

Original article

A novel inhalation allergen present in the working environment of beekeepers

Background: Inhalation allergies, caused by allergens from various kinds of pollen, house dust mites, animal epithelium, and mould fungi, are strongly increasing in frequency. In 2.6% of the cases the allergen source remains unidentified. The present paper describes a so far unknown inhalation allergy which was observed in the case of a patient working with hives.

Methods and results: The allergen was characterized by immunoblotting, enzyme-linked immunosorbent assay inhibition, and isoelectrofocusing, using the serum of the patient. It is present in both the bee bodies and the larvae, has a molecular mass of 13 kDa, and an isoelectric point of 5.85. It is thermolabile and does not cross-react with allergens from birch, mugwort and timothy grass pollen, mould fungi, or bee venom. The N-terminal amino acid sequence of allergen from larvae was determined to be ²QIEELKTRLHT¹². A similar allergen of 13 kDa was also found in *Varroa* mite accompanying bee populations.

Conclusion: Honey bees (including the larva stadium) and *Varroa* mite contain a 13-kDa protein causing an allergic reaction. Presently, there is no evidence whether the case described is a singular phenomenon or whether this allergen is a more common inducer of allergies among subjects exposed to honey bees. However, a bee and *Varroa* mite allergy has to be considered for beekeepers after exclusion of known inhalation allergies.

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Inhalation allergies are caused by a number of different allergens. Most of the allergic subjects simultaneously react to more than one allergen. In Germany, the most common inhalation allergies are caused by various kinds of pollen (86%), animal epithelia (55%), house dust mites (38%), and mould fungi (13.5%). In 2.6% of all cases, the allergen responsible for the inhalation allergy remains unidentified (1).

In this paper, we report on a case of a so far unknown inhalation allergy occurring in the working environment of a beekeeper. Working with hives, the male patient had contact not only with bees, larvae, and propolis (a resinous fungicidal and bactericidal substance collected by bees from the buds of trees and flowers), but also with common bee parasites such as *Ascosphaera apis* fungus and *Varroa* mite. We prepared extracts from all these materials, identified the allergen(s), and investigated possible cross-reactions with pollen, bee venom, and mould fungi.

Material and methods

Case report

The patient H.F. is a 54-year-old male beekeeper. The patient had been working with honey bees for decades. In 1985, he showed the

first allergic symptoms such as rhinitis with sneezing, conjunctivitis, and pharyngeal irritations. The complaints were observed approximately 30 min after initiating to work with the hives. The patient disestablished his apiary, whereupon the complaints disappeared. In 1997, he re-started to work with honey bees. Since this time, he suffered from the same allergic symptoms as before. Since 1999, he additionally developed a shortage of breath. All symptoms except for the conjunctivitis were weakened remarkably by applying a dust shield mask. The allergic history did not indicate that the patient suffered from a bee venom, pollen, or honey allergy.

Serum

The serum of the allergic patient (H.F.) and a serum pool of 20 healthy (control) subjects, obtained from our outpatient department, were investigated.

Determination of the bee parasitic fungus and its *in vitro* cultivation

The hives of the patient were infected by a fungus. The species of this parasite was determined as follows: the fungus was isolated from mummified bee larvae (supplied by the beekeeper patient) by careful scraping and classified microscopically according to Alexopoulos and Mims (2) and Spiltoir and Olive (3).

After separation from the mummified larvae, the fungus was cultivated at room temperature on sterile malt agar plates (4).

Extracts

Extracts were prepared from:

- dry bee bodies, mummified larvae, propolis from the beekeeper patient's hives (all infected by a fungal parasite);
- living bee (complete bee bodies), living larvae, living *Varroa* mites, and propolis from uninfected hives (obtained from the Institute of Apidology, University of Jena);
- parasitic fungus from the hives and the *in vitro* cultivated fungus.

A total of 1 g bee bodies, larvae, or propolis were homogenized in 10 ml of 0.125 M NH_4HCO_3 buffer (pH 8) for 10 min at 4°C by means of a high-speed rotating-blade homogenizer (ultraturrax). Correspondingly, 0.2 g of both the isolated and cultivated fungus or 0.1 g of extensively washed *Varroa* mites were prepared in 2 or 1 ml, respectively. The homogenates were centrifuged for 30 min at 20 000 g and lyophilized.

