
ANALYSIS OF LYMPHOCYTE RESPONSE TO CHIRONOMID MIDGE ANTIGENS IN ASTHMATIC AND NON-ASTHMATIC INDIVIDUALS

Toru EDAHIRO, Nobuo OHTA, Hiroyuki MATSUOKA, Akira ISHII, Yoshio TANIZAKI1, Hikaru KITANI1, Taiji KUNITOMI2, Shin NOONO3 and Keiko TACHIBANA3

Department of Parasitology, Okayama University Medical School, 2-5-1, Shikata-cho, Okayama 700, 1Department of Medicine, Institute for Environmental Medicine, Okayama University Medical School, Misasa, Touhaku-gun, Tottori 682-02, 2Department of Pediatrics, Okayama University Medical School, Okayama 700, and 3Department of Pediatrics, Tamano City Hospital, 2-3-1, Uno, Tamano, Okayama 706

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SUMMARY: Chironomid antigens are currently one of the important allergens for bronchial asthma in Japan. We evaluated in vitro responses of peripheral blood lymphocytes (PBLs) to chironomid antigens and compared these responses with serum IgE levels. PBLs from adult asthmatic patients showed stronger proliferation in response to the extract of adult midges of Chironomus yoshimatsui compared with healthy adults. On the other hand, elevated PBL responses of child asthmatic patients to chironomid antigens were not observed. There was no significant correlation between PBL proliferation and the serum IgE level. Our results might suggest that elevated PBL proliferation in response to chironomid allergens has somehow important pathogenic roles in adult cases although this does not correlate directly with specific IgE production.

枝廣 徹・太田伸生・松岡裕之・石井 明(岡山大学医学部寄生虫学教室 岡山市鹿田町2-5-1)
谷崎勝朗・貴谷 光(岡山大学医学部環境病態研究施設 鳥取県東伯郡三朝町山田827)
国富泰二(岡山大学医学部小児科学教室 岡山市鹿田町2-5-1)
濃野 信・立花敬子(玉野市民病院小児科 岡山県玉野市字野2-3-1)
INTRODUCTION

Chironomidae, consisting of nearly 5,000 species, are the insects spread all over the world. Their aquatic larvae are distributed widely in all types of inland waters. Formerly they were thought to be merely nuisance insects, however, hypersensitivities to them have been reported since 1920s (1-5). Dried extracts of the larvae are known to induce immediate-type hypersensitivity reactions to fish-food factory workers or fish-hobbyists in Europe (1,2). Adult chironomid antigen was found to be one of the most important causative allergens in asthma in Japan (6). Chironomid midges are the most common airborne allergens in out-door environment in Okayama Prefecture (7), and few inhabitants can be free of contact with the insect allergens. Recent epidemiological studies clarified that the incidence of hypersensitivity to the chironomid is next to that of house dust mite.

Anti-chironomid IgE antibodies were commonly detected in Japanese asthmatics; however the age distribution is uneven (6). Susceptibility to chironomid-hypersensitivity seems to be depend on an unknown age factor(s) since nearly 50% of adult asthmatics (>18 year) had specific IgE, while it was detected in only less than 6% of child patients (<12 year) (4,6,8).

There are tight evidences showing that cellular immunity is involved in allergic reactions in humans. Atopic patients showed strong in vitro proliferative responses to grass pollen (9,10), ragweed pollen (11,12) or house dust mite antigens (13,14). In the light of these observations, this study was conducted to examine for peripheral blood lymphocyte (PBL) responses to two chironomid species, *Tokunaga-yusurika akamusi* and *Chironomus yoshimatsui*, both of which are abundant in Okayama, Japan and important candidates for the causes of asthma. We used soluble extracts of adult midges of *T. akamusi* (TAA) and *C. yoshimatsui* (CYA) and compared PBL proliferation in response to those soluble antigens between asthmatic and non-asthmatic populations and between adult and child ones. Based on the results, we discuss possible involvement of chironomid antigens-induced cellular responses in asthmatic diseases in Okayama, Japan.

MATERIALS AND METHODS

Donors: We tested 76 patients with bronchial asthma who had not received corticosteroid therapy or immunotherapy. As controls, we tested 52 asymptomatic random healthy volunteers. The frequency of skin test-positive persons to TAA
was 43.8% (7/16) in patients and 40.6% (13/32) in asymptomatic controls. The 128 subjects were divided into four groups: [1] 36 asymptomatic subjects of over 16 years (normal adults); [2] 22 asthma patients of over 16 years (asthmatic adults); [3] 16 asymptomatic subjects of under 15 years (normal children); and [4] 54 asthma patients of under 15 years (asthmatic children).

**Lymphocyte preparation:** Peripheral blood lymphocytes (PBLs) were prepared from fresh heparinized blood by the method of Ficoll-Conray (specific gravity = 1.077) (15).

