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DETECTION OF INADEQUATE LABELLING AND CONTAMINATION AS CAUSES OF ALLERGIC REACTIONS TO FOOD

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Discrepancies between food content and label can lead to adverse reaction in people with hypersensitivity to particular food ingredients. Over a period of more than 10 years, 171 cases of adverse reaction to food have been registered. In all cases the offending foods were compound products where the ingredients were either hidden to the consumer as a cause of inadequate labelling (73 cases) or because the food was contaminated with the ingredient (64 cases). In 34 of the cases, the ingredient was inadvertently consumed. The largest number of cases is caused by milk, followed by tree nuts, peanuts and egg. However, the most severe reactions were caused by peanuts and soy, followed by hazelnuts and milk. Soy has caused severe anaphylactic reactions leading to death in peanut allergic individuals consuming meatballs, hamburgers and kebab containing high amounts of soy protein.

The presence of the offending ingredient was confirmed by analysis of the food item by immunological and DNA-based methods. In some of the cases we were able not only to detect the causative protein, but also to estimate the dose causing the allergic reaction. The doses range from less than 1 mg, causing emergency treatment, to several hundreds of mg causing stomach pain, vomiting and diarrhoea. A dose equivalent to 60 mg of casein in a sausage caused fatal anaphylaxis in a 15-year-old girl and a dose equivalent to 5.6 mg of peanut protein caused fatal anaphylaxis in a 22-year-old man.

Keywords: food allergy, anaphylaxis, protein detection, estimated dose, severe food reactions

In Western countries up to 1–2% of the total population suffer from clinically proven food allergies. Among children, the prevalence is even higher, with up to 8–10% being allergic. The symptoms of food allergy may range from mild urticaria to life-threatening anaphylactic shock. In the USA, a rate of approximately 150 deaths per year was calculated to be caused by allergic reactions to hidden allergens in food (BOCK et al., 2001). Foods are more often involved in anaphylactic reactions than other items. In hospital-based series of patients with anaphylaxis, foods were involved in 25–60% of all anaphylactic reactions in which the cause has been established with reasonable certainty (YOCUM & KHAN, 1994; KEMP et al., 1995; NOVEMBRE et al., 1998). In a study from northern England, the most severe anaphylactic reactions in 172 patients were reported (PUMPHREY & STANWORTH, 1996). Of these, 103 were related to foods, 28 to bee or wasp venom and 15 to drugs. The most common foods responsible for more than 90% of allergic reactions are cows milk, egg, fish and crustaceans, peanut, soy bean, cereals

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and tree nuts and products of these as recognised by the Codex committee (KJELKEVIK et al., 1997). The European Commission also recognises sesame seed, celery and mustard as import sources for adverse reactions and thus for food labelling (EUROPEAN COMMISSION, 2003). Although cow milk and egg white cause most anaphylactic reactions during infancy, the situation is different in older children and adults. A survey study (EUROPEAN COMMISSION, 1998) compiling data from five EU member states showed that among 613 anaphylactic reactions caused by foods, peanuts are the most frequent causative agent, followed by tree nuts and fish.

Allergic individuals have to strictly avoid the consumption of the allergenic food. However, studies have demonstrated that allergic reactions occurred even when people knew about their allergy and tried to avoid the causative food (FOUCARD & MALMHEDEN YMAN, 2001). The access to methods to detect proteins that might cause allergic reactions is important, both for food manufacturers and for food control authorities (POMS et al., 2004). Up to now, mainly immunological methods based on specific antibodies have been used. Recently, also techniques for the detection of DNA have been described using polymerase chain reaction (PCR) as a complement to protein methods. However, currently the DNA method is only qualitative or semi-quantitative. For quantitative purposes, only protein methods can be used.

The aim of this study is to compile data regarding incidence and types of adverse reactions to food in Sweden, to evaluate methods for the identification of the causative protein in food and to estimate the dose, which might be involved in eliciting the allergic reaction.

