it is suggested that CRTH2-mediated pathway may induce pathology without regulating local production of these proinflammatory cytokines.

Outcomes of pollinosis were compared between sensitized/challenged CRTH2$^{-/-}$ mice and nonsensitized/single-challenged CRTH2$^{-/-}$ mice. The levels of Cry j 1-specific IgE (0.159 ± 0.044 vs 0 ± 0 OD at 450 nm: $p = 0.003$), Cry j 1-specific IgG1 (0.638 ± 0.163 vs 0 ± 0 OD at 450 nm: $p = 0.004$), nasal eosinophila (66.4 ± 8.2 vs 6.6 ± 1.1 cells/field: $p = 0.005$), and IL-4 production by submandibular lymph node cell (72.8 ± 31.1 vs 6.7 ± 3.8 pg/ml: $p = 0.004$) were significantly higher in sensitized and subsequently challenged CRTH2$^{-/-}$ mice as compared with nonsensitized and single-challenged CRTH2$^{-/-}$ mice. However, the frequencies of sneezing (1.7 ± 0.5 vs 0.6 ± 0.2 times in 10 min: $p = 0.088$) and rubbing (12.3 ± 2.6 vs 10.2 ± 3.1 times in 10 min: $p = 0.516$) were similar between two groups, suggesting that CRTH2 is particularly essential for the development of nasal symptoms.

Effect of ramatroban on Cry j 1-induced pollinosis

As seen in CRTH2-deficient mice, treatment with ramatroban significantly reduced several indicators of pollinosis including sneezing, Cry j 1-specific IgG1 production, and Cry j 1-induced IL-4 production by submandibular lymph node cells as compared with the control treatment (Fig. 8, *A*, *D*, and *G*). Although the differences did not reach to the statistical level, other parameters such as nasal rubbing, Cry j 1-specific IgE production, nasal eosinophilia, and Cry j 1-induced IL-5 production were also reduced by the treatment with ramatroban (Fig. 8, *B*, *C*, *F*, and *H*).

Discussion

In the present study, we analyzed the pathophysiological effects of nasal exposure to Cry j 1 in BALB/c mice. Mice sensitized with Cry j 1 without adjuvants showed not only allergic symptoms such as sneezing and rubbing but also produced Cry j 1-specific IgE and IgG1 and displayed nasal eosinophilia. Additionally, submandibular lymph node cells isolated from these mice produced IL-4 and IL-5 in recall response to Cry j 1. These results suggest that intranasal sensitization with Cry j 1 induces pollinosis in BALB/c mice.

To investigate the initiation of allergic rhinitis in vivo, administration of Ags via the natural route (i.e., through the nostril) is desirable. In fact, it is known that administration of Ags through different routes results in different degrees of IgE production (27, 28). Also, murine models of allergic rhinitis have been generated by intranasal or aerosol-mediated sensitization (8, 29), but these models generally employ adjuvants such as cholera toxin, which have immunoregulatory effects that may distort the physical sensitization (30, 31). Therefore, we and others have established murine models of allergic rhinitis by intranasal sensitization with Ags including *Schistosoma mansoni* egg Ag, phospholipase A_2 from honeybee venom, extracts of *Aspergillus fumigatus*, OVA, and trimellitic anhydride in the absence of adjuvants (5–7, 9, 32). We think that our current model is the first in which murine pollinosis was induced by intranasal sensitization with pollen allergen in the absence of an adjuvant. This model may be useful not only for understanding the pathophysiology of pollinosis but also for developing and/or testing new therapies for allergic rhinitis, especially JCP.

BALB/c mice sensitized with Cry j 1 showed an increase in the expression of CRTH2 mRNA in the nasal septum compared with control mice. This agrees with our recent report demonstrating that the amount of CRTH2 mRNA in nasal mucosa is significantly higher in patients with allergic rhinitis than in control subjects not showing hypertrophy of inferior turbinates (12). These results suggest that the expression of CRTH2 may play a role in the pathogenesis of allergic rhinitis both in humans and in mice. In fact, it is known that the expression of CRTH2 in eosinophils and CD4$^+$ T cells is elevated in atopic patients (33–35). CRTH2 is expressed by eosinophils and a subset of CD3$^+$ T cells in nasal mucosa, especially in patients with allergic rhinitis (23). Because a mAb against murine CRTH2 that can be used for immunohistochemistry is not currently available, we could not investigate the phenotype of cells expressing CRTH2 in mice.

The pathophysiology of allergic rhinitis was clearly impaired in CRTH2$^{-/-}$ mice. Following repeated intranasal sensitization and nasal challenge with Cry j 1, CRTH2$^{-/-}$ mice displayed reduced nasal symptoms, production of Cry j 1-specific IgE and IgG1, and nasal eosinophilia compared with WT mice. Additionally, submandibular lymph node cells from Cry j 1-sensitized CRTH2$^{-/-}$ mice produced significantly less IL-4 and IL-5 in response to Cry j 1 than those from WT mice. We think that the present results are the first demonstration of the in vivo role of CRTH2 in the initiation of Th2 responses in the upper airway.

We also found that Cry j 1-specific IgE and IgG1 but not IgG2a production was impaired in CRTH2$^{-/-}$ mice. Ag-specific IgE/IgG1 and IgG2a production is known to be positively regulated by Th2 and Th1 responses, respectively, in mice (36). Thus, our results indicate that signals mediated by CRTH2 selectively enhance Th2-type Ab production. The decreased production of IL-4 by submandibular lymph node cells from CRTH2$^{-/-}$ mice in response to Cry j 1 restimulation supports this result because IL-4 plays a critical role in IgE synthesis in vivo (37). Although whether CRTH2 activation directly leads to IL-4 production in mice remains unclear, recent investigations have demonstrated that PGD_2 causes the preferential induction of IL-4 production by Th2 cells in humans by binding to CRTH2 (38, 39). Additionally, our recent report showing that CRTH2 signals up-regulate CD40L in resting human Th2 cells supports our conclusions because the engagement of CD40 by CD40L is also essential for IgE isotype switching (39, 40).

After intranasal sensitization with Cry j 1, CRTH2$^{-/-}$ mice developed a weaker eosinophilia than did WT BALB/c mice. This suggests that CRTH2 mediates local eosinophil recruitment in this model, which agrees with reports showing that CRTH2 activation leads to changes in eosinophil shape, chemotaxis, and degranulation in vitro (16, 18, 41). Additionally, recent investigations have revealed that CRTH2 plays a proinflammatory role in eosinophil chemotaxis into inflamed tissue in vivo (11, 12, 21, 24, 42). On the other hand, submandibular lymph node cells from WT and CRTH2$^{-/-}$ mice produced similar amount of IL-5 after intranasal sensitization with Cry j 1. It is well known that IL-5 plays a critical role in eosinophilic inflammation, especially in mice (43). Although little is known about whether CRTH2 activation enhances IL-5 production in mice, CRTH2 activation on Th2 cells is known to induce IL-5 production in humans (38, 39). One explanation of why nasal eosinophilia was reduced in CRTH2$^{-/-}$ mice irrespective of IL-5 production is that cognate interaction between PGD_2 and CRTH2 on eosinophils may have an additive effect on local eosinophil recruitment, primarily due to the action of IL-5. In fact, in a mouse model of asthma, nebulized DK-PGD_2, a CRTH2 agonist, exacerbates eosinophilic lung inflammation without changes in IL-5 content in lung (21).

