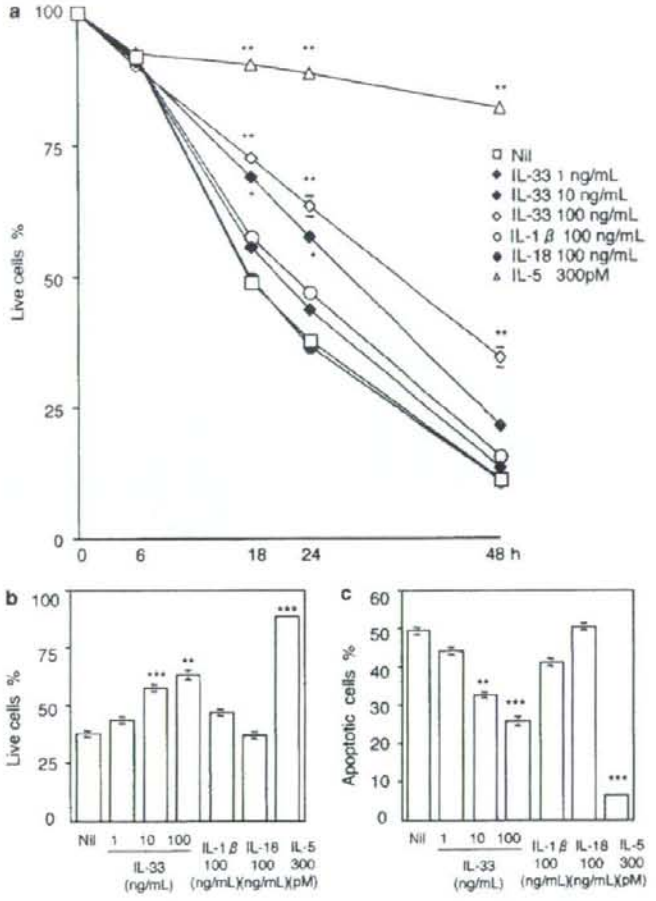

Figure 5 IL-33 enhances survival of eosinophils. (**a**) Time course of survival of human eosinophils. Highly purified eosinophils were cultured in medium alone or in the presence of IL-33 (1, 10 and 100 ng/ml), IL-1β (100 ng/ml), IL-18 (100 ng/ml) or IL-5 (300 pM) for the indicated times. The cells were analyzed by double staining with annexin V and PI. Live cells were negative for both annexin V and PI. Data are expressed as percentages of total cell numbers. (**b**, **c**) Viable (**b**) and apoptotic eosinophils (**c**) after 24-h incubation. Early apoptotic cells were defined as annexin V-positive and PI-negative. Bars represent the s.e.m. ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* medium alone.

IL-5, which also affects mediator synthesis and release by eosinophils.[23,24]

IL-33 is a recently identified cytokine belonging to the IL-1 family. It has increasingly been thought that IL-33 may be involved in the pathogenesis of Th2-polarized inflammation. Schmitz *et al*[14] demonstrated that IL-33 induces Th2-polarized cells to produce Th2 cytokines such as IL-5 and IL-13. In addition, *in vivo* exposure to IL-33 causes histological changes in the lungs and gastrointestinal tract, including eosinophilic and mononuclear cell infiltration, increased mucus production, and epithelial cell hyperplasia and hypertrophy.[14] Although the principal source of IL-33 in Th2-related allergic diseases has yet to be determined, this cytokine can reportedly be produced by many cell types including epithelial cells and smooth muscle cells.[25] Thus, IL-33 secreted by various cell types may act collectively to induce allergic inflammation through Th2 cell differentiation and a direct effect on Th2 effector cells, including eosinophils.

Among the IL-1 family cytokines we tested, IL-33 was the only one that activated eosinophil functions. In previous studies, IL-18, another IL-1 family cytokine, has been demonstrated to induce IL-8 production by eosinophils and to enhance antigen-induced eosinophil recruitment in mouse airways.[26,27] IL-1β has also been reported to enhance

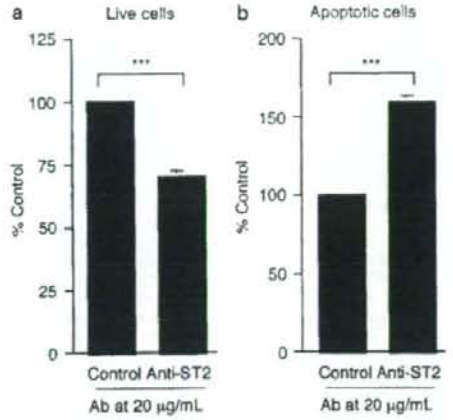

Figure 6 Effect of anti-ST2 antibody on IL-33-induced eosinophil survival. Highly purified eosinophils were cultured with IL-33 at 10 ng/ml and anti-ST2 neutralizing antibody at 20 μg/ml for 24 h. Live (**a**) and apoptotic (**b**) cells were analyzed by flow cytometry. Data are expressed as percentages of corresponding cells cultured with control antibody. Bars represent the s.e.m. ($n = 5$). ***$P < 0.001$ *vs* control.

