

American cockroach Cr-PI allergen induces lymphocyte proliferation and cytokine production in atopic patients

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Summary

Background Previous studies have shown cockroach-induced antigen-specific IgE-mediated asthma. In cockroach-infested areas, more than 50% of asthmatic subjects may have positive skin reactions to this allergen. Partial purified Cr-PI allergen from American cockroaches contains allergens with molecular weights of 72 and 78 kDa; however, little is known about its effect on the lymphocyte proliferation and cytokine production.

Objective IgE synthesis is known to be regulated by interleukin-4 (IL-4) and interferon gamma (IFN γ). Therefore, we studied Cr-PI allergen-induced cytokine production in atopic patients and healthy normal controls to understand each factors' role in the disease.

Methods Peripheral blood mononuclear cells (PBMC) from cockroach skin-sensitive patients and controls were stimulated with mitogen and Cr-PI for proliferative response and cytokine production. Cr-PI antigen-specific T-cell cultures of atopic patients and healthy normal controls were used to test Cr-PI-induced proliferation and cytokine mRNA expression.

Results PMBC of atopic subjects showed a significantly ($P < 0.01$) higher stimulation index for Cr-PI induced proliferation ($SI = 11.8 \pm 3.7$) when compared with that of non-atopic subjects ($SI = 4.1 \pm 0.8$) and cord bloods ($SI = 2.1 \pm 0.4$). Cr-PI-induced IL-4 was observed only in the PBMC of atopic patients, whereas Cr-PI-induced IFN γ was detected in both atopic patients and normal controls. Likewise, Cr-PI-induced IL-4 mRNA expression in T-cell cultures was detected in all atopics but only one of nine controls.

Conclusion IL-4 mRNA expression and IL-4 production in PBMC and T-cell cultures of atopic patients showed good correlation with clinical symptoms, skin-reactivity, specific IgE and proliferative response to Cr-PI. These results suggests that cockroach allergen may be a hidden cause of asthma and other atopic diseases.

Keywords: atopic, cockroach allergen, PBMC, T-cell culture, lymphoproliferation, IL-4, IFN γ , mRNA

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Introduction

Inhalant allergens are known to induce immediate bronchospasm in asthmatic subjects [1–3]. Elevated

cockroach-specific IgE antibodies have been detected in the sera of allergic patients and more than 50% of asthmatic patients in cockroach-infested areas show a positive skin reaction to cockroach allergens [4–6]. Furthermore, cockroach antigen-specific IgE can induce asthma in sensitive patients [4,7]. Two allergenic fractions (Cr-PI) and Cr-PII) have been isolated from

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crude American cockroach (*Periplaneta americana*) extract (CRa-A). From the Cr-PI fraction, protein components of 72 and 78 kDa have been identified as major allergens [8]. Subsequently, a panel of monoclonal antibodies was produced and they reacted strongly with the 72 kDa and weakly with 78 kDa proteins of *P. americana* [9]. Recently, biologically active mRNA specific for proteins corresponding to Cr-PI [10] and cDNA clones encoding 72 kDa and 78 kDa allergens of American cockroaches have been isolated [11]. These findings suggest that cockroach allergen may also be a hidden allergic factor in patients with asthma [12].

Recent studies indicate that IgE synthesis is regulated by interleukin-4 (IL-4) and interferon- γ (IFN γ). IL-4 is important in the induction of IgE synthesis in peripheral blood mononuclear cells (PBMC), while IFN γ down-regulates IL-4-induced IgE secretion and negatively affects the development of TH₂ cells [13–15]. IL-4 producing TH₂ cells, it has been suggested to be present in allergic patients and involved in the elevated IgE production [16,17]. Aberrant cytokine productions in PBMC of allergic patients have been reported to involve both non-specific mitogens such as phytohaemagglutinin (PHA), phorbol esters and specific allergens such as mite and grass pollen [18–22]. Higher levels of IL-4 and lower levels of IFN γ have been observed in the PBMC of atopic patients, when compared with that of nonatopic healthy donors [19,21]. However, there have been no previous reports regarding cockroach allergen induced proliferative response and cytokine production in atopic subjects. The present study examines the Cr-PI-specific proliferative response in PBMC of CRa-A-sensitive atopic and normal subjects. The production of IL-4 and IFN γ as well as their gene expressions, in fresh PBMC and T-cell lines, were analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) assays.