1. Material and methods

1.1. Food samples and clinical data

Since 1993, all physicians in Sweden have been asked to report life-threatening and fatal allergic reactions caused by food as they occurred (FOUCARD & MALMHEDEN YMAN, 1999). Suspected food samples are collected by doctors and dieticians, sufferers and their families, and food control authorities and food manufacturers. The samples are sent to the National Food Administration for analysis. In some of the cases the allergic individuals were allergic to two or more proteins and the analysis had to confirm which protein was present. Data regarding the severity of the reaction, the estimated dose and the age and gender of the sufferer are also collected. In some of the death cases, gastric juice was also analysed.

1.2. Antisera

Antiserum to several animal and vegetable proteins were produced in rabbits (AgriSera AB, Vännas, Sweden). The antisera used for the detection of food proteins include antiserum to milk proteins (casein, beta-lactoglobulin, alpha-lactalbumin) and egg proteins (ovalbumin, ovomucoid, conalbumin). For the detection of tree nuts, antisera to

hazelnut, Brazil nut, cashew, pistachio, pecan, walnut and almond were produced and for the detection of sesame seeds, an antiserum to sesame protein was raised. Different legumes were detected by antisera to peanut, to pea protein and to lupine. Some of the proteins used for immunisation were commercially available and could be used directly for the production of antiserum. Other proteins, like those from nuts, were purified (KLEIN et al., 1985). The specificity of the different antisera had to be confirmed before use. Overlapping specificity between different nut antisera is common and had to be eliminated by adsorption of the antiserum, e.g. antiserum to hazelnut was incubated with walnut and pecan extracts at +4 °C overnight, followed by centrifugation at +4 °C, 20 000 g.

1.3. Rocket immunoelectrophoresis

Rocket immunoelectrophoresis was performed on 4.5×5.2 cm GelBond plastic sheets covered with 1.8 ml of agarose containing 10–20 µl of rabbit antiserum (MALMHEDEN YMAN et al., 1994). Samples and references (in general 2 g) were extracted in 10 ml Tris buffer, pH 8.6, at 40 to 50 °C. For full extractability of dark chocolate samples, addition of the polymer polyvinylpyrrolidone (PVP) (LOOMIS, 1974) or gelatine (KECK-GASSENMEIER et al., 1999) is necessary to bind tannins, a natural component in cocoa. Two µl of samples and references were applied to each well in the antibody containing gel. Electrophoresis was performed on a PhastSystem™ electrophoresis equipment for 60 min at 100 V, 25 mA and 7 W at 15 °C. After electrophoresis, the gels were soaked in saline and water before being stained by Coomassie Brilliant blue R-250. The detection level may vary somewhat depending on the antiserum, but for most proteins is at the level of 10–20 ppm.

1.4. Immunoblotting

Samples (2 g) were extracted with 10 ml of Tris glycine buffer, pH 8.5, containing 2% SDS and 0.1% DTT. Twenty µl of the extracts were applied on a SDS polyacrylamide gel, 4–12% (NuPAGE, Bis-Tris gel) and the food proteins were separated by electrophoresis. The separated proteins were then transferred to a nitrocellulose sheet using Novex X cell II blotting equipment (Invitrogen, USA). The proteins were first stained with Ponceau S, destained and then immunostained. The immunoreactive bands were visualised by addition of the different rabbit antisera, followed by addition of a second antibody (goat anti rabbit IgG), a peroxidase linked antibody (PAP-complex) and substrate for the enzyme (JANSSEN et al., 1987).

1.5. Enzyme immunoassays

Soy protein was quantified with a commercially available enzyme immunoassay (EIA) (Tepnel BioSystems Ltd., UK), using a polyclonal antibody in a competitive assay. The detection limit was 0.2% soy protein.

Gluten analysis was performed with an EIA (Transia Plate gluten, Diffchamb SA, France) using a monoclonal antibody to omega-gliadin. The detection limit was 20 ppm gluten.

1.6. Lysozyme activity

Lysozyme, a protein from egg white with a bacteriolytic activity, was measured by the addition of *Micrococcus lysodeikticus* to the suspected food samples. The lysis of the cells was followed by measurement of the change in optical density at 450 nm (BIRKKJÆR et al., 1982).

