eosinophils was significantly induced only when eosinophils were coincubated with neutrophils and stimulated with GRO-α (migrated eosinophils: 0.6 ± 0.3 by medium control, 1.2 ± 0.4 by a coincubation with neutrophils, P = n.s.; 0.8 ± 0.7 by GRO-α alone, P = n.s.; 7.1 ± 2.3 by a combination of GRO-α and coincubation with neutrophils, $P < 0.05$ versus the other three conditions, $n = 4$; Figure 2A). LTB_4 (100nM), which is chemotactic for both eosinophils and neutrophils, directly induced the TBM of eosinophils; however, the TBM of eosinophils was enhanced when both eosinophils and neutrophils were coincubated (migrated eosinophils: 1.0 ± 0.8 by medium control, 1.2 ± 0.9 by a coincubation with neutrophils, P = n.s.; 12.9 ± 4.6 by LTB_4 alone, $P < 0.05$ versus control; 23.6 ± 6.0 by a combination of LTB_4 and coincubation with neutrophils, $P < 0.05$ versus the other three conditions, $n = 5$; Figure 2B). Finally, eotaxin (3 nM), a CC chemokine that selectively stimulates chemotactic response of eosinophils, induced the TBM of eosinophils (migrated eosinophils: 2.9 ± 0.7 by medium control versus 59.5 ± 3.8 by eotaxin alone, $P < 0.01$). Coincubation with neutrophils did not modify the TBM of eosinophils in response to eotaxin (migrated eosinophils: 62.3 ± 4.5, P = n.s. versus eotaxin alone, $n = 5$; Figure 2C). Similar results were obtained when RANTES, another CC chemokine, was an activator ($n = 3$, data not shown).

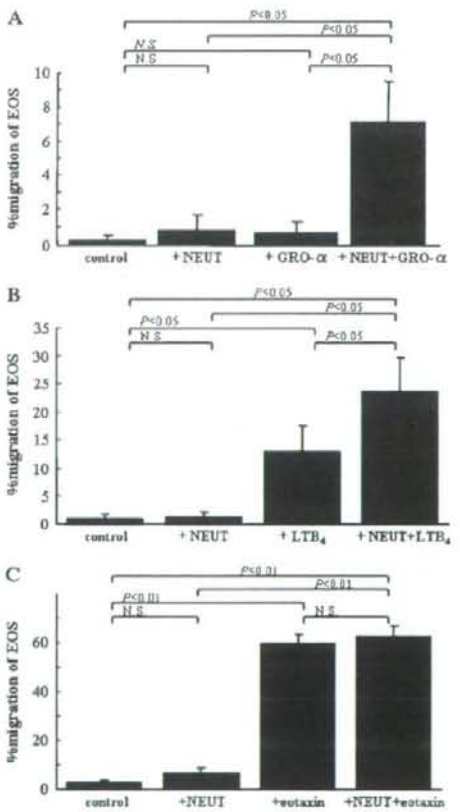

Figure 2. Effects of neutrophils and a variety of agonists on the TBM of eosinophils. Molecules used were GRO-α (10 nM, *A*), LTB_4 (100 nM, *B*), and eotaxin (10 nM, *C*). The means ± SEM of four to five experiments using cells from different donors are shown. N.S., not significant.

MMP-9, LTB_4, PAF, and TNF-α Are Involved in the Augmentation of Eosinophil TBM by IL-8–Stimulated Neutrophils

The results shown the above sections suggest that neutrophils act as regulators to affect TBM of eosinophils. Suppression of neutrophil-derived protease may lead to the suppression of TBM of eosinophils. To test this hypothesis, we inhibited MMPs, which work when cells digest the basement membrane during intrusion. An MMP-9 inhibitor (10 μM) could inhibit the augmented TBM of eosinophils in the presence of IL-8–activated neutrophils (Figure 3A). Similar results were observed when GM1489, an inhibitor of MMPs, was tested ($n = 5$, data not shown).

Activated neutrophils may secrete stimulatory molecules for eosinophils and thus augment their TBM as observed above. LTB_4, PAF, and TNF-α are the representative molecules secreted by neutrophils and are capable of activating eosinophils. To investigate whether they are involved, we inhibited the activity of these molecules and observed whether the TBM of eosinophils was suppressed. Reagents used were BIIL260, an LTB_4 receptor antagonist; WEB2086, a PAF receptor antagonist; and an anti–TNF-α monoclonal antibody (anti–TNF-α mAb, clone Mab11, mouse IgG1, 3 μg/ml). All of these reagents partially suppressed the TBM of eosinophils in the presence of IL-8–activated neutrophils but showed no effect in the absence (Figures 3B–3D). Similar results

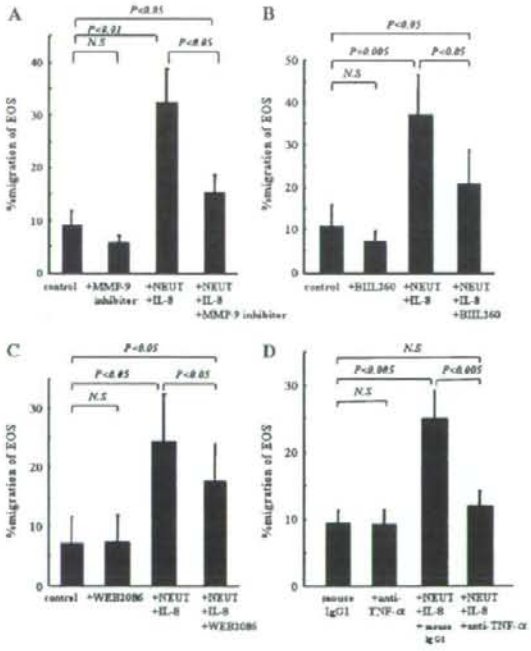

Figure 3. Effects of MMP-9 inhibitor (10 μM, *A*); BIIL260, an LTB_4-antagonist (1 μM, *B*); WEB2086, a PAF-antagonist (10 μM, *C*); and an anti–TNF-α antibody (clone Mab11, isotype mouse IgG1, 3 μg/ml; *D*) on the TBM of eosinophils in the presence of neutrophils stimulated by IL-8. The means ± SEM of five to seven experiments using cells from different donors are shown.

were obtained in the studies using AA861, a 5-LO inhibitor that suppresses the production of LTB_4, and WEB2070, another PAF receptor antagonist (data not shown). These results indicate that the augmentation of eosinophil TBM due to IL-8–stimulated neutrophils is partly mediated by LTB_4, PAF, and TNF-α. The effects of these inhibitors on TBM of neutrophils alone or eosinophils alone were also examined: IL-8 (10 nM)–induced TBM of neutrophils was not modified by inhibitors for MMP9, LTB4, PAF, or TNF-α ($n = 6$, P = n.s., data not shown). Similarly, eotaxin (3 nM)-induced TBM of eosinophils was not modified by inhibitors for MMP9, LTB4, or PAF ($n = 6$, P = n.s., data not shown). Anti–TNF-α mAb slightly but significantly reduced eotaxin-induced TBM of eosinophils (migrated eosinophils: 48.1 ± 3.5 by isotype mouse IgG1 versus 39.3 ± 2.4 by eotaxin alone, $n = 6$, $P = 0.02$), suggesting that TNF-α may act as an autocrine activator and partly contribute to the TBM of eosinophils in this system. Finally, pretreatment of only neutrophils, but not eosinophils, with the various inhibitors did not modify the subsequent TBM of eosinophils in the presence of IL-8–activated neutrophils ($n = 3$, P = n.s., data not shown).

