

Identification of β -Tubulin Isoform V as an Autoantigen in Allergic Rhinitis by a Proteomic Approach

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Abstract: Autoantibodies to IgE and β 2-adrenergic receptor have been reported in patients with allergic rhinitis. To investigate whether autoimmunity in allergic rhinitis is directed to such limited molecules or directed to a wide range of self proteins, we here attempted to survey autoantigens/autoantibodies comprehensively, using proteomics. Specifically, we separated proteins extracted from peripheral blood mononuclear cells by 2-dimensional electrophoresis and then detected autoantigens by subsequent western blotting with sera from patients with allergic rhinitis. As a result, we detected multiple autoantigens, some of which were further identified by mass fingerprinting. Next, we confirmed antigenicity of one of the identified autoantigens, β -tubulin isoform V (β -tubV), using a recombinant protein and then measured prevalence of the anti- β -tubV autoantibodies. As a result, 52% of the tested patients with allergic rhinitis were found to possess anti- β -tubV autoantibodies. Our study indicates that autoimmunity is a common phenomena and β -tubV is one of the major autoantigens in allergic rhinitis.

Key words: β -Tubulin, Autoantigen, Allergic rhinitis, Proteomics

Type I allergic rhinitis, an inflammatory condition of upper airways, often impairs quality of life of sufferers, even though it is not a life-threatening disease (25). For example, the disease results in decreased productivity and potential absenteeism from work or schooling (2). Prevalence of allergic rhinitis appears to be increasing. Possible factors for this would be increases in airborne pollution, increases in dust mite populations, changes in living and dietary conditions, control of infectious diseases and vaccinations (4, 15, 22, 23).

On pathogenesis of allergic rhinitis, major interest has been centered on immunological disorders of massive production of allergen-specific IgE antibodies and pro-allergic mediators including histamine (9, 15). However, the role of autoimmunity has been also implicated recently (16, 21, 24, 26, 27). Specifically, autoantibodies to IgE and β 2-adrenergic receptors were reported in patients with allergic rhinitis (24, 27). Possible pathological roles of these autoantibodies have not been con-

firmed yet; rather, it remains even to be resolved whether autoimmunity in allergic rhinitis is directed selectively to IgE and/or β 2-adrenergic receptors or directed to a wide range of self proteins. To address this question here we surveyed autoantibodies/autoantigens in allergic rhinitis using a proteomic approach. Specifically, proteins extracted from human peripheral blood mononuclear cells (PBMC) were separated by two-dimensional electrophoresis (2DE), and then were subjected to western blotting (WB) with serum samples from patients with allergic rhinitis. Then detected autoantigens were identified by mass fingerprinting. One of the identified proteins was found to be β -tubulin isoform V (β -tubV), which was found to be recognized as an autoantigen in 52% of the tested patients with allergic rhinitis by WB using recombinant β -tubV.

Abbreviations: AR, allergic rhinitis; BPB, bromophenol blue; 2DE, 2-dimensional electrophoresis; IEF, isoelectric focusing; MBP, maltose binding protein; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PBMC, peripheral blood mononuclear cells; PDI, protein disulfate isomerase; RAST, radioallergosorbent test; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; β -tubV, β -tubulin isoform V; WB, western blotting.

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Materials and Methods

Patients. Twenty-five patients with allergic rhinitis (mean age 29.8, 13 males and 12 females) were enrolled in this study. All of the subjects fulfilled the following 6 criteria for allergic rhinitis: 1. Presence of nasal obstruction, rhinorrhoea and sneezing for at least 1 hr per day for the last 3 months. 2. Positive radioallergosorbent test (RAST) to aeroallergens by a score of $2 <$. 3. No previous nasal or sinus surgery. 4. No pregnancy or possible drug-induced rhinitis. 5. Normal sinus X-ray appearance. 6. No systemic treatment with steroids. Serum samples were obtained from the patients in April, May, or June. In RAST, all the patients here were positive for at least one of the four tested common aeroallergens of house-dust, mite, cedar pollen, and hogweed/ragweed pollen as shown in Table 1. Further, they did not show disease conditions other than allergic rhinitis. Serum samples from age- and sex-matched healthy donors, who were confirmed to be negative in RAST for the above allergens with no clinical symptoms, were used as a control. Scoring of severity in allergic rhinitis was made by the criteria reported previously (14). This research was carried out in accordance with the human experimentation guidelines of our institution and the Helsinki Declaration.

