Contact Dermatitis, 1996, 34: 101–105
Printed in Denmark . All rights reserved

Copyright © Munksgaard 1996
CONTACT DERMATITIS
ISSN 0105-1873

# Long-lasting allergic patch test reactions to nickel sulfate: analysis by nickel quantification and immunocytochemistry

J. Handley, D. Todd, O. Dolan, D. McMaster[1], M. Walsh[2], G. Allen and D. Burrows
Department of Dermatology, Royal Victoria and Belfast City Hospitals, Belfast
[1]Department of Medicine, Queens University, Belfast
[2]Department of Pathology, Royal Victoria Hospital, Belfast, Northern Ireland, UK

We previously showed the median duration of positive patch test reactions to nickel sulfate (5% pet.) was 9 days, and defined as long-lasting (LLAPTR) the 14.3% of reactions that persisted for 17 days or longer. The pathomechanisms of LLAPTR are unclear, but may involve either localized antigen persistence or abnormal downregulation of the cellular immune response. In this study, we compared (a) the nickel concentration and (b) the immunocytochemical nature of the local immune reaction, between biopsies from LLAPTR ($n$=8) and normally resolving allergic patch test reactions (NRAPTR) ($n$=8) to nickel sulfate. The concentration of nickel in LLAPTR (median 0.56 μg/g, range 0.25–3.87 μg/g, mean 0.83 μg/g, 95% CI 0.35–1.31) and NRAPTR (median 0.58 μg/g, range 0.2–1.85 μg/g, mean 0.88 μg/g, 95% CI 0.02–1.74) was similar. Activated T lymphocytes, expressing surface IL-2 receptor, HLA DR, DR alpha 1, DP, DQ, and CD2>CD8>CD4 antigens, were seen throughout the dermis and occasionally infiltrating the suprabasal layer of the epidermis in all biopsies. CD1 and HLA DR, DR alpha 1, DP, and DQ-expressing Langerhans cells were present throughout the epidermis and occasionally seen in the papillary dermis. HLA DR, DR alpha 1, DP, and DQ antigen expression were also seen on the surface of non-dendritic cells in the epidermis (probably either keratinocytes or T lymphocytes) and vascular endothelial cells in the papillary dermis. There were no significant qualitative or quantitative differences in the immunocytochemical nature of the localized immune reaction between LLAPTR and NRAPTR. These findings suggest that the pathomechanism of LLAPTR to nickel sulfate is unlikely to be explained simply on the basis of nickel concentration or the nature of the localized immune reaction at the patch test site.

*Key words:* nickel sulfate; long-lasting patch test reactions; immunological basis. © Munksgaard, 1996.

*Accepted for publication 5 June 1995*

Duration of allergic patch test reactions is not well documented. Kanerva stated that most such reactions resolved within 5–10 days (1). We have shown that duration of positivity varies for different antigens, e.g., median duration of positive reactions to nickel sulfate, fragrance mix, potassium dichromate, cobalt and colophony was 9, 11, 7, 29 and 10.5 days, respectively (2). With regard to nickel sulfate, the duration of patch test positivity showed a bimodal distribution with 2 peaks (median 9 days, range 3–17, and median 30 days, range 21–48). We defined the 14.3% of reactions that remained positive for 17 days or longer as long-lasting allergic patch test reactions (LLAPTR) (2). The pathomechanisms behind LLAPTR are unknown, but may involve localized antigen persistence or defective downregulation of the cell-mediated immune response. Such downregulatory mechanisms are poorly characterized, but may be mediated by suppressor T lymphocytes, anti-idiotypic antibodies, cytokines and HLA class II antigen (Ia)-expressing epidermal keratinocytes (3–5). With regard to the latter, we have previosly demonstrated no difference in keratinocyte expression of HLA DR, DP and DQ antigens, between LLAPTR and NRAPTR, for a variety of different antigens including nickel sulfate (6). In the present study, we aimed to investigate further the pathogenesis of LLAPTR to nickel by comparing (a) the tissue concentration of nickel and (b) the immunocytochemical nature of the localized immune response, between biopsies from LLAPTR and NRAPTR.

