

Production-scale purification of the recombinant major house dust mite allergen Der f 2 mutant C8/119S

Satoshi Koyanagi,^{1,*} Toshio Murakami,² Toshihiro Maeda,³ Kenjiro Kawatsu,² Hiroshi Okamura,¹ Yoshiaki Oda,³ Yoshinobu Miyatsu,¹ Keishin Sugawara,¹ and Hiroshi Mizokami^{1,2,3}

Biologics Development & Production Department, The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1 Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan,¹ Applied Research Department, The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1 Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan,² and Clinical Development Department, The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1 Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan³

Received 16 March 2010; accepted 18 May 2010

A WHO position paper states that allergen immunotherapy is an effective treatment for allergic diseases, and well characterized allergens should be used in immunotherapy. The house dust mite is a major cause of allergic disease. However, the biological activity of the mite extracts currently used cannot be clearly determined, since these extracts contain various impurities. The use of recombinant allergens can avoid this problem. However, there remains a risk of contamination by other impurities, such as host cell-derived proteins (HCPs). Advanced purification techniques are thus required to remove these contaminants. C8/119S is a mutant of the major house dust mite allergen Der f 2, and is expressed and accumulated as an inclusion body in *Escherichia coli*. The C8/119S was refolded and purified through three column chromatography steps. Using this method, we could obtain about 2 g of the purified C8/119S in one purification batch. This amount is equivalent to 100,000 of the maintenance doses required for immunotherapy based on the WHO position paper. The purity of the C8/119S was 99% or more. The antigenicity of HCPs in the C8/119S was examined by passive cutaneous anaphylaxis assays. When the C8/119S was administered at 40 µg/kg, no local anaphylaxis was observed. C8/119S was thus highly purified with an extremely low level of impurities, and our procedure was shown to be an effective advanced production-scale purification process for this Der f 2 mutant. In this study, we established an advanced purification processes for C8/119S, then characterized the purified C8/119S and evaluated its purity.

© 2010, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** *Dermatophagoides*; Der f 2; C8/119S; *Escherichia coli*; Immunotherapy; Antigenicity; PCA]

Anti-allergic or anti-histaminic agents are used for the treatment of allergic diseases such as allergic asthma, allergic rhinitis, allergic dermatitis, and allergic conjunctivitis. However, these agents are symptomatic, and thus cannot achieve a radical cure for allergic diseases. Immunotherapy, in which causative antigens of allergic diseases are injected, is believed to be the sole means of achieving a radical cure of allergic diseases (1–5). A WHO position paper published in 1998 stated that allergen immunotherapy was an effective treatment for patients with allergic rhinitis/conjunctivitis or allergic asthma, and the use of well characterized and standardized allergen vaccines makes it possible to define an optimal maintenance dose in the range of 5–20 µg of major allergen per injection (6). The therapeutic mite allergen vaccines currently used are extracted from the mite *Dermatophagoides*. Their biological activity cannot be clearly determined, since they contain not only major allergens but also a variety of impurities derived from mites. Therefore, it is difficult to prepare allergen vaccines with consistent titers for injection at the

effective maintenance doses recommended by the WHO position paper. The limitation of source materials from mites makes obtaining a stable supply of a large quantity of allergens difficult. Allergen vaccines are usually subcutaneously injected in order to minimize potential symptoms in allergic patients. However, if the vaccines are contaminated with impurities, they can newly induce immune responses to the impurities and cause serious adverse side effects. There is thus a need for highly purified allergen vaccines in order to ensure safety during immunotherapy.

Several major mite allergens (Der f 1, Der f 2, Der p 1, Der p 2, etc.) have already been identified and expressed by recombinant techniques (7–12). Because they are not contaminated by mite-derived impurities, the recombinant allergens can be stably supplied as safe allergen vaccines. However, there remains a risk of contamination by other impurities, such as host cell-derived proteins (HCPs) and culture-media contaminants that arise from the recombinant allergen production processes. Because these impurities might induce unintended antigenicity, advanced purification techniques are required to remove them.

