

Upregulation of the Axonal Growth and the Expression of Substance P and its NK1 Receptor in Human Allergic Contact Dermatitis

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Nerve fibers and sensory neuropeptides substance P and calcitonin gene-related peptide (CGRP) have been reported to be involved in allergic contact dermatitis (ACD). In the present study, we investigated the general innervation (using antibody against protein gene product 9.5, PGP 9.5), axonal growth (using antibody against growth associated protein, GAP-43), CGRP, and substance P with its receptor neurokinin 1 (NK1), in positive epicutaneous reactions to nickel sulphate from nickel-allergic patients, at the peak of inflammation, 72 hr after challenge with the antigen. There was an increased ($p < 0.01$) number of GAP-43 positive fibers in the eczematous compared with control skin, indicating an increased axonal growth already at 72 hr postchallenge. Double staining revealed a coexpression of CGRP and GAP-43 on dermal nerve fibers. There was no difference in the number of substance P and CGRP positive nerve fibers between eczematous and control skin. However, semiquantification analyses showed an increased expression of substance P positive inflammatory cells, being CD3, CD4, or CD8 positive, and NK1R positive inflammatory cells, being tryptase or CD3 positive. These results indicate a contribution of regenerating nerve fibers and substance P to the contact allergic reaction.

Keywords Allergic Contact Dermatitis, Axonal Growth, CGRP, NK1 Receptor, Substance P.

INTRODUCTION

Allergic contact dermatitis (ACD) is a common skin inflammatory disorder that results from contact with a low molecular weight chemical (hapten). Epidermal Langerhans cells (LCs) contribute to ACD by internalizing and processing the hapten molecules, migrating to draining lymph nodes, and presenting the hapten-protein complex to T lymphocyte (sensitization). Re-exposure to the same antigen activates the sensitized T lymphocytes to release their inflammatory mediators, which promote the inflammatory response and result in the characteristic ACD reaction.⁽¹⁾

The interaction between the nervous and immune systems has been well documented.⁽²⁾ In the skin, this interaction takes place through a cross-talk between the sensory nerves, autonomic nerves, and immune cells via the action of neuromediators that are released by skin nerves and the immune cells *per se*, and their corresponding receptors.^(3,4) For example, the function of LCs, key players in ACD, has been reported to be affected by neuropeptides from cutaneous nerves.^(5,6)

The sensory neuropeptides calcitonin gene-related peptide (CGRP), a 37 amino acid peptide, and substance P, an 11 amino acid peptide, are both released in the skin and are involved in the pathogenesis of allergic contact dermatitis.⁽⁷⁾ Upon release, both neuropeptides are capable of inducing a series of inflammatory responses including vasodilation, plasma extravasation, and cytokine production.⁽⁸⁾ Substance P mainly exerts its effects via neurokinin 1 receptor (NK1R).⁽⁹⁾ NK1R is widely distributed in the central and peripheral nervous systems and has been targeted for therapeutic applications via the action of NK1R antagonists.⁽¹⁰⁾

Reducing of cutaneous innervation or deleting CGRP and substance P-containing nerves have been reported to abolish contact sensitivity.⁽¹¹⁾

In a previous study we found an increase in axonal growth 24 hr after the elicitation of murine allergic contact dermatitis.⁽¹²⁾ In the present investigation we studied the contribution of the sensory neuropeptides and innervation to the human contact allergic reaction, at the peak of inflammation, 72 hr after antigen application.

MATERIALS AND METHODS

Patients

Patients with positive patch test reactions (grade 3+, according to the scoring system recommended by the International Contact Dermatitis Research Group) to 5% nickel sulphate (Sigma-Aldrich, St Louis, MO, USA) were included in the study. Biopsies were taken from positive patch test reactions and from the noninvolved contralateral skin of the back of 13 patients.

The ethical committee at Karolinska University Hospital, Solna, approved the study.