The Conditioned Medium from IL-8-Stimulated Neutrophils Induces the TBM of Eosinophils

To further examine whether the neutrophils transmigrated to IL-8 produce chemoattractants for eosinophils, neutrophils were added to the upper compartment of a chamber with a Matrigel-coated transwell insert and either the control medium or IL-8 (10 nM) was added to the bottom compartment. After a 2-h incubation in 5% CO_2 at 37°C, the bottom compartments were centrifuged at 4°C for 20 min at 700 × *g*. The supernatants were gently recovered and then examined for their ability to induce the TBM of eosinophils. The conditioned medium from a combination of IL-8 and neutrophils significantly induced the TBM of eosinophils as compared with the control medium or the condition medium from neutrophils in the absence of IL-8 (migrated eosinophils: 7.0 ± 0.6%, $P < 0.05$ versus the other two conditions, $n = 5$).

Anti-CXCR2 Antibody Does Not Modify the Augmentation of Eosinophil TBM by IL-8–Stimulated Neutrophils

The augmented TBM of eosinophils observed with IL-8–stimulated neutrophils may be a consequence of modification of the CXCR2, an IL-8 ligand, which is expressed on activated eosinophils (24). Although addition of the anti-CXCR2 antibody partly attenuated the TBM of neutrophils in response to IL-8 (% inhibition: 53.3 ± 8.6%, $n = 5$, $P < 0.01$ versus isotype control), the augmented TBM of eosinophils in the presence of IL-8 and neutrophils was not significantly modified by this antibody ($n = 5$, P = n.s., data not shown). An anti-CXCR1 antibody provided similar results ($n = 2$, data not shown).

DISCUSSION

We showed that neutrophils stimulated with chemoattractant such as IL-8 augment the TBM of eosinophils. The reaction kinetics of the augmented TBM of eosinophils traced that of neutrophils. The augmented TBM of eosinophils by a combination of neutrophils and IL-8 was inhibited by an MMP-9 inhibitor, an LTB_4 receptor antagonist, PAF-antagonists, or an anti–TNF-α mAb. LTB_4, a chemotactic factor for both neutrophils and eosinophils (18, 25), by itself induced the TBM of eosinophils, and this TBM is augmented by the presence of neutrophils. There results provide a mechanism for our previous observation that a positive correlation between the concentrations of neutrophils and eosinophils in sputum from subjects with severe asthma (10), and suggest that neutrophils can regulate the accumulation of eosinophils through these mechanisms in the airways of asthma.

The mechanism by which neutrophils enhance the TBM of eosinophils is important. We showed that the inhibition of MMP-9 effectively suppresses the augmented TBM of eosinophils. There is evidence that IL-8 stimulates the release of MMP-9 from neutrophils (26). Moreover, the cellular source of MMP-9 may include eosinophils (27). The MMP-9 inhibitor, which is a selective inhibitor of MMP-9 (IC50 = 5 nM) (28), did not modify the TBM of neutrophils to IL-8 or eosinophils to eotaxin, suggesting that MMP-9 is not required for TBM induced by these ordinary and potent chemoattractants in these experimental conditions. Nonetheless, our results suggest that digestion of membrane by MMP-9 is involved in the mechanisms of augmented TBM of eosinophils induced by IL-8–stimulated neutrophils. The process of TBM of eosinophils would also require the presence of an activator that acts as either chemotactic or chemokinetic for eosinophils. Zuurbier and colleagues (29) reported that eosinophil migration across monolayers of lung epithelial cells in response to complement fragment 5a (C5a), but not to RANTES, PAF, or IL-8, was increased in the presence of neutrophils. They explained that neutrophils, but not eosinophils, rapidly inactivated C5a and decreased the activity of C5a that had diffused into the upper compartment, and thereby maintain a proper C5a chemotactic gradient in their trans-epithelial migration model. In contrast, our study suggested that neutrophils enhanced the TBM of eosinophils, at least in part, via the generation of activators for eosinophils: the inhibition of LTB_4, PAF, or TNF-α actions partially suppressed TBM of eosinophils. In addition to LTB_4, PAF is a chemotactic factor for both neutrophils and eosinophils (18, 24). The pharmacologic inhibitors may reduce migration of neutrophils and subsequently reduce migration of eosinophils, instead of acting to disrupt the effects of neutrophil-derived mediators on eosinophils. From this point of view, we observed that IL-8–induced TBM of neutrophils in this system was not modified by inhibitors for LTB4 or PAF. Furthermore, pretreatment of neutrophils with the various inhibitors did not modify the subsequent TBM of eosinophils in the presence of IL-8–activated neutrophils. TNF-α is not a chemotactic agent for neutrophils or eosinophils itself, but has been shown to be an activator for functions of both neutrophils and eosinophils such as adhesion or respiratory burst (25, 30). Our results that TNF-α is involved in the augmented TBM of eosinophils suggest that the activation of effector function(s) of either neutrophils or eosinophils may be sufficient to augment the TBM of eosinophils. We observed that anti–TNF-α mAb slightly but significantly reduced eotaxin-induced TBM of eosinophils, but not IL-8–induced TBM of neutrophils, suggesting some role of this cytokine as an autocrine activator for eosinophil migration in this system.

Increased concentrations of LTB_4 (31, 32), PAF (33), and TNF-α (34) have been reported in the airways of asthma. Our results suggest that neutrophils are activated via a combination of IL-8 and basement membrane and generate these mediators. In this context, we found that the conditioned medium from a combination of IL-8 and neutrophils, but not neutrophils alone or medium alone, induced the TBM of eosinophils, indicating that the neutrophils transmigrated to IL-8 produce chemoattractant(s) that result in subsequent migration of eosinophils. Taken together, the augmented TBM of eosinophils is likely a complex of the effects of both MMP-9, on the basement membrane, and eosinophil activators and chemoattractants such as LTB_4, PAF, and TNF-α.

Our assay simulates the initial phase of inflammation involving neutrophils and eosinophils, in which cells are stimulated and

migrate out of blood vessels to accumulate at the inflammation site. In the later phase of inflammation, the expression of surface receptors of cells may be modified to become responsive to molecules that they were initially unresponsive. A good example is the expression of CXCR2, an IL-8 ligand, in activated eosinophils (24). The augmented TBM of eosinophils in the presence of IL-8 and neutrophils was not significantly modified by anti-CXCR2 antibody, ensuring in this assay that eosinophils are activated not directly by IL-8 but via the activation of neutrophils. However, this antibody partly attenuates the TBM of neutrophils to IL-8. Similar results were observed with an anti-CXCR1 antibody. These results suggest that migration of neutrophils is not a sole contributing factor for the augmented eosinophil migration. Not only MMP-9 or chemoattractants released from transmigrated neutrophils, but also the eosinophil activators released from neutrophils activated by IL-8 and integrin-mediated signaling before transmigration, may be important in the subsequent migration of eosinophils. We speculated that a complex consisting of this priming process, and release of MMP-9 and chemoattractants from migrated neutrophils, results in the eventual manifestation of the enhanced transmigration of eosinophils.

