

*IgE antibodies against midge and moth found in Japanese asthmatic subjects and comparison of allergenicity between these insects

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The specific IgE antibodies to moth (*Bombyx mori*) and midge (*Chironomus yoshimatsui*) were measured by the Pharmacia CAP system in 51 house-dust-mite-sensitive asthma patients. None of these patients had definite histories of exposure to these insects or apparent evidence of insect-induced asthma symptoms. The RAST-inhibition assay was performed to investigate cross-allergenicity between these two insects. Furthermore, IgE immunoblotting was done to study the IgE-binding components in moth and midge extracts. Thirty (59%) of these patients showed positive IgE antibodies to moth, while 25 (49%) showed positive IgE antibodies to midge. Those frequencies of positivity were similar to that for Japanese cedar pollen, which is well known to cause allergy. A significant correlation ($r=0.863$) was observed between IgE antibody titers of these two insects. The results from the RAST-inhibition assay indicated cross-allergenicity between these insects and also the existence of species-specific allergens. Fifteen IgE-binding components in moth extract were observed. The most frequent IgE-binding protein was the 79-kDa (84.2%), followed by the 72-kDa (78.9%), the 82-kDa (57.9%), and the 76-kDa (57.9%) proteins. Those were considered to be major allergens in moth. Twenty-four IgE-binding components in midge extract were observed. However, no IgE-binding protein to which over 50% of patient sera reacted was observed. These results suggest that these two insects may be considered to bear important allergens and that there is cross-allergenicity between these insects as well as species-specific allergens.

Key words: butterfly; immunoblotting analysis; insect allergy; midge; moth; RAST-inhibition assay.

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There is a wide variety of insects in our environment (1). It has been reported that insects such as moths, midges, and butterflies, besides the well-established house-dust mite, can cause symptoms of allergic rhinitis and bronchial asthma (2–5).

Insects such as the moth and the butterfly had already been shown to cause symptoms of bronchial asthma in 1932 by Parlato (4). In 1987, Kino & Oshima showed that the incidence of positive intracutaneous reactions to moth and butterfly were as high as those to house dust and *Candida*

and that approximately one-third of nonselected asthmatic patients had specific IgE to these insects in their sera (2). Furthermore, the bronchial responsiveness of patients to proteins derived from these insects was proven by provocation tests (2).

The midge was also reported to have caused asthma attacks in 1950 (3). In Japan, there are a number of reports showing that many types of midges cause bronchial asthma (6–9). For example, Ito et al. have reported that the midge allergen is one of the components of house dust and that there is little cross-allergenicity between midge and mite (9). There are many reports on the major allergens from midge and cockroach (10–12). However, few reports on allergen analysis of moth are available.

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Baldo & Panzani found IgE-binding bands with molecular weights in the region of approximately 30 kDa and above in two types of moth extract (13).

In this study, using the Pharmacia CAP System, we aimed to investigate the incidence of insect-specific IgE antibodies in asthmatic subjects sensitive to house-dust mite, and to analyze the cross-reactive and species-specific allergens in moth and midge by immunochemical methods.

Material and methods

Subjects

The group consisted of 51 patients with bronchial asthma who were selected from the Outpatient Asthma Clinic of Toyoko Hospital and the Institute of Medical Science, St Marianna University School of Medicine. All of these patients showed positive IgE antibody against house-dust mite. Twenty-nine were women, and 22 were men. The average age for men was 38.4 years, and for women, 40.9 years (range: 18–73 years).

Allergens

The allergens were extracted from the wings of the moth *Bombyx mori*, from the whole body of the butterfly *Pier rapae*, and from the whole body of the midge *Chironomus yoshimatsui*. An amount of 0.5 g of each material, after being defatted, was incubated in 12.5 ml of 0.1 M phosphate buffer (pH 7.4) with shaking, at 4°C for 2 h. The mixture was centrifuged at 10 000 g for 30 min, and the supernatant was filtered (TOYO 4A) and desalted on PD10 columns (Pharmacia Biotech). The protein concentration of each extract (1 : 35 w/v) was determined by protein assay kit (Bio-Rad Laboratories). The results were 732 µg/ml for *B. mori*, 1438 µg/ml for *P. rapae*, and 931 µg/ml for *C. yoshimatsui*. These extracts were then used for the RAST-inhibition assay as an original inhibitor. The butterfly *P. rapae*, common in Japan, was used as another Lepidoptera species whose allergenicity could be compared with that of moth extract.