## Materials and Methods

Patients were recruited from patch test clinics in the Royal Victoria and Belfast City Hospitals. All were tested with the European standard series and showed a + or greater response (according to standard ICDRG criteria) to nickel sulfate (5% pet.) at either 2 or 3 days. The positive reaction was circled with indelible ink and patients were reviewed 14 or 15 days later (i.e., 17 days after application of patch tests). LLAPTR were defined as those showing + or greater response, and NRAPTR as those reactions that showed no visible response at this time. After informed consent was obtained, all patients had 4 mm punch biopsies taken from the centre of the encircled reaction. Stiefel disposable punches were buried to the hilt, the resultant sample including skin and subcutis removed and divided in half. One half was sent for estimation of tissue nickel concentration, using atomic absorption spectrophotometry, the other half for indirect immunofluorescent staining with a panel of monoclonal antibodies.

### *Tissue nickel concentration estimation by atomic absorption spectrophotometry*

Prior to study commencement, the sensitivity of the atomic absorption spectrophotometry technique was confirmed by analysing 4 mm punch biopsies from exposed skin of the back ($n=4$) and from skin underlying a standard nickel sulfate 5% pet. patch test applied to the upper back 2 days after application) ($n=4$) from volunteers attending the patch test clinic. Nickel was detectable in all specimens underlying patch test (concentration range 1.85–3.67 $\mu$g/g), while negligible concentrations were found in unexposed skin.

All analyses in the study were performed single-blind by one of the authors (DM). Biopsies were dried in aluminium foil for 2 h at 100°C and then weighed into glass tubes. 50 $\mu$l of nitric acid was added to each biopsy and the tubes heated in Driblock with temperatures ramped from 100–130°C. Digestion was continued until virtually all the nitric acid had evaporated, but stopped before charring occurred. 200 $\mu$l of nickel-free water was added to each tube and the nickel content of each solution analysed by atomic absorption spectrophotometry, as previously described (8). All samples were assayed in 1 run, which included 4 digestions prepared from a standard reference material (Orchard leaves, National Bureau of Standards, Washington DC). The certified nickel content of Orchard leaves is 1.3, $\mu$g/g (SD 0.2) and our results on the 4 reference digestions were mean 1.12 $\mu$g/g (SD 0.08). Statistical analysis was by Wilcoxon signed rank test to significance $p<0.05$.

### *Indirect immunofluorescence*

Detailed methodology has been previously described (7). Briefly, tissue was snap frozen liquid nitrogen at −70°C. Serial 5 $\mu$m sections were cut on a cryostat at −20°C, dried and incubated with a panel of monoclonal antibodies. The following antibodies were used: HLA DR, HLA DR alpha 1, HLA-DP, HLA DQ (all Becton-Dickinson), CD2 (T11), CD4 (T4), CD8 (T8), CD1 (T6) and IL2R (all Dako Ltd). The cell specificity for these antibodies in peripheral blood and epithelium is as follows: HLA-DR, HLA-DR alpha, HLA-DP, HLA-DQ (Langerhans cells, monocytes, macrophages, B lymphocytes and activated T lymphocytes), CD2 (pan T lymphocyte), CD4 (T helper/inducer lymphocyte), CC8 (T cytotoxic/suppressor lymphocyte), CD1 (Langerhans cell) and IL2R (activated T lymphocyte). Sections were then incubated with monoclonal fluorescein-labelled antisera, mounted in aqueous medium and viewed by fluorescent microscopy. Analysis was performed single-blind by the same pathologist (M.W.)

Quantitative grading of cell surface antigen expression was by viewing a representative high power field within the epidermis and dermis using the following grading system

0=no fluorescent staining
+=1/2 cells showing fluorescent staining
++=3–10 cells showing fluorescent staining
+++=> 10 cells showing fluorescent staining

per high power field

For the purposes of qualitative analysis, biopsies were reported as positive if staining was graded as + or greater. Statistical analysis was by Fisher exact probability and Mann Whitney $U$-tests to significance $p<0.05$.