Neutrophils may actively participate in the development of airway disease of asthma. Our results suggest that neutrophils migrated to IL-8 may lead eosinophils to accumulate in the airways of asthma and possibly aggravate this disease. Therefore, therapies that suppress functions or accumulation of neutrophils may be effective for severe asthma. Further study will be warranted to elucidate the detailed roles of neutrophils in the pathophysiology of the disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Eosinophils Do Not Enhance the Trans-Basement Membrane Migration of Neutrophils

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Key Words
Neutrophils · Eosinophils · Interleukin-8 · Cell migration

Abstract

Background: There is increasing evidence that both neutrophilic and eosinophilic inflammation persist in the airways of patients with severe asthma. We have reported a positive relationship between the concentrations of eosinophils and neutrophils in sputum from severe asthmatics, suggesting a possible role of eosinophils in regulating neutrophilic inflammation. The aim of this study was to investigate whether activated eosinophils modify the trans-basement membrane migration (TBM) of neutrophils. ***Methods:*** Eosinophils and neutrophils were isolated from peripheral blood drawn from healthy donors. The TBM of neutrophils in response to a variety of chemoattractants was evaluated in the presence or absence of eosinophils by using the chambers with a Matrigel®-coated Transwell® insert. ***Results:*** As expected, eotaxin (10 nM) and RANTES (10 nM), but not IL-8 (10 nM), induced the TBM of eosinophils. On the contrary, only IL-8 induced the TBM of neutrophils. When eosinophils were coincubated with neutrophils and stimulated with IL-8, the TBM of eosinophils was significantly augmented. On the other hand, when neutrophils were coincubated with eosinophils and stimulated with eotaxin or RANTES, the TBM of neutrophils was not modified. ***Conclusions:*** Neutrophils migrated by IL-8 may lead eosinophils to accumulate in the airways of patients with severe asthma. On the other hand, it is unlikely that eosinophils migrated by chemoattractants such as CC chemokines regulate neutrophilic inflammation.

Introduction

Eosinophils are inflammatory cells predominantly found in the airways of patients with asthma and therefore possibly contribute to the pathophysiology observed in asthma [1–4]. The mechanism by which eosinophils accumulate in the airways is a complex process which is mainly regulated by cytokines, chemokines, and adhesion molecules. This process is likely to be inhibited by corticosteroid treatment via the reduced productions of cytokines and chemokines from corticosteroid-sensitive cells such as Th2 cells. In a subgroup of patients with asthma such as those with severe asthma, the accumulation of neutrophils is found in their airways even in the absence of apparent infection [5–8]. Based upon the European network study for understanding mechanisms of severe asthma (ENFUMOSA), patients with severe asthma have greater percentage of neutrophils in sputum and evidence of the continuing release of eosinophil-derived mediators in comparison to mild to moderate asthmatics,

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suggesting that both neutrophilic and eosinophilic inflammation persist in their airways [9]. Consistently, we observed a positive correlation between the numbers of neutrophils and eosinophils in induced sputum from severe persistent asthmatics that had been treated with systemic corticosteroids [10]. We have previously reported that when eosinophils were coincubated with neutrophils and stimulated with IL-8 in vitro, the trans-basement membrane migration (TBM) of eosinophils was significantly induced [11]. This provides a hypothesis that neutrophils may be a controlling factor for regulating the accumulation of eosinophils in the airways of patients with severe asthma who are treated with corticosteroids. This hypothesis is challenged by the possibility that eosinophils are autonomically regulating the accumulation of neutrophils. The aim of this study was to obtain the supporting evidence for the first hypothesis by investigating whether activated eosinophils modify the TBM of neutrophils.

Material and Methods

Reagents

Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyl Biotec (Auburn, Calif.). Percoll® was purchased from Pharmacia (Uppsala, Sweden). HBSS was purchased from Gibco BRL (Grand Island, N.Y., USA). IL-8, eotaxin, and regulated upon activation, normal T cell expressed, and secreted (RANTES) were purchased from R & D Systems (Minneapolis, Minn., USA). LTB4 was purchased from Cayman Chemical (Ann Arbor, Mich., USA). O-Phenylenediamine (OPD) and BSA were obtained from Sigma (St. Louis, Mo., USA). The acetoxy methyl ester of 2′-7′-bis (2-carboxy-ethyl)-5(6)-carboxyfluorescein (BCECF-AM) was purchased from Dojin Laboratory (Kumamoto, Japan).

Preparation of Neutrophils and Eosinophils

Neutrophils and eosinophils were isolated from peripheral blood collected from nonatopic healthy donors whose eosinophil content was less than 5% of their peripheral leukocytes [11]. The numbers of males and females were comparable among donors, with similar age distributions ranging from 23 to 35 years. Informed consent was obtained prior to the collection of each blood sample. Neutrophils and eosinophils were separated by the combination of Percoll density gradient centrifugation and negative immunomagnetic bead selection as previously described [12]. Briefly, 40 ml of dextran were added to 160 ml of heparinized blood, and erythrocytes were removed as sediments. The remaining suspension of leukocytes was layered onto Percoll gradients of 1.080, 1.085, and 1.090 g/ml in density. After centrifugation at 700 *g* for 20 min, neutrophils (purity exceeded 95%) were collected from 1.085/1.090 g/ml interface and were then suspended in HBSS containing 0.2% BSA (HBSS/BSA buffer). Following the removal of Percoll, red blood cells in the pellet were lysed by hypotonic shock and were then removed by washing with cold PBS. The remaining cells were washed with 4°C HBSS supplemented with 2% NCS (HBSS/NCS), were incubated with anti-CD16 antibody-coated magnetic beads for 30 min at 4°C and were then filtered with a column containing steel wool placed in a magnetic field (Miltenyl Biotec). Eosinophils (>98% purity and >99% viability), which passed through the column, were collected and washed, and the number of cells was adjusted to 2.5×10^5 cells/ml by using the HBSS/BSA buffer.

