

## Pathogenesis of Orally Induced Flare-up Reactions at Old Patch Sites in Nickel Allergy

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The flare-up reaction of old patch test sites following oral intake of antigen have been shown to be site and antigen specific reactions. Using enzyme and immunohistochemistry, the morphology of 6- to 8-week-old patch test sites, before and after oral challenge with nickel, was investigated. Before oral challenge, small scattered perivascular cell infiltrates consisting of macrophages, mast cells, T-lymphocytes, and OKT6<sup>+</sup> dendritic cells were found in old patch test sites. In early and strong flare-up reactions combined with systemic toxicoderma-like reactions, polymorphonuclear leukocytes and lysed granular fragments from these cells were prominent. In slower flare-up reactions with little systemic involvement, T-lymphocytes predominated. We conclude that cells, probably macrophages, which are able to secrete inflammatory mediators promoting chemotaxis for polymorphonuclear leukocytes and/or T-lymphocyte proliferation, may play an important role in initiation of the flare-up reaction. *Key words: Contact dermatitis; Flare-up reactions; Nickel; Micromorphology.* (Received May 22, 1984.)

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Following systemic exposure to a specific or cross-reacting hapten in hypersensitive patients, flare-up reactions may occur in previously positive patch test and contact dermatitis sites together with maculo-papular toxicoderma-like rash and an increased number of palmar vesicles (1). The flare-up reactions in 4- to 6-week-old patch test sites after ingestion of nickel have been shown to be both site and antigen specific reactions (2). Clinical and histopathological investigation 24 hours after ingestion of allergen demonstrated a reaction comparable to acute eczema (2). The morphology and pathogenesis of similar flare-up reactions have been investigated in guinea pigs (3, 4). Intravenous injection of potassium dichromate in sensitized animals showed polymorphonuclear leukocytes to be the dominant cells in the Arthus-like reaction (3). In another study, 2,4-dinitrobenzenesulfonic acid sodium salt (DNBSO<sub>3</sub>NA) was injected intravenously into 2:4 dinitro-1-chlorobenzene (DNCB) hypersensitive animals; the histopathology was mostly eczema-like (4). We undertook the following study to examine the morphologic changes of flare-up reactions in old patch test sites in humans to try to better understand the cells participating in the reaction.

### MATERIALS AND METHODS

#### *Subjects*

Five volunteers (mean age 42, range 29-70, 4 females, 1 male) hypersensitive to nickel, enrolled after giving informed consent. None had a history of hand eczema.

### Patch testing

The subjects were patch tested (12 mm Finn chambers) with equal amounts of 2.5% nickel sulphate (NISO<sub>4</sub>) in petrolatum on four sites on the outer part of the upper arm (3 subjects), or buttock (2 subjects). After 48 hours, the subjects removed the patches and 24 hours later, the test reactions were read and scored (erythema + infiltration and papules (+++), erythema + strong infiltration and vesicles (++++)). At this time, the margin of the reactions was marked with an indelible pen. The subjects kept this marking visible throughout the study. The markings were rechecked after 3–4 weeks. No application of topical preparations of any kind, or overt sunlight exposure to the test areas was allowed during the study.

### Oral provocation and punch biopsy

Six to eight weeks after primary patch testing, the subjects swallowed a capsule containing 25 mg nickel sulphate (5.6 mg Ni<sup>+</sup>). At the same time, a 4 mm punch biopsy was taken from the center of an earlier test site and from adjacent normal skin. Six, twelve, and twenty-four hours later, the clinical reactions were recorded and a new 4 mm punch biopsy taken from a test site if a clinical reaction was present. At 24 hours, a 4 mm punch biopsy was taken from adjacent normal skin. All biopsies were taken under local anesthesia with 1% lidocaine.

### Histology technique

The skin specimens were bisected. One part was immediately frozen in liquid nitrogen (LN<sub>2</sub>), stored in LN<sub>2</sub> until embedded in OCT compound (Lab-Tek Products) and cryosectioned. Five µm thick sections were picked up on alcohol-cleaned and formol/gelatin coated glass slides and immediately fixed in cold acetone. After air-drying, the slides were placed in an air tight box at -70°C. Additional sections, 10 µm thick, were cut to identify Langerhans' cells (LC). One 5 µm thick section was fixed by dipping in ether/alcohol (1:1), air-dried, and stained with hematoxylin and eosin.