Two-dimensional electrophoresis (2DE) and western blotting (WB). Mononuclear cells were separated from peripheral blood of healthy volunteers by gradient. The cell pellet, re-suspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS), was frozen in liquid nitrogen and thawed at 37 C five times. After centrifugation at 4 C for 30 min, the supernatant was collected and its protein concentration was determined using the Bradford method. The supernatant was stored at -80 C until use.

The extracted proteins were diluted in a rehydration buffer (8 M urea, 2% CHAPS, 2.8 mg/ml DTT, trace of bromophenol blue (BPB)) containing 0.5% immobilized pH gradient (IPG) buffer (pH range 3–10; Amersham Pharmacia Biotech, Sweden) and loaded onto 7 cm Immobiline Drystrips (Amersham Pharmacia Biotech) in the IPG reswelling tray (Amersham Pharmacia Biotech) at room temperature overnight as described elsewhere (20). Up to 400 μ g of the extracted proteins was applied onto the drystrip gels for western blotting and up to 1,000 μ g for the analysis by MS. Isoelectric focusing (IEF) was performed in a horizontal electrofocusing apparatus (MultiPhor II; Pharmacia Biotech) according to the manufacturer's instructions. After IEF, the IPG strips were equilibrated in the first equilibration solution (10 mg dithiothreitol per 1 ml) sodium dodecyl sulfate

(SDS) equilibration buffer (1.5 M Tris-Cl, pH 8.8; 6 M urea, 30% glycerol, 2% SDS), and then in the second equilibration solution (25 mg iodoacetamide per 1 ml SDS equilibration buffer). The equilibrated strips were placed on top of 12.5% SDS polyacrylamide gel electrophoresis (PAGE) slab gels and sealed with 0.5% lower melt gel, and then the second-electrophoresis was performed with 40 mA constant current in separating gel at 20 C.

After the electrophoresis, the SDS-PAGE gels were stained with Coomassie Brilliant Blue (CBB) or used for protein transfer onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany).

In-gel digestion, mass determination and mass fingerprinting. Protein spots on the gel stained with CBB, which corresponded to the positive spots on the WB membranes, were recovered. The recovered gel fragments were washed in double distilled water for 15 min, de-colored in 50 μ l de-coloring solution (0.1 M ammonium hydrogen carbonate, 50% methanol) at 40 C for 15 min, and cut into small pieces. The gel pieces were re-hydrated in 20 μ l trypsin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (0.1 pmol/ μ l trypsin in 50 mM Tris-HCl) and incubated at 37 C. The digested peptides were extracted from the gel pieces using trifluoroacetic acid (TFA) and acetonitrile. Specifically, the digested products were supplemented with 50 μ l of 0.1% TFA/50% acetonitrile, vortexed, and sonicated for 10 min. After centrifugation, the supernatant was recovered. After two more cycles of this extraction, a similar extraction was performed with 50 μ l of 0.1% TFA/80% acetonitrile. The collected supernatant was centrifuged again, filtered, and concentrated down to 50 μ l in an evaporator. The peptide sample solution was stored at -20 C until mass spectrometry analysis. The masses of the digested peptides in the samples were determined using mass spectrometer (LCQ Advantage, Thermoquest Inc.). A list of the determined peptide masses was compiled for the mass fingerprinting using the Mascot software program (Matrix Science Ltd., London, U.K.), in which the NCBI protein databases were searched.

