QOL in a Japanese patient population with SAR during the peak cedar pollinosis season. The RQLQ and WPAI-AS instruments are well-established tools for assessing QOL as an outcome measure in clinical trials in the United States of America and Europe [5–7]. Both Questionnaires were recently translated and validated for use in a Japanese population, as QOL measurements will soon be adopted as an outcome measure for clinical trials [9]. The results presented here support the use of the Japanese versions of RQLQ and WPAI-AS Questionnaires for assessing the impact of SAR symptoms on QOL and work productivity in Japan.

In conclusion, this is the first clinical study to show that fexofenadine HCl 60 mg b.i.d. improves overall QOL and work productivity in Japanese patients with SAR during the peak cedar pollinosis season, using the recently validated RQLQ and WPAI-AS Japanese instruments.

References

1 Committee of Allergic Rhinitis Practice Guideline Preparation. Allergic Rhinitis Practice Guideline, ed 4. Tokyo, Life Science, 2002, chap 2, p 8.
2 Okuda M: Epidemiology of Japanese cedar pollinosis throughout Japan. Ann Allergy Asthma Immunol 2003;91:288–296.
3 Bousquet J, Duchateau J, Pignat JC, Fayol C, Marquis P, Mariz S, Ware JE, Valentin B, Burtin B: Improvement of quality of life by treatment with cetirizine in patients with perennial allergic rhinitis as determined by a French version of the SF-36 questionnaire. J Allergy Clin Immunol 1996;98:309–316.
4 Blaiss MS: The role of outcome studies in allergy and clinical immunology. Allergy Asthma Proc 1996;17:355–358.
5 Juniper EF, Guyatt GH: Development and testing of a new measure of health status for clinical trials in rhinoconjunctivitis. Clin Exp Allergy 1991;21:77–83.
6 Reilly MC, Zbrozek AS, Dukes EM: The validity and reproducibility of a work productivity and activity impairment instrument. Pharmacoeconomics 1993;4:353–365.
7 Reilly MC, Tanner A, Meltzer EO: Work, classroom and activity impairment instruments. Clin Drug Invest 1996;11:278–288.
8 Okuda M, Crawford B, Juniper E, Leahy M: Preparation of Japanese versions of the Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) and Work Productivity and Activity Impairment-Allergy Specific Questionnaire (WPAI-AS) (in Japanese). Arerugi 2003; 52(suppl 1):70–86.
9 Crawford B, Okuda M, Leahy M: Psychometric validation of a Japanese translation of the RQLQ and WPAI-AS (abstract). J Allergy Clin Immunol 2003;111:428.
10 Bousquet J, van Cauwenberge P, Khaltaev N, ARIA Workshop Group, World Health Organization: Allergic rhinitis and its impact on asthma. J Allergy Clin Immunol 2001;108(suppl 5):S147–S334.
11 Bronsky EA, Falliers CJ, Kaiser HB, Ahlbrandt R, Mason JM: Effectiveness and safety of fexofenadine, a new nonsedating H1-receptor antagonist, in the treatment of fall allergies. Allergy Asthma Proc 1998;19:135–141.
12 Bernstein DI, Schoenwetter WF, Nathan RA, Storms W, Ahlbrandt R, Mason J: Efficacy and safety of fexofenadine hydrochloride for treatment of seasonal allergic rhinitis. Ann Allergy Asthma Immunol 1997;79:443–448.
13 van Cauwenberge P, Juniper E, and the STAR Study Investigating Group: Comparison of the efficacy, safety, and quality of life provided by fexofenadine hydrochloride 120 mg, loratadine 10 mg, and placebo administered once daily for the treatment of seasonal allergic rhinitis. Clin Exp Allergy 2000;30;891–899.
14 Nsouli SM: Treatment of allergic rhinitis: Fexofenadine versus loratadine (abstract P40). Ann Allergy Asthma Immunol 1998;80:109.
15 Kittner B, Austin M, McKenzie D: Pooled analysis of the effects of once daily fexofenadine on nasal congestion and other SAR symptoms (abstract 228). Allergy 2001;56(suppl 68):78.
16 Baba K, Kittner B: Fexofenadine is effective and well tolerated in seasonal allergic rhinitis: Results from a placebo-controlled study in Japan (abstract 523). J Allergy Clin Immunol 2001;107:S159.
17 Paul E, Berth-Jones J, Ortonne J-P, Stern M: Fexofenadine hydrochloride in the treatment of chronic idiopathic urticaria: A placebo-controlled, parallel-group, dose-ranging study. J Dermatol Treat 1998;9:143–149.
18 Finn AF Jr, Kaplan AP, Fretwell R, Qu R, Long J: A double-blind, placebo-controlled trial of fexofenadine HCl in the treatment of chronic idiopathic urticaria. J Allergy Clin Immunol 1999;104:1071–1078.
19 Abdelaziz MM, Devalia JL, Khair OA, Bayram H, Prior AJ, Davies RJ: Effect of fexofenadine on eosinophil-induced changes in epithelial permeability and cytokine release from nasal epithelial cells of patients with seasonal allergic rhinitis. J Allergy Clin Immunol 1998;101: 410–420.
20 Paolieri F, Battifora M, Riccio AM, Bertolini C, Cutolo M, Bloom M, Ciprandi G, Canonica GW, Bagnasco M: Terfenadine and fexofenadine reduce in vitro ICAM-1 expression on human continuous cell lines. Ann Allergy Asthma Immunol 1998;81:601–607.
21 Marone G, Gentile M, Petraroli A, De Rosa N, Triggiani M: Histamine-induced activation of human lung macrophages. Int Arch Allergy Immunol 2001;124:249–252.
22 Tanner LA, Reilly M, Meltzer EO, Bradford JE, Mason J: Effect of fexofenadine HCl on quality of life and work, classroom, and daily activity impairment in patients with seasonal allergic rhinitis. Am J Managed Care 1999;5: S235–S247.
23 Juniper EF, Guyatt GH, Griffith LE, Ferrie PJ: Interpretation of rhinoconjunctivitis quality of life questionnaire data. J Allergy Clin Immunol 1996;98:843–845.
24 Cockburn IM, Bailit HL, Berndt ER, Finkelstein SN: Loss of work productivity due to illness and medical treatment. J Occup Environ Med 1999;41:948–953.
25 Ministry of Health, Labor and Welfare of Japan: Monthly Labor Survey. April 1, 2002.
26 Thompson AK, Finn AF, Schoenwetter WF: Effect of 60 mg twice-daily fexofenadine HCl on quality of life, work and classroom productivity, and regular activity in patients with chronic idiopathic urticaria. J Am Acad Dermatol 2000;43:24–30; erratum in: 2001;45: 960.
27 Meltzer EO, Casale TB, Nathan RA, Thompson AK: Once-daily fexofenadine HCl improves quality of life and reduces work and activity impairment in patients with seasonal allergic rhinitis. Ann Allergy Asthma Immunol 1999;83:311–317.

Allergy 2005: 60: 211 217

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ALLERGY
DOI: 10.1111/j.1398-9995.2005.00660.x

Original article

Characterization of allergen-specific monocyte-derived dendritic cells generated from monocytes by a single-step procedure: effect on naïve and memory T cells

Background: Dendritic cells are one of the most potent antigen-presenting cells and when pulsed with allergen can modulate allergen-specific T-cell responses. We sought to establish a single-step method by which to generate allergen-specific monocyte-derived dendritic cells (MoDCs).
Methods: *Dermatophagoides farinae* (Df)-prepulsed MoDCs were generated from monocytes by culturing with Df in the presence interleukin (IL)-4, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)-α simultaneously. Df-prepulsed MoDC were incubated with autologous naïve and memory T cells in the absence of recall antigen, then proliferation and cytokine production by T cells was determined.
Results: Generation of allergen-prepulsed MoDCs was confirmed by examining expression of surface molecules. Df-prepulsed MoDC selectively induced proliferation of Df-specific T cells in the absence of recall antigen. Under these conditions, Df-prepulsed MoDCs augmented but did not alter the cytokine production profile. In addition, Df-prepulsed MoDCs activated naïve T cells leading to proliferation and selective production of IFN-γ in allergic patients but not in healthy subjects.
Conclusions: These results suggest that Df-prepulsed MoDC generated from monocytes by a simple single-step manipulation can induce Df-specific cellular responses from both naïve and memory T cells in the absence of recall antigen, and these cells potentially can be utilized as immune adjuvants in allergen-specific immunotherapy.

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Key words: allergen; cytokine; dendritic cells; memory; naïve; rhinitis; T cell.

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Accepted for publication 4 May 2004

Allergic rhinitis is strongly associated with expansion of T-helper (Th)2-type immune responses (1). Differentiation and activation of antigen (Ag)-specific T cells require two signals provided by Ag-presenting cells (APCs) (2). Dendritic cells (DCs) are the most potent APCs, and both myeloid and plasmacytoid DC exist in the human nasal mucosa and increase following allergen exposure (3, 4). DC can lead to the activation of not only memory but also naïve T cells in an Ag-specific manner (5–7). In addition, depending on the type of stimulation they receive, DCs can induce the immune deviation (8, 9).

Monocyte-derived DCs (MoDCs) are considered a useful immunotherapeutic tool (10–16). Vaccination with peptide-pulsed MoDCs augments the delayed type hypersensitivity (DTH) responses associated with the regression of metastasis in melanoma (13). In addition, immunosuppressive strategies utilizing DCs have been developed in several diseases such as graft-*vs*-host disease (GVHD) and autoimmune diseases (14–16).

Monocyte-derived DCs can take up allergens and induce allergen-specific T-cell responses (6, 7, 17–20), and have been shown to result in greater production of interleukin (IL)-4 and/or IL-5 by autologous T cells from allergic patients when compared with nonallergic subjects. On the contrary, the effects on proliferation and IFN-γ production are still disputed (6, 7, 18–20). In most of the investigations, however, MoDCs were first created by treatment with IL-4 and GM-CSF followed by uptake of Ag.

For future use of DCs as allergen-specific immunotherapy, it would be ideal to produce allergen-specific DCs by simple manipulation. The aim of the present study is to establish a simple single-step process by which to generate allergen-specific MoDCs. We pulsed peripheral blood monocytes with allergen while simultaneously stimulating with IL-4, GM-CSF and tumour necrosis factor (TNF)-α;

Abbreviations: APC, antigen-presenting cell; CM, culture medium; Df, *Dermatophagoides farinae*; GM-CSF, granulocyte-macrophage colony-stimulating factor; MoDC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; TCL, T-cell lines.

no further manipulation is needed to establish allergen-specific MoDCs following this protocol. We investigated the duration of Ag presentation by these allergen-prepulsed MoDCs as well as surface molecule expression and activation of naïve and Ag-specific memory T cells, and compared the ability of Ag presentation by allergen-prepulsed DC with DC generated before Ag simulation. Our observations indicate that allergen-prepulsed MoDCs generated by our one-step procedure has the potential to be a novel tool for allergen-specific immunotherapy.

Methods

Subjects

Twelve Japanese patients (mean 29.2 years old) with perennial allergic rhinitis and seven healthy control subjects (mean 29.1 years old) were examined. All patients showed an elevation of serum IgE specific for *Dermatophagoides farinae* (Df) by CAP-RAST (Pharmacia, Uppsala, Sweden). Informed consent was obtained from each subject. None of the subjects used immunosuppressive drugs or underwent immunotherapy during the study.

Antigen

Crude extract of Df was provided by Torii Co (Tokyo, Japan) (21). Purified protein derivative (PPD) was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). Protein concentration of Ag was determined by bicinchoninic acid assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Generation of Df-specific, MoDCs

Peripheral blood mononuclear cells (PBMCs) in RPMI 1640 (Sigma chemical Co., St Louis, MO, USA) supplemented with 10% FCS (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine (Gibco, Grand Island, NY, USA) [culture medium (CM)] were incubated in a tissue culture flask at 37°C for 30 min in a 5% CO_2 atmosphere. After washing, 1×10^6/ml adherent cells were incubated in CM containing 10 μg/ml Df and 10 ng/ml GM-CSF (Peprotech EC, London, UK), 10 ng/ml IL-4 (Peprotech EC) and 10 ng/ml TNF-α (Promega, Madison, WI, USA). After 7 days of culture, cells corresponding to the MoDC-enriched fraction were harvested, washed and used for subsequent experiments.