C8/119S is a mutant of the major mite allergen Der f 2. Of the 130 amino acids constituting C8/119S, Cys 8 and Cys 119 have each been replaced by Ser residues in order to disrupt one of three disulphide

* Corresponding author. Biologics Development & Production Department, The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1, Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan. Tel.: +81 968 37 3100; fax: +81 968 37 3616.

E-mail address: koyanagi@kaketsuken.or.jp (S. Koyanagi).

bonds in wild-type Der f 2. C8/119S is expected to have low risk of anaphylaxis in immunotherapy (13–16). In a previous study, we established a large-scale culture process for expressing C8/119S (17). In this study, we established an advanced purification processes for C8/119S, then characterized the purified C8/119S and evaluated its purity.

MATERIALS AND METHODS

Expression of C8/119S in *Escherichia coli* The expression plasmid pWU11-C8/119S was constructed by integrating the C8/119S gene downstream of trp promoter. We established a recombinant *E. coli* expressing C8/119S, which we designated pWU11-C8/119S/HB101 (17). A single colony of this recombinant *E. coli* was selected, inoculated into 200 ml of LB medium, and grown to an optical density at 600 nm (OD₆₀₀) of three or more. Cells were suspended in cultured medium with 20% glycerol added, dispensed into 1 ml vials and stored at –80 °C as a master cell bank. For preparation of the working cell bank, one vial of the master cell bank was inoculated into 1 l of LB medium, cultured, and stored in a similar fashion.

One of the working cell banks was inoculated into 1 l of LB medium and grown to an OD₆₀₀ of three or more, and the cultured medium was inoculated into 250 l of the production culture medium supplemented with 2% glucose in a 300 l-bioreactor (F-02; Hitachi Ltd., Tokyo). After the initial glucose was consumed, 50 l of 60% glucose solution supplemented with nutrients was added to coincide with the cell proliferation. During the growth period, the pH value was maintained at 7.0 using a 25% ammonia solution, and the dissolved oxygen level was controlled from 20% to 40% of the air saturation value by controlling the agitation speed and aeration. Expression of C8/119S was started by thermal induction. The culture was continued for 12 h after the induction (17).

Purification of C8/119S Recombinant *E. coli* cells were collected from the culture medium by using a micro filter membrane (Asahi Kasei, Tokyo) and suspended in 20 mM Tris–HCl, pH 8.5. Next, lysozyme was added to the solution at a final concentration of 0.6 g/l and left overnight at 10 °C or less. The resulting mixture was fed into a French press to crush the cells. The solution was concentrated by a micro filter membrane to remove the cell debris and diluted with 20 mM Tris–HCl, pH 8.5. Then, in order to collect the inclusion bodies, the resulting solution was centrifuged and the inclusion bodies were recovered as the precipitate. The inclusion bodies with a wet weight of about 1,000 g were obtained by a 300 l-scale culture. An aliquot of inclusion bodies with a wet weight of 100 g was dissolved in 10 l of 20 mM Tris–HCl, pH 8.5, containing 10 mM cysteine and 8 M urea (denaturing buffer). The protein concentration was about 1 mg/ml. The dissolved solution was diluted into a 10-fold volume of 20 mM Tris–HCl, pH 8.5, containing 3 mM cysteine and 0.3 mM cystine (refolding buffer) and left to stand for four overnights at 10 °C or less. The pH of the refolding solution was adjusted to 8.5 and the resulting solution was concentrated by using an ultrafiltration membrane (Asahi Kasei) to 1/10 of the starting volume and purified by anion-exchange chromatography (DEAE-Toyopearl 650 M; Tosoh Co., Tokyo). A 2 l-DEAE-column was equilibrated with 20 mM Tris–HCl, pH 8.5, containing 50 mM sodium chloride. The refolding solution was passed through the column, and the through fraction was collected. Next, hydrophobic chromatography (Butyl Sepharose FF GE Healthcare, Piscataway, NJ, USA) was performed. A 2 l-Butyl Sepharose FF-column was equilibrated with 20 mM phosphate buffer, pH 7.2, containing 3 M sodium chloride. Then the above eluate, which was dialyzed with 20 mM phosphate buffer, pH 7.2, containing 3 M sodium chloride, was applied. The C8/119S was eluted with 20 mM phosphate buffer, pH 7.2. Additional anion-exchange chromatography (QAE-Toyopearl 650 M; Tosoh Co.) was performed to obtain highly purified C8/119S. A 1 l-QAE-column was equilibrated with 20 mM Tris–HCl, pH 8.5, containing 2 M urea. Then the above eluate, which was dialyzed with 20 mM phosphate buffer, pH 7.2, containing 2 M urea, was applied. The C8/119S was eluted with a linear gradient of sodium chloride from 0 to 100 mM (20 column volumes). The eluate fraction was dialyzed with 10 mM phosphate buffer, pH 7.2, containing 140 mM sodium chloride (PBS) and aseptically filtered to obtain a solution of purified C8/119S.