## Results

We analysed biopsies from 16 patients with allergic patch test reactions to nickel sulfate, 8 LLAPTR and 8 NRAPTR. The mean age of patients in each group was similar; 34.1 versus 32.2 years respectively, though there were more males in the LLAPTR group (4/8 versus 0/8, $p<0.05$).

Grade of positivity at 2/3 days is shown in Table 1. There was no correlation between grade of positivity and duration of patch test reaction.

Tissue nickel concentrations are depicted graphi-

*Table 1.* Grade of positivity of allergic patch test reactions to nickel sulphate at 2/3 days

| Patient no. | NRAPTR | LLAPTR |
|---|---|---|
| 1 | + | ++ |
| 2 | ++ | ++ |
| 3 | + | + |
| 4 | ++ | ++ |
| 5 | + | ++ |
| 6 | + | + |
| 7 | ++ | + |
| 8 | +++ | + |

Grades 0–+++ according to ICDRG criteria

cally in Fig. 1. The tissue concentration of nickel did not differ significantly between biopsies from LLAPTR (mean 0.88 μg/g, 95% CI 0.02–1.74) and NRAPTR (mean 0.83 μg/g, 95% CI 0.35–1.31).

Indirect immunofluorescence staining showed the following features in all biopsies: (a) CD1 surface staining on Langerhans cells distributed evenly throughout the epidermis (93.8% of biopsies) and scattered sparsely in the papillary dermis (68.8% of biopsies); (b) CD4, CD2, CD8 and IL2R-expressing T lymphocytes throughout the dermis (12.5%, 43.8%, 37.5%, and 18.8% of biopsies), infiltrating occasionally into the suprabasal layer of the epidermis (18.8%, 18.8%, 12.5% of biopsies);(c) HLADP, DQ, DR, DR alpha 1 expression on the surface of Langerhans cells (62.5%, 43.8%, 100%, and 100% of biopsies) distributed evenly throughout the epidermis and on the surface of T lymphocytes infiltrating throughout the dermis (56.3%, 56.3%, 93.8 and 100% of biopsies); and (d) HLA class II antigens (either DP, DQ, DR, or DR alpha 1) were seen occasionally on non-dendritic cells in the epidermis, probably either keratinocytes or T lymphocytes (62.5% of biopsies), dermal capillary endothelial cells (31.3% of biopsies) and dermal dendritic cells, probably Langerhans cells or tissue macrophages (31.3% of biopsies).

Table 2 summarizes the qualitative, and Table 3 the quantitative, comparisons in immunocytochemical staining between LLAPTR and NRAPTR. Although CD2 and HLADP were detected on dermal T lymphocytes more frequently in LLAPTR (62.5 versus 25% and 75 versus 37.5% of biopsies, respectively), and a higher proportion of dermal T lymphocytes expressed CD2 on their surface in LLAPTR (median grade of staining 2+ versus 0), these differences were not statistically significant. Otherwise, there were no significant qualitative or quantitative differences in the proportions of (a) CD4, CD8, CD2, IL2R-expressing lymphocytes, (b) Langerhans cells or (c) class II HLA antigen-expressing T lymphocytes, Langerhans cells, keratinocytes and capillary endothelial cells, in either the epidermis or dermis between LLAPTR and NRAPTR.

## Discussion

These findings show evidence of a localized cell-mediated immune reaction at the site of positive patch test reactions to nickel sulfate, consistent with previous studies (1, 6). In summary, we showed activated HLA class II antigen-expressing CD2, CD4,

