

Original article

A new recombinant mosquito salivary allergen, rAed a 2: allergenicity, clinical relevance, and cross-reactivity

Background: Mosquito salivary proteins cause allergic reactions in humans. The allergenicity, clinical relevance, and species cross-reactivity of a new 37-kDa recombinant mosquito (*Aedes aegypti*) salivary allergen, rAed a 2, were evaluated.

Methods: rAed a 2 was expressed using a baculovirus/insect cell system and purified. Its allergenicity was examined using an enzyme-linked immunosorbent assay (ELISA), ELISA inhibition tests, immunoblots, and skin tests. Epicutaneous tests with the allergen, mosquito whole body extracts, and mosquito bite tests were performed on 48 volunteers. Serum rAed a 2-specific immunoglobulin E (IgE) was measured in individuals with positive mosquito saliva-specific IgE and negative controls.

Results: Both immunoblots and ELISAs demonstrated that rAed a 2 bound to the IgE of mosquito-allergic individuals. The binding could be inhibited by the addition of a natural mosquito preparation. Furthermore, rAed a 2 induced immediate and delayed skin reactions. Ten per cent of 31 participants with a positive mosquito bite test had positive skin reactions to rAed a 2, compared with 32% for mosquito whole body extract. None of the participants with a negative bite test showed positive reactions to either of the two extracts. Forty-three per cent of individuals with positive saliva-IgE had positive rAed a 2-IgE. rAed a 2 was a species-shared allergen, being present in the saliva of the 11 species studied.

Conclusions: rAed a 2 has identical antigenicity and biologic activity to its native form. It can be used in the diagnosis of mosquito allergy, and is an important species-shared antigen.

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Due to the lack of availability of mosquito salivary preparations, allergic reactions to mosquito bites are under-diagnosed and under-treated (1). Mosquito bites commonly trigger an immediate wheal and flare and/or delayed induration in the skin, but in individuals who are allergic to mosquito saliva, large local reactions and even systemic reactions such as generalized urticaria, angioedema, asthma, and anaphylaxis may occur (2–9). The mechanisms underlying allergic reactions to mosquito bites involve immunoglobulin E (IgE) antibodies, lymphocyte proliferation, and probably IgG antibodies as well (1, 2, 7, 10). Immunotherapy with injections of mosquito whole body extracts may prevent allergic reactions to subsequent bites (3–6, 11, 12), however, mosquito whole body extracts, currently available commercially, contain few salivary proteins and many nonsalivary proteins (13) that may cause new sensitizations and side effects. Recent advances in clinical immunology involving recombinant DNA technology have produced many recombinant allergens and their

innovative derivatives (14–19). Utilization of molecular biologic techniques to produce pure mosquito salivary allergens will make the diagnosis and treatment of mosquito allergy more accurate and safe, and will facilitate the development of innovative allergen immunotherapy possible.

Mosquito salivary antigens have been identified by immunoblot analyses. A number of mosquito antigens with molecular masses ranging from 14 to 126 kDa have been reported in various mosquito species (20–22). To date, only four recombinant mosquito salivary proteins, designated rAed a 1 (23), rAed a 2, rAed a 3 (24), and rAed a 4 (25) have been identified as allergens. Aed a 2, a 37-kDa protein belonging to the D7 family of mosquito salivary proteins with unknown function, is found in the adult female salivary glands of the mosquito *Aedes aegypti* (26). In this study, we expressed rAed a 2 using a baculovirus/insect cell system, purified it, and examined its allergenicity, clinical relevance, and cross-reactivity with 11 mosquito species.

Materials and methods

Materials

The pVL1392 vector containing the Aed a 2 cDNA and rabbit anti-rAed a 2 were prepared as described previously (26). *Spodoptera frugiperda* (Sf9), a High-Five insect cell line, and Grace's insect media were purchased from Invitrogen (San Diego, CA). Ex-cellTM 405 serum-free medium was obtained from JRH Biosciences (Lenexa, KS). Other materials were purchased from Pharmacia Biotech (Uppsala, Sweden), Sigma Chemicals (St Louis, MO) or VWR Scientific of Canada Ltd, unless otherwise specified. Goat anti-human IgE was a gift from Dr N. F. Adkinson Jr, The Johns Hopkins Allergy and Asthma Centre (Baltimore, MD). Other antibodies and their conjugates were purchased from Jackson ImmunoResearch Lab. Inc. (West Grove, PA).

