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Specific IgE and IgG Responses and Cytokine Profile in Subjects with Allergic Reactions to Biting Midge *Forcipomyia taiwana*

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Key Words
Bites, insect · Cytokine · *Forcipomyia taiwana* · Immune response · Midge · Protease

Abstract
Background: *Forcipomyia taiwana* is a tiny blood-sucking midge whose habitat covers large parts of Taiwan and southern China. Female midges bite during the day, causing intense pruritis and swelling in allergic individuals. In this study, we investigated the immune responses of different allergic reactions to midge bites. ***Methods:*** *F. taiwana* (midge)-specific IgE, -IgG and -IgG subclasses were examined by ELISA in 62 human subjects. Peripheral blood mononuclear cells (PBMC) from 6 subjects with solely delayed reactions (SDR) to midge bites and 6 nonallergic controls (NAC) were cultured with midge extract at various time points and assayed. Proliferation of PBMC was measured by MTT assay. Expression of cytokine mRNA was measured by real-time PCR and protein levels by cytometric bead immunoassay or ELISA. Protease activity in midge extract was determined by the Azocoll method. ***Results:*** Midge-specific IgE among subjects with an immediate reaction were significantly elevated compared to SDR and NAC subjects. There were no differences in the level of midge-specific-IgG, -IgG_1, -IgG_2, -IgG_3 and -IgG_4 among subjects with different biting reactions. Midge extract elicited significantly more PBMC proliferation, higher expression of IFN-γ, IL-10, IL-6 and TNF-α in SDR subjects than in NAC. Protease activity was detected in midge extract. Protease inhibitors E64 and pepstatin suppressed midge-extract-induced IL-8 production. ***Conclusions:*** Our results suggest that an immediate reaction to midge bites is IgE-mediated. IFN-γ, IL-6 and TNF-α are involved in delayed reactions to midge bites. A protease-activated pathway may also be involved in the intense, itchy reactions to midge bites.

Introduction

Forcipomyia taiwana is a tiny (1–1.5 mm) blood-sucking midge which is widely found in urban and suburban habitats in Taiwan and southern China [1, 2]. Like mosquito bites, midge bites can cause allergic reactions. During the day, groups of *F. taiwana* attack exposed parts of the human body [3], causing intense pruritis and swelling in allergic individuals.

There are 2 types of reactions after midge bites: (1) immediate reaction, in which a large local swelling develops within 1 h of bites, and (2) delayed reaction, in which intense itching papules and vesicles develop 6–24 h after

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bites. The delayed-type lesions may turn centrally necrotic several days later and last for weeks or even months. We have previously reported that about 59.1% of exposed subjects develop hypersensitivity reactions to midge bites [4]. Among the midge-allergic subjects, 14% developed a solely immediate reaction, 43% developed an immediate reaction followed by delayed reactions and 43% developed a solely delayed reaction. Subjects with insect allergy frequently curtail outdoor activities because of the annoying allergic reactions, leading to a significant worsening of their quality of life [5].

Our previous study identified the 24-, 35/36- and 64-kDa proteins as major IgE-binding allergens of this midge [4]. However, the immune mechanism of delayed reactions to midge bites has not been well studied. Delayed reactions to midge bites usually last for weeks or even months, causing an even greater impact on the patient's quality of life than the rapidly resolved immediate reaction. Except for a few studies that focused on the immune mechanisms of mosquito bites [6–12], to the best of our knowledge, there have been no reports in the medical literature regarding the immune responses to midge bites in humans. In the present study, we examined specific IgE and IgG responses in subjects with allergic reactions to *F. taiwana* and cytokine changes in subjects with delayed reactions in order to further understand the immune mechanisms involved.

Materials and Methods

Preparation of Whole Body Midge Extract

Female *F. taiwana* were collected by the human bait method using special collectors designed by Dr. C.S. Chen's laboratory, Department of Life Science, Tunghai University, Taichung, Taiwan. One thousand female midges were ground and suspended in 5 ml of PBS, ultrasonicated for 30 min at 4°C and centrifuged at 8,000 *g* for 15 min. The supernatant was collected, filtered through a 0.22-μm filter, aliquoted and stored at –70°C. The protein concentration was determined with a Bradford assay reagent (Bio-Rad, Hercules, Calif., USA) [13].