CRTH2$^{-/-}$ mice displayed a significantly lower frequency of both sneezing and nasal rubbing after the nasal challenge compared with the WT mice. Several molecules, including IL-5, CD80/CD86, H1, and CD39, have been shown to contribute to these symptoms via different mechanisms (38, 44–46). The present result suggests that activation of CRTH2 is also involved

in the symptoms of nasal hyperreactivity. In humans, nasal challenge with PGD_2 induces a sustained nasal obstruction but not sneezing or rhinorrhea (47). Whether murine mast cells express CRTH2 is not well known, and further investigations are needed to determine whether the effect of CRTH2 on nasal hyperreactivity is due to the control of Th2 responses or to a direct effect on mast cells.

Treatment with ramatroban, a CRTH2/TP dual antagonist, induced a reduction in several indicators of JCP such as sneezing, Cry j 1-specific IgG1 production, and Cry j 1-induced IL-4 production. It is known that ramatroban suppresses allergic responses including nasal signs both in vivo and in vitro (11, 24, 46, 48). For example, ramatroban significantly inhibited sneezing and nasal rubbing induced by Ag in actively sensitized C57BL/6 mice and guinea pigs (46, 48). Our present results are consistent with these reports and support the findings seen in $CRTH2^{-/-}$ mice that suggest a proinflammatory role of CRTH2 in allergic rhinitis. On the other hand, treatment with ramatroban was less effective than CRTH2 deficiency in all parameters of investigation. One of the possible reasons is that ramatroban antagonizes not only CRTH2 but also TP. Since it is not fully elucidated whether signals through TP, especially in mice, are proinflammatory or antiinflammatory in allergic rhinitis, simultaneous blockage with TP may affect changes of the outcomes induced by CRTH2 antagonism.

In conclusion, we developed a novel model of murine allergic rhinitis that mimics pollinosis. Additionally, we found that CRTH2 plays an essential role in the initiation of allergic rhinitis in mice. These results suggest that this murine model will be useful for elucidating the pathophysiology of allergic rhinitis, especially JCP. These observations may provide a basis for developing therapeutic approaches for managing allergic rhinitis, specifically by inhibiting PGD_2-CRTH2 interactions in the nose of individuals with allergic rhinitis.

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Disclosures

The authors have no financial conflicts of interest.

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ORIGINAL ARTICLE

Aging Exacerbates Restraint Stress-Induced Inhibition of Antigen-Specific Antibody Production in Mice

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ABSTRACT

Background: We have recently found that exposure to acute restraint stress suppresses antigen-specific antibody production, including IgE, in a murine model of allergic rhinitis. Although age-related alterations in immune responses are known, it remains unclear whether aging modulates the antibody production under stressful conditions. In this study, we set out to determine the effects of aging on antibody production under acute restraint stress in mice.

Methods: Both young and aged CBA/J mice were repeatedly sensitized intranasally with phospholipase A2 (PLA2) without adjuvants. Restraint stress was applied using uniform cylinders once a week for a continuous 8 h period, on 5 occasions in total. Blood samples were taken at 0, 20 and 30 days after primary sensitization, and production of PLA2-specific antibodies and levels of IL-4, IFN-γ, IL-10 and IL-1β in sera were determined by ELISA.

Results: Repeated intranasal sensitization with PLA2 induced PLA2-specific IgE, IgG1 and IgG2a production in aged mice. We found that exposure to restraint stress significantly inhibited production of PLA2-specific IgE, IgG1 and IgG2a in aged mice. In addition, antibody production under restraint stress decreased significantly in aged mice when compared with young mice. No IL-4, IFN-γ, IL-10 or IL-1β were detected in sera from non-stressed or stressed aged mice.

Conclusions: Aging exacerbates the immunosuppressive role of acute restraint stress in antigen-specific antibody production in mice.

KEY WORDS

aged mouse, immunosuppression, phospholipase A2, restraint stress, specific antibody

INTRODUCTION

It is known that aging is associated with a reduced immune function, so called immunosenescence, in both humans and animals.[1-4] For example, a shift in lymphocyte population from conventional T cells to NK cells and extrathymic T cells is observed in human centenarians.[1] Changes in the proportion of T cell subsets, in addition to increases in memory T cells, impairment of response to mitogens and other stimuli, and alterations in cytokine production also occur with aging.[2-4]

In terms of humoral immunity, it is known that pro-B cells in old mice are impaired in their capacity to rearrange themselves to both D to J and V to DJ gene segments in mice.[5] In addition, serum IgE levels and antigen-specific IgE production are known to decline with age in humans.[6,7]

Exposure to physical, neurological, or emotional stress can also affect both innate and acquired immune responses.[8-10] For example, exposure to acute stress modulates antigen-specific T cell responses.[11] We have recently reported that inhibition of antigen-specific antibody production was confirmed using a type of restraint stress following intranasal sensitization with phospholipase A2 (PLA2) in mice.[12] How-

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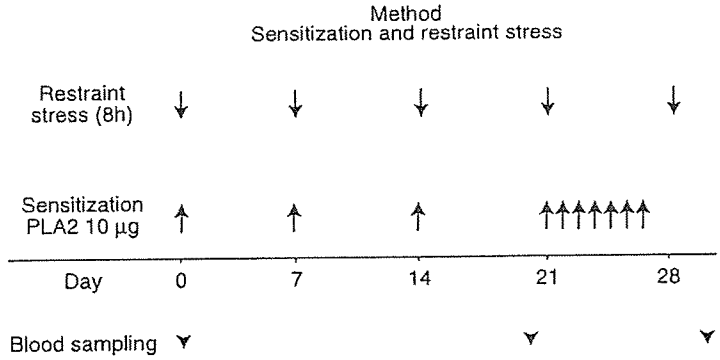

Fig. 1 Treatment schedule; Mice were intranasally sensitized to 10 μg of PLA2 in 20 μl saline. Sensitization was repeated in the same manner. Following sensitization, restraint stress was applied to mice using a single transparent cylindrical chamber and repeated once every week, for a total of 5 applications. Blood samples were taken from each tail vein at 0, 20, and 30 days after primary sensitization.

ever, little is known whether aging affects stress-induced alterations in humoral immune responses.

In this study, we compared stress-induced inhibitions of antibody production between aged and young mice in an intranasal sensitization model. As physical restraint is occasionally used in geriatric care in order to prevent bed fall in hospitals,[13] the results presented here may provide a basis for evaluating the risk of restraint stress on humoral immunity in elderly patients.

METHODS

ANIMALS

Nine-week old female, young adult mice (18–20 g) and 17- month old female, CBA/J strain mice (26–30 g) (Charles River Japan, Yokohama, Kanagawa, Japan) were used in this study. Mice were maintained in an animal house according to the guidelines of the Animal Study Committee of the Kagawa Prefectural College of Health Sciences. All animals were housed in groups of 3, each in an opaque polycarbonate mouse cage (30 × 20 × 30 cm) with access to food and water ad libitum, and were maintained on a 12-hour light-dark cycle for 2–3 weeks before the experiments began. The temperature in the animal house was maintained at 25℃.