Analysis of C8/119S SDS-PAGE was carried out as follows. Samples were mixed with SDS buffer and an aliquot of mixed sample, equivalent to 0.5 µg of C8/119S, was applied to SDS-PAGE gel (Funakoshi, Tokyo). After electrophoresis, the gel was silver-stained. The mass of C8/119S was analyzed by MALDI-TOF/MS (BIFLEX III; Bruker, Billerica, MA, USA). Protein calibration standard I (Bruker) was used as a standard in the mass spectrometry analysis. The purity of the C8/119S was analyzed by high performance liquid chromatography (HPLC) with a size exclusion (SE) column (G3000SWXL; Tosoh Co.). SE-HPLC was done with a mobile phase of 0.1% TFA in 60% acetonitrile at 1 ml/min with measurement of absorbance at 280 nm. The analysis time was set at 20 min, the sample concentration was approximately 500 µg/ml, and the sample volume was 100 µl. The bacterial endotoxin content was determined using an Endospecy set, Toxicolor set, and Endospec ES test (Seikagaku Co., Tokyo) following the instructions of the manufacturer. Limit of detection of the bacterial endotoxin was 0.002 EU/ml.

Preparation of host cell-derived proteins (HCPs) A recombinant *E. coli* pWU-11/HB101 transformed by plasmid pWU-11 which did not contain the C8/119S gene was cultured in a 300 l-bioreactor, and inclusion bodies were recovered using the same method with the C8/119S preparation. A 100 g of inclusion bodies was dissolved in

denaturing buffer and diluted into a 10-fold volume of refolding buffer, then left to stand for four overnights at 10 °C or less. The pH of the refolding solution was adjusted to 8.5 and the resulting solution was concentrated with an ultrafiltration membrane up to an appropriate concentration and used as HCPs.

Preparation of guinea pig and rabbit anti-HCP sera Four-week-old male Hartley guinea pigs were purchased from Japan SLC Inc. (Hamamatsu). HCPs were mixed with alum as an adjuvant and subcutaneously injected at 0.5 mg/head five times at 2-week intervals. The second immunization with HCPs, at 0.35 mg/head, was intravenously performed at 2 weeks after the last immunization. Blood samples were collected and the guinea pig anti-HCP sera were separated 1 week after the second immunization (Fig. 1).

Male JW rabbits were purchased from KBT Oriental (Saga). HCPs were mixed with alum as an adjuvant and subcutaneously injected at 2 mg/head four times at 2-week intervals. The second immunization with HCPs, at 2 mg/head, was intravenously performed at 2 weeks after the last immunization. Blood samples were collected and the rabbit anti-HCP sera were separated 1 week after the second immunization (Fig. 1).