Processing of Specimens

Biopsies were fixed in 10% formalin with 0.2% picric acid for 1–2 hr, then rinsed in 0.1 M phosphate buffered saline (PBS) containing 10% sucrose for at least 24 hr and stored at -70°C . They were later embedded in Tissue-Tek (Zoeterwoude, The Netherlands) and cut (14 μm thick) using a Dittes cryostat (Heidelberg, Germany). Sections were then mounted on SuperFrost/Plus glass slides (Menzel-Glaser, Freiburg, Germany) and kept at -70°C until immunohistochemistry. Sections were cut at approximately 100 μm intervals to include areas with different degree of inflammation.

Immunohistochemistry

For routine immunohistochemistry polyclonal rabbit antibodies to PGP 9.5 (protein gene product 9.5, a general nerve marker; dilution 1:10,000; Ultracclone, Cambridge, England), GAP-43 (growth associated protein-43, an axonal growth marker; dilution 1:1,500; Chemicon, Temecula, CA, USA), CGRP and substance P (both 1:10,000; Bachem, St. Helens, England), and NK1R (dilution 1:5,000; Abcam, Cambridgeshire, UK), were used.

As secondary antibodies, biotinylated goat antirabbit or horse antimouse antibodies (both diluted 1:200; Vector, Burlingame, CA, USA) were used, and to visualize the staining the fluorochrome Cy2 (1:2,000; Amersham Biosciences, Uppsala, Sweden) was added. Briefly, the primary antibodies were applied overnight at $+4^{\circ}\text{C}$ and were followed by the secondary biotinylated antibodies for 40 min, after which the fluorochrome was added for 40 min. Then the sections were mounted with glycerol/PBS (10:1) containing 0.1% paraphenylenediamine before being covered with glass slips.

As a control the primary antibodies were omitted whereas in case of CGRP and substance P preadsorption was also performed with the respective peptides (Bachem) at a concentration of 10^{-6} mol/L. In both cases the controls were negative.

Double Staining

A monoclonal antibody directed against GAP-43 (Sigma-Aldrich, Stockholm, Sweden) was incubated at a dilution 1:10,000 overnight at $+4^{\circ}\text{C}$, followed by a biotinylated horse antimouse antibody (1:200) and then Texas Red (1:2,000; Vector). Polyclonal substance P or CGRP antibodies (both 1:800) were then added, followed by the addition of FITC-conjugated swine antirabbit antibody (1:40; DakoCytomation, Glostrup, Denmark).

Sections were also incubated with the polyclonal rabbit antisubstance P and anti-NK1 receptor antibodies, followed by biotinylated goat antirabbit secondary antibody (1:200) and Texas Red. Monoclonal antibodies to tryptase (1:5,000; Chemicon), CD3, CD4, or CD8 (dilution 1:80; Becton Dickinson, San Jose, CA, USA) were added to study the colocalization with substance P, and were then followed by the donkey antimouse FITC (fluorescein isothiocyanate)-conjugated antibody (dilution 1:150; Jackson ImmunoResearch Lab, West Grove, PA, USA).

Microscopy

Sections were examined using a Nikon Eclipse E 800 (Yokohama, Japan) microscope equipped for epifluorescence. Cy2 fluorescent structures were visualized with a B-1E filter cube (excitation at 465–495 nm), and for Texas Red a G-1B filter cube (excitation at 540–580) was used. Analyses were performed on coded slides (three observers: R.A., A.G., and H.E.). Immunoreactive nerve-like structures were examined and enumerated manually. Four sections per slide were counted and the average number of nerves was calculated. Those nerves that were found to extend through the dermoepidermal junction were counted within the layer that contained the largest part of the nerve.

In the case of substance P and NK1R positive cells, due to difficulties to visualize individual positive cells, semiquantification was performed, grading a low +, medium ++, or high +++ number of cells. Colored images were generated using a digital video camera system (Nikon DXM 1200, Yokohama, Japan) attached to the fluorescence microscope and connected to a PC.

Statistics

For statistical calculation the Student's *t*-test was used. A *p* value of <0.05 was regarded significant.

