

Mite Allergen Induces Allergic Dermatitis with Concomitant Neurogenic Inflammation in Mouse

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Pathogenesis of atopic dermatitis involved the interactions of immune and neuroendocrine systems. Here we describe a mouse model for atopic dermatitis with concomitant neurogenic inflammation, by epicutaneous sensitization with a dust mite allergen. Allergen patching resulted in localized dermatitis characterized by pronounced epidermal hyperplasia and spongiosis, which was associated with infiltration of eosinophils and neutrophils, degranulated mast cells, CD4⁺ and CD8⁺ T cells, and dendritic cells. There was increased innervation of calcium gene related peptides and substance P in inflamed skins, interactions between nerve fibers and mast cells were seen, indicating the coexis-

tence of neurogenic inflammation. Splenic T cells produced T helper 2-polarized cytokines in response to allergen stimulation *in vitro*, indicating systemic allergen sensitization. This is the first report of a mouse model of eczema, accompanied by neurogenic inflammation, which shows close resemblance to human allergic diseases. This work supports the notion that the skin is an important site for the initiation of primary allergen sensitization. Besides, this model may also be useful for study of other stress-associated neuroinflammatory skin disorders such as neurogenic pruritus and psoriasis. Key words: hypersensitivity/inflammation/skin. *J Invest Dermatol* 121:289–293, 2003

Atopic dermatitis (AD) is a common chronic relapsing inflammatory skin disease afflicting up to 10 to 15% of the population in industrialized countries (Rudikoff and Lebwohl, 1998). Both clinical and epidemiologic studies indicate that AD is closely associated with respiratory allergies, also known as the atopic triad (atopic eczema, rhinitis, and asthma), usually manifesting with eczema in early life followed by the development of respiratory allergies later on (Hanifin and Rajka, 1980). The pathogenesis of AD is multifactorial, and genetically determined immunologic aberrations are thought to play a central part in the pathogenesis of chronic AD. The mechanisms involved in disease development are not well understood. In addition, there is some evidence that T cell responses to inhalant allergens, such as house dust mite allergens and pollens or food allergens are crucial to the propagation of AD (Werfel and Kapp, 1998). Recent studies indicate that both T helper (Th)2 (interleukin (IL)-4, IL-5, IL-13) and Th1 (interferon (IFN)- γ) type cytokines contribute to the pathogenesis of skin inflammation in AD and the biphasic cytokine expression pattern was therefore proposed (Thepen *et al*, 1996; Spergel *et al*, 1999).

It is known that exacerbation of AD can be provoked by stress-associated and abnormal local expression of neuropeptides (Anand *et al*, 1991). Inflammatory changes mediated by sensory nerves have been reported in the skin and other tissues (Baluk, 1997). Increasing evidence indicated that the neuropeptides released by sensory nerves such as c-fibers in the skin are capable

of activating specific cutaneous target cells located in close proximity of the activated nerve fibers to induce a range of inflammatory activities (Baraniuk *et al*, 1990; Ansel *et al*, 1996). For example, mast cell degranulation can be induced by neuropeptides such as substance P (SP) released by cutaneous nerve (Ebertz *et al*, 1987; Sugiura *et al*, 1992). In addition, it has been shown that SP can induce keratinocytes and mast cells to produce IL-1 and tumor necrosis factor- α , respectively (Brown *et al*, 1990; Ansel *et al*, 1993).

Given the complexity of the immunopathogenesis of AD, a good animal model would provide important insights into the potential mechanisms contributing to disease studies. A murine model is a good research tool as the murine immune system is well characterized and the research reagents for murine works are readily available. To date several murine models have been reported for human AD, most of them are not induced by specific protein antigens, and therefore not useful for the study of disease mechanisms. There have been two reports of murine AD models that involved the use of protein antigens (Hsu *et al*, 1996; Spergel *et al*, 1998). The main drawback of these models, however, is that the protein antigens used are not relevant to human AD. As numerous recent studies have suggested that house dust mite allergens play a direct pathophysiologic role in AD (Maeda *et al*, 1992; Kimura *et al*, 1998), the use of house dust mite allergen to establish a murine model for AD is a logical and more desirable choice.