Materials and methods

Patients and specimens

During an 8-month period (January–August 1994), 114 patients were selected from the allergy clinic at Taichung Veterans General Hospital, Taichung. All patients with allergic symptoms, including asthma, allergic rhinitis and urticaria underwent skin-prick tests (SPT) with commercial American cockroach (1:20 w/v, Greer Laboratories, Lenoir, NC, USA) in addition to 48 other common inhalant allergens. Eleven normal subjects were included as controls. The mean age of the patients was 35 years, with a range of 23–68 years. Allergen extract (0.02 mL) was pricked intradermally with a 27-gauge disposable

needle. Physiological phosphate-buffered saline (PBS) containing 50% glycerol and histamine-PBS solution (1.0 μ g/mL) were used as negative and positive controls, respectively. The result was read after 15 min by measuring erythema and weal diameters. Interpretation was based on the following criteria: (–) no weal or erythema or same size as negative control, (1+) weal <5 mm, erythema <10 mm, (2+) weal 5–7 mm, erythema >20 mm, (3+) weal 7–10 mm, erythema >20 mm, (4+) weal >10 mm or pseudopods, erythema >20 mm. Subjects with a positive SPT were given a fluoroallergo-sorbent test (FAST) to determine the concentration of serum anti-CRa IgE antibodies.

FAST

Optimal concentrations of crude CRa-A (4.0 μ g/well) were determined by checkerboard titration and coated onto blank microtitre wells (3M Diagnostic Systems Inc., Santa Clara, CA, USA) in our laboratory. Cockroach-specific IgW antibody levels in atopic subjects, were determined by an enzyme immunoassay kit (IgE FAST-Plus Test, 3M Diagnostic System). The test was performed according to the manufacturer's instruction [4]. A class-scoring system based on specific IgE concentration was assigned according to the manufacturer's recommendation: class 0 <0.35 IU/mL; class I 0.35–0.99 IU/mL; class II 1.0–2.99 IU/mL; class III 3.0–6.99 IU/mL; class IV 7.0–16.9 IU/mL; class V 17.0–43.99 IU/mL; and class VI >44.0 IU/mL.

Allergen preparation

American cockroach (*P. americana*) allergens were extracted with CoCa's solution and Cr-PI was separated from CRa-A, by sephadex G-150 SF gel filtration, as described [8]. Protein concentrations were assayed by the method of Bradford, using bovine serum albumin as a standard [23].

Culture methods

PBMC from patients and healthy donors were prepared from heparinized whole blood by Ficoll-paque (Pharmacia, Piscataway, NJ, USA) gradient separation. The cells were washed three times and suspended with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, Utah), 2 mM glutamine, 25 U/mL penicillin and 25 μ g/mL streptomycin in all experiments. For antigen-specific T-cell culture, PBMC (3×10^6 /mL) were suspended in AIM-V medium (GIBCO/BRL, Gaithersburg, MD, USA) and stimulated with Cr-PI (20 μ g/mL) for 5 days at 37°C and 5%

Table 1. Skin-test reactivity of cockroach allergen in atopic patients and normal controls

Subjects	(number)	Allergen skin-test					(% Positive)
		0	1+	2+	3+	4+	
Atopics	114	80	25	4	2	3	(29.8%)
Normal	11	10	1	0	0	0	(9.1%)

Atopic patients (age 21–69, mean 35 years) including asthma, allergic rhinitis, urticaria were selected from the allergy clinic at Taichung Veterans General Hospital. Age-matched normal volunteers of both sex were included as controls.

