

## Short communication

# Expression of the American cockroach Per a 1 allergen in mammalian cells

**Background:** Cockroach allergens are one of the major etiologic risk factors for developing IgE-mediated allergic respiratory illness throughout the world. Per a 1 is a cross-reactive allergen of American and German cockroaches. This study aimed to investigate the expression of a recombinant American cockroach (*Periplaneta americana*) Per a 1, C42, allergen in mammalian COS-1 cells.

**Methods:** The COS-1 cells and *Escherichia coli* were used to express the *P. americana* C42 allergen. Recombinant proteins were purified with hydroxylapatite and DE52 chromatography. Biologic reactivities of recombinant proteins were examined by direct IgE binding and IgE inhibition studies with the enzyme-linked immunosorbent assay (ELISA).

**Results:** C42 was successfully expressed in the mammalian COS-1 cell as a 50-kDa secreted protein, and purified from the culture medium. The specific human IgE antibodies against recombinant C42 from either *E. coli* (C42-*E. coli*) or COS-1 (C42-COS-1) were compared by ELISA with 12 sera from Per a 1 and C42 skin-test-positive patients. All atopic sera contained specific IgE antibodies to C42 from either *E. coli* or COS-1. Moreover, recombinant C42-COS-1 bound higher levels of serum IgE than recombinant C42-*E. coli* among C42-sensitive atopic patients, and a statistically significant difference ( $P < 0.01$ ) was found between them. In addition, recombinant C42-COS-1 as an inhibitor revealed higher inhibition of IgE binding to natural Per a 1 than recombinant C42-*E. coli*.

**Conclusions:** The biologically highly reactive recombinant C42 produced in the COS-1 cell provides an alternative expression system and will facilitate studies on the immune response of asthma patients to cockroach allergens.

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Asthma has become a major health problem throughout the world (1). The rising trends of prevalence and mortality rate occur not only in Western countries, but also in some countries of the Far East such as Taiwan (2, 3). Aerosolized particles derived from cockroaches induce respiratory allergy (4). The major domiciliary cockroaches are the American and German cockroaches. A high prevalence of cockroach hypersensitivity in atopic (20–55%) and asthmatic (49–60%) populations has been documented worldwide (5–8), including Taiwan (atopic, 50.7%; asthmatic, 57.5%) (9). Per a 1 of the American cockroach, which comprises a family of isoallergens (10) containing internal repeat sequences, is the cross-reactive allergen between species. These molecules were found to have significant sequence identities with a German cockroach (*Blattella germanica*) Bla g Bd90K (11) and ANG12. ANG12 is a probable secretory protein with unknown function, which is induced in the midgut of female *Anopheles gambiae* after a meal. Recently, an additional member of the *P. americana* Per a 1 allergen family, C42, has been defined at molecular

level, characterized, and shown unequivocally to be the cross-reactive allergen between *P. americana* and *B. germanica* (12). Recombinant C42 is a 50-kDa protein and sequence homology searches revealed 27% identity to ANG12 and 71% identity to Bla g Bd90K. Both Bla g Bd90K and C42 allergens contain several internal repeats, and the crude extract from *B. germanica* was able to inhibit the binding of IgE to C42.

Cloning eukaryotic genes in *E. coli* is the best way to obtain a large amount of reasonably well-purified allergens for clinical use. To date, the best construct encoding cockroach allergens has been recombinant proteins from various vectors expressed in *E. coli*. As an alternative, we investigated the FLAG system to express *P. americana* C42 allergen in the mammalian COS-1 cell.

## Material and methods

Expression and transfection of mammalian cells

The cDNA encoding the full-length clone C42 was

excised from the plasmid pSPORT1-C42 by double-digestion with *Sall* and *XbaI* (12). The *Sall/XbaI* was then cloned into pFLAG-CMV-1 (Eastman Kodak, New Haven, CT, USA) and digested with the same enzymes to generate pFLAG-CMV-1-C42. The resulting plasmid was transformed into *E. coli* DH5 $\alpha$  for amplification. The host strain carrying inserts was plated on Luria-Bertani medium plus 50  $\mu$ g/ml ampicillin according to the supplier's instructions, and plasmid DNA of the ampicillin-resistant colonies was isolated to identify the positive clones. For expression in mammalian cells, COS-1 cells (ATTC CRL 1650) were maintained in Dulbecco's modified Eagle mediums/F-12 (Life Technologies, Gaithersburg, MD, USA) supplemented with 0.5% fetal bovine serum and 9.5% TCM<sup>TM</sup> (ICN Biomedicals, Inc., Aurora, OH, USA), a serum-free medium, to approximately 75% confluence in 9-cm<sup>2</sup> culture dishes. The cells were transfected with 8.0  $\mu$ g of vector DNA with DEAE-dextran and chloroquine (Sigma Chemical, St Louis, MO, USA), as described by Kriegler (13). At 24 h after transfection, the culture medium was removed and fresh culture medium supplemented with 1.0  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and 1.0  $\mu$ g/ml antipain was then added, and incubation was continued at 37°C for another 48 h. The culture medium was then recovered, and fresh medium containing protease inhibitors was added. At 144 h, the culture medium was recovered and pooled with the other samples.