Mosquitoes and mosquito saliva and salivary gland preparations

The *Ae. aegypti* colony used for mosquito bite tests was obtained from the Department of Entomology at the University of Manitoba and maintained in our laboratory. The saliva or salivary gland extracts from *Ae. aegypti*, *Ae. vexans*, *Ochlerotatus triseriatus*, *Culex tarsalis*, *Culiseta inornata*, and *Culex quinquefasciatus* were prepared as described previously (22). The salivary gland extracts of *Ae. albopictus*, *Ae. togoi*, *Cx. pipiens*, and *Anopheles sinensis* were provided by Dr Sulan Liu (Shanghai Medical University, China). The saliva of *Ae. communis* was provided by Dr Henrikki Brummer-Korvenkontio (National Public Health Institute, Helsinki, Finland). Mosquito *Ae. aegypti* whole body extract was purchased from Hollister-Stier (Miles Canada Ltd, Etobicoke, ON, Canada). *Ae. aegypti* head and thorax extracts were prepared for the use of enzyme-linked immunosorbent assay (ELISA) inhibition tests (27).

Participants and serum samples

This project was approved by The University of Manitoba Faculty Committee on the use of human subjects in research and the participants gave written, informed consent before study entry. Forty-eight healthy adults with a history of mosquito bites and skin reactions of various sizes, ranging from none to large, were recruited for epicutaneous tests with rAed a 2 and mosquito whole body extract, and a bite test from a live laboratory-reared mosquito. Participants did not take antihistamines for 1 week before testing.

For rAed a 2-specific IgE (rAed a 2-IgE) measurement, 21 serum samples with positive mosquito saliva-specific IgE (saliva-IgE) were obtained from physicians in allergy clinics across North America. Twenty control sera from individuals who had negative mosquito bite tests were obtained from our previous study (10). Mosquito saliva-IgE was measured in these samples as described previously (28), and was considered positive when its value was higher than the cutoff level determined by the geometric mean + 1 SD of the controls.

Construction of recombinant baculovirus, expression, purification, and determination of isoelectric point

Recombinant baculoviruses carrying the Aed a 2 cDNA were generated by cotransfection of *S. frugiperda* (Sf9) insect cells with the transfer vector pVL1392/Aed a 2, together with linearized baculovirus AcMNPV DNA using the cationic liposome method. Pure recombinant viral stocks were prepared as described previously (23,

29). To increase the yield of the expressed protein, High-Five cells cultured in EX-cellTM 405 media (JRH Biosciences) were used to replace the Sf9 cells. After infection with the recombinant virus, cultures were harvested when maximum expression occurred, then centrifuged twice (23, 29).

Purification of rAed a 2 was achieved by ion exchange chromatography with DEAE Sephacel. The supernatant was concentrated, dialyzed, and added into a DEAE Sephacel column. After washing with Tris buffer, bound proteins were eluted from the column with a linear gradient of 0–0.5 M NaCl in 40 ml of Tris buffer. Protein concentrations in the fractions were monitored using optical density (OD) at 280 nm (OD₂₈₀) and rAed a 2 levels were monitored using an ELISA, in which fractions were coated on the plates and then incubated with rabbit anti-rAed a 2 antibodies. Positive fractions were pooled, desalted, lyophilized, and stored at –70°C. The isoelectric point of purified rAed a 2 was determined using a Model III mini IEF cell (Bio-Rad, Mississauga, Canada) according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed to identify the immunogenicity and cross-reactivity of rAed a 2 (23, 29). Briefly, proteins in purified rAed a 2 and *Ae. aegypti* saliva (as a positive control) were separated by SDS-PAGE under reducing conditions and then transferred to nitrocellulose membranes. The membranes were first incubated with rabbit anti-rAed a 2, then with alkaline phosphatase-conjugated goat anti-rabbit IgG, or sequentially incubated with mosquito-allergic human sera (1:50), monoclonal anti-human IgE and alkaline phosphate-conjugated goat anti-mouse IgG. Color was developed by incubation of the membranes with substrate. In the identification of cross-reactivity, mosquito salivary gland or saliva preparations from 11 species (1–2 µg/lane) were loaded into the gel. Membranes were incubated with rabbit anti-rAed a 2 followed by incubation with conjugated goat anti-rabbit IgG.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA was developed using the previously described techniques (28) to detect serum rAed a 2-IgE. Microplates coated with rAed a 2 (10 µg/ml) were incubated with human serum samples (1:20). After IgE binding, plates were incubated with goat anti-human IgE (1:2000), followed by incubations with enzyme-conjugated rabbit anti-goat IgG (1:2000) and then the substrate. When color was fully developed, optical absorbency was read at a wavelength of 410 nm. All serum samples were assayed in duplicate. After standardization against a reference serum, results were expressed using optical density at 410 nm (OD₄₁₀).