REAGENTS

ELISA plates were purchased from Corning (Corning, NY, USA). Purified rat anti-mouse IgE was purchased from Biosource (Camarillo, CA, USA), extraAvidin-peroxidase conjugate, PLA2, carbonate buffer and fetal calf serum from Sigma (St. Louis, MO, USA), tetramethylbenzidine substrate from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA), phospholic acid from Wako Pure Chemical Industries (Osaka, Japan), peroxidase-conjugated goat anti-mouse IgG1/IgG2a monoclonal antibody from Boehringer-Mannheim (Indianapolis, IN, USA) and biotin (long-arm) N-hydroxy succinimide ester from Vector Laboratories (Burlingame, CA, USA). It is known that endotoxin contamination suppresses allergen-induced immunologic responses including IgE Production on mice.[14] Contamination of endotoxin was negligible as determined using an Endospec assay kit (Seikagaku Kogyo, Tokyo, Japan) in accordance with the manufacturer's instructions.

SENSITIZATION OF MICE

Mice (n = 6–8 per group) were sensitized by nasal administration of 20 μl of saline containing 10 μg of PLA2 using a microsyringe (Hamilton, Reno, NV, USA). PLA2 was carefully given as 7–8 drops of aqueous solution into each nostril in turn. Sensitization was repeated in the same manner after 1 and 2 weeks. On day 21 and on the following 7 consecutive days, the same amount of PLA2 was given in the same manner. Blood samples were taken from the tail vein on days 0, 20, and 30 after primary sensitization (Fig. 1).

INDUCTION FOR RESTRAINT STRESS

Following sensitization, restraint stress was applied to mice (n = 6–8 per group) using a single transparent polymethylmethacrylate cylindrical chamber (20 mm diameter, 100 mm long) commonly used for drawing blood from mice. This chamber was placed horizontally in the mouse cage, and the mice were maintained therein for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of 5 occasions (Fig. 1). Control mice were maintained in their cages without food and water at the same time. Three separate experiments were performed to confirm reproducibility.

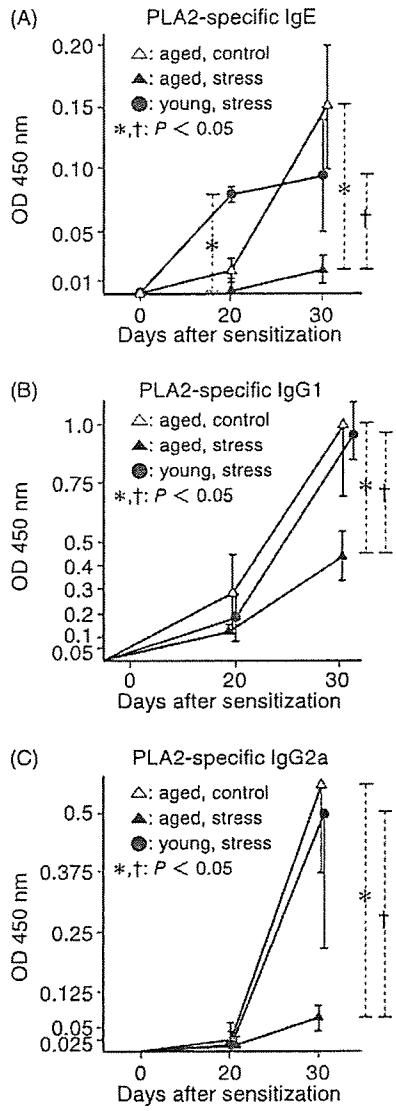

Fig. 2 Effect of restraint stress on PLA2-specific IgE (**A**), IgG1 (**B**) and IgG2a (**C**) production in aged and young mice. Both aged ($n = 9$, closed triangle) and young ($n = 9$, closed circle) were placed in a cylindrical chamber for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of five occasions. Control aged mice ($n = 9$, open triangle) were maintained in their cages without food and water at the same time. Blood samples were taken on days 0, 20 and 30 after primary sensitization, and levels of PLA2-specific antibodies were determined by ELISA. Results are expressed as mean ± SEM. Data are representative of 2 separate experiments. $*P < 0.05$ between stressed aged group and control aged group. $\dagger P < 0.05$ between stressed aged group and stressed young group.

PLA2-SPECIFIC IgE, IgG1, AND IgG2a IN SERUM

Serum levels of PLA2-specific IgE, IgG1 and IgG2a were determined using ELISA.[12,14] Titers for specific IgE were estimated as mean optical density (OD) at 450 nm of 1 : 4 diluted sera. Titers for specific IgG1 and IgG2a were estimated as mean OD at 450 nm of 1 : 100 diluted sera.

TOTAL IgE, IgM, AND IgG IN SERUM

Serum levels of total IgE in serum were measured as described previously.[14] The detection limits of this system was 0.3 ng/ml. The levels of total IgM and total IgG were measured using ELISA Quantitation Kit (Bethyl Lavoratories, Inc., Montgomery, TX, USA). The detection limits for IgM and IgG in this system were 0.4 and 0.4 ng/ml, respectively.

CYTOKINE DETERMINATION

Concentration of IL-4, IFN-γ, IL-10 and IL-1β in sera were measured using Opt EIA sets (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). The detection limits for IL-4, IFN-γ, IL-10 and IL-1β in this system were 10, 60, 15 and 30 pg/ml, respectively.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean (SEM) for each subject group. Statistical analysis was performed using Student's unpaired t- test to compare titers of PLA2-specific IgE, IgG1 and IgG2a for restrained and control groups. Values of $p < 0.05$ were considered to indicate a statistically significant difference.

RESULTS

EFFECT OF RESTRAINT STRESS ON ANTIGEN-SPECIFIC ANTIBODY PRODUCTION IN AGED MICE

Production of PLA2-specific IgG1 was seen 20 days after the first intranasal sensitization in control aged mice, and production of PLA2-specific IgE and IgG2a, 30 days after the first sensitization. In aged mice under restraint stress, impaired production of these 3 antibodies was observed. On day 30, aged mice under stress produced significantly lower amounts of PLA2-specific IgE, IgG1 and IgG2a as compared with non-stressed aged mice ($P < 0.05$) (Fig. 2A, B, C).

EFFECT OF AGING ON RESTRAINT STRESS-INDUCED INHIBITION OF ANTIGEN-SPECIFIC ANTIBODY PRODUCTION

We then compared PLA2-specific antibody production under restraint stress between young and old mice. Young mice under stress produced PLA2-specific IgE and IgG1 20 days after the first sensitization, and produced PLA2-specific IgG2a 30 days after sensitization. The level of PLA2-specific IgE on day 20 was significantly less in aged mice under stress than young mice, and the difference could still be ob-

