

Lactoferrin, myeloperoxidase, lysozyme and eosinophil cationic protein in exudate in delayed type hypersensitivity

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A skin window technique was used to study the morphology of leucocytes in upper dermis and exudate during nickel challenge in patients with contact allergy to nickel. Contact allergic patients and healthy volunteers tested with a skin window without addition of nickel to the chamber medium served as controls. The morphology of the leucocytes in dermis was studied in biopsies taken 8, 24, or 48 h after skin window application, and in a parallel test the morphology of the exudate was examined by sequential collection of the chamber medium during a 48 h period. The infiltrate in dermis of contact allergic patients with nickel challenge in the chamber medium showed a time-dependent increase of mononuclear cells, eosinophils and basophils and a concomitant decrease of polymorphonuclear granulocytes, characteristic of a combined specific and unspecific inflammation. The morphology of the exudate in contact allergic patients exposed to nickel showed a dominance of polymorphonuclear granulocytes throughout the study period, while mononuclear cells, eosinophils and basophils were detected at a much lower quantity and with a considerable delay. Further, we studied the kinetics of the leucocyte granule proteins: lactoferrin, myeloperoxidase, lysozyme and eosinophil cationic protein in exudate fluid in a parallel test. A significant higher flux was found for all during the second day of allergen exposure compared to contact allergic patients without allergen challenge as well as normal volunteers. The increased protein fluxes were not accompanied by an increased flux of polymorphonuclear granulocytes in the exudate. An unexpected difference in the kinetic profiles between the secondary granule proteins lactoferrin and lysozyme was observed in patients with and without nickel challenge as well as in controls, indicating a difference in diffusion or degradation of the two proteins before entering the chambers.

Key words: cellular morphology; eosinophil cationic protein; lactoferrin; lysozyme; myeloperoxidase; nickel allergy.

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Allergic contact dermatitis is a delayed type hypersensitivity (DTH) reaction (24) elicited by epidermis penetration of an antigen in sensitized individuals. According to studies on contact sensitivity in mice (10) the effector phase of DTH consists of a cascade of steps that are dependent on the sequential action of two types of antigen specific Ly-1⁺ T lymphocytes. The first of these steps occurs within 2 h of challenge and is dependent upon antigen-binding, anti-

gen-specific T cell factors that sensitize the tissues for a vasoactive step. This allows an interaction between antigen presenting HLA-DR cells (monocytes and Langerhans cells) and Ly-1⁺ effector T-cells of classic 24 to 48 h DTH responses to enter the tissues and produce chemoattractant lymphokines (19). Evidence indicates that dermal and epidermal infiltrate can depend on chemotactic factors (8, 15, 18, 30), some of which can attract granulocytes

found in the early infiltrate (9). Chemotactic activated granulocytes can then degranulate (3, 4, 29).

The purpose of the present study was to examine the cellular infiltrate in dermis and exudate in humans during the development of contact sensitivity to nickel. For these observations we used an improved skin chamber technique with nickel added to the chamber medium, which allows us to study the morphology of the exudate in skin windows. In a parallel test the skin window chambers were removed at fixed intervals and biopsies were taken for the study of the cellular infiltrate in upper dermis. Furthermore, the kinetics of the flux of polymorphonuclear granulocytes (PMN) and four granule proteins have been measured in exudate during the development of contact sensitivity to nickel to evaluate the effector phase of granulocyte activation.

PATIENT AND METHODS

The control group consisted of five healthy controls (all females) mean age 28 (age range 23–32) with no eczema or history of allergic contact dermatitis. Patch tests for nickel sulphate, potassium dichromate and cobalt chloride were negative. The patients studied (8 females, 2 males, mean age 37 years, age range 20–65) all had shown allergic contact dermatitis to nickel which was clinically verified by the history and patch tests. Patch testing was performed according to recommendations of the International Contact Dermatitis Group with 5.0% nickel sulphate in petrolatum. In all cases the nickel patch tests were strongly positive (erythema, oedema and vesicles). At the time of investigation the eczema was inactive or mildly active. In none of the patients was the forearm involved and the eczema never exceeded 5.0% of total skin surface. Neither of the patient groups were undergoing medical treatment at the time of the investigation and systemic glucocorticoids had not been administered for at least 6 months and no other anti-inflammatory drugs (including topical glucocorticoids) had been used for at least 3 days. One patient had psoriasis and one

patient suffered from seborrheic dermatitis and multiple sclerosis. None of the patients had any signs or history of atopic disease. All patients had normal blood leucocyte and differential counts prior to investigation.