Radioallergosorbent test (RAST)

RASTs for IgE antibodies to house-dust mite (*Dermatophagoides pteronyssinus*), Japanese cedar (*Cryptomeria japonica*), moth wing (*B. mori*), and midge (*C. yoshimatsui*) were carried out with the Pharmacia CAP System (14). Results were expressed in U/ml. In the study, IgE antibody values above 0.35 U/ml were considered positive.

RAST inhibition

Two sera with positive IgE antibody for both moth and midge and two sera which were positive only for midge or moth were used in the RAST-inhibition assay. The allergenicity of the extracts from moth wing, butterfly, and midge were studied by RAST inhibition with commercially available ImmunoCAP for moth and midge.

The allergen extracts (1 : 35 w/v) were serially diluted (1/10/4) with 0.1 M phosphate buffer (pH 7.4), and 30 µl of each dilution was mixed with 20 µl of patient serum and preincubated at room temperature with continuous shaking for 2 h. As a control, 30 µl of the same buffer was used instead of allergen extract. Then, the residual specific IgE antibodies in the mixture were measured with ImmunoCAP of moth or midge by the Pharmacia CAP System.

SDS-gradient PAGE

The lyophilized, crude extracts of moth and midge used for SDS-gradient PAGE were reconstituted at a concentration of 5 mg dry weight/ml. SDS-gradient PAGE was performed essentially as described by Neville (15). The extracts were treated with an equal volume of the sample buffer containing 3.7% SDS and 4.0% 2-β-mercaptoethanol and were boiled for 5 min. Both extracts were applied on the stacking polyacrylamide gels (6%) on top of polyacrylamide gradient gels (7.5–20%) at an approximate concentration of 8 µg protein/mm gel. The electrophoresis was run at 6 mA per gel for 18 h with 0.04 M Tris-borate (pH 8.64) containing 0.1% SDS and 0.42 M Tris-HCl (pH 9.18) for upper and lower reservoir buffers, respectively. The molecular masses of the allergen components were estimated by protein standard (LMW calibration kit, Pharmacia Biotech).

Immunoblotting

The separated allergen extract and the protein standard in the gradient gel were transferred to a nitrocellulose (NC) membrane (Hybond-C, 0.45 µm, Amersham) with blotting buffer (25 mM Tris, 192 mM glycine, and 20% methanol) in a Trans-Blot cell, by electrophoretic forces at 0.4 A for 4 h. After the transfer, unoccupied protein-binding sites were blocked by incubation with 0.05 M phosphate buffer (pH 7.4), containing 0.5% Tween 20, at 37°C for 30 min. The blotting gel and a part of the blotted strips were stained with Coomassie brilliant blue R-250 in 30% methanol and 7% acetic acid to evaluate the effectiveness of transfer. The NC strips were then further cut into

5-mm-wide strips and incubated with 2 ml of patient serum, diluted 1 : 4 or 1 : 2, in a phosphate buffer (pH 7.4) at room temperature on a shaker for 18 h.

The NC strips were washed, incubated with ^{125}I -labeled antihuman IgE (Phadebas RAST tracer, Pharmacia Diagnostics), and diluted 1 : 5 on a shaker at room temperature for 4 h. The ^{125}I -labeled bands were detected on an x-ray film (hyperfilm-MP, Amersham) after exposure at -70°C for 72 h (16).

Results

Incidence of moth- and midge-specific IgE antibodies

In 51 sera obtained from patients with house-dust-mite-sensitive allergic asthma, specific IgE antibody to moth was detected in 30 sera (59%), and to midge in 25 sera (49%). Fig. 1 shows the correlation between specific IgE antibody titers to moth and midge. There was a significant positive correlation between IgE antibody titers to these two insects ($r=0.863$; $P<0.0001$). However, seven moth-positive sera were negative to midge, while two midge-positive sera were negative to moth.