The propolis extracts contained about 0.3 mg/ml protein, the other extracts between 4 and 10 mg/ml protein, as determined by the method of Bradford (5).

Lyophilized extracts from birch, timothy, and mugwort pollen, as well as from bee venom and the mould fungi *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarium*, and *Penicillium notatum* were obtained from Allergopharma, Reinbek, Germany.

Skin and sera tests

The prick tests were performed with commercial test solutions (birch, mugwort, timothy grass pollen, bee venom, *Alternaria alternata*, *Aspergillus fumigatus*, *C. herbarium*, *P. notatum*), purchased from Allergopharma. In addition, self-prepared extracts from the bee parasite fungus, as well as from infected and uninfected bee bodies, larvae, and propolis were tested. Their protein concentration was adjusted to 100 µg/ml with phosphate buffer saline (PBS) containing 50% (v/v) glycerol, followed by sterile filtration. Histamine dihydrochloride and physiological sodium chloride solution served as positive and negative controls, respectively.

The determination of total IgE and specific IgE to birch, mugwort, and timothy grass pollen, as well as bee venom and honey, were performed using a standard nephelometric procedure (Dade Behring BN II) and the Pharmacia CAP system, respectively.

SDS-PAGE and isoelectrofocusing

Electrophoretic separation was performed using 8–18% polyacrylamide gels (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at nonreducing conditions, applying 10 µg protein/lane.

Polyacrylamide gel isoelectric focusing was carried out using Ampholine PAGplates 3.5–9.5 (Amersham Pharmacia Biotech AB). The samples (15 µg/lane) were focused at 1500 V for 2 h.

For subsequent immunoblotting or amino acid sequencing, the proteins were transferred to nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membranes (Schleicher & Schuell, Dassel, Germany) at 10 V and 200 mA/gel for 75 min. To prevent non-specific binding, the membranes were then treated for 30 min with PBS containing 0.5% Tween 20.

Immunoblot and immunoblot inhibition

For the detection of specific IgE antibodies, NC strips (after SDS-PAGE and after isoelectrofocusing or IEF) were incubated

overnight at 4°C while shaking with 100 µl of either the patient serum or the negative serum pool (diluted with PBS/Tween to a total volume of 500 µl). Thereafter, the NC strips were treated for 1 h each with monoclonal mouse anti-human IgE AP conjugate (1 : 1000; BD-Biosciences, Pharmingen, Heidelberg, Germany) and finally developed with 4-nitroblue tetrazolium (Sigma, St Louis, MO) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

For immunoblot inhibition, 100 µl of patient serum were preincubated for 1 h at room temperature with 50 µg protein of the different extracts in a total volume of 500 µl (adjusted by addition of PBS/Tween). Thereafter, this serum was transferred to the NC strips and handled as described for the immunoblot.

Enzyme-linked immunosorbent assay inhibition (ELISA)

Microtitre plates (Nunc, Maxi Sorp, Roskilde, Denmark) were coated overnight at 4°C with the extract from uninfected larvae (20 µg/ml) and washed with PBS/0.1% Tween 20 blocking solution. The patient serum (diluted 1 : 5) was preincubated for 1 h at room temperature with extracts from uninfected larvae (nonheated or preheated for 5 min at 95°C) and with extracts from propolis of both uninfected and infected hives. The plates were loaded with 50 µl of the preincubated serum or 50 µl of nonpreincubated serum and incubated for 1 h at room temperature. Specific IgE was determined by incubation for 1 h with AP-conjugated monoclonal mouse anti-human IgE (1 : 1000; Pharmingen). The ELISA was developed with *p*-nitrophenyl phosphate (Sigma) as a substrate, and the optical density was measured at 405 nm.

Amino acid sequencing

The N-terminal amino acid sequences of protein samples were determined by automated Edman degradation on the Applied Biosystems model 494 sequencer (Applied Biosystems, Foster City, CA).

