

EDITORIAL

Peptidase allergens, occludin and claudins. Do their interactions facilitate the development of hypersensitivity reactions at mucosal surfaces?

Few issues of *Clinical and Experimental Allergy* arrive without some work connected with the identification and characterization of agents that cause the development of hypersensitivity conditions. In this issue, Shen and colleagues describe a 34-kDa allergen from *Aspergillus fumigatus* [1]. IgE reactivity to this allergen was detectable in a significant proportion of serum samples from patients with asthma, and amongst those samples that were known to be reactive to *Aspergillus fumigatus* extracts the frequency of reactivity to this allergen was high [1]. Microsequencing indicates that the IgE-reactive protein described by Shen and colleagues is a serine peptidase [1], adding to the list of allergens from diverse sources that are known or suspected to have proteolytic activity. Furthermore, the authors of this paper show that this serine peptidase from *A. fumigatus* is cross-reactive with allergens from *P. notatum* and *P. oxalicum* [1]. These findings prompt further speculation on the possible connection between proteolytic activity and the interaction of *A. fumigatus* with human hosts, and invite a general revisitation of the role of proteolytic activity as a potentially important component of developing hypersensitivity reactions to a wide range of antigens.

A. fumigatus is a widely occurring airborne fungus which has small spores that facilitate its inhalation. Normally with little effect in healthy individuals, *A. fumigatus* has significant potential to be pathogenic, saprophytic or allergenic depending on the immunological status of the host and/or the presence of other clinical conditions. In the immunocompromized, invasive aspergillosis can result from exposure to vegetative *A. fumigatus*. In people who are immunologically competent, but who have lung tissue damage due to tuberculosis or sarcoidosis, *A. fumigatus* can behave as a saprophyte, resulting in aspergillomata.

In asthma and cystic fibrosis, *A. fumigatus* is encountered as an allergen. Approximately 20% of asthma patients develop positive IgE-dependent skin test reactions to *Aspergillus* [2–4], whereas 31–59% of patients with cystic fibrosis show reactivity [5–7]. In the two serum libraries screened by Shen *et al.* 27% and 34%, respectively, of the sera showed reactivity to *A. fumigatus* extracts [1], generally consistent with the previously observed occurrence. In a subset of asthma or cystic fibrosis allergic bronchopulmonary aspergillosis (ABPA) can develop in those who are sensitized to *A. fumigatus*. ABPA is a

hypersensitivity disease of the lung that results in chronic inflammation of the proximal bronchi. As defined by the classic work of Pepys and colleagues, ABPA involves a type III hypersensitivity reaction [8] presumably superimposed on a type I reaction because the presence of both reagenic and precipitating antibody is essential to elicit the lesions characteristic of the condition in a primate model [9].

The potential pathogenicity of *A. fumigatus* has prompted concerted interest in the molecular mechanisms involved in its virulence. Extracts of *A. fumigatus* contain many components, some being cytotoxic proteins with allergenic properties (e.g. restrictocin, Asp f1, an 18-kDa ribotoxin). Others are secreted peptidases, with representation from molecules with metallopeptidase (Asp f5), serine peptidase (Asp f13), aspartic peptidase (Asp f10) and dipeptidyl peptidase activity (Asp f11). Although early studies suggested that proteolytic activity released by *Aspergillus fumigatus* might be crucial to its invasive activity, current evidence does not support a major role for peptidases in this process, or at best suggests that considerable redundancy exists in the repertoire of *Aspergillus* secreted peptidases [10].

Targeted gene disruption has been used to create single mutants of *Aspergillus* that are negative for alkaline serine peptidase activity, metallopeptidase activity, aspartyl peptidase activity, or double mutants lacking both alkaline serine and metallo-peptidase activity. These single and double mutants are as lethal in mice as wild type *Aspergillus*, suggesting that these particular peptidases do not exert a critical influence in the development and progression of invasive aspergillosis [10]. This raises the question of whether the peptidases secreted by *Aspergillus* have any role to play in the development of human responses to this fungus. As noted above, enzymatic activity is a feature of several *Aspergillus* allergens, including the one now reported by Shen and colleagues (Asp f18) [1]. Of the enzymatic activities detected as allergens, proteolytic activity predominates, although allergens with oxidase activity are also known (e.g. Asp f6). Could it be that secreted peptidases are more relevant to the development of hypersensitivity than the rampant fungal colonization and destruction of blood vessels seen in invasive aspergillosis, or is it mere happenstance that these enzymes are allergens? At present, this question is unanswered, but