Preparation of recombinant proteins. According to the reported nucleotide sequence of β -tubulin isoform V, we prepared two DNA primers to amplify the cDNA fragment that encoded the entire protein coding region of β -tubulin V by PCR. The nucleotide sequences of the two primers are as follows:

The amplified cDNA fragment was subcloned into a plasmid expression vector of pMAL-eHis, a derivative from pMAL-c2 (New England Biolabs, Inc.). Using this construct, recombinant β -tubulin V proteins was produced in *Escherichia coli* (DH5 α) (Toyobo Co., Ltd.,

Osaka, Japan) as a fusion protein with maltose binding protein (MBP). The recombinant protein was purified from the bacterial cell lysate according to the histidine- Ni^{+} affinity purification as described previously (13).

Western blotting (WB). Western blotting was performed as follows. In 1DE-WB, 5 μg of a purified fusion protein or MBP as a control was separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a nitrocellulose membrane. Proteins separated by 2DE as described above were also transferred onto a nitrocellulose membrane for WB. After blocking with PBS containing 1% BSA and 0.1% Tween 20 for 2 hr and washing in PBS with 0.1% Tween 20 for 5 min 3 times, the membrane was then incubated with each serum sample for 2 hr. The serum sample was diluted to 1:100 with PBS containing 1% BSA and 0.1% Tween 20, and was incubated with 2,000 $\mu\text{g}/\text{ml}$ of bacterial lysate and purified proteins containing non-recombinant pMAL-eHis products for 2 hr at room temperature in advance. The membrane was then washed 5 times in PBS with 0.1% Tween 20, and the bound antibodies were reacted with horseradish peroxidase-conjugated goat anti-human IgG (Zymed Laboratories, San Francisco, Calif., U.S.A.)

diluted to 1:3,000 with PBS containing 1% BSA and 0.1% Tween 20 for 1 hr. The bound antibodies were visualized with diaminobenzidine.

Results

Detection of Self Proteins Reactive to Sera from Patients with Allergic Rhinitis

We first tried to understand the overall profiles of autoimmunity in patients with allergic rhinitis. To achieve this aim, we detected autoantibodies/autoantigens by means of 2DE and subsequent WB using 6 serum samples from 3 patients with allergic rhinitis and 3 healthy donors. The 3 patients (no. 1–3 in Table 1) were randomly selected from the patients who were positive for all 4 tested allergens in RAST. Proteins extracted from human PBMC were separated by 2DE, by which the proteins migrated into their specific positions according to their unique pI values and molecular weights (Fig. 1a). Next the separated proteins were transferred onto membranes and then were reacted with each of the 6 serum samples by WB. Results from the analysis of the 3 patients with allergic rhinitis and a representative result of the analysis from the healthy donors are shown

Table 1. Profiles of patients with allergic rhinitis enrolled in this study

Pt. no.	Age	Sex	Severity	HD	Mite	Cedar	Ragweed/ hogweed
1	19	M	1+	+	+	+	+
2	43	F	2+	+	+	+	+
3	23	M	1+	+	+	+	+
4	26	M	2+	—	—	+	—
5	23	F	3+	+	+	+	—
6	61	F	1+	+	+	+	—
7	34	F	2+	—	—	+	—
8	6	F	4+	+	+	+	—
9	8	F	2+	—	—	+	—
10	44	F	1+	+	+	—	—
11	55	F	2+	—	—	+	—
12	35	F	1+	—	—	+	—
13	55	M	1+	—	—	+	—
14	24	M	1+	+	+	+	—
15	31	F	2+	+	+	+	—
16	44	M	2+	+	+	+	+
17	27	M	1+	+	+	+	+
18	24	F	1+	+	+	+	—
19	24	M	1+	—	—	+	—
20	21	M	1+	+	+	+	—
21	24	M	1+	+	+	—	—
22	24	F	1+	+	+	+	—
23	25	M	1+	+	+	+	—
24	22	M	1+	—	—	+	+
25	23	M	1+	—	—	+	—

Pt. no.: patient number, HD: house dust. Severity of allergic rhinitis was scored by the criteria reported previously (14).

in Fig. 1, b–d and e–g, respectively. We found 23 protein spots reactive to at least one of the serum samples from the patients with allergic rhinitis. Eleven out of the 23 protein spots were not reactive to any of the healthy serum samples. The positions of the 11 spots on the 2DE gel are shown in Fig. 1h and their reactivity is summarized in Table 2. The detection of multiple autoantigens here indicates that autoimmunity is a common phenomenon directed toward various self proteins in allergic rhinitis.

