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Epicutaneous exposure to proteins and skin immune function

The skin has a sophisticated and highly orchestrated immune system. The ability of proteins encountered at skin surfaces to access that immune system remains controversial, however. In this article the question considered is whether proteins encountered epicutaneously (on the skin) at abraded or tape-stripped skin surfaces, but also at sites where the skin is intact, can engage with the cutaneous immune system to provoke and regulate responses. The available evidence suggests that epicutaneous exposure to foreign proteins is able to elicit immune and allergic responses, and that encounter with protein via this route may favour the development of selective Th2 responses and allergic sensitisation. It is also clear that proteins can modify immunological function when delivered topically and that intact skin may provide an effective route of exposure for active immunotherapy of allergic disease. An appreciation that epicutaneously applied proteins can interact with the skin immune system, even when delivered at intact skin sites, opens up important opportunities for immunotherapy, local immune modulation and the treatment of inflammatory skin diseases. It also indicates that this route of exposure must be considered as part of the safety assessment and risk management of protein-induced allergic sensitisation.

Key words: allergy, IgE, protein, skin immune system, skin penetration, Th2 response

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The capacity for pleiotropic immune responses in the skin

The skin is much more than a barrier. It is now well established that the skin is immunologically extremely active and is populated by a variety of cellular vectors of innate and adaptive immune function. In fact, the composition of the cutaneous immune system is such that there is the potential for a wide variety of stimulatory and immunoregulatory responses to antigen encountered in the skin. This is illustrated most clearly by a very brief consideration of the phenotypic and functional heterogeneity of skin dendritic cells (DC) within the epidermis (Langerhans cells; LC) and dermis (d DC).

Dendritic cells were recognised first as professional antigen presenting cells with the ability to initiate antigen-driven T lymphocyte responses. However, it is now appreciated that DC represent important orchestrators of immune function with the ability also to regulate and shape responses. This is also true of DC in the skin.

Until relatively recently, the generally accepted paradigm for LC function was that they represented epidermal sentinels forming a trap for antigens encountered at skin surfaces. The view was that LC were able to recognise, internalise and process antigen, and then, following mobilisation and migration, to provoke the activation of antigen-sensitive T lymphocytes in regional lymph nodes [1]. However, with

remarkable foresight, the late Wayne Streilein, in 1989, was persuaded by his studies in mice to conclude that LC "are sufficient but not required" for effective skin sensitisation [2]. With the availability now of a series of engineered mice in which discrete functional subpopulations of epidermal LC and dermal dendritic cells (dDC) can be selectively targeted and ablated, there is compelling evidence that skin sensitisation can proceed in the absence of epidermal LC [3, 4], although the requirement for different populations of cutaneous DC for the acquisition of sensitisation is, to some extent, dependent on the local availability of inducing hapten [5, 6]. The DC that initiate sensitisation in the absence of LC are Langerin⁺ dDC. LC are also Langerin⁺, but can be distinguished phenotypically from Langerin⁺ dDC; thus LC in mice are CD11b^{hi}, CD103⁻ and Ep-CAM^{hi}, whereas Langerin⁺ dDC are CD11b^{lo}, CD103⁺ and Ep-CAM^{lo} [7]. There are also other differences. Thus, compared with LC, Langerin⁺ dDC are short-lived and re-populate in the skin more quickly after ablation. Moreover, Langerin⁺ dDC traffic more rapidly to regional lymph nodes than do LC following mobilisation [8]. However, it is important to appreciate that even Langerin⁺ dDC are not themselves mandatory for driving skin sensitisation. Thus, mice lacking the *Bat3* transcription factor, and as a consequence also lacking Langerin⁺ dDC, display normal skin sensitization [9]. It would appear, therefore, that both LC and Langerin⁺ dDC have the potential to induce sensitisation but that there is not an absolute requirement for either population.