RESULTS

GAP-43 and PGP 9.5

In the epidermis the number of GAP-43 immunoreactive fibers (Figures 1a,1b) were increased ($p < 0.01$), being 28.4 ± 10.0 (mean $\pm$ SD) fibers per section in eczematous skin in contrast to 16.6 ± 6.8 in control skin. In the dermis, the number of GAP-43 positive regenerating axons almost doubled ($p < 0.01$) in the eczematous, 16.6 ± 6.0 , compared with the control, 9.4 ± 5.4 , skin (Figure 2).

There was no difference in the number of PGP 9.5 positive fibers (Figure 1c) between the eczematous, 165.3 ± 42.1 , and control skin, 165.6 ± 70.4 (total number given for epidermis and dermis).

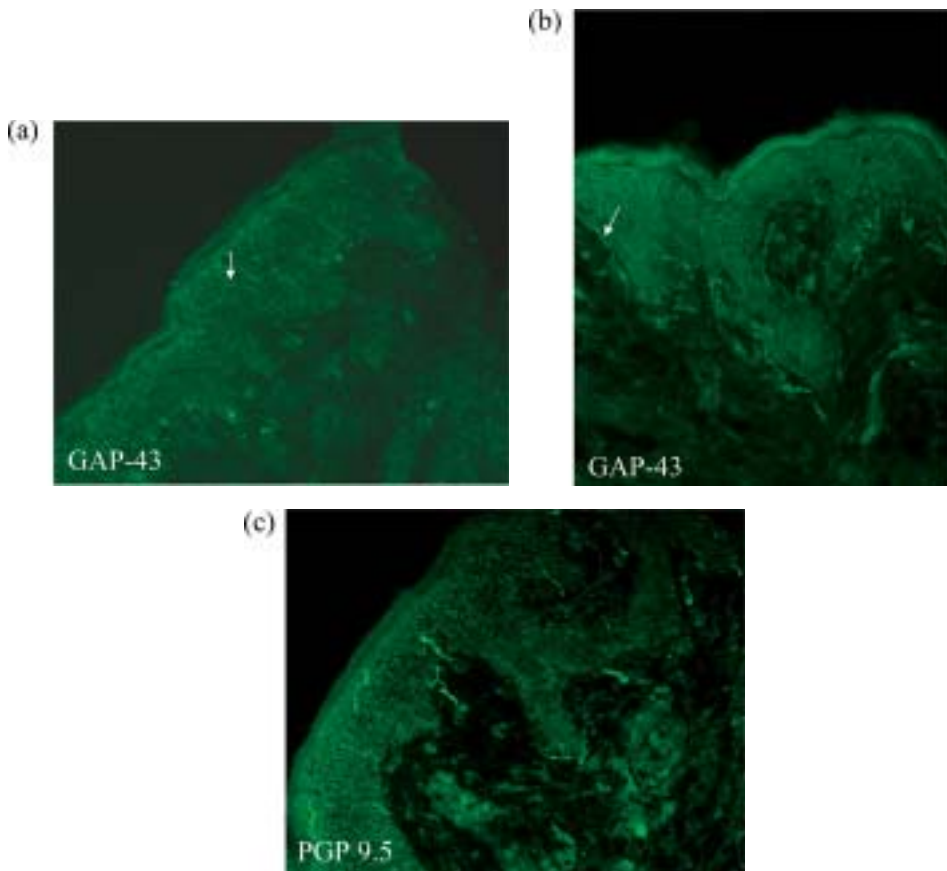


Figure 1: GAP-43 positive fibers in contact eczematous (arrow in (a)) and control skin (arrow in (b)), and PGP 9.5 positive fibers (c) in eczematous skin. (magnification $\times 200$).