CO₂ in a culture dish. Human recombinant IL-2 (Genzyme Diagnostic, Cambridge, MA, USA) 20 U/mL and irradiated feeder cells (1×10^6 /mL) were added to the culture, and allowed to incubate for 9 days [24]. PBMC or T-cell cultures (1×10^6 cells/mL) were stimulated with mitogens or Cr-PI for 24 h to isolate RNA.

Proliferation assay

PBMC (2×10^6 cells/mL) of atopic patients and healthy controls were incubated with 1 µg/mL PHA (Wellcome Diagnostics, Hartford, USA) or Cr-PI (5 µg/mL) in AIM-V medium for 6 days at 36°C in an atmosphere of 5% CO₂ [25]. ³H-thymidine (TdR) (0.5 µCi) was added to each well of the 96-well microtitre plate during the final 24 h of incubation. Cultures were harvested, followed by determination of ³H-TdR incorporation by scintillation counter (Packard TRI-CARB 2200 CA, Downer Grove, IL, USA). The stimulation index (SI) values were calculated as follows: SI = CPM in the presence of cockroach allergens/CPM in the absence of cockroach allergens.

Cytokine production

PBMC or T-cell cultures (1×10^6 /mL) were stimulated with 10 µg/mL Cr-PI allergen, 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and ionomycin (200 ng/mL) or 5 µg/mL PHA for 24 to 96 h at 37°C and 5% CO₂. Supernatants from cell culture were harvested by centrifugation and stored at –70°C until assayed.

IL-4 and INFγ assay

Determination of cytokine production in each sample was performed with by commercial enzyme-linked immunosorbent assay (ELISA) kits. Supernatants were assayed for cytokine production with an IL-4 kit from R&D System Human PBMC (2×10^6 /mL) from FAST-positive atopic patients were studied for proliferative response to Cr-PI over a period of 6 days. Cr-PI-specific lymphoproliferation in T-cell cultures of atopic patients and controls was dose-dependent (Fig. 1). The PBMC of atopic subjects had a significantly ($P < 0.01$) higher stimulation index (SI = 11.8 ± 0.4) (Table 2).

Table 2. Comparison of Cr-PI-induced proliferation in PBMC of atopic and healthy normal subjects

Group (number)	Medium only	PHA (1µg/mL)	Cr-PI (5µg/mL)	Stimulation index
Atopic				
FAST > 3 (5)	316 ± 85	31466 ± 7190	3235 ± 751	11.8 ± 3.7*
FAST > 3 (7)	433 ± 68	35958 ± 7051	1438 ± 407	3.5 ± 0.7
Non-atopic (9)	314 ± 21	35420 ± 3202	1643 ± 322	4.1 ± 0.8
Cord blood (17)	841 ± 168	44022 ± 4631	1492 ± 322	2.1 ± 0.4

*PBMC (2×10^6 cells/mL) from atopic patients and healthy controls were incubated with PHA or Cr-PI for 6 days at 37°C in an atmosphere of 5% CO₂. ³H-TdR (0.5 µCi) was added to the microtitre plate during the final 24 h incubation. Data are expressed as the mean cps of triplicate ³H-TdR incorporation. $P > 0.01$.

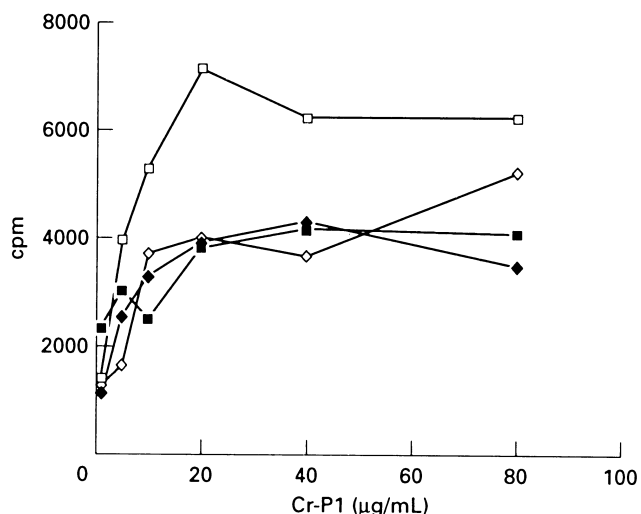


Fig. 1. Dose-dependent Cr-PI-induced proliferation in T-cell cultures of atopic patients (P) and healthy normal controls (N). Cell proliferation was expressed as the mean cpm of ^3H -TdR incorporation from triplicate samples.

