

Important Animal Allergens Are Lipocalin Proteins: Why Are They Allergenic?

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Key Words

Allergen · Allergenicity · Animal allergen · Epitope · Immune response · Lipocalin · T cell · Tolerance

Abstract

Major respiratory allergens of dogs, mice, rats, horses and cows belong to the lipocalin group of proteins. The sequence identity of lipocalins is often less than 20%, but they contain between one and three structurally conserved regions and their three-dimensional structures are similar. Lipocalins share common biological functions, predominantly related to the transport of small hydrophobic molecules, such as vitamins and pheromones. Immune reactivity to lipocalin allergens is not well known. In Bos d 5, the IgE-binding epitopes are spread along the molecule, whereas in Bos d 2, the C terminus appears to contain the human B cell epitopes. Bos d 5 contains several murine T cell epitopes. No information is available on human T cell epitopes. The maximal number of epitopes an allergic patient's T cells could recognize in Bos d 2 was five. Three of the epitopes were colocalized in the structurally conserved regions of lipocalins. Interestingly, one of the epitopes was recognized by the T cells of all patients and the computer predictions suggested that there would be an epitope in the corresponding parts of human endogenous lipocalins. The proliferative responses of peripheral blood mononuclear cells of Bos d 2-allergic subjects to Bos d 2 were weak. The T cell response was Th2-dominated. To explain these observations, we have pro-

posed that the allergenicity of lipocalins may be a consequence of molecular mimicry between lipocalin allergens and endogenous lipocalins at the T cell level.

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Lipocalin Family of Proteins

Signature and Sequence Homology

The name lipocalins was proposed in 1987 [1] for a group of functionally and structurally related proteins. Since then, the number of proteins fulfilling the criteria of lipocalins has increased steadily [for a review, see ref. 2]. Later, the structural properties of two other groups of proteins were found to be similar to those of lipocalins, and the calycin superfamily was designated to comprise lipocalins, fatty acid-binding proteins and the avidin/streptavidin group [3, 4].

Three criteria are considered for the inclusion of a protein in the lipocalin group: sequence homology, biological function and structural similarity (<http://www.expasy.ch/cgi-bin/nicedoc.pl?PDOC00187>, Sep. 26, 1999). The signature sequence of lipocalins from the Prosite data base is defined as follows:

[DENG]-x-[DENQGSTARK]-x(0,2)-[DENQARK]-[LIVFY]-{CP}-G-{C}-W-[FYWLRH]-x-[LIVMTA]

A Prosite search of the Swiss-Prot data base with this signature sequence detected 70 lipocalins (<http://www.expasy.ch/cgi-bin/prosite-search-ac?ps00213>). In addition, three partial sequences and 27 proteins which were missed by the search could be identified as lipocalins by other cri-

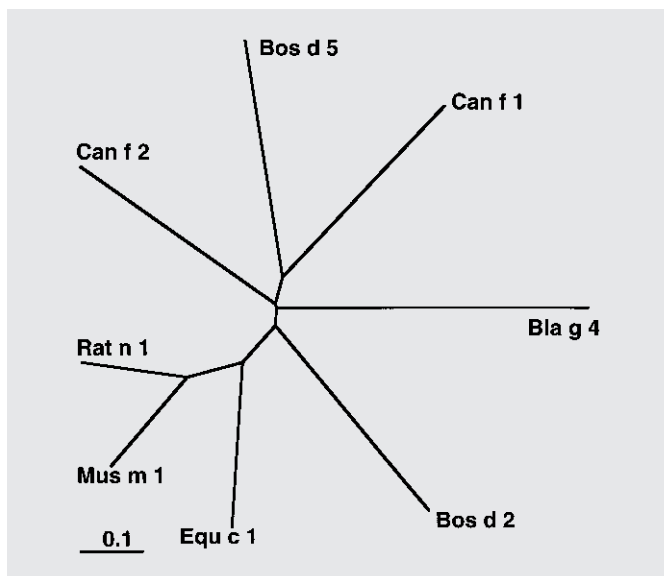


Fig. 1. Phylogenetic relatedness of eight lipocalin allergens. Analysis was performed with the ClustalW program [5] at the European Bioinformatics Institute (<http://www2.ebi.ac.uk/clustalw/>) and the resulting tree was visualised with the Tree View program [6].

teria. As can be expected from the signature, the overall identity between lipocalins varies and is often less than 20%. However, homology is apparent as distinguishable conserved sequence motifs. The most conserved motif is the GXW triplet, which is present in all lipocalins. The so-called kernel lipocalins share three conserved sequence motifs, as opposed to outlier lipocalins, which have only one or two of the motifs (<http://www.expasy.ch/cgi-bin/nicedoc.pl?PDOC00187>, Sep. 26, 1999).