In ELISA inhibition tests, a pooled serum with a high titer of rAed a 2-specific IgE was diluted (1:20) and then incubated with an *Ae. aegypti* head and thorax extract at different dilutions for 1 h at 37°C. Incubation of the diluted serum with saline served as a positive control. After incubation, serum samples were assayed for rAed a 2-IgE as described above.

Mosquito bite tests and epicutaneous tests

An *Ae. aegypti* bite test was performed (10). After the bites, immediate local reactions were read at 20 min and delayed reactions were read at 24 h. An average wheal diameter of ≥4 mm or flare diameter of ≥15 mm was considered to be a positive immediate reaction, while an induration diameter of ≥4 mm was taken to be a positive delayed reaction.

Epicutaneous tests were performed 3 cm apart on the volar surface of the forearms with: rAed a 2 (1 mg/ml in 50% glycerine), commercial *Ae. aegypti* whole body extract (in 50% glycerine), histamine (1 mg/ml) and 50% glycerine (30). A wheal of ≥ 3 mm and/or a flare of ≥ 10 mm, measured 20 min after testing, were considered to be a positive reaction. An induration diameter of ≥ 3 mm, measured 24 h after the tests, was taken to be a positive delayed reaction.

Data analysis

Analysis of data was performed using SAS software (SAS Institute, Carey, NC). For group comparisons, pooled equal variance *t*-tests were used. Chi-square tests were used to compare the positive percentages between groups.

Results

Construction, expression, identification, and purification

Expression of rAed a 2 in the culture medium was confirmed by immunoblot analysis. Both rAed a 2 expressed in the culture medium of High-Five cells and the native Aed a 2 present in *Ae. aegypti* saliva were visualized after immunoblotting with either mosquito allergic-human serum or rabbit anti-rAed a 2 (Fig. 1), indicating that rAed a 2 had identical allergenicity as natural Aed a 2. In the purification, rAed a 2 was not adsorbed in the matrix of DEAE Sephacel, while all contaminating proteins were bound to the matrix. The purified rAed a 2 presented as a single bend on the

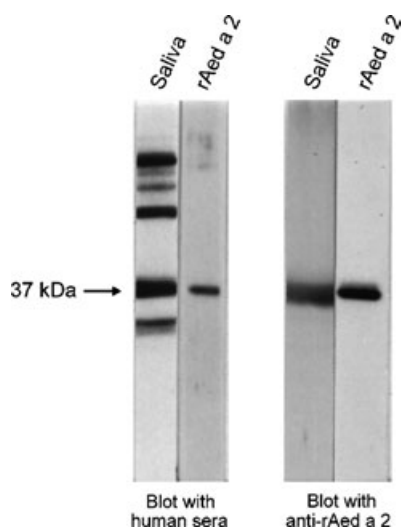


Figure 1. Identification of Aed a 2 by immunoblot. Native Aed a 2 present in *Aedes aegypti* saliva and rAed a 2 expressed in the culture medium of High-Five cells were separated from other proteins using SDS-PAGE. Proteins were then transferred to nitrocellulose filters, which were incubated with either mosquito-allergic human serum (left panel) or rabbit anti-rAed a 2 (right panel).

SDS-PAGE and the isoelectric focusing (IEF) gel with an isoelectric point of 9.0 (data not shown).