Subjects

The project was reviewed and approved by the institutional review board of Taichung Veterans General Hospital. Recruitment of volunteer human subjects who had been exposed to *F. taiwana* bites was done through 2 channels: advertisement and the allergy clinic of our hospital. They were interviewed by a nurse regarding details of their reactions to midge bites and the skin lesions caused by midge bites in natural settings were examined by a physician (Y.-H.C.). Subjects were then skin-tested with the midge extract after giving their written informed consent. For the skin test we used midge extract at a concentration of 200 μg/ml in PBS containing 50% glycerol, with epicutaneous sterile disposable Sharp Test® applicators (Greer Laboratories, Lenoir, N.C., USA). Histamine (1 mg/ml) and 50% PBS-glycerol were used as positive and negative controls, respectively. All skin test results were read 20 min after placement for immediate wheal-and-flare reaction. A response with a wheal or an erythema 3 mm larger in size than that produced by the negative control was considered positive. 30–50 ml of blood was drawn after the skin test.

Subjects were divided into 3 groups based on their clinical symptoms and skin lesions: (1) those with an immediate reaction, regardless of the presence of delayed reactions; (2) those with solely delayed reactions, and (3) those who tolerated and had no reaction to *F. taiwana* bites. They constituted the nonallergic control group.

Detection of Midge-Specific IgE, IgG and IgG Subclasses

Indirect ELISA was used to determine the *F. taiwana* (midge)-specific IgE, IgG and IgG subclasses. After choosing optimal conditions by using checkerboard titration, microtiter plates (Nunc, Roskilde, Denmark) were coated with 3 μg/well midge extract in triplicate and incubated at 37°C for 2 h. In general, 3 washings were performed between each step. After blocking with 1% goat serum, the plates were incubated with 1:10 dilution of sera from subjects and incubated for 2 h at room temperature. After washing, antibody binding was detected using alkaline phosphatase-labeled mouse anti-human IgE (BD Pharmingen, San Diego, Calif., USA), IgG, IgG_1, IgG_2, IgG_3 or IgG_4 antibodies (Zymed Laboratories, San Francisco, Calif., USA) respectively, along with ρ-nitrophenyl phosphate (Sigma, St. Louis, Mo., USA) as substrate. The optical density (OD) was determined at 405 nm on a microtiter plate reader (Tecan, Grödig, Austria).

Preparation of PBMC

Peripheral blood mononuclear cells (PBMC) from subjects were separated from heparinized blood by centrifugation through Ficoll-Paque solution (Pharmacia, Uppsala, Sweden). After 3 washings with PBS, the cells were suspended in conditioned media composed of RPMI (Sigma) supplemented with 2 g/ml sodium bicarbonate, 2 mM L-glutamine, 5 mM HEPES buffer, 200 U/ml penicillin, 200 μg/ml streptomycin and 10% heat-inactivated autologous human serum.

Cell Culture and PBMC Proliferation by MTT Assay

PBMC (2×10^5) were cultured in 96-well flat-bottomed culture plates in duplicate at 37°C in an incubator with 5% CO_2 with: (1) media alone; (2) 0.2 μg/ml concanavalin A; (3) 0.3 μg/ml midge extract; (4) 3 μg/ml midge extract, or (5) 30 μg/ml midge extract. After 24 and 48 h of culture, 20 μl (5 mg/ml) of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; Sigma) was added into each well and incubated for an additional 1 h. 100 μl of dimethyl sulfoxide was added to dissolve the formazan crystals in viable cells. The absorbance of the converted dye was read at a wavelength of 570 nm with background subtraction at 650 nm with a microtiter plate reader (Tecan). Data were presented as a stimulation index by the following calculation: level of $OD_{570\,nm}$ in medium with midge extract/level of $OD_{570\,nm}$ in medium only at same time point [14].

Cell Culture for Cytokine Analysis

PBMC (1×10^6) were cultured in 12 × 75 mm BD Falcon™ cell culture tubes (BD Biosciences, Franklin Lakes, N.J., USA) in

duplicate in 1 ml of media alone, with concanavalin A (0.2 μg/ml) or midge extract (3 μg/ml) for 24, 72 and 144 h. Phorbol 12-myristate-13-acetate (PMA, 50 ng/ml) was then added and incubated for an additional 24 h. The samples were then pooled from the duplicate cultures and harvested. The cells were centrifuged and preserved in RNAlater (Ambion, Foster City, Calif., USA) for RNA extraction. The cell-free supernatants were stored at –70°C until further analysis.