In this study, we report the establishment of a murine model for allergic dermatitis with AD-like symptoms using an house dust mite allergen from the mite species *Dermatophagoides pteronyssinus* (Der p) through epicutaneous patching. This allergen, designated as Der p 8, is a glutathione-S-transferase of Der p mites and shown to have a 40 to 50% frequency of IgE reactivity with mite allergic patients (O'Neill *et al*, 1994). This is a first report of the induction of strong systemic Th2-skewed immune responses, as well as allergic and neurogenic inflammation of the skin of sensitized mice through epicutaneous sensitization with a mite allergen of clinical importance.

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Abbreviations: AD, atopic dermatitis; DDC, dermal dendritic cell.

MATERIALS AND METHODS

Mice and antigens Six to eight week old female BALB/C mice from the Sembawang Laboratory Animals Center, National University of Singapore, were used in the experiments. Recombinant Der p 8 expressed in *Pichia pastoris* was used as a sensitizing antigen. Animal experiments were performed according to Institutional guidelines for Animal Care and Handling, National University of Singapore.

Antibodies Rat and hamster monoclonal antibodies used for immunofluorescence, FACScan analysis, and magnetic cell sorting (MACS) were anti-DEC205 (NLDC-145, Serotec Ltd, Oxford, UK), biotin-conjugated anti-CD3e (145-2C11), anti-CD8 α (53-6.7), fluorescein isothiocyanate-conjugated anti-CD4 (RM4.5), anti-IFN- γ (XMG-2.1), phycoerythrin-conjugated anti-CD4 (RM4.5), anti-CD8 α (53-6.7), and anti-IL-4 (BVD4-1D11). Monoclonal antibodies used in enzyme-linked immunosorbent assay (ELISA) were rat antibodies to mouse IL-4 (BVD4-1D11), IL-5 (TRFK5), IFN- γ (R4-6A2), and biotin-conjugated rat antibodies to mouse IL-4 (BVD6-24G2), IL-5 (TRFK4), and IFN- γ (XMG1.2). The recombinant mouse IL-4, IL-5, and IFN- γ were used as standards in sandwich ELISA. All monoclonal antibodies and reagents were purchased from PharMingen (San Diego, California) unless otherwise specified. Rabbit polyclonal anti-SP and anti-calcitonin gene related peptides were purchased from DiaSorin Inc. (Stillwater, Minnesota). Cy-chrome-conjugated streptavidin, fluorescein isothiocyanate-conjugated streptavidin, and streptavidin microbeads were used for fluorescence studies and cell sorting.

Sensitization and challenge of mice Epicutaneous sensitization of mice was performed as described by Wang *et al* (1996) and Spergel *et al* (1998). Briefly, 50 μ g of Der p 8 in 100 μ L of phosphate-buffered saline (PBS) or PBS alone was applied to 1 cm² gauze, which was patched to the skin with a transparent dressing and further secured with an elastic bandage. The patch was applied for 4 d and then removed. Seventeen days later, an identical patch was reapplied to the same skin site. This procedure was repeated twice over a period of 50 d. A group of mice received two patches of PBS and were challenged with Der p 8 at the last patch.

T cell culture in vitro Cells were culture with RPMI-1640 supplemented with 10% heat-inactivated bovine calf serum, 2 mM L-glutamine, antibiotics (100 U per mL penicillin and 100 μ g per mL streptomycin) (Hyclone Laboratories, Logan, Utah), 1 mM sodium pyruvate, and 5.5×10^{-2} mM 2-mercaptoethanol (Life Technology, Grand Island, New York). The spleen cells were cultured in 24-well plates in the presence of Der p 8 (10 μ g per mL) for 3 d. Recombinant IL-2 was added to the cultured cell on days 3, 5, and 7. On day 10, the cells were harvested. For characterizing the cytokine profile of T cell subsets, cells were separated into two populations by AutoMACS (Miltenyi Biotec GmbH Bergisch Gladbach, Germany) and the purified CD4⁺ or CD8⁺ T cells (1×10^5 cells per well) were stimulated with immobilized anti-CD3 (10 μ g per mL) and anti-CD28 (2 μ g per mL) in 96 well U-bottom plates for 24 h.