Flowcytometric analysis

The method of flowcytometry has been described previously (22). The following monoclonal antibodies were used throughout the study: flourescein isothiocyanate (FITC)-conjugated anti-CD1a, anti-CD54 or anti-CD80; phycoerythrin (PE)-conjugated anti-CD86, anti-CD40, anti-CD83 or anti-CD14; Cy-chrome-conjugated anti-human leucocyte Ag (HLA)-DR (Becton Dickinson, Mountain View, CA, USA).

Cell preparation

CD45RA+, CD14+, and CD19+cells were separated from PBMCs using antibody (Ab)-coated magnetic microbeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. When microbeads coupled to anti-CD45RA, anti-CD14 and anti-CD19 mAb were used, >95, 95 and 85% of the sorted cells were CD45RA+, CD14+ and CD19+ by flowcytometry, respectively. Df and PPD-specific T-cell lines (TCLs) were generated as described previously (23). Flowcytometric analysis revealed that >97% cells in Ag-specific TCLs expressed both CD45RO and CD4.

Ag-specific T-cell responses

Monocyte-derived DC-driven proliferation and cytokine production by naïve and Ag-specific memory T cells was tested as described previously (23). In brief, 1×10^5 of either CD45RA+ cells or Ag-specific TCLs suspended in 200 μl CM were co-cultured with serial dilutions of 30 Gy-irradiated autologous APCs in the presence or absence of recall stimulation with 10 μg/ml Df. CD45RA+ cells and Df-specific TCLs were cultured for 7 and 3 days, respectively.

Cytokine production assays and determination

Levels of IL-4 and IFN-γ in culture supernatants were measured by Opt EIA™ sets (Becton Dickinson) according to the manufacturer's instructions. The detection limits in these assays were 5 pg/ml for IL-4 and 10 pg/ml for IFN-γ.

Statistical analysis

Data were analysed through use of the paired *t*-test or Mann–Whitney's *U*-test. $P < 0.05$ was considered statistically significant. Values were given as mean ± standard deviation (SD).

Results

Effect of Df prepulse on the expression of surface molecules on MoDCs

Peripheral blood mononuclear cell-derived monocytes from allergic patients were cultured with GM-CSF, IL-4 and TNF-α in the presence or absence of Df for 7 days. They expressed CD1a, HLA-DR, CD54, CD80, and CD86, but expression of CD14 was downregulated, suggesting maturation into MoDC (Fig. 1). Although expression of CD1a, HLA-DR, CD54, CD80, and CD86 was similar between Df-prepulsed and non-prepulsed MoDCs, expression of CD40 was significantly upregulated in Df-prepulsed MoDCs ($P = 0.005$) (Fig. 2).

Roles of Df-prepulsed MoDCs in the activation of Ag-specific memory T cells in the absence of recall stimulation

Four Df-specific CD4+ TCLs were generated from peripheral blood of two donors (T.T. and S.O.). Df-prepulsed MoDCs could induce the proliferation of all TCLs in the absence of recall Ag (Fig. 3A). However, Df-prepulsed MoDC did not induce the responses of PPD-specific TCLs without recall stimulation (data not shown). This activation occurred even at a MoDC to T-cell ratio of 1 : 25, and the maximal activation was observed when the

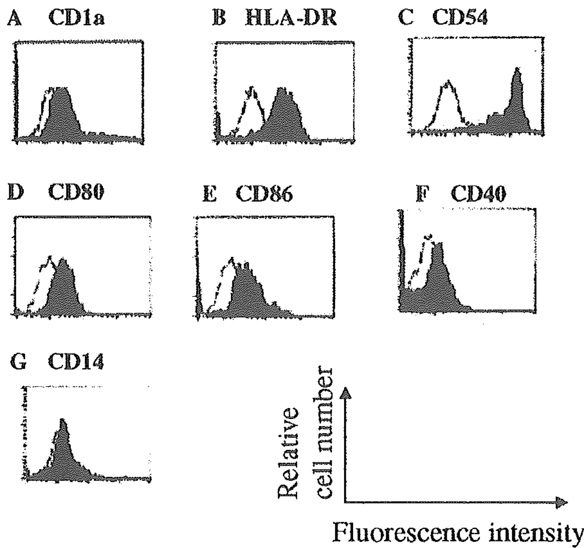

Figure 1. Phenotypic characterization of MoDC. Monocytes from allergic patients were incubated with 10 ng/ml IL-4, 10 ng/ml GM-CSF and 10 ng/ml TNF-α for 7 days. The expression of surface molecules were analysed by flowcytometry. Bold and fine line represent the intensity with and without fluorescence-conjugated mAb, respectively.

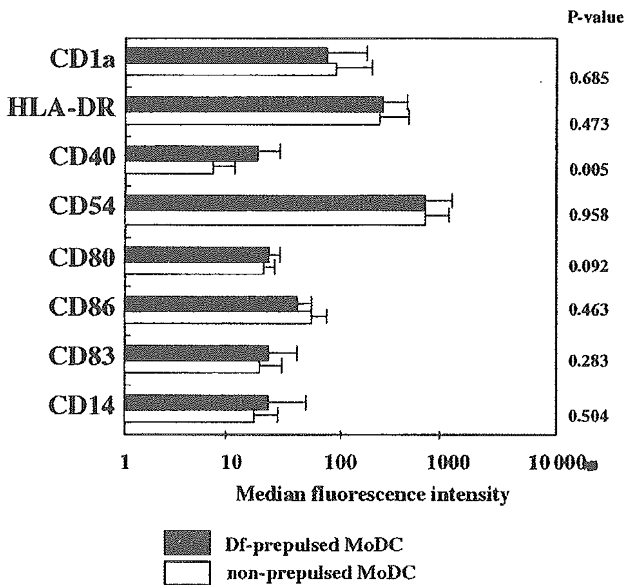

Figure 2. Effect of prepulse with Df on the expression of surface molecules in MoDC. Monocytes from allergic patients ($n = 7$) were incubated with 10 ng/ml IL-4, 10 ng/ml GM-SCF and 10 ng/ml TNF-α in the presence (closed bar) or absence (open bar) of 10 µg/ml Df for 7 days. Then the generated MoDC were harvested, and the expression of surface molecuoles were analysed by flowcytometry. *P*-value was obtained through use of paired *t*-test.

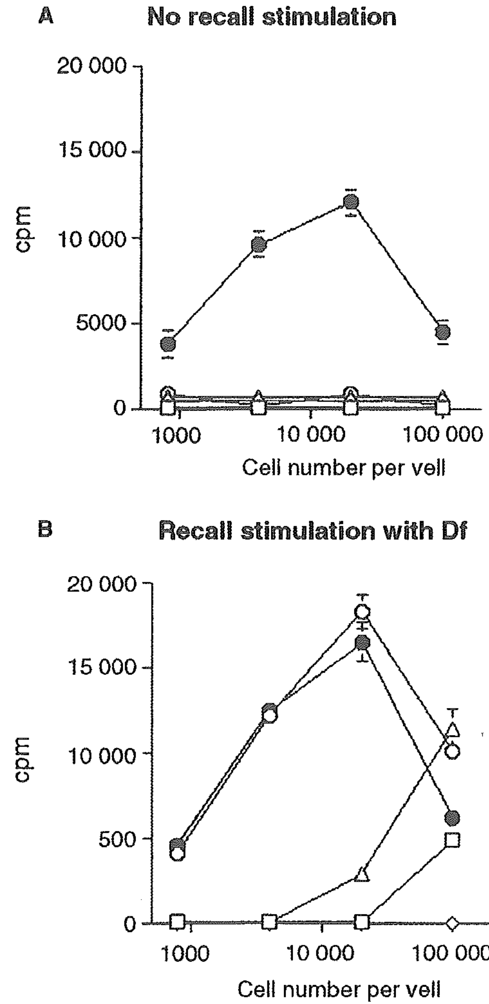

Figure 3. Proliferative responses of Df-specific TCL by APCs with or without recall stimulation; 2×10^4 Df-specific TCL (TF-4) were incubated with serial numbers of 30 Gy-irradiated PBMC (open square), B cell (open diamond), monocyte (open triangle), Df-nonpulsed MoDC (open circle) or Df-prepulsed MoDC (closed circle) in the absence (A) or presence (B) of 10 µg/ml Df for 72 h. Cell proliferation was estimated by mean uptake of ^{3}H-thymidine (count per minute) in triplicate culture. Bars indicate mean ± SD. Results were concordant with data from other TCLs such as TF-3, OD-1 and OD-2.

ratio was 1 : 1. Non-prepulsed MoDC, B cells, monocytes and PBMC did not induce the responses (Fig. 3A). With recall Ag stimulation, both Df-prepulsed and non-prepulsed MoDCs exhibited similar abilities to induce Df-specific T-cell proliferation (Fig. 3B).

Effect of Df-prepulsed MoDCs on cytokine production by Ag-specific memory T cells

Two TCLs (TF4 and OD2) were defined as Th0, as they produced detectable amounts of both IL-4 and IFN-γ in

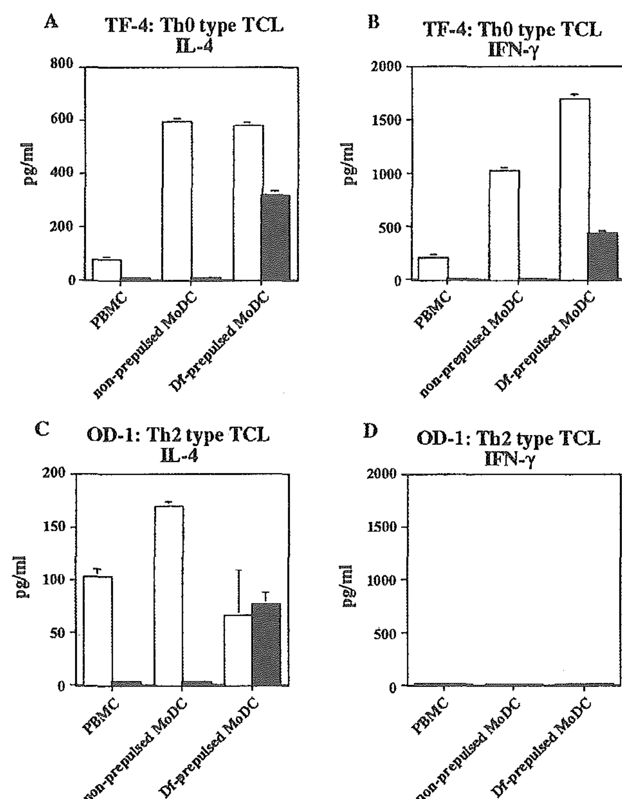

Figure 4. Effect of Df-prepulsed MoDC on cytokine production by Df-specific memory TCL; 2×10^4 of Th0-type (TF-4; A and B) or Th2-type (OD-1; C and D). TCL-specific for Df were incubated with 30 Gy-irradiated PBMC, non-prepulsed MoDC or Df-prepulsed MoDC in the presence (open bar) or absence (closed bar) of 10 μg/ml Df for 65 h. After incubation, supernatants were collected and IL-4 (A and C) and IFN-γ (B and D) were determined by means of enzyme-linked immunosorbent assay (ELISA). Results indicate mean ± SD of triplicate wells. Data are representative for two separate experiments, and concordant with data from TF-3 and OD-2 TCLs.

response to Df when irradiated PBMCs were used as APC. The other TCLs (TF3 and OD1) were defined as Th2 as they produced IL-4 but not IFN-γ in the same stimulation. In Th0 TCLs, Df-prepulsed but not non-prepulsed MoDCs induced production of both IL-4 and IFN-γ in the absence of recall Ag (Fig. 4A,B). In Th2 TCLs, only Df-prepulsed MoDCs induced IL-4 but not IFN-γ production in the absence of recall Ag (Fig. 4C,D). Furthermore, we compared cytokine production by Ag-specific CTLs in response to Df-prepulsed MoDCs and non-prepulsed MoDCs in presence of recall Ag. We found no differences in stimulatory ability between Df-prepulsed MoDCs and non-prepulsed MoDCs (data not shown).