One way to express relationships between lipocalins is a phylogenetic analysis based on sequence similarity. The complete sequences of eight lipocalin allergens are currently available from the Swiss-Prot data bank. Figure 1 shows the phylogenetic relatedness of these allergens. The rodent allergens Mus m 1 and Rat n 1 comprise a closely related pair, whereas the cockroach allergen Bla g 4 has little resemblance with the mammalian allergens. A comparison based on the partial sequences of Equ c 2 suggests that Equ c 2 might in fact be more closely related to Bos d 2 than Equ c 1 [7].

Structural Features

The second characteristic of lipocalins is the common tertiary structure. Figure 2 shows a view of the three-dimensional structure of four representative lipocalins. The structure of recombinant (r) Bos d 2 (fig. 2a) demonstrates all the structural elements characteristic of lipocalins [8].



Fig. 2. Molecular models of two lipocalin allergens and two human lipocalins. **a** Bos d 2. **b** Mus m 1. **c** Human ApoD. **d** Human RBP. The RasMol 2.6-ucb 1.0 program was used to create the models using coordinates from the Brookhaven Protein Data Bank.

The structure was determined by using X-ray diffraction at 1.8 Å resolution and was then refined to a crystallographic R factor of 18.4%. A short N-terminal region of the protein was disordered and was not included in the model. The central β -barrel comprises eight antiparallel β -strands (A–H). The structure also contains a long α -helix (green in fig. 2) between the last two β -strands H and I, a 3_{10} -helix between β -strands A and B, and a C-terminal 3_{10} -helix. The structure is monomeric without any glycosylation or other observable posttranslational modifications. The presence of two disulfide bridges was revealed by the electron density. The bridge between Cys44 and Cys48 is located in the loop between strands B and C. The Cys63–Cys154 bridge is located between the N terminus of the β -strand D and the C terminus of the protein, a structure shared by several lipocalins. The pocket formed by the β -strands defines the ligand-binding site of lipocalins. The type of ligand is regulated by the amino acids lining the ligand-binding pocket resulting in some degree of specificity. On the other hand, retinol, for example, binds to several lipocalins [9] and an odorant-binding protein (OBP) can bind several different organic molecules [10].

Of the lipocalins with known three-dimensional structures, the mouse major urinary protein (1MUP) [11] and bovine OBP (1OBP) [12] have the highest sequence identities with Bos d 2, i.e. 20 and 26%, respectively. Figure 2 shows a three-dimensional comparison of Mus m 1 and Bos

d 2 with two human lipocalins, apolipoprotein D (ApoD) and retinol-binding protein (RBP). Despite the different origins and functions, the structural similarity is obvious. The localization of the conserved sequences in the three-dimensional structure reveals that preservation is concentrated in the β -sheets, and most of the variation occurs in the intervening loop regions.

While most known proteins of the lipocalin family exist as monomers, the structure of β -lactoglobulin was characterized, as a dimer [13], and later refined by several other groups. In β -lactoglobulin, one of the β -strands forms an important part of the dimer interface, which contains a number of hydrogen bonds. The proteins exist as a dimer at neutral pH but as a monomer at acidic pH. A property that separates Bos d 5 (β -lactoglobulin of cow's milk), Bos d 2 and Mus m 1 (MUP6) from several other lipocalin allergens is the absence of glycosylation. Four lipocalin allergens Can f 1, Can f 2, Equ c 1 and Bla g 4, have putative N-glycosylation sites located on the protein surface.

An analysis of the structural relationships between lipocalins is presented by Sansom et al. [14]. They used the three-dimensional profiles of six crystallized lipocalins to search protein libraries for sequences compatible with the fold of each of the lipocalins. The program they used gives a similarity score (Z-score: the number of standard deviations above the mean score of all sequences of similar length). They found that almost all lipocalins gave significant scores against one or more of the six profiles. They suggested that the scores against different profile probes may define lipocalin subfamilies which may not be clear from sequences alone. However, scores also gave false-positive and false-negative results.

Biological Functions

The majority of the mammalian lipocalins are produced in the liver or secretory glands. They are present in various body fluids and secretions, and originally they were characterized as carriers of small hydrophobic molecules. However, as the number of proteins included in the list of lipocalins has increased, so has the variety of biological functions associated with these proteins. A detailed survey of the biological features of lipocalins is presented in a recent review by Flower [2].