IL-4 and $\text{INF}\gamma$ production induced by Cr-PI

In the following experiments we compared the effects of Cr-PI allergen, PMA and ionomycin and PHA on IL-4

and $\text{INF}\gamma$ production in PBMC. Atopic patients showed significantly higher IL-4 production in PBMC, either induced by PMA and ionomycin or Cr-PI, when compared with healthy normal controls after incubation for 48 h (349 ± 132 vs 75 ± 19 pg/mL, $P \leq 0.01$ and 9 ± 6 vs 0 pg/mL, $P < 0.01$; respectively) (Table 3). Using a commercially available ELISA kit with a sensitivity of 5 pg/mL, IL-4 production was not detected in either unconcentrated or 10-fold concentrated culture supernatants of Cr-PI-treated PBMC of healthy normal controls. In contrast, Cr-PI induced $\text{INF}\gamma$ production in PBMC could be easily detected in the culture supernatants by a commercially available ELISA kit with a sensitivity of 0.03 IU/mL. However, no significant difference in $\text{INF}\gamma$ production in PBMC, induced by PMA and ionomycin or Cr-PI among normal controls and the atopics (1321 vs 1161 IU/mL and 46 vs 17 IU/mL, respectively; $P > 0.05$). PHA induced a similar trend with slightly lower levels of IL-4 and $\text{INF}\gamma$ production than PMA and ionomycin, in both atopic and non-atopic subjects (data not shown).

IL-4 and $\text{INF}\gamma$ mRNA expression

Since IL-4 production was low or could not be detected in the culture supernatants of Cr-PI-inducing PBMC, we

Table 3. IL-4 and $\text{INF}\gamma$ production in PBMC of atopic and non-atopic subjects

Subject	Disease	Clinical skin		FAST Class	IL-4 (pg/mL)		IFN γ (iU/mL)	
		Severity	reactivity		PMA + I	Cr-PI	PMA + I	Cr-PI
Atopic patients								
LK	Asthma	Severe	Multiple	IV	170*	10	790	2
TS	Asthma	Severe	Multiple	II	230	30	819	10
HY	Rhinitis	Severe	Multiple	III	420	0	1452	242
LM	Urticaria	Moderate	Multiple	I	826	5	1894	10
LS	Urticaria	Moderate	Multiple	II	550	0	851	177
X $\pm$ SE					439 ⁺ $\pm$ 132	8 ⁺ $\pm$ 6	1161 $\pm$ 221	46 $\pm$ 38
Normal controls								
LF		None	None	0	105	0	1267	10
WS		None	None	0	17	0	1101	30
TC		None	None	0	55	0	689	3
WJ		None	None	0	44	0	735	3
JK		None	None	0	160	0	3700	100
LH		None	None	0	121	0	1081	3
PS		None	None	0	14	0	1211	1
KJ		None	None	0	44	0	823	3
YC		None	None	0	93	0	1178	3
X $\pm$ SE					85 $\pm$ 25		1446 $\pm$ 507	25 $\pm$ 17

* PBMC (1×10^6 cells/mL) were stimulated with PMA (50 ng/mL) and ionomycin (200 ng/mL) or Cr-PI (10 $\mu\text{g/mL}$) and incubated for 48 h. $^+P < 0.01$.