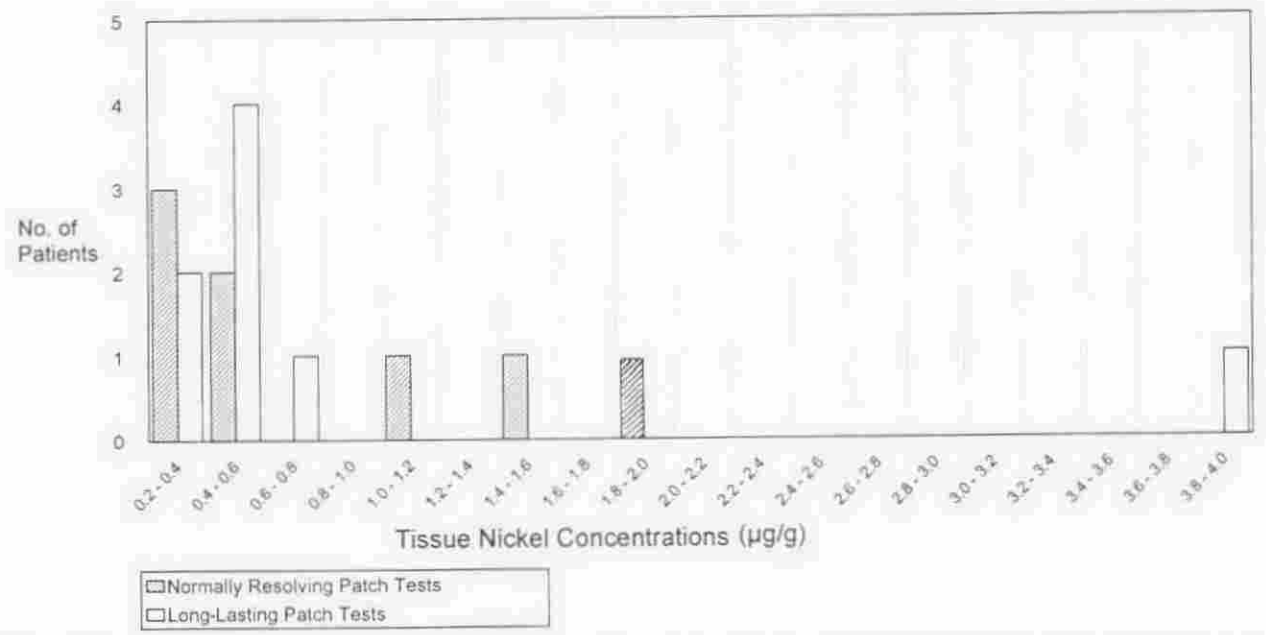


*Fig. 1.* Tissue nickel concentrations in long-lasting and normally resolving allergic patch test reactions to nickel sulfate (5% pet.).

*Table 2A.* Qualitative comparative in epidermal immunofluorescent staining between LLAPTR and NRAPTR

| | No of biopsies showing positive staining (or more) | |
|---|---|---|
| Antigen | LLAPTR | NRAPTR |
| (a) Langerhans cells (dendritic) | | |
| HLA DP | 6 | 4 |
| HLA DQ | 3 | 4 |
| HLA DR | 8 | 8 |
| HLA DR alpha 1 | 8 | 8 |
| CD1 | 8 | 7 |
| (b) T lymphocytes/keratinocytes (non dendritic cells) | | |
| All HLA class II antigens | 5 | 5 |
| CD4 | 0 | 3 |
| T8 | 0 | 3 |
| T11 | 0 | 3 |
| IL2R | 0 | 2 |
| No. of biopsies | 8 | 8 |

*Table 2B.* Qualitative comparison in dermal immunofluorescent staining between LLAPTR and NRAPTR

| | No of biopsies showing positive staining (+or more) | |
|---|---|---|
| Antigen | LLAPTR | NRAPTR |
| (a) dendritic cells (langerhans cells, macrophages) | | |
| All HLA antigens | 3 | 2 |
| CD1 | 7 | 4 |
| (b) T lymphocytes | | |
| HLADP | 6 | 3 |
| HLADQ | 5 | 4 |
| HLADR | 8 | 7 |
| HLADR alpha 1 | 8 | 8 |
| CD4 | 1 | 1 |
| CD8 | 4 | 2 |
| CD2 | 5 | 2 |
| IL2R | 1 | 2 |
| (c) capillary endothelial cells | | |
| All HLA antigens | 2 | 3 |
| No. of biopsies | 8 | 8 |