Trans-Basement Membrane Migration

The TBM of neutrophils and eosinophils was examined using a modified Boyden's chamber method [11, 13]. The study was conducted in duplicate. Briefly, neutrophils were suspended in the loading buffer with BCECF-AM at a final concentration of 1 μM and were then incubated for 30 min at 37°C in a dark chamber [11, 14]. Under this condition, the generation of superoxide anion from neutrophils was not modified by BCECF-AM [unpubl. observation]. Labeled neutrophils (0.5×10^5 cells), labeled eosinophils (0.5×10^5 cells), or a combination thereof (0.5×10^5 cells plus 0.5×10^5 cells) in a 200-μl medium was added to the upper compartment of a chamber with a Matrigel®-coated Transwell® insert (pore size 3 μM; Becton Dickinson Labware, N.Y., USA). Either the control medium (500 μl) or a medium which contained one of the activators (IL-8, eotaxin, RANTES, and LTB4) was added to the lower compartment of the chamber. After a 2-hour incubation in 5% CO_2 at 37°C, the medium in the upper compartment and the inserts in between were gently removed. The peroxidase activity of eosinophils in the medium in the lower compartment was determined, and the number of migrated eosinophils was calculated from the activity of the standard media which contained known numbers (5×10^3, 1.5×10^4, 5×10^4, 1.5×10^5, and 5×10^5 cells) of eosinophils. To determine the peroxidase activity of eosinophils, the medium was incubated with a substrate (1 mM OPD, 1 mM H_2O_2, and 0.1 % Triton X-100 in Tris-HCl, pH 8.0) for 30 min at room temperature [12, 13]. The reaction was stopped by adding 50 μl of 4 N H_2SO_4, and absorbance at 490 nm was determined [12, 13]. The numbers of migrated neutrophils were determined by the measurement of fluorescence in the medium using the Fluoromark® (Bio-Rad Laboratories, Calif., USA) microplate fluorometer [11, 13]. Viability of both eosinophils and neutrophils after migration exceeded 98% by trypan blue exclusion.

Statistical Analysis

Values are expressed as mean ± SEM. Student's t test was used to compare two groups, and repeated-measures analysis of variance (ANOVA) with Scheffé's constants was used to compare more than two groups. $p < 0.05$ was considered statistically significant.

Results

To reconfirm whether stimulated neutrophils affect the TBM of eosinophils, we coincubated a mixture of eosinophils and neutrophils in the presence or absence of IL-8, a CXC chemokine which selectively stimulates the chemotaxis of neutrophils. As previously reported, nei-

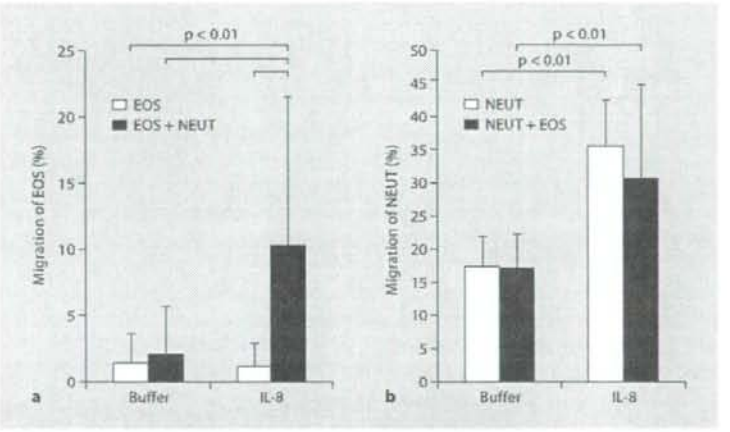

Fig. 1. Effects of IL-8 (10 nM) on the trans-basement membrane migration (TBM) of eosinophils (**a**) and neutrophils (**b**). Open bars represent the TBM of each cell type alone, and closed bars represent the TBM in the presence of both cell types. The means ± SEM of 10 experiments using cells from different donors are shown.

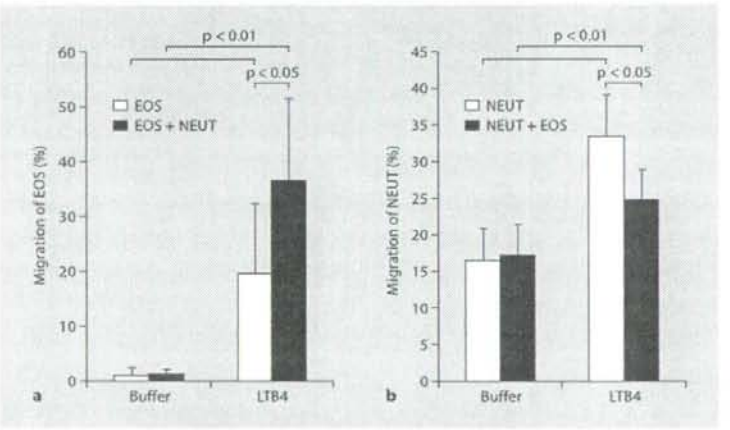

Fig. 2. Effects of LTB4 (30 nM) on the TBM of eosinophils (**a**) and neutrophils (**b**). Open bars represent the TBM of each cell type alone, and closed bars represent the TBM in the presence of both cell types. The means ± SEM of 8 experiments using cells from different donors are shown.

ther coincubation with neutrophils nor stimulation with IL-8 (10 nM) alone induced the TBM of eosinophils (% migrated eosinophils: 1.4 ± 2.2 with the medium control, 2.1 ± 1.8 by coincubation with neutrophils, p = n.s., 1.2 ± 3.6 by IL-8 alone, p = n.s., n = 10). However, when eosinophils were coincubated with neutrophils and stimulated with IL-8, a significant TBM of eosinophils was observed (% migrated eosinophils: 10.3 ± 11.2, $p < 0.01$ vs. other three conditions, n = 10; fig. 1 left panel). Although IL-8 (10 nM) significantly induced the TBM of neutrophils regardless of the presence of eosinophils, the IL-8-induced TBM of neutrophils was not modified by coincubation with eosinophils (% migrated neutrophils: 17.6 ± 4.5 with the medium control, 17.5 ± 6.9 by coincubation with eosinophils, p = n.s., 35.0 ± 4.8 with IL-8 alone, $p < 0.01$, 30.0 ± 14.2 by coincubation with eosinophils and stimulated with IL-8, $p < 0.01$, n = 10; fig. 1 right panel).

LTB4 (30 nM) alone induced the TBM of eosinophils (% migrated eosinophils: 0.9 ± 1.3 with the medium control, 1.1 ± 0.9 by coincubation with eosinophils, p = n.s., 19.4 ± 12.9 by LTB4, $p < 0.01$, n = 8). This TBM of eosinophils was augmented by coincubation with neutrophils (% migrated eosinophils: 36.6 ± 15.0, $p < 0.05$ vs. LTB4 alone, n = 8; fig. 2 left panel).

LTB4 (30 nM) alone induced the TBM of neutrophils (% migrated neutrophils: 16.4 ± 4.5 with the medium control, 17.1 ± 4.2 by coincubation with eosinophils, p = n.s., 33.3 ± 5.6 by LTB4, $p < 0.01$, n = 8). Unexpectedly, this TBM of neutrophils induced by LTB4 was attenuated

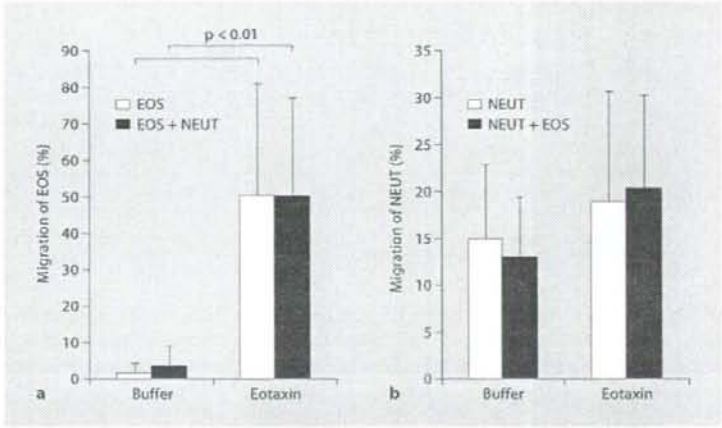

Fig. 3. Effects of eotaxin (10 nM) on the TBM of eosinophils (**a**) and neutrophils (**b**). Open bars represent the TBM of each cell type alone, and closed bars represent the TBM in the presence of both cell types. The means ± SEM of 7 experiments using cells from different donors are shown.