RNA Extraction and cDNA Synthesis

Total RNA from PBMC was extracted using RNAzol reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, Calif., USA). The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer (Beckman DU7400). cDNA was synthesized from 1 μg of total RNA in a 20-μl reaction mixture containing 2.5 μM of oligo-dT primer, 1 mM deoxyribonucleoside triphosphates (dNTP), 5 μM dithiothreitol, 40 U of RNaseOUT RNase inhibitor, and 15 U of ThermoScript reverse transcriptase (Invitrogen). Reverse transcription was performed at 50°C for 60 min followed by a heat denaturation step at 85°C for 5 min with a thermocycler (Perkin-Elmer, Irvine, Calif., USA).

Cytokine mRNA Expression by Real-Time PCR

mRNA levels were quantified by subjecting cDNA to TaqMan PCR analysis using a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Überlingen, Germany). Predesigned sequence detection reagents specific for human IFN-γ, IL-10, IL-6 and TNF-α were purchased from Applied Biosystems. To normalize each sample for RNA content, we used β-actin as a control gene. Each probe has a fluorescent reporter dye (FAM) linked to its 5′ end and a downstream quencher dye (TAMRA) linked to its 3′ end. Each 20-μl PCR reaction mix included 1 × TaqMan Universal Master Mix with AmpliTaq Gold DNA polymerase, 1 × probe/primer mix and 50 ng cDNA. Amplification conditions consisted of 45 cycles of 95°C for 15 s and 60°C for 1 min after incubation at 95°C for 10 min. The threshold cycle C(t), the cycle number at which there was a detectable increase above background fluorescence, was determined for each sample using GeneAmp software. The results were expressed as fold changes between medium control and midge extract-stimulated cells after correcting by the housekeeping gene.

Cytokine Proteins in Culture Supernatants Using Cytometric Bead Immunoassay

Culture supernatant was analyzed simultaneously for 11 different cytokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IFN-γ, TNF-α and TNF-β, using the bead-based FlowCytomix (Bender MedSystems, Vienna, Austria) as described previously [15, 16]. Following a final wash, the contents of each well were suspended in 200 μl of assay buffer, placed in 12 × 75-mm polystyrene tubes, and then read on a FACScan™ flow cytometer (BD Biosciences). Cytokine concentrations were calculated by comparison with a standard curve for each cytokine derived from the cytokine standards assayed in the same manner.

Detection of Protease Activity in Midge Extract by Azocoll Method

Protease activity was determined by the Azocoll (Sigma) method as described previously [17]. Protease activity from partially purified cockroach allergen Cr PI [18, 19] was measured concomitantly for comparison. A different concentration of subtilisin (Sigma) from *Bacillus subtilis* was used as a standard.

Effects of Protease Inhibitors on Midge Extract-Induced IL-8 Release by A549 Cells

The A549 airway-derived epithelial cell line, purchased from the Bioresource Collection and Research Center, Taiwan, was grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. For the experiments, the cells were seeded in 6-well culture plates and grown to 80% confluence. To test the effect of protease inhibitors, midge extract was pretreated for 15 min at 37°C with or without the inhibitors before being added to A549 cells for 24 h. Culture supernatants were collected and stored at –70°C until assayed. The following protease inhibitors were used: phenylmethane sulfonyl fluoride (PMSF; 0.01–0.4 nM), E-64 (5–200 ng) and pepstatin (5–200 ng). All protease inhibitors were obtained from Sigma and dissolved in ion-free PBS (Dulbecco's PBS; Gibco, Invitrogen) before use. The concentration of IL-8 in the culture supernatants were measured using an ELISA kit (Bender) according to the manufacturer's instructions.

Statistical Analysis

Data in the figures are presented as mean ± standard error of mean (SEM) unless otherwise specified. Medians and ranges were described in the text. Differences of means were compared using a nonparametric Mann-Whitney U test. Analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, Ill., USA). A p value less than 0.05 was considered statistically significant.

Results

Clinical Data and Midge-Specific IgE and IgG Levels

Sixty-two subjects were enrolled and subgrouped according to their reactions to midge bites. As shown in table 1, 20 subjects had an immediate reaction with or without a delayed reaction, 12 subjects had solely delayed reactions (SDR) and 30 subjects had a tolerance to midge bites, and so were nonallergic controls (NAC). Sera were collected and tested for midge-specific IgE, IgG and IgG subclasses. The mean level of midge-specific IgE was significantly elevated among subjects with an immediate reaction to midge bites, regardless of whether they had subsequent delayed reactions or not (OD = 0.37 ± 0.06, median 0.31, range 0.1–1.08). Comparably low levels of midge-specific IgE were found in SDR (OD 0.10 ± 0.01, median 0.10, range 0.05–0.16) and NAC subjects (OD 0.07 ± 0.01, median 0.06, range 0.05–0.09; fig. 1a). There were no differences with regard to the level of midge-specific IgG, IgG_1, IgG_2, IgG_3 and IgG_4 among the 3 groups (fig. 1b).