The hydrophobic ligands transported by lipocalins comprise molecules with wide-ranging functions. Retinol (carried by RBP) is an example of a vitamin ligand [15]. Pheromones bound to lipocalins in the urine of rodents and in secretions are associated with sexual behavior and territory marking [16–18]. The corresponding lipocalins include the major urinary proteins (MUPs) of rodents and aphro-

disin. These lipocalins also have pheromone activity. Human ApoD is present in several tissues and is apparently able to transport several different ligands. In the secretions of the axillary sweat glands, ApoD participates in the production of personal odor [19]. On the other hand, lipocalins present in nasal and oral secretions bind molecules associated with odor or taste perception (OBPs and von Ebner's gland proteins, respectively) [10, 20]. Lipocalins are also found in invertebrates, e.g. involved in coloration (bilin-binding proteins) [21].

For some of the transport functions of lipocalins it is necessary that the lipocalin is able to attach to specific receptors on the target cell surface. For instance, RBP and α_1 -microglobulin (A1M) possess this property [22, 23]. Lipocalins are usually monomers or homodimers (β -lactoglobulin), but they can also form complexes with other proteins; e.g., RBP is present in serum as a complex with transthyretin and ApoD with lecithin-cholesterol acyltransferase [24, 25].

Some lipocalins are involved in stress and inflammatory reactions. Glycodelin or placental protein 14 (PP14), neutrophil lipocalin (NGAL), α_1 -acid glycoprotein (AGP) and A1M are acute-phase proteins and seem to have immunomodulatory properties [26–28]. The lipocalin C8 γ is a part of the component C8 in human complement [29].

Enzymatic activity is not a common property of lipocalins. However, glutathione-independent prostaglandin D₂ synthase has been classified as a lipocalin [30]. A number of lipocalins may also act as regulators of cell growth and proliferation [2].

Lipocalins as Allergens

The allergens of mammalian origin later to be identified as lipocalins have been studied extensively for more than 20 years. The current list of mammalian lipocalin allergens includes mouse Mus m 1, rat Rat n 1, dog Can f 1 and Can f 2, horse Equ c 1 and Equ c 2 and cow Bos d 2 (<ftp://biobase.dk/pub/who-iuis/allergen.list>, Sep. 26, 1999). They are spread as aeroallergens. Cat allergens are an interesting exception; neither Fel d 1 nor a newly discovered cat allergen, a cysteine protease inhibitor, are lipocalins, although the latter has a motif partially conserved in Can f 1 and Can f 2 [31]. On the other hand, an insect allergen, Bla g 4 of the *Blattella germanica* cockroach, is a lipocalin [32]. In addition, an important food allergen, Bos d 5, is a lipocalin.

Persons working in laboratory animal facilities constitute a risk group for allergy to rodents [33]. The predominant allergen responsible for sensitization to mice was

found in mouse urine [34], but it is also present in pelt extracts and serum [35]. The allergen, a prealbumin, was earlier called Ag1 [36] or MA1 [37]. The current designation for this allergen is Mus m 1. Mus m 1 has been equated with MUP, which had already been described in the 1960s as a complex of urinary proteins made in the liver under hormonal control [38]. Currently, seven proteins with different but highly identical amino acid sequences are listed as mouse MUP in the Swiss-Prot data bank. MUP6 is listed as Mus m 1; the others would represent isoallergens or variants of Mus m 1. At the genome level, the group is even larger, consisting of about 35 genes giving rise to differentially sliced mRNAs [39]. MUP mRNA has also been found in several secretory tissues [40].

Rats are another source of allergens causing work-related allergies among exposed persons [34, 41]. Rat n 1, the predominant rat allergen, is also a urinary protein, and in fact, highly homologous to mouse MUP. Two cross-reacting major allergens, a prealbumin and α_{2u} -globulin (α_2 -eu-globulin), were originally identified in rat urine [42]. They had different sizes of 17 kD and 15 kD and pI values of 4.2–4.4 and 5.1–5.5, respectively. In 1986, the International Union of Immunological Societies' subcommittee for allergen nomenclature assigned the code Rat n I for the prealbumin and Rat n II for the α_{2u} -globulin [43]. More recently, the committee has decided that amino acid sequences should be the basis of the nomenclature [44], and consequently the more correct names are Rat n 1.01 for the prealbumin and Rat n 1.02 for the α_{2u} -globulin [45–47].

