

Kinetics of neuronal contribution during the development of a contact allergic reaction

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Abstract The nervous system contributes to allergic contact dermatitis (ACD). Elucidation of the implication of the nervous system during different stages of ACD could be of therapeutic value. Our aim was to study the kinetics and contribution of the nervous system to ACD by investigating innervation and expression of neuropeptides in skin biopsies obtained at 0, 6, 24, 48 and 72 h post-challenge. Biopsies were stained using antisera against protein gene product (PGP) 9.5, growth associated protein (GAP)-43, substance P and its receptor (R) neurokinin (NK)-1, NKA and NK-2R, and calcitonin gene-related peptide (CGRP). GAP-43-immunoreactive (ir) nerves revealed a time-dependent increase that was more pronounced at 48 and 72 h, while PGP 9.5-ir nerves remained unaltered. Substance P-, NKA- and CGRP-ir nerves at 0 and 6 h were significantly higher compared to later time points, whereas NKA-, NK-1R- and NK-2R-ir cells were lower. A dramatic rise in cell numbers was noted at 24 h. Our findings demonstrate the implication of nerves and sensory neuropeptides during the kinetics of ACD and suggest a possibility to target this system at an early time point for therapy.

Keywords Allergic contact dermatitis · Nerve fibres · Substance P · Neurokinin A · Calcitonin gene-related peptide · Neurokinin receptors

Introduction

Allergic contact dermatitis (ACD) is a common inflammatory skin disorder that is characterized by redness, papules and vesicles, followed by scaling and dry skin [11]. This T cell-mediated disorder has two phases, sensitization and challenge. Sensitization is initiated by the dendritic cell priming of T lymphocytes after the exposure to an antigen. Re-exposure to the same antigen elicits the challenge phase, which leads to a cascade of inflammatory responses that make up the hallmark of ACD [25].

The interaction between the nervous and immune systems has been well documented by literature reports [3, 19], and furthermore the neuroimmune interaction in ACD has been earlier described (for review see Ref. [17]). This is clearly seen in the cross talk between cutaneous nerves and immune cells, where sensory c-fibres are shown to release neuromediators such as tachykinins and calcitonin gene-related peptide (CGRP), which modulate the actions of immune cells via targeting specific receptors expressed by these cells leading to the initiation of neurogenic inflammation [4, 8, 13, 20, 26]. On the other hand, immune cells per se are known to synthesize and release neuromediators that activate target receptors and contribute to neurogenic inflammation (for review see Ref. [21]).

One of the most recognized nerve markers is protein gene product (PGP) 9.5, which is a ubiquitin carboxyl-terminal hydrolase that is abundantly present in unmyelinated epidermal c-fibres [27]. Another protein that labels regenerating nerve fibres is the axonal growth marker, growth associated protein-43 (GAP-43). Our previous work demonstrated that GAP-43 axonal expression was altered at the peak of inflammation in contact hypersensitivity in mice, 24 h after challenge with the antigen [6] and in humans at 72 h post-challenge [5].

Substance P and neurokinin A (NKA) are members of the tachykinin family that are known to be implicated in murine

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ACD [18]. Substance P and NKA have an agonizing effect on preferentially neurokinin-1 (NK-1) receptor (R) and NK-2R, respectively [14, 15]. Both NK-1 and NK-2 receptors belong to the superfamily of G protein-coupled membrane receptors (GPCRs) [1]. NK-1R and NK-2R activation increases vascular permeability, plasma extravasations and leukocyte infiltration, which cause skin inflammation (e.g. for review see Ref. [9]). In a previous report [5], we have shown the expression of both substance P and NK-1R on mast cells and lymphocytes in human ACD skin at 72 h.

CGRP belongs to a family of calcitonin peptides. This sensory neuropeptide stimulates vasodilation, plasma extravasation and cell degranulation. CGRP was found to have an inhibitory role in contact hypersensitivity [2, 24].

Tachykinins and CGRP are actively involved in mast cell-derived cutaneous inflammation [22]. These neuropeptides can also modulate functions of keratinocytes, Langerhans cells, dermal microvascular endothelial cells and other infiltrating immune cells [16].

In this report, we have been investigating the kinetics of inflammation in ACD, with respect to the innervation and expression of substance P, NKA and CGRP, as well as the NK-1 and NK-2 receptors, during development of the contact allergic reaction at 0, 6, 24, 48 and 72 h after challenge with nickel sulphate.

Subjects and methods

Subjects/challenge

Ten nickel allergic female patients (mean age 47.3 years, range 21–66), who had a history of nickel allergy and who had earlier been developing allergic reactions (grade 3+, according to the scoring system recommended by the International Contact Dermatitis Research Group, ICDRG) to 5% nickel sulphate (Sigma-Aldrich, St Louis, USA), participated in this study. Five percent nickel sulphate in petrolatum was applied in five Finn chambers (EPITEST LTD. OY, Tuusula, Finland) on the back of the patients, in a vertical line. The reactions were read at the designated time points (0, 6, 24, 48 and 72 h) by the investigator (H. E.) and were recorded as 0, 1+, 2+ or 3+. Immediately after each reading, 3 mm skin biopsies were obtained from the patch tested areas on the back.

The patients gave their informed consent and the study was approved by the Local Ethical Committee at Karolinska University Hospital, Stockholm, Sweden.