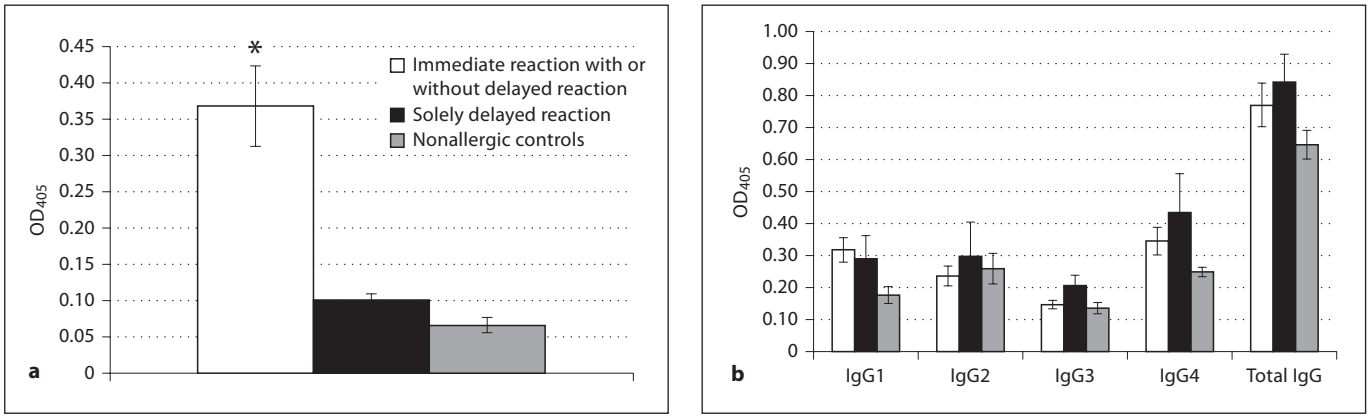


Fig. 1. Midge-specific IgE (**a**) and IgG subclasses (**b**) in individuals with different allergic reactions to midge bites and nonallergic subjects by ELISA. Results are expressed as mean ± SEM from 20 midge-allergic subjects with immediate reaction, with or without delayed reaction, 12 subjects with solely delayed reaction and 30 nonallergic subjects. * $p < 0.05$ versus nonallergic subjects.

Table 1. Patient demographics

Reaction after midge bite	Immediate allergic reaction ± delayed reaction (n = 20)	Solely delayed reaction (n = 12)	Nonallergic control (n = 30)
Age (mean ± SD), years	32.1 ± 2.47	37.75 ± 2.54	29.1 ± 2.01
Gender, n			
Male	4	2	15
Female	16	10	15
SPT to crude midge extract			
Wheal size (mean ± SD), mm	6.24 ± 0.38	0.25 ± 0.18	0 ± 0
Erythema (size ± SD), mm	15.82 ± 2.12	0.33 ± 0.22	0.09 ± 0.06

