

Oral administration of a mite allergen expressed by zucchini yellow mosaic virus in cucurbit species downregulates allergen-induced airway inflammation and IgE synthesis

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Background: Sublingual-swallow immunotherapy in house dust mite-related asthma has a good safety profile and improves respiratory function and bronchial hyperreactivity. Zucchini yellow mosaic virus (ZYMV) is envisaged as a promising viral vector for expressing large quantity of foreign proteins in cucurbit species.

Objective: We sought to investigate whether oral feeding of dust mite allergen expressed by ZYMV in a cucurbit species can suppress allergen-induced inflammation and IgE synthesis.

Methods: An infectious plant virus clone, p35SZYMV2-26, that contains the full-length cDNA to the genomic RNA of a Taiwan isolate of ZYMV, driven by the cauliflower mosaic virus 35S promoter, was engineered as an *in vivo* viral vector to express *Dermatophagoides pteronyssinus* group 5 allergen (Der p 5) in cucurbit species. Female BALB/c mice were intraperitoneally sensitized with *Escherichia coli* bacteria-expressed Der p 5 and orally treated with the virus-expressed Der p 5 (vDer p 5) extracted from the recombinant virus-infected squash plants. Der p 5-specific immunoglobulins were measured by ELISA, and bronchoalveolar lavage assays were used to measure airway inflammation.

Results: Infectivity assays and immunoblotting revealed that large quantities of free-form vDer p 5 are produced in the recombinant virus-infected squash plants. The recombinant virus carried and expressed the Der p 5 allergen in squash plants for at least 1 year after numerous passages. In animal tests, squash extract containing vDer p 5 inhibited Der p 5-specific IgE synthesis and airway inflammation.

Conclusion: Our results suggest that oral feeding with allergen produced by the plant viral vector provides a novel approach for the therapy of allergic asthma. (J Allergy Clin Immunol 2004;113:1079-85.)

Key words: Allergic asthma, zucchini yellow mosaic virus, allergen, oral tolerance

Abbreviations used

BALF: Bronchoalveolar lavage fluid
bDer p 5: Bacteria-expressed *Dermatophagoides pteronyssinus* group 5 allergen
bGFP: Bacteria-expressed green fluorescent protein
CP: Coat protein
Der p 5: *Dermatophagoides pteronyssinus* group 5 allergen
ORF: Open reading frame
vDer p 5: Virus-expressed *Dermatophagoides pteronyssinus* group 5 allergen
vGFP: Virus-expressed GFP
ZYMV: Zucchini yellow mosaic virus

Feeding protein antigens into animals can downregulate systemic immune responses and induce tolerance.¹ There has been interest in its potential to modulate autoimmune, inflammatory, and allergic disorders.² Despite immunosuppressive cytokines such as TGF- β and IL-10 being found abundantly in the intestine when induced by oral tolerance, the actual mechanism by which they occur remains controversial.³ Subcutaneous or intradermal injections of inhalant allergen extracts provoke local or systemic reactions and have provided an effective method of immunotherapy for patients with allergic rhinitis and asthma. However, anaphylaxis develops occasionally and leads to death in rare cases.^{4,5} Oral or sublingual immunotherapy has been an effective and safer measure.⁶ The efficacy of the sublingual-swallow route has been ascribed to the use of higher dosage and partial oral tolerance mechanisms.^{7,8}

Zucchini yellow mosaic virus (ZYMV), a member of plant potyvirus, is deleterious to many cucurbit species, including cucumber, squash, melon, and watermelon.⁹ Because of the genome organization and proteolytic processing strategy of all potyviruses, ZYMV has been engineered as an expression vector for synthesis of foreign proteins in cucurbits. The target protein is synthesized as a part of the viral polypeptide and produced in an equal molar ratio as with all viral proteins.^{10,11}

In this article, we demonstrate the use of the infectious cDNA clone p35SZYMV2-26, which contains the full-length cDNA of the genomic ss(+)RNA of a Taiwan

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isolate of ZYMV (TW-TN3),¹² to test the feasibility of expressing the green fluorescent protein (the viral vector-expressed GFP; vGFP) reporter gene. Subsequently, the open reading frame (ORF) of the group 5 allergen of house dust mite *Dermatophagoides pteronyssinus* (Der p 5) was inserted into the ZYMV vector and expressed in squash plants (the viral vector expressed Der p 5 protein; vDer p 5). Der p 5 is a clinically important major allergen that reacts to >50% of allergic patients.¹³ In this study, we intended to provide an example to explain that the allergen can be expressed by plant virus vector. The histidine-tagged vGFP and vDer p 5 proteins were purified by affinity column chromatography, and the stability of the expression of foreign proteins by the vector was analyzed. We further evaluated the effect of oral administration of vDer p 5 extract in downregulating allergic inflammation *in vivo* by using a mouse model of allergic airway inflammation elicited by Der p 5 clinically relevant to human asthma.¹⁴ Our results suggest that oral feeding with allergen produced by a plant viral vector provides a new approach for the therapy of allergic asthma.