Processing of the specimens

The biopsies were fixed in 4% formalin with 0.2% picric acid for 1 h and then rinsed in 0.1 M phosphate-buffered saline

(PBS) containing 10% sucrose for at least 24 h and stored at -70°C . They were later embedded in Tissue-Tek (Zoeterwoude, The Netherlands) and cut (14 μm thick) using a Microm cryostat (Heidelberg, Germany). The sections were then mounted on SuperFrost/Plus glass slides (Menzel-Glaser, Freiburg, Germany) and kept at -70°C until immunohistochemistry, employing the streptavidin–biotin technique.

Immunohistochemistry

The sections were left to dry at room temperature for 20–30 min before being washed in PBS.

Commercial polyclonal rabbit antisera against PGP 9.5 (catalogue number RA95101; dilution 1:10,000; UltraClone, Cambridge, England), GAP-43 (AB5220; 1:10,000; Chemicon, Temecula, CA, USA), substance P (T-4107; 1:10,000), CGRP (T-4243; 1:10,000), NKA (T-4445; 1:1,000), all from Bachem (San Carlos, California, USA), NK-1R (NB300-119C; 1:5,000; Novus Biologicals, Littleton, CO, USA) and NK-2R (SP4506P; 1:2,000; Acris Antibodies, Herford, Germany) were incubated overnight at $+4^{\circ}\text{C}$. The following morning, the sections were incubated for 40 min at room temperature with a biotinylated goat anti-rabbit antibody (BA-1000; 1:200; Vector, Burlingame, CA, USA). Then the fluorochrome Cy2 labelled streptavidin (PA42001; 1:2,000; Amersham Pharmacia Biotech, Uppsala, Sweden) was incubated for 40 min at room temperature to visualize the staining. Finally, the sections were mounted with glycerol/gelatin (Merck, Darmstadt, Germany) and glass coverslips were applied.

As controls, the primary antibodies were omitted or normal rabbit serum IgG (X 936; Dako, Glostrup, Denmark) was used in the same dilutions as the primary antibodies, which abolished the staining.

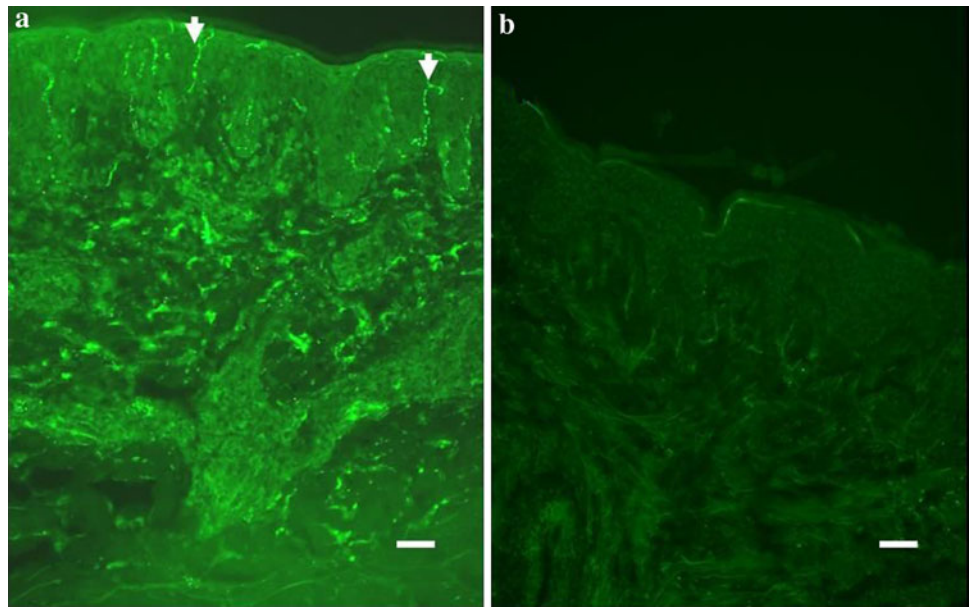
Double staining

To characterize NKA- and NK-2R-positive cells double staining was performed on six biopsies (two per each time point) obtained at 24, 48 and 72 h. NKA and NK-2R polyclonal antibodies were incubated, at similar concentrations as above, overnight at $+4^{\circ}\text{C}$. The following day, staining was continued as above, then monoclonal mouse antisera against tryptase (MAB1222; 1:1,000; Chemicon) and CD3 (347340; 1:50; Beckton-Dickson, San Jose, CA, USA) were incubated in the refrigerator until the next morning. Finally, the anti-mouse Alexa Fluor 594 (A21203; 1:750; Invitrogen, Eugene, Oregon, USA) was applied to visualize the staining.

Microscopy

Microscopic examination of the sections was performed using a fluorescent Zeiss Axioskop 2 MOT microscope (Carl Zeiss AB, Stockholm, Sweden). Cy2 fluorescent

Fig. 1 Micrograph showing PGP 9.5-positive nerve fibres (arrows in **a**) in a contact allergic skin lesion obtained at 72 h. Control abolished staining with normal rabbit serum IgG seen in **b**. Scale bars 20 μ m



structures were visualized with a B-1E filter cube (excitation at 465–495 nm), while Alexa Fluor 594 was visualized with a G1-B filter cube (excitation 540–580 nm).