RAST-inhibition assays (Fig. 2)

In the inhibition assay with both moth and midge ImmunoCAP in patient no. 4, all three extracts inhibited the response in a dose-dependent manner. In the inhibition assay with serum from patient no. 12, the response was inhibited by the homologous extract to ImmunoCAP, but not by the heterologous extracts. In serum from patient no. 51, which was positive to midge alone, midge extract inhibited the response to midge ImmunoCAP, but the moth and butterfly extract did not inhibit the response. No response was obtained by all three inhibitors with moth ImmunoCAP. In contrast, the response of serum from the patient positive to moth alone, with moth ImmunoCAP, was inhibited by moth and butterfly extract, but not by midge extract. No response was obtained from all three extracts when tested with midge ImmunoCAP.

Immunoblotting analysis

The 19 patients who were positive to moth IgE in RAST were positive in the immunoblotting studies of extract from moth (Fig. 3). The IgE immunoblots showed a total of 15 IgE-binding bands in moth extract. The most frequent band was the 79-kDa protein (84.2%), followed by the 72-kDa (78.9%), 82-kDa (57.9%), and 76-kDa (57.9%) proteins

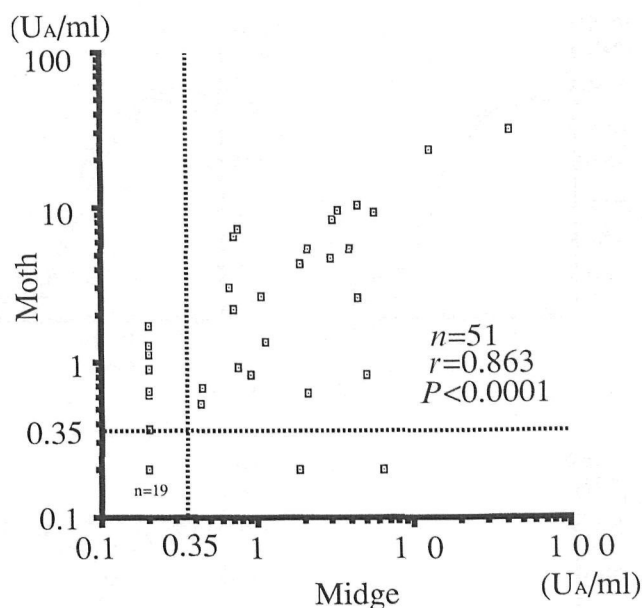


Fig. 1. Correlation between specific IgE antibody titers to moth and midge.

(Table 1). These were considered to be the major allergens of moth extract.

Fig. 4 shows the IgE immunoblot profile of midge extract of 19 patients who were positive against midge in RAST. Twenty-four components were found in midge extract. The most frequent bands were the 73 kDa (36.8%) and the 55 kDa (36.8%), but there was no component which showed more than 50% reactivity in tested sera (Table 2).

Discussion

Insects such as the moth, the butterfly, and the midge cause asthma symptoms (1). In 1987, Kino

Table 1. Results of IgE immunoblotting of moth allergen with 19 moth-positive sera

Molecular mass (kDa)	No. of positive patients	Positive frequency (%)
140	1	5.3
130	3	15.8
86	1	5.3
82	11	57.9
79	16	84.2
76	11	57.9
72	15	78.9
55	3	15.8
42	1	5.3
38	2	10.5
35.5	3	15.8
32	2	10.5
27	2	10.5
22.5	1	5.3
14	3	15.8