Mosquito bite tests and epicutaneous tests with rAed a 2 in 48 volunteers

Thirty-one of 48 participants had a positive immediate reaction to the mosquito bite, and 17 had a negative mosquito bite test. Among the 31 bite-test positive participants, 10 had a positive immediate reaction to the mosquito whole body extract. Three of 10 participants also had a positive immediate reaction to rAed a 2 ($P < 0.44$). None of the bite-test negative subjects had a positive reaction to either rAed a 2 or the whole body extract.

Thirty-three of 48 participants had a positive delayed reaction to a mosquito bite. Four of 33 bite-positive participants had a positive delayed reaction to the whole body extract. One of four participants also had a positive delayed reaction to rAed a 2. None of the bite-test negative participants showed a delayed reaction to either rAed a 2 or the whole body extract.

ELISA inhibition tests

To determine whether the ELISA was specific to the detection of rAed a 2-specific IgE and if the natural Aed a 2 in the mosquito head and thorax extract can block the binding of human IgE to the rAed a 2, ELISA inhibition tests were performed. The mosquito head and thorax extract successfully inhibited the binding of Aed a 2-specific IgE in the human serum to the rAed a 2 coated on the plates in a dose dependent manner (Fig. 2). This indicates that the ELISA was specific for the measurement of rAed a 2-specific IgE and that the rAed a 2 has identical immunologic properties to its native form in the mosquito preparation.

rAed a 2-IgE in 21 serum samples with a positive mosquito saliva-IgE

rAed a 2-IgE was measured in 21 individuals who had a positive saliva-IgE and 20 control individuals who had negative mosquito bite tests. The geometric mean level of rAed a 2-IgE was significantly higher in the individuals with positive saliva-IgE (0.23 ± 0.03) than in the controls (0.09 ± 0.03 , $P < 0.0008$). Using the geometric mean + 1 SD of the controls as a positive cutoff level, 43% (nine of 21) of saliva-IgE-positive individuals had positive rAed a 2-IgE, while only 5% of the controls had positive rAed a 2-IgE ($P < 0.01$) and positive saliva-IgE (Fig. 3). More importantly, the levels of rAed a 2 correlated significantly with the levels of saliva-IgE ($r = 0.553$, $P < 0.0001$) (Fig. 4).

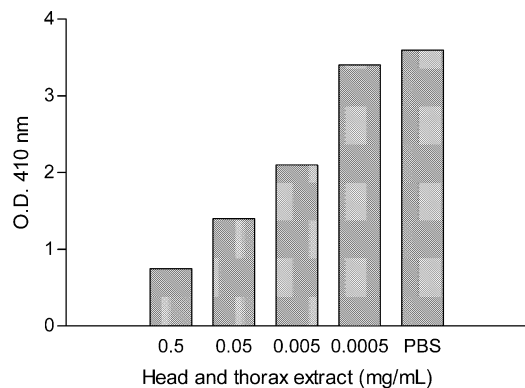


Figure 2. Inhibition of the binding of Aed a 2-specific IgE to rAed a 2 by addition of a mosquito head and thorax extract. Serum with a high level of Aed a 2-specific IgE was incubated with various dilutions of a mosquito head and thorax extract, and then assayed for the ability to bind the rAed a 2 coated on a 96-well plate using an ELISA.

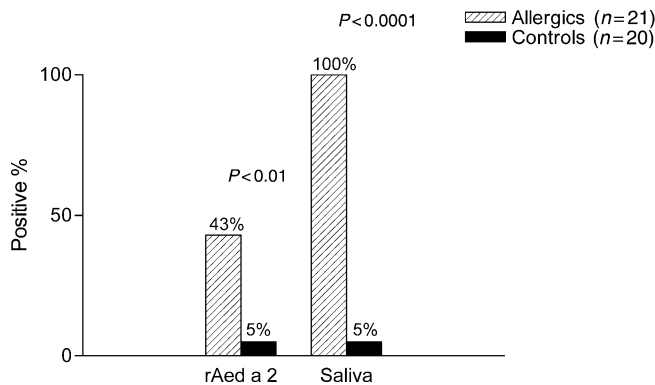


Figure 3. Positivity of serum rAed a 2-specific IgE. Serum rAed a 2 was measured by an ELISA in 21 mosquito-allergic individuals who had a positive saliva-specific IgE and in 20 controls who had a negative mosquito bite test. The geometric mean + 1 SD of the controls was used as a positive cutoff level.