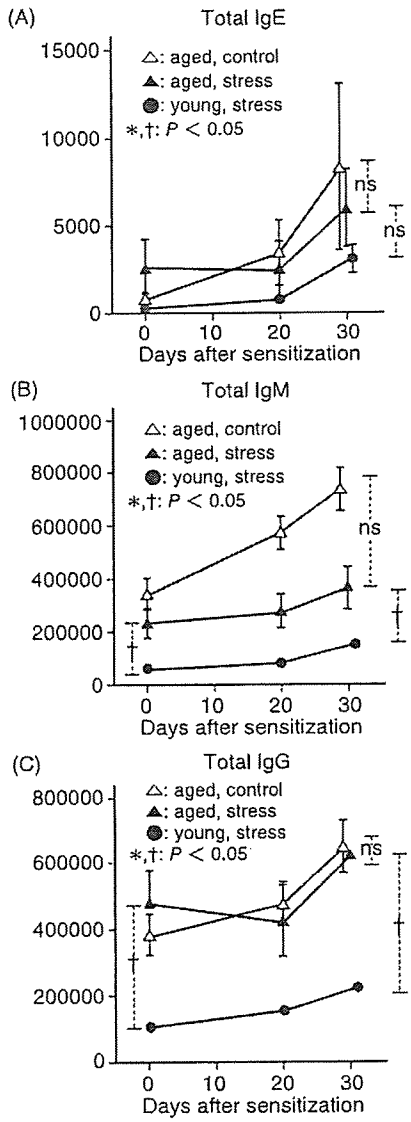

Fig. 3 Effect of restraint stress and/or aging on levels of total IgE (**A**), IgM (**B**), and IgG (**C**) in sera. Both aged (closed triangle) and young (closed circle) mice were placed in cylindrical chambers for a continuous 8-hour period without food or water. This manipulation was performed once a week, on a total of five occasions. Control aged mice (open triangle) were maintained in cages without food and water at the same time. Blood samples were taken on days 0, 20 and 30 after primary sensitisation, and levels of total Ig were determined by ELISA. $*P < 0.05$ between stressed aged group and control aged group. $\dagger P < 0.05$ between stressed aged group and stressed young group.

served on day 30 ($p < 0.05$). In addition, a significant reduction in the production of both PLA2-specific IgG1 and IgG2a was seen on day 30 in aged mice as compared with young mice ($p < 0.05$) (Fig. 2A, B, C).

EFFECTS OF RESTRAINT STRESS ON SERUM LEVELS OF IL-4, IFN-γ, IL-10 AND IL-1β

Serum levels of IL-4, IFN-γ, IL-10 and IL-1β were determined in mice with and without restraint stress. None of these cytokines were detected in sera from non-stressed or stressed aged mice. In addition, these cytokines were not detected even in sera from stressed young mice.

EFFECTS OF AGING AND/OR STRESS ON LEVELS OF TOTAL IgE, IgM, AND IgG IN SERA

Levels of total IgE, total IgM, and total IgG in sera did not differ between stressed aged and non-stressed aged groups. On the other hand, levels of serum total IgM and IgG but not IgE were significantly lower in the stressed young group compared with the stressed aged group throughout the experimental period (Fig. 3).

DISCUSSION

Reductions in T-cell function in aged mice have been shown to reduce IgE antibody production by impairing differentiation of IgE-containing progenitor B cells into IgE antibody-producing plasma cells.[16] These age-associated reductions in immune function, and T-cell function in particular, are thought to affect the function of helper B cells and suppress indirect antibody production response.

In aged mice, various effects of stress in the immune system, and particularly in T cells, have been investigated in previous studies. For example, Kanno *et al.* reported in a study of restraint stress on mice that atrophy of the thymus and decreases in splenic T cells were observed after exposure to stress. However, young mice showed a rapid recovery of the immune function after 1 week, while the aged mice never recovered.[17] However, little is known whether aging can affect stress-induced humoral responses despite the fact that aging and stress share similar effects on immune function.[18]

We previously reported that the humoral immune system in young mice was suppressed by restraint stress in the early stages of antibody production following intranasal sensitization with PLA2.[12] In this study we have further demonstrated that, although repeated intranasal sensitization with PLA2 induced PLA2-specific IgE, IgG1 and IgG2a in aged CBA/J mice, exposure to restraint stress significantly inhibited production of PLA2-specific antibodies. In addition, the present study found that aged mice underwent even more marked suppression of antibody production than young mice under restraint stress. These results suggest for the first time that aging and stress have a synergic effect on the impairment of humoral immunity, and more importantly, that aging exacerbates stress-induced inhibition of humoral re-

sponses. None or only slight differences in antibody production were found between the aged control group and young stressed group. This may be due to an aging effect, and may suggest that the impact of aging on antibody production in our model resembles that of restraint stress seen in young mice.

Restraint stress suppresses both PLA2-specific IgG1 and IgG2a production in aged mice. It is known that IgG1 and IgG2a is Th2 and Th1-type IgG isotype, respectively.[19] Fukui *et al.* reported that restraint stress significantly suppressed both Th1- and Th2-type immune responses in mice.[10] It has also been reported by Dhabhar *et al.* that B cells show a greater stress-induced decrease than T cells.[20] These reports support our findings, suggesting that restraint stress suppresses both Th1- and Th2-type humoral responses in aged mice. Defective induction of functional Th2 cytokine responses has been reported in aged mice[21] in addition to Th1 type immune response being important for the protection against intracellular pathogens such as viruses, mycobacterium and protozoan parasites.[22] Thus susceptibility to impair Th1-type immune responses by restraint stress in elderly patients may increase the risk of suffering from infectious diseases by intracellular pathogens.

The levels of total IgE, total IgM, and total IgG in sera did not differ between stressed aged and non-stressed aged groups. This result suggests that restraint stress selectively affects antigen-specific antibody production in aged mice. Interestingly, levels of serum total IgM and IgG but not IgE were significantly lower in the stressed young group compared with the stressed aged group. This may be due to baseline differences, as serum total IgM and IgG in aged groups were higher than in young groups even before intranasal sensitization. Long-term life in the animal house under a conventional environment may increase serum total IgM and IgG levels.

Although no IL-4, IFN-γ, IL-10 or L-1β was detected in sera from non-stressed aged mice or stressed aged mice, the mechanisms involved in the suppression of antibody production in aged mice under stress have not been clearly elucidated.

Other studies have examined the application of restraint stress, and further studies are needed to clarify the direct or indirect involvement of endocrinological neuronal pathways in the initiation of allergic rhinitis.[23-25] Accumulation of findings from a wide field of research focusing on the immune system and including the nervous endocrine systems is necessary.

In conclusion, we have shown that restraint stress impaired antigen-specific antibody production, especially in aged mice, and aging displays a strong impact on stress-induced inhibition of humoral immune responses. These observations may provide a basis for the management of care for elderly patients with physical restraints. In modern life, both the young and elderly are exposed to various forms of stress.[26] Our study suggests stress as one of the mechanisms for the epidemiological finding that serum IgE levels and antigen-specific IgE production decline with age in humans.[6,7]

ACKNOWLEDGEMENTS

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総説

スギおよびヒノキ花粉アレルゲンに結合する糖タンパク質糖鎖の構造特性と免疫活性

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抄　録

花粉症の原因物質，花粉アレルゲンはその殆どが糖タンパク質であり，哺乳類には存在しないβ1-2キシロースとα1-3フコースを有する植物抗原性糖鎖（*N*-グリカン）が結合する場合が多い。本研究では植物抗原性*N*-グリカンの花粉症発症への関与を解明するため，代表的なスギ花粉アレルゲンCry j1，Jun a1およびヒノキ花粉アレルゲンCha o1に結合する*N*-グリカンの構造解析，植物抗原性*N*-グリカンとIgEの結合性および*N*-グリカンの免疫活性測定を行った。その結果，花粉アレルゲンCry j1，Jun a1にはLewis a エピトープ（Galβ1-3 (Fucα1-4) GlcNAcβ1-）を有する植物抗原性*N*-グリカンが結合していることを初めて明らかにした[1-3)]。更に植物抗原性*N*-グリカンはIgEに対する直接のエピトープには成り得ないが，スギ花粉症患者Th2細胞からのIL-4産生を有意に抑制することを見出し，植物抗原性*N*-グリカンが花粉症の有効な治療薬となる可能性を示唆した[4)]。