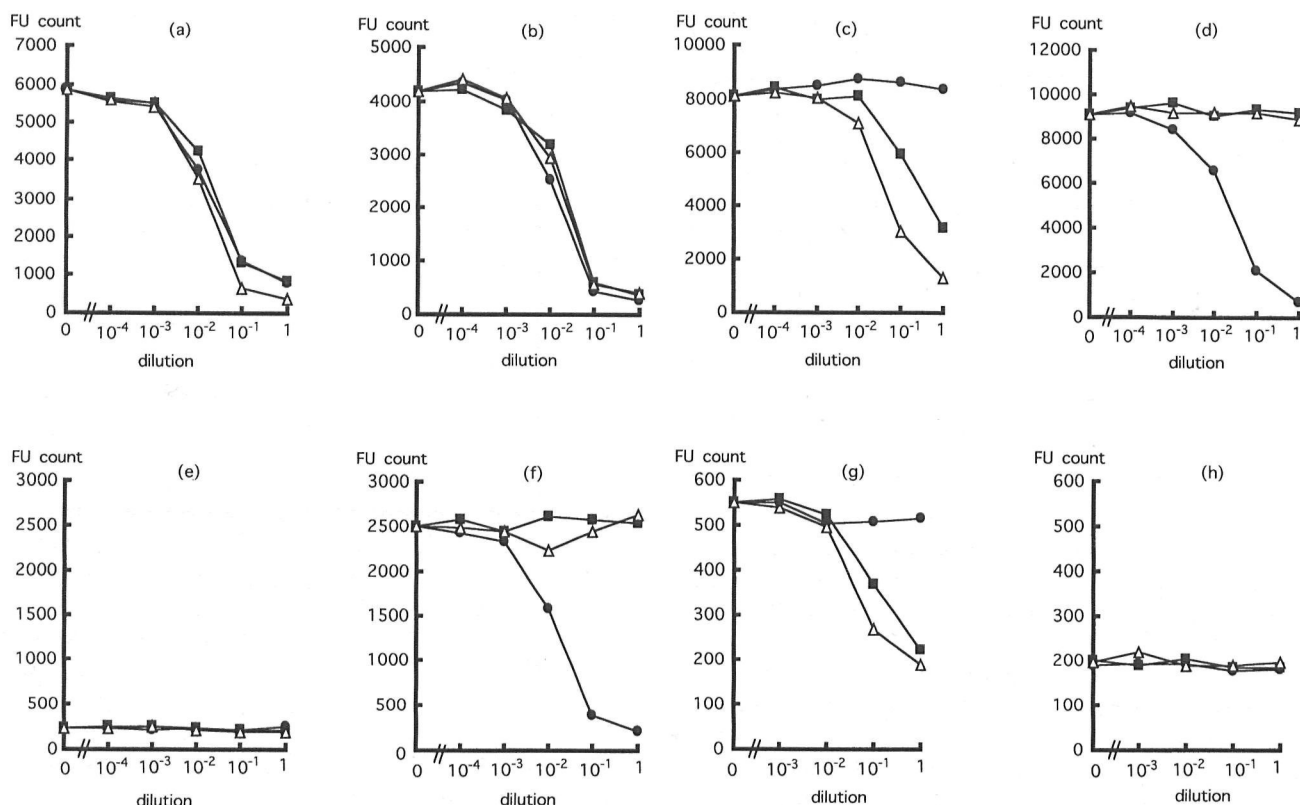


Fig. 2. Results of RAST-inhibition assays. a) Moth RAST with serum from patient no. 4 (moth IgE: 23.4 U/ml); b) midge RAST with serum from patient no. 4 (midge IgE: 12.8 U/ml); c) moth RAST with serum from patient no. 12 (moth IgE: 31.0 U/ml); d) midge RAST with serum from patient no. 12 (midge IgE: 41.6 U/ml); e) moth RAST with serum from patient no. 51 (moth IgE: <0.35 U/ml); f) midge RAST with serum from patient no. 51 (midge IgE: 6.4 U/ml); g) moth RAST with serum from patient positive to moth alone (moth IgE: 6.4 U/ml); h) midge RAST with serum from patient positive to moth alone (midge IgE: <0.35 U/ml). Concentration of three inhibitors was 1 : 35 (w/v) at dilution 1. Patient nos. are same as those in Figs. 3 and 4. Sera in g) and h) were not used in IgE immunoblotting studies. FU count: photometric units for measuring fluorescence intensity in Fluoro Count 96. Inhibitors: moth (—Δ—), midge (—●—), butterfly (—■—).

& Oshima showed that the incidence of positive intracutaneous reactions to moth and butterfly were as high as those to house dust and *Candida*, and that approximately one-third of nonselected asthmatic patients had specific IgE to these insects in their sera (2). Furthermore, the bronchial responsiveness of asthmatic patients with positive IgE to proteins derived from these insects was proven by provocation test (2). Midge was also reported to cause asthma attacks in 1950 (3–5).

In Japan, a number of reports have shown that many types of midges can cause bronchial asthma (6–8). For example, Ito et al. have reported that midge allergen is one of the components of house dust and that there was little cross-allergenicity between midge and mite (9).