METHODS

Construction of ZYMV–Der p 5 recombinant plant virus

The previously constructed infectious clone p35SZYMV2-26,¹² which is driven by a cauliflower mosaic virus 35S promoter to generate *in vivo* infectious transcript, was used as a vector to carry the ORF of GFP (BD Biosciences Clontech, Palo Alto, Calif) between the P1 (P1 protein) and HC-Pro (helper component proteinase) coding regions of ZYMV. The multiple cloning sites (*Nco*I, *Sph*I, *Apa*I, *Mlu*I, *Kpn*I, and *Sac*II) were created flanking the N-terminus and C-terminus of the GFP coding region by PCR with designed primers. A hexahistidine (histidine tag) and a Nla protease cleavage motif of ZYMV (S-V-R-L-Q/S) were also created by PCR mutagenesis at the C-terminal end of GFP ORF. The new viral vector harboring the reporter GFP with multiple cloning sites, a histidine tag, and an Nla protease cleavage motif was designated p35ZYMVGFPHis (Fig 1).

The Der p 5 ORF was amplified from the total RNA of mite crude extract by RT-PCR and cloned in pGEX-2T-Der p 5.¹⁵ The primers PDP5-5eSph (5'-TAGCATGCATGAAATTCATCATTGCTTCT-3') and MDP5-3eKpn (5'TTGGTACCAACTTCAATCTTTT-AACACGTGC-3') were used to introduce *Sph*I and *Kpn*I sites (underlined) to the 5' and 3' termini of the Der p 5 ORF, respectively, by PCR. The amplified fragment was cloned into *Shp*I-*Kpn*I digested p35SZYMVGFPHis to create p35ZYMVDerp5 (Fig 1).

Plant inoculation

The systemic host plants of *Cucurbita pepo* L var Zucchini (squash) at 2 cotyledons stage and local lesion host plants of *Chenopodium quinoa* Willd with 4 fully expanded leaves were used for infectivity assay of various constructs. Individual plasmids (1 µg) containing recombinant clones were used to infect *C quinoa* plants by mechanical rubbing on leaves.¹² Seven days post inoculation (dpi), single lesions were isolated and mechanically transferred to zucchini squash. Inoculated plants were kept in a temperature-controlled greenhouse (23°C–28°C) for observation of symptom development.

Western blot analysis

The GFP reporter was also expressed by pET36b in bacteria (Novagen, Madison, Wis; bacteria-expressed GFP; bGFP) and

purified by gel purification.¹² Similarly, Der p 5 protein was expressed by pGEX-2T in bacteria (Promega, Madison, Wis; bacteria-expressed Der p 5; bDer p 5) and purified by glutathione-S-transferase (GST)-affinity column chromatography.¹⁵ Production of polyclonal antisera to bGFP or bDer p 5 followed the method described previously.¹⁶ The engineered protein expressed by ZYMV in infected squash plants was monitored by Western blotting. Extracts from equal amounts (0.01 g) of leaf tissue collected at 7 dpi were loaded, separated on a gel (12.5%), and transferred onto nitrocellulose membrane. Antisera to ZYMV coat protein (CP),¹² bGFP, or bDer p 5 for immunoblot analyses were used at a 1:5000 dilution.

Isolation of histidine-tagged proteins from infected plants

Histidine-tagged vGFP and vDer p 5 expressed by the ZYMV recombinants ZYMV-GFPHis (derived from the construct p35SZYMVGFPHis) and ZYMV-Derp5 (derived from the construct p35SZYMVDerp5), respectively, were purified by affinity column chromatography. Infected squash leaves (20 g) were harvested 8 to 10 dpi, and target proteins in plant extracts were isolated with nickel–nitrilotriacetic acid (Ni-NTA) agarose column chromatography by manufacturer's protocol (Qiagen, Valencia, Calif). The isolated vGFP and vDer p 5 in 10 µL of the consecutive 250-µL eluted fractions were analyzed by SDS polyacrylamide gel (12.5%) electrophoresis and stained with Coomassie brilliant blue R-250 (Sigma, St Louis, Mo) using BSA (NEB, Beverly, Mass) protein as standard. The concentrations of purified proteins were determined by Image Gange version 2.54 software (Fuji Photo Film Co, Minato-Ku, Tokyo, Japan).