Duration of Ag presentation by Df-prepulsed MoDCs

Monocytes were incubated with Df in the presence of GM-CSF, IL-4 and TNF-α for 1, 2 and 4 weeks, thereafter cells were harvested and co-cultured with TCLs. MoDCs harvested in the first week displayed the most potent Ag presentation. This ability was reduced when the cells were incubated for longer periods, however, cells harvested at 4 weeks could still induce proliferation and cytokine production by TCLs. However, non-prepulsed MoDC did not display the ability during the periods observed without recall Ag (Fig. 5).

Role of Df-prepulsed MoDCs in the activation of naïve T cells in the absence of recall stimulation

We co-cultured Df-prepulsed MoDCs with CD45RA+ T cells from either allergic patients or healthy subjects (Fig. 6). The proliferation of naïve T cells to Df-prepulsed MoDCs was significantly higher in allergic patients than healthy subjects (Fig. 6A). IL-4 production was not present in either group (Fig. 6B). However, Df-prepulsed MoDCs induced IFN-γ production only in allergic patients (Fig. 6C).

Discussion

In the present study, we characterized the ability of Ag-prepulsed MoDCs to present Ag to both naïve and Ag-specific T cells. The most novel aspect of our method to generate Ag-specific MoDCs was to pulse monocytes with Ag and cytokine cocktails simultaneously at the beginning of culture. Although several methods by which to pulse DCs with Ag have been reported, these systems require multiple manipulations (19, 20). Our method may be beneficial for the future use of immunotherapy because of its simplicity.

Several reports have demonstrated the phenotypic differences in DCs between allergic patients and healthy donors in the presence or absence of Ag (7, 19, 20). By our method, Df-prepulsed DCs expressed higher levels of CD40 when compared with non-prepulsed DC. Engagement via CD40 is known to induce IgE isotype switching, eosinophil survival and cytokine release by memory T cells (24–26). Thus our results suggest that Df-prepulsed MoDC may augment Df-specific T-cell responses via enhanced expression of CD40.

Monocyte-derived DCs prepulsed with Df at the beginning of their generation could activate Ag-specific memory T cells in the absence of recall Ag. This capacity was seen at MoDC to T-cell ratios ranging from 1 : 25 to 5 : 1, thus falling in line with previous reports of strong responses at a DC to T-cell ratio of $\leq$ 1 : 10 (6, 7, 19, 20). Stimulator/responder ratio affects Th1 or Th2 differentiation from naïve T cell (9). In addition, DCs have been shown to induce immune deviation (27, 28). Df-prepulsed MoDC elicited similar patterns of IL-4 and IFN-γ production by Df-specific TCLs at several DC to T cell ratios ranging from 1 : 625 to 5 : 1. Immune deviation to a Th1 response may be useful for prevention of or

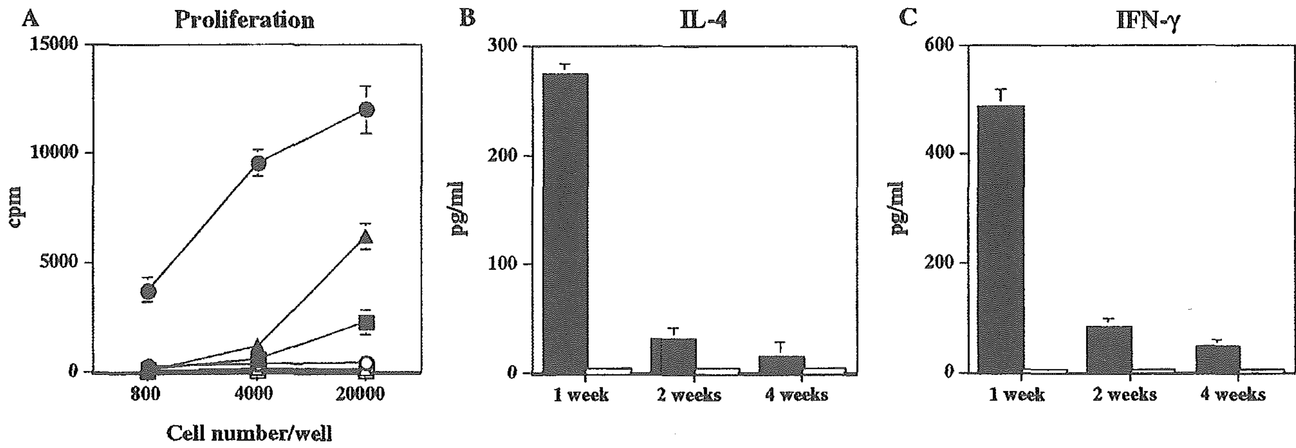

Figure 5. Duration of Ag-presenting capacity of Df-prepulsed Mo-DC. Monocytes from allergic patient (TT) were cultured with (closed symbols and bars) or withtout (open symbols and bars) 10 μg/ml Df together with 10 ng/ml IL-4, 10 ng/ml GM-SCF and 10 ng/nl TNF-α. 1 (cricle), 2 (triangle) and 4 (square) weeks after the beginning of culture, the generated MoDC were harvested, washed and irradiated for 30 Gy. Then serial numbers of MoDC were cultured with autologous 2×10^4 Df-specific TCLs (TF-4) without recall stimulation for 72 h. Cell proliferation of TCLs was measured by incorporation of ^{3}H-thymidine (A). Results indicate mean cpm ± SD of triplicate cultures. And after incubation for 65 h, supernatants were collected, and IL-4 (B) and IFN-γ (C) were measured by means of ELISA. Results indicate mean cpm ± SD of triplicate wells. Data are representative for two separate experiments, and concordant with data from OD-2 TCL.

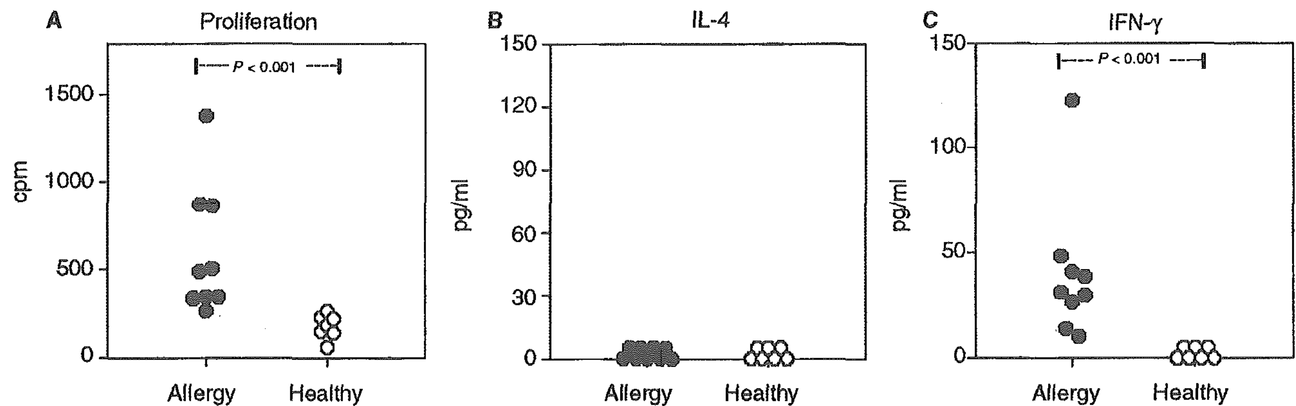

Figure 6. Proliferation and cytokine production of naïve T cells induced by Df-prepulsed MoDC without recall stimulation. Df-prepulsed MoDC from nine allergic patients with perennial rhinitis (closed circle) and seven healthy subjects (open square) were generated as described above; 30 Gy-irradiated 2×10^4 Df-prepulsed MoDC were co-cultured with the same number of autologous CD45RA+ cells for 7 days. ^{3}H-thymidine was added, and incorporation of ^{3}H-thymidine was measured as described above. After 3 days incubation, supernatants were collected, and IL-4 (B) and IFN-γ (C) were determined by means of ELISA. *P*-value was obtained through use of Mann–Whitney's *U*-test.

immunotherapy for type I allergy (27, 28). However, our results suggest that Df-prepulsed MoDCs produced by our method have no potential to induce immune deviation of Df-specific memory T cells.

One of the unique properties of DCs is that they can activate naïve T cells (5, 6). The present study showed that Df-prepulsed MoDCs activate naïve T cells which leads to not only to proliferation but also to IFN-γ production in allergic patients but not in healthy subjects (Fig. 6). However, Bellinghausen et al. (6) reported that a Th2 cytokine profile was induced in naïve T cells from atopic but not from nonatopic donors after stimulation with autologous allergen-pulsed DCs. Recent investigations revealed that DCs may not have an intrinsic capacity to direct either Th1 or Th2 cell development, but rather might be modified by external factors such as dose of Ag and a range of stimuli including pathogen-derived products and inflammatory mediators (29, 30). Thus the difference between these studies may arise from the methods used to generate allergen-pulsed DCs such as

the use of TNF-α at the biginning of culture and the concentration of cytokines loaded. Our method appears to have a beneficial advantage for the future use as allergen-specific immunotheraphy as Df-prepulsed MoD-Cs generated by our method can promote the immune deviation into Th1 responses in naïve T cells from allergic but not healthy subjects (27, 28).

In conclusion, we have provided *in vitro* evidence that MoDCs prepulsed with Df at the beginning of their generation display an ability to stimulate both memory and naïve T cells in the absence of recall Ag. This capacity can last up to 4 weeks after loading with Df. In addition, Df-prepulsed MoDC can promote allergen-specific Th1 responses from naïve T cells only in allergic patients. These cells have obvious interest and utility in the study of allergy diseases and potentially could even be utilized as immune adjuvants in allergen-specific immunotherapy.

Acknowledgments

The authors thank Megumi Maeda and Tazuko Fujiwara for excellent assistance, and Yuko Okano for editorial assistance. This work was supported in part by the grants from Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 14704043) and Research on Allergic disease and Immunology of Ministry of Health, Labor and Welfare (no. 14210301).

