

Arbeiten aus dem Paul-Ehrlich-Institut
11th International Paul-Ehrlich-Seminar, Bad Homburg 2005
Verlag Chmielorz

Standardization of Animal Epithelia

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Summary

The standardization of animal epithelia is warranted for an accurate diagnosis and safe and efficacious treatment of allergic respiratory diseases induced by the inhalation of mammalian aeroallergens. We have compared several sources of raw materials of cat, hamster, goat and rabbit hair and epithelia to establish differences between the protein and allergenic composition of these extracts. The main differences in these raw materials was that “epithelia” were supplied as a mixture of hair and epithelia previously treated with acetone, and that the hair was supplied and used untreated. A possible influence of the age of rabbits on the composition of rabbit hair extracts was also evaluated. Overall, important differences were detected in the composition of epithelia *versus* hair extracts. Epithelia extracts contained more irrelevant proteins and, in most cases, less amounts of major allergens. Important differences were also detected in the composition of the extracts prepared with hair of young *versus* adult animals. In general, the extracts derived from young animals contained less major allergen and more albumin than those derived from older animals.

A greater effort should be made to identify the ideal sources of animal skin derived allergen extracts to provide the allergologists with better extracts for the diagnosis and treatment of allergic respiratory diseases. There is also a need for a consensus on the terminology applied to animal skin derived extracts. The use of the term dander extracts seems to be more appropriate. These extracts contain more relevant allergens and fewer albumins.

Introduction

The inhalation of epithelial derived allergens is a common cause of acute and chronic allergic respiratory diseases worldwide. The most important, and better studied allergens, are derived from cats, dogs, horses and cows. Allergic sensitization may occur at home, or at the workplace [1,2]. The introduction of exotic pets in the home

environment has also increased the number of mammalian species to which allergic sensitization has been described [3-5]. Occupational allergy to rodents is very common among laboratory workers. The most common offending agents are guinea pigs, rabbits, mice and rats. Rodent allergens can also be transported on clothes [5], as has been described for cats [7]. Exposure to these allergens in homes can pose a significant risk of sensitization for children. Sheep and goats are also animals to which allergic sensitization has been described [8]. While allergy to goat and cow milk has been widely studied [9,10], allergy to goat and sheep dander has not been widely analyzed. Goat and sheep belong to the order *Artiodactyla*, suborder *Ruminantia*; family *Bovidae*, which includes antelopes, gazelles, cattle, sheep, goats and relatives; subfamily *Caprinae* (sheep, goats, serows, chamois) [11]. Cross-reactivity between deer, cow, horse and goat allergen extracts has been described [12,13]. In European countries, sensitization to roe deer (*Capreolus capreolus*) has been described [14] and cross-reactivity among roe deer, goat and cow allergens could be due the lipocalins (20 to 25 kDa) [15]. Lipocalins are one of the most representative groups of allergens in mammalian species. The lipocalin superfamily constitutes a phylogenetically conserved group of more than 40 proteins that function in the binding and transport of a variety of physiologically important ligands. Members of this family cover diverse functions as carriers of retinoids, odorants, chromophores, pheromones and sterols [16]. In recent years, several important mammalian allergens have been purified, characterised and sequenced and their cross-reactivity analysed. A list of some of these allergens is presented in Table 1 (adapted from “www.allergen.org” and “www.allergome.com”).

Due to the increasing rate of sensitization to animal aeroallergens in occupationally and non-occupationally exposed individuals, standardized extracts are needed to better diagnose and treat these patients. Based on clinical and laboratory findings, sensitized patients, who cannot avoid the contact with the allergen, are good candidates for allergen immunotherapy.

Special precautions for the preparation of mammalian extracts

In recent years, there has been concern about the spread of the bovine spongiform encephalopathy across Europe and the United States of America (USA) and the potential contamination of skin derived allergen extracts. Precautions must be taken to avoid the presence of infectious agents in these extracts. The European Pharmacopoeia 5th Edition, volume 1, contains a section (Section 5.2.8) entitled: “Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products”. In this section it is clearly stated that “derivatives of wool and hair of ruminants, such as lanolin, and wool alcohols derived from hair shall be considered in compliance with this chapter, provided the wool and hair are sourced from live animals” [17]. The hair samples should be shipped to the laboratory together with a certificate of analysis in which a Doctor in Veterinary Medicine states that the hair was collected from healthy animals free of infectious diseases and infectious agents. When the hair is collected from cows, sheep, goats or other ungulates, it should be done from herds in which no BSE had been declared previously.