Extraction of vDer p 5 for animal test

Leaves of squash plants (250 g) infected with ZYMV-Derp5 were harvested 10 dpi and homogenized in a blender with twice sample volumes of water. The homogenate was clarified by centrifugation at 5000g for 10 minutes, filtered through Miracloth (Calbiochem, La Jolla, Calif), and centrifuged again at 40,000g for 30 minutes. The supernatants were lyophilized, and the concentrated powder was distributed into vials for animal testing. A total of 1.4 kg of infected leaves was used.

The amount of vDer p 5 in lyophilized powder was determined by ELISA by using the antiserum prepared to bDer p 5. A 10-fold serial dilution of lyophilized powder containing vDer p 5 (from 200 mg/mL to 20 µg/mL) was prepared in 0.1-mol/L sodium phosphate buffer (pH 9.6). The microtiter plates were coated with 100 µL diluted vDer p 5 and incubated at 37°C for 2 hours. After washing and blocking, aliquots of 100 µL rabbit anti-bDer p 5 IgG diluted 1:2000 in PBS containing 1% BSA were used to detect vDer p 5. Goat antirabbit IgG-conjugated alkaline phosphatase (1:2000, Zymax 81-6122) was used as the secondary antibody. The plates were finally incubated with 1 mg/mL p-nitrophenyl phosphate disodium salt (Sigma) for 30 minutes, and the color reaction was measured at 405 nm. A standard curve using purified bDer p 5 diluted from 1.25 mg/mL to 78.125 µg/mL was exploited to estimate the amount of vDer p 5 in the extract.

Animals and study protocol

Female BALB/c mice between 6 and 8 weeks old were obtained from the animal breeding center of the College of Medicine, National Taiwan University, Taipei (originated from The Jackson Laboratory, Bar Harbor, Me). They were divided into 4 groups for each experiment. At least 12 mice were used in each experiment. The animal model is summarized in Fig 2, and the protocols for each treatment are described in Table I. Mice were first sensitized by intraperitoneal injection of 10 µg bDer p 5 (10 µL) mixed with 4 mg aluminum hydroxide (90 µL; Wyeth Pharmaceuticals, Punchbowl, Australia). Seven days after sensitization, mice were treated with