For PGP 9.5 analysis, one representative image of a microscopic field from each biopsy was stored by using a digital video camera system (Nikon DXM 1200, Yokohama, Japan) attached to the fluorescence microscope and connected to a PC. Then the nerves were analysed by using the software Easy Image Analysis (Bergström Instruments, Solna, Sweden) and the percentage of the area fraction (ratio of specifically stained fluorescent/non-fluorescent areas) was calculated.

The image analysis programme was only used for PGP 9.5-positive nerve fibres because the programme was unable to recognize a specific threshold where the positive signal from the other antigen labelled immunoreactive (ir) nerves could be detected, due to the weak contrast in the signal between the positive nerves and surrounding tissue. Hence, GAP-43-, substance P-, CGRP- and NKA-ir nerve fibres were enumerated manually by two examiners (H. E. and R. A.). NK-1, NK-2 receptors and NKA-positive cells in the epidermis and papillary dermis were also enumerated manually by the investigators (H. E. and J. L.). Immunoreactive nerves or cells were counted in four sections per coded slide, and then the means were calculated. Nerves crossing the dermo-epidermal junction were only counted within the layer that contained the longest part of the nerves.

Statistics

For statistical analysis, the Student's *t* test or Mann–Whitney *U* test was applied. A *p* value of <0.05 was regarded as being significant.

Results

Patch test reactions

The earliest reactions appeared at 24 h on eight patients, showing 1+. At 48 h, one patient showed a 1+ reaction, whereas seven patients had 2+ and the remaining two patients acquired 3+ reactions. Eight patients at 72 h had 3+ reactions, while the remaining two acquired 2+.

PGP 9.5

The area fractions of PGP 9.5-immunoreactive (ir) nerve fibres (Fig. 1a) in the epidermis and dermis, individually and combined together, did not show any significant difference at the different time points. The epidermal area fractions recorded at 0, 6, 24, 48 and 72 h were 2.2 ± 1.7 (mean $\pm$ SD), 2.1 ± 1.4 , 2.2 ± 1.9 , 1.9 ± 1.5 and 1.9 ± 1.6 , respectively.

GAP-43

The total (epidermis + dermis) numbers of GAP-43-ir nerve fibres were found to be increased ($P < 0.05$) in a time-dependent manner with the lowest numbers being recorded at the early time points (Fig. 2a, b). Also, epidermal GAP-43-positive nerve fibres (Fig. 2c) were found to be lower ($P < 0.01$) at 0 and 6 h [medians 14 (quartile deviation 13) fibre/section and 23 (20) respectively] compared to 48 h, median 45 (34) and 72 h, median 72 (46). At 24 h, the median for epidermal GAP-43-labelled nerves was 35 (46).