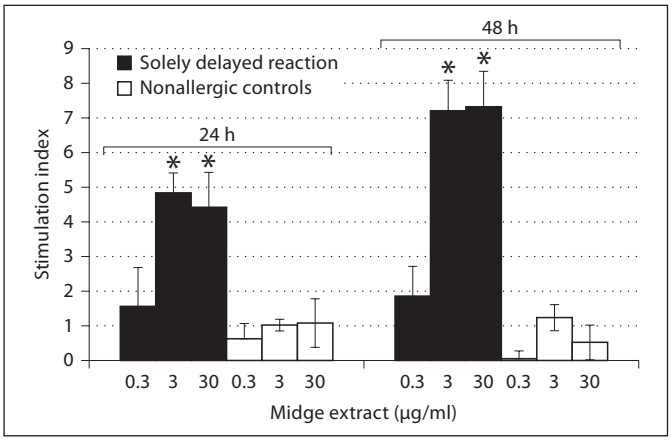


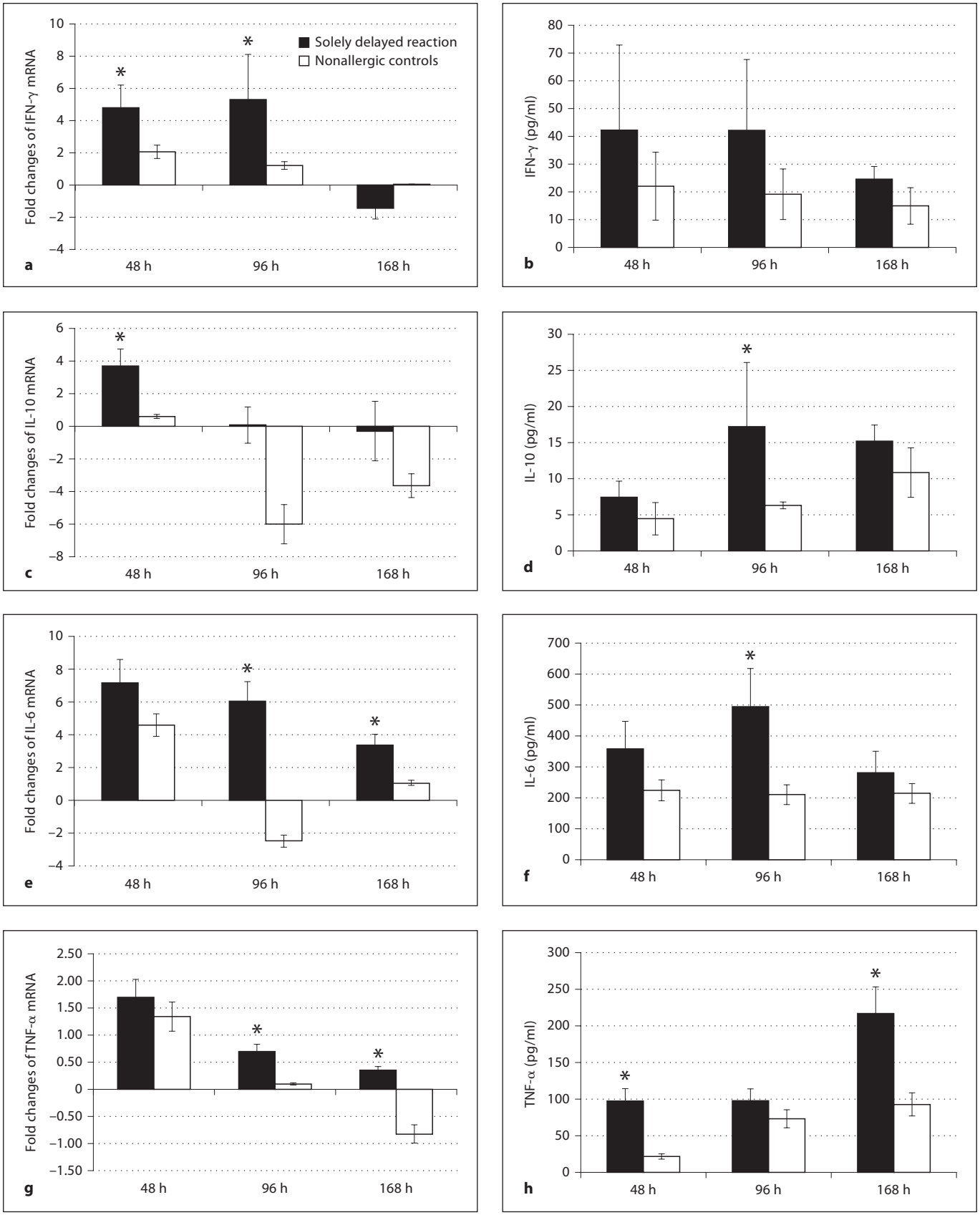
Fig. 2. PBMC proliferation from 6 midge-allergic subjects with solely delayed reaction and 6 nonallergic subjects after stimulation with various concentrations of midge extract. * $p < 0.05$ versus nonallergic subjects.

Proliferation of PBMC

PBMC from 6 SDR subjects and 6 NAC subjects were stimulated with different concentrations of midge extract and then cell proliferation was determined by MTT assay. We found that PBMC from SDR subjects markedly proliferated in response to midge extract compared to NAC at 3 μg/ml at 24 h [stimulation index 4.83 ± 2.44 vs. 1.02 ± 0.16, median (range) 4.43 (0.42–18.13) vs. 0.85 (0.33–1.35)] and 48 h [stimulation index 7.22 ± 2.78 vs. 1.24 ± 0.38, median (range) 5.09 (1.94–22.48) vs. 0.98 (0.49–2.00)]. A higher concentration of 30 μg/ml gave no further increase of cell proliferation, as shown in figure 2.