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In addition to functional redundancy among cutaneous DC with regard to the acquisition of sensitization, the other realization has been that LC have immunoregulatory functions. Selective ablation of epidermal LC has been shown to potentiate the acquisition of skin sensitisation and responses to other antigens, the implication being that LC serve as natural negative regulators of skin immune function [3, 4, 10]. In fact, it has been proposed that LC are pre-committed to immunoregulatory functions in the skin, with the purpose of maintaining local peripheral tolerance [11]. It appears that this negative regulatory activity is dependent upon cognate interaction of LC with CD4⁺ T lymphocytes and is mediated, at least in part, through the production of interleukin (IL) 10 [12, 13]. Other mediators (such as transforming growth factor β ; TGF- β) and the induction of other cell populations, including T regulatory (Treg) and NKT cells, may also be important [12-15]. The hypothesis is that, during the initiation of cutaneous immune responses (to skin sensitising chemicals at least – contact allergens being the most commonly used antigens in such studies), initial immune activation is effected by antigen-bearing Langerin⁺ and Langerin⁻ dDC that arrive first in draining nodes, generating hapten-specific CD4⁺ and CD8⁺ T lymphocytes. Somewhat later, LC with acquired antigens reach the same nodes, where, following cognate interaction with CD4⁺ cells, they become fully activated, secrete IL-10, and inhibit the expansion of responsive T lymphocytes [13, 16].

A potentially important consideration is the selectivity of the negative regulatory activity of LC. As indicated above, much of our understanding of the function of LC derives from investigations of the induction of skin sensitisation; a response that is generally of Th1-type. There is a view that LC may favour the development of selective Th2-type responses [17], and it could be argued, therefore, that one role for LC is to support the elaboration of Th2 responses to antigenic signals from (for instance) parasites, while limiting and constraining Th1 responses. Possibly of relevance to this speculation is that fact that, in mice, the kinetics of LC mobilisation in response to Th1-inducing and Th2-inducing chemical allergens differ, with a delayed migration of LC from the epidermis in response to Th2 allergens that is dependent upon IL-10 [18]. However, there may be additional levels of complexity to accommodate within any consideration of LC function. Recent investigations in mice have revealed that epidermal LC may be required for the development of Th17 cells and that this is antagonised by Langerin⁺ dDC. The argument is that the balance achieved between the opposing influences of LC and dDC may impact upon the phenotype of induced immune responses and on the characteristics of the local cytokine microenvironment [19].

Although from the foregoing descriptions it will be apparent that cutaneous DC play central, and frequently decisive, roles in the initiation and regulation of skin immune responses, it is important to emphasise that there are other cells, molecules and cellular-molecular interactions that are also influential [20-23].

The key point is that there is available in the skin the complex cellular machinery necessary to initiate, sustain and regulate pleiotropic immune responses to antigens encountered locally. As illustrated above, many chemicals can readily gain access to the epidermis and dermis. Such chemicals may provoke immunological priming and the

acquisition of allergic sensitisation, or may in other ways influence the skin immune system.

What is more contentious is whether, and to what extent, topically applied proteins in intact skin may gain access to the viable epidermis (and other skin compartments), such as to induce immune responses or influence immune function. That is the question addressed here.

Epicutaneous exposure to proteins

It is not disputed that proteins can provoke skin immune responses of one kind or another if there is an appropriate delivery system in place; such as, for instance, intradermal injection.

However, what has been less certain is the extent to which proteins encountered at intact skin surfaces may gain access to the viable epidermis and beyond, such as to provoke or modulate immune responses. To rephrase this, the issue to be addressed is whether epicutaneous ('onto' the skin) exposure to proteins can be associated with the initiation, regulation and/or modification of local immune responses. In tackling this question it must be acknowledged that there is some lack of consistency in what is described in the literature as being 'epicutaneous' administration. Exposures recorded as epicutaneous can in reality use administration through abraded or taped-stripped skin sites and, even if tape stripping or deliberate abrasion is not used in animal studies, not uncommonly the exposure site is shaved and that may itself cause some local disruption of barrier function. In addition, it is also the case that administration can use some form of occlusion that will serve to augment contact with the protein [24]. This degree of uncertainty about what constitutes truly intact skin must be borne in mind. However, while acknowledging such uncertainty, the important question to be addressed is whether proteins encountered at apparently intact skin sites have the potential to engage with the cutaneous immune system.

Here the ability of topically applied proteins to: (a) provoke skin immune responses, (b) induce allergic sensitisation, (c) effect allergen-specific immunotherapy and (d) modulate the skin immune system, are each considered briefly by example.

Immune activation following epicutaneous exposure to proteins

The first question to be addressed is whether epicutaneous exposure to proteins will elicit an adaptive immune response. Although in most instances some form of barrier disruption has been employed, it is clear that topical exposure to foreign proteins can provoke humoral and cell-mediated immune responses [25, 26]. While this has implications for vaccination, the more interesting question from a clinical perspective is whether topical exposure to proteins is able to cause the acquisition of allergic sensitisation.