Dairy farming is an occupation associated with exposure to allergens from cows. Rare in many other countries, asthma caused by cow dander is a significant occupational problem in small family farms in Finland [48]. Three major allergenic proteins were identified in cow dander as well as in other tissue and body fluids by Prahl et al. [49], and these were later designated as Bos d 1, Bos d 2 and Bos d 3. In a more recent series of studies, cDNAs of three allergens were cloned from the skin epithelium of cows [50–52]. One of the latter allergens was found to be a member of the lipocalin family [52] and shown to be identical with the Bos d 2 of Prahl et al. [53]. Bos d 2 is apparently made by the apocrine sweat glands [54], but a similar, probably related allergen is also present in cow urine [55]. Furthermore, there seem to be several isoforms of Bos d 2 [56]. Therefore, it is likely that Bos d 2 belongs functionally to the same group of pheromone carriers as MUPs.

In contrast to cow dander allergy, allergic reactions and intolerance to cow's milk are common disorders in children worldwide [57]. Bos d 5 (β -lactoglobulin) is one of the three predominant allergens present in milk [58, 59].

Exposure to allergens of furred animals can also occur in the home environment. The two most popular pets, cats and dogs, are sources of potent allergens. As mentioned above, no lipocalin cat allergens have been found so far, but both Can f 1 and Can f 2 are lipocalins.

Hair/dander and saliva are the major sources of dog allergens; in contrast to rodents, dog urine does not seem to contain significant amounts of allergens [60]. A dander allergen originally called Ag13 [61], D4 [62] or Ag8 [63] was the first canine allergen purified and characterized sufficiently to warrant the official name of Can f 1 [64, 65]. It was cloned in 1997 from the parotid gland and was shown to have a high degree of homology and a tissue distribution similar to von Ebner's gland proteins, closely related lipocalins in saliva of several species [66]. Can f 1 is a major allergen; in the same study 75% of subjects allergic to dogs had IgE antibodies against rCan f 1 [66].

Can f 2, another lipocalin allergen in dog dander, was described as dog allergen 2 by de Groot et al. [60]. It was cloned in 1997, also from the parotid gland [66]. It is apparently a minor allergen, since in that study only about 25% of allergic patients had anti-Can f 2 IgE antibodies [66].

Persons coming into contact with horses may develop allergies, and studies on horse dander allergens have a long history [67]. Two allergens are currently recognized in the official nomenclature ([ftp://biobase.dk/pub/who-iuis/allergen.list](http://biobase.dk/pub/who-iuis/allergen.list), Sep. 26, 1999), and they are both lipocalins. Equ c 1 is the predominant allergen. It was probably recognized by Stanworth [67] and was named Ag 6 by Løwenstein et al. [68]. The current name was proposed by Dandeu et al. [69] and the corresponding cDNA was cloned in 1996 [70]. In addition to dander, it is also present in saliva, and in a much lower concentration in urine [71].

The horse dander allergens Ag6, Ag9 and Ag11 were originally designated Equ c I, Equ c II and Equ c III, respectively, following the nomenclature rules of that time [72]. More recently, N-terminal amino acid sequences of two dander allergens were found to be related to lipocalins and proposed to be designated variants of Equ c 2 according to the current rules of nomenclature [7]. A sequence comparison showed that Equ c 2 might be the horse counterpart of Bos d 2 [7].

Cockroaches are a source of allergens in apartment buildings of lower socioeconomic level in inner cities [73–75], where IgE antibodies against cockroach have been detected in 22–26% of asthma patients as compared to 4.5–6% in comparison groups [76, 77]. Numerous allergenic proteins are present in whole-body extracts of cockroaches and in their excrement [78]. The lipocalin allergen Bla g 4 was cloned from *B. germanica* in 1995, and the

prevalence of IgE antibodies to recombinant Bla g 4 in patients was found to be 40–60% depending on the assay method [32].

Immunological Features of Lipocalin Allergens

B Cell Epitopes

Different strategies can be employed for determining the IgE-binding epitopes of allergens. Over the last decade, several studies have examined antibody binding by fragments of Bos d 5. A more detailed analysis with synthetic peptides revealed six regions involved in IgE binding [79]. The segment recognized by all patients was located at amino acids 97–108. A recent study with tryptic peptides located the predominant IgE epitopes in the regions of amino acids 41–60, 102–124 and 149–162 [80]. Two other major epitopes were identified at amino acids 25–40 and 92–100; the corresponding regions were also identified by Ball et al. [79] as minor epitopes. The epitopes of Ball et al. [79] at 97–108 and that of Sélo et al. [80] at 92–100 correspond to the conserved motif 2 of lipocalins and include the sequence DTDY. This sequence is found, for instance, in human RBP, human PP14 and several allergens including Bos d 5 and Mus m 1, but is not present in Bos d 2, which is an outlier lipocalin. Furthermore, a monoclonal antibody has been described which recognizes peptides from Bos d 5 and RBP comprising this sequence [81].