Allergen	Function	kDa	Origin
<i>Felis domesticus</i>			
Fel d 1		38	Saliva
Fel d 2	Albumin	20 – 30	Dander/serum/urine
Fel d 3	Cystatin	11	
Fel d 4	Lipocalin	22	
<i>Canis familiares</i>			
Can f 1	Lipocalin	20	Dander/saliva
Can f 2	Ca-binding S100 homologue	11	Dander/saliva
Can f 3	α -lactoalbumin	14.2	Dander/saliva/serum
Can f 4	β -lactoglobulin	18.3	Dander
<i>Bos domesticus</i>			
Bos d 2	Lipocalin	20	Dander
Bos d 3	Ca-binding S100 homologue	11	Dander
<i>Equus domesticus</i>			
Equ c 1	Lipocalin	25	Skin
Equ c 2	Lipocalin	18.5	Skin
Equ c 3	Albumin	67	Skin/milk
Equ c 4		17	Skin
Equ c 5		17	Skin
<i>Mus musculus</i>			
Mus m 1	Lipocalin	19	Urine
Mus m 2		28	Dander/hair
<i>Rattus norvegicus</i>			
Rat n 1	Lipocalin	17	Urine
<i>Oryctolagus cuniculus</i>			
Ory c 1	Lipocalin	19	Dander/saliva/urine
Ory c 2		21	Dander/saliva/urine
<i>Cavia porcellus</i>			
Cav p 1	Lipocalin	20	Dander/saliva/urine
Cav p 2	Lipocalin	17	Skin

Table 1. Summary of important mammalian allergens

Selection of raw materials for the preparation of skin derived mammalian extracts

Mammalian allergens can occur in a variety of sources, including hair, skin, glands (sebaceous, salivary, anal [18]), saliva, urine, faeces, serum, milk and meat. We have focussed our review mainly on skin derived allergens. The selection of the optimal raw material has been the subject of numerous publications [19-24]. Besides the regular *in vitro* and *in vivo* evaluations of the allergen extracts involving skin prick in Europe, or intradermal testing in the USA, the standardization of any allergen extract begins with the standardization of the raw material. A revision of the terminology used in the literature to describe skin derived allergen sources disclosed a variety of terms including hair, epithelium(a), dander, dandruff, skin scrapings, epithelial scrapings, pelt, cat washes, wool, fur and hides. We believe that at this point, a brief definition of some of these terms is appropriate to better understand this terminology and better choose the appropriate name. Epithelium is a tissue composed of a layer of cells. The outermost layer of our skin is composed of dead squamous epithelial cells. Dander is a scaly or shredded dry skin from the coat, or feathers of various animals. In most cases, it is of an allergenic nature. Dander is similar to dandruff, which is a condition in which white scales of dead skin are shed by the scalp. Hair is the filamentous outgrowth of the epidermis found in mammals. Pelt is the skin of an animal with the fur or hair still on it, whereas fur is the thick coat of soft hair covering the skin of a mammal, such as a fox or a beaver.

The use of different raw materials to produce these extracts is confusing and in some cases, these names may represent the same product. However, from the 1920s, the dander, or particles from the pelt attached to the hair, has been widely assumed to be the vector of the allergic response [25-27]. While in the USA, the suppliers of raw material mostly supply pelt, epithelia, or hair of a variety of mammalian species, the suppliers in Europe include in their lists a larger number of products, including hair, whole skin, skin scrapes and dander. Hair of different dog breeds is also offered. These differences in the terminology and offer of raw materials are also reflected in the list of licensed allergen extracts registered in the USA *versus* Europe. In the USA, 2 companies have standardized cat hair and cat pelt extracts and 5 companies, standardized cat hair extracts [28]. In Europe, the terminology used to name standardized extracts of animal epithelia varies more widely, and according with the list of raw materials available [29].

Important issues, which have not been considered when selecting raw materials for allergen extracts, are the sex and the age of the animals. It has been demonstrated that Fel d 1 production is greater in male than in female cats [30] and that this allergen is under hormonal control and, therefore, should be present in greater quantities in adult than in younger animals [31,32]. In skin biopsies, Fel d 1 has been found in epithelial squamous cells, within the epidermis, on the surface of the epidermis and hair follicles and in sebaceous gland cells. Fel d 1 seems to be produced by sebaceous cells and, to a lesser extent, by basal squamous epithelial cells. It is mainly stored on the surface of the epidermis and fur [33]. Although the exact function of Fel d 1 is not firmly established, the close association of Fel d 1 with skin sebaceous

glands and anal sacs with holocrine and lipid secretion functions, and the homology of chain I of Fel d 1 with some steroid-binding proteins, suggests a possible physiological role for Fel d 1 in the regulation of lipids on skin and cat fur [18].