HCP-ELISA An ELISA plate (Nalge Nunc, Roskilde, Denmark) was coated with 10 µg/ml of guinea pig anti-HCP polyclonal antibody at 4 °C overnight. After washing the plate three times with PBS containing 0.05% Tween 20 (PBST), 200 µl per well of Block Ace (Dainippon Sumitomo Seiyaku, Osaka) was added to the plate for blocking at 37 °C for 1 h. After washing the plate three times with PBST, 100 µl portions of standard HCPs solutions and sample solutions appropriately diluted were added to each well, and the plate was left to stand at 37 °C for 2 h. After washing the plate three times with PBST, 100 µl of a rabbit anti-HCP polyclonal antibody (1 µg/ml) was added to the wells and the plate was left to stand at 37 °C for 1 h. After washing the plate three times with PBST, 100 µl of a donkey anti-rabbit IgG antibody labeled with horseradish peroxidase was added to the wells and the plate was left to stand at 37 °C for 1 h. After washing the plate three times with PBST, 100 µl of TMB solution (Sigma-Aldrich, St. Louis, MO, USA) was added to the wells and allowed to react for 10 min with shielding from light. The reaction was stopped by adding 100 µl per well of 0.3 N sulfuric acid, and absorbance at 450 nm was measured to calculate the amount of HCPs.

Preparation of anti-C8/119S sera Male JW rabbits were purchased from KBT Oriental. C8/119S was mixed with alum as an adjuvant and subcutaneously injected at 2 mg/head four times at 2-week intervals. The second immunization with C8/119S, at 2 mg/head, was performed intravenously at 2 weeks after the last immunization. Blood samples were collected and the sera were separated at 1 week after the second immunization (Fig. 1).

Passive cutaneous anaphylaxis (PCA) assay Seven- to nine-week-old male Hartley guinea pigs were purchased from Japan SLC Inc. Anti-HCP sera were diluted with saline solution in a four-fold dilution series, and 0.1 ml of the resulting solution was intradermally injected into a clipped region of the back (B-1 and B-2 groups). Anti-C8/119S sera (B-3 group) and PBS (B-4 group) were used as positive and negative controls, respectively. C8/119S was dissolved in saline solution to obtain 0.5 mg/ml concentrations, and 0.5% Evans blue (Wako Pure Chemical Industries, Tokyo) was added. At 24 h after the anti-HCP sera injection, 40 µg/kg of C8/119S was intravenously administered. After 30 min, the animals were exsanguinated, the skin was removed, and the blue test sites were observed. The long and short diameters of the blue sites were measured, and judged positive when the average of the diameters was 5 mm or more (18,19).

RESULTS

Evaluation of purification steps The inclusion bodies with a wet weight of about 1,000 g were recovered from 300 l of culture

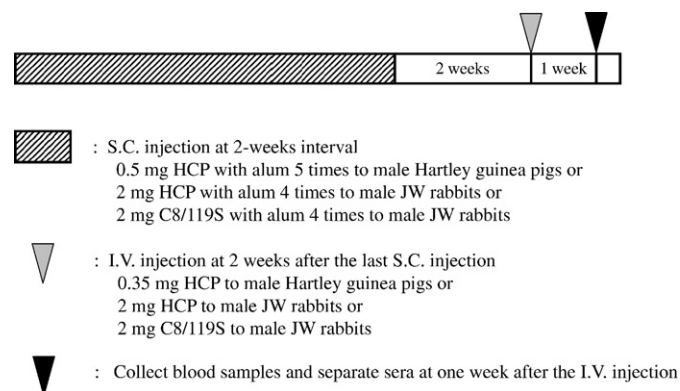


FIG. 1. Experimental schedule for the preparation of guinea pig and rabbit anti-host cell-derived protein sera, and rabbit anti-C8/119S sera.