Cytokine Profile of SDR Subjects

PBMC from 6 SDR subjects and 6 NAC subjects were stimulated with midge extract for 48, 96 and 168 h. As shown in figure 3a, c, e and g, mRNA from SDR subjects


Solely delayed reaction
Nonallergic controls
Fold changes of IFN-γ mRNA
IFN-γ (pg/ml)
Fold changes of IL-10 mRNA
IL-10 (pg/ml)
Fold changes of IL-6 mRNA
IL-6 (pg/ml)
Fold changes of TNF-α mRNA
TNF-α (pg/ml)
48 h
96 h
168 h
a
b
c
d
e
f
g
h

3

expressed more IFN- γ, IL-10, IL-6 and TNF-α than NAC subjects at 48, 96 and 168 h. The median fold changes (range) are as follows (SDR vs. NAC).

IFN-γ at 48 h: 4.66 (1.22 to 9.99) versus 2.06 (–1.03 to 4.76), at 96 h: 4.89 (–1.44 to 16.68) versus 1.38 (–7.06 to 5.03), and at 168 h: –1.36 (–4.06 to 1.21) versus –1.03 (–3.94 to 7.57).

IL-10 at 48 h: 3.22 (1.33 to 7.62) versus 0.08 (–2.19 to 1.36), at 96 h: –0.57 (–2.20 to 4.32) versus –2.26 (–35.26 to 10.20), and at 168 h: 0.02 (–5.37 to 6.19) versus –3.65 (–15.78 to 6.41).

IL-6 at 48 h: 7.63 (–5.98 to 19.43) versus 2.68 (–1.45 to 12.55), at 96 h: 2.20 (–2.60 to 29.65) versus –1.69 (–19.4 to 10.03), and at 168 h: 1.85 (–1.59 to 13.74) versus 1.05 (–1.43 to 6.36).

TNF-α at 48 h: 1.61 (1.01 to 2.71) versus 1.34 (1.04 to 1.88), at 96 h: 0.87 (–1.93 to 2.95) versus –0.30 (–4.26 to 1.82), and at 168 h: 1.2 (–0.35 to 2.38) versus –0.90 (–4.20 to 2.08).

As shown in figure 3b, d, f and h, PBMC from SDR subjects secreted more IFN-γ, IL-10, IL-6 and TNF-α than NAC subjects at 48, 96 and 168 h. The differences reached statistical significance at 96 h in IL-10 and IL-6 and at 48 h and 168 h in TNF-α. The mean fold changes (range) were as follows (SDR versus NAC).

IFN-γ at 48 h: 51 pg/ml (24–109) versus 22 pg/ml (5–43), at 96 h: 43 pg/ml (29–94) versus 21 pg/ml (5–31), and at 168 h: 26 pg/ml (25–64) versus 16 pg/ml (5–24).

IL-10 at 48 h: 8 pg/ml (5–10) versus 7 pg/ml (2–10), at 96 h: 19 pg/ml (16–39) versus 7 pg/ml (6–8), and at 168 h: 16 pg/ml (8–23) versus 14 pg/ml (4–25).

IL-6 at 48 h: 298 pg/ml (104–835) versus 210 pg/ml (35–346), at 96 h: 340 pg/ml (190–866) versus 215 pg/ml (80–222), and at 168 h: 244 pg/ml (51–698) versus 208 pg/ml (16–298).

TNF-α at 48 h: 91 pg/ml (63–263) versus 16 pg/ml (8–42), at 96 h: 89 pg/ml (67–257) versus 68 pg/ml (10–196), and at 168 h: 213 pg/ml (102–635) versus 87 pg/ml (9–152).

Fig. 3. Cytokine mRNA and protein expression from PBMC of 6 SDR and 6 NAC subjects. PBMC were cultured with midge extract (3 μg/ml) for various time points and mRNA expression was quantified by real-time PCR and assayed cytokine proteins by cytometric bead immunoassay. **a** IFN-γ mRNA. **b** IFN-γ protein. **c** IL-10 mRNA. **d** IL-10 protein. **e** IL-6 mRNA. **f** IL-6 protein. **g** TNF-α mRNA. **h** TNF-α protein. Data are mean ± SEM. * $p < 0.05$ versus nonallergic subjects.