mediator release from IgE-stimulated eosinophils and adhesiveness of eosinophils to endothelium.[28] Thus, the IL-1 family cytokines are considered to be important proinflammatory cytokines in allergic inflammation. The IL-1 family member most closely related to IL-33 is reported to be IL-18.[14] In clear contrast to IL-33, the main cellular source of IL-1β and IL-18 seems to be hematopoietic cells. Moreover, IL-1β and IL-18 are not considered to be selective Th2-related cytokines, as they can also promote Th1-associated responses. On the basis of our present study, IL-33 seems to have different roles from IL-1β and IL-18. Thus, IL-33 promotes Th2-associated responses, at least partly through direct activation of eosinophils, and this action seems unique among IL-1 family cytokines.

An IL-1 receptor family member, ST2, has been shown to function as an important effector molecule of Th2 responses in a number of experimental settings,[8,11] and it is an active receptor for IL-33. It has been demonstrated that expression of ST2 on Th2 cells is induced by proinflammatory substances, including TNF, IL-1α, IL-1β, IL-6, IL-5 and PMA, and importantly, crosslinking of ST2 provided a costimulatory signal for Th2 cells and directly induced Th2 cell proliferation and type 2 cytokine production.[29,30] Thus, ST2 might be important in the pathogenesis of diseases of the Th2 phenotype.

In the present study, real-time PCR analysis of eosinophils revealed that eosinophils express mRNA for ST2, although the expression level of mRNA was lower than that of mast cells which have already been reported to express ST2 on their cell surface.[10] Although surface expression of ST2 on eosinophils was hardly detectable, ST2 was shown to be present in the cells by intracellular flow cytometric analysis. At present, we do not know whether presumably faint levels of surface ST2 would suffice for triggering cell activation signals, or whether intracellular ST2 in eosinophils is also involved in cell activation by IL-33. However, based on the successful blocking of IL-33's effects by IL-33-neutralizing antibodies, we can reasonably say that ST2 protein expressed by eosinophils has functional relevance. In our present study, a high-affinity ST2 ligand, IL-33, induced a strong eosinophil adhesion response with efficacy higher than that of IL-5. The IL-1β, TNF-α, IFN-γ, IL-5 and IL-4 inflammatory cytokines upregulate the expression of adhesion molecules on the endothelium and eosinophils and increase eosinophil binding to endothelial cells.[31] On the basis of the findings of this study, IL-33, like other proinflammatory cytokines, also upregulated expression of CD11b, a component of Mac-1, on eosinophils. Therefore, the enhancement of eosinophil adhesiveness by IL-33 is at least partially due to this enhanced expression of CD11b. Neutralization experiments confirmed that β1 and β2 integrins are critically involved in the adhesion process of IL-33-treated eosinophils. Also, IL-33 prolonged the life span of eosinophils, although the effect was weaker than that of IL-5. We have shown that the survival-enhancing effect of IL-33 is not due to autocrine production of IL-4, IL-5 or GM-CSF, since neutralization of those cytokines did not affect IL-33-induced prolongation of eosinophil survival. On the other hand, at relatively high concentrations (10–20 μg/ml) anti-ST2 antibody was fairly efficient at inhibiting IL-33-induced upregulation of CD11b expression and survival of eosinophils, but those abrogating effects were not complete. We speculate that, in eosinophils, a small amount of IL-33 that can bind to cell-surface ST2 even in the presence of anti-ST2 antibody may act efficiently to modulate cell functions. Another possibility is that, in eosinophils, intracellular ST2 may not be completely blocked by anti-ST2 antibody. In any case, our results indicate that ST2 is an important receptor through which IL-33 mediates most, if not all, of various aspects of eosinophil activation.

Eosinophils are considered to be the most prominent cells at sites of allergic inflammation. Tissue eosinophils are believed to contribute to exacerbation of inflammation by an autocrine or paracrine mechanism. Therefore, marked reduction of tissue-infiltrated eosinophils is considered to be a promising therapeutic target for allergic diseases. On the basis of the findings of our present study, neutralization of ST2 abolished the effect of IL-33 on eosinophil activation indicating that IL-33 affected eosinophil function through ST2 although ST2 protein levels were low in eosinophils. In addition, in the clinical setting, elevated ST2 protein levels were reported in the sera of patients with asthma exacerbation, and the severity of asthma exacerbation correlated with the levels of serum ST2.[13] Furthermore, it has been demonstrated in a murine asthma model that administration of recombinant ST2 fusion protein attenuated eosinophilic inflammation of the airway and suppressed IL-4 and IL-5

production.[11] Thus, the IL-33–ST2 pathway may be actively involved in the pathogenesis of eosinophil-related allergic diseases such as asthma and eosinophilic gastroenteritis. In this context, several studies have shown that inhibition of the ST2-related signal pathway leads to abrogation of allergic diseases.[8,11,12]

In summary, we have explored for the first time the receptor expression and functions of IL-33 in human eosinophils, and we found that this cytokine potently activates eosinophils. In combination with previous reports by others, our results strongly suggest that the IL-33–ST2 pathway may be critically involved in the pathogenesis of allergic diseases. The effects of IL-33 on other inflammatory cells also need to be explored in detail; these information will help us understand the detailed mechanisms underlying clinical allergic diseases.