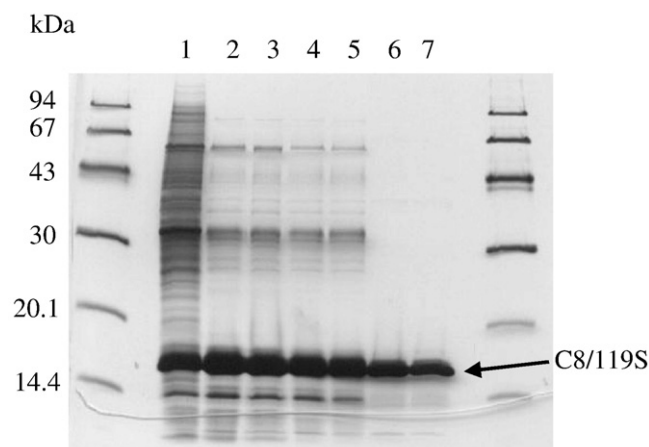


FIG. 2. SDS-polyacrylamide gel electrophoresis. Lane 1, refolding solutions; lanes 2 and 3, pass-through fraction on DEAE-Toyopearl chromatography; lanes 4 and 5, eluted fraction on Butyl-Sepharose chromatography; lanes 6 and 7, eluted fraction on QAE-Toyopearl chromatography. The gel was silver-stained.

medium. An aliquot of inclusion bodies with a wet weight of 100 g containing about 15 g of C8/119S was dissolved in the denaturing buffer, and refolding was performed by dilution of denaturing agent. After refolding, C8/119S was purified by DEAE-Toyopearl chromatography. In this step, most of the impurities derived from the culture process were captured by the DEAE resin, and C8/119S and aggregated C8/119S were passed through the DEAE column under equilibrium conditions of 20 mM Tris-HCl, pH 8.5, containing 50 mM sodium chloride (Fig. 2). In the Butyl-Sepharose FF chromatography step, C8/119S was recovered in the eluate concentrated to about five times the applied sample solution, which made it easy to exchange the buffer condition to apply the next chromatography step. In the QAE-Toyopearl chromatography step, aggregated C8/119S and residual impurities were removed and about 2 g of purified C8/119S was obtained (Table 1).

We performed the procedure four times in order to produce four purified batches to evaluate the ability of the purification process to remove impurities. The results of the SE-HPLC analysis of the purified C8/119S are shown in Table 2. The purity of the C8/119S monomer was 100% in FB-002, FB-006 and FB-007. In the FB-003, we detected 0.3% of C8/119S aggregate. The chromatogram of SE-HPLC is shown in Fig. 3. The purified C8/119S was tested to determine the bacterial endotoxin content, which was found to be less than 0.01 EU/mg (Table 3). The mass of C8/119S was measured with MALDI-TOF/MS. The result was 14130.98 (Fig. 4), and matched to the molecular weight (14133.03) calculated from the amino acid sequence shown in Fig. 5.

TABLE 1. Purification of C8/119S.

Purification step	C8/119S concentration (μg/ml)	Volume (l)	C8/119S amount (g)	Percentage yield (%)	Purity (%)
Refolding	90	160	14.4	100	–
DEAE Toyopearl Chromatography	700	10	7.0	49	–
Butyl Sepharose Chromatography	4,000	1.5	6.0	42	83
QAE Toyopearl Chromatography	750	3.0	2.3	16	100

The C8/119S concentration was calculated by ELISA using anti-Der f 2 antibody, and the purity was determined by size exclusion chromatography analysis. Percentage yield was calculated as follows. (Percentage yield, %) = (C8/119S amount at each step) * 100% / (C8/119S amount at refolding step).

TABLE 2. Results of the SE-HPLC analysis.

Batch no.	Purity of C8/119S monomer
FB-002	100%
FB-003	99.7%
FB-006	100%
FB-007	100%

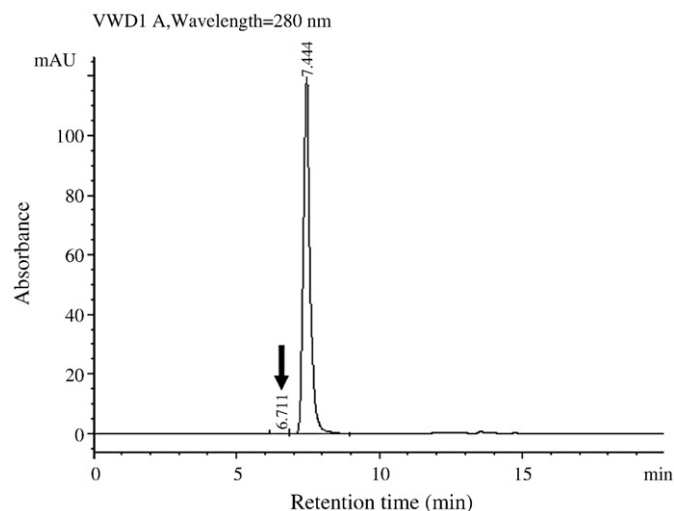


FIG. 3. The chromatograms of SE-HPLC. The sample is purified C8/119S, and the batch number is FB-003. The arrow shows C8/119S aggregate.