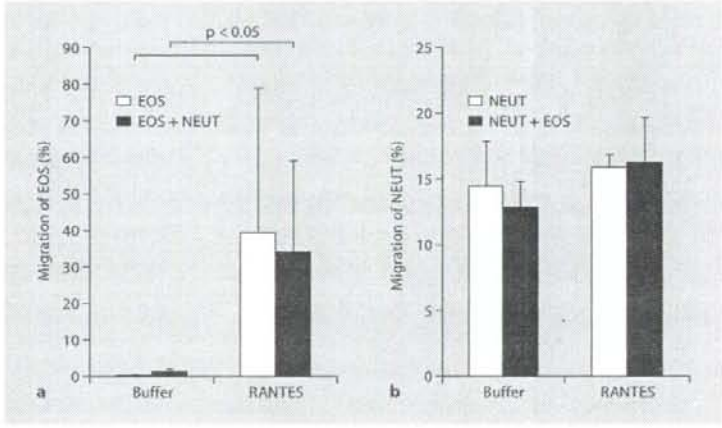

Fig. 4. Effects of RANTES (10 nM) on the TBM of eosinophils (**a**) and neutrophils (**b**). Open bars represent the TBM of each cell type alone, and closed bars represent the TBM in the presence of both cell types. The means ± SEM of 3 experiments using cells from different donors are shown.

by coincubation with eosinophils (% migrated neutrophils: 24.7 ± 4.1 by coincubation with eosinophils and stimulated with LTB4, $p < 0.05$ vs. LTB4 alone, $n = 8$; fig. 2 right panel).

Eotaxin (10 nM) induced the TBM of eosinophils which was not modified by neutrophils, as previously reported (% migrated eosinophils: 1.6 ± 2.7 with the medium control, 3.4 ± 5.6 by coincubation with neutrophils, p = n.s., 50.6 ± 30.6 with eotaxin alone, $p < 0.01$, vs. control, 50.3 ± 27.0 by coincubation with neutrophils and stimulated with eotaxin, $p < 0.01$, $n = 7$; fig. 3 left panel). Neither eotaxin alone nor the combination of eosinophils and stimulation with eotaxin induced the TBM of neutrophils (% migrated neutrophils: 15.0 ± 7.9 with the medium control, 13.1 ± 6.3 by coincubation with eosinophils, p = n.s., 19.0 ± 11.9 with eotaxin alone, p = n.s., 20.5 ± 9.9 by coincubation with eosinophils and stimulated with eotaxin, p = n.s., $n = 8$; fig. 3 right panel).

Similarly, RANTES (10 nM) induced the TBM of eosinophils which was not modified by neutrophils (% migrated eosinophils: 0.3 ± 0.1 with the medium control, 1.4 ± 0.7 by coincubation with neutrophils, p = n.s., 39.5 ± 39.9 with RANTES alone, $p < 0.05$, 34.5 ± 24.7 by coincubation with neutrophils and stimulated with RANTES, $p < 0.05$, $n = 3$; fig. 4 left panel). Neither RANTES alone nor the combination of eosinophils and RANTES induced the TBM of neutrophils (% migrated neutrophils: 14.5 ± 3.3 with the medium control, 12.9 ± 2 by coincubation with eosinophils, p = n.s., 16.0 ± 0.9 with

RANTES alone, p = n.s., 16.3 ± 3.4 by coincubation with eosinophils and stimulated with IL-8, p = n.s., n = 3; fig. 4 right panel).

Discussion

In this study, we reconfirmed that, when eosinophils are coincubated with neutrophils and stimulated with either IL-8 or LTB4, a significant TBM of eosinophils is introduced, as previously reported [11]. This supports the hypothesis that neutrophils may be a controlling factor for regulating the accumulation of eosinophils in the airways of patients with severe asthma. Under this experimental condition, however, we confirmed that the IL-8-induced TBM of neutrophils is not modified by coincubation with eosinophils. Surprisingly, this TBM of neutrophils induced by LTB4 was attenuated by eosinophils. CC chemokines eotaxin and RANTES induced the TBM of eosinophils which was not modified by neutrophils, as previously reported [11]. On the contrary, neither CC chemokine alone nor the combination of eosinophils and CC chemokine induced the TBM of neutrophils.

We did not expect that the LTB4-induced TBM of neutrophils is attenuated by coincubation with eosinophils. Although the exact mechanism of this event remains unknown, the combination of LTB4 and eosinophils possibly results in the enhanced production of factors that inhibit either neutrophil migration or LTB4 actions. One of the possible candidate substances responsible for this event may be lipoxins, bioactive eicosanoids that are formed during cell-to-cell interactions between activated inflammatory cells including eosinophils [15]. For example, lipoxin A4 can attenuate neutrophil migrative response [16] and also inhibits the leukotriene B4-induced generation of inositol 1,4,5-trisphosphate from neutrophils [17]. Therefore, lipoxin A4 might be generated from eosinophils during the TBM in response to LTB4, thus attenuating the TBM of neutrophils by LTB4. This issue should be further investigated in a future study.

The mechanism by which the lack of enhancement of neutrophil TBM by eosinophils acts remains to be elucidated. Eosinophils are capable of generating a variety of factors, including lipid mediators, cytokines, and chemokines, which may activate the TBM of neutrophils [1]. However, it is plausible that the interaction between eosinophils and chemoattractants-basement membrane complexes fails to generate sufficient concentrations of neutrophil-activating or chemotactic molecules under the conditions of the present study.

Corticosteroids, the mainstream of long-term treatment for asthma, are not sufficient to suppress the activation of neutrophils [18, 19], suggesting that neutrophils continue to activate inflammation and thus play a role in the pathophysiology of severe asthma. Neutrophils are likely to be exposed to a variety of inflammatory mediators in the airways of asthmatic patients. For example, IL-8 is found in bronchoalveolar lavage fluid and serum obtained from patients with asthma [20–22], and therefore this chemokine may be an essential molecule responsible for the development of neutrophilic inflammation in asthma. Activated neutrophils can secrete a variety of mediators, e.g. matrix metalloproteinases (MMPs), LTB4, and platelet-activating factor (PAF), as well as a variety of cytokines, which can induce the digestion of basement membrane or the migration or activation of eosinophils [23, 24]. From this point of view, we observed that the TBM of eosinophils induced by the combination of neutrophils and IL-8 in vitro is inhibited by MMP-9 inhibitors, LTB4 antagonists, or PAF-receptor antagonists [11]. On the other hand, our results demonstrated that neutrophil migration was not enhanced under the conditions in which eosinophil TBM is strongly occurred, suggesting that eosinophils do not regulate the trans-basement membrane migration of neutrophils.