Skin chamber technique

A dermabrasion was made on the inner surface of both forearms of patients with nickel hypersensitivity and on the right forearm of controls, as previously described (13, 14). In brief, an epidermal abrasion was performed with a surgical scalpel to visualize the network of the corium without causing any bleeding. The denuded area varied in size between 1.5 and 6 cm². A sterile chamber of epoxy material was sealed over the skin window with glue (Ducocement Dupont de Nemours SA., Belgium).

Chamber media and collection procedure

The chamber medium was autologous serum obtained 24 h prior to the investigation under strictly sterile conditions and left at 4°C. The chamber applied to the right forearm of the eczema patients and controls was filled with 0.5 ml autologous serum containing 10 mM EDTA immediately after the application and after each collection. The chamber medium used in the chamber applied to the left arm of the nickel allergic patients consisted of 0.5 ml autologous serum with 10 mM EDTA and 10 mM nickel sulphate. The collection of chamber medium was routinely performed at 3, 6, 9, 12, 17, 22, 24, 27, 30, 33, 36, 39, 41, 46 and 48 h after abrasion. The leucocytes were harvested from the chamber, resuspended and counted in a modified Neubaur haemocytometer. Smears of the collected cells were stained (May-Grünwald-Giemsa) and differential count performed by counting 500 cells (Table 1). The migration of polymorphonuclear cells (PMN) was expressed as the mean of the logarithm to the total numbers of PMN related to the skin window area, i.e. PMN flux. The concentration of the migrated cells was expressed as the cell number in the exu-

date volume, which is the collected volume minus the chamber medium volume (0.5 ml serum).

Patients and technique used for evaluation of cellular infiltrate in dermis

In the parallel skin chamber experiment, nine patients with verified allergic contact dermatitis to nickel and six control subjects without previous eczema were studied. Biopsy specimens (diameter 2 mm) were taken from the abraded area 8, 24 or 48 h after the performance of the skin window, and only one biopsy was taken from each skin lesion. The biopsies were fixed in 10% neutral phosphated buffered formalin and embedded in glycol methacrylate and polyethyleneglycol. Sections 3 µm thick were stained (haematoxylin-eosin and May-Grünwald-Giemsa) (20) and several sections were cut from each specimen. Four hundred blood derived cells were counted in upper dermis just beneath the abraded epidermis (1000 × oil-immersion objective) in each biopsy. The classification of mononuclear and polymorphonuclear cells were done according to Groth & Skoog (10).

Lactoferrin, myeloperoxidase, lysozyme and eosinophil cationic protein determinations

Radioimmunoassays of lactoferrin (LF), myeloperoxidase (MPO), lysozyme (L) and eosinophil cationic protein (ECP) were performed on aliquots of the chamber content after removal of the migrated cells as previously described (16, 25, 26). Concentrations were corrected for the exudate volume and expressed as mg per l volume. The flux for each protein was obtained by calculating the total amount of protein in the cell free exudate within 1 cm² of skin window area.

Statistics

The fluxes of PMN, LF, L, MPO and ECP were evaluated by two-way analysis of variance of the logarithmic observations, but only observations with an interval of 3 h between collections were included for statistical analysis. Comparisons between patients and controls were done by Wilcoxon's two-sample test. Extract *P*-values in Wilcoxon's one- and two-sample tests were done for small numbers of observations by a special computer program (12).