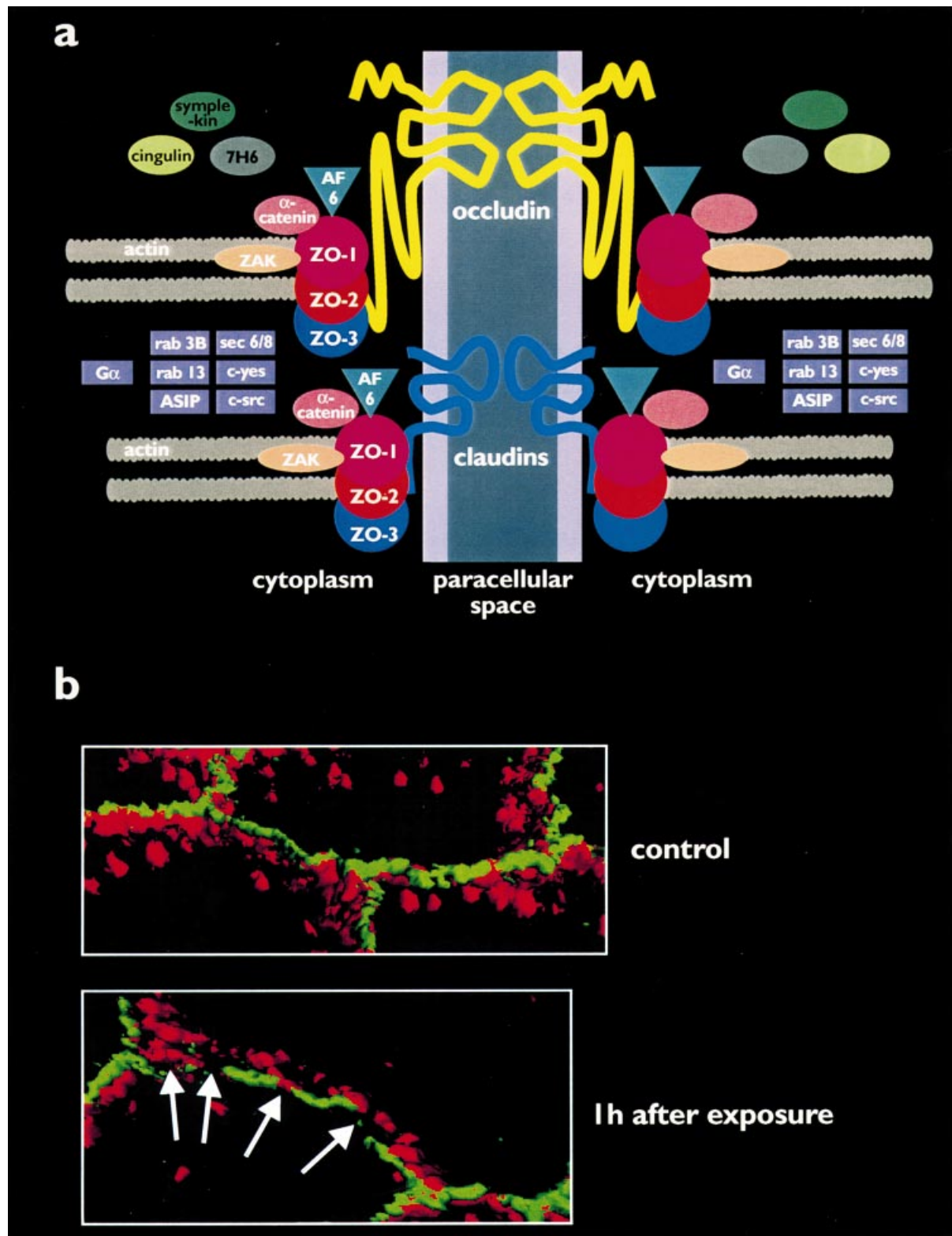


Fig. 1. (See next page for caption)

some potentially interesting parallels exist between *A. fumigatus* peptidases and another set of allergens in which peptidases have significant representation.