References

1. Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS et al. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage colony stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. J Immunol 1992;**148**:2390–2394.
2. Liu Y, Linsley Y. T cell costimulation. Curr Opin Immunol 1992;4:265–270.
3. Jahnsen FL, Lund-Johansen F, Dunne JF, Farkas L, Haye R, Brandtzaeg P. Experimentally induced recruitment of plasmacytoid (CD123high) dendritic cells in human nasal allergy. J Immunol 2000;**165**:4062–4068.
4. Till SJ, Jacobson MR, O'Brien F, Durham SR, KleinJan A, Fokkens WJ et al. Recruitment of CD1a+ Langerhans cells to the nasal mucosa in seasonal allergic rhinitis and effects of topical corticosteroid therapy. Allergy 2001;**56**:126–131.
5. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. Cell 2001;263–266.
6. Bellinghausen I, Brand U, Knop J, Saloga J. Comparison of allergen-stimulated dendritic cells from atopic and nonatopic donors dissecting their effect on autologous naïve and memory T helper cells of such donors. J Allergy Clin Immunol 2000;**105**:988–996.
7. Bellinghausen I, Brand U, Steinbrink K, Enk AH, Knop U, Saloga J. Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells. J Allergy Clin Immunol 2001;**108**:242–249.
8. Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, Malefy R et al. Reciprocal control of T helper cell and dendritic cell differenciation. Science 1999;**283**:1183–1186.
9. Tanaka H, Demeure CE, Rubio M, Delespesse G, Sarfati M. Human monocyte-derived dendritic cells induce naïve T cell differentiation into T helper 2 (Th2) or Th1/Th2 effectors: role of stimulator/responder ratio. J Exp Med 2000;**192**:405–411.
10. Akagawa KS, Takasuka N, Nozaki Y, Komuro I, Azuma M, Ueda M et al. Generation of CD1+RelB+ dendritic cells and tartrate-resistant acid phosphatase-positive osteoclast-like multinucleated giant cells from human monocytes. Blood 1996;**88**:4029–4039.
11. Tsai V, Southwood S, Sidney J, Sakaguchi K, Kawakami Y, Appella E et al. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary *in vitro* immunization with peptide-pulsed dendritic cells. J Immunol 1997;**158**:1796–1802.
12. Chapuis F, Rosenzwajg M, Yagello M, Ekman M, Biberfeld P, Gluckman JC. Differentiation of human dendritic cells from monocytes *in vitro*. Eur J Immunol 1997;**27**:431–441.
13. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4:328–332.
14. O'Rourke RW, Kang SM, Lower JA, Feng S, Ascher NL, Baekkeskov S et al. A dendritic cell line genetically modified to express CTLA4-IG as a means to prolong islet allograft survival. Transplantation 2000;**69**:1440–1446.
15. Kim SH, Kim S, Evans CH, Ghivizzani SC, Oligino T, Robbins PD. Effective treatment of established murine collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express IL-4. J Immunol 2001;**166**:3499–3505.
16. Morita Y, Yang J, Gupta R, Shimizu K, Shelden EA, Endres J et al. Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. J Clin Invest 2001;**107**:1275–1284.
17. van den Heuvel MM, Vanhee DD, Postmus PE, Hoefsmit EC, Beelen RH. Functional and phenotypic differences of monocyte-derived dendritic cells from allergic and nonallergic patients. J Allergy Clin Immunol 1998;**101**:90–95.
18. Sung SJ, Taketomi EA, Smith AM, Platts-Mills TA, Fu SM. Efficient presentation of house dust mite allergen Der p 2 by monocyte-derived dendritic cells and the role of beta 2 integrins. Scand J Immunol 1999;**49**:96–105.
19. De Wit D, Amraoui Z, Vincart B, Michel O, Michils A, Van Overvelt L et al. Helper T-cell responses elicited by Der p 1-pulsed dendritic cells and recombinant IL-12 in atopic and healthy subjects. J Allergy Clin Immunol 2000;**105**:346–352.
20. Hammad H, Charbonnier AS, Duez C, Jacquet A, Stewart GA, Tonnel AB et al. Th2 polarization by Der p 1-pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. Blood 2001;**98**:1135–1141.
21. Dandeu JP, Le Mao J, Lux M, Rabillon J, David B. Antigens and allergens in Dermatophagoides farinae mite. II. Purification of AG f1, a major allergen in *Dermatophagoides farinae*. Immunology 1982;**46**:679–687.

22. Nakada M, Nishizaki K, Yoshino T, Okano M, Masuda Y, Ohta N et al. CD86 (B7-2) antigen on B cells from atopic patients shows selective, antigen-specific upregulation. Allergy 1998;**53**:527–531.
23. Okano M, Kino K, Takishita T, Hattori H, Ogawa T, Yoshino T et al. Roles of carbohydrates on Cry j1, the major allergen of Japanese cedar pollen, in specific T cell responses. J Allergy Clin Immunol 2001;**108**:101–108.
24. Oettgen HC. Regulation of the IgE isotype switch: new insights on cytokine signals and the function of epsilon germline transcripts. Curr Opin Immunol 2000;**12**:618–623.
25. Bureau F, Seumois G, Jasper F, Vanderplasschen A, Derty B, Pastoert PP et al. CD40 engagement enhances eosinophil survival through induction of cellular inhibitor of apoptosis protein 2 expression: possible involvement in allergic inflammation. J Allergy Clin Immunol 2002;**110**:443–449.
26. Rogers NJ, Jackson IM, Jordan WJ, Lombardi G, Delikouras A, Lechler RI. CD40 can costimulate human memory T cells and favors IL-10 secretion. Eur J Immunol 2003;**33**:1094–1104.
27. Brand U, Bellinghausen I, Enk AH, Jonuleit H, Becker D, Knop J et al. Allergen-specific immune deviation from a TH2 to a TH1 response induced by dendritic cells and collagen type I. J Allergy Clin Immunol 1999;**104**: 1052–1059.
28. Hattori H, Okano M, Yamamoto T, Yoshino T, Yamashita Y, Watanabe T et al. Intranasal application of purified protein derivative suppresses the initiation but not the exacerbation of allergic rhinitis in mice. Clin Exp Allergy 2002;**32**:951–959.
29. de Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells *in vitro* and diverse Th cell-polarizing signals. J Immunol 2002;**168**:1704–1709.
30. Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu Y-J et al. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. J Exp Med 2003;**197**:101–109.

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2005;136:142–147
DOI: 10.1159/000083321

Received: April 5, 2004
Accepted after revision: September 30, 2004
Published online: January 12, 2005

Immunosuppressive Effect of Restraint Stress on the Initiation of Allergic Rhinitis in Mice

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Key Words
Allergic rhinitis · IgE · IgG1 · IgG2a · Immunosuppression · Mouse model · Nasal sensitization · Phospholipase A2 · Restraint stress

Abstract
Background: Exposure to acute stressors modulates both innate and acquired immune function. However, little is known about whether stress has the potential to modulate the pathogenesis of allergic rhinitis. ***Objectives:*** To determine the effects of acute restraint stress on the initiation of allergic rhinitis in a murine model. ***Methods:*** CBA/J mice were repeatedly intranasally sensitized with phospholipase A2 (PLA2) from honeybee venom without adjuvant. Restraint stress was applied using uniform cylinders once a week for a continuous 8-hour period, on five occasions in total. Production of PLA2-specific antibodies and degree of nasal and blood eosinophilia were compared between stressed and control mice. ***Results:*** Repeated intranasal sensitization with PLA2 induced PLA2-specific IgE and marked eosinophilia in both the nose and blood in CBA/J mice. Exposure to restraint stress significantly inhibited production of PLA2-specific IgE, IgG1 and IgG2a. Conversely, the stress exerted no significant effect on eosinophilia. ***Conclusions:*** Exposure to acute restraint stress inhibits antigen-specific antibody production, but not local or systemic eosinophilia. The results of this study suggest that acute stress has the potential to modulate the initiation of allergic rhinitis.

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Introduction

Exposure to physical, neurological, or emotional stress can affect both innate and acquired immunity [1–4]. In general, stressor exposure can increase many measures of innate immunity, such as natural killer cell activity and nitric oxide synthesis [5, 6]. Exposure to acute stress also modulates features of acquired immune functions such as antigen-specific T-cell responses [7, 8]. Such stresses reportedly suppress Th1 responses, since Th1 cells selectively express β2-adrenergic receptor [9]. Controversial results have been reported regarding the effect of stressor exposure on the production of antigen (i.e. keyhole limpet hemocyanin)-specific IgG or IgM [10, 11]. Although exposure to stress affects the pathophysiology of allergic disease, little is known regarding the effect of stress on the initiation of allergic rhinitis [12, 13]. This may be due to a lack of adequate animal models reflecting the pathogenesis of allergic rhinitis.

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1018–2438/05/1362–0142$22.00/0

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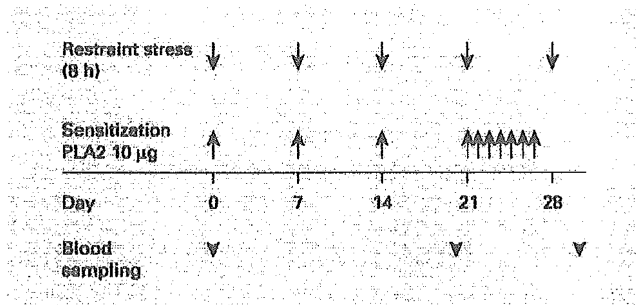

Fig. 1. Treatment schedule. Mice were intranasally sensitized to 10 µg of PLA2 in 20 µl of saline. Sensitization was repeated in the same manner. Following sensitization, restraint stress applied to mice using a single transparent cylindrical chamber was repeated once every week, on a total of five occasions.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

We have previously generated a murine model of allergic rhinitis using PLA2, a major allergen in venom from the honeybee, as a sensitizing allergen [14, 15]. In this model, CBA/J mice display production of PLA2-specific IgE and nasal eosinophilia on repeated intranasal sensitization with PLA2 without adjuvants. The present study sought to determine for the first time the role of restraint stress on the pathogenesis of allergic rhinitis in vivo using this murine model.

Methods

Animals

Young adult (10 weeks old) female CBA/J mice (Charles River Japan, Yokohama, Japan) weighing about 18–20 g were used in this study. Mice were maintained in an animal house according to the guidelines of the Animal Study Committee at 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 experiments began. Temperature in the animal house was maintained at 25 °C.

Reagents

ELISA plates were purchased from Corning, (Corning, N.Y., USA). Purified rat anti-mouse IgE was purchased from Biosource (Camarillo, Calif., 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), goat anti-mouse IgG1/IgG2a monoclonal antibody peroxidase conjugate from Boehringer-Mannheim (Indianapolis, Ind., USA) and biotin (long-arm) N-hydroxy succinimide ester from Vector Laboratories (Burlingame, Calif., USA). 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/group) were sensitized by nasal administration of 20 µl of saline containing 10 µg of PLA2 using a microsyringe (Hamilton, Reno, Nev., USA). PLA2 was carefully administered 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 administered in the same manner. Blood samples were taken from the tail vein on days 0, 20, and 30 after primary sensitization (fig. 1).

Induction of Restraint Stress

Following sensitization, restraint stress was applied to mice (n = 8) using a single transparent polymethylmethacrylate cylindrical chamber (20 mm diameter, 100 mm long) commonly used for drawing blood from mice. This chamber was placed in a horizontal position 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 five occasions (fig. 1). Control mice were maintained in their cages without food and water at the same times. Three separate experiments were performed to confirm reproducibility. One mouse escaped from the cylinder during the restraint period, and was excluded from analysis.

PLA2-Specific IgE, IgG1, and IgG2a in Serum

Serum levels of PLA2-specific IgE were determined using sandwich ELISA, and PLA2-specific IgG1 and IgG2a were detected using indirect ELISA as described elsewhere [14, 15]. Results are expressed as endpoint titers, where the endpoint equals the final serum dilution yielding an absorbance twice the background. Absorbance was measured at 450 nm using an automatic microplate reader (BioRad, Hercules, Calif., USA).

To determine whether restraint stress affected antigen-specific immune responses, differences in values of PLA2-specific IgE, IgG1 and IgG2a serum levels were compared between stressed and control animals.

Histological Examination

Twelve hours after drawing the final blood sample, mice were killed by cervical dislocation. The nasal cavity with paranasal sinuses was removed, fixed in 10% formalin and decalcified. Coronal nasal sections were stained using hematoxylin/eosin and Luna staining techniques. Eosinophils in the nasal mucosa were counted under light microscopy.

Blood Eosinophils in Mice

Eosinophils were counted in a blood smear stained using May-Grünwald Giemsa.

Statistical Analysis

Data are expressed as means ± 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 statistically significant.

