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Lysozyme, a new allergen in donkey’s milk

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TO THE EDITOR:

In addition to its cosmetic properties, well known since antiquity, donkey’s milk (DM) has gained attention as a substitute for cow’s milk (CM) in CM allergic patients\(^1\). Different mammalian milks have been investigated in order to find valid alternatives of CM, which is highly cross-reactive with goat’s and sheep’s milk proteins, but not with camel, horse milk (HM) and DM, which are tolerated by more than 80\% of CM allergic children\(^1,2\). However, anaphylactic reactions to DM were reported in few of these children\(^2\) and in one adult\(^3\).

We report two cases of allergy to DM. The first patient (patient 1) is a 9-year-old girl referred for an acute generalized urticaria after the first application of a moisturizing cream containing DM (La Zane Attitude, Overijse, Belgium). She never ingested DM, but had used soap with DM as ingredient before. The girl had a history of previous anaphylactic reactions to peanut and tree nuts. She is not allergic to horse or donkey dander and never reacted to CM, goat or sheep’s cheese. Prick-to-prick (PTP) were positive with DM and the culprit cream La Zane Attitude. Mean weal sizes measured 19 and 10.5 mm respectively. The skin tests to CM, goat’s milk and sheep’s yogurt were negative.

The second patient (patient 2) is a 33-year-old woman who presented at the emergency room with angioedema involving the lips, left eyelid and hands. Symptoms occurred within a few minutes after tasting a couple of DM drops in order to check its temperature before feeding her child with it. The skin symptoms were associated with general discomfort, xerostomia and sweating. The patient had a
history of atopic dermatitis and moderate persistent allergic rhinitis, with allergies to house dust mite, poaceae pollen, cat and dog dander. She reported previous mild exacerbations of dermatitis due to DM-based cosmetics (Dhal, Italy) but she never reacted to CM or other food allergens. PTP were positive with DM (7 mm) and the Dhal DM-based creams (6 mm with hand cream, 7 mm with face cream).

In order to characterize the allergen(s) of DM, SDS-PAGE and IgE-immunoblot were performed with milk from donkey, horse, cow, goat and camel as described\(^4\). Both patient sera detected a strong band at 14 kDa in DM and a weaker band at the same molecular weight in HM (Figure 1A-1B). No IgE-reactivity was detected in cow, camel and goat milk. The 14 kDa protein band in DM was subjected to mass spectrometry and could be identified as donkey lysozyme (sequence coverage 96%). To confirm IgE-reactivity to lysozyme, the protein was purified from DM and HM (Figure 2A). As a first step, whey was obtained by adjusting the milk to pH 4.0 using 2N HCl. Whey was heated at 70 °C for 15 min. Lysozyme was purified by cation exchange chromatography using 20 mM NaH\(_2\)PO\(_4\) pH7 and a linear NaCl gradient (0-500 mM) (Resource S, GE Healthcare, Buckinghamshire, UK). Protein identity was confirmed by mass spectrometry.

An IgE-immunoblot inhibition experiment was performed in order to confirm the identity of the IgE-reactive band (Figure 1C). Incubation with purified donkey or horse lysozyme completely abolished IgE binding to the respective milk whey. Purified donkey and horse lysozyme was used in PTP test, ELISA and basophil activation assay. Skin test with patient 1 was positive at 1 μg/ml for donkey (3 mm) and horse (7 mm) lysozyme. Mean wheal size of patient 2 was 9 mm for donkey and 7 mm for horse lysozyme. Specific IgE to donkey and horse lysozyme were quantified for both patients (Figure 2B) by ELISA as described\(^4\). No reactivity to chicken egg lysozyme could be detected. The sera of 3 CM allergic patients with a skin prick test to DM ≤ 3 mm were tested negative for donkey, horse and egg lysozyme (data not shown). A basophil activation assay was performed with whole blood from patient 1, lysozyme and whey preparations from cow, donkey and horse (Figure 2C) using the FlowCAST kit (Bühlmann, Basel, Switzerland). Basophil reactivity was very strong with donkey and horse lysozyme and whey, negative with cow whey. Two atopic controls without history of food allergy to milk did not show any basophil reactivity to milk products (data not shown).
We have identified lysozyme as allergen in two cases of allergy to DM and/or DM containing cosmetic products. Both patients were also reactive to HM, but tolerant to CM. IgE-reactivity to DM and HM can be explained by the high sequence identity (99%) of both lysozymes. Lysozyme, whose antimicrobial properties are promoted by cosmetic manufacturers, is a thermostable protein that can therefore withstand the industrial processes of cosmetic production. It is present in high quantities in DM (up to 4 g/L) and HM (up to 1.4 g/L) whereas in CM, camel and goat, lysozyme is only found in the microgram range. Sequence identity of donkey lysozyme with cattle lysozyme is 53%. Although this percentage of sequence identity could point to a potential cross-reactivity, it is unlikely that patients with specific IgE to donkey or horse lysozyme will react to CM, given the low content of lysozyme in CM. None of our patients showed an IgE-reactivity to CM in immunoblot (Figure 1B). They also did not react to Gal d 4, chicken egg lysozyme (Figure 2B). Gal d 4 has 44% amino acid sequence identity with donkey lysozyme. Donkey and horse lysozyme have been named Equ a 6 and Equ c 6 by the WHO/IUIS Allergen Nomenclature Sub-Committee.

Even though serum albumin and beta-lactoglobulin have been described as allergens in DM, (www.allergome.com), their role in DM allergy has never been demonstrated. For the first time we have strong evidence that DM lysozyme is involved in allergic reactions to DM in patients without cross-reactions to CM. In our cases, the allergy was induced through a percutaneous pathway, since they never ingested DM before. This is consistent with previous data, describing how cosmetics containing food proteins could be a risk for subsequent development of allergic reactions in patients with skin barrier dysfunctions such as in patient 2 and in very similar case reports of food allergy to DM and HM.

In conclusion, we identified lysozyme as a molecule responsible for allergic reactions to DM. The high cross-reactivity with HM lysozyme, is suggesting caution when using HM in DM allergic subjects and vice versa. Moreover, we confirm the risk of the percutaneous sensitization associated with DM cosmetics, in particular in patients with atopic dermatitis.
Conflicts of Interest

None

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Figure 1: Detection of IgE-reactive proteins in donkey and horse’s milk. A) Separation of milk proteins by SDS-PAGE and staining with Coomassie Blue, B) IgE immunoblot of milks and patient sera P1 and P2, C) Immunoblot inhibition: patient sera P1 and P2 were incubated with immunoblotted donkey whey (Dw) without inhibitor (-) or after addition of 50 µg purified donkey (DL) or horse (HL) lysozyme. M, molecular weight standard, D, donkey, H, horse, C, cow, G, goat, Ca, camel’s milk.

Figure 2: Purification of lysozyme from milk and confirmation of IgE binding by immunoassay and basophil activation test. A) Lysozyme was purified from donkey (D) and horse (H) whey by ion exchange chromatography and purity of isolated donkey (DL) and horse (HL) lysozyme was assessed by Coomassie stained SDS-PAGE. B) Specific IgE to donkey, horse and chicken lysozyme was quantified by ELISA. C) Basophils of patient 1 were sensitized with increasing quantities of whey and purified lysozymes. Relative cell counts of CD63+ basophils were determined by flow cytometry. Anti-FcεRI is a positive control of basophil activation.