TABLE 3. Endotoxin content in C8/119S.

Batch no.	Endotoxin content
FB-002	0.0031 EU/mg
FB-003	0.0046 EU/mg
FB-006	<0.002 EU/mg
FB-007	<0.002 EU/mg

Limit of detection of the bacterial endotoxin was 0.002 EU/ml.

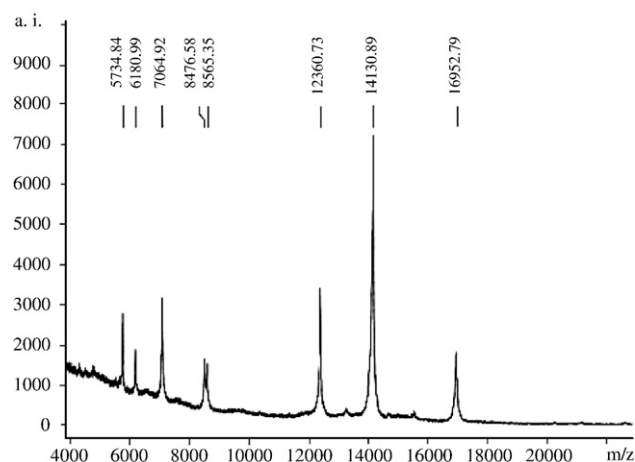


FIG. 4. Mass spectrometric pattern of purified C8/119S. Protein calibration standard I (Bruker) was used as a standard.

MDQVDVKDSANNEIKKVMVDGCHGSDPCI
 HRGKPF[—]TLEALFDANQNTKTAKIEIKASLD
 GLEIDVPGIDTNACHFMKCP[—]LVKGQYDAK
 YTNWVPKIAPKSENVVTVKLVGDNGVLAS
 AIATHAKIRD

FIG. 5. Amino acid sequence of Der f 2 mutant C8/119S. C8/119S is comprised of 130 amino acids, and its molecular weight calculated from amino acid sequence is 14133.03. The solid lines show the disulfide bonds.

Evaluation of the HCP content in C8/119S We performed an HCP-ELISA test to determine the levels of HCPs in the C8/119S. The content of HCPs in purified C8/119S was less than 0.5% which was equivalent to 100 ng per dose, based on the maintenance dose described in the WHO position paper (Table 4). In addition, the antigenicity of HCPs in purified C8/119S was evaluated by PCA assays, and the results are shown in Table 5. We used anti-HCP sera for the PCA assay at dilutions from 1:8 to 1:1024. No test sera challenged with 40 µg/kg of C8/119S exhibited local anaphylaxis (B-1, B-2 groups). All four of the test sites were judged positive in the positive control (B-3 group), and no test sites were judged positive in the negative control (B-4 group).

DISCUSSION

C8/119S expressed in *E. coli* was purified through three column chromatography steps. Under the equilibrium condition of DEAE chromatography, most impurities derived from culture were captured by DEAE resin but C8/119S was passed through the DEAE column. The impurities could be easily and efficiently removed by this process. However, aggregated C8/119S remained in the fraction passed through the DEAE column. To separate the C8/119S monomer and aggregates effectively, we evaluated the effect of denaturing agent in the mobile phase of QAE chromatography. We confirmed that it was possible to separate C8/119S and these aggregates by addition of urea to the mobile phase of QAE chromatography. On SDS-PAGE and SE-HPLC analysis, the amount of the aggregates and the degradation products were very little in the purified C8/119S. Moreover, the endotoxin level was also very low. To confirm whether purified C8/119S was complete molecule, we performed mass spectrometry analysis. The result was that the mass of C8/119S measured with MALDI-TOF/MS matched to the molecular weight calculated from the amino acid sequence. We confirmed that purified C8/119S was a complete molecule that had a target all amino acid sequence. We could obtain about 2 g of the purified C8/119S in one purification batch. This amount is equivalent to 100,000 of the maintenance doses required for immunotherapy based on the WHO position paper.