Cytokine ELISA All the antibodies used for detection of cytokines in the cultured supernatant were purchased from Pharmingen (Pharmagen, San Diego, CA). The ELISA was performed according to the manufacturer's instructions.

Intracellular staining Cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 in the presence of 3 μ M of monensin (Sigma, St Louis, Missouri) for 10 h. After staining of anti-CD4 or anti-CD8 monoclonal antibodies, cells were fixed and permeabilized with 0.1% saponin (Sigma) and further stained with IL-4 and IFN- γ .

Histocytochemistry and immunocytochemistry For histologic examination, specimens were obtained from the patched skins at day 50 after sensitization and fixed in 10% buffered neutral formalin immediately. After embedding in the paraffin, sections of 4 μ m were cut and stained with hematoxylin and eosin. For immunofluorescence staining, the skins obtained from the patched area were embedded in Tissue-Tek O.C.T. compound, snap-frozen in liquid nitrogen and 10 μ m sections were prepared. These sections were further incubated with 5% normal mouse serum before the first antibodies were applied. The fluorescence was viewed under the Zeiss laser scanning confocal microscope. For staining of neuropeptides, mice were perfused with 4% paraformaldehyde, embedded in (Tissue-Tek) O.C.T. compound (Sakura Finetek USA, Inc., Torrance, California) and 20 μ m sections were prepared. Sections were stained by an avidin-biotin complex method

(Vector Laboratories, Inc., Burlingame, California) and counterstained by methylene green.

Data analysis All data were represented as mean $\pm$ SEM. Mast cells in the dermatitis layers were quantified from fields of 400 $\times$ magnification. All the results were analyzed by two-tailed Student's t test.

RESULTS AND DISCUSSION

Mite allergen induces severe dermatitis Histologic examination showed features of dermatitis with epidermal hyperplasia and spongiosis in the area of skin patched with Der p 8 (**Fig 1A**). A significant lichenification and excoriation could be observed in the thickened skin (**Fig 1B**). Eosinophilia and neutrophilia were seen in the dermis and subcutaneous layers (**Fig 1C**). To rule out the irritant effect of Der p 8, PBS patched mice were challenged with Der p 8 at the last patch (**Fig 1D**). It showed similar histology as PBS patched mice. An inflammatory T cell infiltrate is one of the main histologic characteristics of AD and increasing evidence suggests that T cells and the cytokines they release play a pathologic role in AD (Yamaguchi *et al*, 1991; Hamid *et al*, 1996; Akdis *et al*, 1999; Herz *et al*, 1998). In our data, the most dominant infiltrating cell type in the epidermis and dermis layers of the affected skin was the mononuclear cell, and most of these were CD4⁺ and CD8⁺ T lymphocytes (**Fig 2**). Our preliminary results (data not shown) showed that the cultured cutaneous T cells from the inflamed skin produced significant levels of IL-4 and IL-10, as well as high levels of IL-5 and IL-13. The presence of these Th2-like cytokines as well as the eosinophilia of the inflamed skin strongly suggest that a Th2-skewed immune response has been elicited and is associated with the pathophysiology of AD seen in our model.

Langerhans cells and dermal dendritic cells (DDC) are potent antigen-capturing and presenting cells, and play an important role in the pathogenesis of AD (Mudde *et al*, 1990). The Der p 8 patched skin showed a prominent increase in the number of Langerhans cells/dendritic cells at the junctions of dermis and epidermis of the skin (**Fig 2**; as indicated by DEC205⁺/DEC205⁺CD8⁺ cells). It has been reported that DDC are rapidly recruited from blood into the inflamed skin. These DDC could serve as an immediate source of antigen-presenting cells to CLA⁺ T cells in the inflamed skin. Although the precise role of these DDC remains speculative, it is tempting to