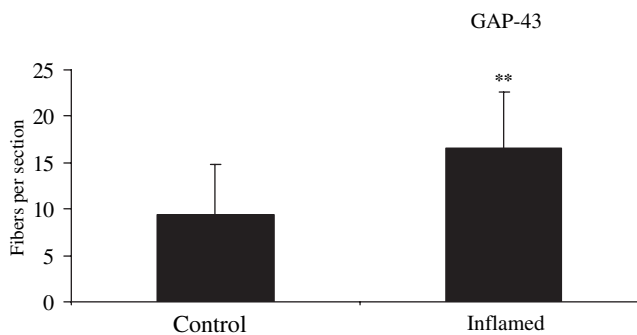


Figure 2: Graph shows the number of dermal GAP-43 immunoreactive fibers being increased in the inflamed compared with control skin. Results are presented as mean $\pm$ SD. ** $P < 0.01$

Sensory Neuropeptides and NK1 Receptor

The number of CGRP immunoreactive nerve fibers (not shown) in the papillary dermis showed a tendency to be slightly decreased in the eczematous, 5.5 ± 2.9 fibers/section, compared with control skin, 7.1 ± 4.0 , whereas there was no difference between the number of substance P positive fibers (Figure 3a) in the inflamed, 4.2 ± 1.7 , and control skin, 4.4 ± 2.1 . CGRP immunoreactive nerve fibers were positive for GAP-43 in contrast to substance P fibers (Figures 4a,4b). This coexpression was also detected in nerve bundles.

Semiquantification of substance P- (Figure 3a) and NK1R positive (Figure 3b) papillary dermal mononuclear cells showed an increase in the eczematous (+++) in contrast to control (+) skin. Moreover, substance P positive cells were observed to intrude into the epidermis. NK1R immunoreactivity was generally higher in basal keratinocytes in inflamed compared with control skin (Figure 3b). The intensity of this immunoreactivity also appeared to be increased with more intense inflammation.

By double staining, the substance P positive cells were found to be tryptase, CD3, CD4 or CD8 positive (not shown), whereas NK1R cells were tryptase or CD3 positive (Figures 5a,5b, shown for tryptase).

DISCUSSION

In the present investigation we report the implication of the nervous system in allergic contact dermatitis to nickel, at the peak of inflammation, 72 hr after challenge with nickel sulphate. We found an increased number of regenerating nerve fibers and an increase in substance P and NK1R positive cells, in the contact eczematous compared to control skin.

Several studies have reported an increase in tissue innervation during an inflammatory response.⁽¹³⁾ In a study by Kinkelin et al.⁽¹⁴⁾ an increase was found in the length of GAP-43 positive fibers in contact allergic reactions 96 hr after antigen application on the human skin. Our results indicate a contribution of the nervous system in humans already at 72 hr after application of the antigen. The results are consistent with our findings from the murine contact eczematous model, in which axonal growth was increased at the peak of inflammation (24 hr postchallenge).⁽¹²⁾

Colocalization of CGRP and GAP-43 immunoreactive nerve fibers was apparent, in which the GAP-43 positive fibers were the most abundant. This finding indicates an earlier sprouting of CGRP compared with substance P-containing nerve fibers. In a report by Wu et al.⁽¹⁵⁾ a colocalization of CGRP and GAP-43 fibers was also reported in the setting of adjuvant arthritic rats.

No significant difference in the number of CGRP and substance P immunoreactive nerves in inflamed versus control skin was found, but there was an increased immunoreactivity for substance P nonneuronal cells in the inflamed skin, indicating a nonneuronal contribution of substance P to the allergic reaction.