1.7. Real Time PCR

DNA extraction from food samples and references were performed using the Qiagen DNeasy® tissue kit with some modifications. All PCR amplifications were performed with 50 ng of total DNA. For detection of soy and peanut, specific primers and Taqman probes for the lectin gene and for ArahII gene, respectively, were used. For the specific detection of DNA from different tree nuts and seeds, a real-time PCR (polymerase chain reaction) method in conjunction with SYBr Green I and melting curve analyses has been developed. For hazelnut, an amplicon length of 182 base pair was used (HOLZHAUSER et al., 2000). The target sequence is coding for the major hazelnut allergen Cor a1. Specific target sequences encoding for the 2S storage proteins Ber e1 and Jug r1 were chosen for Brazil nut and walnut amplification. For almond, the target sequence encoding for lipoxygenase detected almond but not apricots (SANDBERG & MALMHEDEN YMAN, 2004). The detection level was 0.01% DNA.

2. Results

2.1. Food responsible for adverse reactions

Most of the cases of adverse reactions to food during the more than 10-year registration period were caused by milk (44 cases), followed by tree nuts (30 cases), peanuts (29 cases) and eggs (21 cases). This is illustrated in Fig. 1A based on 154 cases, excluding gluten and lactose reactions, which account for totally 16 of the 171 reported cases, and one case caused by the red dye karmin (E 120). Legume products account for totally 52 cases, which is one-third of the total number of reactions. Of the reactions caused by legumes, peanuts account for 29 cases (55.8%), soy was involved in 17 (32.7%) and pea or lupine in 6 cases (11.5%).

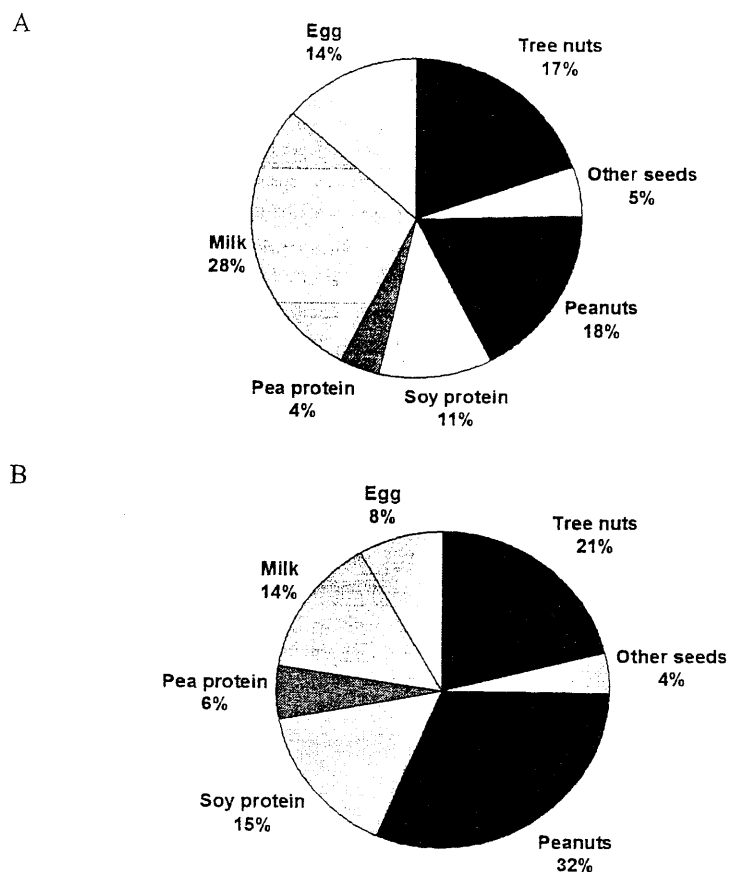


Fig. 1. A) The relative distribution of allergic reactions to various food based on 154 reported cases during a 10-year period; B) The relative distribution of the most severe food reactions based on 72 severe cases selected from the group reported in A

It is interesting to note that five of the 21 cases (24%) reported from egg allergic individuals were caused by lysozyme, a preservative declared as E1105 and used during cheese processing. Lysozyme is also used in wine industry for clearing purposes.

Hazelnut accounts for 20 (67%) of the cases caused by tree nuts, while almond/apricots account for 5, Brazil nuts for 3 cases and walnut and coconut, one case each. A significant number (4 cases) was caused by sesame seed, while sunflower seed, linseed and pine nuts account for one case each.