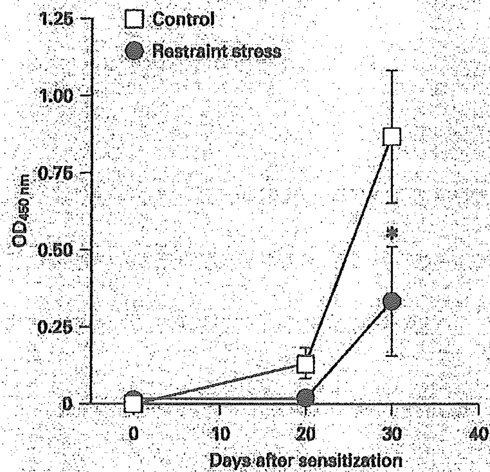

Fig. 2. Effect of restraint stress on PLA2-specific IgE production. Production of PLA2-specific IgE in mouse serum was significantly increased 30 days after the first sensitization. The level of PLA2-specific IgE was significantly decreased in stressed mice compared with control mice on day 30 (* $p < 0.05$). Data are representative of three separate experiments.

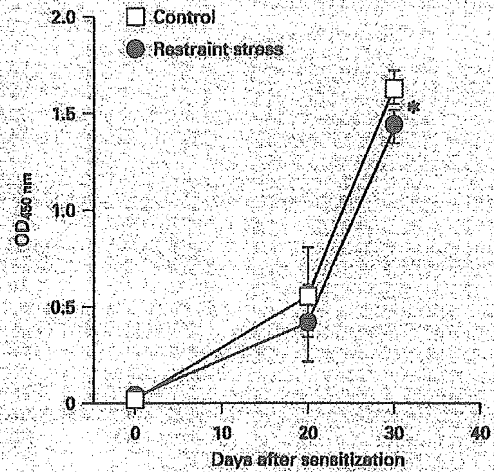

Fig. 3. Effect of restraint stress on PLA2-specific IgG1 production. Production of PLA2-specific IgG1 in mouse serum was significantly increased 30 days after the first sensitization. The level of PLA2-specific IgG1 was significantly decreased in stressed mice (* $p < 0.025$). Data are representative of three separate experiments.

Results

Effect of Restraint Stress on Antibody Production

Production of PLA2-specific IgE, IgG1 and IgG2a in mouse serum after PLA2 nasal sensitization was observed 20 days after the first nasal sensitization, and significant increases in serum IgE, IgG1 and IgG2a levels were detected 30 days after the first sensitization (fig. 2–4). As shown in figure 2, levels of PLA2-specific IgE were significantly lower in stressed mice than in control mice on day 30 ($p < 0.05$). Similarly, levels of PLA2-specific IgG1 and IgG2a were significantly decreased in stressed mice compared to control mice ($p < 0.025$ and $p < 0.05$, respectively) (fig. 3, 4).

Effect of Restraint Stress on Eosinophilia in the Nose and Blood

After sensitization with PLA2, the number of eosinophils that infiltrated the mucous membranes of the mouse nasal septum was significantly increased, in line with our previous reports [15]. Stressed mice tended to display a lower degree of nasal eosinophilia compared with control mice, but this difference was not significant ($p > 0.05$).

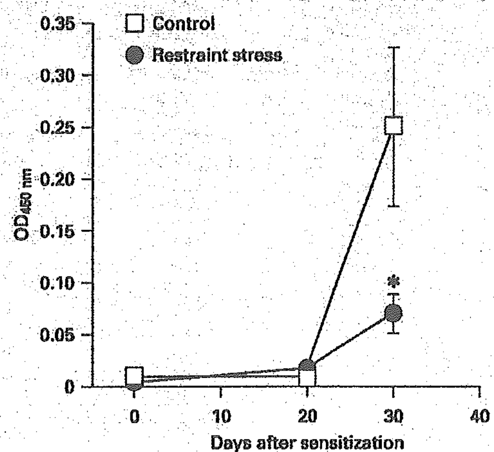

Fig. 4. Effect of restraint stress on PLA2-specific IgG2a production. Production of PLA2-specific IgG2a in mouse serum was significantly increased 30 days after the first sensitization. The level of PLA2-specific IgG2a was also significantly decreased in stressed mice (*$p < 0.05$). Data are representative of three separate experiments.

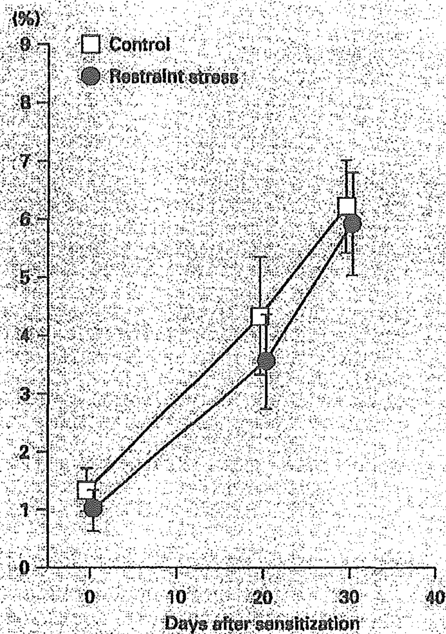

Fig. 5. Effect of restraint stress on blood eosinophilia. After sensitization with PLA2, blood eosinophil counts increased significantly (day 20, 4.3%, $p < 0.005$; day 30, 6.2%, $p < 0.0001$) after the first sensitization (day 0, 1.3%). In contrast, in restraint-stressed mice, eosinophil counts did not significantly change compared with control mice on days 20 and 30. Data are representative of three separate experiments.

Mean eosinophil counts in stressed and control mice were 21.86 ± 4.46 (10 × 40) and 27.50 ± 5.87 eosinophils/high power field, respectively.

Eosinophil concentration in blood after sensitization with PLA2 increased significantly (day 20, 4.3%, $p < 0.005$; day 30, 6.2%, $p < 0.0001$) from the first sensitization (day 0, 1.3%). No significant inhibition was seen in mice under restraint stress (day 20, 3.6%; day 30, 5.9%) (fig. 5).

Discussion

Many studies have shown that stress can affect IgE production [3, 16–20]. In humans, increased levels of serum IgE are induced by acute stressors such as burns and trauma [16, 17]. More recently, chronic caregiver stress in early childhood has been reported to upregulate IgE production in children [13]. Conversely, several kinds of stressors can suppress IgE production in rodents [3, 18, 19]. However, mild inescapable electric foot-shock stress can augment IgE production in rats [20]. Given these findings, stress should be categorized as either acute stress or chronic continuous stress, and immune reactions should thus be analyzed under each stress condition. The present finding that PLA2-specific IgE production is significantly decreased in stressed CBA/J mice following intranasal sensitization with PLA2 without adjuvants is consistent with earlier reports, and suggests that acute restraint stress plays an inhibitory role in IgE production in a murine model of allergic rhinitis.

In addition, production of both PLA2-specific IgG1 and IgG2a was also inhibited in stressed mice. Interleukin-4 is widely accepted as facilitating development of Th2 responses and inducing IgG1 and IgE production in mice. In contrast, interferon-γ induces a Th1 response that is associated IgG2a production [21]. Although we did not examine cytokine production by nasal mononuclear cells due to the in vitro cytotoxicity of PLA2, our results suggest the possibility that mice under restraint stress display impaired development of both Th1 and Th2 responses to intranasal sensitization with PLA2. Cortisol is known to be able to shift T cells toward a Th2 phenotype [22]. However, Fukui et al. [3] documented that restraint stress significantly suppressed both Th1- and Th2-type immune responses in mice, and this suppression was also closely correlated with the inhibition of interleukin-4 and interferon-γ production. Furthermore, Dhabhar et al. [23] reported that B cells showed greater stress-induced decreases than T cells. This latter report may support our finding, and the current results may suggest that polarized antibody production was not induced under restraint stress in our murine model of allergic rhinitis.

No significant change in nasal eosinophilia was identified in mice under acute restraint stress, as compared with control mice. Several reports have demonstrated that stress enhances eosinophilia in peripheral blood, sputum and bronchoalveolar lavage fluids [24–26]. Although our results were not consistent with these reports, this is the first report to focus on the effect of stress on eosinophil infiltration into the nose. Our results may suggest that eosinophil infiltration into the nose is more resistant to stress than the lower airways. In fact, a recent investigation revealed organ-specific regulation such as the expression of corticotropin-releasing factor receptor 1 following exposure to stress [27]. In addition, several reports have demonstrated that the degree of nasal eosinophilia does

not always parallel IgE production in murine models of allergic rhinitis [15, 28, 29].

A wide range of immunological, endocrinological and neuronal pathways are known to mediate and modulate systemic stress responses [30–32]. Okimura et al. [31], for example, reported that stress-induced production of antibody was stopped by extirpation of the suprarenal glands. Various studies have also revealed that plasma levels of catecholamines, corticosterone and ACTH are increased by the application of restraint stress [33, 34]. Further studies are needed to clarify the direct or indirect involvement of endocrinological and neuronal pathways in the initiation of allergic rhinitis in this model.

In summary, acute restraint stress inhibits antibody production, but not eosinophilia in the murine model of allergic rhinitis. Since the duration, frequency and degree of experienced stress represent important determinants of the impact on health and illness [12], future investigations using various kinds of stresses will help to further our understanding of the roles of stress in the initiation of allergic rhinitis. However, the observations presented herein may provide a new basis for future prophylactic and therapeutic approaches to the management of allergic rhinitis.

Acknowledgments

We wish to thank Tazuko Fujiwara for technical assistance and Yuko Okano for editorial assistance. This work was supported in part by grants for Research on Allergic Disease and Immunology by the Ministry of Health, Labor and Welfare (No. 14210301 to M.O.). This paper was presented in part at the World Allergy Organization Congress XVIII ICACI, Vancouver, on September 10, 2003.

References

1 Kusnecov AW, Rabin BS: Stressor-induced alterations of immune function: Mechanisms and issues. Int Arch Allergy Immunol 1994; 105:107–121.

2 Dabhar FS, McEwen BS: Stress-induced enhancement of antigen-specific cell-mediated immunity. J Immunol 1996;156:2608–2615.

3 Fukui Y, Sudo N, Yu XN, Nukina H, Sogawa H, Kubo C: The restraint stress-induced reduction in lymphocyte cell number in lymphoid organs correlates with the suppression of in vivo antibody production. J Neuroimmunol 1997;79:211–217.

4 Loureiro I, Wada CY: Influence of stress on IgE production. Physiol Behav 1993;53:417–420.

5 Irwin M, Brown M, Patterson T, Hauger R, Mascovich A, Grant I: Neuropeptide Y and natural killer cell activity: Findings in depression and Alzheimer caregiver stress. FASEB J 1991;5:3100–3107.

6 Campisi J, Leem TH, Fleshner M: Acute stress decreases inflammation at the site of infection: A role for nitric oxide. Physiol Behav 2002;77: 291–299.

7 Fleshner M, Bellgrau D, Laudenslager ML, Watkins LR, Maier SF: Stress-induced changes in the mixed lymphocyte reaction is dependent on macrophages but not on shifts in the phenotypes. J Neuroimmunol 1995;56:45–52.

8 Benneau RH, Brehm MA, Kern AM: The impact of psychological stress on the efficacy of anti-viral adoptive immunotherapy in an immunocompromised host. J Neuroimmunol 1997;78:19–33.

9 Ramer-Quinn DS, Baker RA, Sanders VM: Activated T helper 1 and T helper 2 differentially express the beta-2 adrenergic receptor: A mechanism for selective modulation of T helper 1 cytokine production. J Immunol 1997;159: 4857–4867.

10 Karp JD, Smith J, Hawk K: Restraint stress augments antibody production in cyclophosphamide-treated mice. Physiol Behav 2000;70: 271–278.

11 Moraska A, Campisi J, Nguyen KT, Maier SF, Watkins LR, Fleshner M: Elevated IL-1b contributes to antibody suppression produced by stress. J Appl Physiol 2002;93:207–215.

12 Wright RJ, Rodriguez M, Cohen S: Review of psychosocial stress and asthma: In integrated biopsychosocial approach. Thorax 1998;53: 1066–1074.

13 Wright RJ, Finn P, Contreras JP, Cohen S, Wright RO, Sataudenmayer S, Wand M, Perkins D, Weiss ST, Gold DR: Chronic caregiver stress and IgE expression, allergen-induced proliferation, and cytokine profiles in a birth cohort predisposed to atopy. J Allergy Clin Immunol 2004;113:1051–1057.