Results

Determination of the bee parasitic fungus

Mummified larvae were observed to be covered by grey mould, consisting of mycelia and many large fructifications. The latter were spherical or polyhedron-shaped synasci and had diameters between 62 and 85 µm. The spore balls within the synasci were also in the shape of spheres with 10–16 µm diameters. Free spores were hyaline and ellipsoid (3–4 µm length and 1–1.5 µm width). According to the features described for ascomycetes in the literature (2, 3) the fungus was definitely classified as *Ascosphaera apis*.

The microscopic features of the *in vitro* cultured fungus corresponded with those of the fungus in the hives in all details.

Skin tests and serological investigations

The prick test results with birch, timothy grass, and mugwort pollen, as well as bee venom, mould fungi, and infected propolis were inconspicuous. In contrast, positive skin reactions were observed with the extracts from

infected bee bodies, infected larvae, and *Ascosphaera apis* from the hives. Positive results were also obtained with the extracts from uninfected materials (bee bodies, larvae, propolis; Table 1).

Table 1. Skin prick test results and CAP classes of the serum from the allergic patient

Extracts	SPT	CAP classes
Bee bodies, infected	++	n.d.
Larvae, infected	+	n.d.
<i>Ascosphaera apis</i> from beehive	++	n.d.
Propolis, infected	–	n.d.
Bee bodies, uninfected	++	n.d.
Larvae, uninfected	+	n.d.
Propolis, uninfected	+	n.d.
Birch pollen	–	0
Timothy pollen	–	0
Mugwort pollen	–	0
Bee venom	–	2
Honey	n.d.	0
<i>Alternaria alternata</i>	–	n.d.
<i>Aspergillus fumigatus</i>	–	n.d.
<i>Cladosporium herbarium</i>	–	n.d.
<i>Penicillium notatum</i>	–	n.d.
Sodium chloride solution	–	n.d.
Histamine	+	n.d.

SPT, skin prick test; –, negative reaction; +, weal >3 mm; ++, weal >5 mm; n.d., not determined.

The total serum IgE was 439 IU/ml. No specific IgE to the allergens was detected with the exception of antibodies to bee venom of CAP class 2 (Table 1).

Allergen characterization

Following SDS-PAGE, the protein composition of the larva and bee body extracts was visualized by silver staining (Fig. 1A, lanes 1 and 2). By immunoblotting, an IgE-binding protein was detected at 13 kDa in the bee body and larvae extracts, both infected (Fig. 1B, lanes 1 and 2) and uninfected (lanes 3 and 4), in the extract of *Ascosphaera apis* from the hives (lane 6) as well as in the extract of *Varroa* mite (lane 7). In contrast, the extract from *Ascosphaera apis* cultured *in vitro* did not bind IgE (lane 5). The control serum did not show a positive reaction. The IgE binding to uninfected larva (Fig. 1B, lane 4) was inhibited by both bee body and both larva extracts, by the *Ascosphaera apis* extract from the hives, and also by the *Varroa* mite extract (Fig. 1C, lanes 1–6). In contrast, IgE binding was not inhibited by the extracts from *Ascosphaera apis* cultured *in vitro*, from birch, timothy grass, and mugwort pollen, from bee venom, or from *Alternaria alternata*, *Aspergillus fumigatus*, *C. herbarium*, and *P. notatum* (Fig. 1C, lanes 7–15). The immunoblot for the uninfected larvae after IEF revealed an IgE reaction at pI 5.85 (Fig. 2).