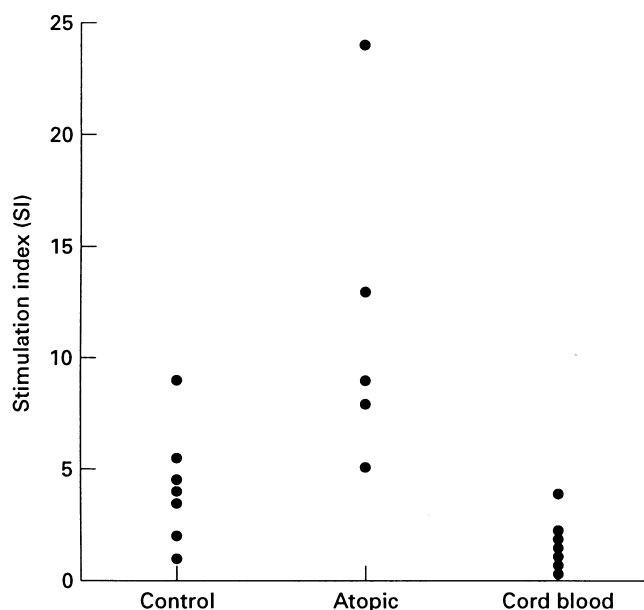


Fig. 2. Stimulation index of Cr-PI-induced proliferation in PBMC of atopic patients, healthy normal controls and cord blood. The SI of the atopic patient group was significantly higher than in other groups, $P < 0.01$, using Mann-Whitney rank test.

examined IL-04 gene expression in PBMC and T-cell cultures of atopic and healthy normal subjects. RNA was isolated from Cr-PI-stimulated PBMC at various time intervals. cDNA was reverse-transcribed and subjected to PCR with primers specific for IL-4, $\text{INF}\gamma$ and β -actin. The results are shown in Table 4 and Fig. 2. IL-4 mRNA level was induced by PHA or PMA and ionomycin in PBMC of both atopic and healthy normal subjects. However, IL-4 mRNA was detected in the Cr-PI-stimulated T-cell cultures of all atopics but only one (JK) healthy normal subject, although there was a similar level of β -actin at 24, 48 or 96 h after Cr-(Minneapolis, MN) and an $\text{INF}\gamma$ kit from MED-GENIX (Fleurus, Belgium) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction

Ribonucleic acid (RNA) was isolated by a commercial kit, Ultraspec RNA (Biotecx Lab. Inc., Houston, TX, USA). The extracted RNA from treated cells ($1 \times 10^6/\text{mL}$) was resuspended in diethyl pyrocarbonate (DEPC) treated water and incubated at 65°C for 5 min. After cooling, $1 \mu\text{g}$ of each sample was subjected to first-strand

Table 4. IL-4 and $\text{INF}\gamma$ mRNA expression by T-cell lines in atopic and non-atopic subjects

Subject	Disease	FAST Class	IL-4		IGN γ	
			PMA + I	Cr-PI	PMA + I	Cr-PI
<i>Atopic patients</i>						
LK	Asthma	IV	Positive*	Positive	Positive	Positive
TS	Asthma	II	Positive	Positive	Positive	Positive
HY	Allergic rhinitis	III	Positive	Positive	Positive	Positive
LM	Urticaria	I	Positive	Positive	Positive	Positive
LS	Urticaria	II	Positive	Positive	Positive	Positive
<i>Normal controls</i>						
LF		0	Positive	Negative	Positive	Positive
WS		0	Positive	Negative	Positive	Positive
TC		0	Positive	Negative	Positive	Positive
JK		0	Positive	Positive	Positive	Positive
WJ		0	Positive	Negative	Positive	Positive
LH		0	Positive	Negative	Positive	Positive
PS		0	Positive	Negative	Positive	Positive
KJ		0	Positive	Negative	Positive	Positive
YC		0	Positive	Negative	Positive	Positive

*T-cell cultures (1×10^6 cells/mL) from atopic patients and normal healthy donors were stimulated with PMA (50 ng/mL) and ionomycin (200 ng/mL) or Cr-PI (10 $\mu\text{g}/\text{mL}$) and incubated for 24 h. IL-4 and $\text{INF}\gamma$ mRNA expressions were determined by RT-PCR.