In conclusion, neutrophils which accumulated in the airways of patients with severe asthma may contribute to the development of eosinophilic inflammation in severe asthma, while eosinophils are not likely to control neutrophil accumulation. Therefore, therapeutic strategies against neutrophils and/or neutrophil-induced eosinophil accumulation may be beneficial for a subgroup of asthmatic patients, especially for those with severe disease which is resistant to the corticosteroid treatment.

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綜　説

喘息治療とH5N1感染
—Early Interventionの提言—

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Key words: cessation from the long term medication in adult asthma —— early intervention for asthma —— early intervention for human H5N1

はじめに

実際の講演は，学会誌抄録の題名と異なり，上記の演題名とした．

筆者がここ10年間取り組んできた臨床研究を中心に述べる．

喘息の薬物療法には，ガイドラインに示される標準的な治療法によって，飛躍的に進歩し，大多数の患者のぜんそく症状はコントロールされやすくなっている．しかし，喘息発症の原因はいくつか想定されるものの，発症は止められず国内外とも罹患者数は増加し続け，呼吸器疾患の中では第一位を占め，患者自身の負担，社会的，医療経済的負担は増大し続けている．これに対して筆者は，これらの負担を減ずることが出来ないかという立場で臨床研究を行って来た．つまり，①寛解導入を目的とした成人喘息発症早期に集中的な介入療法を行いその正否を検証する，②成人慢性喘息の長期薬物療法からの離脱が可能か，可能ならばその指標は？というテーマである．①の臨床研究の発想と実行は，1993年に発表されたHaahtelaらの喘息に対するearly interventionの論文[1]に触発されたものであるが，私達の方法は，軽症喘息患者のみならず，重症，急性発症型を含めたすべての喘息を対象とすべく，治療法を強化し，いわば集中的治療法をとった．結論的に言えば，早期介入療法の有用性は高いと思われるが，確定する為には多施設共同無作為臨床研究で結論を出すことが必要であり，喘息治療の発展の一つの契機になればと思う．同時にアレルギーとは分野が異なるものの，筆者は新興・再興感染症にここ数年間関与してきた．その中から，SARSやH5N1の新興の急性重症感染症の治療に対してもこのearly interventionが疾患の治療に重要な戦略であることを強く認識し，会長講演とした次第である．いずれも未だ道半ばであるが，講演内容の要点を以下にまとめる．

成人喘息における早期介入療法[2]

現在，気管支喘息は気道炎症であり，炎症が放置されるとすべての症例ではないがリモデリングが進行し，不可逆性を増して重症化し，慢性の病態を完成させていくと考えられている．現在われわれが治療対象としている多くの気管支喘息は，罹病期間が長く，悪循環に陥った完成された病態であるため現在の治療法によっては，根治，寛解

利益相反（conflict of interest）に関する開示：著者は本論文の研究内容について他者との利害関係を有しません．
PROPOSAL OF EARLY INTERVENTION FOR ADULT ASTHMA REMISSION AND HUMAN H5N1 INFECTION
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Disease Control and Prevention Center, International Medical Center of Japan
Abbreviations: BOOP "bronchiolitis obliterans organizing pneumonia", COP "cryptogenic organizing pneumonia"
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が困難になっていると思われる．そうであるならば，逆に気道のリモデリングが出現する前の気管支喘息を早期に診断し治療をする，いわば early intervention を実施すれば，気管支喘息は慢性化することなく，寛解か根治させる可能性があるのではないかと考えた．

early intervention を治療戦略にするとき，大きく3つの課題があると思われる．先ず，何を目標にするのか？—寛解かコントロールか．どの位早期でなければならないか？そして適切な治療（強度，期間）は？である．これらの課題に答えるべく，1997 年より寛解（1 年間無症状で経過するという当研究での仮の定義とする）に持ち込むための，早期介入寛解試験を実施した．

一般に，喘息の診断には依然としてゴールドスタンダードはないと言われている．国際的及び本邦の各種ガイドラインの気管支喘息の診断基準をまとめると，おおむね，1)気流制限の可逆性としてピークフローの 20% 以上の日内変動，あるいは1秒量の 12% 以上の日内変動，2)気道過敏性の存在，3)繰り返す呼吸困難の症状を有すること，4)他疾患の除外となっている．本研究で設定した早期診断のための診断基準は，①ピークフローが 15% の変動，②気道過敏性，メサコリン-PC_{20}が 8000 より低値，という具体的カットポイント値を設定した．これら①，②は，GINA2002[3]にコンセンサスを得ている．③有症期間としては，具体的気道症状が1カ月以上続くものとした．気道症状とは，咳，喀痰，呼吸困難，喘鳴である．この項目は，GINA 等のガイドラインとのコンセンサスは得ていないが，急性気管支炎等の急性気道炎症は通常1カ月以内に何らかの治療か，自然に治まるという臨床的経験による．繰り返す呼吸困難が現在の重要な喘息定義の一つとして唱えられているが，本項にこだわると慢性化したものを喘息とすることになり早期診断の概念と主旨に反する．更に加えて，ガイドラインと同様，④他疾患を除外するものとした．これらの①～④項目をすべて満たした場合喘息と診断した．鑑別を要する疾患，あるいは合併疾患として，咳喘息，急性・慢性の気管支炎，肺気腫，細気管支炎，気管支結核，ブロンコレア，GERD，ABPA(M)，PIE，CSS，精神的咳及び呼吸困難が挙げられるが，GERD と喘息の合併例以外は，本試験の対象から除外した．

次に，どの位の強さの薬物治療が必要であるかを考察させる，一つの症例を示したい（Fig. 1）．重症例として，当初プレドニゾロン（PSL）10mg/day 8 日間そして吸入ステロイドと気管支拡張剤を許容最大量使用している，発症 4 カ月の症例に，再度 PSL を 0.5mg 体重/kg/日を 14 日間投与すると，ピークフロー値は更に上昇することを観察している．本例は一例にすぎないが他の多くの例から，気管支喘息においてピークフローや呼吸機能の最大値を得るためには，一定期間の全身ステロイド投与と気管支拡張薬の投与が必要であることを経験している．そしてステロイド治療期間（吸入の局所療法を含めた期間）については，ステロイド療法が寛解に導く他の肺疾患，たとえば COP（Cryptogenic Organizing Pneumonia，かつては BOOP; Bronchiolitis Obliterans Organizing Pneumoniaと命名）や，好酸球性肺炎（急性・慢性）などにおける治療期間を参考とし，約 4 カ月前後（18 週）と設定した．