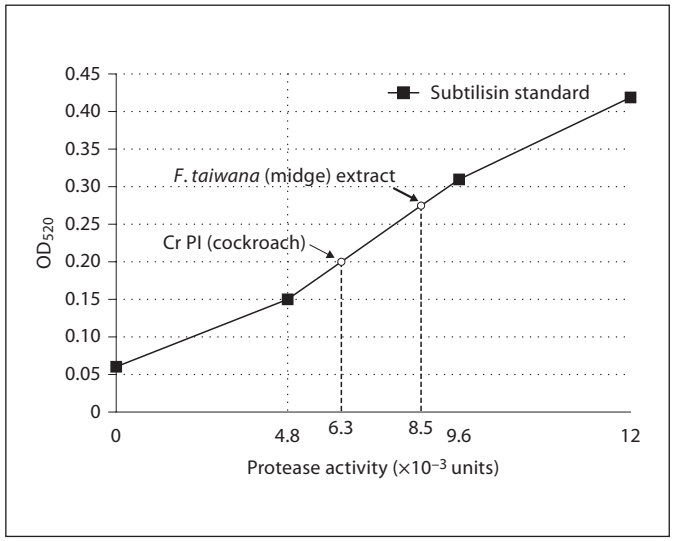


Fig. 4. Protease activity in midge extract (20 μg/ml) by the Azocoll method. Thick and thin arrows indicate the estimated protease activity of midge extract (8.5×10^{-3} units) and Cr PI (cockroach allergen, 6.3×10^{-3} units), respectively.

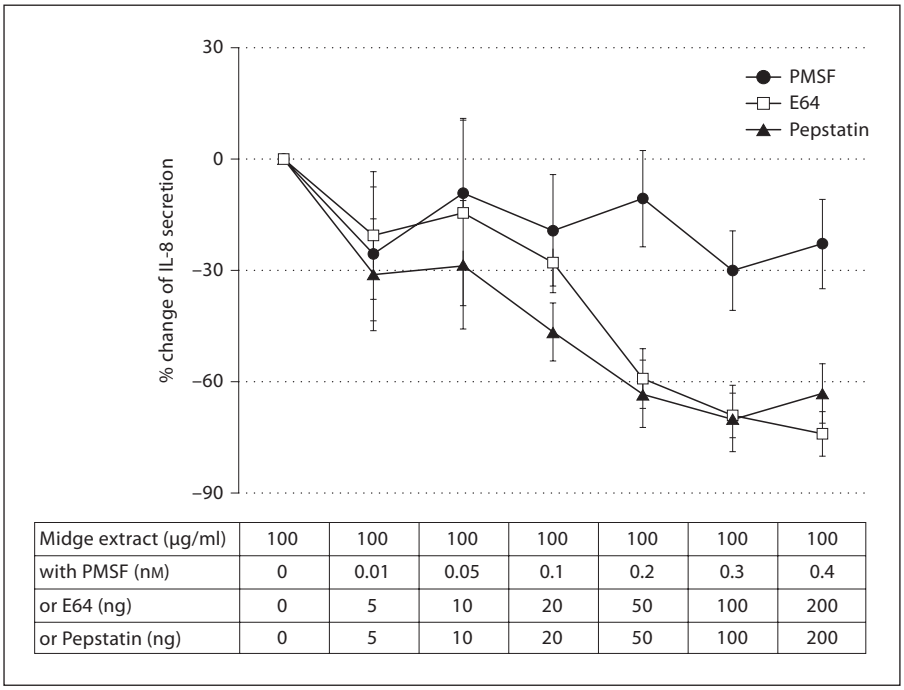
IL-8 (13,102.38 pg/ml vs. 11,200.43 pg/ml at 48 h) and IL-2 (60.27 pg/ml vs. 70.52 pg/ml at 48 h) increased in both SDR and NAC subjects, with no significant differences. IL-4, IL-5, IL-12p70 and TNF-β were not detected in the culture supernatant (data not shown).

Protease Activity in Midge Extract

Whole body midge extract stimulated IL-8 production at high levels from PBMC in both the midge-allergic and nonallergic subjects. A similar pattern has been reported in cockroach allergens that contain protease activity [20, 21], thus we decided to determine the protease activity in midge extract and used cockroach allergen Cr PI for comparison. Protease activity was detected in midge extract by the Azocoll method. The protease activity was higher in the midge extract (8.5×10^{-3} units) than in cockroach allergen Cr PI (6.3×10^{-3} units; fig. 4).

Inhibition of Midge-Extract Induced IL-8 Secretion by Protease Inhibitors from A549 Cells

IL-8 production from A549 cells stimulated by midge extract was suppressed by E-64 and pepstatin in a dose-dependent manner but not PMSF, as shown in figure 5.