14 Okano M, Nishizaki K, Satoskar AR, Yoshino T, Masuda Y, Harn DA: Involvement of carbohydrate on phospholipase A_2, a bee-venom allergen, in vivo antigen-specific IgE synthesis in mice. Allergy 1999;55:811–818.

15 Yamamoto T, Okano M, Ono T, Nakayama E, Yoshino T, Satoskar AR, Harn DA, Nishizaki K: Gender-related differences in the initiation of allergic rhinitis in mice. Allergy 2001;56: 525–531.

16 Gleich GJ, Dunnette SL, Volence FJ, Mani MM: Quantification of serum IgE in patients with burns. Clin Allergy 1979;9:133–139.

17 Chelazzi G, Pinotti G, Nicora C, Rossi D, Senaldi G: Increased total serum IgE concentration in patients. J Clin Pathol 1985;38:1309–1310.

18 Ito Y, Mine K, Ago Y, Nakagawa T, Fujiwara M, Ueki S: Attack stress and IgE antibody production in rats. Pharmacol Biochem Behav 1983;19:883–886.

19 Loureiro I, Mota I: Decrease in passive cutaneous anaphylaxis reactions in rats secondary to stress. Braz J Med Biol Res 1993;26:719–723.

20 Persoons JH, Berkenbosch F, Schornagel K, Thepen T, Kraal G: Increased specific IgE production in lungs after the induction of acute stress in rats. J Allergy Clin Immunol 1995;95: 765–770.

21 Snapper CM, Paul WE: Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;236:944–947.

22 Ramirez J, Fowell DJ, Puklavek M, Simmonds S, Mason D: Glucocorticoids promote a Th2 cytokine response by CD4+ T cells in vitro. J Immunol 1996;156:2406–2412.

23 Dhabhar FS, Miller AH, McEwen BS, Spencer RL: Effects of stress on immune cell distribution. Dynamics and humoral mechanisms. J Immunol 1995;154:5511–5527.

24 Buske-Kirschbaum A, Gierens A, Holling H, Hellhammer DH: Stress-induced immunomodulation is altered in patients with atopic dermatitis. J Neuroimmunol 2002;129:161–167.

25 Liu LY, Coe CL, Swenson CA, Kelly EA, Kita H, Busse WW: School examinations enhance airway inflammation to antigen challenge. Am J Respir Crit Care Med 2002;165:1062–1067.

26 Joachim RA, Quarcoo D, Arck PC, Herz U, Resz H, Klapp BF: Stress enhances airway reactivity in an animal model of allergic bronchial asthma. Psychosom Med 2003;65:811–815.

27 Radulovic M, Weber C, Spiess J: The effect of acute immobilization stress on the abundance of corticotropin-releasing factor receptor in lymphoid organs. J Neuroimmunol 2000;103:153–164.

28 Okano M, Yoshino T, Nishizaki K, Satoskar AR, Brombacher F, Satoskar AA, Abe M, Takeda Y, Harn DA: Interleukin-4-independent production of Th2 cytokines by nasal lymphocytes and nasal eosinophilia in murine allergic rhinitis. Allergy 2000;55:723–731.

29 Watanabe T, Okano M, Hattori H, Yoshino T, Ohno N, Ohta N, Sugata Y, Orita Y, Takai T, Nishizaki K: Roles of FcγRIIB in nasal eosinophilia and IgE production in murine allergic rhinitis. Am J Respir Crit Care Med 2004;169:105–112.

30 Cruse JM, Keith JC, Bryant ML, Lewis RE Jr: Immune system-neuroendocrine dysregulation in spinal cord injury. Immunol Res 1996;15:306–314.

31 Okimura T, Ogawa M, Yamauti T, Sasaki Y: Stress and immune responses: IV. Adrenal involvement in the alteration of antibody responses in restraint-stressed mice. Jpn J Pharmacol 1986;41:237–245.

32 Borysenko M, Borysenko J: Stress, behavior, and immunity: Animal models and mediating mechanisms. Gen Hosp Psychiatry 1982;4:59–67.

33 Kventnansky R, Hukuhara K, Pacak K, Cizza G: Endogenous glucocorticoids restrain catecholamine synthesis and release at rest and during immobilization stress in rats. Endocrinology 1993;133:1411–1419.

34 Plotsky PM, Meaney MJ: Early, postnatal experience alters hypothalamic corticotrophin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Mol Brain Res 1993;18:195–200.

Biosci. Biotechnol. Biochem., **69** (1), 137–144, 2005

JSBA

Occurrence of Lewis a Epitope in *N*-Glycans of a Glycoallergen, Jun a 1, from Mountain Cedar (*Juniperus ashei*) Pollen

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Received August 27, 2004; Accepted November 5, 2004

We have determined the structures of *N*-glycans linked to major allergens in the mountain cedar (*Juniperus ashei*) pollen, Jun a 1. First, two kinds of the pollen glycoallergen (Jun a 1-A and Jun a 1-B) were purified from partially purified Jun a 1 by cation exchange chromatography. The *N*-glycans were liberated by hydrazinolysis from the two glycoallergens and the resulting sugar chains were *N*-acetylated and then coupled with 2-aminopyridine. Three pyridylaminated sugar chains were purified by reversed-phase HPLC and size-fractionation HPLC from Jun a 1-A and Jun a 1-B respectively. The structures were determined by a combination of *exo*- and *endo*-glycosidase digestions, two dimensional sugar chain mapping, and electrospray ionization mass spectrometry (ESI-MS) analysis. Structural analysis indicated that Lewis a epitope (Galβ1–3(Fucα1–4)GlcNAcβ1–) occurs in the *N*-glycans of the pollen allergens.

Key words: *N*-glycan; Lewis a epitope; pollen allergen; mountain cedar pollen; *Juniperus ashei*

Recently, it has been found that many allergens from pollens or other plant materials are *N*-glycosylated and that the *N*-glycans linked to such glycoallergens often belong to antigenic plant complex type structures consisting of β1–2 xylose and/or α1–3 fucose residues.[1–4] However, it is still obscure whether such antigenic *N*-glycans are directly involved in pollen or plant food allergies. In our previous report,[5] we found that the free plant complex type *N*-glycans ($GlcNAc_2$-$Man_3Xyl_1Fuc_1GlcNAc_2$, $Man_3Xyl_1Fuc_1GlcNAc_2$) did not inhibit the binding of a pollen glycoallergrn (Cry j 1) to IgEs from the pollinosis patients. This result suggested that antigenic *N*-glycans themselves must not be major epitopes for the antibodies but the antibodies may recognize a conformation constructed by the peptide moiety around the glycosylation site and the *N*-glycan. Furthermore, we have found that such antigenic *N*-glycan in free form can suppress the production of IL 4 from T-cells of pollinosis patients, which had previously been stimulated by the allergen. These observations suggest that the antigenic *N*-glycans of many pollen allergens can be a candidate of glyco-drugs for pollinosis therapy.[5]

Fichette-Lainè *et al.* first reported that Lewis a antigen unit (Galβ1–3(Fucα1–4)GlcNAcβ–) occurs in *N*-glycans of plant glycoproteins.[6] In fact, it has been reported that an allergen of Japanese cedar pollens, Cry j 1, also contains β-galactosyl and α-fucosyl residues at the non-reducing end of the biantennary plant complex type structure ($GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$),[3,4] but the structure of *N*-glycans linked to another pollen allergen, Jun a 1, from mountain cedar (*Juniperus ashei*) pollens remains to be determined. Midoro-Horiuti *et al.*, have cloned a cDNA of Jun a 1 and found that Jun a 1 possessed a high level of amino acid sequence homology with Cry j 1.[7] In this report, therefore, we analyzed the structures of *N*-glycans linked to Jun a 1 to confirm the ubiquitous occurrence of the Lewis a epitope in plant *N*-glycans. The structural analysis clearly indicated that the Lewis a epitope occurs in the *N*-glycan moiety of mountain cedar pollen allergen, Jun a 1.

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Abbreviations: PA-, pyridylamino; RP-HPLC, reverse-phase HPLC; SF-HPLC, size-fractionation HPLC; ESI-MS, electrospray ionization mass spectrometry; FPLC, fast protein liquid chromatography; Hex, hexose; HexNAc, *N*-acetylhexosamine; Deoxhex, deoxyhexose; Pen, pentose; M3FX, Manα1–6(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc-PA; MFX, Xylβ1–2Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc-PA; ^{GN}M3FX, GlcNAcβ1–2Manα1–6(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc-PA; GN2M3FX, GlcNAcβ1–2Manα1–6(GlcNAcβ1–2Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc-PA

Materials and Methods

Materials. An Asahipak NH2P-50 column (0.46 × 25 cm) was purchased from Showa Denko (Tokyo, Japan), and a Cosmosil 5C18-AR column (0.6 × 25 cm) from Nacalai Tesque (Kyoto, Japan). GN2M3FX and M3FX were prepared from the glycoproteins of oil palm pollens and ricin D.[8,9] ^{GN}M3FX was prepared from glycoproteins of oil palm pollens[9] and *Ginkgo biloba* pollens.[10] α-Mannosidase (Jack bean) and β1–3/6 specific β-galactosidase (recombinant expressed in *E. coli*) were purchased from Sigma (St. Louis, MO, U.S.A.). β-*N*-acetylglucosaminidase (*Diplococcus pneumonia*) was from Boehringer (Mannheim, Germany). α1–3/4 Specific α-fucosidase (*Streptomyces* sp. 142) and Lacto-*N*-biosidase (*Streptomyces* sp. 142) were from Takara (Kyoto, Japan).

Purification of Jun a 1. The partially purified Jun a 1, which was prepared by the method of Midoro-Horiuti *et al.*,[11] were further purified by a cation exchange FPLC using a Mono S column (Amersham Pharmacia Biotech, Upsala, Sweden). The absorbed proteins were eluted by a linear gradient of NaCl from 0 M to 0.5 M in 10 mM Na–acetate buffer, pH 4.0. Although two major components (fractions A and B) were separated on the column, as shown in Fig. 1-I, these components showed almost the same molecular weight (about 43 kDa) on SDS–PAGE (Fig. 1-II). Since these fractions were recognized by an antiserum against Jun a 1, for structural analysis of *N*-glycans, we used these two proteins (Jun a 1-A for fraction A and Jun a 1-B for fraction B) separately.

Preparation of pyridylaminated N-glycans from Jun a 1-A and Jun a 1-B. *N*-Glycans were released by hydrazinolysis (100 °C, 12 h, in 200 µl of anhydrous hydrazine) from the lyophilized glycoallergens (about 700 µg of Jun a 1-A and 980 µg of Jun a 1-B). After *N*-acetylation of the hydrazinolysate with saturated ammonium bicarbonate (400 µl) and acetic anhydride (20 µl), the acetylated hydrazinolysate was desalted using Dowex 50 × 2 resins. Pyridylamination of the sugar chains was done by the method of Natsuka and Hase.[12] Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus with a Jasco 821-FP Intelligent Spectrofluorometer, using the Shodex Asahipak NH2P-50 column (0.46 × 25 cm) and the Cosmosil 5C18-AR column (0.6 × 25 cm). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.05% TFA linearly from 0 to 10% at a flow rate of 1.2 ml/min. In the case of size-fractionation HPLC using the Asahipak NH2P-50 column, the PA-sugar chains were eluted by increasing the water content in the water–acetonitrile mixture from 36% to 62% linearly in 60 min at a flow rate of 0.7 ml/min.

Electrospray ionization (ESI) mass spectrometry. ESI-MS analysis of PA-sugar chains was done as described in our previous reports,[13,14] using a Perkin Elmer Sciex API-III triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source.