Evaluation of skin derived allergen extracts from different animals and sources

While the *in vivo* and *in vitro* techniques to standardize allergen extracts are well established in Europe and the USA, there is no standard method, or agreement, to prepare mammalian skin derived allergen extracts. We undertook a project to elucidate differences in the allergenic profiles of extracts prepared using different raw materials. Cat hair, "cat antigen", cat epithelia and cat dander, hamster hair and hamster epithelia, goat hair and goat epithelia, and guinea pig hair and guinea pig epithelia were purchased from different commercial sources. The main differences in these raw materials was that the epithelia were supplied as a mixture of hair and epithelia previously treated with acetone, and that the hair was supplied untreated. These materials were extracted and analysed following standard operating procedures at Laboratorios LETI, SL, Madrid, Spain. We also evaluated if the sex or age of the rabbits could influence the allergen composition of these extracts.

Collection of hair

Live animals were shaved using an electric shaver. The collected hair was stored in sealed plastic bags until further analysis. Afterwards, the hair was incubated in acetone (1:40 weight/volume) for 16 hours, and the dander separated after sequential sieving, using vacuum, through a stainless steel 1 mm mesh size sieve and a Whatman number 1 filter. This procedure was repeated several times. The dander collected on the filters was air-dried. Figure 1 shows the appearance of the collected cat dander and of the purchased cat epithelia.

Preparation of the extracts

The collected dander was extracted, 1:40 weight/volume (w/v), in PBS 0.01 M (pH 7.4) for 16 hours at 4°C, centrifuged at 16,000 g for 30 minutes, dialyzed overnight against bi-distilled water, sterile-filtered, frozen and freeze-dried. This same procedure was used for the extracts purchased from commercial sources.

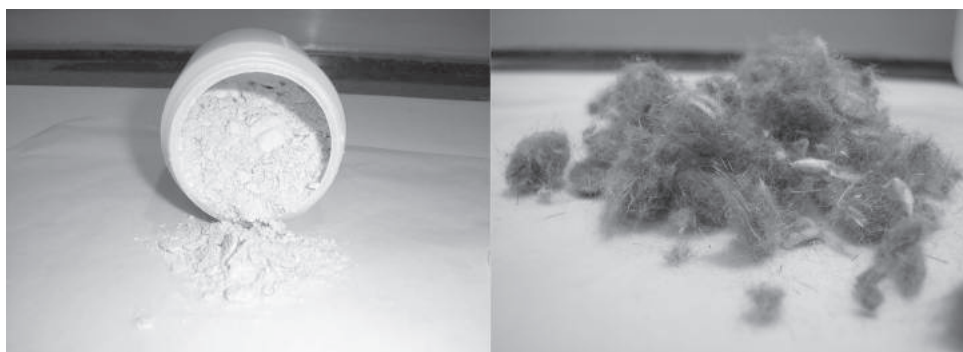


Fig. 1. Cat dander versus "epithelia".

Allergenic and antigenic profiles of these extracts

Figure 2 shows the protein profile of the different cat allergen extracts. Table 1 shows their respective Fel d 1 levels. Cat Antigen (lane 5), Cat Dander (lane 6) and Cat Epithelia (lane 7) extracts showed less Fel d 1 content per mg of freeze-dried material than the extracts prepared from cat hair prepared by the methods previously described. However, a great band corresponding to cat albumin was detected in the cat epithelial extract. This band was practically absent in cat dander extracts.

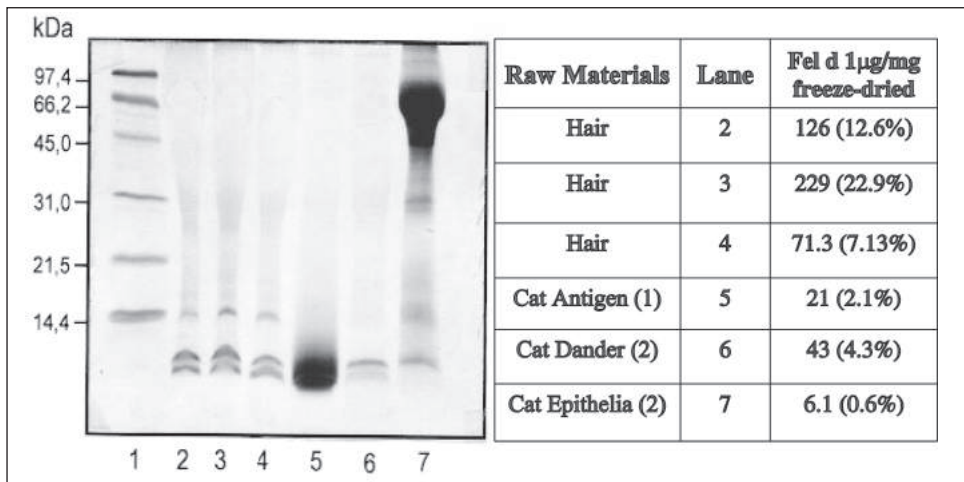


Fig. 2. SDS-PAGE and Fel d 1 levels of cat extracts prepared with different raw materials.