#### Purification of secreted recombinant C42

The pooled culture medium (yield typically 1.0 mg recombinant protein/l of culture) was concentrated by 80% ammonium sulfate precipitation and dialyzed against buffer A (50 mM sodium phosphate, pH 7.4, and 1.0 mM EDTA). The dialyzed culture medium was applied to a hydroxylapatite column equilibrated with the same buffer. The column was washed with buffer A, and the proteins were eluted by 50 mM to 500 mM sodium phosphate gradient. The fraction containing C42, which migrated at 50 kDa on SDS-PAGE gels, was pooled, concentrated, and then dialyzed against buffer B (20 mM Tris-HCl [pH 7.4], 50 mM NaCl, and 0.1 mM EDTA). The pooled fraction was then loaded onto an anion exchange DE52 (Whatman International, Kent, UK) column equilibrated with buffer B. After washing of two column volumes with the same buffer, proteins were eluted with 50 mM to 2.0 M NaCl gradient. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA using anti-Per a 1 monoclonal antibodies (mAbs) (12, 14), and fractions that contained C42 protein were pooled, dialyzed against phosphate-buffered saline (PBS), concentrated, and then frozen at -70°C.

#### Expression in *E. coli* and purification of recombinant protein

C42 in pET 21 was overexpressed in *E. coli* BL21 (DE3), and the purification of recombinant protein was performed as previously described (12).

#### Cockroach extracts and preparation of Per a 1

Crude American cockroach extract (CRa-A) was prepared by extracting the whole body of insects with Coca's solution in our laboratory (15). Per a 3 and Per a 1 were purified from the CRa-A by gel filtration (15) and preparative isoelectric focusing (14), respectively.

#### SDS-PAGE and immunoblotting

Protein samples were loaded on a 5% polyacrylamide stacking gel above a 12% separating gel, and the gel was run with discontinuous buffer by Laemmli's method (16). After electrophoresis, gels were fixed and stained with 0.2% Coomassie brilliant blue R-250. For immunoblotting, gels were transferred electrophoretically to polyvinylidene difluoride membranes (Millipore Corp., Marlborough, MA, USA) (17), and immunodetection was performed as previously described (12).

#### Direct binding of IgE and binding inhibition to Per a 1

Purified recombinant C42 (0.5  $\mu$ g/well) from either *E. coli* or COS-1 or Per a 1 (0.5  $\mu$ g/well) in coating buffer (0.1 M sodium carbonate, pH 9.6) was coated onto each well and incubated at 37°C for 2 h for direct ELISA. After incubation, the wells were washed five times with PBS-0.01% Tween (PBST) and then filled with 1% normal goat serum and incubated for 2 h at room temperature. The wells were washed and incubated overnight at 4°C with either 1:10 dilution of patient serum for direct binding or with different concentrations of CRa-A, natural Per a 1, Per a 3, recombinant C42-COS-1, and recombinant C42-*E. coli* which had been preincubated for 2 h with the patient serum pool (1:10) at room temperature for inhibition binding. Twelve sera from natural Per a 1- and recombinant C42-skin-test-positive atopic and non-atopic patients (using natural Per a 1 as antigen) were used for direct binding. Atopic sera constituted the patient serum pool for inhibition studies. All sera were diluted in PBST containing 1% bovine serum albumin (BSA). After washing, peroxidase-labeled goat antihuman IgE (1:100, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) in PBST containing 1% normal goat serum was reacted with the wells for 2 h at room temperature. The excess antibodies were washed, and the reactions were developed and determined as previously described (12).

## Results

### Transient transfection and expression time course

The vectors, pFLAG-C42 and control pFLAG-BAP, were transfected into COS-1 cells under the optimized conditions, and the medium was collected 48 h after transfection. The transfection efficiency was examined by checking the amount of secreted allergens in the culture medium by ELISA using anti-Per a 1 mAbs. In order to obtain maximal expression and secretion, we then measured the amount of secreted allergens at various time points. The amount of secreted allergen peaked at 72 h after transfection and leveled off for another 72 h. Cell viability decreased rapidly 6 days after transfection. Therefore, we collected cultured medium twice, at 72 and 144 h after transfection, for the purification.