ACKNOWLEDGEMENTS

We thank Ms Chise Tamura for her skilled technical assistance and excellent secretarial work. Thanks are also extended to Ms Yasuko Asada for secretarial help, and Dr Koichi Hirai and Dr Toshiharu Nakajima for fruitful discussion on this manuscript. This work was supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan, and grants-in-aid from the Ministry of Health, Labor and Welfare of Japan, and a Long-range Research Initiative (LRI) grant from the Japan Chemical Industry Association.

DISCLOSURE/DUALITY OF INTEREST

We have no duality of interest to declare.

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帝京医学雑誌 31(2)：75～86（2008）

■原　著■

気管支喘息と胃食道逆流との関連についての臨床的・実験的検討—QUEST問診票に基づく胃食道逆流診断と治療的介入の効果—

竹　澤（矢野）　智　湖

帝京大学医学部内科学講座

背景・目的：気管支喘息では高率に胃食道逆流（以下GERD）が合併し，GERDは喘息増悪因子として位置づけられている。簡便なGERD診断法として，QUEST問診表が開発されており，気管支喘息患者におけるGERD合併を，QUEST問診票を用いて診断・治療することの有用性を検討した。また，GERDによる喘息増悪機序として，気道内への酸微量誤嚥の関与が想定されているため，酸曝露が気道上皮細胞株の生存や活性化におよぼす影響を*in vitro*で検討した。

対象と方法：気管支喘息患者88名におけるQUEST陽性率を調査した。さらに，QUEST陽性の気管支喘息患者19名に，オメプラゾール20 mgを4週間投与し，QOL，喘息症状，呼吸機能を評価した。また，低pHで気道上皮細胞株BEAS-2Bを培養し，生存とサイトカイン・ケモカイン産生に及ぼす影響を検討した。

結果：気管支喘息患者におけるQUEST陽性率は54.5％であった。QUESTスコアは喘息重症度と正相関し，使用薬剤よりも喘息重症度と強く相関していた。オメプラゾール投与によって，QUESTスコアと咳嗽が有意に改善し，QOLも向上した。*In vitro*の検討では，酸曝露4時間後より気道上皮細胞死が惹起され，PDGF-BB分泌が増強した。

結論：気管支喘息患者におけるGERD合併を，QUEST問診票を用いて診断・治療する診療モデルの妥当性が示唆された。また，酸曝露による気道上皮細胞死，PDGF-BB分泌亢進が喘息増悪に関与している可能性が示唆された。

キーワード：気管支喘息，胃食道逆流，QUEST問診票，咳嗽，気道上皮細胞

はじめに

胃食道逆流症（Gastroesophageal Reflux Disease，以下GERD）は胃食道逆流による身体的合併症や，逆流関連症状により健康な生活を障害しているものを指す[1]。身体的合併症とは，食道炎などの局所病変に加え，喘息，肺炎，嗄声，咽頭炎などの合併症を指し，健康な生活の障害とは，胸焼けなどの自覚症状とそれに由来するQualite of Life（以下QOL）の低下を指す[1]。そして，週に2回以上の症状があれば，QOLに悪影響を与えるとされる[1]。

気管支喘息にGERDが高頻度に合併することは1960年代より報告されている[2]。疫学的には，喘息患者の40％にびらん性食道炎があることや，80％以上に異常逆流を認めることが報告されている[3]。GERDが喘息病態を悪化させる機序としては酸の微量誤嚥や，逆流に伴う迷走神経反射によ

（指導：大田　健教授，長瀬洋之講師）
学位申請論文

る気道抵抗の増大などの関与が想定されており，気管支喘息増悪因子として，GERD合併の有無を検討することは，気管支喘息の治療管理の点からも重要である。

しかしながら，呼吸器外来における日常診療では，GERDの診断は必ずしも容易ではなく，増悪因子として検索されていないことも多い。GERDの診断は，胸やけ，呑酸などの定型的症状に関する詳細な問診を基本とするが，確定診断には24時間食道pHモニタリングが有用である。また，気管支喘息とGERDの関連を検討した報告においても，食道pHモニタリングや上部消化管内視鏡検査に基づくGERD診断が行われている。しかしながら，これらの検査は比較的侵襲が大きく，結果も迅速には得られにくい。