はじめに

花粉症や食物アレルギーを引き起こす植物由来のアレルゲンは，アスパラギン残基のアミド窒素に糖鎖が*N*-グリコシド結合した糖タンパク質である場合が多く，それら糖鎖のほとんどが植物に特徴的なβ1-2結合キシロースとα1-3結合フコースを有するアスパラギン結合型糖鎖（*N*-グリカン）である[5-8)]。このような哺乳動物には存在しない植物に特徴的な*N*-グリカンは，哺乳動物にとって強い抗原性を示すことが知られている[9, 10)]。そこで本研究では，花粉アレルゲンに結合している植物*N*-グリカンと花粉症発症との相関を明らかにする研究の一環として，スギ花粉アレルゲン（Cry j1，Jun a1）およびヒノキ花粉アレルゲン（Cha o1）に結合している*N*-グリカンの構造特性と抗原性糖鎖の免疫活性について解析した。

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方　法

1．スギ，ヒノキ花粉アレルゲンに結合する*N*-グリカンの糖鎖構造解析

日本スギ花粉アレルゲン（Cry j1），アメリカイトスギ花粉アレルゲン（Jun a1）およびヒノキ花粉アレルゲン（Cha o1）からヒドラジン分解（100℃，12時間）によって糖鎖を切り出し，*N*-アセチル化，ピリジルアミノ化により蛍光標識糖鎖（PA-糖鎖）を調製した[11]。各々のPA-糖鎖は逆相及びサイズフラクショネーションHPLCによって精製した後，２次元糖鎖マッピング，ESI-MS分析，MS/MS分析及びグリコシダーゼ消化により構造解析を行った。

2．Cry j1特異的IgEの植物*N*-グリカンによる競合阻害ELISA

スギ花粉抽出物に特異的なIgE抗体が陽性の日本スギ花粉症患者45名（７～81歳，平均33.60±19.61歳）のうち，スギ花粉アレルゲンCry j1に対するIgE抗体が陽性であった40名について競合阻害ELISAを行った。阻害作用は，植物抗原性*N*-グリカン（$Man_3Xyl_1Fuc_1GlcNAc_2$および$GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$）と非抗原性*N*-グリカン（$Man_9GlcNAc_2$）について検定した。ELISAプレートはラット抗ヒトIgEモノクローナル抗体でコートし，ブロッキング後，血清サンプルを添加し37℃で２時間反応させた。予め混合しておいたビオチン化Cry j1（終濃度6 nM）と*N*-グリカン（終濃度0，30，300，3000 nM）を加え，37℃で２時間IgEとCry j1の結合を行い，続いて37℃で１時間アビジンHRPとの反応後，TMB基質による発色を行った。反応は５％リン酸で停止させ450 nmの吸光度を測定した。

3．植物*N*-グリカンのCry j1特異的なTh2細胞への作用

７名のスギ花粉症患者（21－43歳，平均31.29±8.48歳）由来末梢血単核球からCry j1に特異的なT細胞株を樹立した。*N*-グリカンは植物抗原性*N*-グリカン（$Man_3Xyl_1Fuc_1GlcNAc_2$）と非抗原性*N*-グリカン（$Man_9GlcNAc_2$）を用い，Th2細胞をCry j1で再刺激する際に，0.5，5，50 μMになるように添加し，トリチウムチミジンの取り込み量を指標とした細胞増殖，ELISA法によるIL-4産生，IFN-γ産生の測定を行った。

結　果

1．Cry j1，Jun a1およびCha o1に結合する*N*-グリカンの構造的特徴

表１にまとめたように，スギ花粉アレルゲンCry j1，Jun a1およびヒノキ花粉アレルゲンCha o1は植物抗原性*N*-グリカンのコア構造となる$Man_3Xyl_1Fuc_1GlcNAc_2$の非還元末端側にβ1-2結合した$GlcNAc_{1\sim2}Man_3Xyl_1Fuc_1GlcNAc_2$を主に有していた。更にCry j1とJun a1については，これらの*N*-グリカンの非還元末端側にLewis aエピトープ（Galβ1-3（Fucα1-4）GlcNAcβ1-）を有する植物抗原性*N*-グリカンが結合しており，その存在比はCry j1，約50%，Jun a1，約25%であった。それに対して，ハイマンノース型*N*-グリカンはCha o1のみから約11%（$Man_9GlcNAc_2$，$Man_7GlcNAc_2$）見出された。

2．Cry j1特異的IgEと植物*N*-グリカンの結合性

植物抗原性*N*-グリカンおよび非抗原性*N*-グリカンは，いずれについても殆どの花粉症患者由来IgEとCry j1との結合を著しく阻害しなかった（図２）。しかしながら40人中９人は植物抗原性*N*-グリカン（$Man_3Xyl_1Fuc_1GlcNAc_2$および$GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$）によって結合が阻害されており，植物抗原性*N*-グリカンはメジャーなIgEエピトープには成り得ないけれどもマイナーなIgEエピトープとなることが示唆された。

表1．スギ花粉アレルゲン Cry j1，Jun a1 およびヒノキ花粉アレルゲン Cha o1 に結合している*N*-グリカンの構造（文献[1-3]を改編）

Proposed Structures	Relative amount (%)			
	Cry j1	Jun a1 (A)	Jun a1 (B)	Cha o1
GlcNAcβ1-2{Manα1-6, Manα1-3}Manβ1-4GlcNAcβ1-4GlcNAc (Xylβ1 on 2, Fucα1 on 3)	nd	nd	3%	nd
GlcNAcβ1-2Manα1-6, GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc (Xylβ1 on 2, Fucα1 on 3)	47%	75%	76%	89%
Fucα1-4{GlcNAcβ1-2Manα1-6, Galβ1-3 GlcNAcβ1-2Manα1-3}Manβ1-4GlcNAcβ1-4GlcNAc (Fucα1 on Gal, Xylβ1 on 2, Fucα1 on 3)	38%	23%	21%	nd
Galβ1-3GlcNAcβ1-2Manα1-6, Galβ1-3GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc (Fucα1 on Gal, Xylβ1 on 2, Fucα1 on 3)	15%	2%	nd	nd
Manα1-2Manα1-6, Manα1-3{Manα1-6, Manα1-2Manα1-3}Manβ1-4GlcNAcβ1-4GlcNAc	nd	nd	nd	2%
Manα1-2Manα1-6, Manα1-2Manα1-3{Manα1-6, Manα1-2Manα1-2Manα1-3}Manβ1-4GlcNAcβ1-4GlcNAc	nd	nd	nd	9%

Nd: not detected.