### Purification of COS-C42

Larger-scale transfections were performed, and cultured media were collected at 72 h and 144 h after transfection to generate sufficient proteins for the purification. Pooled COS-C42 culture medium was first applied onto a hydroxylapatite column, and anti-Per a 1 mAbs based ELISA- and immunoblotting-positive fractions were pooled. After the application to hydroxylapatite, most of the BSA was removed, but it still remained the predominant protein (Fig. 1A and B, lane 3). The amount of C42 was enriched after the DE52 column and

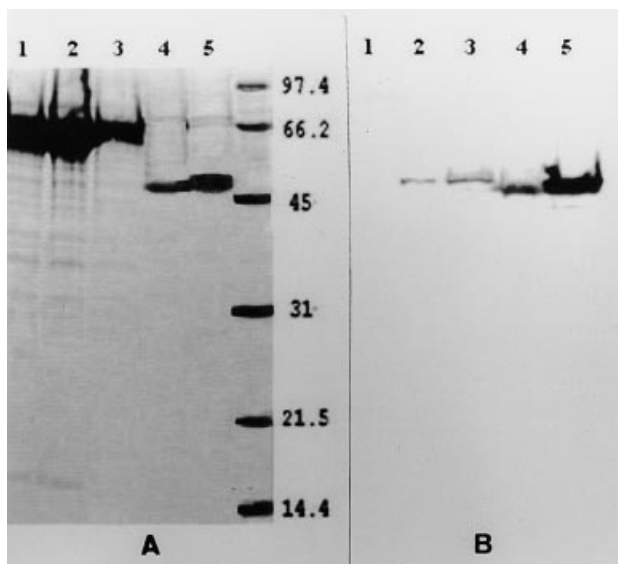


Figure 1. SDS-PAGE (A) and immunoblotting (B) of C42 purification. Proteins were stained with Coomassie brilliant blue (A), or probed with anti-Per a 1 mAbs (B). Lanes 1–5) culture medium only (lane 1), medium from COS-1 cells transfected with pFLAG-C42 (lane 2), pooled protein fractions after use of hydroxylapatite column (lane 3), C42 after DE52 purification (lane 4), and C42 purified from *E. coli* (lane 5). Numbers at center indicate sizes of protein markers.

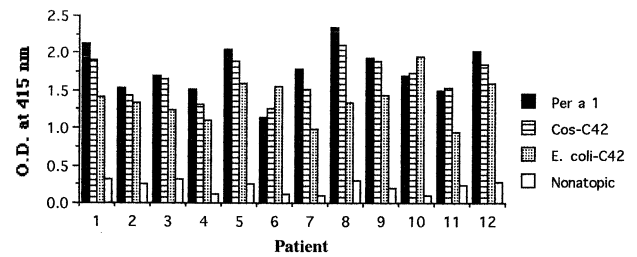


Figure 2. Comparison of recombinant C42-COS-1 and C42-*E. coli* immunoreactivity with IgE-binding ELISA.

could be detected on Coomassie brilliant blue-stained SDS-PAGE and immunoblotting (Fig. 1A and B, lane 4).

### IgE-binding and inhibition studies

The specific human IgE antibodies against recombinant C42 from either *E. coli* or COS-1 were compared by ELISA with 12 sera from Per a 1- and C42-skin-test-positive patients and 12 sera from nonatopics. All atopic sera contained specific IgE antibodies to natural Per a 1, and recombinant C42 from either *E. coli* or COS-1. The optical densities (OD) ranges were 1.523–2.122, 1.318–2.116, and 0.946–1.958 for the atopic sera with natural Per a 1, recombinant C42-COS-1, and recombinant C42-*E. coli*, respectively, and the OD range was 0.104–0.324 for nonatopic with natural Per a 1 (Fig. 2). The mean absorbances and standard deviations of natural Per a 1, recombinant C42-COS-1, and recombinant C42-*E. coli* and nonatopic were  $1.777 \pm 0.329$ ,  $1.678 \pm 0.265$ ,  $1.373 \pm 0.285$ , and  $0.215 \pm 0.087$ , respectively. A significant statistical difference ( $P < 0.01$ ) was found between atopic and nonatopic ( $P < 0.01$ ), natural