We started our studies on the IgE binding of Bos d 2 by using recombinant fragments [82]. Two overlapping fragments were produced as fusion proteins in *Escherichia coli*. In an immunoblot assay, the sera of 3 out of 12 patients reacted with the C-terminal fragment of Bos d 2, whereas no reactions were observed against a fragment comprising the N-terminal part of the molecule. In ELISA, low levels of reactivity were detected, mainly against the C-terminal part when the fragments were used as antigens. In ELISA inhibition, both fragments were completely negative. These experiments showed that a preparation containing two overlapping fragments of the allergen would have a greatly reduced IgE-binding capacity while presenting the complete amino acid sequence of the protein to T cells.

To get a closer look at how the integrity of the molecule affected IgE binding, a more refined procedure was used. It has been reported that the disruption of intramolecular disulfide bridges interferes with the IgE binding of mite allergens [83–86]. We applied the same principle to Bos d 2. A set of mutant recombinants was prepared, including point mutations and a four-amino-acid deletion which eliminated one of the disulfide bridges. The fusion proteins were tested

for IgE binding [87]. The loss of the disulfide bridge caused a significant decrease in IgE binding when analyzed by ELISA inhibition. Since the loss of a disulfide bridge probably causes only a minor change in conformation, it was concluded that the IgE epitopes are highly dependent on the three-dimensional structure. However, the results for immunoblotting and skin prick tests were less clear-cut, and one lesson to be learned from our experiments is that the method used to test mutant or otherwise modified allergens for IgE binding affects the results and has to be taken into account.

As mentioned above, Bos d 2, Bos d 5 and Mus m 1 are nonglycosylated. Therefore, glycosylation does not seem to be critical for the allergenicity of lipocalins. However, the carbohydrate moiety may affect IgE binding [88]. Our own experience suggests that this is also true for the glycosylated lipocalin allergen Can f 2 [89]. SDS-PAGE electrophoresis of recombinant Can f 2 produced in *Pichia pastoris* showed two proteins reacting with IgE antibodies. Since deglycosylation with endoglycosidase-F made the band with the higher apparent molecular weight disappear, it was assumed that both glycosylated and nonglycosylated forms of Can f 2 were made in *P. pastoris*. In an ELISA inhibition test, considerably more deglycosylated Can f 2 was needed to achieve the inhibition level of untreated rCan f 2. Therefore, the carbohydrate moiety seemed to affect IgE binding, but this observation needs to be confirmed.

T Cell Reactivity

Very little information is available on human or murine T cell responses to lipocalin allergens. We have recently shown that patients with an intense clinical allergy to Bos d 2 mounted weak proliferative responses against the allergen [90]. The poor responsiveness was also reflected in the difficulty of inducing Bos d 2-reactive T cell clones. However, once established, the clones exhibited strong proliferative responses upon stimulation with Bos d 2. Using Bos d 2-specific T cell lines and clones, we showed that the allergen contains a limited number of epitopes. The total number of T cell epitopes detected was seven and the maximal number an individual's T cells could recognize was five. One of the epitopes, defined as epitope G, situated at the C-terminal α -helix (fig. 2a), was especially interesting because it was recognized by the T cells of all the patients studied. The clones specific for this epitope seemed to be predominantly of the Th2/0-like phenotype. Epitope predictions based on amphipathicity have suggested that endogenous human lipocalins should contain an epitope in the area corresponding to epitope G in Bos d 2 [90].

Human T cell epitopes of Bos d 5 are not known, but murine T cell epitopes in three different strains of mouse

have been determined [91–93]. As expected, the results were strain-dependent but the three studies also showed slightly variable results with BALB/c mice. Nonetheless, the studies agreed that for BALB/c mice, Bos d 5 contains at least three or four regions with T cell-stimulatory capacity, roughly corresponding to amino acids 42–47, 67–76, 107–117 and 134–148 [91–93]. The most vigorous responsiveness was observed against the second region, but the last region, which corresponds to the C-terminal α -helix (amino acids 130–140) and is therefore equivalent to epitope G of Bos d 2, also induced a good response [92, 93].