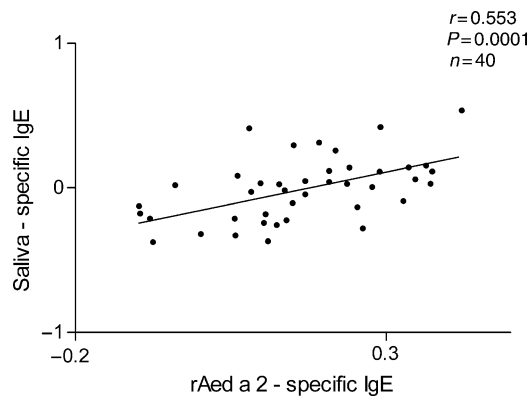


Figure 4. Correlation of serum rAed a 2-IgE and saliva-specific IgE levels (logarithmic transformed values) in 21 mosquito allergic individuals and 20 negative controls.

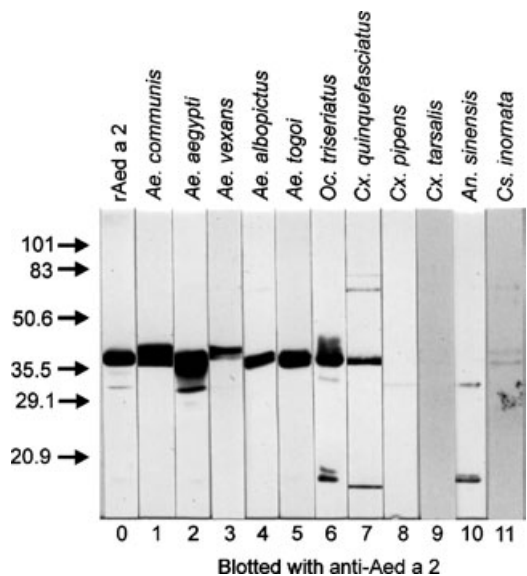


Figure 5. Examination of the presence of Aed a 2 in the salivary preparations of 11 mosquito species. Proteins in the saliva or salivary gland preparations were separated by SDS-PAGE and then transferred to nitrocellulose filters. The filters were probed for the presence of Aed a 2 using rabbit anti-rAed a 2.

Analysis of shared immunogenicity of Aed a 2 in 11 mosquito species

A 37-kDa protein moiety recognized by rabbit anti-rAed a 2 was found in all five *Aedes* species studied, *Oc. triseriatus*, and *Cx. quinquefasciatus*, but was not present in *Cx. pipiens*, *Cx. tarsalis*, *An. sinensis*, and *Cs. inornata* (Fig. 5). This indicates that these polypeptides in other mosquito species, especially, members of the genus *Aedes*, share common antigenic structures with Aed a 2 of *Ae. aegypti*. The species with smaller molecular weight, found in *Oc. triseriatus*, *Cx. quinquefasciatus*, and *An. sinensis* extracts, may represent denatured or short members of the D7-related protein family (31) that share some common antigenic structure with Aed a 2.

Discussion

In the saliva of *Ae. aegypti*, the most common mosquito pest worldwide, there are at least eight allergens (22), and new ones are being discovered (32). The Aed a 2 antigen is the original protein (26) that defines a large family of D7-related proteins. A large number of cDNAs corresponding to these proteins have been isolated and characterized from salivary gland-specific libraries of a variety of hematophagous insects (31). Two types of D7 proteins have been identified, the so-called long (molecular weights in the 30+ kDa range) and short (molecular weights in the 16–20 kDa range) forms. It is not surprising that Aed a 2 antibody

cross-reacts with saliva or salivary gland extracts from other mosquitoes (Fig. 5). Most of the cross-reactivity with short molecular weight forms is in *Cx. quinquefasciatus*, *An. sinensis* and *Oc. triseriatus*, although there also seems to be some reactivity with the higher molecular weight forms in *Cx. quinquefasciatus*. Our data confirm the general antigenicity of this family of salivary gland proteins.