Subcutaneously injected C8/119S will be used as an allergen vaccine for the treatment of allergic diseases. Therefore, even if impurities are removed to a level that is difficult to be detected by physical analysis, it must not be assumed that no impurities are present. Residual HCPs that might induce an immune reaction would

TABLE 4. Residual HCP contents in C8/119S.

Batch no.	Residual HCP (%)
FB-002	0.2%
FB-003	0.1%
FB-006	0.4%
FB-007	0.3%

TABLE 5. Results of HCP antigenicity by passive cutaneous anaphylaxis (PCA) assay.

Group	Sensitizing sera	Challenging antigen	Positive ratio			
B-1	Anti-HCP sera (1:8 to 1:512)	C8/119S (40 µg/kg)	1:8	1:32	1:128	1:512
B-2	Anti-HCP sera (1:16 to 1:1024)		1:16	1:64	1:256	1:1024
B-3	Anti-C8/119S sera (1:1024)		0/2	0/2	0/2	0/2
B-4	PBS (1 ml/kg)				4/4	0/4

The batch number of the C8/119S test sample used as a challenge antigen was FB-002.

be highly undesirable. Thus we evaluated the antigenicity of the remaining HCPs by PCA assay. For this evaluation, we used 40 µg/kg of C8/119S. This dose was equivalent to 100–400 times the maintenance dose per kilogram of weight recommended for immunotherapy in the WHO position paper. No local anaphylaxis was observed in the test sera. It was suggested that the HCPs content in the C8/119S was low enough not to induce anaphylactic reaction in the PCA assay. Taken together, these findings indicated that C8/119S was highly purified and that the level of HCPs remaining in the purified C8/119S was extremely low.

We established an advanced production-scale purification process for C8/119S, which is a mutant of the major mite allergen Der f 2. The safety and effectiveness of C8/119S are expected to be confirmed in future studies.