Immune Defense System and Allergy

The principal task of the immune system is to recognize and eliminate harmful exogenous invaders. To be able to do that, the immune system goes through a complex process of maturation and adaptation. One of the essential properties of the immune response, distinction between self and non-self, is initially acquired in the thymus, resulting in the elimination of T cells with high-affinity T cell receptors (TCRs) for self [94–96]. The autoreactive peripheral T cells with low-affinity TCRs rarely cause disease but it is supposed that they may do so in genetically predisposed individuals under appropriate conditions, e.g. in association with infections [97, 98]. However, if the infectious agent (or an allergen) closely resembles the host's self, an effective defense may be problematic. If a strong Th1-deviated immune response, which is important in antimicrobial defense [99], were launched against the cross-reacting agent, it could be deleterious to the host itself. For example, there is a considerable amount of data suggesting that Th1 cells play an important role in autoimmune pathology [99–101]. In the situation where an exogenous ligand resembles self but would have superagonist properties, i.e. could induce a stronger (heteroclitic) response than the native ligand [102, 103], the Th1-type response could be expected to be a true hazard. It is conceivable that a superagonist could efficiently activate silent autoreactive T cells, as the study of Zügel et al. [104] suggests. Another possibility for the harmful activation of the immune system could be offered by proteins containing both homologous and foreign sequences, the latter being able to break the tolerance to a conserved self protein, as shown by Dalum et al. [105]. What role the homologous parts of the protein may play will be discussed below. Nevertheless, it is intriguing to speculate that a Th2-deviated immune response might represent a safer alternative than a Th1-deviated response against invaders resembling self.

The concept presented above is compatible with the fact that the immune response against helminths is Th2-deviated [106]. Helminths are complicated eukaryotic organisms [107], and it seems reasonable to assume that they contain proteins which cross-react with human proteins. For example, it has been reported that the IgE-binding epitope sequences of a *Schistosoma japonicum* antigen exhibited similarities with those of known allergens, including lipocalins [108]. In addition, several allergens appear to be conserved in evolution [109]. For example, some allergenic proteins of *Aspergillus fumigatus* have been reported to show a high degree of sequence identity and similarity to human counterparts [110]. Therefore, the common feature of immune responses against helminths and allergens, Th2 dominance, may be a way to reduce the likelihood of tissue destruction. In accordance with this view are the observations that strong proliferative or Th1-deviated immune responses against helminths are associated with more severe forms of the diseases [111–113]. In a mouse model of *Schistosoma mansoni* infection, mice incapable of down regulating the Th1-type response showed exacerbated hepatocyte damage [114]. In experimental mouse models of asthma, allergen-specific Th1 cells were observed to cause severe airway inflammation [115, 116].

Because it is well known that Th2 cells play a crucial role in allergic diseases [117, 118], it is logical to infer that factors which favor Th2 differentiation promote the development of allergy. For example, the lack of bacterial or viral infections which are known to induce Th1-type responses [99], has been associated with a risk of atopic disorders [119, 120]. Although the role of allergens in favoring sensitization (Th2 differentiation) has been the subject of discussion [121–125], it seems that only a part of the antigenic components of an allergen source bind IgE [126]. Observations like this suggest that allergenic molecules themselves may have an intrinsic capacity to induce IgE synthesis. Enzymatic activity was the property first proposed as a characteristic of these molecules. Enzymatic activity has been thought to interfere with the immune system by a variety of mechanisms favoring sensitization [124, 127]. There is, however, disagreement as to whether the allergenicity of certain enzymes is specifically dependent on their enzymatic activity [128–131]. We have recently presented another hypothesis for allergenicity, in an attempt to explain the observations we made with Bos d 2. We proposed that the allergenicity of lipocalins may be associated with the adaptation of the immune system to the presence of endogenous lipocalins [132].

Lipocalin Allergens and the Immune System

As mentioned above, the immune system encounters lipocalins both as exogenous allergens and endogenous proteins. Although the overall sequential identity between lipocalins is not high (about 20%) they share characteristic conserved sequence motives [2]. Sometimes the similarity can be considerably higher, as with endogenous PP14 [133] and the food allergen Bos d 5 (43% amino acid identity). In relation to Bos d 2, our results indicated that the human immune response was dichotomous, in that patients exhibited high levels of specific IgE whereas the allergen's capacity to induce proliferative responses of peripheral blood mononuclear cells was weak. Furthermore, experimental data and epitope predictions suggest that endogenous and allergenic lipocalins may have epitopes in the corresponding parts of the molecules [90]. Taking these observations into account, an important issue to be addressed is how the immune system functions and how it is regulated when exposed to proteins which resemble self but are still foreign.