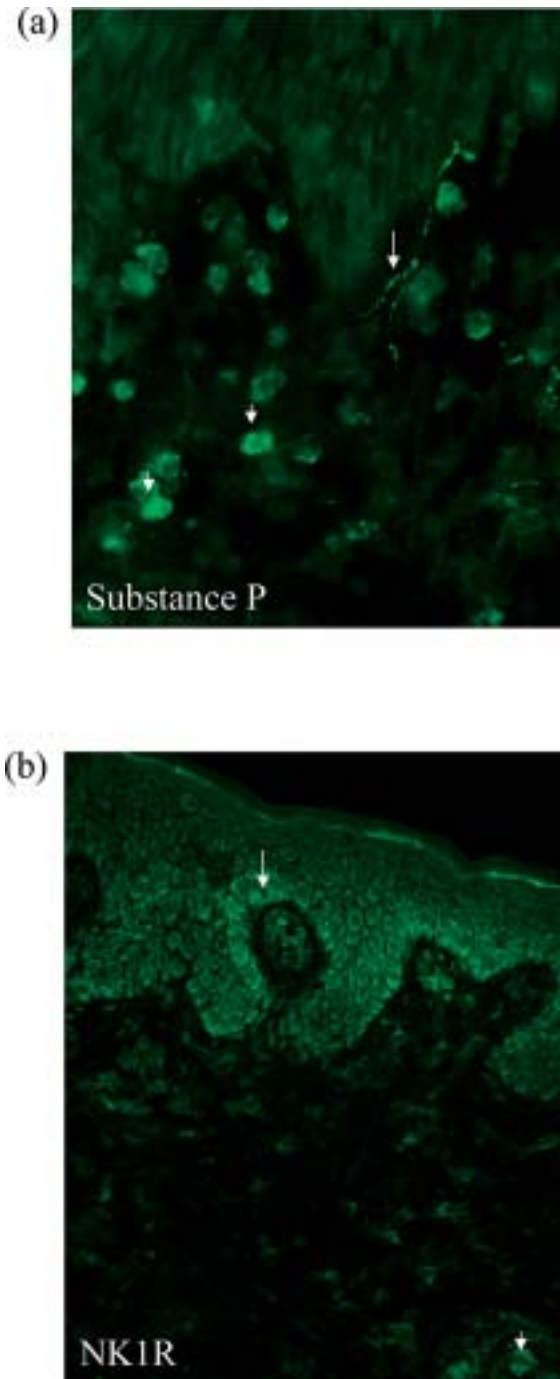


Figure 3: Substance P positive fiber (arrow in (a)) and cells (arrowheads in (a)) in contact eczematous skin. NK1R immunoreactivity in the inflamed basal epidermis (arrow in (b)). Arrow-head in (b) defines NK1R positive inflammatory cell ($\times 400$ for (a) and $\times 200$ for (b)).