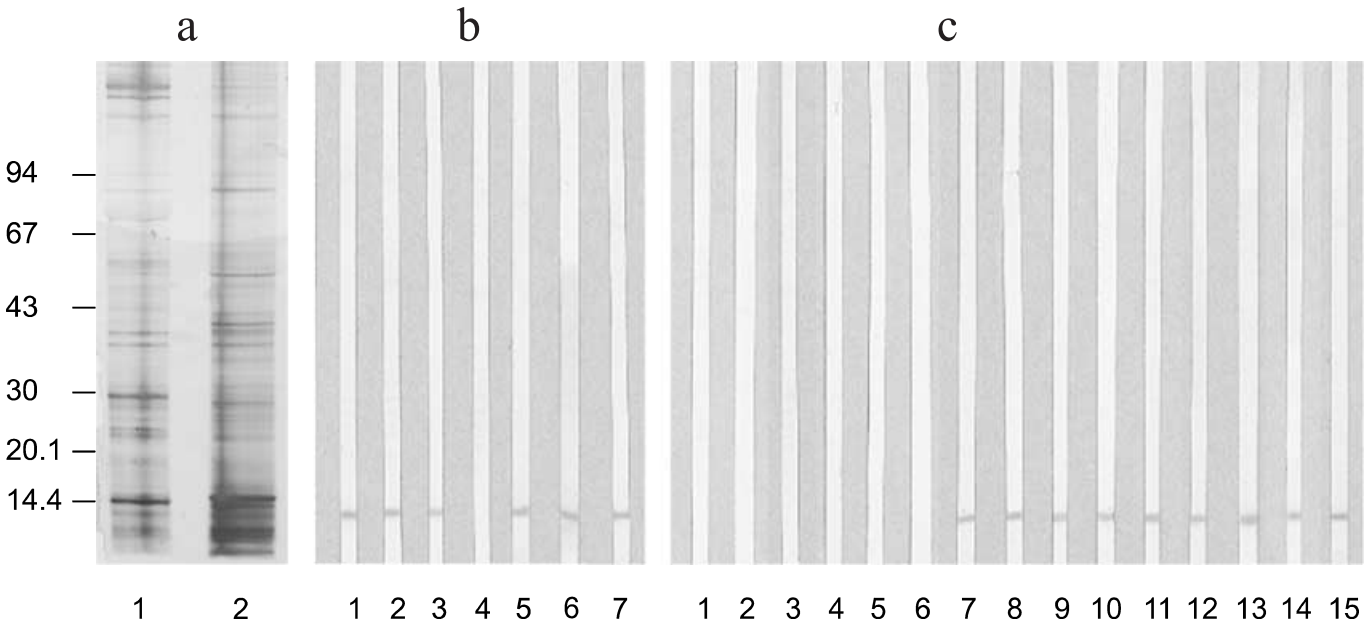


Figure 1. (A) Protein composition of the larva (lane 1) and bee body (lane 2) extracts, as visualized by silver staining. (B) Immunoblot after SDS-PAGE for the extracts of bee bodies, larvae from infected hives (lanes 1 and 2), of bee bodies, larvae from uninfected hives (lanes 3 and 4), of *Ascosphaera apis* cultivated *in vitro* and from hives (lanes 5 and 6), and of *Varroa* mites (lane 7). (C) Immunoblot inhibition of IgE binding to uninfected larva extract (solid phase) by different extracts: infected bee bodies (lane 1), infected larvae (lane 2), uninfected bee bodies (lane 3), uninfected larvae (homologous inhibition, lane 4), *Ascosphaera apis* from hives (lane 5), *Varroa* mite (lane 6), *Ascosphaera apis*, cultivated *in vitro* (lane 7), birch pollen (lane 8), timothy pollen (lane 9), mugwort pollen (lane 10), bee venom (lane 11), *Alternaria alternata* (lane 12), *Aspergillus fumigatus* (lane 13), *Cladosporium herbarium* (lane 14), and *Penicillium notatum* (lane 15).

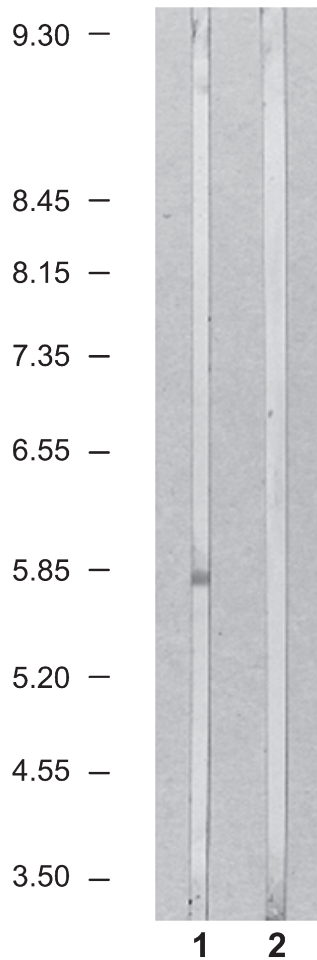


Figure 2. Immunoblot after IEF with serum from the allergic patient (lane 1) and with control serum (lane 2). Solid phase: extract from uninfected larvae.