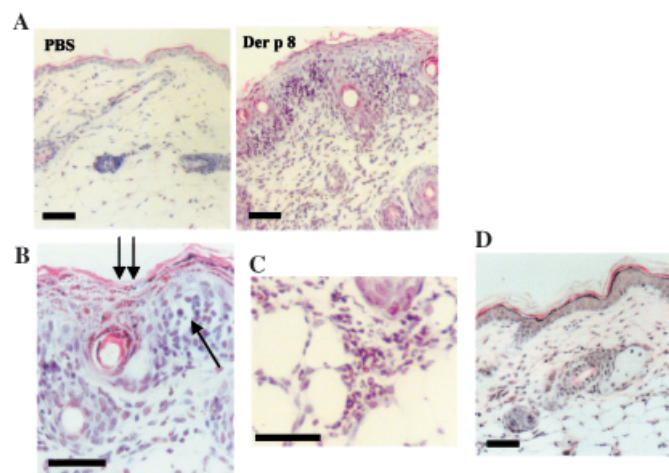


Figure 1. Skin histopathology induced by Der p 8. (A) Histology of PBS and Der p 8 patched skin. (B) Der p 8-patched skin showing hyperplasia, spongiosis (single arrow) and lichenification (double arrow). (C) Der p 8 patched skin showing the infiltration of mononuclear cells, eosinophils, and neutrophils. (D) Histology of PBS patched/Der p 8 challenged skin. Scale bar: 50 μ m.

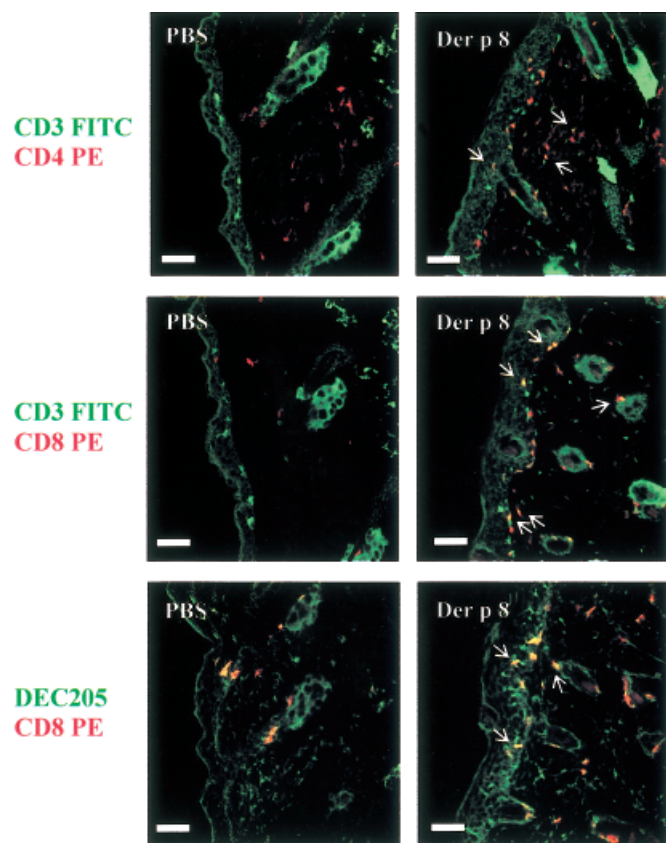


Figure 2. Der p 8 sensitization induced infiltration of T cells and dendritic cells. Skin sections were stained with fluorescein isothiocyanate labeled α -CD3, phycoerythrin labeled α -CD4, phycoerythrin labeled α -CD8, and biotinylated α -DEC205 plus Streptavidin phycoerythrin as indicated. The green color indicated CD3⁺ or DEC205⁺ cells. The single positive CD4⁺ or CD8⁺ cells are indicated by red color. The double positive cells are indicated by orange to yellow color (as indicated by arrows). Scale bar: 50 μ m.

hypothesize that DDC play a part in the development of systemic sensitization.