Identification of the Detected Autoantigens

We next tried to identify the 11 proteins by mass-fingerprinting. Specifically, peptides extracted from the corresponding gel spots after digestion with trypsin, were subjected to mass measurement and computer searching. We thus successfully identified 5 out of the 11

protein spots (spot no. 3, 4, 5, 6, and 22). Names, MW, and pI values of the identified proteins and a representative data set of the mass fingerprinting are shown in Table 3. The protein of spot no. 5 was β -tubulin isoform V (β -tubV) with an estimated molecular weight of 49.6 kDa and a pI value of 4.8. Since β -tubV was recognized by 2 out of the 3 tested serum samples from the patients with allergic rhinitis, we next focused on β -tubV for a further analysis.

Frequency of the Autoantibodies against β -TubV in Patients with Allergic Rhinitis

To confirm antigenicity of β -tubV, we prepared a recombinant β -tubV protein as a fusion protein with MBP (β -tubV-MBP, Fig. 2). We separated the purified β -tubV-MBP as well as MBP alone as a control by 1D SDS-PAGE and then tested reactivity to the serum sam-

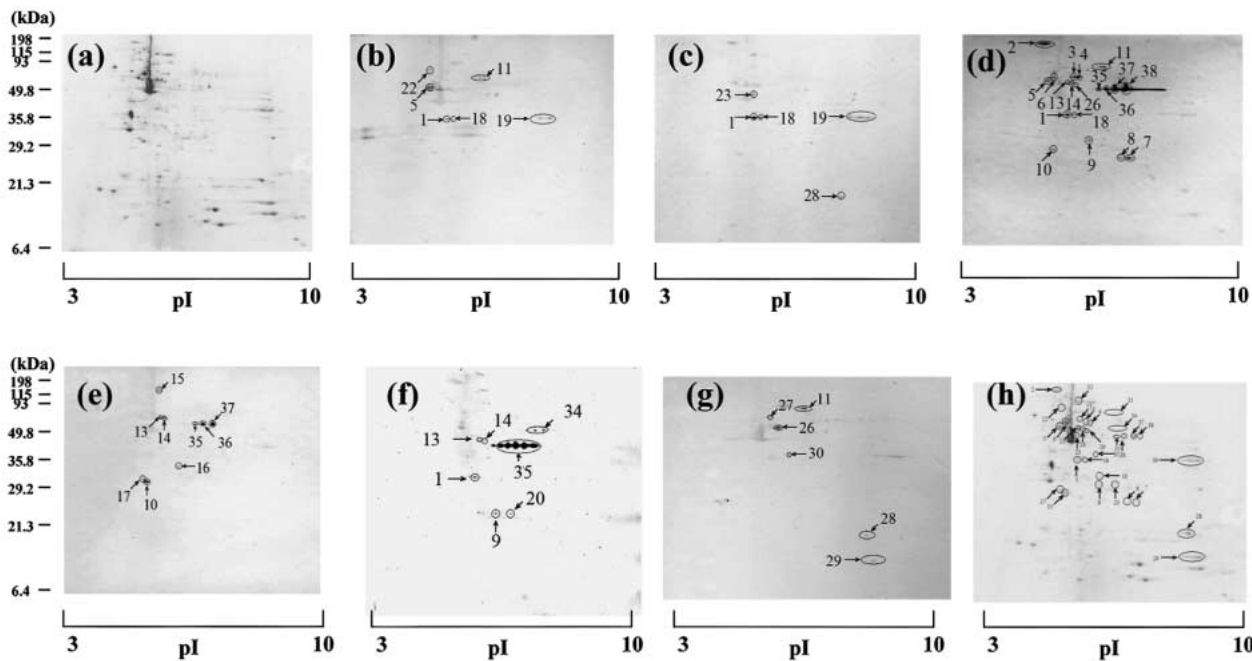


Fig. 1. Detection of autoantigens in patients with allergic rhinitis by 2DE and western blotting. Total proteins extracted from PBMC were separated by 2DE and visualized by Coomassie Brilliant Blue (a) or were transferred onto nitrocellulose membranes for western blotting after 2DE separation. In the western blotting, the proteins were reacted with each of six serum samples from 3 patients with allergic rhinitis and 3 healthy donors. All the results of western blotting from the 3 patients and 3 healthy donors are shown in (b)–(d) and (e)–(g), respectively. Location of all the positive spots on western blotting is identified on the 2DE gel (h).