簡便なGERD診断法として，Questionnaire for the Diagnosis of Reflux Disease（以下QUEST）[4]や，Frequency Scale for the Symptoms of GERD（FSSG）[5]といった問診表が開発されている。QUEST問診票は1998年に開発され[4]，胸焼けなどの典型的なGERD症状をスコア化し，高い特異度，感度を有している。消化器疾患領域ではGERD診断に広く用いられているが，気管支喘息患者において，GERDの典型的症状がいかに修飾されるかについては不明であり，気管支喘息患者においてQUEST問診票の有用性を検証する必要がある。しかしながら，現時点ではQUEST問診票のみに基づいて気管支喘息患者におけるGERDの合併を診断し，GERD治療の気管支喘息病態への効果を検討した報告はない。

そこで，今回の研究目的は，1）気管支喘息患者における，QUEST陽性率を調査すること，2）気管支喘息患者におけるGERD合併を，QUEST問診票を用いて診断・治療することの有用性を検討し，日常診療に応用可能な診療体系を確立すること，とした。具体的には，QUEST陽性喘息患者にプロトンポンプ阻害薬（proton pump inhibitor，以下PPI）を投与し，QOL，喘息症状，呼吸機能を評価した。

さらに，GERDによる気管支喘息増悪の機序として，気道内への酸微量誤嚥の関与が想定されている。気道上皮細胞は，外界への最前線に位置し，種々のサイトカイン，ケモカインの産生を介して気道炎症形成に関与することが明らかとなっているが，酸曝露による気道上皮細胞傷害や炎症性メディエーター産生へ及ぼす影響に関しては不明な点が多い。そこで，気道上皮細胞株を用いて，塩酸による低pHの及ぼす影響を *in vitro* で検討し，GERDによる気管支喘息増悪の一機序を検討することとした。

対象と方法

本研究計画は，帝京大学医学部倫理委員会の承諾を得た。研究に参加する際は，研究内容を文書で説明し，同意書への署名を得た。

1．気管支喘息患者におけるQUEST陽性率の調査

帝京大学医学部附属病院の呼吸器・アレルギー外来に気管支喘息にて通院中の連続受診患者94名を対象にQUEST問診票を配布し，88名から有効回答を得た（平均年齢50.7±1.7歳，男性：41名，女性：47名）。QUEST問診票は7問からなり，逆流症状，誘因，発現・消失パターンに重み付けをしたスコアが配点されており，最高点は18点である。カットオフ値を4点以上とすると，感度70％，特異度は46％，カットオフ値を6点以上とすると，感度54％，特異度は60％と報告されている[4]。気管支喘息の診断および重症度判定は，喘息予防・管理ガイドラインに基づいて行った[6]。

2．QUEST陽性気管支喘息患者に対するプロトンポンプ阻害薬投与の効果

QUEST問診票の有効回答を得た88名のうち，下記のクライテリアを満たし，試験参加の同意を得た安定期気管支喘息患者19名を対象とした。（1）QUESTスコア4点以上，（2）H_2阻害薬，PPIを内服していない，（3）気管支喘息治療薬を4週間以上変更せずに使用している，（4）4週間以上気管支喘息発作を認めない，（5）経口ステロイド薬を内服していない。19名（平均年齢57.0±2.9歳，男性：8名，女性：11名）の患者背景を**表1**に示す。喘息罹患年数は，17.2±2.5年，喘息重症度はステップ2：12名，ステップ3：7名であった。

PPI投与開始前の4週間を観察期間とし，観察期間終了後にオメプラゾール20 mgの投与を1

表1 プロトンポンプ阻害薬投与対象例の患者背景

患者	年齢(歳)	性別	喘息病型	罹患年数(年)	喘息ステップ	QUEST スコア
1	44	F	ND	41	2	6
2	37	F	A	14	2	6
3	67	M	NA	12	2	8
4	75	M	NA	16	2	8
5	55	F	NA	4	3	8
6	56	F	A	20	3	8
7	57	F	NA	27	2	8
8	50	F	A	10	2	9
9	62	M	NA	22	3	11
10	68	M	A	14	2	11
11	60	F	ND	11	3	12
12	75	M	NA	15	3	15
13	36	M	A	26	2	6
14	65	F	A	12	2	10
15	72	F	NA	9	2	12
16	70	M	ND	7	3	6
17	47	F	A	21	2	9
18	46	F	A	43	3	6
19	41	M	ND	3	2	11