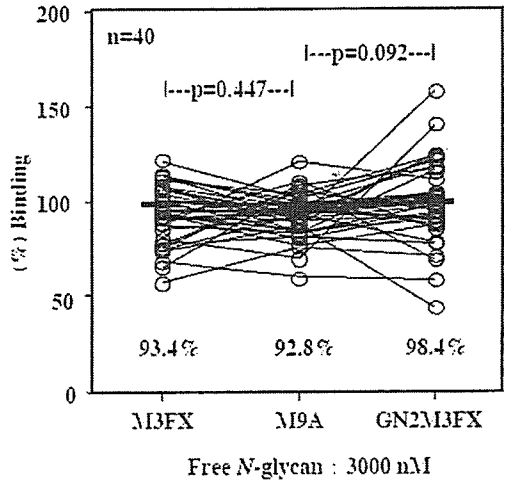

図1．植物*N*-グリカンによるスギ花粉アレルゲンCry j1とスギ花粉症患者IgEとの結合阻害効果　M3FX, $Man_3Xyl_1Fuc_1GlcNAc_2$; M9A, $Man_9GlcNAc_2$; GN2M3FX, $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$. (文献[4]より引用)

3．植物*N*-グリカンの細胞性免疫活性

植物抗原性*N*-グリカン（$Man_3Xyl_1Fuc_1GlcNAc_2$）は，スギ花粉症患者由来Cry j1特異的なT細胞増殖およびTh2型サイトカインであるIL-4産生を濃度依存的に抑制した（図3-2）。一方，非抗原性*N*-グリカン($Man_9GlcNAc_2$)はT細胞の細胞増殖やサイトカイン産生にはなんら影響を与えなかった（図3-1）。

考　察

スギ花粉アレルゲンCry j1，Jun a1およびヒノキ花粉アレルゲンCha o1に結合している*N*-グリカンの構造解析を行った結果，スギ花粉アレルゲンからはLewis aエピトープを有する植物抗原性*N*-グリカンを含む4種類の*N*-グリカンを，ヒノキ花粉アレルゲンからはハイマンノース型*N*-グリカンを含む3種類の構造を同定できた（表1）。Cry j1，Jun a1およびCha o1はアミノ酸配列の相同性が高いため[12]*N*-グリカンの構造は非常に類似していた。しかしながらLewis aエピトープを有する構造はアレルゲンによって存在比が大きく異なっており，それぞれの花粉における種々のグリコシルトランスフェラーゼ[13, 14]の発現程度が影響していると推察された。

更に植物抗原性*N*-グリカンはCry j1に特異的なIgEのメジャーなエピトープにはならないが（図2），Cry j1特異的なTh2細胞の増殖やIL-4産生を抑制することを明らかにした（図3）。これらの植物抗原性*N*-グリカンがIgEの直接的なエピトープであるか，長い間議論の的であったが[15-17]，本研究結果によりメジャーなエピトープにはならないことが示唆され，糖鎖とその周辺のペプチドが構築する立体構造を考慮する必要があると考えられた。

また，植物抗原性*N*-グリカン（$Man_3Xyl_1Fuc_1GlcNAc_2$）が花粉症患者Th2細胞のCry j1特異的なIL-4産生を抑制することから花粉症治療薬としての可能性が示されており，今後のIL-4産生抑制の分子メカニズム解明が期待さ

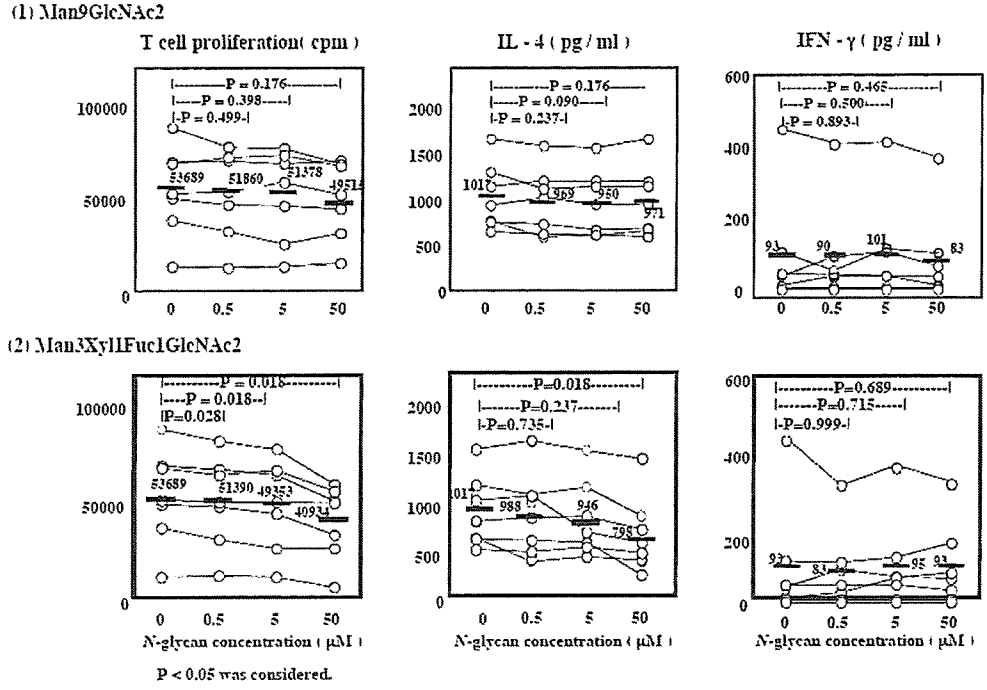

図2．Cry j1特異的Th2細胞に対する植物*N*-グリカンの免疫抑制活性（文献[4]より引用）

れる。加えて，スギ花粉アレルゲンから同定されたLewis aエピトープを有する*N*-グリカンは分泌型の糖タンパク質からも見出されており[6, 8, 12, 18]，免疫活性や植物における生理学的機能の解明に興味が持たれる。

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Structural Features and Immunoactivity of *N*-Glycans Linked to Cedar and Cypress Pollen Allergens.

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Abstract

Many plant allergens such as pollen allergens are glycosylated and bear highly antigenic *N*-glycans with β1-2 xylosyl and α1-3 fucosyl residues. As a first step to reveal immunological activity of antigenic plant *N*-glycans involved in pollinosis, we first analyzed the structural features of *N*-glycans linked to cedar and cypress pollen allergens, Cry j1 (Japanese cedar), Jun a1 (mountain cedar), and Cha o1 (Japanese cypress). The structural analysis showed that all pollen allergens bear highly antigenic *N*-glycans, $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$, as a major structure. In the case of cedar pollen allergens, Cry j 1 and Jun a 1, we revealed that the Lewis a epitope (Gal β1-3 (Fuc α1-4) GlcNAc β1-) structure occurred at non-reducing end of oligosaccharides. Next, we examined immunological activities of the antigenic *N*-glycan such as reactivity towards IgE from pollinosis patients or effect to T-cell responses. Although plant complex type *N*-glycan didn't inhibit the binding of IgE to Cry j1, the *N*-glycan suppressed the production of IL-4 from Cry j1-specific Th2-cells. This result suggested plant complex type *N*-glycans may be useful as glycodrug for pollinosis therapy.

key word: *N*-glycan; pollen allergens; Lewis a epitope; IL-4; pollinosis

Ⅳ．耳鼻科
3）アレルギー性鼻炎の新しい治療薬開発の現状
－免疫療法薬を中心に－

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アレルギー性鼻炎に対して現在開発が進んでいる免疫療法薬は，抗原特異的な治療薬と非特異的な治療薬に大別できる。抗原特異的な治療薬はアレルゲンエキスの開発ということになるが，非アナフィラキシー型の治療エキスとしてペプチド，化学修飾アレルゲン，キメラタンパクなどが展開されている。さらに治療効率を高めるアジュバントとして，MPLやCpG-DNAが利用されている。抗原非特異的な治療薬としては，アレルギー性鼻炎の病態関連分子を標的とした抗体医薬や衛生仮説を背景とした微生物ワクチンなどが期待されている。