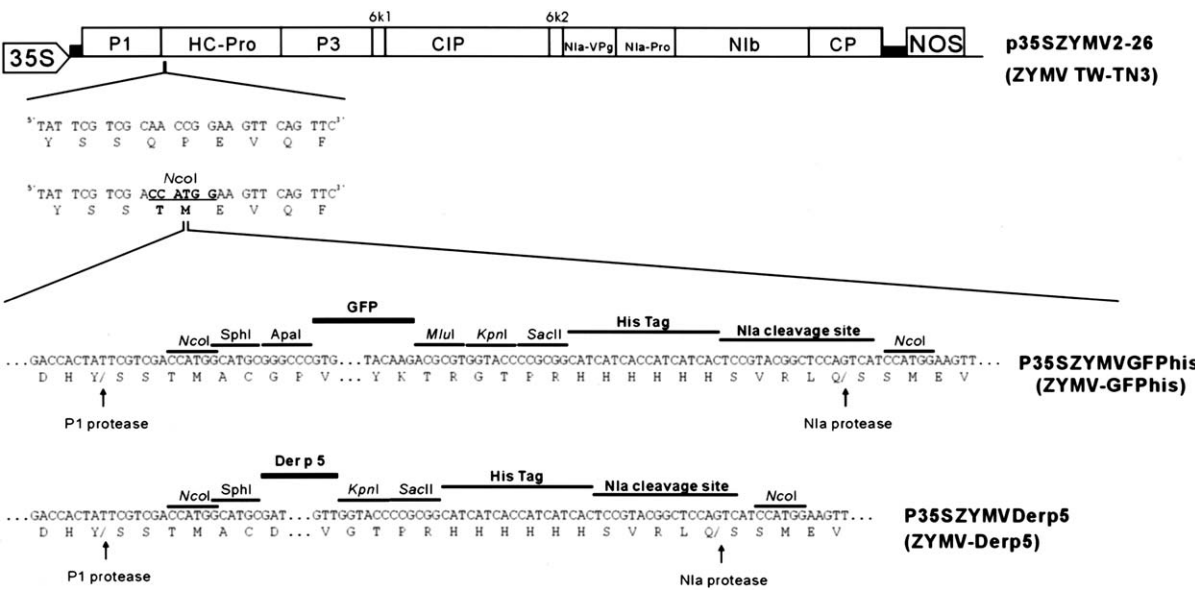


FIG 1. Diagram of the nucleotide and amino acid sequences in the modified regions of the viral vectors derived from a Taiwan strain (TW-TN3) of ZYMV. The corresponding recombinant viruses generated by each construct are shown in parentheses.

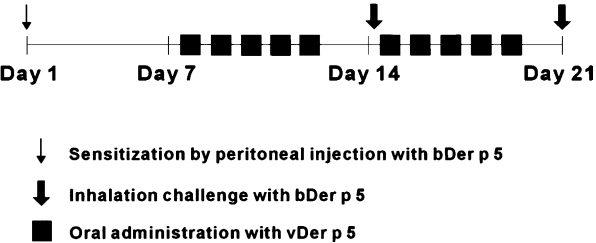


FIG 2. The animal study protocol for immunotolerance of dust mite allergen Der p 5. Methods of sensitization, oral administration, and inhalation challenge of mice are indicated.

vDer p 5—containing lyophilized powder dissolved in 400 μ L PBS once a day for 10 days by using a syringe fit with an oral feeding needle. The treated mice were divided into 2 groups: the low-dose group, 20 mg lyophilized powder containing vDer p 5/100 g body weight/d; and the high-dose group, 200 mg powder in PBS/100 g body weight/d. Control groups included mice orally treated with PBS only or with 20 g powder from squash infected with ZYMV vector/100 g body weight/d. Fourteen and 21 days after the initial sensitization, mice were exposed to an aerosol of 0.1% bDer p 5 purified from *Escherichia coli* for 20 minutes.¹⁴ Aerosols were generated with an ultrasonic nebulizer (Devlbiss, Somerset, Pa). The mean mass diameter of the aerosol was <4 μ m. Eight hours after last inhalation challenge, the bronchoalveolar lavage fluids (BALFs) and sera were collected.

Determination of Der p 5—specific IgG and IgE

The amounts of Der p 5—specific IgG and IgE were determined by ELISA. Protein high-binding plates were coated with 100 μ L per well of purified bDer p 5 diluted in coating buffer (0.1 mol/L NaHCO₃, pH 8.2) at a concentration of 5 μ g/mL. After overnight incubation at 4°C, plates were washed 3 times and blocked with 3% (wt/vol) BSA in PBS buffer at 25°C for 2 hours. Sera were used at 1:100 dilution for

IgG and 1:10 dilution for IgE determinations in duplicate. After overnight incubation at 4°C, either biotinylated rat antimouse IgE mAb (R35-72; BD Biosciences Pharmingen, San Diego, Calif) or rat antimouse IgG mAb (R12-4; BD Biosciences Pharmingen) diluted in 0.05% gelatin PBS buffer was added and incubated for an additional hour. Avidin-alkaline phosphatase conjugate was then added (1:1000) and incubated at 25°C for 1 hour. After 6 washes, color reaction was initiated with the addition of p-nitrophenyl phosphate (1 mg/mL) disodium salt (Sigma). Plates were read at 405 nm in a microplate autoreader (Metertech, Taipei, Taiwan). Readings were referenced to a standard serum pooled from 6 mice that received an initial intraperitoneal injection of 10 μ g bDer p 5 with 4 mg aluminum hydroxide and a booster injection with the same dose 21 days later. The standard serum was normalized as 100 ELISA units/mL.

Bronchoalveolar lavage and cell counting

After measurement of lung function parameters, mice were lavaged with 5 $\times$ 0.5-mL aliquots of 0.9% sterile saline solution through a polyethylene tube introduced through a tracheostomy. Lavage fluid was centrifuged (500 g for 10 minutes at 4°C), and cell pellets were resuspended in 0.5 mL Hank balanced salt solution. Total cell counts were made by adding 10 μ L cell suspension to 90 μ L 0.4% trypan blue and counting under a light microscope in a Neubauer chamber. Differentiated cell counts were made from cytospin preparations stained by Leu stain. Cells were identified and differentiated into eosinophils, lymphocytes, neutrophils, and macrophages by standard morphologic techniques. A total of 500 cells for each sample was counted under 400-fold magnification, and the percentage and absolute number of each cell type were determined.