M：男性，F：女性，A：アトピー型，NA：非アトピー型，ND：Not determined

カ月行った。オメプラゾールは朝食後，1日1回の内服とした。観察期間，内服期間を通して，気管支喘息治療薬は変更せずに継続した。

評価項目はQUESTスコア，呼吸機能，QOL，朝および夕のピークフロー，症状点数とし，PPI内服前後で検討した。QOLは，疾患特異的なAsthma Quality of Life Questionnaire（以下AQLQ）日本版と疾患非特異的なMedical Outcomes Study 36-Item Short-Form Health Survey（以下SF-36）日本版を用いて評価した。ピークフローは携帯用ピークフローメーター（Mini-Wright，Clement Clarke，Harlow，UK）で測定した。症状点数は，日本アレルギー学会の方法で[7]，喘息日誌に記録された呼吸困難感，喘鳴，咳嗽の程度から0～9点の間でスコア化した。咳嗽（強い咳：1点，弱い咳：0.5点），痰量（多い：2点，少ない：1点，認めない：0点），痰の切れ（悪い：1点，良い：0点）もおのおのスコア化し，観察期間と内服期間のそれぞれ最終の1週間のスコアを合計して算出した。QOLに関しては17名，呼吸機能検査，喘息日誌は13名が評価可能であった。

3．酸曝露が気道上皮細胞株の生存およびサイトカイン産生に及ぼす影響

1）気道上皮細胞培養

気道上皮細胞株は，正常人の気道上皮細胞をアデノウイルスとSV40のハイブリットにより腫瘍化した細胞株であるBEAS-2Bを用いた。まず，BEAS-2BをLHC-9培養液（GIBCO，Grand Island，USA）で培養し，Confluentに達するまで約1週間増殖させた。その後，trypsin-EDTAで剥離し，2×10^4個（in200 μl/well）を96穴プレート（Iwaki，Tokyo）に分注した。培養液は1％ペニシリン・ストレプトマイシン（GIBCO）および5％ウシ胎児血清（Fetal Calf Serum，以下FCS，JRH bioscience，USA）を添加したDMEM/F-12（GIBCO）を用い，Confluentに達するまで，37℃下，CO_2インキュベーター中で3日間培養した。

2）酸曝露が気道上皮細胞からの炎症性メディエーター産生に及ぼす影響

Confluentに達したBEAS-2B細胞の培養液を，pH 3，4，5，6，6.5，7，7.5に調整した培養液に置換してさらに培養後に上清を回収し，－80℃で保存した。培養液は1％ペニシリン・ストレプトマイシンおよび0.1％ウシ血清アルブミン

(Bovine Serum Albumin, 以下BSA)を添加したDMEM/F-12 (DMEM/F-12-0.1% BSA)を1N HClを用いてpHを調整して用いた。培養上清の検討は，デュプリケートないし，トリプリケート由来のデータを平均して1パッセージのデータとした。

上清中サイトカイン・ケモカイン濃度は，Human Cytokine 27-Plex Panel Kit (BIO-RAD, Hercules, USA)を用い，蛍光マイクロビーズアレイシステムであるLuminex® (Hitachi, Tokyo)で測定し，27種類のターゲットの濃度を測定した。各ターゲットに対する特異的一次抗体には，ターゲット毎に異なる蛍光強度で標識された27種類のビーズが結合しており，ビオチン結合特異的二次抗体，ストレプトアビジン-PEでターゲットの濃度を検出した。すなわち，50 μlの上清をビーズ結合一次抗体と30分反応させ，洗浄後二次抗体と30分反応，さらに洗浄後，ストレプトアビジン-PEと10分反応させ，Luminex®で測定した。プレート壁への抗体付着を防ぐために，サンプルは0.5%以上の蛋白を含有させた。スタンダードは0.5% BSAを含有するDMEM/F-12で溶解し，また培養上清には，2.5% BSAを含有するDMEM/F-12でBSAを添加した。

測定に際しては，低pH下で抗体反応を行うと，抗体の結合性が低下する可能性を考え，抗体反応条件を検討した。スタンダード溶解液のpHを3～7.5に調整したところ，pH 4以下で抗体反応させると，測定値が低値となったため，pH 7に戻した状態で抗体と反応させた。さらに，低pH下で蛋白を放置した場合，分解される可能性を考え，各種ターゲットの蛋白を含有するスタンダードをpH 3～7.5に調整した溶解液中で48時間孵置し，回収後にpH 7に戻して抗体と反応させた。IL-4, IL-6, IL-7, IL-9, IL-13, IL-17, PDGF-BB, MCP-1, RANTES, Eotaxin, MIP-1β, IL-8, IP-10は，pH 3に孵置後もpH 7で孵置した際と同等の結果が得られ，低pH下でも分解されないことが示唆された。以上から，上記のターゲットについて，上清をpH 7に調整後に抗体と反応させてLuminex®で測定した。

3) 酸曝露が気道上皮細胞生存に及ぼす影響

pH 3～7の各pHで培養後のBEAS-2B細胞の培養上清を回収後，PBS200 μlで付着細胞を洗浄し，PBS5 μlおよびトリプシン-EDTA20 μlを各wellに分注してBEAS-2Bを剥離した。この後各wellをPipes A (25 mM Pipes, 119 mM NaCl, 5 mM KCl, 0.03%ヒトアルブミンを含有) 200 μlで2回洗浄して細胞を回収した。細胞生存の解析は，MEBCYTO apoptosis Kit (MBL, Nagoya)を用いて行った。死細胞にはFITC標識annexin Vが結合することを利用して生細胞の分画をフローサイトメトリー (FACS Caliber, Becton Dickinson, USA)を用いて定量した。