At least four groups of denominated allergens from house dust mites are peptidases [11]. The group 1 allergens are cysteine peptidases, whilst those of groups 3, 6 and 9 are serine peptidases [11]. Recently there has been considerable interest in the possible relationship between the enzymatic activity of these allergens and early events in the development of allergic sensitization. Most work has been performed with Der p 1 as an experimental paradigm [12], although reports are now emerging on the serine peptidase allergens. Mice sensitized by intraperitoneal injection of Der p 1 develop an immunological response that is less vigorous when the Der p 1 is rendered catalytically inactive using a cysteine peptidase inhibitor [13]. Similar data have been obtained with the archetypal cysteine peptidase, papain, which itself is an occupational allergen [14].

Both these animal studies suggest that catalytic activity might have some influence on the development of the immunological response. However, Der p 1 and papain are most commonly encountered as inhaled allergens and when delivered by this route they may be diluted in tissues and the circulation before such catalysis could take place. However, enzymatic activity may be of considerable significance when these allergens impact upon the airway mucosa. The epithelial lining of the airways is a developmentally specialized barrier that must be crossed for inhaled allergens to encounter dendritic antigen presenting cells [11,12]. Recent studies have shown that application of house dust mite faecal pellets to epithelial monolayers results in a striking disassembly of tight junctions (TJs) under conditions that mimic natural exposure to this source of allergens [12].

TJs are macromolecular assemblies formed from transmembrane adhesion proteins (occludin and the

claudins) and intracellular proteins such as ZO-1, -2 and -3 (Fig. 1). They are expressed apically at epithelial surfaces, such as the airways mucosa, where homo- and heterotypic interactions between the extracellular domains of adhesion proteins on adjacent cells leads to an intercellular seal being formed. Maintenance of these intercellular seals is vital to maintaining the integrity of the epithelial barrier in the airways. Occludin and the claudins have a generically similar topography at the cell surface in having cytoplasmic NH₂- and -COOH termini and two extracellular loop domains that are presumed to be critical to TJ adhesion. The extracellular exposure of these adhesion domains renders TJs potentially susceptible to perturbation by external agents that can disrupt or disassemble the TJ adhesion proteins [12]. As TJs normally limit access to paracellular channels, a consequence of their disassembly is an increased permeability of the epithelial barrier, allowing allergens to be delivered to dendritic antigen presenting cells [12]. The potential significance of allergen-induced TJ cleavage is obvious since transepithelial delivery of Der p 1 is not detectable until these junctions open [12].

Purified Der p 1 mimics this effect of the 'natural' allergen mixture [12,15], as do serine peptidase allergens from house dust mites [16]. Detailed examination of the events that occur reveals a mechanism with two main components. Firstly, the allergens attack specific extracellular cleavage sites in the TJ adhesion proteins [12,15]. Secondly, they trigger clearance of the cytoplasmic plaque protein, ZO-1, from TJs by a combination of intracellular proteolysis and intracellular redistribution [12,15]. A putative cleavage site for Der p 1 is highly conserved in the first extracellular domain of occludin (and its NH₂-terminal splice variant, occludin 1B), and the claudin family of TJ adhesion proteins (Fig. 2) [12]. Serine peptidases in house dust mite faecal pellets also cause cleavage of TJ adhesion proteins, although the actual