Statistical analysis

To assess changes of IgE and IgG levels and cells in the BALF after bDer p 5 challenges, repeated measures for ANOVA were performed to compare the differences among groups. After analysis of variance, Duncan multiple range tests ($P < .05$) were used to differentiate differences between experimental and control groups.

TABLE I. Protocol of the animal test of 4 experimental groups and procedures performed for each group*

Group	N	Body weight (g)	Sensitization	Treatment	Aerosol challenge
C	12	28 ± 1.1	bDer p 5	PBS	PBS
NC	12	30 ± 1.5	bDer p 5	PBS	bDer p 5
Mock	12	31 ± 1.5	bDer p 5	ZYMV (20 mg/100 g/d)	bDer p 5
Low-dose	18	29 ± 1.2	bDer p 5	vDer p 5 (20 mg/100 g/d)	bDer p 5
High-dose	18	32 ± 1.4	bDer p 5	vDer p 5 (200 mg/100 g/d)	bDer p 5

*Female BALB/c mice were divided into 4 groups for experiment. bDer p 5 was used for sensitization and aerosol challenge. *C*, Control group with PBS aerosol challenge. *NC*, Control group with sensitization and bDer p 5 aerosol challenge, but orally administrated with PBS only. *Mock*, Orally administrated with lyophilized powder from squash infected with ZYMV vector (20 mg/100 g/10 d). *Low-dose*, Orally administrated with 20 mg lyophilized squash powder containing vDer p 5 (20 mg/100 g/d for 10 days). *High-dose*, Orally administrated with 200 mg/100 g/d for 10 days.

RESULTS

Expression of vGFP and vDer p 5 by recombinant ZYMV

The construct p35SZYMVGFPHis was introduced into plants of *C. quinoa* by mechanical inoculation. Local lesions developed on the inoculated leaves 7 dpi. The construct p35SZYMVGFPHis generated the recombinant virus ZYMV-GFPHis that was established through single lesion transfer from *C. quinoa* to squash plants. ZYMV-like symptoms were noticed on noninoculated leaves of plants infected with ZYMV-GFPHis 4 to 6 dpi.

Illumination of symptomatic noninoculated upper leaves of ZYMV-GFPHis-infected squash plants with long-wavelength UV light revealed strong green fluorescence that was not observed in leaves of healthy plants or in those of TW-TN3-infected plants (data not shown). Western blot analyses of extracts from systemically infected squash leaves showed that ZYMV-GFPHis was able to produce a free form of vGFP and a vGFP/HC-Pro fusion protein as judged from its molecular size (Fig 3, A, upper box). The quantity of viral CPs in plants infected with ZYMV-GFPHis was similar to that in plants infected with the wild-type TW-TN3 (Fig 3, A, lower box).

Virus recombinant ZYMV-Derp5 derived from p35SZYMVDerp5 induced local lesions on *C. quinoa* plants 7 dpi and induced mosaic symptom on squash plants 4 to 6 dpi. The systemic symptoms of ZYMV-Derp5 infection on squash became prominent yellow mosaic 7 to 9 dpi, similar to symptoms induced by the wild-type TW-TN3. ZYMV-Derp5 was also able to produce a free-form vDer p 5 and a fusion vDer p 5/HC-Pro (Fig 3, A, upper box), and its CP quantity in squash was also similar to that in plants infected with wild-type TW-TN3 (Fig 3, A, lower box). Our results indicate that the TW-TN3 is an efficient viral vector for expressing vGFP and vDer p 5, and the accumulation of the recombinant viruses was similar to that of the wild-type ZYMV.

Isolation and concentration of foreign proteins expressed by the viral vector

Virus-expressed GFP with histidine tag was readily purified by Ni-NTA affinity column chromatography as monitored by bGFP-specific polyclonal antibodies in immunoblot analysis (Fig 3, B, lanes 2-4). The yield of

purified vGFP was estimated as 3.7 µg per gram of the leaf tissue by Image Gange software analysis. Also, vDer p 5 was purified from infected squash leaves by using Ni-NTA affinity column (Fig 3, B, lanes 6-8), yielding 1.5 µg vDer p 5 per gram of leaf tissues. In Fig 3, B, a minor band at 79 kd was also detected from the GFP gel and considered the unprocessed fusion form of GFP/HC-Pro compared with Fig 3, A. The minor band detected from the vDer p 5 gel was considered the dimer of vDer p 5 according to its molecular size.