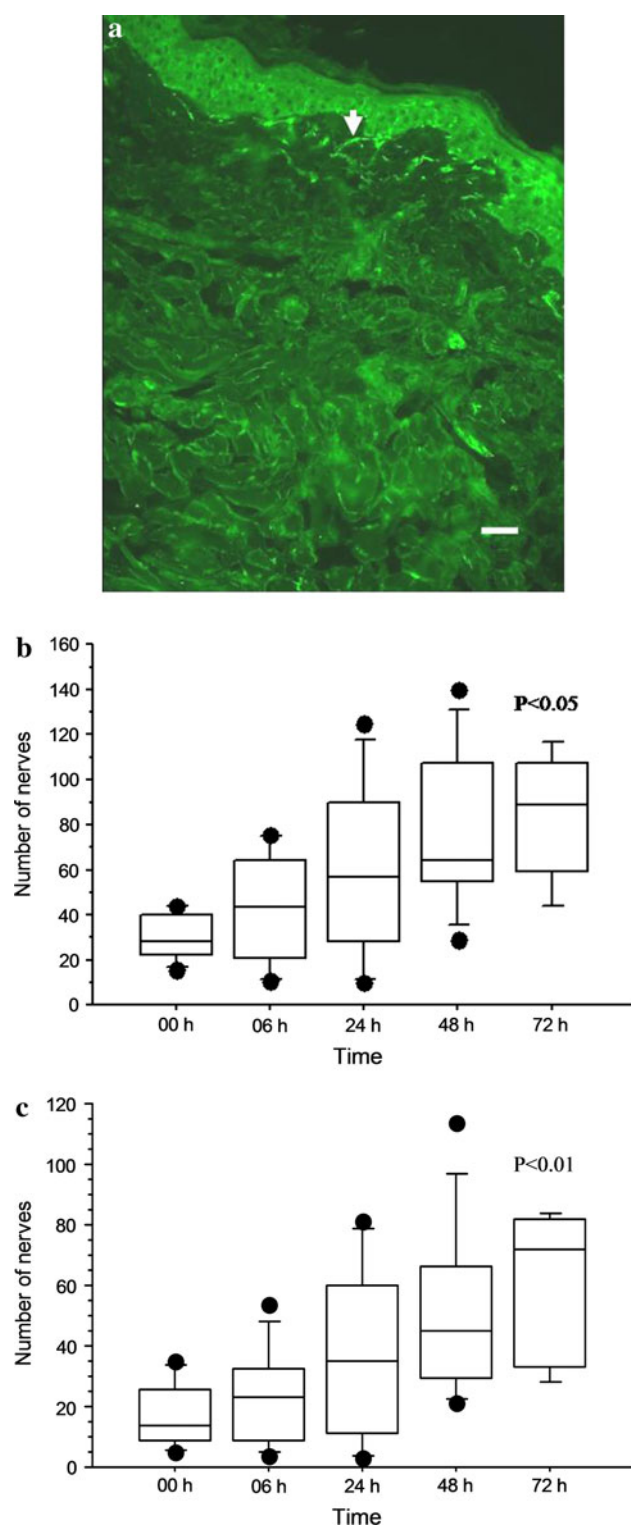


Fig. 2 Micrograph depicting GAP-43-positive nerve fibres in allergic contact dermatitis skin (arrow in **a**). Graphs showing the total numbers of GAP-43-positive fibres (**b**) and epidermal fibres (**c**). Scale bar 20 μ m

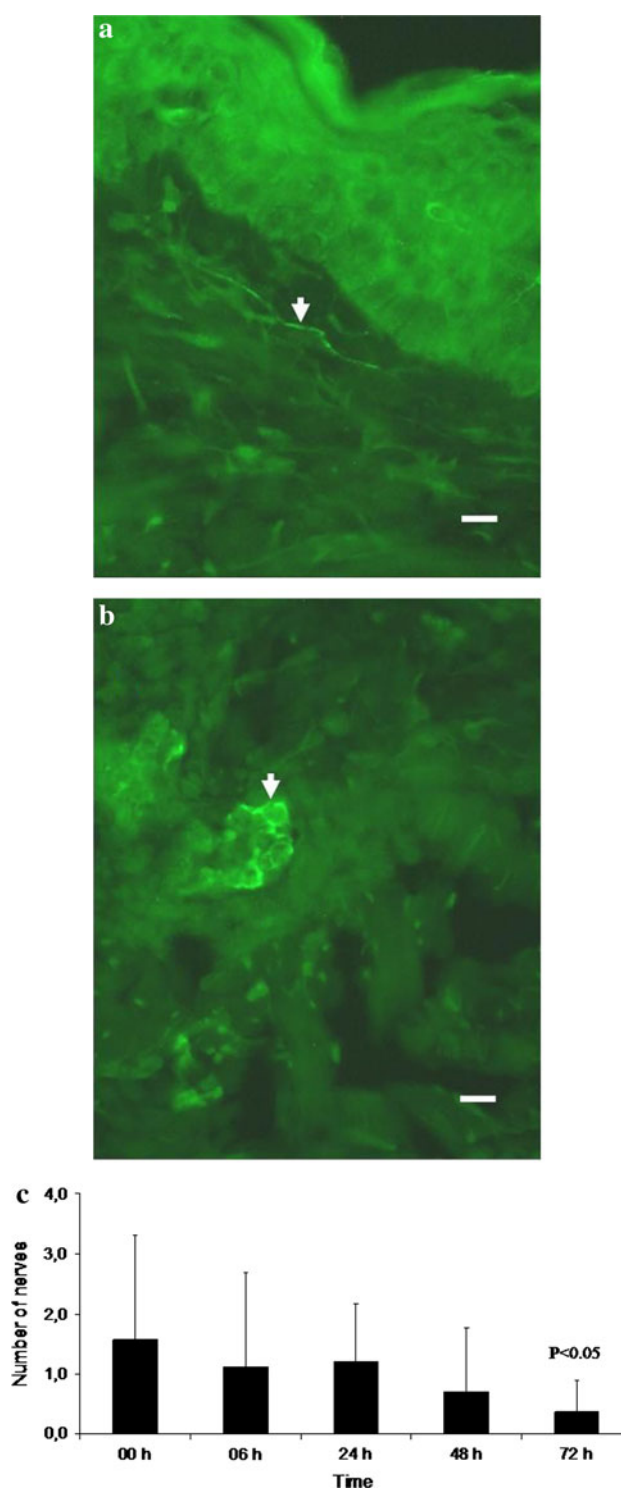


Fig. 3 Allergic contact dermatitis skin micrographs depicting substance P-positive nerve (arrow in **a**) and cells (arrow in **b**). The numbers of substance P-positive nerve fibres is shown in **c**. Scale bars 10 μ m