*Table 3A.* Quantitative comparison in epidermal immunofluorescent staining between LLAPTR and NRAPTR

| | Median grade of biopsy staining (0–3+) | |
|---|---|---|
| Antigen | LLAPTR | NRAPTR |
| (a) Langerhans cells (dendritic) | | |
| HLADP | 1+ | 1+ |
| HLADQ | 0 | 0 |
| HLADR | 1+ | 1+ |
| HLADR alpha 1 | 1+ | 1+ |
| CD1 | 2+ | 2+ |
| (b) T lymphocytes/keratinocytes (non dendritic) | | |
| all HLA antigens | 0 | 0 |
| CD4 | 0 | 0 |
| CD8 | 0 | 0 |
| CD2 | 0 | 0 |
| IL2R | 0 | 0 |
| No. of biopsies | 8 | 8 |

*Table 3B.* Qualitative comparison in dermal immunofluorescent staining between LLAPTR and NRAPTR

| | Median grade of biopsy staining (0–3+) | |
|---|---|---|
| Antigen | LLAPTR | NRAPTR |
| (a) dendritic cells (langerhans cells, macrophage) | | |
| all HLA antigens | 0 | 0 |
| CD1 | 1+ | 1+ |
| (b) T lymphocytes | | |
| HLADP | 2+ | 0 |
| HLADQ | 1+ | 1+ |
| HLADR | 2+ | 2+ |
| HLADR alpha 1 | 2+ | 2+ |
| CD4 | 0 | 0 |
| CD8 | 1+ | 0 |
| CD2 | 1+ | 0 |
| IL2R | 0 | 0 |
| (c) capillary endothelial cells | | |
| all HLA antigens | 1+ | 1+ |
| No. of biopsies | 8 | 8 |

CD8 and IL2R-positive lymphocytes scattered throughout the dermis and infiltrating into the suprabasal layers of the epidermis, and Langerhans cells evenly distributed throughout the epidermis and infiltrating into the papillary dermis. HLA class II antigen expression was also seen on scattered keratinocytes and capillary endothelial cells. Although lymphocytes within the dermis expressed HLADP and CD2 more frequently in LLAPTR than NRAPTR, these differences were not statistically significant. Overall, no qualitative or quantitative differences in nickel concentration were found between LLAPTR and NRAPTR.

These results confirm the findings of a previous immunocytochemical study, in which we reported no differences in keratinocyte HLA class II antigen expression between LLAPTR and NRAPTR to various patch test allergens including nickel sulfate (6). Both these studies have a number of limitations, namely: (a) their cross-sectional nature; specifically, analyses in this study were at day 17 after patch test applications (primarily with a view to detection of differences in nickel concentration), whereas analysis at an earlier stage, e.g., 2 or 3 days, may have a higher chance of detecting differences in the nature of local immune infiltrates (the downside of this may be that biopsy at this time may interfere with the natural course of duration of the patch test reaction); (b) small patient numbers, hence low study power and relatively high chance of type 1 statistical error; (c) cut point qualitative analysis and relative insensitivity of the grading system used in quantitative immunocytochemical analysis meant that subtle differences in

the nature of the immune reaction between LLAPTR and NRAPTR could have been missed.

In addition, atomic absorption spectrophotometry used in the present study measures only overall tissue nickel concentration, and thus any localized concentration differences of nickel around hair follicles, sweat or sebaceous glands, which may possibly have accounted for persistent patch test reactions, would not have been detected.

Accepting their need for confirmation, our findings suggest that LLAPTR to nickel are unlikely to be explained simply on the basis of overall tissue nickel concentration or localized histological and immunocytochemical features at the patch test site, although the non-significant increase in CD2 and HLADP-expressing lymphocytes in the dermis of LLAPTR found in this study warrants further investigation.