Inhibition of allergen-specific IgE synthesis

A total of 21 g lyophilized powder was prepared from 1.4 kg of leaves of squash infected with ZYMV-Derp5. It was concentrated about 68 times from the harvested leaf tissue (wt/wt). By comparing with the standard curve of the ELISA readings of the bDer p 5, the amount of vDer p 5 was estimated at 6.4 mg per gram of lyophilized powder. Because the average weight of the mice treated was ~30 g, each mouse in the low-dose treatment group received ~40 µg vDer p 5 daily, and each mouse in the high-dose group received ~400 µg.

We evaluated the *in vivo* efficacy of the oral administration of vDer p 5 to determine whether a protective response to inhalation allergen challenge was functionally significant. Both mock-treated and vDer p 5-treated mice received 2 inhalation challenges with allergen bDer p 5 at 2 and 3 weeks after intraperitoneal sensitization (Fig 2). The presence of anti-Der p 5 IgE in the serum 3 weeks after the second allergen challenge was determined by ELISA. Der p 5-specific IgE increased significantly in the PBS-treated group compared with the control group, which was sensitized with bDer p 5 but without treatment and aerosol challenge (Fig 4). In contrast, vDer p 5-treated mice in both the low-dose and high-dose groups showed >50% inhibition of Der p 5-specific IgE synthesis (Fig 4). The Der p 5-specific IgE level of the control group orally administrated with powder from squash plants infected with ZYMV vector was similar to that of the control group orally treated with PBS (data not shown). Thus, oral administration of vDer p 5 expressed by ZYMV in squash inhibited an *in vivo* allergen-specific IgE synthesis efficiently in an allergen-specific manner. There was no significant difference among specific IgG levels among groups (Fig 4).