ペプチド／化学修飾アレルゲン／アジュバント／抗体医薬／微生物ワクチン

はじめに

現時点でアレルギー性鼻炎の治癒，または長期寛解を期待できる唯一の治療法は特異的免疫療法(減感作療法)である。しかしながら，長期にわたる治療を要する上に効果発現が遅い点や，稀にアナフィラキシーなどの重篤な副反応を生じることから，一般の普及に至っていないのが現状である。現在，これらのデメリットを改善すべく，治療用抗原エキスやアジュバントの開発が進んでいる。また基礎的な検討を介してアレルギー性鼻炎の制御に重要な分子が明らかになりつつあり，非特異的な免疫療法として抗体医薬や微生物ワクチンなどの臨床研究が展開されている(表1)。本稿では，古典的な免疫療法に代わりうる，より即効性でかつ安全性が高く新しい根治療法となりうる治療薬を紹介し，その臨床応用の可能性および考えられる問題点について概説する。

Ⅰ．特異的免疫療法薬の開発

1．治療用抗原エキスの開発

WHO見解書などのガイドラインでは，抗原特異的免疫療法においては一回あたり5～20 μgの抗原投与が推奨されている。一方，高用量の抗原投与はアナフィラキシーなどの重篤な副反応を生じるリスクを高める。そこで高い安全性をもちア

表1　研究開発が進んでいる免疫療法薬

特異的免疫療法薬	非特異的免疫療法薬
・治原用抗原エキスの開発 　・ペプチド 　・化学修飾アレルゲン 　・キメラタンパク　など ・アジュバントの開発 　・Monophosphoryl lipid (MPL) 　・DNA 　・Alum 　・リポソーム　など	・抗体医薬 　・抗 IgE 抗体 　・抗 CCR4 抗体　など ・微生物ワクチン 　・プロバイオティクス 　・抗酸菌製剤 (*M. vaccae* など)　など

レルゲン投与量を増加させるための戦略として，原則としてアナフィラキシーをおこさないアレルゲンエキスの開発が進められている。

1) ペプチド

免疫応答の司令塔であるT細胞はアレルゲン全体を認識するのではなく，抗原提示細胞に捕捉されアミノ酸 10 個前後に分解されたアレルゲンペプチド (T細胞エピトープ) を MHC (major histocompatibility complex) クラスⅡ分子と共に認識する。ペプチド免疫療法とはこのT細胞エピトープとなるペプチドを用いた免疫療法である (図1)。IgE (immunoglobulin E) はアレルゲンの3次元的な構造を認識するとされ，アミノ酸残基 15 前後のペプチドは，通常 IgE に認識されない。また抗原やペプチドの高用量投与は免疫寛容を誘導することが実験的・臨床的に知られている。

スギ花粉症においては，Cry j 1 および Cry j 2 上の主要T細胞エピトープが同定され，これらをアルギニンダイマーではさみ直列につないだ多重ペプチド (Cry- コンセンサス) が合成され，臨床試験が進められている[1]。

一方，ペプチド療法の問題点もいくつか挙げられている。例えば個々の患者T細胞が認識するペプチドが必ずしも同一でないことがあり，将来はオーダーメイドのペプチド療法が必要かもしれない。さらにネコアレルゲンペプチド (ALLERVAX® CAT) など現在までに示された臨床治験ではペプチド投与による気道症状の発現が少なからずみられることから，アナフィラキシーを完全に克服する戦略，例えば後述する抗 IgE 抗体との併用療法の構築も必要であろう[2]。

2) 化学修飾アレルゲン

グルタルアルデヒドなどによりアレルゲンを重合 (polymerization) させると，IgE への結合性が著明に減弱する。従って短期間に高用量の投与が期待されている。

カモガヤとオリーブのみに花粉症を有する鼻炎患者を対象にした二重盲検試験が報告されている[3]。投与の翌シーズンにおいてグルタルアルデヒド重合アレルゲンワクチンを投与した群 ($n = 28$) ではプラセボ群 ($n = 25$) に比較して，花粉飛散期の症状スコアおよび薬物スコアが有意に低値を示した ($p < 0.001$)。さらに花粉飛散期の QOL (quality of life) もグルタルアルデヒド重合アレルゲンワクチン投与群で有意に良好であった ($p < 0.05$)。

MHC (major histocompatibility complex)　IgE (immunoglobulin E)　QOL (quality of life)

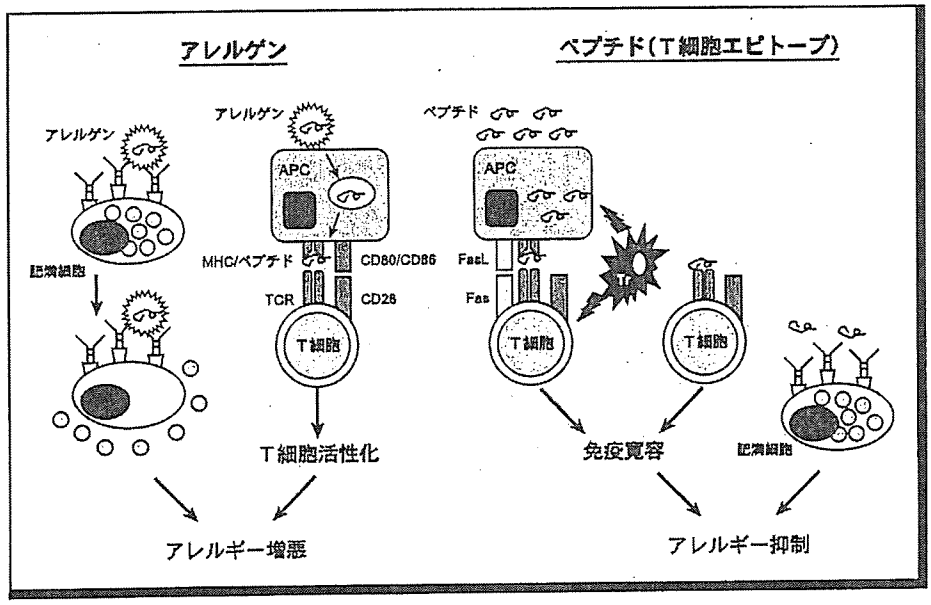

図1　ペプチド免疫療法の作用機序
アレルゲンペプチドの投与は，原則的には肥満細胞の脱顆粒をおこすことなく，免疫寛容を誘導する．

多くの報告で全身的な副反応はみられないか軽度であり，10倍量換算の投与でも安全性は通常のアレルゲンエキスと同等であるとの報告もみられる[4]。一方，通常法を含む他の免疫療法との有効性の比較を行った報告はほとんどなく，化学修飾による有効性の改善に関しては今後の検討が必要である。