The ELISA inhibition showed that the larva extract largely lost its immune reactivity after heating to 95°C (Fig. 3). Compared to the untreated extract, approximately 15 times more extract had to be applied to achieve a 50% inhibition. The inhibition of the IgE binding to larva proteins by both propolis extracts was approximately 40 times lower (at 50% inhibition) than the homologous inhibition.

The amino acid sequences of both the larva and the *Varroa* mite 13-kDa allergens were determined at the N-terminus. The sequences are identical with the exception of the isoleucine at position 3, which is absent in the mite protein (Table 2).

A computer search in the Swiss protein database with the 11 and nine residues determined for the larva and the mite allergen, respectively, did not reveal any protein with 100% identity. On the basis of eight residues identical in both sequences (EELKTRLH), an alignment was found in a worm (*Caenorhabditis elegans*) protein with 75% identity. Shorter fragments (six residues) showed up to 100% identity

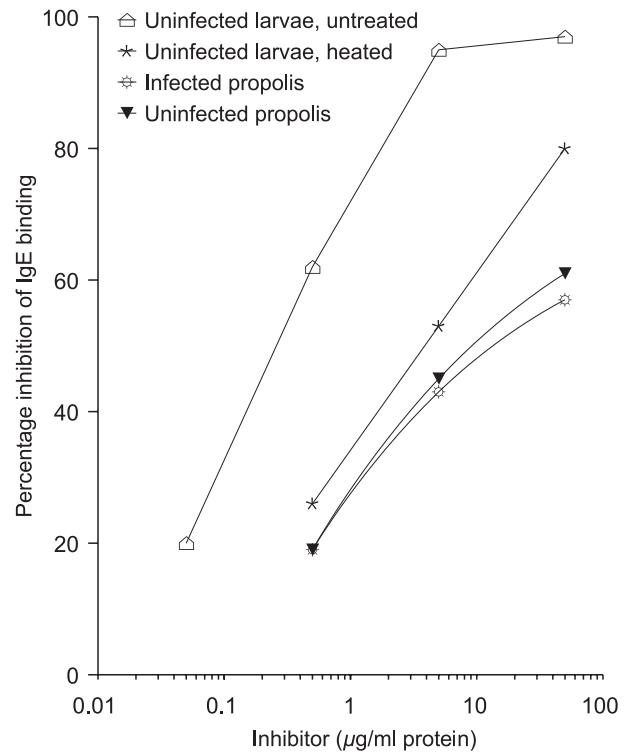


Figure 3. ELISA inhibition of IgE binding to extract from uninfected larvae (solid phase) by untreated or heated extract from uninfected larvae (homologous) and by propolis extracts from infected and uninfected larvae.

with proteins from other organisms, e.g. bacteria (Table 2). However, all these proteins are not known as allergens (result of computer searches in <http://www.ncbi.nlm.nih.gov/blast> and <http://www.allergome.org>).

Discussion

The patient history suggested an allergy to compounds of bee bodies, larvae, propolis, or of the bee parasites *Ascosphaera apis* fungus or *Varroa* mite. However, it had to be taken into consideration that these materials could be contaminated mutually. To distinguish between reactions caused by the materials originating from the bees and the fungus, we performed tests with extracts from bee bodies and larvae taken from hives that were not infected by *Ascosphaera apis* and, additionally, with *in vitro* cultivated *Ascosphaera apis*.

It is commonly known that exposure to mould fungi can cause allergic sinusitis, rhinitis, or asthma (6, 7). *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, and *Mucor* represent only some of the fungi with allergenic activity (6–11). Therefore, we initially suspected an allergy to the bee parasite fungus infecting the hives of the allergic beekeeper. Bee mycoses are usually caused by two fungal species: by the mould fungus *Aspergillus flavus*

Table 2. N-terminal sequences of the 13-kDa larva and mite allergens as compared with proteins of other organisms (computer search in the Swiss protein database using the residues EELKTRLH present in both allergens)