Table 1

Cellular morphology in the exudate at 12, 24 and 42 h after start of skin window

	PMN (%)	Lymphocytes (%)	Monocytes (%)	Basophils (%)	Eosinophils (%)
12 hours					
Healthy volunteers	98.1 (95.3 - 99.2)	1.6 (0.8 - 4.4)	0 (0 - 0.3)	0 (0 - 0.6)	0 (0 - 0)
Nickel-allergics without nickel	97.2 (95.4 - 99.4)	1.3 (0 - 3.6)	0.4 (0 - 3.2)	0.2 (0 - 0.6)	0 (0 - 0.2)
Nickel-allergics with nickel	98.0 (91.0 - 99.2)	1.3 (0.4 - 4.5)	0.4 (0 - 2.5)	0.2 (0 - 2.0)	0 (0 - 0)
24 hours					
Healthy volunteers	94.4 (93.6 - 98.6)	3.8 (1.0 - 5.4)	0.2 (0 - 1.8)	0.2 (0 - 0.8)	0 (0 - 0.2)
Nickel-allergics without nickel	97.3 (87.6 - 98.6)	1.7 (0.4 - 11.4)	0.6 (0 - 1.4)	0.1 (0 - 1.8)	0 (0 - 0.2)
Nickel-allergics with nickel	94.0 (85.2 - 97.2)	3.0 (0.8 - 9.0)	0.6 (0.2 - 1.6)	1.5 (0.4 - 3.0)*	0.3 (0 - 6.6)*
42 hours					
Healthy volunteers	97.4 (97.0 - 99.8)	1.8 (0.2 - 2.4)	0.2 (0 - 0.6)	0.2 (0 - 0.4)	0 (0 - 0)
Nickel-allergics without nickel	96.7 (92.8 - 99.6)	1.6 (0.2 - 6.0)	1.0 (0 - 2.3)	0.2 (0 - 1.6)	0 (0 - 0)
Nickel-allergics with nickel	86.2 (73.8 - 97.8)*	7.1 (0 - 21.6)*	0.8 (0.4 - 1.6)	2.3 (1.6 - 6.8)*	0.3 (0 - 6.8)*

Median value and observed range by the counting of 500 cells at each collection. Difference between groups are indicated by * *P* < 0.05 (Wilcoxon's rank sum test).

RESULTS

Clinical response and morphology of the chamber cells and the infiltrate in upper dermis

After removal of the left chambers with nickel added to the medium, all but two patients showed erythema, infiltration and papules or vesicles. The two remaining patients had erythema, but no vesicles. During the study five patients complained of itching in the chamber with nickel. When the right chambers were removed, none of the patients except one showed signs of eczematous reaction. This patient reacted with vesicles, erythema and infiltration even in the right skin window. A subsequent patch test with chamber material proved negative. No signs of eczema were present in the control group.

The microscopic evaluation of the exudate cells at 12, 24 and 48 h after start of the skin window experiment is shown in Table 1. A significant increase in the percentage of lymphocytes, eosinophils, basophils and a concomitant decrease in neutrophils was observed from the 24th hour to the end of the observation period in patients exposed to nickel in the medium. Table 2 shows the microscopic cell counts of the infiltrate in upper dermis evaluated from

sections cut from biopsies taken 8, 24 or 48 h after start of the parallel skin window experiment. An increase of mononuclear cells and a decrease of neutrophil cells were detected from the 8th hour to the 48th hour in contact allergic patients with nickel added to the medium. The percentages of eosinophils and basophils were increased after the 24th hour. Statistical comparison between the patient groups and control group were not performed because of the low numbers in each group.

Flux of PMN, LF, MPO, L and ECP in skin window exudate

Controls and patients with and without nickel in the chamber medium showed identical kinetics of the PMN flux during the observation period (Fig. 1). The PMN flux increased rapidly during the initial 9 h with only minor variations during the following 30 h.

In parallel, the flux of LF, MPO, L, or ECP in the exudate showed identical kinetics between the three patient groups during the initial 15 h (Fig. 2). From the 15th hour to the end of the study period a higher flux of LF, MPO, L and ECP ($P < 0.01$) was observed in patients with nickel added to the chamber medium compared to patients without nickel challenge and

Table 2

Morphology of the infiltrate in dermis underlying the skin window at 8, 24 and 48 hours after start of skin window.

	PMN (%)	Mononuclears (%)	Basophils (%)	Eosinophils (%)
8 hours				
Healthy volunteers (2)	90 (86 - 94)	8 (6 - 10)	0 (0 - 1)	2 (1 - 3)
Nickel-allergics without nickel (3)	91 (85 - 94)	7 (5 - 11)	0 (0 - 1)	2 (2 - 4)
Nickel-allergics with nickel (3)	82 (76 - 84)	14 (10 - 18)	2 (1 - 4)	2 (1 - 3)
24 hours				
Healthy volunteers (2)	87 (82 - 92)	8 (6 - 10)	2 (1 - 2)	3 (2 - 5)
Nickel-allergics without nickel (3)	83 (80 - 89)	9 (7 - 12)	3 (2 - 4)	4 (3 - 6)
Nickel-allergics with nickel (3)	50 (44 - 58)	34 (30 - 40)	8 (5 - 10)	7 (6 - 9)
48 hours				
Healthy volunteers (2)	85 (80 - 92)	10 (7 - 11)	2 (2 - 3)	3 (2 - 4)
Nickel-allergics without nickel (3)	85 (79 - 91)	10 (7 - 13)	2 (1 - 3)	3 (2 - 5)
Nickel-allergics with nickel (3)	45 (38 - 50)	37 (33 - 42)	9 (6 - 11)	8 (6 - 10)

Mean value and observed range by counting 400 cells. The number of persons in each group is indicated in parenthesis.