2.2. Foods involved in the most severe reactions

The most severe reactions (Fig. 1B) were caused by peanuts (22 cases) and tree nuts (16 cases), followed by soy (10 cases). However, also milk (10 cases) and egg (6 cases) were involved in severe reactions.

Typical foods where peanuts have been detected are bakery products, chocolate and ice cream. In most of the cases, the products were mislabelled. Pieces of peanuts or peanut flakes were clearly seen but labelled as almond or hazelnut. The falsification could only be identified by analysis of the flakes and pieces. Recently, also Asian foods account for a growing number of reactions to peanuts. However, in these foods usually no pieces of peanuts are seen, since the ingredient mainly is a paste of peanuts used as a spice, i.e. Satay mein paste. For tree nuts, the situation is different from that of peanuts. Tree nuts are most often detected as a contaminant in foods. Typical products, where hazelnut or hazelnut paste (soft nougat) is found are chocolate and ice creams covered with chocolate or with fillings of chocolate, bakery products and breakfast cereals.

2.3. Foods causing fatal anaphylaxis

Thirteen of the reactions to food caused fatal anaphylaxis. Eight of these cases have already been described in detail (FOUCARD & MALMHEDEN YMAN, 1999). Five additional deaths have occurred. One young man, 22 years of age, had a meal at a Thai restaurant, became ill shortly after, with vomiting, lost consciousness and was declared dead upon arrival at the hospital. Post-mortem analysis of the gastric juice revealed a peanut protein concentration of 0.028% in the gastric content. The young man had a well-known allergy to peanuts, hazelnut, milk and egg and was informed by the waitress that none of these ingredients should be in the dish. Only peanut was detected upon analysis, not milk, egg or hazelnut proteins. The cause of death of a 6-year-old girl was not possible to verify. She died after having lunch at school but no food was taken for analysis and post-mortem examination was not done. One case led to the death of a peanut allergic 30-year-old man. An empty peanut bag was found nearby the dead person and pieces of peanuts were confirmed by DNA-analysis in the gastric juice. Out of twelve confirmed cases, peanuts were the cause of four deaths and another four were caused by soy. Hazelnut and milk caused two deaths each.

The majority of those who died in anaphylactic shock were children and youngsters. Their ages varied between 6 and 22 years. Many of them suffered from mild to moderate asthma, two cases were classified as severe. Combination with exercise or intake of cold beverages were probably aggravating factors in some of the cases.

2.4. Estimation of the dose of the incriminated food

In many of the cases reported to the National Food Administration, we have been able not only to detect the causative protein but also to estimate the dose causing the allergic reaction.

For the milk protein casein (Table 1), the dose ranged from less than 1 mg of casein in candies, causing emergency treatment of a 6-year-old girl, to several hundreds of mg causing stomach pain, vomiting and diarrhoea. A dose equivalent to 60 mg of

casein in a sausage caused fatal anaphylaxis in a 15-year-old girl and a dose equivalent to 36 mg of casein in chips caused fatal anaphylaxis in a 10-year-old boy.

For the egg protein ovalbumin (Table 2), a dose of 10 mg gave stomach pain in a 17-year-old girl and 20 mg in a cevapcici gave an anaphylactic reaction requiring emergency treatment of a 20-year-old female.

For the hazelnut protein corylin (Table 3), a dose of about 1 mg of corylin in chocolate caused asthma, vomiting, urticaria, breathing difficulties and emergency treatment in both children and adults. An estimated dose of 6 mg gave an anaphylactic reaction in a 13-year-old girl. A dose of 7 mg Brazil nut protein caused anaphylaxis and emergency treatment in a female aged 38.

For legumes, the dose for soy protein varied from about 10 mg up to several hundreds of mg (Table 4) and the same applies to pea protein. However, the dose of peanut protein, causing severe reactions, has been more difficult to calculate, since peanut flakes can be unevenly distributed in a piece of cake or bread. In one case of reaction to chocolate only, the peanut protein (paste) was evenly distributed and the dose could thus be estimated to 1.1 mg. This dose caused severe breathing difficulties and urticaria in a 3-year-old girl after consuming a piece of chocolate from an Advent calendar. From one of the last victims with fatal outcome, a 22-year-old man, we were able to quantify the concentration of peanut protein to 0.028 percent in the gastric content. From these data, the dose could be estimated to 5.6 mg peanut protein.