**Antigens:** Crude soluble antigens extracted from adult worms of chironomid midges, *T. akamusi* (TAA) and *C. yoshimatsui* (CYA), were prepared by the method described elsewhere (8). We tested purified protein drivative (PPD, Nihon BCG Seizo Co., Japan) as a positive control antigen, and a soluble egg antigen of *Schistosoma japonicum* (SEA) (16) as a negative control. Protein concentration was determined by Lowry's method (17), and all antigens were cryopreserved at −20 C before testing. The single lot of each antigen was used for the present study.

**In vitro lymphocyte proliferation assays:** PBLs were cultured in 96-well flat-bottomed microtiter plates (Corning Co., Corning, CA) in 0.2 ml of RPMI 1640, supplemented with 10% heat-inactivated pooled human male sera (HS), 100 µg/ml streptomycin, 100 U/ml penicillin, 20 mM L-glutamine, in the presence or absence of antigens. The optimal conditions and antigen doses for antigen-driven PBL proliferation were determined in the previous experiments (18). In brief, 1 × 10⁵ of PBL were cultured at 37 C in humidified 5% CO₂ and 95% air for 7 days. We used antigens at concentrations of 10 µg/ml for TAA, 7.5 µg/ml for CYA, 2 µg/ml for PPD, and 5 µg/ml for SEA. Wells were pulsed with 1 µCi of [³H]-thymidine (specific activity = 10 Ci/mmol) (ICN Radiochemicals, Irvine, CA) for the final 16 hr, and [³H]-thymidine incorporation was assessed by liquid scintillation spectrometry.

**Serum levels of TAA-specific IgE in asthmatic and healthy individuals:** Chironomid-specific serum IgE levels were measured by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (8).

**Statistical analysis:** Statistical significance was tested by χ² test or by Fisher's direct method (19). Analysis of data was performed with ln Δ cpm calculated as follows:

\[
\ln \Delta \text{ cpm} = \ln (\text{test cpm} - \text{antigen-free control cpm})
\]

When we observed negative control responses which were widely varied from the others, we rejected them from analysis of data by the criteria of Smirnoff's rejection method (20). By comparing responses to positive and negative control antigens, we decided a cutoff point for dividing positive and negative responses to chironomid antigens.
RESULTS

In vitro PBL Responses to PPD and SEA

PBL from individuals in group I, (normal adults) showed response to PPD with ln Δ cpm from 8.1 to 11.8 (10.40 ± 0.78) and to SEA from 6.1 to 9.1 (7.75 ± 0.69) (Fig. 1). Then we decided the value of 8.99 as a cutoff point to divide the individuals of group I into negative and positive responders, as this cutoff point gave the maximum validity. By the same criterion, 9.25 was decided as a cutoff point for group II (Fig. 2). In groups III and IV, PPD was not sufficient as a positive control antigen, because not all children had been immunized with tuberculin in

Fig. 1. In vitro PBL proliferative response to PPD and SEA (A), TAA (B) and CYA (C) in healthy adult control group (group I). A cutoff point to divide the individuals into negative and positive responders was decided at 8.99

Fig. 2. In vitro PBL proliferative response to PPD and SEA (A), TAA (B) and CYA (C) in asthmatic adult group (group II). The cutoff point was at 9.25.
Fig. 3. In vitro PBL proliferative response to PPD and SEA (A), TAA (B) and CYA (C) in healthy child control group (group III). The cutoff point was at 7.93.

Fig. 4. In vitro PBL proliferative response to PPD and SEA (A), TAA (B) and CYA (C) in asthmatic child group (group IV). The cutoff point was at 9.28.

Japan. Therefore, a cutoff point was decided only from the responses to SEA. In group III, PBL reactivity to SEA ranged from \( \ln \Delta \text{cpm} 6.79 \) to \( 7.77 \) (mean = 7.32 ± 0.30). We decided 7.93 (mean plus 2SD) as a cutoff point for group III (Fig. 3) and 9.28 for group IV (mean \( \ln \Delta \text{cpm} = 8.08 \pm 0.60 \)) (Fig. 4).

**PBL Response to Chironomid Antigens in Adult Population**

Out of 36 healthy adult controls, 12 (33%) were classified into high responders to TAA and 15 (42%) to CYA (Fig. 1). Out of 22 adult asthmatics, only one (4.5%) was a high responder to TAA, while 15 (68%) were high responders to CYA (Fig. 2). The frequency of high responders to CYA among the patients was
significantly higher than that among healthy control adults (p<0.05), while the frequency of high responders to TAA among the patients was lower than that among controls (p<0.05). When we compared the mean values of ln Δ cpm, only responses to CYA were significantly elevated (9.03 for asthmatics vs. 6.18 for controls) (p<0.05). The mean responses to TAA were not different between the two groups.