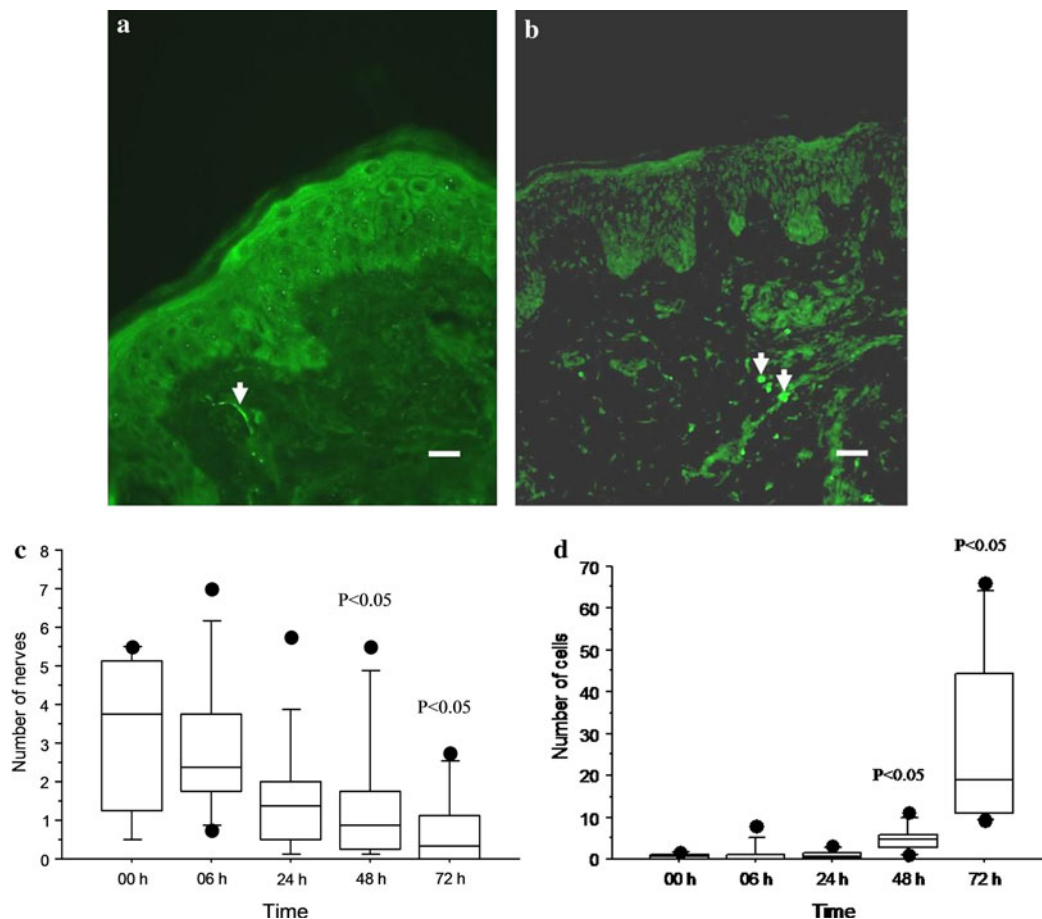


Fig. 4 NKA-positive nerve fibre (arrow in **a**) and cells (arrows, **b**). Graphs showing NKA-positive nerves (**c**) and cells (**d**). Scale bars 10 μ m in **a** and 20 μ m in **b**

Substance P

Substance P immunoreactivity was noted on nerve fibres and mononuclear cells (Fig. 3a, b). The number of substance P-ir nerve fibres revealed a time-dependent decrease, 0 h (1.6 ± 1.7), 6 h (1.1 ± 1.6), 24 h (1.2 ± 1.0), 48 h (0.7 ± 1.1) and 72 h (0.4 ± 0.5), which was most evident when comparing 0 and 24 h with 72 ($P < 0.05$, Fig. 3c). Epidermal substance P-positive nerve fibres were seen and more fibres were seen in the dermis.

Substance P-positive mononuclear cells were observed in the epidermis and dermis of the contact allergic skin. However, there was no significant difference in the total number of these cells, 6.4 (10.3), 5.8 (9.9), 8.4 (8.2), 9.5 (21.3) and 10.6 (16.6), recorded at 0, 6, 24, 48 and 72 h, respectively, but a tendency towards an increase at the later time points was observed.

NKA

The expression of NKA was seen on cutaneous nerve fibres and inflammatory cells (Fig. 4a, b). The numbers of NKA-ir nerve fibres (Fig. 4c) demonstrated a time-dependent

decrease ($P < 0.05$) when compared with the numbers of fibres at the early time points [0 and 6 h (median 3.8; quartile deviation 3.5) and 2.4 (1.9), respectively] with 48 and 72 h [0.9 (1.4) and 0.3 (0.8) respectively]. At 24 h, the number of NKA-positive fibres was 1.4 (1.4).

The numbers of NKA-positive cells were lower ($P < 0.05$) at 0 and 6 h in contrast to 48 and 72 h (Fig. 4d).

CGRP

CGRP was expressed on cutaneous nerve fibres (Fig. 5a), but not on immune cells. These nerve fibres (Fig. 5b) were gradually decreased ($P < 0.05$) when compared with their numbers (21.9 ± 16.5 and 22.9 ± 16.3) at the onset of inflammation (0 and 6 h respectively) with numbers (16.8 ± 10.1 , 10.3 ± 8.8 and 4.1 ± 4.3) at the later time points (24, 48 and 72 respectively).

NK-1R

NK-1R-positive cells were observed in the papillary dermis (Fig. 6a–c). The numbers of NK-1R-ir cells were found to