4．統計

喘息重症度とQUESTスコアの相関解析にはSpearman順位相関係数の検定を行った。QUESTスコアを規定する背景因子の解析は，ステップワイズ重回帰分析を行った。PPI治療前後の各種パラメーターの比較はWilcoxon符号付順位和検定を用いた。BEAS-2Bを用いた検討における多群間の比較には，一元配置分散分析法 (one-factor ANOVA)を用い，有意差を認めた場合，Fisher's PLSD検定で群間の比較を行った。結果は平均値±標準誤差で示した。いずれの検定においても$p<0.05$をもって有意と判定した。

結　果

1．気管支喘息患者におけるQUEST問診票陽性率

まず，気管支喘息患者にQUEST問診票を配布し陽性率を調査した。88例中4点以上は48例 (54.5%)，6点以上は36例 (40.9%)であった。また，気管支喘息重症度とQUESTスコアとは強い正相関を示した (**図1A**, $p<0.01$)。QUEST陽性率と気管支喘息重症度との相関も検討したが，QUESTスコアのカットオフを4点以上，6点以上とした場合の双方で強い正相関を示した (**図1B**, $p<0.01$)。

QUESTスコアを規定する背景因子について検討する目的で，QUESTスコアを目的変数とし，年齢，性別，気管支喘息重症度，β_2刺激薬，テオフィリン薬，吸入ステロイド薬，経口ステロイド薬の各薬剤使用を説明変数としたステップワイズ重回帰分析を行った。予測式として，QUESTスコア=2.22x気管支喘息重症度ステップ−0.971 (r^2

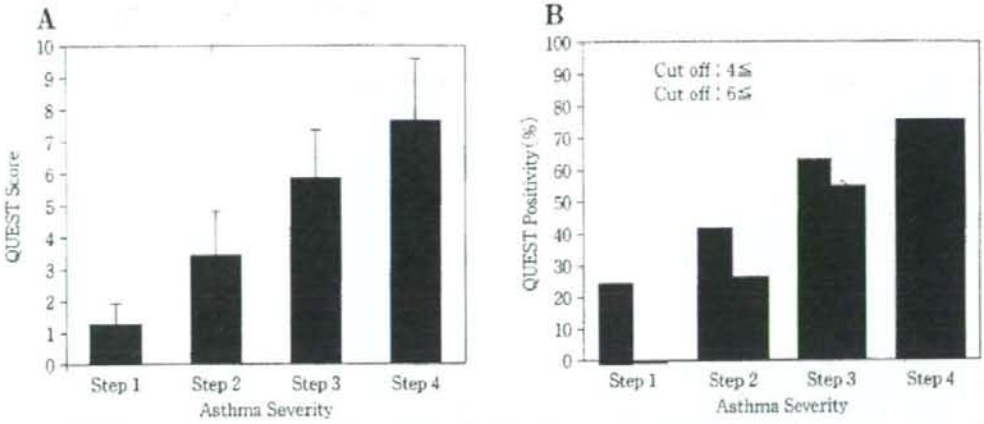

図1 気管支喘息重症度とQUEST score (A) およびQUEST陽性率 (B) との関連
A：気管支喘息重症度とQUEST scoreは有意な正相関を認めた ($p<0.05$)．
B：気管支喘息重症度とQUEST陽性率も，Cut off4点以上 ($p<0.05$)，6点以上 ($p<0.05$) の双方で正相関を示した ($n=88$，Step 1 ($n=8$)，Step 2 ($n=37$)，Step 3 ($n=39$)，Step 4 ($n=4$))．

表2 PPI投与前後の呼吸機能検査，喘息症状の変化 ($n=13$)

		Baseline	Post-treatment	p
呼吸機能検査	FEV1.0 (L)	1.99±0.14	1.94±0.14	0.067
	% FEV1.0	87.9±4.7	84.8±5.1	0.106
	ピークフロー（朝）(l/min)	309.9±24.1	303.2±26.1	0.058
	ピークフロー（夕）(l/min)	313.9±21.1	314.3±23.8	0.913
喘息症状	痰量	0.7±0.2	0.5±0.1	0.236
	咳嗽	2.9±1.1	1.1±0.3	*0.036
	夜間点数	2.8±1.7	1.5±0.6	0.753
	症状点数	5.1±1.8	4.5±1.5	0.799

=0.120，$p=0.0006$) が得られ，使用薬剤よりも気管支喘息重症度がQUESTスコアに強く相関する因子として採用された。

2．QUEST陽性気管支喘息患者に対するプロトンポンプ阻害薬投与の効果

安定期気管支喘息患者において，PPI投与前後で各種パラメーターを比較した（**表2**）。QUESTスコアはPPI治療前後で有意に改善した（**図2**：8.9±0.6→6.2±1.0，$p<0.01$）。呼吸機能検査では，1秒量，%1秒量（1秒量／予測1秒量x100），朝のピークフロー値は，有意差には達せず，夕のピークフロー値も改善を認めなかった。喘息症状に関しては，夜間症状，日内症状，痰量のいずれも，有意差には達しなかった。