The other half of the 4 mm punch biopsy was fixed in phosphate buffered (pH 7.4) 3% paraformaldehyde at 4°C for 12–14 h, washed for 1 h with 0.1 M phosphate buffer containing 3% sucrose (4°C), and post-fixed in 2% 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Sigma) in 0.1 M phosphate buffer (4°C). The tissue was then washed in buffer, dehydrated in acetone, and embedded in glycol methacrylate as previously described (5). Thin sections (2–3 µm) were cut and stained with hematoxylin and eosin, and Azure II. Additional sections were tested for peroxidase (perox), α-naphthyl acetate esterase (ANAE), and naphthyl AS-D chloroacetate esterase (CAE) as previously described (5).

### Immunohistochemical staining procedures of frozen sections

We performed immunohistochemistry with mouse monoclonal antibodies which identify T-lymphocyte subsets (T11, T4, T8), B-lymphocytes (B1), HLA-DR antigen (I2) (Coulter Immunology, Florida) and Langerhans' cells (OKT6) (Ortho Diagnostic, Systems, New Jersey). *In situ* binding of these antibodies was labeled by a modification of the avidin-biotin peroxidase complex method described by Hsu et al. (6). Preliminary experiments with different dilutions of each primary antibody in Tris-HCl/saline buffer resulted in the following optimal dilutions: T11, 1:40; T4, 1:20; T8, 1:20; I2, 1:160; OKT6, 1:40. Appropriate dilution of normal ascites fluid (Bethesda Research Laboratories, Gaithersburg, MD) incubated instead of primary antibody served as control for each of the antibodies used.

Subsets of T lymphocytes were identified by their partial or total brown-colored rim staining. B lymphocytes and I2+ cells were identified by rim staining and diffuse granular cytoplasmic stain. Intraepidermal OKT6+ cells with a clear nuclear space and at least 2 dendrites were identified in 1.5–2 mm of epithelium at 400× magnification and expressed as LC/mm (10 µm thick sections). Statistical differences were calculated by paired *t*-tests.

## RESULTS

### Clinical reaction

The primary patch test reading for all 5 subjects showed reactions of at least grade (+++). In each subject all 4 sites were of the same intensity. At the time of oral challenge, all test sites had returned to normal with the exception of one patient where a slight post-inflammatory hyperpigmentation without infiltration was visible 6 weeks after primary patch testing. Three of the five subjects reacted to orally administered nickel with more or