There are a number of other possibilities that may account for persistent patch test reactions. Patients with LLAPTR may be intrinsically more allergic to nickel and mount stronger patch test reactions, which, as part of a normal physiological spectrum, take longer to resolve. In our opinion, this is unlikely as: (a) no correlation between initial grade of patch test response at 2/3 days and duration was seen in this or our previous studies (2) (6); and (b) duration of nickel patch test reactions shows a bimodal and not normal distribution, with peaks at 9 and 30 days, suggesting a separate pathomechanism for long-lasting reactions. Alternatively, localized pockets of nickel, possibly within hair follicles, sebaceous and sweat glands, phagocytes, keratinocytes, and extracellularly within both the epidermis and dermis, may act as a persistent source of immune activation in LLAPTR. The present study may well have failed to detect any such localized concentration variations between LLAPTR and NRAPTR, providing the overall tissue nickel concentration was unaffected. As standard histochemical staining is too insensitive to detect the very low concentrations of nickel found in patch test reactions, other techniques that preserve tissue architecture, such as electron and immunoelectron microscopy, would be required to address this issue further. Nickel exposure at sites distant from the patch test site, for example jewellery or foodstuffs, may also have played some role in persistence of patch test reactions, a factor not addressed in this study.

It is also possible that patients with LLAPTR may have subtle defects in downregulation of their cellular immune system. Such downregulatory mechanisms are not well characterized, but may involve abrogation of the immune response by suppressor T lymphocytes, HLA class II expressing keratinocytes, cytokines, and anti-idiotypic antibodies, which inhibit binding of T cell receptors with HLA class II antigen-restricted hapten on antigen-presenting cells. The results of this and our previous study (6) provide no evidence that lack of either suppressor T lymphocytes or HLA class II-expressing keratinocytes are involved in LLAPTR, although they do not exclude functional defects of these cells. The possible rôle of cytokines in LLAPTR, such as IFN gamma and markers of endothelial activation that may contribute to erythema, such as ICAM-1, VCAM and E-selectin, requires further investigation. An alternative investigative approach might be to isolate T cells from long-lasting and normally resolving patch test sites and investigate their in vitro cytokine profiles.

In summary, the results of this study provide no explanation of the pathomechanisms behind LLAPTR to nickel sulfate. Elucidation of the aetiology of persistent patch test reactions is important, as it may further our understanding of the delayed-type hypersensitivity response and possibly have therapeutic implications.

## References


1. Kanerva L, Estlander T, Jolanki R. Immunohistochemistry of lymphocytes and langerhans cells in long lasting allergic patch tests. *Acta Dermato-venereologica* 1988; *68:* 116–122.
2. Alderdice D, Todd D, Gore H, Burrows D. An investigation to determine the duration of allergic patch test reactions. In: *Proceedings of 1st Congress of the European Society of Contact Dermatitis*, Brussels, 1992.
3. Gaspari AA, Jenkins MC, Katz SI. Class II MHC bearing keratinocytes induce antigen specific unresponsiveness in hapten specific TH1 clones. *J Immunol* 1988; *141:* 2216–2220.
4. De Panfilis G, Manara G C, Ferrari C, Torresani C, Rowden G. Subjects of keratinocytes and langerhans cells express epitopes associated with suppressor-inducer capabilities in resting human epidermis. *Immunol* 1990; *69:* 622–625.
5. Belsito D V. The immunological basis of patch testing. *J Amer Acad Dermatol* 1989; *21:* 822–829.
6. Todd D J, Handley J, Walsh M, Dolan O, Burrows D. Keratinocyte expression of class II MHC antigens in long-lasting allergic patch test reactions. *Contact Derm* 1992; *26:* 22–26.
7. Handley J M, Maw R D, Horner T, Lawther H, Walsh M, Dinsmore W W. A placebo controlled observer blind immunocytochemical and histologic study of epithelium adjacent to anogenital warts in patients treated with systemic interferon alpha in combination with cryotherapy versus cryotherapy alone. *Genitourin Med* 1992; *68:* 160–165.
8. McMaster D, Burrows D, Eedy D J, Love A H G. Stimulation of urinary copper excretion by trientine in nickel sensitive patients. *Biol Trace Element Res* 1987; *14:* 65–77.



Address:

*J. Handley*
*Department of Dermatology*
*Ulster Hospital*
*Dundonald, Belfast*
*N. Ireland*
*UK*

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.