Our study using the Pharmacia CAP System clearly demonstrated that IgE antibodies against moth and midge were frequently found in the sera of house-dust-mite-sensitive asthmatic patients. The incidence of positivity of IgE antibody to moth was similar to that of Japanese cedar, which is well

known as a cause of pollinosis (17) (data not shown). These results confirm the findings of other reports. Moth- and midge-specific IgE antibody titers showed no correlation with house-dust-mite titers, suggesting the existence of specific antigenic epitopes, as previously reported (8, 18, 19).

Significant correlation was found in the results between moth and midge by RAST. Those results suggest cross-allergenicity between the insects. However, moth IgE antibody was detected in seven sera negative to midge, and midge IgE antibody was detected in two sera negative to moth. These results suggest the existence of a species-specific allergen in each insect. In order to investigate the cross-allergenicity between the two insects, we performed the RAST-inhibition assay. All three extracts inhibited the response in both moth and midge ImmunoCAP with serum from patient no. 4. The homologous extract to ImmunoCAP inhibited the response, but not the heterologous extract with serum from patient no. 12. Only the homologous extract inhibited the response in midge- or

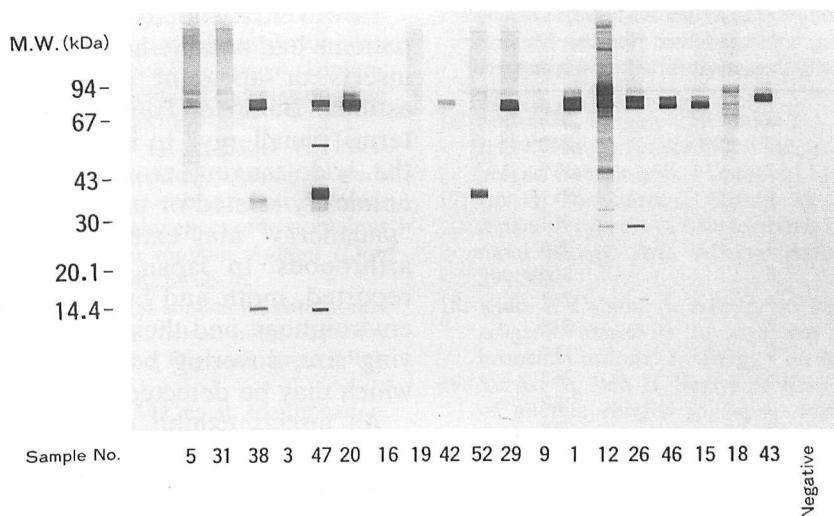


Fig. 3. IgE immunoblotting of moth allergen with 19 moth-positive sera.

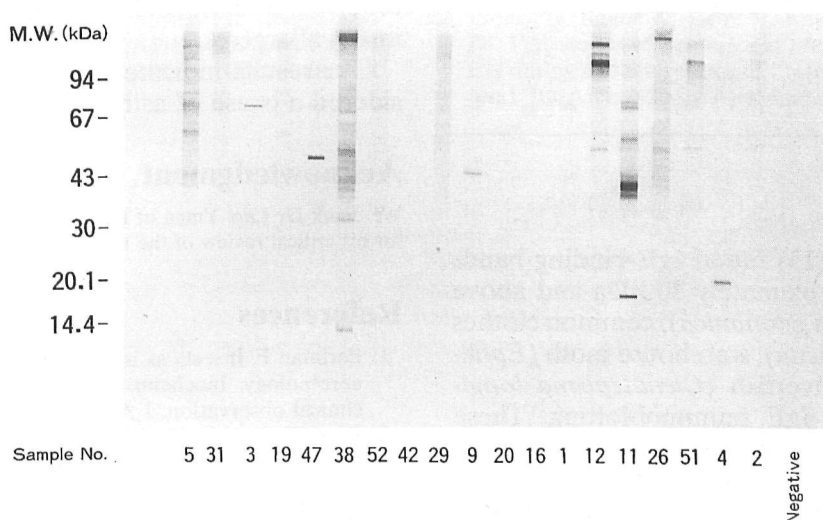


Fig. 4. IgE immunoblotting of midge allergen with 19 midge-positive sera.

moth-positive sera alone. Butterfly extract used as an example of another Lepidoptera species behaved like moth extract.