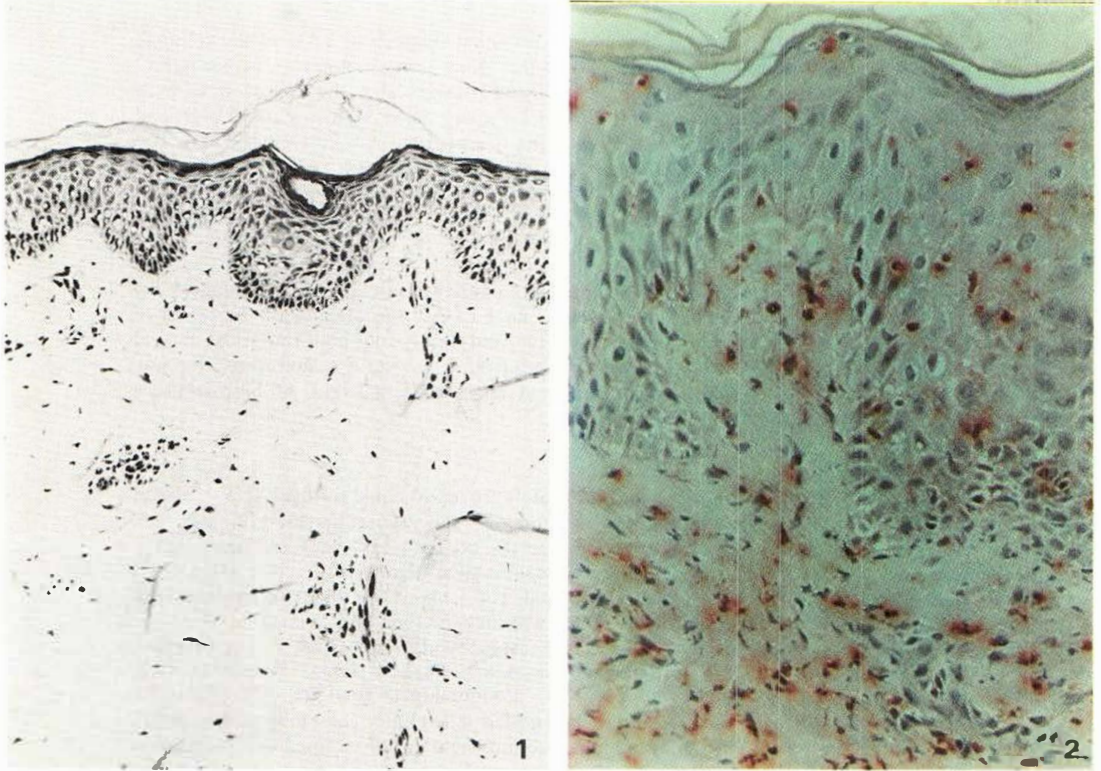


Fig. 1. Increased number of small perivascular dermal cell infiltrates in a 6-week-old positive patch test site (HE,  $\times 100$ ).

Fig. 2. CAE reaction showing positive polymorphonuclear leukocytes and granular fragments intra- and epidermally 6 hours after oral challenge with nickel ( $\times 250$ ).

less pronounced itching, erythematous maculopapular-urticarial-like lesions and flare-up with erythema and infiltration at old test sites. Two of these subjects showed strong and rapid (4–12 h) flare-up of old test sites and a more or less generalized toxicodermat-urticarial-like reaction; the third subject had a weaker and later (12–24 h) onset of flare-up at the old test sites and a less active systemic reaction. Two subjects (female 70, male 30) showed no clinical reactions; punch biopsies were only taken from an old test site and adjacent normal skin just before ingestion of nickel.

#### *Histopathological and enzyme histochemical examination*

Punch biopsies from old test sites taken before oral challenge showed an increased number of scattered small dermal perivascular mononuclear cell infiltrates in all 5 subjects when compared to normal skin (Fig. 1). The infiltrates consisted of lymphocytes (see immunohistochemistry), a modest number of mast cells (CAE and toluidene blue positive), and dermal macrophages (ANAE+); only rare neutrophils (perox. +, CAE+) were seen in the small vascular lumens. The histopathological findings in normal skin 24 h after challenge were similar to the findings in normal skin before challenge.

In the specimens taken 6 h after challenge, a prominent perivascular inflammatory infiltrate and mild edema were observed in the 2 strongly reacting subjects. Besides





Fig. 3. Dermal foci of mainly T-lymphocytes (T-11) 12 hours after challenge with nickel ( $\times 250$ ).

mononuclear cells, the infiltrates contained moderate numbers of neutrophils. Surprisingly, the enzyme histochemical reactions (CAE and perox.) demonstrated a strikingly positive perivascular stain not localized within cells, and many positive granules between bundles of dermal collagen, which we interpreted as residual granules from large numbers of lysed neutrophils. There were modest number of mast cells without demonstrable degranulation in all 3 subjects.

In the specimens taken 12 h after challenge, a pronounced perivascular infiltrate was observed in all 3 reactive subjects. In the 2 strong reacting subjects, the CAE and perox-reaction positivity had extended further into the dermis; in one case, even strong exocytosis into the epidermis was found. Increased numbers of neutrophils could be identified in the stained areas, but the majority of the staining was in granules apparently from lysed cells (Fig. 2). A more monomorphic perivascular infiltrate consisting mainly of T lymphocytes (immunohistochemistry) and without neutrophils was observed in the third subject (Fig. 3). In addition, the perivascular infiltrate contained appreciable numbers of ANAE+ monocytes (up to 20% of the mononuclear cells) in all 3 subjects. Mast cells appeared to be somewhat increased in numbers, but did not show degranulation. Specimens taken 24 h after challenge showed the same type and degree of histopathological findings as at 12 h. Epidermal changes such as spongiosis-vesiculation were absent in all biopsies.