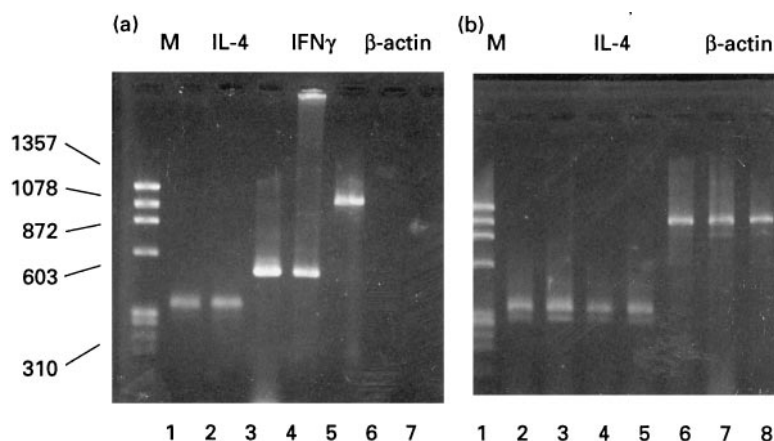


Fig. 3. (a) Analysis of RT-PCR products of cytokine mRNA fractionated electrophoretically in 3% agarose in one atopic patient (TS). Lane 1: DNA (Φ 174) molecular size marker; lane 2: PMA + I; lane 3: Cr-PI; lane 4: PMA + I; lane 5: Cr-PI; lane 6: CR-PI and lane 7: negative control (no cDNA). (b) The Cr-PI-induced PCR products of IL-4 (lanes 2–5) and β -actin (lanes 6–8) in patients (LK, HY, LM and LS). The PCR products of IL-4, IFN γ and β -actin are 456 bp, 494 bp and 1128 bp, respectively.

cDNA synthesis using Moloney murine leukaemia virus (MMLV) reverse transcriptase, oligo-dT₁₅, primer, and dNTP in the presence of RNasin in a 20 μ L reaction at 37°C for 30 min. Polymerase chain reaction (PCR) was performed with 10% of the product of cDNA synthesis, Taq polymerase (2.5 unit per reaction; Perkin Elmer Cetus), 50 pM of each pair of gene-specific primer and 200 μ M dNTP in a reaction buffer (10 mM Tris, 1.5 mM MgCl₂, 50 mM KCl and 0.1 mg/mL gelatine). Primer sequences were 5'ATGGGTCTCACCTCCCAACTG, 3'TCAGCTCGAACACTTTGAATATTTCTCTCTCAT for IL-4 (Clontech, Palo Alto, CA); 5'ATGAAATAT-ACAAGTTATATCTTGGCTTT, 3'GATGGCTCTTC-GACCTCCGAAACAGCAT for IFN γ and 5'ATGG-ATGATGATATCGCCGCG, 3'CTAGAAGCATTT-GCGGTGGACGATGGAGGGGCC for β -actin, as a control for the RNA isolation and transcription. PCR was carried out in a Perkin Elmer Cetus thermocycler for 30 cycles. The samples were initially denatured for 2 min at 95°C. Subsequent denaturation was performed for 2 min at 94°C. Primer annealing was carried out for 2 min at 55°C, and chain extension was allowed to proceed for 2 min at 72°C. The reaction product was visualized by electrophoresis in a 3% agarose gel (consisting of 2% Nusieve, and 1% agarose) and by staining with 0.5 μ g/mL ethidium bromide. Verification of the specific genes was established by their predicted size and restriction enzyme digestion of appropriately sized fragments under UV light.