Table 2. Protein spots that reacted to serum samples from patients with allergic rhinitis

Pt. no.	Spot no.										
	2	3	4	5	6	7	8	18	19	22	23
1	–	–	–	+	–	–	–	+	+	+	–
2	–	–	–	–	–	–	–	+	+	–	+
3	+	+	+	+	+	+	+	+	–	–	–
Healthy1–3	–	–	–	–	–	–	–	–	–	–	+

Table 3. Autoantigens identified by mass-fingerprinting

No.	Protein	pI/MW
3, 4	Protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor [NCBI S55507]	6.1/56.7
5	Tubulin β 5 [NCBI NP_006078]	4.8/49.6
6	Vimentin [NCBI XP_042950]	5.0/53.7
22	DnaK-type molecular chaperone HSPA5 precursor [NCBI A29821]	5.0/72.1

pI: isoelectric point, MW: molecular weight (kDa).

ples used in the above screening by 2DE-WB. As a result, the serum sample of AR3, which positively reacted to the protein spot no. 5, reacted to β-tubV-MBP but not to MBP alone (Fig. 3). This confirmed that protein spot no. 5 was β-tubV. We determined frequency of the autoantibodies to β-tubV using the same recombinant proteins in further numbers of patients with allergic rhinitis by WB. We tested 25 serum samples from patients with allergic rhinitis and 10 from healthy donors by WB. As a result, IgG-type autoantibodies to β-tubV-MBP were detected in 13 (52%) out of the 25 tested patients with allergic rhinitis and none out of the 10 healthy donors, respectively. Representative results of WB are shown in Fig. 3. This indicates that β-tubV is one of the major autoantigens in allergic rhinitis.

Comparison of Clinical Parameters between the Anti-β-tubV Antibody-Positive and -Negative Patients with Allergic Rhinitis

Finally we compared clinical parameters between the anti-β-tubV antibody-positive and -negative group. As summarized in Table 4, the mean age and disease duration showed no significant difference between the two groups. On the RAST values, the anti-β-tubV antibody-positive groups showed lower titers against all four of the tested common aeroallergens of house-dust, mite, cedar pollen, and hogweed/ragweed pollen, although these differences did not meet statistically significant values.

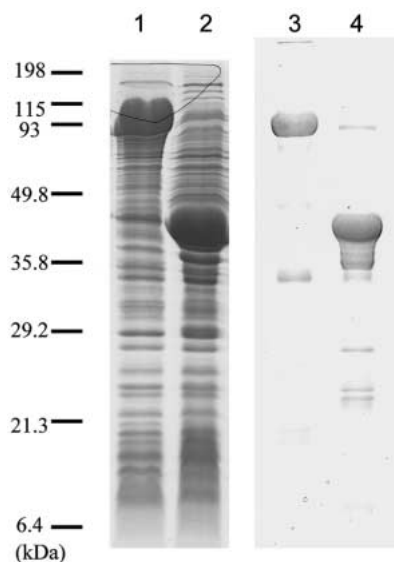


Fig. 2. Preparation of β-tubV as a recombinant MBP fusion protein. cDNA for the entire protein-coding region of human β-tubV was amplified by RT-PCR and then was inserted into pMAL-cHis. The full-length β-tubV was produced as a recombinant MBP fusion protein (β-tubV-MBP) in *E. coli* and purified by using Histidine-N+ affinity (Lane 1; produced β-tubV-MBP before purification, lane 2; produced MBP alone before purification, lane 3; β-tubV-MBP after purification, and lane 4; MBP alone after purification).