#### *Immunohistochemical findings*

The mononuclear infiltrates observed in old test sites before challenge consisted of 60–70% T cells (T11 positive). Of these cells, approximately 50–60% were helper T cells (T4 positive) and 5–10% were suppressor/cytotoxic T cells (T8 positive). Similar infiltrates were found in very small foci in normal skin both before and after oral challenge except that T8 staining was demonstrated in normal skin only after challenge.

Six hours after challenge, a diffuse, granular, brown stain, probably representing endogenous peroxidase, was seen in areas with positive CAE and perox reactions in the 2 early and strongly reacting subjects. In some areas, no T cells were observed, whereas in others with less diffuse staining, T cells were found in the same relation as in old test sites. Twelve hours after challenge, T cell staining was clearly defined, even in foci with

Table I. Number of epidermal LC in old patch test site and normal skin before and after oral challenge

Subjects	Old patch test site before challenge	Old patch test site 6 h after challenge	Old patch test site 12 h after challenge	Old patch test site 24 h after challenge	Normal skin before challenge	Normal skin 24 h after challenge
1. (29 ♀)	34	17	18	—	7	11
2. (42 ♀)	13	9	20	20	5	12
3. (50 ♀)	17	No biopsy	11	10	0	4
4. (30 ♀)	27	No biopsy	No biopsy	No biopsy	17	No biopsy
5. (70 ♀)	28	No biopsy	No biopsy	No biopsy	10	No biopsy
Mean ± SD	23.8±8.6	13.0±5.2	16.3±4.7	15.0±7.0	7.8±6.3	9.0±4.4

endogenous peroxidase. In 2 cases, the T cells were found to have the same phenotypic subset relationship as in old test sites. In the third subject, 75% T11+, 60–65% T4+, and 5–10% T8+ were observed. Compared to earlier specimens, there were no significant changes observed in the specimens taken 24 h after challenge. The I2 antibody (identified on HLA-DR, Ia-like antigen) presented a weak, diffuse granular stain on scattered cells, and cells in perivascular foci. I2+ dendritic cells were seen in papillary dermis at 1:160 antibody dilution, but dendritic cells in epidermis could not be identified at dilution higher than 1:40. These findings were observed both in pre- and postchallenge biopsies.

The B1 antibody did not result in positive staining in any of the specimens from all 5 subjects.

The result of the intraepidermal LC counting (OKT6 positive) is illustrated in Table I. A significant difference was found between old patch test sites and normal skin ( $p=0.006$ ). No significance was found between old patch test sites before and after oral challenge or between flare-up reaction sites at different intervals after challenge.

OKT6+ dendritic cells were observed rather frequently in perivascular infiltrates and scattered in papillary dermis in old patch test sites independent of the flare-up reaction. Normal skin specimens rarely contained OKT6+ dendritic cells in the dermis.

## DISCUSSION

Although the flare-up reactions of old patch test in nickel hypersensitivity sites are variable, these reactions appear to be specific immunological reactions (2). The present study was designed to investigate the mechanisms involved in flare-up reactions of old patch test sites and to provide insight into cutaneous inflammatory reactions. In this study, 3/5 subjects with nickel hypersensitivity showed flare-up reactions when challenged orally with 5.6 mg nickel. The range in reaction pattern was remarkable, from no reaction at all to positive flare-up reactions with slight itching and erythema of old patch test sites in some subjects to a generalized maculo-papular, sometimes urticarial, toxicoderma-like reactions in others. Obviously, some cell or substance is left resting in the skin at the site of previous nickel contact for several weeks as indicated by a positive flare-up reaction of 6- to 8-week-old patch test sites following oral challenge with a specific allergen. Presumably the antigen (nickel) reaches the dermis, reacts with this sensitized cell or antigen-specific substance, and induces the infiltration of the inflammatory cells causing the reaction. The nature of the memory component and the pathogenesis of the resulting inflammatory reaction is unknown.