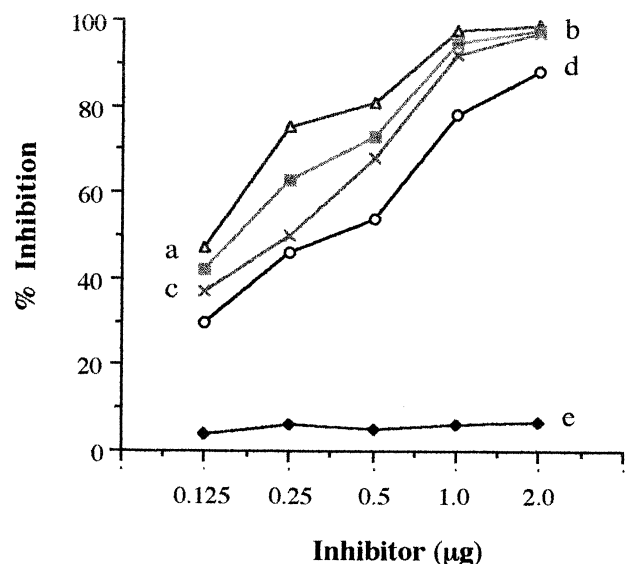


Figure 3. Binding inhibition of IgE to Per a 1 by Per a 1 (a), C42-COS-1 (b), C42-*E. coli* (c), CRa-A (d), and Per a 3 (e). Microtiter plates were coated with Per a 1, and results are average of experiments performed in triplicate as described.

Per a 1 and recombinant C42-COS-1 ( $P < 0.05$ ), natural Per a 1 and recombinant C42-*E. coli* ( $P < 0.01$ ), recombinant C42-COS-1 and nonatopic ( $P < 0.01$ ), and recombinant C42-*E. coli* and nonatopic ( $P < 0.01$ ) with the *t*-test. Moreover, a significant statistical difference ( $P < 0.01$ ) was found between recombinant C42-COS-1 and recombinant C42-*E. coli*. These atopic sera were used for the inhibition of IgE binding to natural Per a 1 and different cockroach allergens tested as inhibitors (Fig. 3). CRa-A, natural Per a 1, recombinant C42-*E. coli*, and recombinant C42-COS-1 inhibited 45%, 75%, 49%, and 64% of IgE binding to natural Per a 1 at 0.25  $\mu$ g, respectively. The lower degrees of inhibition found in CRa-A may have been due to the mixtures of different components in the inhibitor. As expected, no significant inhibition of Per a 3 was observed.

## Discussion

The production of a recombinant protein in *E. coli* is a cornerstone of the biotechnology industry. However, various difficulties are frequently encountered when expressing heterologous genes in this organism (18, 19). To overcome these problems, many studies have expressed heterologous secretory proteins in mammalian cells and have shown that they can be successfully produced and are functional (20). In this study, we first attempted to purify the secreted allergens in the mammalian cell culture medium with protein G-Sepharose immobilized by anti-FLAG M2 mAb. However, we found recombinant C42 protein always copurified with BSA, forming complexes with the allergens and binding to the affinity column. We had reduced the BSA contained in the medium by replacing most of the FBS with TCM, a method which decreased unbound BSA by 90%. We then tried to remove the BSA by passing the culture medium through a Cibacron Blue coupled Sepharose 6 column, a BSA affinity resin which is commonly used for

separating albumin from proteins of interest, but this was also unsuccessful. In order to break the interactions between the allergen and BSA, we used a different ion-exchange column. More than 70% of the BSA was separated from the recombinant C42 by hydroxylapatite chromatography. Purification was achieved after a weak anion-exchanging DE52 column was used to strip off the rest of the BSA.

Over the last 5 years, eight cockroach allergens (10–12, 21–27) have been cloned, all cloned and expressed in *E. coli*. In the present study, we have successfully expressed C42 in a mammalian COS-1 cell, and we purified the protein by ion-exchange chromatography. Immunoblotting revealed that both recombinant C42-COS-1 and recombinant C42-*E. coli* were recognized by anti-Per a 1 mAbs. Moreover, the specific IgE antibodies to both recombinant C42 proteins were detected in all 12 recombinant C42-skin-test-positive subjects by ELISA, and recombinant C42-COS-1 bound significantly higher levels of serum IgE than recombinant C42-*E. coli* in Per a 1- and C42-sensitive atopic patients. Recombinant C42 contains no cysteine nor any potential glycosylation site. The lower degrees of biologic reactivity found in recombinant C42-*E. coli* may be due to the fact that the protein produced in *E. coli* is not post-translationally modified, and protein modifications are usually crucial to activity. The biologically highly reactive recombinant C42 produced in the COS-1 system provides an alternative expression system. The use of mammalian cell-expressed recombinant Per a 1 and other cockroach allergens will facilitate studies on the immune response in patients allergic to cockroach.

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