Table 1. Estimation of the dose of casein (milk protein) involved in eliciting allergic reactions

Food	Amount consumed	Casein* conc.	Estimated dose	Reactions reported	Sex/age
Candies	30 g	0.003%	0.9 mg	Anaphylactic reaction, emergency treatment	f 6y
Biscuit	25 g	0.03%	7.5 mg	Vomiting, breathing difficulties	f 10y
Potato chips	40 g	0.083%	36 mg	Fatal anaphylaxis	m 10y
Ice-cream (soy-based)	5 g	0.2%	10 mg	Swelling of lips and tongue	m 3y
Soy-based infant formula	250 ml	0.004%	10 mg	Asthma attack, vomiting	f 3y
Chocolate	3 g	0.4%	12 mg	Stomach pain, vomiting	m 9y
Chocolate	25 g	0.13%	32 mg	Urticaria, vomiting	f 3y
Chocolate	50 g	0.29%	145 mg	Stomach pain	m 14y
Chocolate	50 g	0.54%	270 mg	Stomach pain	m 14y
Sausage	50 g	0.04%	20 mg	Vomiting, urticaria	m 6y
Sausage	50 g	0.08%	40 mg	Vomiting, breathing difficulties	m 5y
Sausage	100 g	0.06%	60 mg	Fatal anaphylaxis	f 15y
Sausage	10 g	1.1%	100 mg	Urticaria, vomiting, breathing difficulties	f 3y
Sausage	25 g	0.5%	125 mg	Stomach pain	m 6y
Sausage	25 g	1.6%	400 mg	Vomiting, diarrhoea	f 3y
Sausage	25 g	1.7%	425 mg	Stomach pain, vomiting	m 11y

* Casein constitutes 80% of the proteins in milk

Table 2. Estimation of the dose of ovalbumin (egg protein) involved in eliciting allergic reactions

Food	Amount consumed	Ovalbumin* conc.	Estimated dose	Reactions reported	Sex/age
Pasta	100 g	0.01%	10 mg	Stomach pain	f 7y
Cevapcici	100 g	0.02%	20 mg	Anaphylactic reaction, emergency treatment	f 20y
Meat balls	50 g	0.02%	70 mg	Stomach pain, vomiting	m 6y
Cookie	10	1.27%	100 mg	Vomiting, breathing difficulties	f 4y
Hamburger	50 g	0.34%	170 mg	Urticaria, vomiting	m 3y

* Ovalbumin constitutes 54% of the proteins in egg white

Table 3. Estimation of the dose of corylin (hazelnut protein) involved in eliciting allergic reactions

Food	Amount consumed	Corylin* conc.	Estimated dose	Reactions reported	Sex/age
Chocolate	3.6 g	0.02%	0.72 mg	Asthma, urticaria, emergency treatment	m 4y
Chocolate	3.6 g	0.03%	1.1 mg	Vomiting, urticaria	f 3y
Chocolate	6 g	0.016%	1.0 mg	Urticaria, vomiting, breathing difficulties	m 25y
Chocolate	4 g	0.42%	16 mg	Fatal anaphylaxis	f 15y
Wafers	6 g	0.05%	3 mg	Breathing difficulties, urticaria	f 4y
Chocolate	15 g	0.04%	6 mg	Anaphylactic reaction	f 13y
Biscuit	14 g	0.35%	50 mg	Urticaria, swelling of lips	m 14y

* Corylin constitutes about 10% of the hazelnut

Table 4. Estimation of the dose of soy protein involved in eliciting allergic reactions

Food	Amount consumed	Soy protein* conc.	Estimated dose	Reactions reported	Sex/age
Sausage	250 g	0.05%	12.5 mg	Anaphylactic reaction, breathing difficulties	m 5y
Rice noodles	100 g	0.2%	20 mg	Stomach pain	f 25y
Sausage	50 g	0.45%	225 mg	Breathing difficulties, swelling of lips and tongue	m 11y
Sausage	100 g	0.25%	250 mg	Itching, breathing difficulties, vomiting	m 18y
Hamburger	25 g	2.1%	1000 mg	Fatal anaphylaxis	f 9y
Meat balls	50 g	3%	1500 mg	Fatal anaphylaxis	f 17y
Hamburger	50 g	5.75%	2875 mg	Anaphylactic reaction	m 8y
Kebab	50 g	7%	3500 mg	Fatal anaphylaxis	m 9y