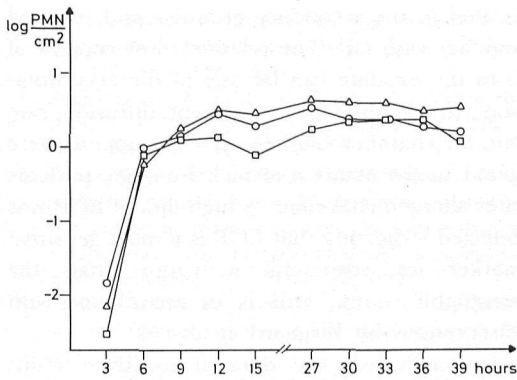


Fig. 1. The mean flux of polymorphonuclear granulocytes (PMN) ($\text{PMN} \times 10^6$ cells) in logarithmic scale. The flux of PMN is expressed as the mean for the number of PMN across 1 cm^2 skin window area at each collection for controls ($\square$ — $\square$), patients allergic to nickel not exposed to nickel ($\circ$ — $\circ$) and the same patients exposed to nickel added to the chamber medium (Δ — Δ).

controls (Fig. 2). An unexpected lack of correlation was detected during the initial 15 h between the kinetics of the flux of LF and L in the exudate from patients and controls. LF and L are located in the secondary granule of PMN and released together during degranulation (31). A correlation analysis between the fluxes of PMN and LF, PMN and MPO, PMN, and L showed no correlations within the groups. The exudate concentration of EPC at 3 h was about 0.3 mg/l for all participants, which is in accordance with a previous report (4). The exudate concentration is 10 times higher than found in normal serum (27), but no eosinophils were detected in the exudate (Table 1). During the second day the ECP concentration rose to about 5.0 mg/l for patients and controls, while less than 1% eosinophils was detected (Table 1). This indicates a different penetrance capacity for eosinophils and ECP into the exudate.

DISCUSSION

The cellular infiltration in dermis from contact allergic patients shows a time-dependent increase of mononuclear, eosinophilic and basophilic cells during allergen challenge; this is indicative of a contact sensitivity reaction. The