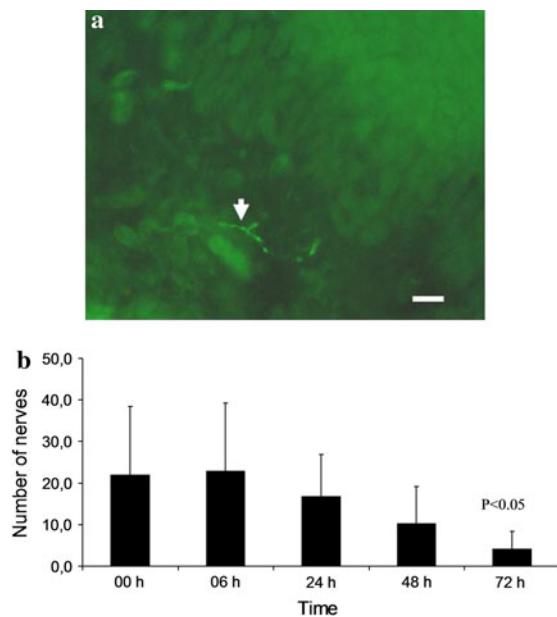


Fig. 5 Micrograph showing CGRP-positive nerve fibre (arrow, **a**). Graph illustrating the numbers of CGRP-positive nerve fibres (**b**). Scale bar 10 μ m

be increased ($P < 0.001$) in a time-dependent fashion: 19.5 (9.0), 19.5 (15.0), 33.5 (21.5), 74 (63.0) and 88 (115.0) at 0, 6, 24, 48 and 72 h, respectively.

NK-2R

NK2-R epidermal immunoreactivity was noted on keratinocytes only in the eczematous in contrast to the non-eczematous skin obtained at 0 h (not shown), but this increased signal was not consistent for all the studied biopsies. NK2-R expression was also seen on epidermal and dermal mononuclear cells (Fig. 7a). The total number of these cells was increased ($P < 0.01$) in a time-dependent manner, from 0.13 (0.6) and 0.13 (0.5) cells/section at 0 and 6 h, respectively, to 1.3 (6.5), 9.0 (4.3) and 23.2 (23.2) at 24, 48 and 72 h, respectively (Fig. 7b).

Double staining

Double staining revealed a co-localization of NKA with tryptase (Fig. 8a–c) and NK2-R with tryptase (not shown) or CD3 (Fig. 9a–c) on immune cells indicating the expression of NKA on mast cells and NK-2R on mast cells and lymphocytes.

Discussion

This study reports an alteration of innervation, expression of sensory neuropeptides and the tachykinin receptors NK-1 and NK-2, during the kinetics of an ACD reaction.

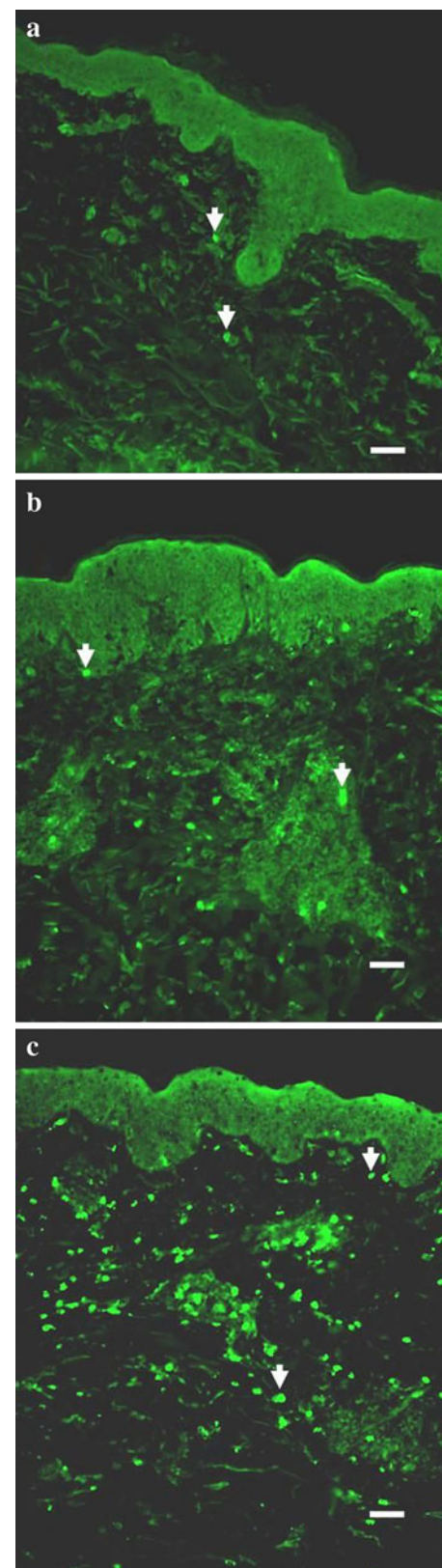


Fig. 6 NK-1R-positive cells in skin biopsies obtained at 0 (arrows, **a**), 48 (arrows, **b**) and 72 h (arrows, **c**). Note the time-dependent increase in the number of cells from being few in the non-eczematous skin to becoming high at the peak of inflammation. Scale bars 20 μ m