There is good evidence that topical exposure to a variety of proteins, including food proteins, latex proteins, plant proteins and industrial enzymes, can be associated with

the development of allergic contact dermatitis or contact urticaria [27-30]. In many instances, reactions, particularly urticarial reactions, are associated with the elicitation of an IgE antibody response, although delayed-type contact hypersensitivity reactions are also described [28-31]. The assumption has been that sensitisation to proteins encountered at skin surfaces is dependent upon, or at least significantly enhanced by, skin damage and compromised barrier function at the site of exposure. However, the interesting point has been made that damage sufficient to permit enhanced access of proteins to viable skin compartments may not even be noticeable to the naked eye [28].

Irrespective of whether or not sensitisation to proteins can be achieved through truly intact skin, it is relevant that epicutaneous exposure to protein allergens can provoke an IgE antibody response. This raises the possibility that skin exposure may be an effective route for systemic sensitisation. Recently, the relevance of skin exposure to food proteins for the development of food allergy has attracted considerable interest.

Using peanut as a model allergenic food, it has been proposed that sensitisation can be acquired by skin contact. Indeed the suggestion is that the skin is in fact more effective for the acquisition of sensitisation to food proteins because dietary exposure (especially in childhood and in advance of exposure via another route) predisposes to the development of oral tolerance [32-35]. The ability of oral exposure to (certain concentrations) of peanut protein to induce immunological tolerance has also been demonstrated in mice [36]. From an immunological perspective there is no reason why skin exposure should not be a relevant route for the development of allergic sensitisation to food proteins. The systemic distribution of IgE antibodies elicited following an encounter with the inducing allergen will result in systemic sensitisation [37].

Moreover, it was also found that epicutaneous exposure of mice to peanut protein, via tape-stripped skin sites, caused allergic sensitisation and prevented the subsequent development of oral tolerance [38]. Taken together, the data available from both human and mouse studies suggest that epicutaneous exposure to protein allergens provides an effective route for the elicitation of Th2 responses, the elicitation of IgE antibodies and the acquisition of sensitisation. Moreover, it appears that, with some protein allergens at least, the induction of a cutaneous immune response to topically applied antigen can effectively prevent the later development of oral tolerance.

On this basis, epicutaneous exposure to foreign protein is clearly able to sustain the induction of immune and allergic responses. It is worth also considering whether exposure to proteins via this route selectively favours the development of allergic responses. With respect to proteins, most evidence points to the skin being an effective route for the elicitation of selective Th2-type responses and IgE antibody production [37], a possibility being that LC favour the preferential induction of Th2 cells [17, 39, 40]. Consistent with this is evidence from studies in mice that epicutaneous exposure to peanut or ovalbumin, via tape-stripped skin (with the stratum corneum removed but with LC intact) caused the preferential induction of Th2 responses [41]. Interestingly, in those same investigations, the observation was made that if antigen was delivered instead by subcutaneous injection, by-passing the epidermis, then responses were of a Th1 type [41].

Notwithstanding the possibility that in some circumstances LC may themselves favour Th2 responses, it is possible that other factors present in skin also promote the elaboration of IgE antibodies and allergic sensitisation. One candidate is IL-13 [42]. Another is thymic stromal lymphopoietin (TSLP), a cytokine produced by epithelial cells at barrier surfaces in response to damage, trauma or encounters with antigens. This cytokine is able to promote and amplify Th2-type allergic responses in the skin (and elsewhere) secondary to effects on local DC [43, 44]. It might be that the production in the skin of TSLP is in fact one manifestation of a broader response by epithelial cells to stress or damage that influences the skin immune system and promotes Th2-type responses [45].

The conclusion drawn is that epicutaneous exposure is an effective route for the initiation of immune responses to protein antigens and favours the acquisition of allergic sensitisation. The question remains, however, whether this picture is true for apparently intact skin or whether some perturbation of barrier function is required. To our knowledge, there have been no systematic comparisons and it is difficult, therefore, to draw firm conclusions. However, we speculate that even on skin surfaces where there has been no deliberate abrasion or tape stripping, and without recourse to occlusion, foreign proteins will have the potential to provoke immune responses. It might be that such responses would be more vigorous if access to the viable epidermis was enhanced by local abrasion but the argument advanced here is that this is not a requirement for initiation of a response *per se*. The other question is whether intact skin will also support the development of Th2-selective allergic responses. Again the contention is that allergic responses can be elicited if a protein allergen is delivered to intact skin. However, it might well be that not only would that response be more vigorous with local abrasion but also that the drive towards Th2 dominance would be more pronounced if there were damage resulting in a stress response by epithelial cells.