Results

Proliferative response

Atopic patients from the Allergy Clinic, Taichung Veterans General Hospital were given a skin-prick test for CRa-A. Thirty per cent (34/114) tested positive, whereas only 9% (1/11) of healthy laboratory volunteers

tested positive (Table 1). Serum anti-CRa IgE antibodies were determined by FAST. PI stimulation. In contrast, IFN γ mRNA expression in T-cell cultures, induced by CR-PI, could be easily detected in both atopic and non-atopic subjects.

Discussion

It has been established for cockroach allergen causes allergic problems in asthmatic subjects living in the inner city [5,6]. The present study showed that atopic subjects had a higher incidence of positive SPT for CRa-A (29% comparable to other reports (33–55%)) [4–6,26]. Cr-PI included a significantly higher SI for proliferation in PBMC of atopic subjects who were SPT-and-FAST-positive for CRa-A, when compared with normal controls (Table 1). These findings are similar to other studies with purified pollen and house dust mite allergens [25,27]. It is perceivable that these reactions can be tentatively correlated to the degree of exposure in atopic patients.

It has been shown that T-cell recognition of aeroallergen may result in both the induction of specific IgE and the activation of mast cells [13,28]. IL-4 is important in the induction of IgE synthesis in B cells. IFN γ down-regulates IL-4-induced IgE and IgC4 secretion, and negatively affects the development of TH₂ cells [14,15]. In this study, IL-4 production in PBMC, induced by PMA and ionomycin, was significantly higher in atopic patients than in non-atopic subjects with a low total IgE and no detectable specific IgE. This agrees with the finding that the IL-4 production in PBMC of allergic patients, induced by phorbol esters or PHA, is higher than in healthy donors [18]. We found that Cr-PI was able to induce IL-4 in two patients with asthma and one with urticaria but not in other atopics or in non-atopics (Table 4). This agrees with the finding of a previous study that IL-4 production in PBMC induced by allergens only

in atopic patients with severe cases [19]. IL-4 producing TH₂ cells appear to be present in allergic patients and involved in the elevated IgE production and other pathophysiologies [16,17]. Thus, the proliferative response to cockroach allergen and IL-4 production in PBMC gives further evidence of the presence of TH₂-like cells in these atopic individuals. If exposure to cockroach allergen is prolonged, these cells may play a role in the initiation and maintenance of an allergic response. In contrast, the present study showed no difference in IFN γ production in PBMC induced by PMA and ionomycin among atopic patients and non-atopic subjects. In addition, Cr-PI was able to induce a similar level of IFN γ production in non-atopic and atopic subjects (Table 2). It is interesting to note that one non-atopic subject (JK) with very high IFN γ production showed a negative SPT, no specific IgE or Cr-PI-induced IL-4. An inverse correlation between IFN γ and specific IgE [13], as well as reciprocal regulatory effect of IL-4 and IFN γ [24] may partially explain this case. The results showed a proportional relationship for IL-4 and IFN γ , suggesting that more than one allergen-specific T-cell population exists in these individuals. Using RT-PCR, we were able to detect Cr-PI-induced IL-4 mRNA expression in T-cell cultures of all five SPT- and FAST-positive atopic patients with asthma, rhinitis or urticaria. However, among the nine SPT- and FAST-negative healthy non-atopic controls, Cr-PI induced IL-4 mRNA expression in T-cell cultures was detected in one (JK). These results are similar to another study that showed the IL-4 expression induced by allergen could not be detected in non-atopics or specific IgE-negative atopic patients [21].

The Cr-PI allergen that reacts with the serum [9] and monoclonal antibodies [10], induces a significant cell proliferation comparable to other major allergens such as house dust mite or grass pollen [18–20,25,27]. The allergen also induces IL-4 production in atopic patients. Furthermore, cytokine expression and production patterns correlate well with clinical symptoms, skin-reactivity, specific IgE and proliferative response in atopic patients. These results indicate that cockroach allergen may be a hidden cause of asthma and other atopic diseases. Future studies of immune response with more patients and defined cockroach Cr-PI allergen, such as recombinant protein, may be benefiting in diagnosis and monitoring patients for therapy.

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