

Dose per unit area – a study of elicitation of nickel allergy

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Background: Experimental sensitization depends upon the amount of allergen per unit skin area and is largely independent of the area size.

Objectives: This study aimed at testing if this also applies for elicitation of nickel allergy.

Patients/methods: 20 nickel allergic individuals were tested with a patch test and a repeated open application test (ROAT). Nickel was applied on small and large areas. The varying parameters were area, total dose and dose per unit area.

Results: In the patch test, at a low concentration [$15 \mu\text{g}$ nickel ($\mu\text{gNi}/\text{cm}^2$)], there were significantly higher scores on the large area with the same dose per area as the small area. At higher concentrations of nickel, no significant differences were found. In the ROAT at low concentration ($6.64 \mu\text{gNi}/\text{cm}^2$), it was found that the latency period until a reaction appeared was significantly shorter on the large area compared to the small area. It was also found that the ROAT threshold (per application) was lower than the patch test threshold.

Conclusion: For elicitation of nickel allergy, the size of the exposed area and therefore the total amount of applied nickel, influence the elicitation reaction at some concentrations, even though the same dose per unit area is applied.

Key words: contact allergy; dose per unit area; elicitation; nickel allergy; patch testing; repeated open application test; ROAT. © Blackwell Munksgaard 2007.

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Experimental studies have established that the dose of allergen per unit area of skin is crucial for the induction of contact allergy. This is based on a few but well-documented experimental studies in healthy volunteers using mostly strong or extremely strong allergens (1). In 1942, Schnitzer concluded, from experimental studies with dinitrochlorobenzene (DNCB) on guinea-pigs, that sensitization was dependent on the concentration and not on the exposed area (2). Magnusson and Kligman substantiated the same by experiments, also on guinea-pigs, using other strong allergens (3). Kligman also showed that in human beings, when the patch test dose of either ammoniated mercury, monobenzyl ether of hydroquinone, nickel sulphate or neomycin sulphate was increased proportionally to the area, keeping the dose per unit area constant, the same number of people were sensitized. At a critical small area, depending on the allergen, fewer people were sensitized (4). One of the pivotal studies was performed on 210 subjects using DNCB. It was

shown that, if the dose per unit area was kept constant, while doubling or halving the area, only a minimal increase in sensitization was found on the larger areas, even though the total dose was higher on the larger areas than on the smaller areas. If the area was kept constant, and the dose per unit area doubled, increasing number of people was sensitized (5). This leads to the conclusion that doubling or halving the area and thereby the number of Langerhans cells, does not influence sensitization, when the amount of allergen per Langerhans cell is the same. By contrast, when the dose per unit area is doubled and thereby the amount of allergen per Langerhans cell is increased, the stimulus of the immune system is increased (6, 7). In a similar study, it was shown that when the same dose per unit area of DNCB was applied to 0.08 and 0.8 cm^2 , fewer people were sensitized on the small area, meaning that at a critical small area the number of Langerhans cells do influence the sensitization rate (8). It was also shown, that when the sensitization dose per unit

area increases, the elicitation dose per area decreases (9).

The concept of the dose of allergen per unit area of skin has been built in predictive animal assays and risk assessment models for skin sensitization (10, 11). It has been assumed – but not studied – that also the dose per unit area of skin is crucial for elicitation. Whether this is true or not is important for diagnosing contact allergy, as well as for preventing clinical disease.

This study aimed at testing, if elicitation of nickel allergy is dependent on the applied amount of nickel per unit skin area ($\mu\text{g}/\text{cm}^2$) or on the total dose of applied nickel in a patch test and a repeated open application test (ROAT) on nickel sensitive individuals.

Materials and Methods

Test subjects

20 subjects with contact allergy to nickel, demonstrated by a positive patch test to nickel sulphate 5% in petrolatum within the last 5 years, at the Department of Dermatology, Gentofte Hospital, University of Copenhagen, Denmark, were enrolled in the study after oral and written consent. The inclusion criteria were age above 18 years, and a former positive standard patch test to nickel, with a positive reaction defined by erythema and homogenous infiltration in the whole test area. The exclusion criteria were active eczema (defined as eczema in a phase of worsening, evaluated by a doctor), systemic immunosuppressive therapy, ultraviolet light on the test area within 3 weeks of the testing, pregnancy, breastfeeding, and not being able to co-operate. The subjects were aged 24–70 years with a mean age of 47.1 years. 18 women and 2 men participated in the study. The study was approved by the local Ethics Committee (KA04100).