13 kDa allergen from larvae	?	Q	I	E	E	L	K	T	R	L	H	T ¹²
13 kDa allergen from <i>Varroa</i> mite		?	Q	E	E	L	K	T	R	L	H ¹⁰	
Hypothetical protein ZK177 from <i>Caenorhabditis elegans</i>				538 E	E	L	K	I	R	L	H ⁵⁴⁵	
33 kDa chaperonin from <i>Vibrio parahaemolyticus</i>				152 E	Q	L	K	T	R	L ¹⁵⁸		
Hypothetical protein AF1731 from <i>Archaeoglobus fulgius</i>				15 E	E	L	K	T	R ²⁰			

?, The first step of degradation is not interpretable.

and the ascomycete *Ascosphaera apis* (12). Whereas *Aspergillus flavus* is relatively rare, *Ascosphaera apis* is an ubiquitous habitant which effectively propagates by spores and is known to cause chalk brood (mummification of the bee brood). Chalk brood is an annoying and insistent disease of bee colonies which is spreading now worldwide and causing immense economic loss (13, 14).

In addition, an extract of *Varroa* mites from a beehive which was not infected by *Ascosphaera apis* was included in the study. *Varroa* mite is known to be the most serious parasite of bee colonies worldwide. It causes abnormalities, reduced weight and longevity of workers and drones, death of young brood, and premature death of adult. Distributing over Europe since 1977, the mite causes enormous loss of managed honey bee colonies (15, 16). Also the hives of the patient were infected by this mite.

In contrast to our initial suspicion that the allergy is caused by the fungus, we identified an allergen of 13 kDa in the bee bodies, larvae and also in the *Varroa* mite, independently of an *Ascosphaera apis* infection. The positive skin test to *Ascosphaera apis* from the hives was presumably due to contamination of the fungus with protein from bee bodies, larvae or *Varroa* mite, since the extracts of all these materials were derived from the infected bee colony of the patient's apiary.

The results from N-terminal sequencing of the 13 kDa allergens of bee larvae and of *Varroa* mites suggest the existence of two proteins with same IgE-binding epitopes. As there is a high sequence homology at the N-terminal ends, we suppose that both proteins are of common origin. A selective deletion of the third amino acid during digestion of the bee allergen (*Varroa* mites feed on bees and larvae) seems to be less probable. Cloning of the corresponding genes from bees and mites might contribute to clarify the relationship between both proteins.

Recently, Callejo et al. (17) reported that propolis is able to cause an IgE-mediated reaction. In our study, the *in vivo* tests with propolis extracts from infected and uninfected hives gave contradictory results (Table 1), whereas the corresponding *in vitro* tests revealed a

comparable but weak inhibition in both cases (Fig. 3). Therefore, we conclude that the propolis also was slightly contaminated by the allergen from bee bodies, larvae, or *Varroa* mite and does not seem to be the cause of the allergy of the beekeeper.

An allergy of the patient to pollen, mould fungi, and honey could be excluded by the patient history, skin tests, and serological investigation (Table 1). Because of the absence of clinically relevant symptoms after bee stings, the IgE level (CAP class 2) of the patient to bee venom does not suggest an IgE-mediated allergy but may be regarded as a sensibilization due to frequent bee stings. We chose birch, timothy grass, and mugwort pollen, as well as mould fungi (*Alternaria alternata*, *Aspergillus fumigatus*, *C. herbarium*, *P. notatum*) as material for immunoblot inhibition, as they play an important role in inhalation allergies, mainly elicited in the outdoor environment. In addition, the allergens of *Alternaria* Alt a 1 and *Cladosporium* Cla h 1 are known to be of comparable molecular size of 13–14 kDa (11). However, there was no cross-reactivity between the 13-kDa protein of bee larvae and any of the allergens mentioned above. We assume that the allergic complaints of the beekeeper were due to inhalation of a fine powder released from brittle mummified larvae and dead bees which was released in the air during the handling of honeycombs in the hives.

Conclusions

The present paper reports on a so far unknown inhalation allergen of bee bodies, larvae, and *Varroa* mite, which is observed in the environment of beekeepers. Presently, there is no evidence whether the case described is a singular phenomenon or whether the bee body and larva allergen from beehive is a more common inducer of allergies among subjects exposed to honey bees. However, our results indicate that this allergen has to be considered as the cause for a beekeeper's inhalation allergy after exclusion of other inhalation allergies, e.g. to pollen or mould fungi.

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