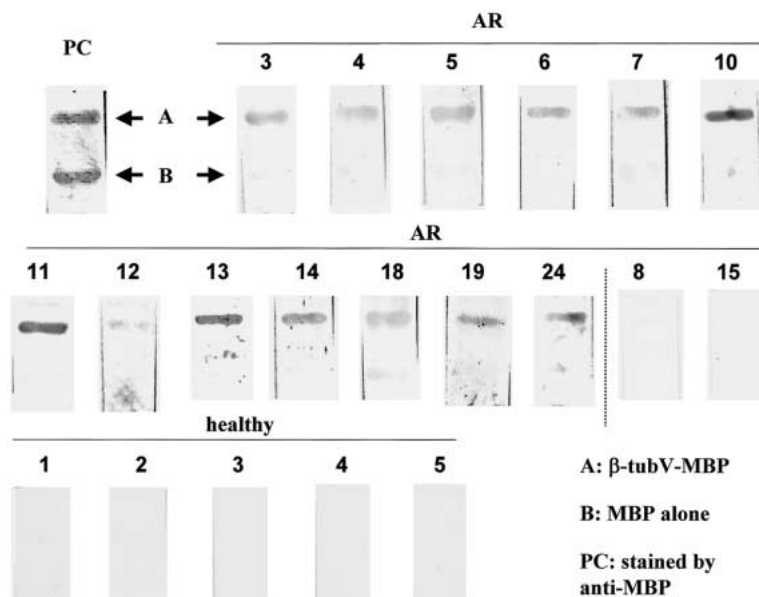


Fig. 3. Western blotting of β-tubV with serum samples from patients with allergic rhinitis. The same amounts of β-tubV-MBP and MBP alone as a negative control, applied into the same lanes, were separated by 12.5% SDS-PAGE and then were transferred onto nitrocellulose membranes. The separated proteins were reacted with serum samples from 25 patients with allergic rhinitis and 10 healthy donors. Results from all 13 serum samples that were positive for the anti-β-tubV antibody, 3 representative results from the antibody-negative patients, and 5 representative results from 10 healthy donors are shown (AR: allergic rhinitis, PC: positive control in which both of β-tubV-MBP and MBP alone were stained with anti-MBP antibodies).

Table 4. Clinical parameters of anti- β -tubV-positive and -negative patients

Parameters	Anti- β -tubV		Significance
	Positive (<i>n</i> = 13)	Negative (<i>n</i> = 12)	
Age (years)	29.8 $\pm$ 16.2	29.8 $\pm$ 11.1	ns
Sex (M:F)	5:8	8:4	ns
Disease duration (years)	4.7 $\pm$ 3.9	3.0 $\pm$ 2.6	ns
RAST/house dust	33.1 $\pm$ 42.2	60.6 $\pm$ 75.1	ns
RAST/mite	11.9 $\pm$ 18.6	19.7 $\pm$ 28.3	ns
RAST/cedar	12.4 $\pm$ 24.6	22.3 $\pm$ 32.4	ns
RAST/ragweed/ hogweed	9.1 $\pm$ 11.4	24.9 $\pm$ 32.4	ns

Ages, disease duration, and RAST titers are shown as mean $\pm$ standard deviation. ns: not significant.

Table 5. RAST for the tested 4 allergens in the anti- β -tubV-positive and -negative patients

RAST		Anti- β -tubV	
		+	-
House dust	+	8	8
	-	5	4
Mite	+	8	8
	-	5	4
Cedar	+	12	11
	-	1	1
Ragweed/hogweed	+	2	4
	-	11	8

The female patients tended to possess the anti- β -tubV antibody more frequently, but this was not statistically significant either. In addition, we compared anti- β -tubV antibody positivity between RAST- positive and -negative patients in each of the tested four allergens, but did not detect correlations (Table 5).

Discussion

Recently, autoantibodies to IgE and β 2-adrenergic receptors have been reported in patients with allergic rhinitis. However, little information has been available on autoimmunity directed to other molecules. Therefore, we used a proteomic approach, a combination of 2DE-WB and the subsequent mass fingerprinting, which has been advanced quite recently. Further, the antigenicity of a detected and identified protein was confirmed by a classical method, use of a recombinant protein. This strategy provided us with successful results.