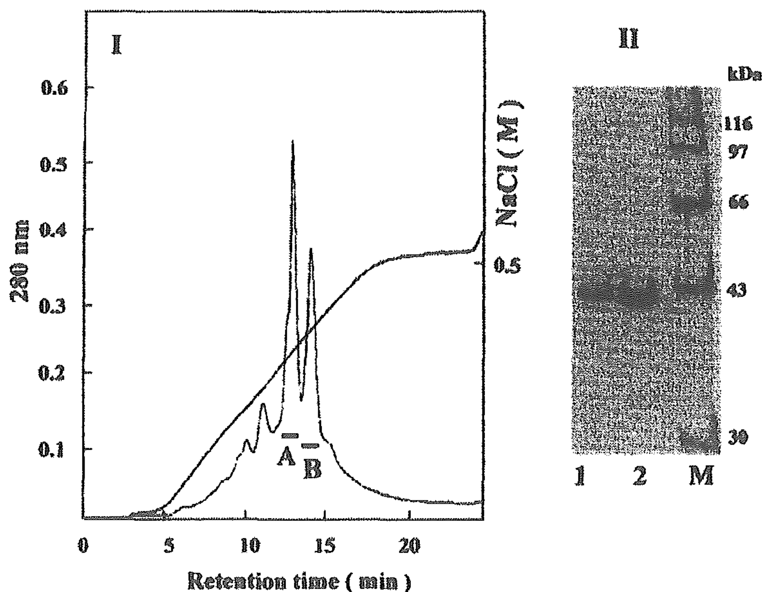

Fig. 1. Mono S-FPLC Profile of Partially Purified Jun a 1.

I, FPLC Profile of Partially Purified Jun a 1. The partially purified Jun a 1 was loaded on a Mono-S column in 10 mM Na–acetate buffer, pH 4.0. The allergen was eluted by a linear gradient of NaCl from 0 M to 0.5 M in 10 mM Na–acetate buffer, pH 4.0. II, SDS–PAGE of Fraction A (Jun a 1-A) and Fraction B (Jun a 1-B) under the reducing condition. Lane 1, Fraction A; Lane 2, Fraction B; M, marker proteins: 1, β-galactosidase (116 kDa); 2, phosphorylase b (97 kDa); 3, bovine serum albumin (BSA, 66 kDa); 4, ovalbumin (43 kDa); 5, carbonic anhydrase (30 kDa).

Glycosidase digestions of PA-sugar chains. Digestions with jack bean α-mannosidase, diplococcal β-*N*-acetylglucosaminidase, and Lacto-*N*-biosidase were done using about 200 pmol of the PA-sugar chains under the conditions described in our previous reports.[8–10] Digestion with the α-fucosidase was done using 200 pmol in 25 mM Na-citrate buffer, pH 5.0 for 16 h. The resulting glycosidase-digests were analyzed by SF-HPLC using the Asahipak NH2P-50 column (0.46 x 25 cm).

Results and Discussion

Purification of PA-sugar chains

First, the PA-sugar chains from Jun a 1-A and Jun a 1-B were partially purified by RP-HPLC as shown in Fig. 2-I. The PA-sugar chains were pooled as indicated by horizontal bars (-A and -B). When some other peaks observed on the chromatograms were analyzed by SF-HPLC, almost all peaks were recovered in the run-through fraction. Hence, we judged that these peaks were not *N*-glycans. As shown in Fig. 2-II, three PA-sugar chains (A, B, and C) from Jun a 1-A and three PA-sugar chains from (D, E, F) Jun a 1-B were purified on the Shodex Asahipak NH2P-50 column. The elution positions of A and E corresponded to those of GN2M3FX on RP-HPLC and SF-HPLC. Although the elution positions of B and F were the same as each other on RP-HPLC and SF-HPLC, they did not correspond to any available authentic PA-sugar chain. Furthermore, the elution positions of PA-sugar chain C on the RP-HPLC and SF-HPLC also did not correspond to any available authentic PA-sugar chain.

Structures of PA-sugar chains A and E

Since the elution positions of PA-sugar chains A and E corresponded to those of GN2M3FX on RP-HPLC and SF-HPLC, the structures of A and E could be estimated with the biantennary plant complex type structure, $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$. The ESI-MS analysis showed a single signal at m/z 836.5 $[(M + 2H)^{2+}]$, suggesting that these PA-sugar chains consist of $(HexNAc)_3(Hex)_3(Deoxyhex)_1(Pen)_1(HexNAc\text{-}PA)$ or $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA. The deduced structure was further confirmed by exoglycosidase digestion. These PA-sugar chains were converted to M3FX with diplococcal β-*N*-acetylglucosaminidase, suggesting that two GlcNAc residues were bound by β1–2 linkage (Fig. 3-II). The product was further converted to MFX by jack bean α-mannosidase diges-

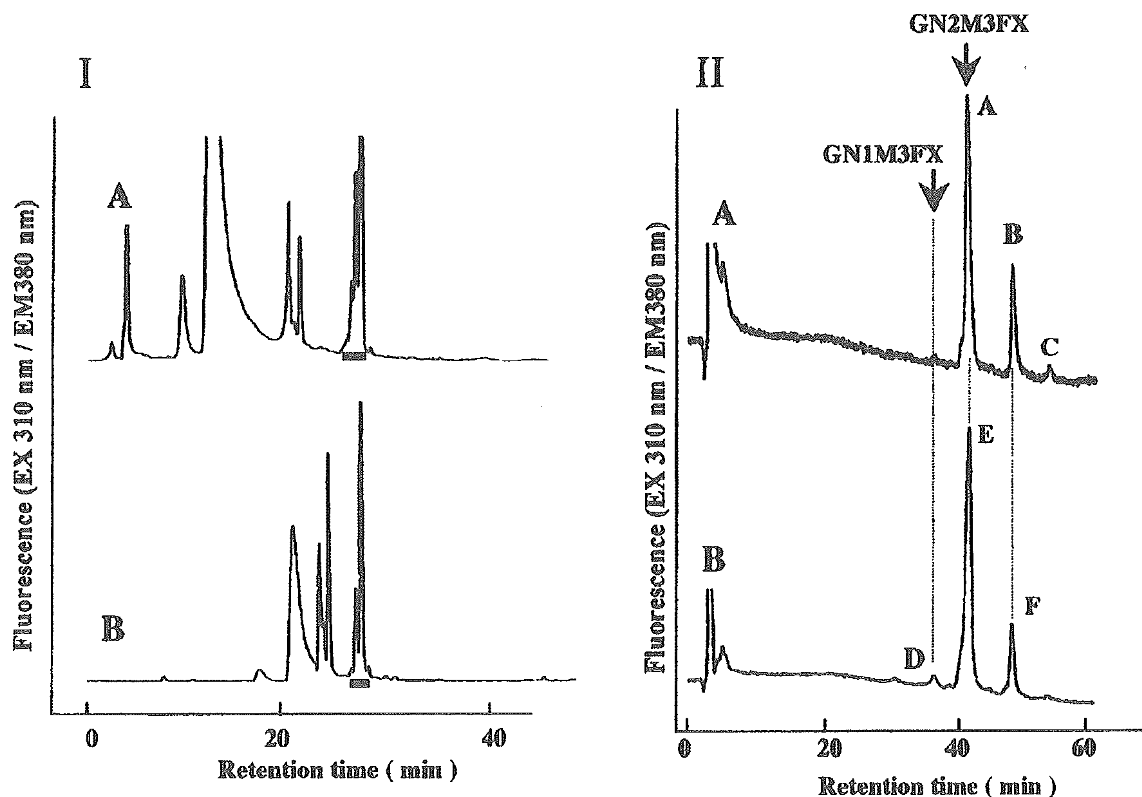

Fig. 2. HPLC Profiles of PA-Sugar Chains from Jun a 1-A and Jun a 1-B.

I, RP-HPLC of PA-sugar chains from Jun a 1-A (A) and Jun a 1-B (B). The PA-sugar chains were pooled as indicated by horizontal bars. II, SF-HPLC of PA-sugar chains pooled in I. A, PA-sugar chains from Jun a 1-A. B, PA-sugar chains from Jun a 1-B. GN2M3FX and GN1M3FX indicate the elution positions of authentic PA-sugar chains, $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$-PA (GN2M3FX) and $GlcNAc_1Man_3Xyl_1$-$Fuc_1GlcNAc_2$-PA (GN1M3FX) respectively.

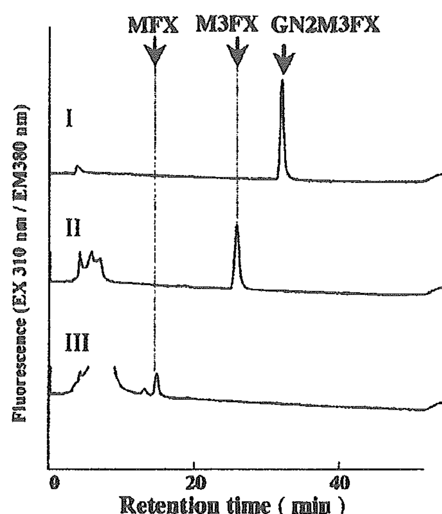

Fig. 3. SF-HPLC of Exoglycosidase Digests of PA-Sugar Chains A and E.

I, PA-sugar chains A and E; II, the diplococcal β-N-acetylglucosaminidase digest of I; III, the jack bean α-mannosidase digest of II. Arrows (GN2M3FX, M3FX, and MFX) indicate the elution positions of authentic PA-sugar chains, $GlcNAc_2Man_3Xyl_1Fuc_1$-$GlcNAc_2$-PA, $Man_3Xyl_1Fuc_1GlcNAc_2$-PA, and $Man_1Xyl_1Fuc_1Glc$-NAc_2-PA respectively.

tion (Fig. 3-III). The data of 2-D sugar chain mapping, exoglycosidase digestion, and ESI-MS analysis suggested that the structure of PA-sugar chains A and E are GN2M3FX as shown in Scheme 1.

Structures of PA-sugar chains B and F

ESI-MS analysis showed a single signal at m/z 990.5 [$(M + 2H)^{2+}$], suggesting that these PA-sugar chains consist of $(Hex)_4(HexNAc)_3(Deoxyhex)_2(Pen)_1$(HexNAc-PA) or $Gal_1Fuc_1GlcNAc_2Man_3Xyl_1Fuc_1Glc$-$NAc_2$-PA (Fig. 4-I). As shown in Fig. 5-II, the PA-sugar chains could be digested with the α1–3/4 fucosidase, suggesting that the fucosyl residue is bound by α1–3 or α1–4 linkage. When the product was treated with diplococcal β-galactosidase, galactosyl residue was not released (Fig. 5-III). On the contrary, the α-fucosidase digest of PA-sugar chains B and F was digested by the β1–3/6 specific galactosidase, as shown in Fig. 5-IV, and the elution position of the product corresponded to that of GN2M3FX. These results indicated that the galactosyl residue was bound to the GlcNAc residue by β1–3 or β1–6 linkage. The de-galactosylated product was converted to M3FX by diplococcal β-N-acetylglucosaminidase digestion (Fig. 5-V), suggesting that two GlcNAc residues were bound to the M3FX structure by β1–2 linkage. To determine the linkage mode of the galactosyl residue, the product obtained by the α-fucosidase digestion of PA-sugar chain B and F (Fig. 5-II) was treated with lacto N-biosidase, which released the Galβ1–3GlcNAc unit but not the Galβ1–4GlcNAc unit from the complex type N-glycans.[15] As shown in Fig. 5-VI, the product obtained by endoglycosidase digestion was eluted at the elution position of GN1M3FX,[10] indicating that the Galβ1–3GlcNAc unit was released by endoglycosidase. From these results of *exo*- and *endo*-glycosidase digestions, it has been found that the fucosyl and galactosyl

Proposed Structures	Peak Name	Jun a 1-A	Jun a 1-B
GlcNAcβ1-2 Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-3 2 3 Xylβ1 Fuc α1	D	nd	3%
GlcNAcβ1-2 Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2 Manα1-3 2 3 Xylβ1 Fucα1	A E	75%	76%
Fucα1-4 { GlcNAcβ1-2Manα1-6 Galβ1-3 { Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1-3 2 3 Xylβ1 Fucα1	B F	23%	21%
Fucα1 4 Galβ1-3GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-3GlcNAcβ1-2Manα1-3 2 3 4 Xylβ1 Fucα1 Fucα1	C	2%	nd

nd: not detected.