Immune modulation following epicutaneous exposure to proteins

The evidence summarised above indicates that epicutaneous exposure to proteins, possibly including exposure via intact skin, is able to support the induction of immune and allergic responses. Here we also consider whether exposure to proteins via this route can modulate or regulate adaptive immune responses.

Allergen immunotherapy is frequently proving to be effective in the treatment of allergic rhinitis and asthma. Attention has focused primarily upon sublingual immunotherapy (SLIT) and subcutaneous immunotherapy (SCIT). However, more recently there has been growing interest in the use of epicutaneous immunotherapy (EPIT) for both food allergy and inhalant allergy [24, 46, 47]. Of particular interest is the observation in mice that effective EPIT and down-regulation of allergic responses require delivery onto intact rather than abraded or tape-stripped skin [48, 49]. The mechanism(s) through which the delivery of protein to intact skin may influence existing allergic responses clearly requires clarification, although the

induction of Tregs has been implicated [48]. Nevertheless, the inference is that proteins can interact directly with cellular components of the skin immune system (probably LC) [50], even in the absence of interventions to perturb barrier function. This potential is also illustrated by investigations into the ability of lactoferrin (LF) to modulate the skin immune system.

Lactoferrin is a 79.5 kD member of the transferrin family of iron-binding proteins. Initial studies in mice revealed that intradermal injection of recombinant murine LF caused the inhibition of LC migration from the epidermis and a reduced accumulation of migrating cells in regional lymph nodes [51]. The migration of LC, in both mouse and humans, is dependent upon the availability of the cytokines' tumour necrosis factor (TNF)- α and IL-1 β . In the absence of either one of these, migration cannot proceed. Moreover, since these cytokines are able to cross-induce expression, local administration of either one into the skin is able to provoke LC mobilisation and migration. It was found that intradermal administration of LF inhibited migration by both chemical contact allergen (oxazolone) and IL-1 β , both of which require the *de novo* local production of TNF- α to cause LC mobilisation. In contrast, LF was without effect on the mobilisation of LC stimulated by intradermal injection of homologous TNF- α (which is independent of a requirement for the production of TNF- α [51]). The interpretation is that LF inhibits the migration of LC in mice secondary to an inhibition of the elaboration of TNF- α . Importantly, it was subsequently found that exactly the same inhibition of LC migration was observed when mice were topically exposed at intact skin sites to murine LF [52].

In subsequent investigations, the impact of homologous recombinant LF on human skin was explored. Human and mouse skin display remarkable similarity with regard to LC mobilisation and migration and the signals required for this [1]. In the first series of human volunteer studies, subjects received topical LF on intact skin prior to treatment at the same site with the contact allergen diphenylcyclopropenone (DPC). It was found that not only did LF inhibit chemical allergen-induced LC migration from the epidermis but it also caused a reduction in the severity of cutaneous inflammation associated with DPC exposure [53]. Additional investigations with human volunteers revealed that, in common with observations in mice, epicutaneous LF was also able to inhibit LC migration in response to IL-1 β [53]. Moreover, the same treatment with LF also caused a significant reduction in the local production of TNF- α caused by injection of IL-1 β [54].

Collectively, these data demonstrate that LF, a comparatively large protein, when applied epicutaneously to intact human (and mouse) skin, can gain sufficient access to the cellular machinery of the skin immune system to down-regulate the induced production (presumably by keratinocytes) of TNF- α and cause the inhibition of LC migration.

Concluding comments

The evidence summarised above indicates that protein delivered epicutaneously, even when exposure is via intact skin sites, can engage with the cutaneous immune system and induce and/or regulate immune responses. An

important observation is that the skin may in fact favour the preferential development of Th2 responses and allergic sensitisation and this indicates that opportunities for epicutaneous exposure (even via intact skin) should be considered carefully as part of the safety assessment and risk management of allergic sensitisation to proteins. In addition, there is growing interest in epicutaneous immunotherapy and it is intriguing that this may be most effective when exposure is via intact skin. The ability of proteins delivered epicutaneously to down-regulate immune responses and to modulate local immune function provides interesting opportunities for the development of new approaches for the treatment of chronic inflammatory skin diseases and skin allergy. ■

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