References

1. Van-Bever, H. P. and Stevens, W. J.: Effect of hyposensitization upon the immediate and late asthmatic reaction and upon histamine reactivity in patients allergic to house dust mite (*Dermatophagoides pteronyssinus*), *Eur Respir J*, **5**, 318–322 (1992).
2. Mchies, J. J., Somville, M. A., Lebrum, P. M., Lebecque, S. J., Jacquemin, M. G., and Saint-Remy, J. M.: Allergic bronchial asthma due to *Dermatophagoides pteronyssinus* hypersensitivity can be efficiently treated by inoculation of allergen-antibody complexes, *J. Clin. Invest.*, **85**, 1024–1035 (1990).
3. Olsen, O., Larsen, K., Jacobsen, L., and Svenden, U.: A one year placebo-controlled double-blind house dust mite immunotherapy study in asthmatic adults, *Allergy*, **52**, 853–859 (1997).
4. Pichler, C., Marquardsen, A., Sparholt, S., Lowenstein, H., Bircher, A., Bischof, M., and Pichler, W. J.: Specific immunotherapy with *Dermatophagoides pteronyssinus* and *D. farinae* results in decreased bronchial hyperreactivity, *Allergy*, **52**, 274–283 (1997).
5. Ewan, P. W., Alexander, M. M., Snape, C., Ind, P. W., Agrell, B., and Dreborg, S.: Effective hyposensitization in allergic rhinitis using a potent partially purified extract of house dust mite, *Clin. Allergy*, **18**, 501–508 (1988).
6. Bousquet, J., Lockey, R., and Malling, H. J.: Allergen immunotherapy: Therapeutic Vaccines for allergic diseases. WHO Position paper, *J. Allergy Clin. Immunol.*, **102**, 558–562 (1998).
7. Thomas, W. R., Stewart, G. A., Simpson, R. J., Chua, K. Y., Plozza, T. M., Dilworth, R. J., Nisbet, A., and Turner, K. J.: Cloning and expression of DNA coding for the major house dust mite allergen Der p 1 in *Escherichia coli*, *Int. Arch. Allergy Appl. Immunol.*, **85**, 127–129 (1988).
8. Chua, K. Y., Stewart, G. A., Thomas, W. R., Simpson, R. J., Dilworth, R. J., Plozza, T. M., and Turner, K. J.: Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1 Homology with cysteine proteases, *J. Exp. Med.*, **167**, 175–182 (1988).
9. Chua, K. Y., Doyle, C. R., Simpson, R. J., Turner, K. J., Stewart, G. A., and Thomas, W. R.: Isolation of cDNA coding for the major mite allergen Der p II by IgE plaque immunoassay, *Int. Arch. Allergy Appl. Immunol.*, **91**, 118–123 (1990).
10. Haida, M., Okudaira, H., Ogita, T., Ito, K., Miyamoto, T., Nakajima, T., and Hongo, O.: Allergens of the house dust mite *Dermatophagoides farinae*—Immunochemical studies of four allergenic fractions, *J. Allergy Clin. Immunol.*, **75**, 686–692 (1985).
11. Yuuki, T., Okumura, Y., Ando, T., Yamakawa, H., Suko, M., Haida, M., and Okudaira, H.: Cloning and expression of cDNA coding for the major house dust mite allergen Der f II in *Escherichia coli*, *Agric. Biol. Chem.*, **55**, 1233–1238 (1991).
12. Yuuki, T., Okumura, Y., Ando, T., Yamakawa, H., Suko, M., Haida, M., and Okudaira, H.: Cloning and sequencing of cDNAs corresponding to mite major allergen Der f II, *Arerugi*, **39**, 557–561 (1990).
13. Nishiyama, C., Yuuki, T., Usui, Y., Iwamoto, N., Okumura, Y., and Okudaira, H.: Effects of amino acid variations in recombinant Der f II on its human IgE and mouse IgG recognition, *Int. Arch. Allergy Immunol.*, **105**, 62–69 (1994).

14. **Nishiyama, C., Fukada, M., Usui, Y., Iwamoto, N., Yuuki, T., Okumura, Y., and Okudaira, H.:** Analysis of the IgE-epitope of Der f 2, a major mite allergen, by in vitro mutagenesis, *Mol. Immunol.*, **32**, 1021–1029 (1995).
15. **Korematsu, S., Tanaka, Y., Hosoi, S., Koyanagi, S., Yokota, T., Mikami, B., and Minato, N.:** C8/119S mutation of major mite allergen Derf-2 leads to degenerate secondary structure and molecular polymerization and induces potent and exclusive Th1 cell differentiation, *J. Immunol.*, **165**, 2895–2902 (2000).
16. **Takai, T., Yokota, T., Yasue, M., Nishiyama, C., Yuuki, T., Mori, A., Okudaira, H., and Okumura, Y.:** Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy, *Nat. Biotechnol.*, **15**, 754–758 (1997).
17. **Koyanagi, S., Maeda, T., Murakami, T., Kawatsu, K., Sugawara, K., Miyatsu, Y., and Mizokami, H.:** Large-scale production of major house dust mite allergen Der f 2 mutant (C8/119S) in *Escherichia coli*, *J. Biosci. Bioeng.*, **106**, 387–392 (2008).
18. **Nagami, K., Matsumoto, H., Maki, E., Motegi, K., Aoyagi, K., Naruse, S., Samura, K., Losos, G. J., and Ikemoto, F.:** Experimental methods for immunization and challenge in antigenicity studies in guinea pigs, *J. Toxicol. Sci.*, **5**, 579–594 (1995).
19. **Watanabe, N. and Ovary, Z.:** Antigen and antibody detection by in vivo methods: A reevaluation of passive cutaneous anaphylactic reaction, *J. Immunol. Methods*, **14**, 381–390 (1997).