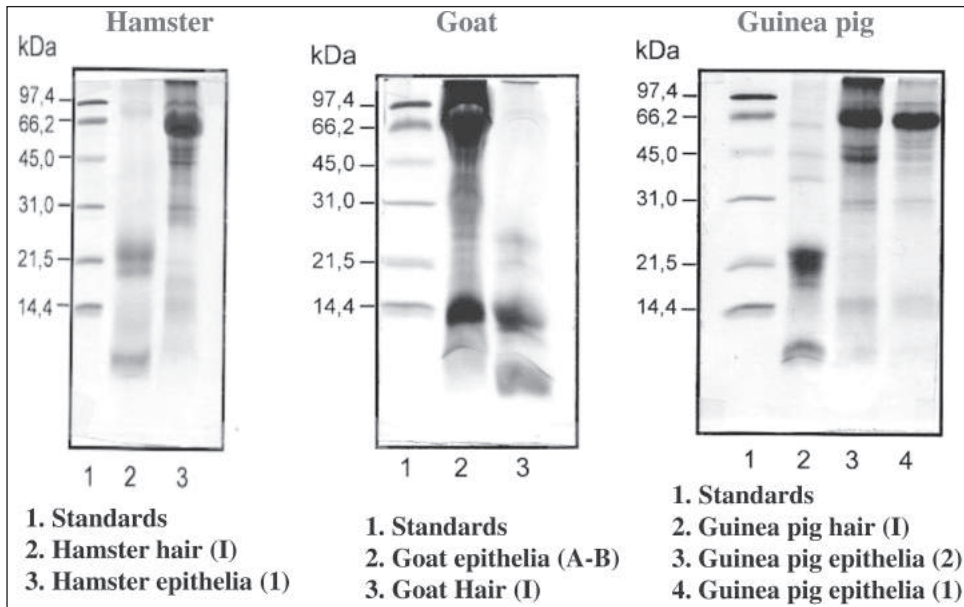


Fig. 3. Other examples of hair versus epithelia.

Figure 3 shows other examples of hair *versus* epithelial extracts, including hamster, goat and guinea pig extracts. In all cases, the epithelial extracts displayed a different protein composition and a higher content in a band with a molecular weight between 66 and 70 kDa, which could correspond to albumin. In the case of the hamster (*Cricetus cricetus*) and guinea pig (*Cavia porcellus*), the two bands at approximately 20 kDa could correspond to the lipocalins, Cav p 1 [34], and to the respective hamster allergen [35].

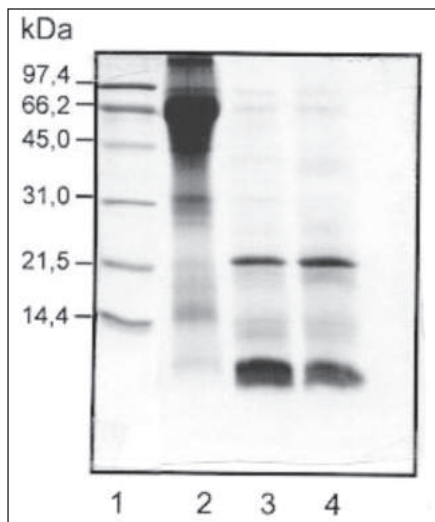


Fig. 4. Rabbit epithelial versus dander extracts. Lane 1: molecular weight markers, lane 2: epithelial extracts, lanes 3 and 4: dander extracts.

Figure 4 shows the results obtained with dander and epithelial rabbit extracts. The results are similar to those obtained with the other species. Since Ory c 1 seems to be present in large quantities in urine, its presence on the hair may suggest cross-contamination [36].

We also evaluated the influence of the age on the allergen composition of rabbit extracts. Figure 5 demonstrates that dander allergen extracts of young rabbits (less than 1 year of age) have a different protein pattern and less major allergen content than extracts of older rabbits (more than 2 years of age).