Fig. 1. (See previous page) Molecular architecture of the TJ complex at the junction of two epithelial cells. **(a)** TJ strands are believed to form copolymers of the transmembrane proteins occludin (coloured yellow) and claudins (coloured cyan). The plaque proteins ZO-1, ZO-2 and ZO-3 (coloured magenta, orange and sky blue, respectively) are related members of the MAGUK family proteins (membrane-associated guanylate kinase homologues) and are localized at the cytoplasmic surface of the TJ complex where they associate with the –COOH termini of occludin and claudins. As well as forming heterodimers with each other and with ZO-3, both ZO-1 and ZO-2 bind actin (coloured grey) and AF-6 (coloured sea green), and probably interact with α -catenin and ZO-associated kinase (ZAK) (coloured pink and sand, respectively). Other cytoplasmic proteins are found associated with TJs, including symplekin, cingulin and 7H6. Amongst these other cytoplasmic accessory proteins are signalling molecules and proteins putatively involved in vesicle formation (coloured pastel blue) which include G-proteins, ASIP, Rab 3b/13 and sec6/8. Shown in **(b)** are parts of the junctional margins of epithelial cells visualized as 3-dimensional isosurface reconstructions following fluorescent antibody labelling of occludin (green staining) and the desmosomal plaque protein, desmoplakin (red). In control cells the staining pattern of occludin is contiguous around cells. However, 1 h after exposure to serine peptidase allergens from *D. pteronyssinus*, note the fragmentation (arrowed) of the staining pattern as the TJs break and the epithelial barrier becomes leakier.



Fig. 2. (See next page for caption)

cleavage sites so far identified differ from those of Der p 1 [16].

Analysis of all the theoretical cleavage sites for the serine peptidases shows strong conservation in occludin and claudins, with a potential bias of the adhesion proteins to chymotryptic (i.e. Der p 6) vulnerability (Fig. 2). In the absence of a 3-dimensional model describing TJ adhesion at the molecular level it is not yet clear whether this proteolytic preference exists in reality as some sites may be sterically hindered when TJs are intact. However, as TJs disassemble by initial proteolysis, previously concealed cleavage sites may be displayed, leading to accelerated catalysis of the TJ adhesion complex. The finding that house dust mite allergens attack adhesion proteins of TJs [12,15] is not only important in understanding how inhalant allergens cause allergy, but it is interesting to note in passing that this discovery actually represents the first molecular level identification of any clinically significant process involving TJs.

Hypothesis

What, if anything, do these experimental findings with house dust mite peptidase allergens suggest about responses to *A. fumigatus*? A simple hypothesis is that TJ disassembly could be a critical response to many antigens, including those of *A. fumigatus*, and that TJ adhesion proteins may be shared substrates of peptidase antigens from a diversity of sources. If correct, it follows that antigens from different sources may interact to degrade these substrates. In the case of allergy and asthma, such interactions would provide a further explanation of the synergies between putative risk factors.

Why then does *A. fumigatus* not always cause invasive aspergillosis if its secreted serine peptidases have the potential to attack TJs and increase paracellular permeability? We propose that this may be explained by the small degree and reversibility of TJ cleavage that may be necessary to drive immunological sensitization in a host susceptible to allergy compared to the grosser destruction required to elicit invasive aspergillosis. The rapid reversibility of TJ cleavage caused by house dust mite allergens

is suggested by radiolabelling studies showing that resynthesis of occludin is initiated on the initiation of TJ cleavage [15]. We postulate that under these conditions rapid resealing of localized bursts in the epithelial barrier would make it difficult for invasive aspergillosis to be established, particularly where mechanisms of innate immunity are fully functional and operate to prevent mycotic colonization.

The general features of the hypothesis above are not without a historical pedigree — it has been speculated previously that changes in tissue permeability could be a component of ABPA [17]. Such conjecture has centred on the possible involvement of *Aspergillus* peptidases in causing bronchiolar injury and the continuous elution of allergens from uncleared spores in causing mast cell degranulation and the liberation of mediators which affect epithelial permeability [17]. The difference now is that molecular identities can be ascribed to potential participants in this subtle process and the necessary molecular markers can be used to undertake a systematic examination of this hypothesis. The intriguing possibility is that a limited repertoire of epithelial cell proteins control a fundamental event in sensitization to inhaled allergens from diverse sources. We await, with interest, the outcome of experiments designed to investigate these specific mechanisms.