実施した研究について述べる．目的は，薬物中止後 1 年間の寛解は可能かどうか，可能な場合はその有意因子は？の検証・検定である．治療法として二つの治療法をとった．一法は喘息の重症度にかかわらず，集中的な治療であり，全身ステロイドを 2 週間投与して 16 週の吸入ステロイドに気管支拡張剤を併用する治療法，他方は症状に応じたガイドラインによる治療法である．この二療法のいずれかを患者自身に選択してもらう前向き研究とした．対象者は 15 歳から 70 歳の発症 2 年以内の先述の診断基準を満たす成人喘息とした．治療導入時急性症状で受診した場合は，集中治療法ではハイドロコーチゾン（300mg/6 時間毎）の静注で始める．18 週の治療が終了した時点で，各種検査を実施し，症状が消失し，薬物治療が中止可能と判断された場合中止し，その後 1 年間の経過を観察した（Fig. 2）．予後によって，寛解，間歇，慢性に分類し，早期介入療法の有用性として予後に寄与する因子を多変量で解析した．

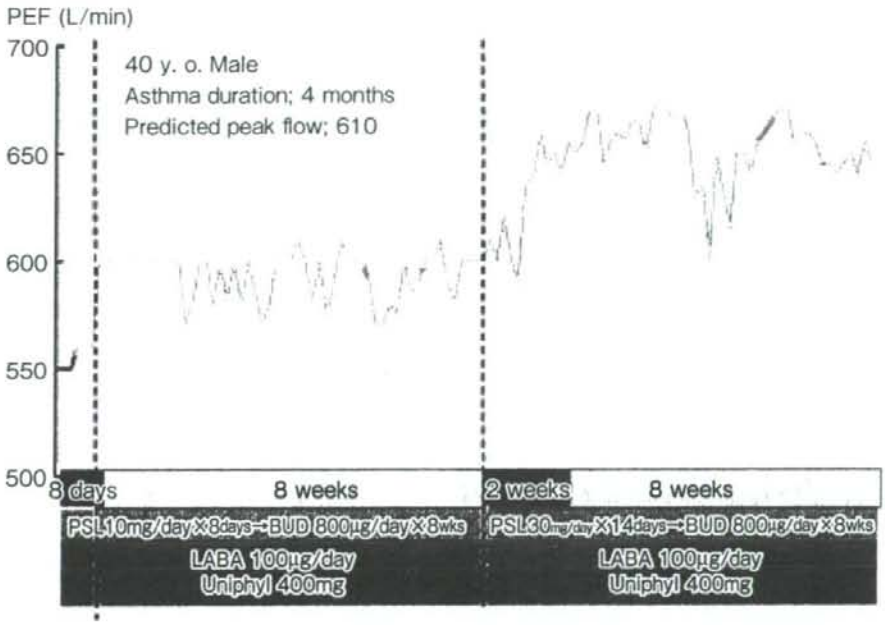

Fig. 1. Necessity of oral corticosteroid intensity for the effective early intervention.

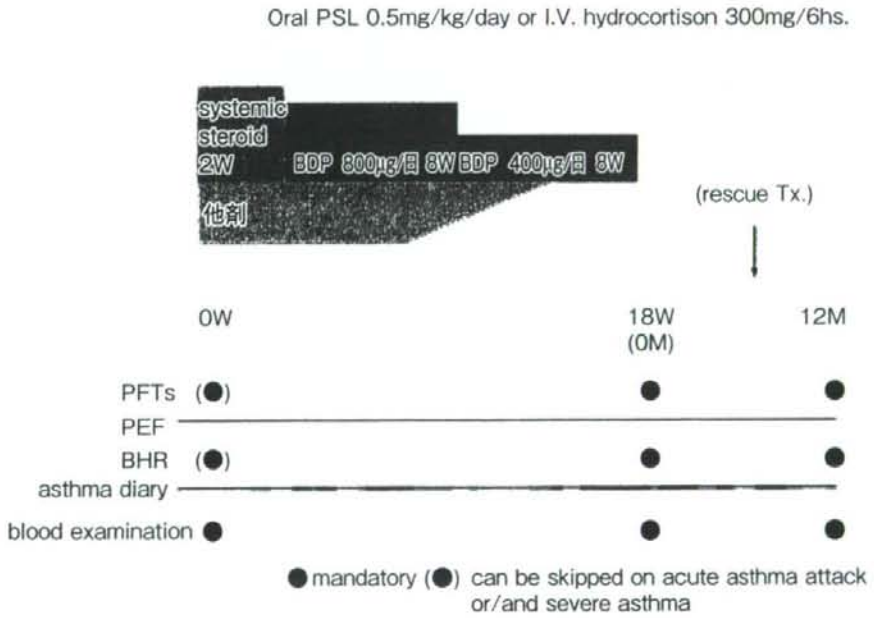

Fig. 2. The protocol for intensive treatment and clinical examinations.

研究対象として登録された成人気管支喘息患者154名の内，集中治療選択者が118名，ガイドライン治療選択者が36名．集中治療の完遂者が86名，脱落者の内，1年後のフォローアップが可能であったものが23名（32名中），ガイドライン治療は完遂者が33名であった．患者背景について，集

中治療群とガイドライン治療群では，集中治療群が重症度が高かったことを除き，両群に優位差は認められなかった．1年後の予後は，両治療群を比較したところ，集中治療群で寛解したのは45例(40%)であり，Fisherのテストでも$p=0.005$と明らかな優位差が認められた．このことより，集中治療法がガイドラインで提唱されている治療法よりも，良好な予後が得られる可能性が高いことが示唆された．

さて，本研究を多変量解析にて予後に寄与する因子を検討したところ，寛解に至る予後因子としては，治療前の背景因子の中で（オッズ比，95% CI，p値)「発症契機の上気道感染の有無」(2.47, 1.05-5.80, $p=0.038$)，「発症期間」(0.82, 0.75-0.89, $p<0.001$)，「集中治療の完遂」(2.31, 1.07-5.01, $p=0.033$）が寛解に至る重要な有意の因子であった．つまり，1年間の寛解にとって，医療側が関与できることは後者2因子である．この2因子を例にとると，発症から治療開始に至るまでの期間が短いことと集中的治療の完遂が大きな寄与因子である．具体的に述べると，発症6カ月以内に治療が開始され，薬物治療が完遂された43例中32例(約70%)の1年間寛解が得られ，発症から治療導入までが3カ月以内の例では，更に高い寛解率が得られた（32例中26例，約80%)．

なお，喘息の病型(アトピー，非アトピー型)や，受診の治療導入時の重症度は寛解導入には無関係であった．

前述の気管支喘息のearly interventionの3つの課題，1. 何を目標とするのかという問いに対して，1年間の寛解(無治療で無症状，呼吸機能が正常，気道過敏性が正常）を保つかなりの例が存在する，2. どのくらい早期でなければならないかという問いには，発症6カ月以内が寛解に入る可能性が高いと，3. どのような治療が適切であるか，の問いに対しては，集中治療(2週間の全身ステロイド投与+16週間の吸入ステロイド及び気管支拡張剤の併用）が標準療法より効果が高い，と言えるが，至適かどうかも含め，より良い治療法の検索は今後の課題であろう．

成人慢性喘息長期薬物療法からの離脱に関する臨床研究

先ず本項が臨床研究となり得る背景と根拠について説明したい．私達が日常の診療している喘息例の多くは慢性化しており，先項の臨床試験の結果からみても早期介入療法の対象となり得ない．しかし逆に罹病期間が長い慢性喘息に対し，早期介入療法とは違った治療戦略を採った場合，寛解を期待できないのだろうかということが次の問いになる．