The RAST-inhibition assay indicated the existence of common and species-specific allergens in the two insects and a similarity of allergenicity in moth and butterfly (Fig. 2).

Significant correlation of IgE antibody titers between moth and midge ($r=0.904$) has been shown by Suzuki et al. (19). Kino et al. (8) (moth and butterfly), Baldo & Panzani (13) (fly, moth, silverfish, cockroach, and beetle), and Lierl et al. (20) (moth, fly, cricket, and grasshopper) found by RAST or RAST-inhibition assay that common and species-specific allergens existed among several insects.

Midge and cockroach allergens have been analyzed, and some major allergens have been identi-

fied (10–12). However, major allergens from moth extract have not been analyzed and identified. Subsequently, we carried out IgE immunoblotting in order to analyze IgE-binding components in moth extract with the sera of 19 patients with positive IgE antibody to moth by RAST. All the patients' sera were positive by IgE immunoblotting. The most common IgE-binding protein was the 79 kDa, followed by the 72, 82, and 76 kDa.

These were considered to be the major allergens in moth extract. In IgE immunoblotting with midge extract, a different profile was obtained from that of moth extract, and no IgE-binding proteins to which over 50% of patient sera reacted were found. Only two patient sera (nos. 4 and 11) reacted to midge proteins with molecular mass in the range 16–32 kDa corresponding to midge hemoglobin (10).

Table 2. Results of IgE immunoblotting of midge allergen with 19 midge-positive sera

Molecular mass (kDa)	No. of positive patients	Positive frequency (%)
125	2	10.5
120	5	26.3
115	2	10.5
105	4	21.1
99	4	21.1
92	3	15.8
83	6	31.6
77	4	21.1
73	7	36.8
66	3	15.8
62	5	26.3
59	3	15.8
55	7	36.8
51	3	15.8
48	1	5.30
46	4	21.1
41	5	26.3
39	1	5.30
32	2	10.5
27.5	2	10.5
20.5	1	5.30
19	1	5.30
18	1	5.30
13.5	1	5.30

Baldo & Panzani (13) found IgE-binding bands in the region of approximately 30 kDa and above in cockroach (*Blattella germanica*), common clothes moth (*Tineola bissellieta*), warehouse moth (*Ephestia cautella*), and silverfish (*Ctenolepisma longicaudata*) extract by IgE immunoblotting. These findings are similar to our results of IgE immunoblotting with moth (*B. mori*) extract. They also demonstrated the similarity of immunoblotting profile in the two moth extracts studied. These results and the findings from the RAST-inhibition assay suggest that each Lepidoptera species may have a large amount of common allergenic determinant.

van Kampen et al. (21) reported that many Japanese and Taiwanese patients had positive IgE antibody to Japanese midge (*Cricotopus sylvestris*), which does not contain hemoglobin, and a few were positive for midge hemoglobin (Chi t 1). They also demonstrated that more Swedish subjects reacted to *Cr. sylvestris* than to Chi t 1. Prelicz et al. reported the existence of hemoglobin antigenicity among midge larvae, pupae, and adults, and declining IgE-binding activities during metamorphosis (22). None of the patients whose sera were used for IgE immunoblotting with midge extract had contact with midge larvae. These patients may react to proteins other than hemoglobin in the adult midge, and those proteins will help to produce IgE antibody.

However, it should be noted that none of our patients had notable histories of exposure to these insects or apparent evidence of insect-induced asthma. Baldo & Panzani (13) have applied the term "panallergy" to insects, a term which means the wide-ranging cross-reactivity between taxonomically related or unrelated insect species. This "panallergy" may extend beyond insects to other arthropods. In Japan, as Kino & Oshima have reported, moth and butterfly are common in the environment, and these insects have scales of varying size, covering both surfaces of their wings, which may be detected in the air (2, 17, 18).

As insects exhibit cross-allergenicity (1), it is possible that when either of these insects sensitizes a patient, the CAP-RAST of another insect will become positive due to the common antigenic determinants. Further allergenic analysis of 76-, 79-, 72-, and 82-kDa components of moth extract is required to complete this comparison with other insect allergens.

Our results indicate that insects should be considered a cause of asthma by allergy clinics.

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