To control immune responsiveness under exposure to self-mimicking agents such as lipocalin allergens, the immune system may exploit several strategies. Because, after thymic deletion, the precursor frequency of autoreactive T cells is low [94, 98, 134], it is likely that the proliferative responses to self-mimicking exogenous antigens are weak. In this regard, it is worth noticing that another animal allergen, cat Fel d 1, which is not a lipocalin, has been reported to induce inconsistent proliferative responses of the peripheral blood mononuclear cells of people allergic to cats [135]. A further factor possibly explaining low proliferative responsiveness with Bos d 2 is the low number of epitopes in Bos d 2. Two of the six Bos d 2-specific T cell lines recognized only one epitope in the allergen [90]. Interestingly, the number of epitopes in Fel d 1 also seems to be limited [136, 137]. It remains to be elucidated whether animal allergens in general contain fewer T cell epitopes than other allergens [138]. This would not be surprising, as immune responses are expected to be directed against the species-foreign regions of a molecule.

The T cells which have escaped thymic deletion and are present in the periphery are controlled by a variety of mechanisms of peripheral tolerance [139, 140]. It has been shown that antigen-specific regulatory CD4⁺ T cells secreting immunosuppressive cytokines can downregulate a pathological immune response in vivo [141]. Anergic T cells are able to suppress T cell responses in vitro provided that the epitope recognized by the anergic T cells is present in the culture [142]. In the context of allergenic molecules, it is interesting to note observations suggesting a role for

self structures in controlling immune responsiveness against exogenous antigens. In an elegant in vivo study, Basu et al. [143] showed that when TCR-transgenic T cells were transferred into mice expressing a transgenic antagonist altered peptide ligand (APL), the proliferative response of the TCR-transgenic T cells upon stimulation with the wild-type (WT) peptide was strongly reduced regardless of whether the TCR-transgenic T cells originated from APL-transgenic or APL-nontransgenic mice. On the other hand, it has been reported that microbial peptides resembling a peptide derived from a myelin basic protein functioned as TCR antagonists, preventing experimental autoimmune encephalomyelitis in mice [144]. Taking into account the similarities between lipocalins, it is conceivable that the presence of endogenous lipocalins may modulate immune responsiveness against lipocalin allergens.

An interesting possibility for controlling immune reactions could be the diversification of a response to less harmful, protective epitopes in the exogenous antigen [145]. Moudgil et al. [146] reported that in an experimental model of human rheumatoid arthritis, i.e. adjuvant arthritis induced by *Mycobacterium tuberculosis* in oil, the T cell response of Lewis rats was observed to diversify to the C-terminal determinants of the bacterial heat-shock protein (HSP) later in the remissive phase of the disease. Importantly, the rats were also observed to mount a strong T cell response against the corresponding determinants in the homologous self protein. The authors of that report discussed the role of Th2 cells in regulating the response. This view is supported by other findings that human self HSP-reactive T cells exhibited Th2 phenotype and that in synovial histology, a Th2-biased phenotype was apparent in remitting cases [147]. The results of the Dutch group with a rat model of adjuvant arthritis [reviewed in ref. 148] support the idea that the regulation of the immune response to mycobacterial HSP was based on the cross-recognition of endogenous and exogenous epitopes by T cells, because only the peptides containing the conserved epitope of *M. tuberculosis* HSP cross-reactive with rat HSP were able to provide protection against the disease. In a model of rheumatoid arthritis with Wistar-Kyoto rats (which are resistant to the disease and have the same MHC II molecules as Lewis rats), the rats were observed to respond soon after an injection of *M. tuberculosis* to the carboxy-terminal protective determinants of HSP, in contrast to the late response of the disease-prone Lewis rats [146]. This finding points out that genetic factors which shape immune responsiveness to the different determinants of the antigen may influence the outcome, i.e. susceptibility to disease. With regard to allergy to Bos d 2, epitope G could represent an epitope with protective or

regulatory capacity in a similar manner as described above. Epitope predictions suggested that endogenous human lipocalins would contain an epitope in the area corresponding to epitope G in Bos d 2. The area coincides with one of the conserved motives in lipocalins. Furthermore, the human immune response against this epitope was Th2/0-dominated [90].