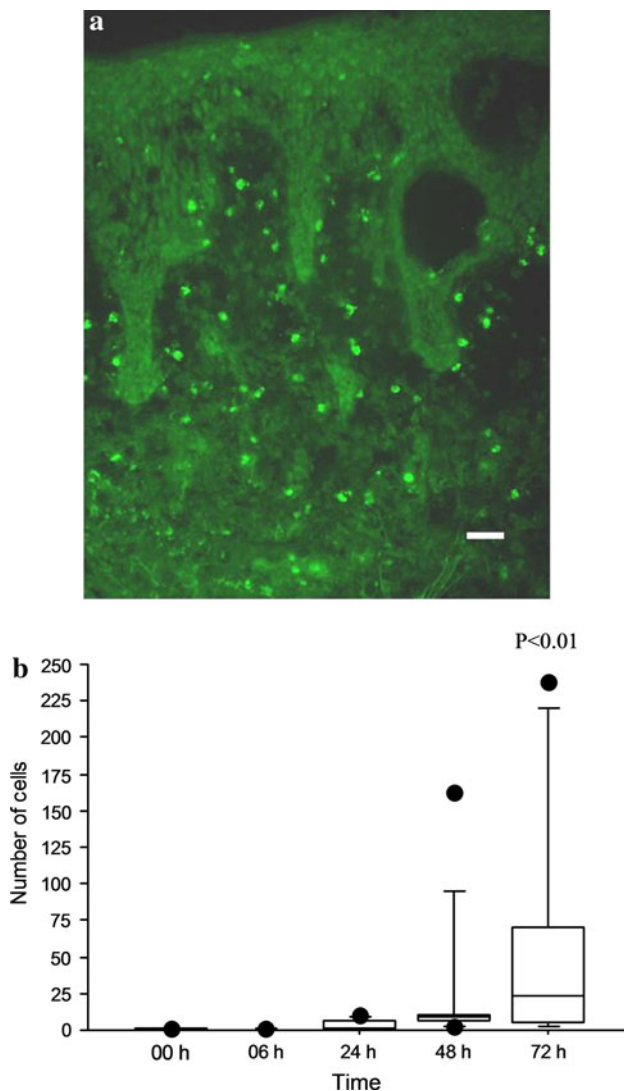


Fig. 7 NK-2R expression on inflammatory cells is seen in **a** (captured at 72 h). Time-dependent increase in the numbers of NK-2R-positive cells is shown in **b**. Scale bar 20 μ m

The elevation in the number of GAP-43-positive nerves at the later stages of inflammation indicates a mechanism of active regeneration of sensory nerves during the different phases of the inflammatory response. This result is consistent with findings by others and our earlier reports that showed an upregulation of axonal growth in contact eczematous skin obtained at the peak of inflammation [5, 12]. In a murine model of ACD [10], cutaneous nerve elongation was shown to be the result of mast cells and a mast cell-derived tumour necrosis factor (TNF). In this present investigation, we further studied the axonal growth at the kinetics of inflammation in human ACD and found a dramatic upregulation of the number of regenerating nerves at 48 h, which was further increased at 72 h compared to the earlier time points.

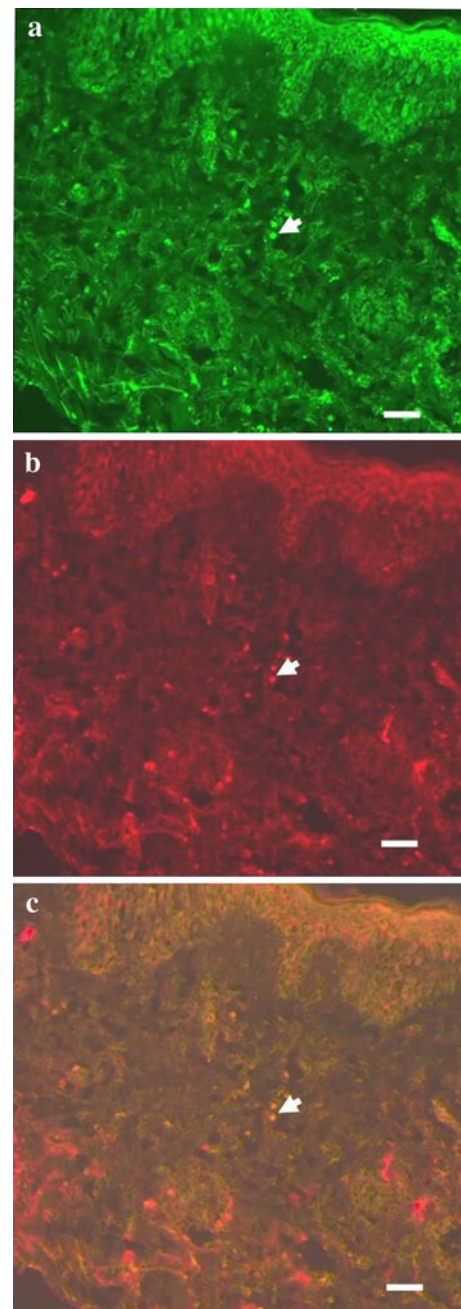


Fig. 8 Double staining micrographs depicting NKA-positive cells (arrow in **a**), appearing green with fluorochrome Cy2, and the same cells shown with red colour (arrow in **b**) expressing tryptase (Alexa Fluor 594). Co-localization (arrow in **c**) seen with the double filter (yellowish-orange colour). Scale bars 20 μ m

Expression of PGP 9.5 on the cutaneous nerves, in both the epidermis and dermis, did not change during the different phases of inflammation, up to 72 h, suggesting a stable release of this protein into the preformed sensory nerves regardless of the kinetics of inflammation. Kinkelin et al. [12] found no difference in the expression of dermal PGP 9.5-ir nerve fibres in the contact eczematous skin compared to control skin. However, the expression of epidermal PGP