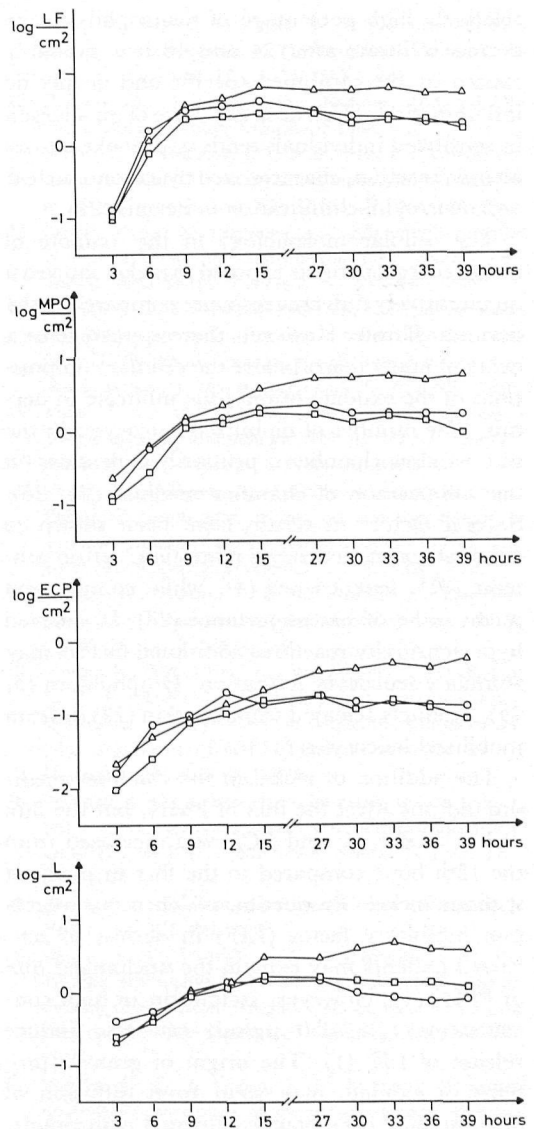


Fig. 2. Average profiles of the flux of lactoferrin (LF), myeloperoxidase (MPO), eosinophil cation protein (ECP) and lysozyme (L) expressed as the mean of the total content of each protein in the cell-free exudate across 1 cm^2 of skin window area measured at each collection.

A. The mean flux of LF ($\text{mg} \times \text{cm}^{-2}$) in logarithmic scale.
B. The mean flux of MPO ($\text{mg} \times \text{cm}^{-2}$) in logarithmic scale.
C. The mean flux of ECP ($\text{mg} \times \text{cm}^{-2}$) in logarithmic scale.
D. The mean flux of L ($\text{mg} \times \text{cm}^{-2}$) in logarithmic scale in the cell-free exudate during the observation period for controls ($\square$ — $\square$), patients allergic to nickel not exposed to nickel in the chamber medium ($\circ$ — $\circ$), and the same patients exposed to nickel added to the chamber medium (Δ — Δ).

relatively high percentage of neutrophils in the dermis infiltrate after 24 and 48 h is probably caused by the combined specific and unspecific inflammation. High dose exposure of an allergen in sensitized individuals tends to provoke a toxic allergic reaction, characterized by a mononuclear and neutrophilic infiltration in dermis (22).

The cellular morphology in the exudate of nickel allergic patients exposed to nickel showed a quantitatively different response compared to the dermis infiltrate. However, there appears to be a delay of many hours before the cellular compositions of the exudate mimics the infiltrate in dermis. The number of mobilized leucocytes in the skin window chamber is primarily dependent on the composition of chamber medium (21, 28). Several factors in serum have been shown to stimulate *in vivo* leucocyte migration: serum proteins (21), leukotrienes (4), while complement seems to be of less importance (28). In delayed hypersensitivity reactions additional factors may stimulate leucocyte migration: lymphokines (5, 15), products released from the skin (23) or from mobilized leucocytes (11).

The addition of nickel to the chamber medium did not affect the flux of PMN, but the flux of LF, MPO, L, and ECP was increased from the 15th hour compared to the flux in medium without nickel. Production of leucocyte migration inhibitory factor (LIF) in dermis of sensitized patients may explain the unchanged flux of PMN (1). However, lactoferrin in high concentrations ($> 250 \mu\text{g/ml}$) can also induce release of LIF (1). The origin of granule proteins in exudate may stem from diffusion of degranulated chemotactic activated neutrophils, monocytes and eosinophils in dermis. It is unknown whether the contact allergic reaction causes a more pronounced degranulation of PMN and eosinophils or a more pronounced degranulation of PMN and eosinophils, or a facilitated diffusion of the granule proteins into the chambers. A direct effect of nickel causing the increased granule flux seems unlikely, since no difference in protein flux values was observed between exposed and unexposed patients during the first day. The different kinetics of the flux of LF and L during the initial 15 hours was unexpected since the majority of L is

located in the secondary granules and released together with LF. The relatively low content of L in the exudate can be due to the accumulation, degradation or a different diffusion rate into the chamber exudate. Few eosinophils were found in the exudate of nickel allergic patients after allergen challenge. A high flux of ECP was detected indicating that ECP is a more sensitive marker for eosinophil activation than the eosinophil count. This is in accordance with observations by Bisgaard et al. (4).

In conclusion, our data demonstrate a different cellular morphology between the infiltrate in dermis and the chamber exudate upon nickel challenge in sensitized patients. Lymphokines released dermis during the contact allergic may account for the reduced mobilization of mononuclear cells, basophils and eosinophils into the chamber exudate. The higher flux of granule proteins in these patients without a concomitant increased mobilization of PMN in the exudate might be caused by a high total amount of eosinophils in dermis, a more pronounced degranulation of PMN and eosinophils, or a facilitated diffusion into the chambers. These observations could indicate a more active role of the PMN in the allergic contact reaction than previously anticipated, possibly by modulating the activities of mononuclear cells (6, 11, 17).

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