Although the hypothesis described above is attractive, it will not be easy to investigate. The principal difficulty we envisage lies in determining what are appropriate levels of *Aspergillus* enzymes that reflect concentrations achieved at the airway lining. Such concerns have largely been overcome in recent *in vitro* modelling of epithelial cell exposures to house dust mite faecal pellets and to the allergen Der p 1 [12,15], but this remains an area that needs to be addressed for other allergens. It is known that extracts of *A. fumigatus* cause epithelial desquamation and evoke cytokine release [18,19], but these are descriptive endpoints whose relevance to *in vivo* exposures remains to be determined.

If the parallel with house dust mite allergens holds true, the serine peptidase allergen, Asp f18 described by Shen and colleagues [1], together with the subtilisin-like alkaline

Fig. 2. (See previous page) Putative cleavage sites for tryptic and chymotryptic serine peptidases in the extracellular domains of claudins and occludin. For the claudin family proteins, a multiple sequence alignment was created using CLUSTALX. Extracellular domain sequences whose positional locations are indicated at the ends of each sequence were deduced logically from computational analysis of the likely positions of transmembrane alpha helices. Putative cleavage sites are indicated by triangles, with red denoting tryptic cuts and blue denoting chymotryptic cuts. For occludin/occludin 1B, the first and second extracellular domains were located as described above. Note that the extracellular domains of occludin and occludin 1B are identical, the latter being an alternative splice variant in which only the intracellular NH₂-terminus is different. Also shown as residues coloured magenta in the first extracellular domain of claudins and occludin is a conserved facile cleavage site identified for Der p 1 [12]. In some members of the claudin family the cleavage site shows conservative substitution (residues shown in green).

serine peptidase (Asp f13) [20] are prime candidates for interaction with TJ adhesion proteins. However, other enzymes from *A. fumigatus* may contribute. Asp f5 is a ~40 kDa collagen-cleaving metallopeptidase [21]. Whilst there is no direct precedent for this enzyme attacking TJs, activation of an endogenous metallopeptidase activity in epithelial cells can lead to disassembly of TJs involving occludin cleavage [22]. Given the exopeptidase nature of the 95 kDa dipeptidyl peptidase IV allergen Asp f11 and the 88 kDa dipeptidyl peptidase V (formerly known as the chymotryptic antigen) [23], it is unlikely that these peptidases target the intact transmembrane loops of TJs. However, they may target other transmembrane proteins that display an extracellular N-terminus, potentially orchestrating other aspects of the epithelial response to *Aspergillus* allergens. Alternatively, like other dipeptidyl peptidases, they might attack cytokines to modify their biological activity.

Not all of the effects of peptidase allergens in epithelia are likely to involve TJ cleavage, and not all effects of allergens in epithelia are likely to be dependent upon proteolytic activity. House dust mite allergens upregulate cytokine expression in epithelial cells by both peptidase and peptidase-independent mechanisms as a result of changes in gene transcription and/or translation [24]. These effects occur in cell lines that are functionally deficient in TJs, suggesting that transduction of these responses involves cleavage of non-junctional cell surface proteins, perhaps yet unidentified receptors for peptidases. Conversely, ligation of known peptidase receptors (the serine peptidase receptors PARs 1–4) does not mimic TJ cleavage evoked by serine peptidase allergens [16]. This notion that some epithelial cell responses to peptidase allergens, such as the crucial process of allergen delivery, depend on cleavage of cell adhesions whereas others may depend on cleavage of other cell surface proteins holds true for other responses, such as apoptosis. In these experiments, the epithelial cell response to allergen is unchanged after deletion of intercellular junctions (Baker, Stewart, Thompson, Garrod & Robinson, unpublished).

There are clearly many questions arising from the notion that inhaled proteins have significant effects on mucosal surfaces which could be highly relevant to understanding antigenic responses in allergic disorders. The report of the vacuolar serine peptidase allergen Asp f18 by Shen and colleagues [1] should act as a stimulus to consider just what effects the many peptidases of *A. fumigatus* may have on human health. If their role is not to drive fungal invasion of tissues, what do they do? Is the answer that they promote a more contained response which initiates and recapitulates hypersensitivity reactions to *Aspergillus* by cleavage of critical targets on epithelial cells? Are some of those critical targets the adhesion proteins of TJs?

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