一方，咳嗽はPPI投与によって有意に改善した（**図3**：2.9±1.1→1.1±0.3，$p<0.05$）。PPI投与前には13例中8例で咳嗽を認めたが，うち6例で投与後に改善した。

また，QOLもPPI投与によって有意に改善した。AQLQでは，感情（**図4A**：5.13±0.26→5.50±0.23，$p<0.05$）および，環境刺激（**図4B**：5.16±0.31→5.65±0.21，$p<0.05$），SF-36では心の健康（mental health）が有意に改善した。（**図4C**：71.8±3.9→78.2±3.0，$p<0.05$）。

3．酸曝露が気道上皮細胞株の生存および炎症性メディエーター産生に及ぼす影響

1）酸曝露が気道上皮細胞生存に及ぼす影響

BEAS-2B細胞を種々のpHで経時的に培養し，細胞生存を検討した結果を**図5**に示す。4時間後の早期より，pH依存性に生細胞比率の低下が認められ，pH5以下では，有意に生細胞比率が減少していた。その後も時間経過に伴って，生細

胞比率は減少し，24時間後には，pH 5以下ではほとんどの細胞が細胞死に陥った。pH 6においても24時間後には有意な細胞死を認め，以後経時的に細胞死は増加した。

2）低pHが気道上皮細胞からの炎症性メディエーター産生に及ぼす影響

BEAS-2B細胞をpH 7で72時間培養後の上清において測定可能であったサイトカイン・ケモカ

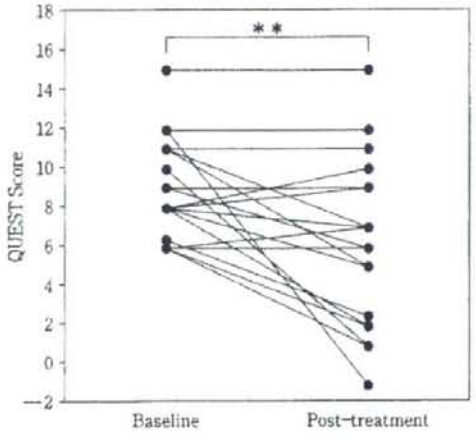

図2 PPI投与によるQUEST scoreの変化（$n=19$，$^{**}p=0.006$）

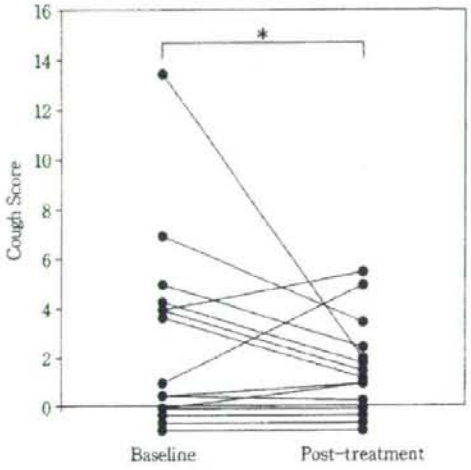

図3 PPI投与による咳嗽症状の変化（$n=13$，$^{*}p=0.036$）。

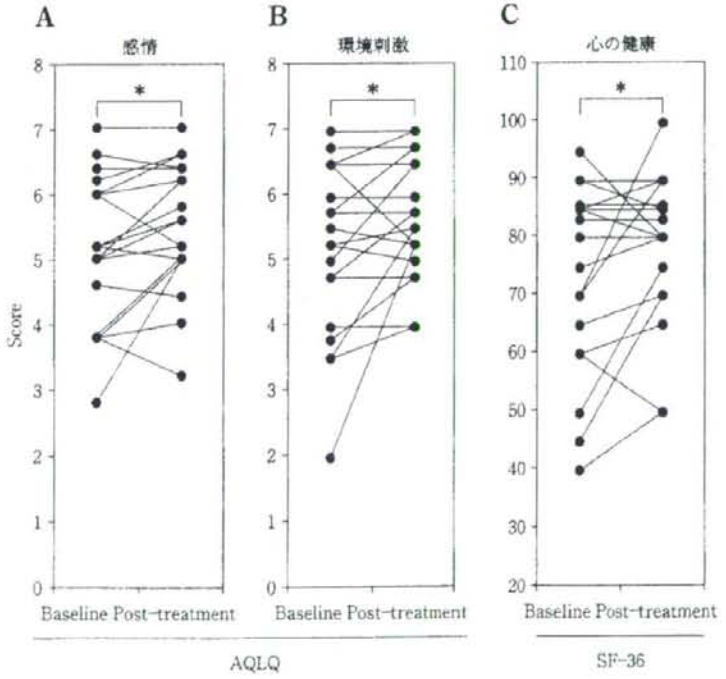

図4 PPI投与によるQOLの変化（$n=19$，$^{*}p<0.036$）。

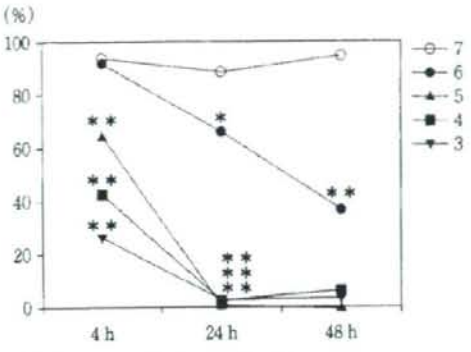

図5 BEAS-2B 生存率の経時的変化
総細胞数を 100 % とし，Annexin V 陰性の生細胞数の占める比率を示した
(n=6，平均値±標準誤差)．各培養時間における pH 7 での生存率と比較して，$^{*}p<0.05$，$^{**}p<0.01$.

インは IL-6, MCP-1, RANTES, MIP-1β, IL-8, IP 10 であった。これらのサイトカイン・ケモカインは pH 依存性に分泌が減弱した。MCP-1, MIP-1β, IL-8, IP 10 は pH 6.5 で，IL-6, RANTES は pH 6 で有意に産生が減弱していた（図 6）。一方，PDGF-BB は，pH 7 で 72 時間培養後には，17.7±9.8 pg/ml と低値であったが，pH 4 より低い pH での培養では，有意に PDGF-BB 分泌が増強した（pH 3：454.5±102.6 pg/ml，$p<0.01$）。時間経過も検討したが，pH 3 では，培養 4 時間後より有意に PDGF-BB 分泌が増強していた（pH 7：0.2±0.0 pg/ml vs pH 3：123.6±14.8 pg/ml，$p<0.01$）。