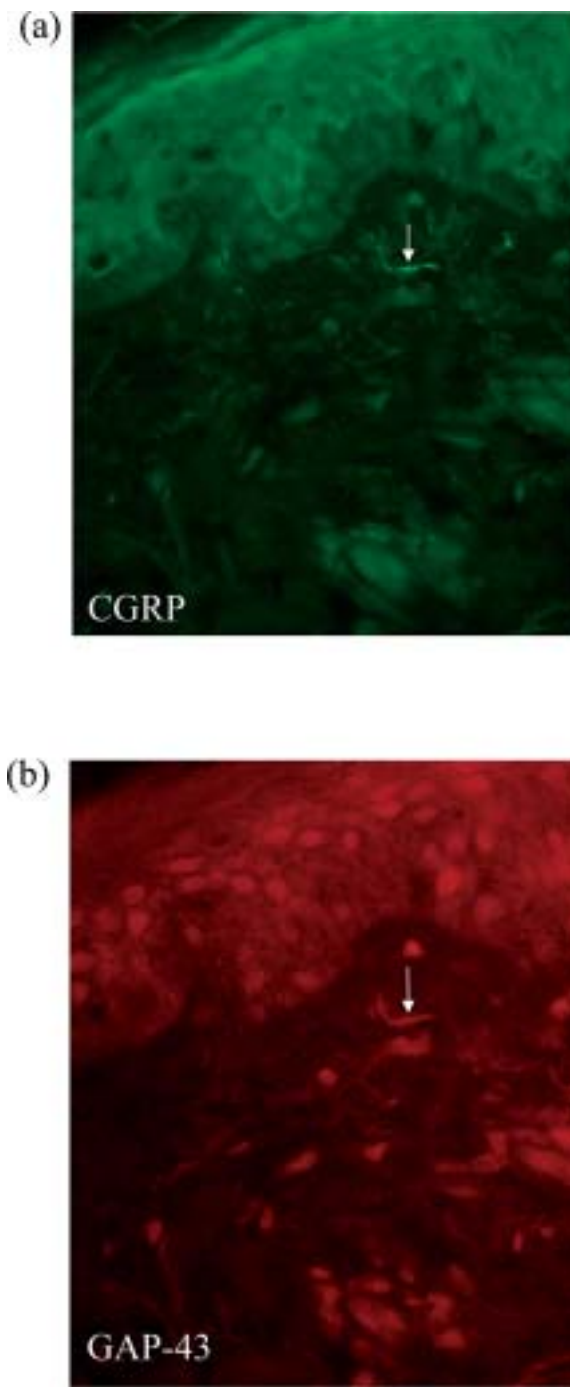


Figure 4: Double staining for CGRP (FITC, arrow in (a)) and GAP-43 (Texas Red, arrow in (b)) positive nerve fibers ($\times 400$).

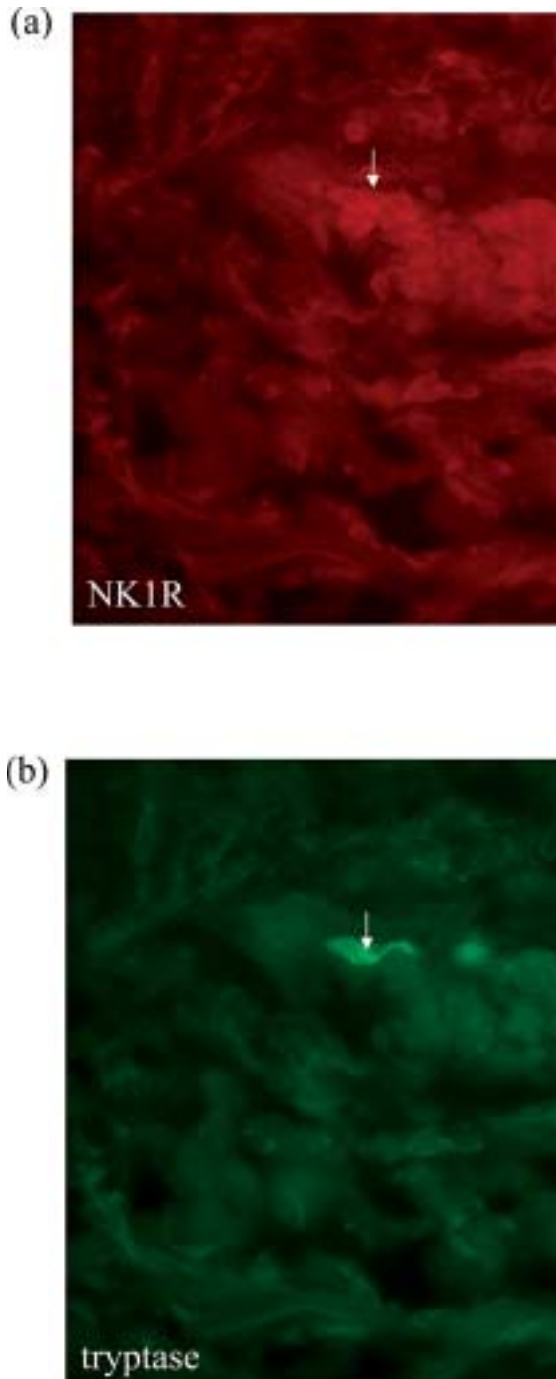


Figure 5: Double staining shows NK1R positive cell (Texas Red, arrow in (a)) being tryptase positive (FITC, arrow in (b)) (x400).

This is also in agreement with the finding of an increased number of NK1R-positive cells. The above-mentioned data are consistent with literature reports of an enhanced murine ACD due to inactivation of neutral endopeptidase and a subsequent increase in substance P,⁽¹⁶⁾ and the inhibition of contact dermatitis in mice lacking the NK1 receptors.⁽⁹⁾

Substance P is known to cause enhancement of ACD via the modulation of immune cellular function such as induction of mast cells degranulation and the release of tumor-necrosis factor- α ,⁽⁷⁾ and modulation of adhesion molecules.⁽¹⁷⁾

CONCLUSION

These results indicate a contribution of the nervous system to the contact allergic reaction in the form of an upregulation of axonal growth as well as nonneuronal substance P and its NK1R.

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