Discussion and conclusions

We have analysed the composition of skin derived allergen extracts from various animal species prepared with raw materials of different sources. We confirmed that these extracts have a different protein and allergen composition. More albumin and high molecular weight components seem to be present in epithelial derived extracts. Cat allergen extracts derived from dander isolated

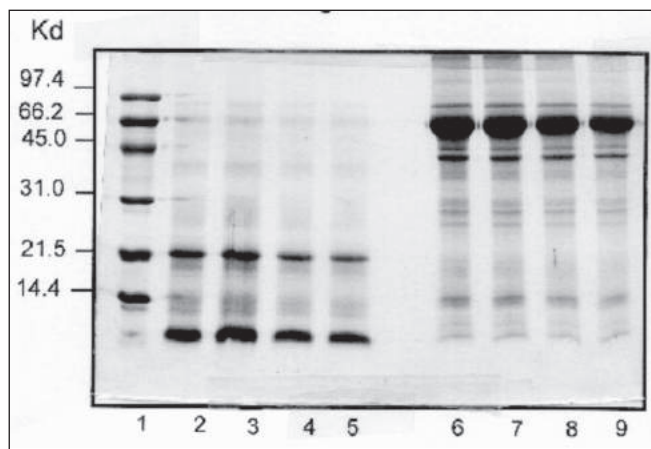


Fig. 5. Different batches of rabbit dander extracts (old versus young rabbits). Lane 1: molecular weight markers, lanes 2-5: dander extract of adult rabbits, lanes 6-9: dander extracts of young rabbits.

from hair after separation by acetone suspension and sieving seem to contain more Fel d 1 and less albumin than epithelia derived extracts. These results confirm previous observations when comparing cat hair with cat pelt extracts [26].

While the source of the dander extracts is well established, epithelia raw material is obtained by scraping the surface of the skin of the animals and, therefore, a greater quantity of albumin is present. One could also speculate that dander derived allergen extracts are more relevant to sensitization, since the Fel d 1 is released from the sebaceous glands and deposited on the surface of the hairs and dander and, therefore, it is collected once the dander is separated from the hair. Similar results were obtained with the other animals, where it was observed that more major allergen (especially lipocalins) and less albumin is present in dander derived extracts.

We identified significant differences in the protein pattern of rabbit dander extracts depending on the age of the animals. The fact that younger rabbits (less than 1 year of age) have a different antigen profile than older rabbits deserves further investigations. This is an important finding, which has implications in the standardization and selection of raw materials for the preparation of rabbit extracts. Studies with other species are also warranted. This different protein pattern may also have an influence on the environmental exposure of the patients and condition a different sensitization pattern in the patient population depending on the age of the animal. Patients who are in contact with young animals may develop a different allergic profile than patients who are exposed to adult animals. Environmental studies should elucidate if mammals are capable of inducing different sensitization patterns depending on their age. Extracts from young rabbits contain less major allergen and more albumin, which is similar to the profile obtained with epithelial extracts. This finding may represent the immature sexual stage of the animals.

Further efforts should also be made to identify and firmly establish the sources of most mammalian aeroallergens and to determine which ones become airborne. Allergen extracts used for the diagnosis and treatment of allergic diseases should contain the most relevant allergens, especially those that become airborne and sensitize individuals.

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Discussion

Falagiani: First, I am curious about results of an Italian allergist who made an epidemiological study on animal sensitization. The results have been that the first in the score was of course cat with regard to the absolute number of sensitized patients followed by dog on the second place. But with regard to the relative rate of sensitization in relation to the number of animals in households, hamster was found to be in the first place. Second, my question is, from the clinical point of view, that some patients state to be allergic to a given cat or dog and not to other animals. Did you ever observe differences among different breeds of animals *in vitro*?

Fernández-Caldas: This is a very good question and it says something that has been discussed for many years. I know that one of the conclusions up to now has been that no dog breed-specific allergens have been identified. But for the cats, it is said that all the normal Mediterranean cats are very similar. But other cat breeds, e.g. the Abyssinian cats or others, may be different.

Falagiani: Personally, I believe that in most cases it is a psychological problem.

de Blay: Do you not think that in Spain and Italy people were eating rabbit at the same frequency? Maybe the way of introducing the allergen could be very important for sensitization. Of course, not only the peak exposure rather than mean exposure, but also the way they eat rabbits could be important too?

Fernández-Caldas: I can tell you that the Spanish patients were eating rabbits. The rabbits were kept outside the houses, but people were getting close while going in and out. In Italy, from what I understand from Dr. Mari, they were kept as pets indoors. So the exposure is different, but I do not know if eating rabbits is protective. But there is a higher amount of IgG binding to allergens from the younger rabbits as compared to the older ones.