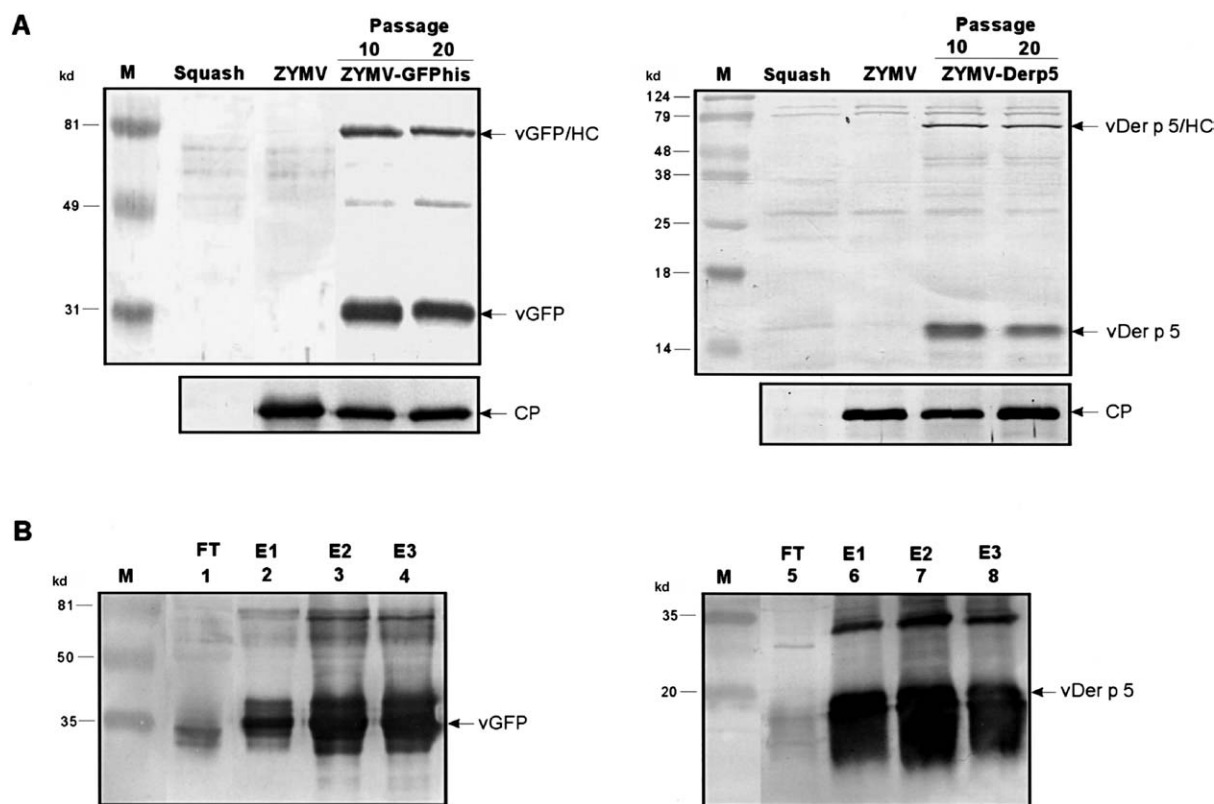


FIG 3. Western blot analyses of vGFP and vDer p 5 and metal-affinity purification of his-tagged proteins from squash plants infected with ZYMV recombinants. **A**, vGFP, vDer p 5, and ZYMV CP in crude sap of recombinant-infected squash plants. **B**, Monitoring of the flow-through (FT) of affinity purification of his-tagged vGFP and vDer p 5. M, Protein markers.

Suppressing Der p 5–induced airway inflammation

To investigate whether oral administration of vDer p 5 could suppress allergen-specific airway inflammation, the number of cells in BALF was used as a measure for the infiltration of cells in the airways. Significantly low numbers of eosinophils and neutrophils in the BALF of vDer p 5–treated mice were observed compared with the mock-treated group ($P < .05$; Fig 5). The numbers of macrophages and lymphocytes of the 2 treated groups were not significantly different from those of the mock-treated control (Fig 5). Our results indicated that bDer p 5 inhalation challenge induced an eosinophilic and neutrophilic cellular infiltrate in the BALF and that this inflammation could be inhibited by orally administered vDer p 5.

DISCUSSION

In this investigation, the infectious cDNA clones of ZYMV TW-TN3 were used as a viral vector to introduce a GFP coding sequence into the ZYMV genome. Results demonstrate that virus replication and movement can easily be monitored by using a UV light excitation in situ and by immunoblotting. The ZYMV-GFPHis and

ZYMV-Derp5 that express the free forms of vGFP and vDer p 5, respectively, were highly stable over the period of a 1-year observation through 20 times transfers, and no deletion variants were noticed (data not shown). Therefore, the ZYMV-base viral vector permits both systemic spread and efficient, stable expression of foreign proteins. It is considered that the stability may be greatly dependent on the nucleotide sequence and the length of the insert.¹⁷

Tobacco etch virus carrying β -glucuronidase (GUS) and plum pox virus carrying GUS that contained a fusion or free-form GUS at the same location as the recombinant viruses constructed in this investigation induced attenuated symptoms.^{18,19} In the case of lettuce mosaic virus, when the GFP or GUS reporter genes were introduced as a free-form protein inserted between the P1 and HC-Pro coding regions, chimeric viruses caused symptomless infection and had low virus accumulation in infected plants.²⁰ Moreover, the Israel isolate ZYMV AG that was grouped in genotype II has been developed as a nonpathogenic viral vector for expression of heterologous proteins.²¹ However, heterologous proteins introduced between the P1 and HC-Pro coding regions of the nonpathogenic vector resulted in nonexpression in plants. These various results may be caused by the natural properties of the corresponding viruses. Our observations indicate that symptom development or virus titers of

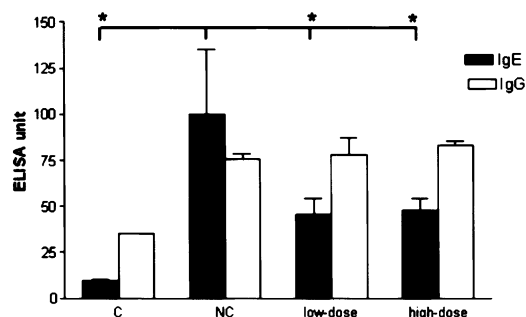


FIG 4. ELISA measurement of Der p 5–specific IgG and IgE in tested mice for the efficacy of the oral administration of vDer p 5. Readings were referenced to the standard serum normalized as 100 ELISA units/mL. Treatment protocols are described in Table I. * $P < .05$ compared with vehicle-treated mice.