Th2 skewed cytokine profiles induced by epicutaneous sensitization of Der p 8 To determine the cytokine profiles of the T cell induced by epicutaneous patching of Der p 8, splenocytes from patched mice were cultured with Der p 8 *in vitro*. As shown in **Fig 3(A)**, splenocytes of Der p 8 patched mice produced higher IL-4 ($p < 0.001$) and IL-5 ($p < 0.001$) as well as lower IFN- γ than the control mice. The data indicated that patching with Der p 8 allergen induced a systemic and predominantly Th2 skewed cytokine profile.

To characterize further the cytokine profile of T cell subsets, we cultured the T cells with Der p 8 *in vitro* for 10 d and analyzed the cells by flow cytometry. As revealed by intracellular cytokine staining, there was a significant increase in the percentages of IL-4 ($4.3\% \pm 2.2\%$ vs $0.8\% \pm 0.3\%$, $p < 0.01$), and IFN- γ ($2.4\% \pm 0.2\%$ vs $1.9\% \pm 0.8\%$, $p < 0.05$) producing cells in the CD4⁺ subset of Der p 8 patched mice (**Fig 3B**). These results were further confirmed by cytokine ELISA. As shown in **Fig 3(C)**, CD4⁺ T cells of Der p 8 patched mice produced a significant amount of IL-4 and IL-5. The amount of IFN- γ produced was 2-fold lower than that of IL-4. A significant amount of IL-13 was also detected by reverse transcription-polymerase chain reaction in these CD4⁺ T cells (data not shown).

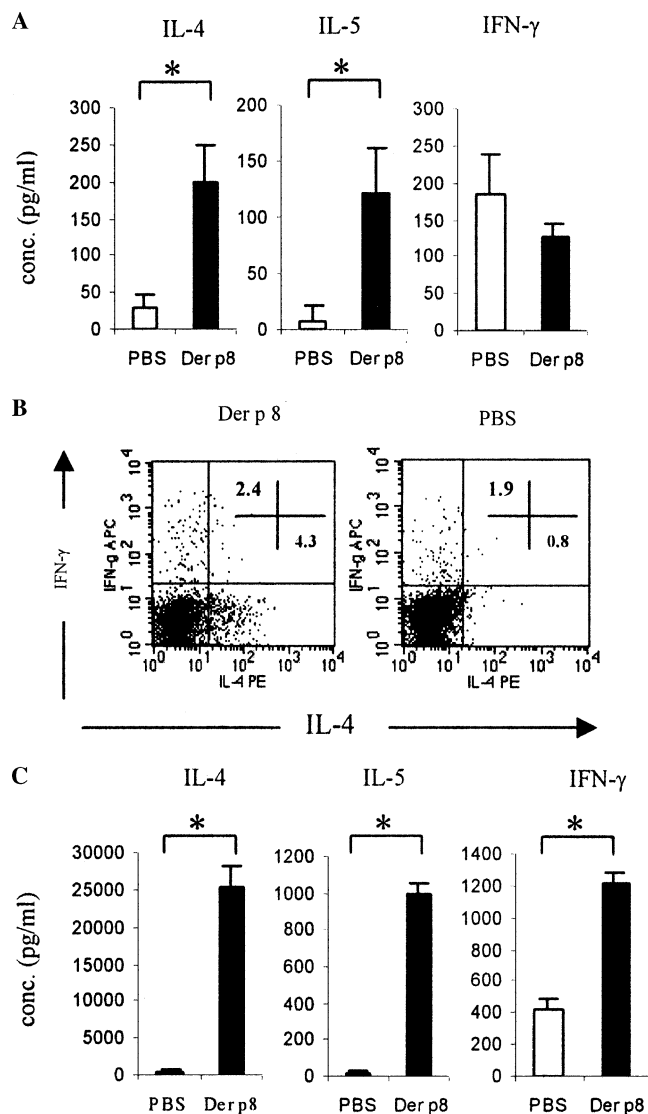


Figure 3. Epicutaneous sensitization of Der p 8 induced Th2 skewed cytokine profiles. (A) Splenocytes from PBS and Der p 8 patched mice were stimulated *in vitro* with Der p 8. The supernatants were collected 72 h after stimulation. Data are expressed as mean $\pm$ SEM ($n = 12$). The cytokine profiles of the purified CD4⁺ T cells were examined by intracellular staining of cytokines ($n = 12$) (B) and ELISA (C). In (C), representative data from six experiments are shown as mean $\pm$ SEM (* $p < 0.001$). The purity of sorted CD4⁺ cells were more than 95% as analysis by FACSscan.