In the first screening of 2DE-WB using serum samples from patients with allergic rhinitis and from healthy controls, 11 protein spots were detected in the allergic rhinitis group but not in the healthy group. But there were found no protein spots that were positive only in the

healthy group. Thus the 11 spots would be candidates for autoantigens in allergic rhinitis. Screening using only three serum samples from patients with allergic rhinitis provided us the 11 autoantigen candidates, indicating that autoimmunity is a common phenomenon in allergic rhinitis.

In the next step of mass fingerprinting, we were able to identify five protein spots out of the 11 spots that were antigenic. Among the five proteins, the proteins of spot no. 3 and 4 were found to be identical, protein disulfate isomerase (PDI) ER60 precursor. Since the spots of no. 3 and no. 4 were located on the 2DE gel with apparently equal molecular weights but with slightly different pI values, the two spots would represent PDI ER60 precursors with different modifications. In the previous report, autoantibodies to PDI were detected in about half of patients with alcoholic liver diseases (16) and also in a spontaneous hepatitis model of Long Evans Cinnamon (LEC) rats (28). Further, PDI was reported to be a target of autoantibodies in systemic lupus erythematosus (16) and in anti-sperm sera from patients with infertility (3). Our data indicates that PDI would be an autoantigen in allergic rhinitis too, even though prevalence of the autoantibodies to PDI has not been determined yet.

Protein spot no. 5 was identified as β -tubV. Since 2 out of the 3 tested serum samples from patients with allergic rhinitis reacted to this spot, we further investigated autoantibodies to β -tubV by preparing a recombinant protein. We demonstrated that 52% of the tested patients with allergic rhinitis were found to possess autoantibodies to β -tubV. The anti- β -tubulin autoantibodies have been reported previously in sera of patients with acute and chronic acquired demyelinating polyneuropathies (5), type I diabetes (19), thyroiditis (11, 18), SLE (1, 6), viral and parasitic infections (8), neuroectodermal tumors (17), and nasopharyngeal carcinomas (10). However, autoantibodies to the isoform V of β -tubulin were identified here for the first time to our knowledge. Also anti-tubulin antibodies in allergic rhinitis have not been reported so far.

Pathological roles of the anti- β -tubV autoantibodies remain to be explicated. However, properties of the tubulin might encourage a reconsideration of their role in the pathogenesis of allergic rhinitis. Tubulin is an integral component of microtubules, and occurs mostly as soluble heterodimers consisting primarily of α - and β -isoforms or as assembled polymers that form microtubules (17). It has been reported that colchicines and vinblastine antagonize the polymerization of tubulin to form microtubules, and both agents inhibit complement- and allergen-triggered release of histamine from basophiles (7). It would, therefore, be interesting to investigate whether the anti- β -tubV antibodies have an

effect on histamine release from normal leukocytes.

In the investigation of the clinical parameters, the anti- β -tubV autoantibody-positive patients tended to show lower RAST titers than the antibody-negative ones, even though the differences were not significant statistically. Immunological disorders of allergic rhinitis may include both allergy and autoimmunity in its early phase and then be concentrated on allergy rather than autoimmunity. Further studies are needed to clarify the relation between allergy and autoimmunity. Alternatively, the anti- β -tubV autoantibody may have regulatory effects on allergy by inhibiting complement- and allergen-triggered release of histamine from basophiles as above mentioned. In addition, it was puzzling that female patients tended to possess the anti- β -tubV autoantibody more frequently. Since it was reported that estradiol and testosterone had opposite effects on microtubule polymerization (12), these hormones may affect antigenic characteristics of β -tubV such as accessibility to autoepitopes. Further studies will be needed to elucidate the mechanisms.

In conclusion, we here applied a proteomic approach to survey autoantigens/autoantibodies in allergic rhinitis and found multiple autoantigens successfully. In particular, the anti- β -tubV antibody was found to be a major autoantigen in allergic rhinitis for the first time to our knowledge. The role of detected autoantibodies including the anti- β -tubV antibody should be investigated in the future.

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