Scheme 1. The Proposed Structures of N-Glycans Linked to Jun a 1-A and Jun a 1-B.

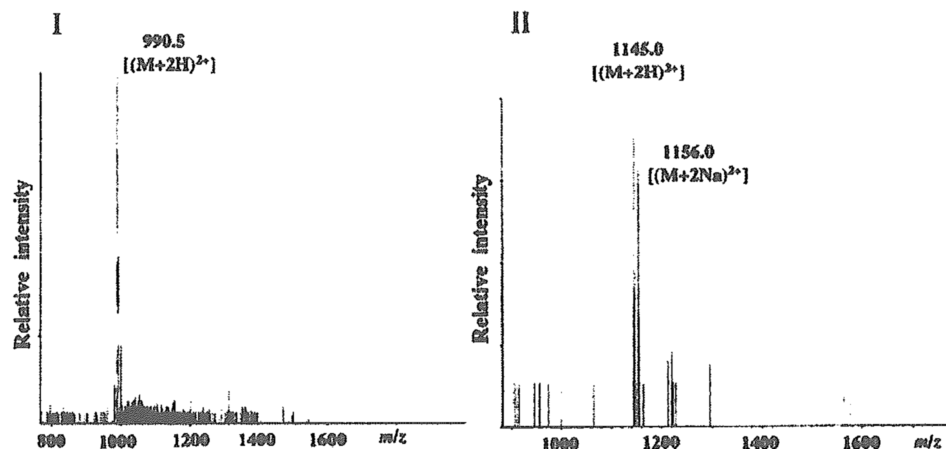

Fig. 4. ESI-MS Spectra of PA-Sugar Chains B and C.
PA-Sugar chains B and C were observed as a double charged ion, $[M+2H]^{2+}$. A sodium adduct ion ($[M+2Na]^{2+}$) was observed for PA-sugar chain C.

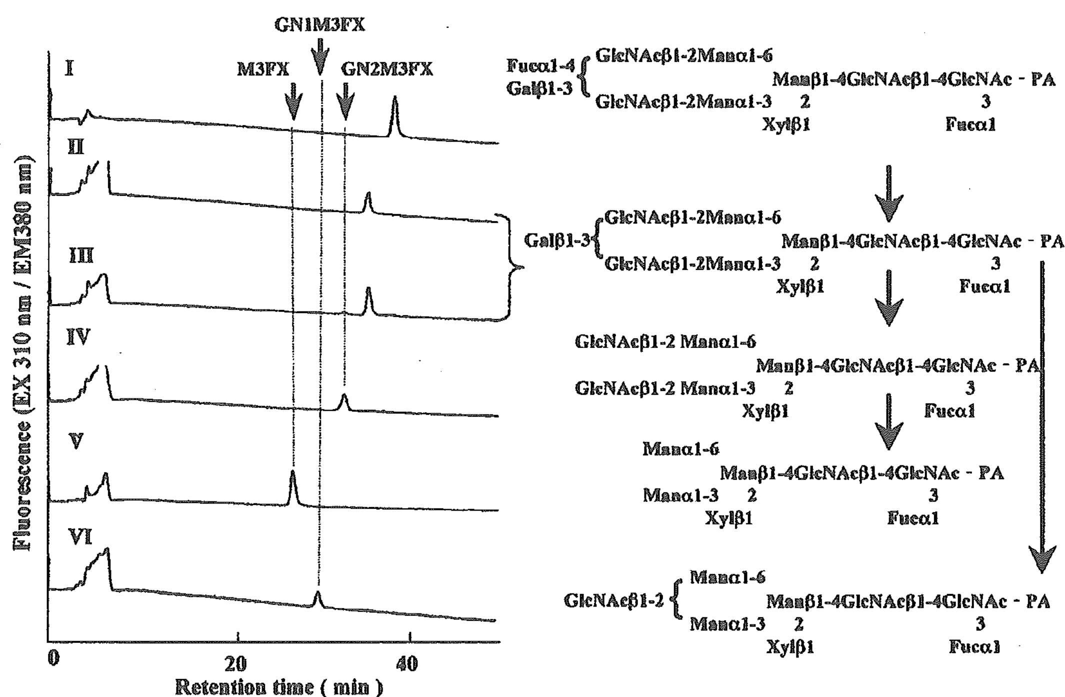

Fig. 5. SF-HPLC of *Exo*- and *Endo*-Glycosidase Digests of PA-Sugar Chains B and E.
I, PA-sugar chains B and E; II, the α1–3/4 specific *Streptomyces* α-fucosidase digest of I; III, the diplococcal β-galactosidase digest of II; IV, the β1–3/6 specific galctosidase digest of III; V, the diplococcal β-*N*-acetylglucosaminidase digest of IV; VI, the lacto *N*-biosidase digest of II.

residues were bound to the outer GlcNAc residue by α1–4 linkage and β1–3 linkage respectively. However, only with these results, it appears no to be enough to determine which mannosyl residue (α1–6 arm Man or α1–3 arm Man) harbors the Lewis a epitope (Galβ1–3 (Fucα1–4)GlcNAcβ1–). Therefore, in Scheme 1, a possible structure of PA-sugar chains B and F is shown.

Structures of PA-sugar chain C

ESI-MS analysis showed two signals at m/z 1145.0 $[(M+2H)^{2+}]$ and 1156 $[(M+2Na)^{2+}]$, suggesting that these PA-sugar chains consist of $(Hex)_5(HexNAc)_3$-$(Deoxyhex)_3(Pen)_1(HexNAc\text{-}PA)$ or $Gal_2Fuc_2GlcNAc_2$-$Man_3Xyl_1Fuc_1GlcNAc_2$-PA (Fig. 4-[II]). When this sugar chain was treated with α1–3/4 fucosidase, fucosyl residues were released (Fig. 6-II), suggesting that the fucosyl residue(s) were bound to the outer GlcNAc residue by α1–3 or α1–4 linkage. When the α-fucosidase digest was treated with diplococcal β-galactosidase, the galactosyl residue was not released (Fig. 6-III). On the

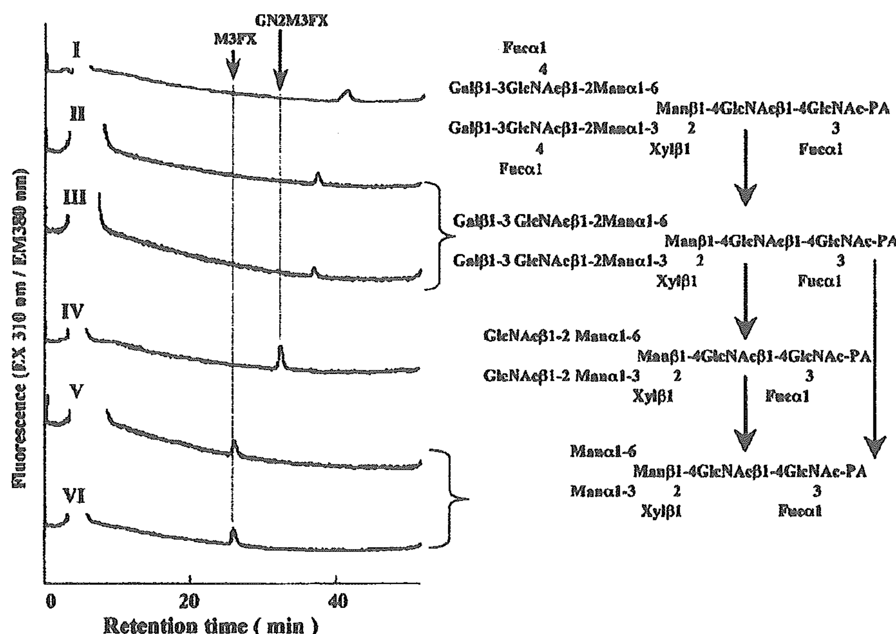

Fig. 6. SF-HPLC of *Exo*- and *Endo*-Glycosidase Digests of PA-Sugar Chain C.
I, PA-sugar chain C; II, the α1–3/4 specific *Streptomyces* α-fucosidase digest of I; III, the diplococcal β-galactosidase digest of II; IV, the β1–3/6 specific galctosidase digest of III; V, the diplococcal β-*N*-acetylglucosaminidase digest of IV; VI, the lacto *N*-biosidase digest of II.

contrary, the galactosyl residue(s) were released from the α-fucosidase digest (Fig. 6-II) by β1–3/6 specific galactosidase digestion, as shown in Fig. 6-IV, and the elution position of the product corresponded to that of GN2M3FX, suggesting that two galactosyl residues were released. These results indicated that the galactosyl residues were bound to GlcNAc residue by β1–3 or β1–6 linkage. The de-galactosylated product was converted to M3FX by diplococcal β-*N*-acetylglucosaminidase digestion (Fig. 6-V), suggesting that two GlcNAc residues were bound to the M3FX structure by β1–2 linkage. To determine the linkage mode of the galactosyl residue, the product obtained by α-fucosidase digestion (Fig. 6-II) was treated with lacto *N*-biosidase. As shown in Fig. 6-VI, the product obtained by the endoglycosidase digestion was eluted at the elution position M3FX, indicating that two Galβ1–3GlcNAc units were released by the endoglycosidase. From these results of *exo*- and *endo*-glycosidase digestions, the structure of PA-sugar chain C is proposed as shown in Scheme 1, indicating that two units of Lewis a epitope occur in the *N*-glycan.

Structure of PA-sugar chain D

Concerning the structure of D, GlcNAc1Man3Xyl1-Fuc1GlcNAc2-PA (GN1M3FX) structure occurs in two isomers; one is Manα1–6(GlcNAcβ1–2Manα1–3)-(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc-PA (^{GN}M3FX), and the other is GlcNAcβ1–2Manα1–6-(Manα1–3)(Xylβ1–2)Manβ1–4GlcNAcβ1–4(Fucα1–3)-GlcNAc-PA (^{GN}M3FX). It has been found that on the ODS column the former structure is eluted before GN2M3FX, and the latter structure is eluted after GN2M3FX. The PA-sugar chain D was eluted slightly after GN2M3FX (PA-sugar chain B) on the ODS column and the elution position of PA-sugar chain D on RP-and SF-HPLC corresponded to those of authentic ^{GN}M3FX (data not shown).[10] When this PA-sugar chain was treated with diplococcal β-*N*-acetylglucosaminidase, one GlcNAc residue was released (Fig. 7-II). The product was further converted to MFX by jack bean α-mannosidase digestion (Fig. 7-III). From these results, the structure of PA-sugar chain D is proposed as shown in Scheme 1.

Structural feature of N-glycans linked to the mountain cedar pollen allergen, Jun a 1

As shown in Scheme 1, we have found that the cedar pollen allergen, Jun a 1, carries plant complex type *N*-glycans containing the Lewis a antigen (Galβ1–3(Fucα1–4)GlcNAcβ1–) unit. Although we have purified two isomers of Jun a 1 (Jun a 1-A and Jun a 1-B) by the cation exchange HPLC, we confirmed that both Jun a 1 molecules harbored Lewis a epitope. In contrast, the complex type *N*-glycans having two Lewis a epitope units (PA-sugar chain C) was found only in Jun a 1-A, and the GN1M3FX structure (PA-sugar chain D) was found only in Jun a 1-B respectively. These observation suggest that the composition of *N*-glycans of Jun a 1-A and Jun a 1-B might be slightly different from each other. Although the elution position of Jun a 1-A on the cation exchange column was different from that of Jun a 1-B (suggesting that the number of glycosylation sites might be different from each other), the molecular