From a theoretical viewpoint, several possibilities exist. First, it is well-established that

macrophages and LC possess antigen processing/presenting abilities (7–11); either macrophages or LC could present the antigen to locally resting, specifically sensitized lymphocytes, inducing release of inflammatory mediators. Another possibility is that inflammatory mediators are released by macrophages or LC directly after activation by the antigen. Next, T lymphocytes and/or mast cells may express specific receptors for the antigen and release their mediators directly, without an initial antigen presentation by macrophages or LC. Finally, local production of antibodies could take place at old patch test sites and formation of antigen-antibody complexes could be the initial triggering factor.

The type and sequence of inflammatory cell infiltration in flare-up reactions may give some information about which of these triggering mechanisms takes place. Six hours after oral challenge, an increased number of polymorphonuclear leukocytes and lysed fragments of these cells were apparent in the two strongly reacting subjects. This suggests a response to polymorphonuclear leukocyte chemotactic mediators. Macrophage-derived Interleukin 1, probably identical to epidermal cell thymocyte-activating factor (ETAF), is chemotactic for polymorphonuclear leukocytes (12). Therefore, antigen activation of specifically primed macrophages and release of Interleukin 1 is a possible explanation. The fact that the polymorphonuclear leukocytes invaded the epidermis in one of the strongly reacting subjects indicates that the secretion of ETAF by keratinocytes may also take place. Also, Interleukin 1-ETAF can activate T cells in the presence of antigen; these activated T cells can then produce a second factor, Interleukin 2, which in turn causes T lymphocyte proliferation (12). This mechanism could explain the histopathological reaction observed in the third reacting subject where no polymorphonuclear leukocytes, but T lymphocytes were found.

Mast cells also release neutrophil chemotactic factors (13), suggesting that this cell could play a role. While a modest number of mast cells were observed in all three reacting subjects, no degranulation was observed, suggesting that mast cells probably do not play an essential role.

The absence of plasma cells or B lymphocytes in all specimens together with the lack of immunoglobulin or complement deposits, examined in an earlier study (2), probably rule out local antibody production.

The question of why some individuals react with flare-up reactions and others do not respond to oral challenge remains unresolved. Increased amounts of small perivascular mononuclear cell infiltrates containing the same cell types, as in the reacting subjects, were also found in the two non-reacting subjects. Nickel is largely absorbed within 2.5 h after oral intake (14, 15), which means the antigen should reach the old patch test site. We can only guess that the capacity to process and/or present the antigen to target cells and generation of essential inflammatory mediators are not turned on in some individuals or, that a higher concentration of antigen is necessary.

In the specimens taken 12 and 24 h after challenge, an increased number of ANAE+, dermal macrophages and T lymphocytes, mainly T helper-cells, were found in all 3 reacting subjects. Estimated by the relatively late arrival of ANAE+ cells into the reaction, it is reasonable to believe that these cells serve primarily as phagocytes. Unfortunately, our methods do not allow us to distinguish between subtypes of macrophage seen in old patch test sites before and after challenge, respectively. Staining with different monoclonal antibodies might reveal macrophage subsets.

The T lymphocytes might be attracted by inflammatory mediators released at the same time as the polymorphonuclear leukocyte chemotactic factors and their role could be moderating or healing. Suppressor-cytotoxic cells were seen in the same low proportion in old patch test sites before and after oral challenge. These cells are also observed in acute allergic patch test reactions in about the same frequency (unpublished observation). They



seem to stay in the old test area for at least 6–8 weeks. However, in the present study they do not seem to be able to suppress a flare-up reaction. If suppressor/cytotoxic cells perform any moderating or suppressing effect on the flare-up reaction is unclear; it seems unlikely that these few cells could be responsible for the absence of flare-up seen in two of the subjects. Suppressor/cytotoxic cells are found sparsely in normal skin after, but not before, oral challenge, which might indicate that they are also activated by oral antigen intake.

The number of epidermal LC was significantly higher in old patch test sites than in normal skin; otherwise no significant difference was found in the number of epidermal LC in the investigated reactions. Since this increased number of LC is a persistent result of previous patch testing, and not a result of positive flare-up reaction, it is questionable if epidermal LC take active part or possess specific antigenic *in situ* memory for the proper reaction.

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