ZYMV-GFP_{his} and ZYMV-Derp5–infected squash plants were not significantly affected. Also, the constructed ZYMV recombinants express large amounts of foreign proteins as well as other genomic products such as CP. Thus, our ZYMV vector is the most efficient potyviral vector so far reported and has a great potential for expressing foreign proteins in cucurbit species. Because the ZYMV has a relatively wide host range, including many economically important cucurbit species, modifications of the viral vectors reported in the current studies as a nonaphid-transmissible and nonpathogenic vector for crops as reported by Arazi et al²¹ will be a great improvement.

Specific immunotherapy with extracts of inhalant allergens to achieve clinical tolerance of the allergens is effective in ameliorating clinical symptoms of respiratory allergic diseases.²² Recent studies suggest that specific immunotherapy may also alter the course of allergic disease by reducing the risk of developing new allergic sensitizations and inhibiting the development of clinical asthma in children treated for allergic rhinitis.²³ The traditional subcutaneous route is burdened by the risk of severe adverse effects. In contrast, the sublingual-oral route is supported by numerous controlled trials and has shown its efficacy in asthma and rhinitis in adults and children.²⁴ Clinical trials and postmarketing surveillance studies show a satisfactory safety profile.²⁵ However, despite the positive reports, there are disappointing results in clinical trials. The principal reason for this failure is a lack of potency of allergens when given by the oral route. In many previous studies, the oral doses of an allergen required for either a serologic or clinical effect were extraordinarily large: 100 to 200 times the cumulative dose required by the subcutaneous route.²⁶ High costs are a serious concern for many human therapeutic proteins, including crude mite extraction. Expensive expression systems such as human or animal cell lines and transgenic animals also raise safety concerns. The ZYMV-based viral vectors reported in this article provide an easy, manageable procedure for production of allergens. Its multiple cloning sites and a histidine tag also provide stable expression of foreign proteins and the convenience for

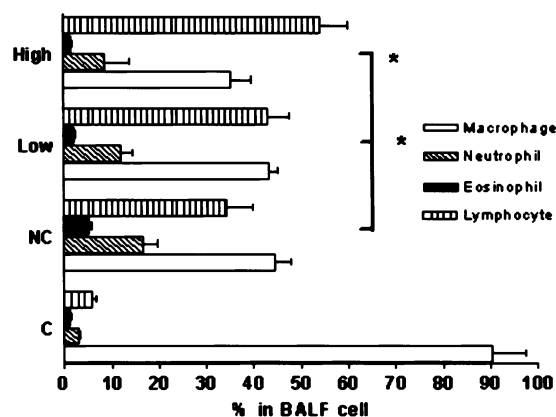


FIG 5. Bronchoalveolar lavage assays for airway inflammation of mice orally treated with ZYMV-expressed dust mite allergen vDer p 5. Numbers of eosinophils, neutrophils, lymphocytes and mononuclear cells in the BALF of Der p 5–sensitized mice treated with low-dose or high-dose vDer p 5 were counted. * $P < .05$ compared with vehicle-treated mice.

protein purification. In this study, we demonstrate that ZYMV-Derp5 mediates the synthesis of large quantity of a soluble vDer p 5 allergen in squash plants and that the soluble vDer p 5 protein can be easily purified by Ni-NTA column chromatography.

Current studies indicate that the ZYMV recombinant infecting cucurbit species is an ideal system to provide large quantities of relatively low-cost soluble proteins for oral tolerance to allergen in the treatment of allergic diseases. The studies also show that the oral administration of the recombinant mite allergen from ZYMV viral vector inhibits the synthesis of specific IgE and downregulates the influx of eosinophils and neutrophils into airway on inhalation challenge. Furthermore, results of oral feeding trials in the studies indicate that proteins produced by ZYMV viral vector retained immunogenic properties after being concentrated from the extract of infected plants. Although this Der p 5–induced murine model could not completely mimic airway pathogenesis of human beings, we did provide evidence that proteins produced by ZYMV viral vector retained immunogenic properties after being concentrated from the extract of infected plants. In summary, we present the first report that a ZYMV viral vector–expressed house dust mite allergen, given as a dietary supplement, inhibits the development of aeroallergen-induced airway inflammation and the synthesis of specific IgE. Further studies are needed to evaluate other recombinant allergens and the possible need for mucosal adjuvants to enhance tolerance. We understand that changes of pulmonary function are the most important endpoint in the treatment of asthma. However, our initial work emphasizes the expression of allergen in this new expression system and oral administration of the expressed product for downregulating the allergen-induced inflammation. We will investigate the working mechanisms and clinical effects in following studies.

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