3）キメラタンパク

より抗原特異的な免疫抑制を誘導する目的で，アレルゲンに抑制シグナルを誘導する分子を遺伝子工学的に結合させたキメラタンパクが考案されている。肥満細胞，好塩基球，あるいは好酸球などには抑制性IgG受容体であるFcγRⅡbが発現している。FcγRⅡbは細胞質内ドメインにITIM (immunoreceptor tyrosine-based inhibition motif) を有しており，この受容体を介するシグナルは肥満細胞/好塩基球の脱顆粒や好酸球性炎症を抑制する[5]。この特性を利用して，ヒトIgGのFc部分にネコ主要アレルゲンであるFel d1を結合させたキメラ抗体gamma-Fel d1（GFD）が考案されている。GFDはFcγRⅡbおよびIgEの両者に結合する。さらにGFDはFel d 1によって誘発される局所および全身反応を抑制し，一方GFD自身はアナフィラキシー反応を誘導しないことから，ネコアレルギーに対する新規治療エキスとしての有効性が示された[6]。本キメラタンパクは，花粉やダニなど他の吸入系アレルゲンへの応用も理論的には可能であり，一層の研究の進展が期待されている。

2．アジュバントの開発

アレルゲンエキスの活性を増強することにより有効性を向上することを目的としている。また投

ITIM (immunoreceptor tyrosine-based inhibition motif)　　GFD (gamma-Fel d1)

与アレルゲン量を減弱できることから，副反応の防止にもつながる。特に，細菌由来のアジュバントのなかには自然免疫の活性化を介してTh2からTh1への免疫偏向を誘導し，有効性をより向上できることが期待できるものがある。Monophospholipidと CpG-DNAがその代表である。

1）Monophosphoryl lipid (MPL)

Monophosphoryl lipid (MPL) はサルモネラ菌（*S. Minnesota* R595株）リポ多糖（LPS）より抽出し，無毒化にしたものである。Toll-like receptor (TLR)-4アゴニストとして働き，Th2からTh1への免疫偏向を誘導する。欧州では，アレルゲン－チロシン－MPL結合エキス（Pollinex® Quattro）が開発され，イネ科花粉やブタクサ花粉などで臨床応用されている。イネ科花粉症を対象とした二重盲検試験（n＝124）では，花粉飛散前にイネ科花粉－MPL結合エキスを投与（4回皮下注射）したところ，花粉飛散期の鼻症状，眼症状および症状薬物スコアを有意に改善している[7]。局所反応はPollinex® Quattro投与群で高率であったが，全身反応は二群の間で差を認めなかった。本邦での開発は未だなく，スギやヒノキなど樹木アレルゲンにおける有効性の検討が望まれる。

2）CpG-DNA

細菌には，樹状細胞などに発現するTLR-9に結合するDNA配列であるCpGモチーフが選択的かつ豊富に含まれる。TLR-9を介する自然免疫はTh1への免疫偏向を強力に誘導するため，アレルギー治療薬としてCpG-DNA，すなわちTLR-9アゴニストを利用したワクチン開発が進められている。

ただしCpG自身は抗原非特異的に作用し，さらに強力なTh1誘導作用による副作用（自己免疫疾患の発生など）が危惧される。そこで抗原特異性を持たせ，かつCpG量を減らすために，CpGとアレルゲンを混合するか，あるいは結合する試みがなされている。海外ではブタクサ花粉症患者を対象にブタクサアレルゲンAmb a 1にCpGを結合させたワクチン（Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate；AIC）を用いた臨床試験が行われている。花粉飛散前に一週おき6回の漸増注射を行うことにより2シーズン目の鼻炎症状の軽快傾向と鼻粘膜局所でのTh1への免疫偏向が誘導される[8]。また最近報告された二重盲検試験では，同様の注射で初年度のみならず2シーズン目の症状をも有意に抑制し，長期にわたる有効性が示された。またAIC投与群ではプラセボ群に比較して，皮膚反応の抑制，飛散期のブタクサ特異的IgE抗体価上昇の抑制，IL（interleukin）-4陽性好塩基球比率の低下がみられた[9]。

Ⅱ．非特異的免疫療法薬の開発

1．抗体医薬

抗体医薬は各種疾患の病態に関連した分子に特異的な抗体を利用した新規治療法である。利点として，作用が標的分子に特異的であること，ヒト化させることにより毒性が少ないこと，血中半減期が長いことなどが挙げられる。一方，欠点としては，高コストであること，および標的となりうる分子が有限（300～400分子）と予想され，対象疾患としては癌，免疫疾患，感染症などに限定していることなどが挙げられる（表2）。アレルギー性鼻炎は免疫疾患のひとつとして，その罹患率の多さからも研究開発のターゲットとなっている。現在までに抗IgE抗体および抗CCR4抗体に

MPL (Monophosphoryl lipid)　LPS（リポ多糖）　TLR (Toll-like receptor)
AIC (Amb a 1-immunostimulatory phosphorothioate oligonucleotide conjugate)　IL (interleukin)

表2　本邦での抗体医薬の現状

一般名	製品名	標的	適応症
		上市ずみ抗体	
トラスツズマブ	ハーセプチン	HER2	乳癌
リツキシマブ	リツキサン	CD20	非ホジキンリンパ腫
バシリキシマブ	シムレクト	CD25	急性拒絶反応
パリビズマブ	シナジス	Fタンパク	RSウイルス感染症
インフリキシマブ	レミケード	TNF-α	関節リウマチ
トシリズマブ	アクテムラ	IL-6	キャッスルマン病
ゲムツズマブオゾガマイシン	マイロターグ	CD33	急性骨髄性白血病
ベバシズマブ	アバスチン	VEGF	結腸・直腸癌
		申請中抗体	
アダリムマブ	ラヒーラ/ヒュミラ	TNF-α	関節リウマチ
オマリズマブ	ゾレア	IgE	気管支喘息
セツキシマブ	アービタックス	EGFR	結腸・直腸癌
		開発中（国内）抗体	
マツズマブ		EGFR	胃・肺小細胞・大腸癌
KW-0761		CCR4	血液癌・アレルギー性鼻炎（欧州）
ゴリムマブ		TNF-α	関節リウマチ
BIW-8405		IL-5	気管支喘息

ついて臨床試験が行われている。

1）抗IgE抗体

アレルギー性鼻炎はI型アレルギーであり，アレルゲン特異的IgE抗体が発症に中心的に作用することは論をまたない。

抗IgE抗体（オマリズマブ）は，血中のIgEレベルを減少させ，IgEの肥満細胞や好塩基球への固着を阻止することによるI型アレルギーの抑制を目的としている。作用機序としては，血中のIgEレベルを減少することの他に，肥満細胞，好塩基球，あるいは樹状細胞のFcεRI発現を抑制することが知られている。

花粉症患者を対象とした検討で，抗IgE抗体の投与によって，花粉飛散期の血中IgEを低下させるのみならず，鼻粘膜内のEPX(eosinophil protein X) 陽性好酸球数やIgE陽性細胞数の有意な減少がみられる[10]。本邦でもスギ花粉症を対象に二重盲検試験が行われた。花粉飛散約1カ月前からの皮下注射によりプラセボ群と比べ有意に鼻症状の改善(軽症化)がみられ，レスキュー薬の使用も有意に少ないことが示された(図2)。一方，注射局所の紅斑や浮腫はプラセボに比べ有意に高率に認め，また因果関係は不明であるが実薬投与群で潰瘍性大腸炎の合併を一例に認めた[11]。I型アレルギー反応を抑える究極の治療法とも言えるが，厳密な意味での根治療法ではなく，また長期

EPX (eosinophil protein X)