以上から，酸曝露によって早期より気道上皮細胞死が惹起され，PDGF-BB 産分泌が増強することが観察された。

考　察

本研究では，気管支喘息患者の QUEST 陽性率は，カットオフ値を 4 点以上とした場合 54.5 %，6 点以上とした場合 40.9 % であり，気管支喘息患者は高率に GERD を合併していることが示唆された。また，QUEST スコアは気管支喘息重症度と正相関すること，QUEST スコアは使用薬剤よりも気管支喘息重症度と強く相関することが示された。さらに，QUEST 陽性患者に PPI を投与すると，QUEST スコアおよび咳嗽は有意に改善し，QOL も向上したことから，気管支喘息患者における GERD 合併を，QUEST 問診票を用いて診断・治療する診療モデルの妥当性が示唆された。

GERD と気管支喘息の関連は 1960 年代から報告されており[2]，喘息患者の 40 % にびらん性食道炎があることや，80 % 以上に異常逆流を認めることが報告されている[3]。既報では，気管支喘息患者における GERD の診断は，比較的侵襲の高い上部消化管内視鏡や pH モニタリングで行われているものが多く，QUEST 問診票を用いた報告は 2 報のみである。気管支喘息患者における QUEST 陽性率は 41～73 % と報告されており[4,5]，われわれの検討でもカットオフ値を 4 点以上とした場合 54.5 % と高率に陽性であった。QUEST 陽性率が気管支喘息患者において，非喘息患者と比して有意に高いかどうかは，非喘息患者における調査が必要であるが，気管支喘息重症度と QUEST スコアが有意な正相関を示したことは，両病態の間に強い関連性があることを示唆している。

QUEST 問診票は，胸焼けなどの典型的な GERD 症状をスコア化しており，消化器疾患領域では広く用いられているが，気管支喘息患者において，GERD の典型的症状がいかに修飾されるかについては不明であり，治療的に介入して QUEST 問診票の有用性を検証した報告はない。今回の検討では，PPI 投与後に逆流症状を示す QUEST スコアは 19 例中 11 例で，また全体としても有意に改善しており，気管支喘息患者においても GERD 診断に QUEST 問診票を適用することの妥当性が示唆された。

また，気管支喘息重症度と GERD 合併率の相関に関する詳細な報告はなかったが，われわれの検討では気管支喘息重症度と QUEST スコアは強い正相関を認め，両病態の関連が定量的に示唆された。さらに，気管支拡張薬である β_2 刺激薬やテオフィリンによる下部食道括約筋の弛緩が，GERD を増悪させる可能性も指摘されているが，興味深いことに，今回行った重回帰分析では，QUEST スコアを最も強く規定する因子は，使用薬剤ではなく気管支喘息重症度であった。Sontag らも，気管支喘息患者において，気管支拡張薬使用の有無は GERD 合併率と関連しなかったことを報告している[3]。これらのことは，薬剤による修飾のみで