Midge extract (μg/ml)	100	100	100	100	100	100	100
with PMSF (nM)	0	0.01	0.05	0.1	0.2	0.3	0.4
or E64 (ng)	0	5	10	20	50	100	200
or Pepstatin (ng)	0	5	10	20	50	100	200

Fig. 5. Effects of protease inhibitors on midge extract-induced IL-8 release from A549 cells. Midge extract (100 μg/ml) was incubated for 15 min at 37°C with different kinds and different amounts of protease inhibitors, PMSF (serine and cysteine protease inhibitor), E64 (papain and cysteine protease inhibitor) or pepstatin (aspartic protease inhibitor), as indicated in the table below the figure, then cultured with A549 cells for additional 24 h. Data are mean ± SEM, n = 3.

Discussion

Allergic reactions to midge bites are a common problem in Taiwan. In this study, we found that the mean midge-specific-IgE was significantly higher in subjects with an immediate reaction, which is consistent with the previous findings of mosquito allergy [22]. In our study, midge-specific IgG and midge-specific IgG_4 were elevated in all 3 groups regardless of their reactions to midge bites. It is possible that the presence of midge-specific IgG and midge-specific IgG_4 only correlates to exposure to midge bites, but not to the presence of allergic symptoms.

It has been demonstrated in a mouse model of mosquito allergy that IL-4 production significantly increased and IFN-γ production decreased [23]. This study suggested a predominant Th2 immune response in mice with delayed skin reactions to mosquito bites. However, in our ex vivo experimental model, midge extract stimulated more IFN-γ, IL-10, IL-6 and TNF-α from PBMC in human subjects with SDR than nonallergic subjects at both mRNA and protein levels. IFN-γ is considered a cytokine indicating Th1 type inflammation and TNF-α and IL-6 are proinflammatory cytokines mainly secreted by innate immune cells [24, 25]. Nevertheless, since we did not study the cellular responses of subjects with solely immediate reactions, we cannot exclude the possibility that IL-4 or other Th2 cytokines participate in the immediate reaction of biting midge allergy.

TNF-α is a key molecule in many inflammatory diseases. The evidence of TNF-α in allergic inflammation has been increasing, especially in refractory asthma [26]. However, the study regarding TNF-α in biting or stinging-insect allergy is limited. Only one previous report has described that human T cell clones specific to bee venom phospholipase A2 produced TNF-α in response to allergen [27]. It has been demonstrated that mast-cell derived TNF-α induced eosinophil survival by autocrine production of granulocyte-macrophage colony-stimulating factor [28]. Our study suggested that TNF-α may play a role in the dense eosinophil infiltration in the skin lesions of subjects with a delayed allergic reaction to biting midge.

IL-10 has been shown to be an anti-inflammatory cytokine produced by regulatory T cells. It inhibits the activation and cytokine production of both Th1 and Th2 cells [29, 30]. It is also considered to play an important role in immune tolerance in allergic inflammation and allergen-specific immunotherapy [31]. However, IL-10 does not seem to be a good indicator for the existence of immune tolerance to midge bites in our study. SDR subjects in our study secreted more IL-10 than the midge-tolerant nonallergic subjects. An increasing secretion of

IL-10 seems to be more of a sign that the immune system 'sees' the allergen and tries to stop the inflammation rather than a successful induction of tolerance to the midge allergy.

In the present study, protease activity was detected in midge extract, and the midge extract-induced IL-8 secretion was suppressed by cysteine and aspartic protease inhibitor. It is known that extracellular endogenous proteases, such as thrombin and trypsin, as well as exogenous proteases from cockroaches [20, 32], mites [33] and mold allergens [34], react with cell surface receptors in the airways and generate leukocyte infiltration and amplify the response to allergens. There has been increasing evidence that protease activation inflammation plays an important role in non-IgE mediated allergic respiratory diseases via activation of protease-activated receptors [35]. The activation of protease activated receptor-2 in skin has been reported to be related to skin itching, recruitment of inflammatory cells and hyper-pigmentation [36–38]. With the detection of strong protease activity in crude *F. taiwana* extracts, we believe that protease activation of inflammation plays an important role in the delayed, long-lasting, intensely itchy, hyperpigmented allergic skin reactions after *F. taiwana* bites. Since protease activation of inflammation is non-specific, it is possible that there is a difference, as yet to be defined, in negative regulatory signals in NAC subjects that mediate the tolerance to midge bites.

In conclusion, our results suggest that an immediate reaction to midge bites is IgE-mediated, whereas IgE may not be the major molecule in delayed reactions. IFN-γ, IL-6, and TNF-α are involved in delayed reactions to midge bites. A protease-activated pathway may also be involved in the intense, itchy reactions to midge bites.

Acknowledgments

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