Production of Th2-like cytokines in CD8⁺ cells of Der p 8 patched mice After 10 d of *in vitro* culture with Der p 8, there was a slight increase in the ratio of CD8⁺/CD4⁺ T cells in the Der p 8 patched mice (0.24 and 0.18 for Der p 8 and PBS patched mice, respectively) (data not shown). Further studies using purified CD8⁺ T cells revealed that CD8⁺ cells of Der p 8 patched mice produced a significant amount of IL-4 ($p < 0.05$) and IL-5 ($p < 0.05$) than control mice (**Fig 4**). These data indicated that a subset of CD8⁺ T cells producing Th2-like cytokines could be induced in Der p 8 patched mice. The induction of IL-4/IL-5 producing CD8⁺ T cells by patching is a novel observation that has not been reported in other animal models for AD. In humans, a pathologic role for CD8⁺ T cells in AD has been suggested (Ishii *et al*, 1998). The frequency of IL-4⁺CD8⁺ T cells correlated with the level of serum IgE in allergic patients. These CD8⁺ T cells enhanced IgE production *in vitro* (Meissner *et al*, 1997). Furthermore, CD8⁺CLA⁺ T cells

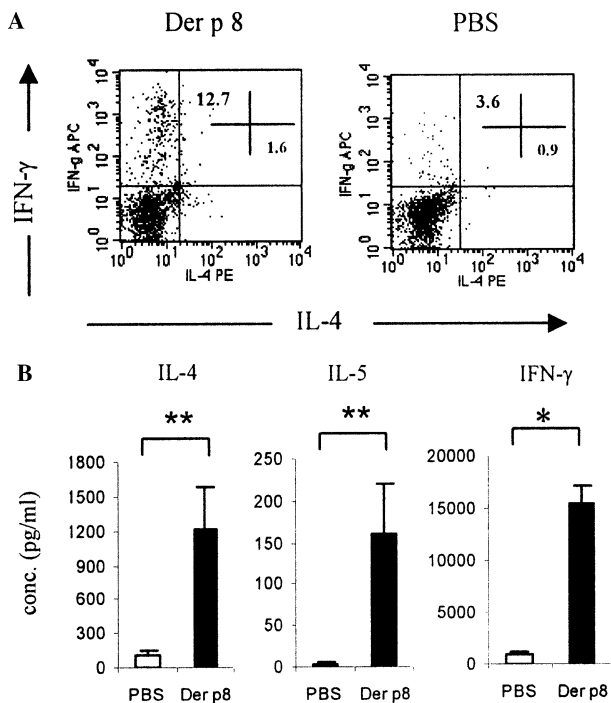


Figure 4. Production of Th2-like cytokines by CD8⁺ T cells in Der p 8 patched mice. CD8⁺ T cells were purified by AutoMACS and the cytokine profiles were examined by (A) intracellular staining of cytokines (n=12) and (B) cytokine ELISA. In (B), representative data from six experiments are shown as mean ± SEM (*p < 0.001, **p < 0.05).

induced IgE production by B cells, mainly through IL-13, and secreted IL-5 that enhanced eosinophil survival by delaying apoptosis of eosinophil (Yamaguchi *et al*, 1991; Akdis *et al*, 1999).