**PBL Reactivity to Chironomid Antigens in Child Population**

Out of 16 healthy children, three (19%) were classified as high responders to TAA and five (31%) to CYA (Fig. 3). Out of 54 child asthmatic patients, two (3.7%) were assigned as high responders to TAA and four (7.4%) to CYA (Fig. 4). The frequency of high responders in the healthy control group to CYA was significantly higher than that in the child asthmatic patient group (p<0.05).

**Correlation between PBL Proliferation and Positive Skin Test in Asthmatic Patients**

Although only a few patients were tested by the skin test, we compared TAA-induced PBL proliferation between skin-test-positive and -negative groups. Results are shown in Fig. 5, and no difference was observed between the two groups.

Fig. 5. Correlation between proliferative lymphocyte response to TAA and the skin test to TAA in asthmatic patients.
Correlation between PBL Proliferation and Specific IgE Level

We determined serum IgE levels to TAA and CYA by ELISA. We tested 27 asymptomatic adults and nine child asthmatics. There was no correlation between PBL proliferation and OD values in ELISA, and r values were -0.39 for TAA and -0.28 for CYA (Fig. 6).

DISCUSSION

The chironomid-induced hypersensitivity has become a health problem in the world (1-5). Nearly 40% of Japanese patients with bronchial asthma have high titers of IgE antibodies specific for the chironomid (4,6). Roles of cell-mediated immunity in allergic diseases have been investigated, and the accumulated data show that elevated PBL proliferation in response to the causative allergens has a biological role(s) in developing clinical symptoms (9-14). In this study, we observed stronger PBL proliferation in response to CYA in adult asthmatic patients than normal controls. Chironomid antigens are common airborne allergens around
Okayama city (7), and it is possible that most inhabitants of Okayama, regardless of asthmatics or not, are sensitized with both TAA and CYA. From such a situation, it is not likely that the difference in strength of PBL proliferation in response to CYA tells us whether antigenic sensitization was enough or not.

It was observed that seasonal allergens induced seasonal fluctuation of PBL proliferation (21). Chironomid antigens are also seasonal, and C. yoshimatsui are dominant in summer in Okayama. We obtained PBL of group I in summer, and of group II mainly in winter. Considering the seasonal factors, we expected that responses to CYA were elevated in group I compared with those in group II. Our results, however, showed that group II had higher response to CYA than did group I. This suggested that the difference in response to CYA between groups I and II was not affected by seasonal factors. From the observation, we would conclude that raised cellular hypersensitivity to CYA might be involved to a certain degree in the biological mechanisms of onset of adult asthmatic diseases in Okayama.

It has been suggested that there is age-dependent susceptibility in chironomid hypersensitivity, since adult asthmatics showed much higher incidence of chironomid-specific IgE than did child patients (6). In stark contrast to adult patients, we observed no elevated cellular response to chironomid allergens in asthmatic children. Our data might support the possibility of the age-related difference in sensitivity to the chironomid allergens.

Not all the asthmatic patients tested in this study had evidence of hypersensitivity to chironomid allergens. To analyze for relationship between PBL proliferation and specific IgE production, we tested two parameters. When we compared PBL proliferation with OD values in the ELISA test, there was no correlation between them. We observed no correlation either between the skin test and cellular proliferation. Although PBL proliferation to CYA was supposed to have pathogenic roles in adult asthmatic patients, this might not have direct relation with elevated IgE production to CYA.

In our previous experiment, chironomid antigens were able to induce CD4+ helper T cell (Th) response both in healthy and allergic individuals (18). Those Th cells are not necessarily related to specific IgE production. In a study of cedar pollinosis, almost all responding T cells of healthy individuals were related to the production of specific IgG but not IgE antibody (22). There might be a functional difference in T cell response between allergic and healthy subjects. Recent reports from several laboratories demonstrated regulatory functions of interleukin 4 (IL-4) in IgG and IgE production. Quantitative difference in IL-4 production seems to determine the class of antibody (23). For onset of allergy, a raised level of allergen-
driven IL-4 production might be very important. Recent research by O'Hehir and colleagues (24) showed that T cell proliferative responses in patients with mite allergy accompanied elevated IL-4 production, while T cell proliferation from skin-test positive healthy donors resulted in no detectable IL-4 production.

Chironomid antigens are considered to have numerous epitopes. In cases of ragweed or cedar pollen allergens, their physicochemical nature is relatively simple, and immunologically important antigenic sites were identified. This might be one of the reasons why raised PBL proliferation to pollen antigens were clearly observed in allergic populations. It is, thus, essential to physicochemically characterize of chironomid allergens to understand the pathogenic roles of the insect antigens in asthma. In serological study, IgE recognition sites of TAA and CYA have been analyzed (25). It will be interesting to analyze T cell recognition sites of chironomid antigens which are important to activate IL-4 production. In line with this strategy, further investigation is now under way.

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