* Soy protein constitutes about 70–90% of soy concentrate/isolate

3. Conclusions

Methods to detect proteins involved in allergic reactions are mainly based on specific antibodies raised in rabbits or sheep. With the quantitative rocket immunoelectrophoresis 10 to 20 ppm are typically detected. Commercial enzyme immunoassays for peanut, hazelnut and egg might be even more sensitive and detect levels down to 1 to 2 ppm. The problem, however, is the availability of commercial antiserum to single proteins. Another problem when using antiserum might be the specificity. Adsorption of the antiserum is normally necessary to reduce overlapping specificity. Some food matrices, like dark chocolate, need special treatment like the addition of polyvinylpyrrolidone (PVP) or gelatine in buffers for full extractability of the proteins.

Recently, by introducing DNA techniques such as real-time PCR and specific primers to detect DNA from tree nuts, legumes, sesame seeds and cereals, we have established a complement to the protein methods. The stability of DNA allows the analysis of heated products where antibody based methods fail. The unique specificity of the target in the DNA methods allows discrimination between different nuts and seeds as well as between cereals, where cross-reactions are seen with immunological methods. The higher risk of contamination with the sensitive DNA methods is almost eliminated by basing the technique on real-time PCR and melting curve analysis. Both protein and DNA methods can thus be used for detection of the presence of nuts, seeds and legumes at low levels. However, only protein assays can detect and quantify allergens. DNA methods can be used as a confirmatory method to protein assays.

So far only few attempts have been made to collaboratively study the immunological methods for detection of proteins that might cause allergic reactions. No method standards are yet available in Europe. The production of reference material would facilitate standardisation of methods.

The dose causing reactions as well as the severity of the reaction may vary with the individual (Tables 1–4). Attempts have been made to estimate the dose in a controlled manner (HOURIHANE et al., 1997). The sensitivity to peanut flower among adults with allergy to peanut was evaluated. Systemic reaction in one individual appeared at a dose of 5 mg peanut protein. Two individuals had mild objective reactions to 2 and 50 mg of peanut protein, respectively. This can be compared with our findings of a dose of 1.1 mg of peanut protein, eliciting a reaction in a 3-year-old girl and 5.8 mg causing the death of a 22-year-old man.

Urticaria and asthma when eating bread covered with chocolate spread was experienced in a 5-year-old boy. The dose was estimated to 56 mg of hazelnut (WENSING et al., 2001), corresponding to about 5.6 mg of hazelnut protein (corylin), since corylin constitutes about 10% of the hazelnut. Our data on hazelnut give values both below and over that level (Table 3).

Allergic reactions to milk-contaminated "non-dairy" products among six milk allergic children have been reported (GERN et al., 1991). The estimated doses of casein causing the reactions were 14–38 mg. An unexpected presence of casein in a salmon product caused anaphylaxis in a 30-year-old woman (KOPPELMAN et al., 1996).

The estimated dose was 10 to 50 mg of casein. These levels are comparable to those we have found during analysis of food involved in allergic reactions to milk (Table 1).

Compilation of data regarding the lowest provoking dose used in food challenge gives figures varying between less than a mg to several hundreds of mg (TAYLOR et al., 2002). For peanut the dose ranged from 0.25 mg to 125 mg peanut protein, for egg the provoking dose varied between 0.13 to 200 mg egg protein and for milk from 0.6 mg to 180 mg milk protein. The very low doses were used for studies in infants and young children. Although these figures in most cases were estimated from general literature data on the protein content of foods rather than determined by analysis of the actual food, they confirm the wide variation in sensitivity between allergic subjects. The data also show that some of the subjects react to doses that are so small that even minute contamination may be enough to elicit serious reactions.

The methods described are examples of useful tools for detection of mislabelling or contamination of food products. Further development of immunoassays and DNA methodology will increase the number of proteins detected and enhance the absolute specificity of the analysis. The recently established working group 12 of TC 275 of CEN (Comité Européen de Normalisation) will elaborate standards in the field of allergens, a work that will increase conformity in analysis and testing and improve quality assurance and food safety.

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