視点を変えるならば，慢性化した喘息がコントロールされた場合いつまで治療するのかということにも関連する．この問いこそ，現在我々が日常の喘息診療で直面している重要な一つの課題かもしれない．本人にとって半永久的に治療を続行すべきだという見解もあるが，患者や医師の立場からみて，目標が設定されない治療にどれだけ意欲が湧くだろうか．この見解では結局は治療のコンプライアンスを低下させ，主張とは逆の結果にならないだろうか．年齢別の，喘息有症率の統計をみると，小児から成人に向かう時に喘息がoutgrowする例が多く存在する．成人でも一旦発症したら一生持続するとは限らない．この十数年間の治療の進歩がこの慢性喘息の予後改善に寄与しているはずである．何故ならば，喘息を慢性疾患と定義し，長期管理に優れた治療法を提供しているとみなされるからである．

つまり臨床研究として本テーマを設定した時，ガイドラインで提唱している治療法そのものを戦略の一部に取り込むべきではないだろうかと考えた．

更に他の基準項目を選択した．現時点では，喘息のコントロールの指標とし，症状の安定化(ACTスコアなどで評価する)，客観的指標としてピークフロー値の安定化，呼気NO濃度，誘発喀痰中の好酸球数など挙げられるが，簡便でどこでもできる方法として，呼吸機能に優位性があるのではないかと考える．その中でもとりわけ経時的な$FEV_{1.0}$の減少率である．

今日の喘息に対する標準的治療法の全盛以前のデータであるが，可部順三郎らが1987年に約40例の喘息において，経年的$FEV_{1.0}$の変移を調べている[4]．$FEV_{1.0}$の低下は重症度とは関係なく軽症喘息でも顕著であり，FlecherらのCOPDのデータと比肩[5]されることを示している．1998年のデンマークのLangeらの更に大規模観察のデータ[6]もある．健常男性・女性の非喫煙者の経年的な$FEV_{1.0}$の低下に比して，喘息では経年的低下が大きいことを示している．1998年なので，吸入ステロイド療法が導入された時代のデータも部分的に反映されていると思われる．これに対して現在の標準的な治療法下では，新たな事態が開けているのではないかという印象を筆者は持っていた．日本の2001年の呼吸器学会誌で，佐々木らが示した日本人の$FEV_{1.0}$の経年的変化（低下）直線[7]を基準にして慢性喘息の$FEV_{1.0}$の変移を調べてみると（薬物の影響を除くべく，薬物中止24時間後の測定）改善する例や低下率が正常化を示す喘息例の存在が確認出来た．先述の可部やLangeらの喘息データとは異なり，これが近年の治療ガイドラインで提唱する治療法の優れた面を示唆するものと考えられる．

以上のことを踏まえ，更に，喘息の罹病期間が1年以上の慢性型で，研究対象のエントリー条件として，安全性と倫理性を期するため，以下の項目を満たすこととした．喘息を喘息有症期間，コントロール期間に分け，有症期間よりもコントロール期間が長いことを1つの条件とした．コントロール期間の治療は，原則これまでの既刊各ガイドラインに基づいた治療で行われた例とした．加えて，$FEV_{1.0}$の低下が標準的低下の10%以内のものとした．薬物治療を呼吸機能検査前24時間中止できる軽症～中等症を対象にした．

2008年6月の時点でエントリー症例の総数は47例，平均現年齢が61歳，発症年齢が50歳で，約10年位の罹病期間を持つ慢性喘息である．コントロール期間/有症期間比が5.9年/3.6年で，コントロール期間が1.6倍長い．そしてコントロール期間の$FEV_{1.0}$の変化量をみたところ，平均量としてはむしろ減少するより改善していることがわかった．このような症例に薬物療法を中止し，1年間の経過を観察している．最終的解析をいずれ近いうちに行う予定である．

高病原性H5N1ウイルスヒト感染症

ここ10年の間に，地球規模で家禽の高病原性インフルエンザH5N1が大規模発生し，家禽類から直接感染したと考えられるヒトへの感染例も多数報告されるようになった．更に本感染症が，パンデミック新型インフルエンザの発生に繋がるのではないかという重大な懸念がますます増大してきている．世界保健機構（WHO）の報告によると，2003年以来，2008年10月まで385例の感染例が確認され，その死亡率は63.1%にのぼる．H5N1型インフルエンザの感染がヒトに起こった場合，短期間に重篤な肺炎を起こし，多くは急性呼吸促迫症候群（ARDS），そして時には多臓器障害（MOF）に陥り高率に死に至る．本症の疫学や，臨床像，治療，予防法等に関する研究報告を総括的にまとめたreview[8]が既に存在するが，本症には，未だ有効な治療法が確立されていないことが深刻な問題であり続けている．

これまでの多くの研究・知見・経験に加えて，我々はベトナム国との共同研究をする機会に恵まれ，実際の症例と直接臨床的に関わりを持つことによって，新たな治療法の開発の可能性を探ってきた．

ベトナムではこれまで，106名のH5N1型インフルエンザのヒトへの感染例が報告されており，うち52名の死亡者数が確認されている．我々はベトナムでこれらの症例を扱った医療機関の臨床的知見を共有し得た[9][10]．また，実際に患者発生時に共同診療を行う遠隔TV会議システムをつなぎ，実行している．これらにより，迅速診断法を既に開発し[11]，ベトナムでは臨床の場で活用し始めた．新たな治療法の実施についても相手側の検討・賛同を得て開始している．私達の研究目的は本疾患を”普通の感染症”にすることであり，同時にパンデミックにも備えることである．これまでの本症との臨床的な関わりから，重篤な急性感染症に対して当然なこととして，患者側の早期受診，医

療側の早期診断，早期治療のいわば early intervention（早期医療介入）が重要で，それらに沿った方法を開発・実践することが本疾患の克服につながると考える[12]．

ヒト H5N1 の臨床像，我々の研究グループが開発した迅速診断キット，実際にベトナムで共同研究として展開している治療プロトコールについて[13]，概説したが，本誌では紙面の都合で割愛する．

さいごに

喘息の治療も，H5N1 に対しても，未だ確固とした根治的治療法が確立していない．しかしながら，両者とも early intervention が基本的で重要な治療戦略であることを提言した．

これらの研究は，次の方々の協力で実施した．（国立国際医療センター呼吸器科）有岡弘子，竹田雄一郎，半田智子，上村光弘，山内康弘，長瀬洋之，小林信之，可部順三郎．（同　検査部）熊澤哲夫．（同　国際疾病センター）川名明彦，泉　信有，高崎　仁．（国立感染症研究所）佐多徹太郎，鈴木和男．（公立学校共済組合関東中央病院）岡　輝明．（ベトナム国立バクマイ病院）Tran Thuy Hanh，Pham Phuong Thuy．事務局：間辺利江．（敬称略）ここに感謝の意を表する．加えて，恩師である宮本昭正先生，可部順三郎先生に長年のご指導・ご高配に心から感謝を申し上げたい．

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