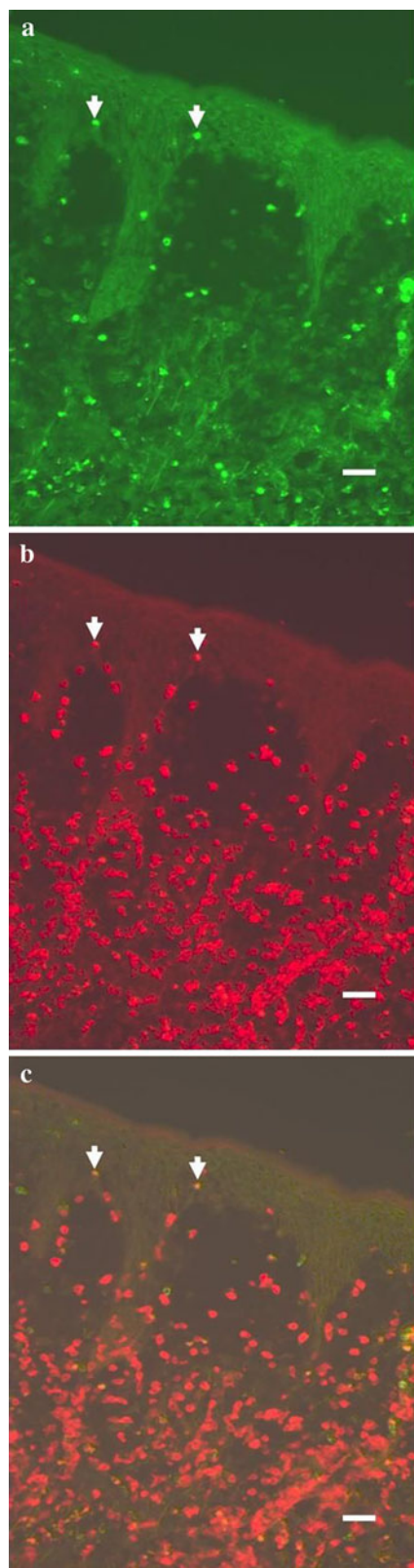


Fig. 9 Co-localization of NK-2R and CD3 is shown. The arrows indicate double stained cells expressing **a** NK-2R (Cy2, green), **b** CD3 (Alexa Fluor 594, red), and **c** NK-2R + CD3 (double filter, yellowish-orange). Note the abundant expression of CD3+ lymphocytes (red) seen in **b** and **c**, whereas not all CD3+ cells express NK-2R. Scale bars 20 μ m

9.5-positive fibres was increased in the contact eczematous skin. The difference in the epidermal PGP 9.5 findings by Kinkelin et al. and our findings could be due to the different methods used to measure the expression of these nerve fibres (object density vs. area fraction).

Several reports have been published on the involvement of sensory neuropeptides in ACD [7, 18, 23]. Thus, the implication of these messengers in established ACD is evident and has earlier been documented. Nevertheless, there is a lack of literature reports investigating the expression of these neuropeptides in humans with regard to the kinetics of a contact allergic reaction. We report here a diminishing number of substance P-, NKA- and CGRP-ir nerve fibres in the contact eczematous skin, which is most evident at the peak of inflammation. This finding contradicts the upregulation of the numbers of regenerating nerves at the later stages of inflammation. A possible explanation of this contradiction could be that the synthesis of these sensory neuropeptides by their neurons in the dorsal root ganglia is accompanied by a slow conducting velocity of the axon terminals in the inflamed skin. Also, a release of these neuropeptides from the nerve endings into the microenvironment to enhance the neurogenic inflammation could have resulted in this diminished expression.

On the other hand, we found an increased number of NKA-positive cells and a tendency towards an increased number of substance P-positive cells in the eczematous skin at the latest stages of the reaction in contrast to non-eczematous skin.

In this study, the numbers of positive cells expressing the NK-1 and NK-2 receptors revealed a time-dependent elevation that gradually increased at the earliest time points of inflammation and reached the maximum at its peak. The above-mentioned findings suggest a non-neuronal involvement of the tachykinins in ACD.

At 24 h, a dramatic raise in the number of NK-1R- and NK-2R-ir cells was noted. This specific phase of the inflammatory response could be pointed out for a possible therapeutic intervention suggesting targeting these tachykinin receptors at an early phase.

ACD is a complex reaction involving different mechanistic responses to noxious stimuli. NKA and NK-2R were shown to have an opposing effect to substance P and NK-1R in murine ACD [18] resulting in a diminished inflammation. Our finding of a higher number of immune cells, such as mast cells, expressing both the ligand NKA and its receptor at the later stages of ACD is in line with the proposed downregulatory function of this neuropeptide. Nevertheless, NK-2R might also exert a dual effect by mediating the pro-inflammatory actions of substance P. In this report, we confirm the expression of NKA on mast cells and NK-2R on lymphocytes, while we have earlier documented, in our previous report [5], the expression of

both substance P and its receptor NK-1R on mast cells and lymphocytes.

Our findings have shown an increase in regenerating nerves, accompanied by a reduction of sensory neuropeptide-positive fibres during the kinetics of an ACD reaction. In addition, we have also suggested a non-neuronal contribution of tachykinins to ACD via the expression of both ligands and receptors on immune cells. We further indicated the specific time points when a dramatic shift in the inflammation is evident. Thus, these findings could suggest the possibility of targeting the neuronal mediators at an early phase of the inflammatory response for therapy of ACD.

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