The flexible recognition of antigen by TCRs able to create qualitatively different signalling to T cells [149] could provide a third mechanism for decreasing the likelihood of harmful immune responses. Evavold and Allen [150] reported in 1991 that while a WT peptide triggered proliferation, interleukin (IL)-4 production and provision of B cell help from a T cell clone, an APL could only induce the latter two phenomena. Subsequently, several studies have shown that the profile of cytokines secreted by T cells can be modified upon stimulation with APLs [151–153]. Although APLs may act as superagonists [102–104], another possibility is that autoreactive T cells with low-affinity TCRs may inefficiently recognize their ligands in self-mimicking allergens inefficiently. For example, with bee venom allergen phospholipase A₂ it has been observed that allergen-specific T cell clones that were suboptimally activated predominantly produced IL-4. Increasing the dose of antigen, i.e. the amount of TCR ligation, shifted the cytokine profile to Th0 type [154]. Interestingly, the clones derived from allergic individuals required higher doses of the antigen than the clones of nonallergic individuals, pointing to the possibility that the T cells of allergic subjects recognized the allergen inefficiently.

There are additional data indicating that the binding of a peptide to MHC II and/or TCR is important for the quality of the T cell response. Pfeiffer et al. [155] reported that the T cells of H-2^s mice primed and then stimulated in vitro with the WT peptide derived from human type IV collagen produced interferon (IFN)- γ and not IL-4 mRNA, while priming with weakly stimulatory APLs with lower binding capacity to I-A^s than the WT peptide resulted in the production of IL-4 and not IFN- γ mRNA upon stimulation with the WT peptide. By increasing the dose of the low-affinity APL in priming, stimulation with the WT peptide induced the production of IFN- γ while IL-4 production was abrogated. In another experiment with H-2^b mice, the authors observed that when the mice were first primed with a superagonist APL having an I-A^b-binding capacity similar to the WT peptide, stimulation with the WT peptide resulted in the production of IFN- γ mRNA, not IL-4 mRNA as was the case if the mice were primed with the WT peptide. The studies of Murray et al. [156] further elucidate the roles of MHC and TCR in governing differential Th1-Th2 respon-

siveness. They observed that H-2^s mice which are Th1 responders to human type IV collagen presented higher densities of the peptide ligand on antigen-presenting cells (APC) than H-2^b mice which are Th2 responders [157–159]. Importantly, the low ligand density on the APCs of Th1-responder H-2^s mice caused a deviation in the response to Th2 dominance, while the high ligand density on APCs of Th2-responder H-2^b mice did not deviate the response to Th1 dominance [160]. The latter result points to the possibility that the T cell repertoire of H-2^b mice may be deficient, lacking T cells capable of recognizing the peptide with high affinity. This view is supported by the observation that against low peptide concentrations at equivalent peptide densities, H-2^s-derived T cell clones proliferated strongly, whereas H-2^b-derived clones did not [156, 159]. The results of another research group using a TCR-transgenic mouse model in an I-E^k background suggested that the concentration of a peptide is a crucial factor for the development of the cytokine pattern of T cells [161]. They proposed a model according to which there would be a threshold for ligands correlating with the percentage of IFN- γ -producing cells and determining the overall Th1/Th2 phenotype of the population. At low concentrations of any ligand, Th2-type responses would predominate, whereas at high ligand concentrations, the response would be Th1-dominated. At intermediate concentrations, both Th1 and Th2 cells would exist [161]. In all, the data presented above indicate that ligands with minor changes, as well as variations in their concentrations, can modify and direct the quality of the immune response. Because lipocalins occur as endogenous proteins and exogenous allergens, it is conceivable that there may be interference between these two groups with regard to T cell recognition. The most straightforward concept is that the T cells of allergic persons recognize allergens inefficiently, either because of low-level presentation by APCs (low ligand density) or because of a deficient T cell repertoire, and this would lead to the suboptimal activation of T cells and secretion of Th2-type cytokines. Whether this type of immune recognition could represent a way to confront proteins containing self-mimicking structures and to avoid harmful responses is not known.

Concluding Remarks

The allergenicity of proteins has long been an intriguing subject. Even though it appears logical that allergens could have an intrinsic capacity to deviate immune response to Th2 dominance, direct evidence to support such a notion, is still missing. The situation has been changing recently be-

cause of the rapid increase in knowledge relating to immunological processes and allergen molecules. In this regard, lipocalin proteins appear especially interesting because all mammalian-derived major allergens, except cat allergens, belong to this group. We have proposed that the allergenicity of lipocalins may be a consequence of molecular mimicry between endogenous lipocalins and exogenous lipocalin allergens at the T cell level. Because relatively little information is available on the characteristics of

immune responsiveness against lipocalins, further studies are urgently needed. It is tempting to speculate that the biological significance of Th2 deviation in allergy and helminth diseases may be the avoidance of deleterious immune reactions against self. Whether allergens represent a true hazard to self or only mislead the immune system to react erroneously when exposed to proteins resembling self remains to be clarified in the future.

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