Baculovirus/insect cell systems have been used extensively for the production of large amounts of recombinant proteins with biologic activity (23, 33). The ability of insect cells to perform many of the post-translational protein modifications found in eukaryotic cells is a major advantage over prokaryotic expression systems. Moreover, the growth of insect cells in serum-free medium makes the purification of the recombinant protein relatively easy using simple laboratory techniques. Proteins expressed using this system can be used in both *in vitro* and *in vivo* tests (14). In contrast, proteins expressed by using prokaryotic expression systems, e.g. *Escherichia coli*, have no carbohydrates and are generally only used in *in vitro* assays (15, 17). The efficiency of expression of baculovirus system varies up to 1000-fold from gene to gene. In our study, about 10 mg of recombinant protein was obtained from 1 litre of culture medium using small-scale batch fermentation in spinner cultures.

rAed a 2, expressed using a baculovirus/insect cell system, has shown to be immunogenic in both *in vitro* and *in vivo* tests. It is extremely difficult to isolate native Aed a 2 from mosquito saliva for a direct comparison with rAed a 2. However, indirect evidence indicates that rAed a 2 has identical immunogenicity and biologic activity to native Aed a 2. First, in Western blotting, rAed a 2 bound to the serum IgE antibodies, which were induced by natural Aed a 2 in mosquito saliva, i.e. the IgE in mosquito allergic sera (Fig. 1, left), and natural Aed a 2 in the saliva was able to bind to the rabbit antibody immunized with rAed a 2 (Fig. 1, right). Secondly, the binding of rAed a 2 to human IgE in ELISA could be inhibited by addition of natural Aed a 2 in mosquito head and thorax extract (Fig. 2). In addition to the above *in vitro* experiments, rAed a 2 itself induced skin immediate and delayed reactions, as the mosquito whole body extract and mosquito bite tests did, indicating that rAed a 2 has biologic activity. Significant correlation of serum rAed a 2-IgE levels with mosquito saliva-IgE levels provides additional evidence supporting its identical immunogenicity to native form in the saliva.

The *in vivo* allergenicity of rAed a 2 has also been investigated in mice (34). Injections of rAed a 2 without adjuvant induced a significant increase in IgE and IgG1 (Th2-driving antibodies), but not IgG2a (Th1-driving

antibody) in BALB/c and C57BL/6 mice. In all sensitized mice, a positive immediate skin reaction was observed. In immunoblot analysis, the mouse IgE antibodies elicited by rAed a 2 bound not only to rAed a 2 but also its natural form in mosquito saliva.

The positivity rate of epicutaneous tests with rAed a 2 (10%) and the mosquito whole body extract (32%) seems to be low in mosquito bite-test positive individuals. In mosquito bite tests, fresh salivary proteins are injected directly into the skin. In our previous experiments, each salivation of a female adult *Ae. aegypti* produces approximately 0.2 µg of saliva, while in epicutaneous tests, each prick introduced about 3×10^{-6} ml of the test solution into the epidermis (30), that is, the equivalent of 0.0030 µg of rAed a 2 or 0.0095 µg of the whole body extract was introduced in each epicutaneous test. The small amount of antigen extract introduced may account for the low positivity rate of both rAed a 2 and the whole body commercial extract. As Aed a 2 is one of eight allergens in the saliva of *Ae. aegypti*, it is to be expected that the positivity rate induced by a single allergen in the saliva would be lower than by the mosquito whole body extract. However, in comparison with the 68-kDa rAed a 1, which induces positive skin reactions in 29% (23) of the participants with a positive mosquito bite test, rAed a 2 is a less common allergen.

Our long-term goal is to clone and express all of the eight allergens identified in the saliva of *Ae. aegypti* (22). Using a cocktail of all the recombinant allergens, we will eventually develop highly sensitive immunoassays for the diagnosis of mosquito allergy. If positive, then using each of the allergens separately will further identify the particular allergen(s) to which the individual is sensitized. Forty-three per cent of individuals with a positive serum IgE to saliva had a positive IgE to rAed a 2, and as a species-shared allergen present in all *Aedes* mosquito studied and other two species, rAed a 2 may have broad applicability in the diagnosis of mosquito allergy.

In conclusion, we report the second recombinant mosquito salivary allergen rAed a 2. In addition, characterization of the third and fourth recombinant salivary allergens of *Ae. aegypti*, rAed a 3 (24), and rAed a 4 (25), are in progress. These recombinant salivary allergens will greatly facilitate the diagnosis and immunotherapy of mosquito allergy.

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