Interaction between neuropeptides and immune response and target cells In comparison with PBS patched mice, Der p 8 patched mice showed a significant increase in the number of mast cells (203.7 ± 11.4 vs 157.4 ± 10.5 , $p < 0.01$, $n = 9$) and degranulated mast cells (37.8 ± 5.5 vs 11.4 ± 2.1 , $p < 0.01$) in the dermis layer (Fig 5A,B). Degranulating mast cells in AD skin release a number of mediators that have profound effects on the pathogenesis of atopic skin (Klein *et al*, 1989; Katayama *et al*, 1992). Furthermore, mast cell degranulation can be induced by neuropeptides such as SP released by cutaneous nerves (Ebertz *et al*, 1987). This interaction between cutaneous target cells and neuropeptides contributes to the neurogenic inflammation seen in AD. We therefore investigated the interaction between the nervous and immune systems in the dermatitis lesions. The presence of neuropeptides, calcium gene related peptide and SP was revealed by immunohistochemical staining of the inflamed skin in mice treated with Der p 8 and undetectable in the control mice (Fig 5C). Numerous nerve fibers ramified into the dermis and appeared to be in close apposition with a number of granular cells that are most likely the mast cells in the dermis (Fig 5D). To our knowledge, this is one of the first animal models that shows the phenomenon of neurogenic inflammation associated with AD. An understanding of these interactions can facilitate the development of more specific treatments for a wide range of chronic neuroinflammatory skin diseases, and the current animal model will serve as an excellent research tool for such studies.

It has been speculated that some genetically determined elements causing an epidermal barrier dysfunction could predispose an atopic individual to allergen sensitization very early in life (Ogawa and Yoshiike, 1993; Taieb, 1999). The synergy of such epidermal barrier breakdown and other immunologic

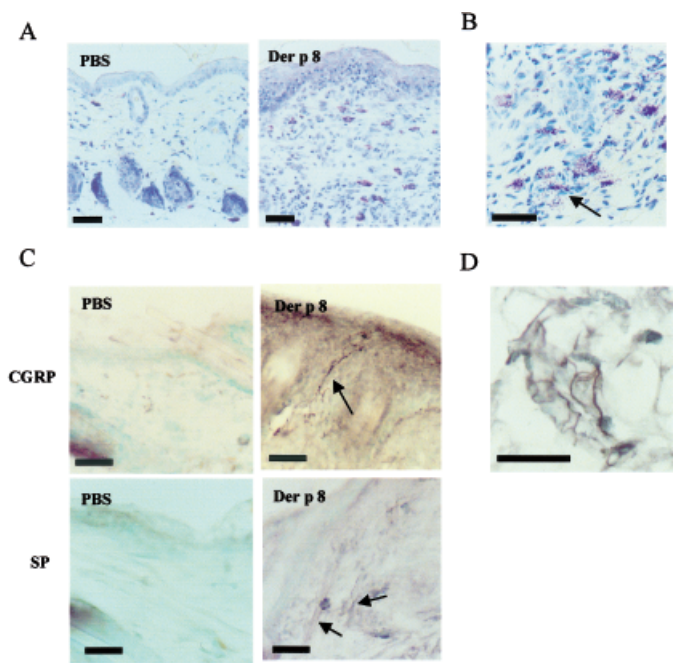


Figure 5. Neurogenic inflammation induced by epicutaneous sensitization. (A) Toluidine staining of mast cells in PBS and Der p 8 patched skin. (B) Degranulated mast cells (indicated by arrow) in Der p 8 patched skin. (C) The presence of immunoreactive nerve fibers ramified from the dermis to the epidermis of the skin of Der p 8 sensitized mice. (D) Numerous granular cells (probably mast cells) were seen in close proximity with the immunoreactive nerve fibers. Scale bar: 50 μ m.

abnormalities could lead to the propagation and enhancement of allergic inflammation associated with the pathogenesis of AD and allergic asthma. Incidentally, the Der p 8-patched mice developed lung allergic inflammation and airways hyperreactivity upon subsequent intratracheal allergen challenge (data not shown). Taken together, the data derived from this animal model support the notion that skin is not only a target site for atopic disease, but it is also an important site for the initiation of primary allergic sensitization to environmental irritants or allergens. Our findings also support the notion that primary prevention of allergic diseases should start very early in life and the problems associated with skin permeability should be addressed for high-risk atopic babies.

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