Study design

Patch testing and ROAT were performed simultaneously on the subjects. The design was the same for the patch test with 4 areas on each side of the back and the ROAT with 4 areas on the forearms. In each case 1 small and 1 large area was exposed to the exact same amount of nickel per unit skin area, and 1 small and 1 large area was exposed to the exact same total amount of nickel (Figs 1 and 2). After termination of the study, the randomization code was broken.

Patch test

Each subject was tested with 8 patches, 4 on each side of the back. On each side, there were 2 small patches and 2 large patches. One side was tested with low concentrations of nickel sulphate in aqua with 10% ethanol and the other side was tested with concentrations 10 times higher. The small patches were small Finn Chambers[®] of 0.5 cm^2 (Epitest Ltd Oy, Tuusula, Finland) on Scanpor[®] (Norgesplaster A/S, Actaris, Norway), and the large patches were large Finn Chambers of 1.13 cm^2 on Scanpor. A micropipette was used to apply the solutions in the Finn Chambers. In the small Finn Chambers, $17.7\text{ }\mu\text{l}$ of different nickel solutions were applied on a filter paper, and in the large Finn Chambers $40\text{ }\mu\text{l}$ of the solutions were applied on an identical filter paper. The filter paper for the large Finn Chambers (Epitest Ltd Oy) was used to produce the filter papers for the small Finn Chambers, in order to have the same thickness of the filter papers in both chambers. The areas were tested with 1.9%, 0.8%, 0.2% or 0.08% $\text{NiSO}_4\cdot 6\text{H}_2\text{O}$ in aqua with 10% ethanol. These concentrations correspond to the doses shown in Table 1 and the relation to the study design is shown in Fig. 1.

The patch test was applied on day 0 (D0), occluded for 2 days, and the reactions were read

	Concentration range A		Concentration range B		
Dose per area	$6.6\text{ }\mu\text{gNi}/\text{cm}^2\text{ Ni}$	$15\text{ }\mu\text{gNi}/\text{cm}^2$	$66\text{ }\mu\text{gNi}/\text{cm}^2$	$150\text{ }\mu\text{gNi}/\text{cm}^2$	Volume
Concentration	0.08%	0.2%	0.8%	1.9%	
Total dose	$3.3\text{ }\mu\text{g}$	$7.5\text{ }\mu\text{gNi}$	$33\text{ }\mu\text{gNi}$	$75\text{ }\mu\text{gNi}$	
	1	3	5	7	$17.7\text{ }\mu\text{l}$
	2	4	6	8	$40\text{ }\mu\text{l}$
Dose per area	$6.6\text{ }\mu\text{gNi}/\text{cm}^2\text{ Ni}$	$15\text{ }\mu\text{gNi}/\text{cm}^2$	$66\text{ }\mu\text{gNi}/\text{cm}^2$	$150\text{ }\mu\text{gNi}/\text{cm}^2$	
Concentration	0.08%	0.2%	0.8%	1.9%	
Total dose	$7.5\text{ }\mu\text{g}$	$17\text{ }\mu\text{g}$	$75\text{ }\mu\text{gNi}$	$169.5\text{ }\mu\text{gNi}$	μgNi

Fig. 1. The study design in the patch test. The concentration and dose per unit area are the same for the small and large area, which are placed on the same vertical axis. The following areas are compared: area 1 with 2, 2 with 3, 3 with 4, 5 with 6, 6 with 7 and 7 with 8. A significant difference was found in the score when area 3 was compared with area 4.

Dose per area	6.64 µg Ni/cm ²	15 µgNi/cm ²	Volume
Concentration	0.67 %	1.5 %	
Total dose	59.76 µgNi	135 µgNi	40 µl
	9	11	
	10	12	90 µl
Dose per area	6.64 µgNi/cm ²	15 µgNi/cm ²	
Concentration	0.67 %	1.5 %	
Total dose	135 µgNi	305.1 µgNi	

Fig. 2. The study design in the ROAT. The doses are dose per application. The concentration and dose per unit area are the same for the small and large area, which are placed on the same vertical axis. The following areas are compared: area 9 with 10, 10 with 11 and 11 with 12. A significant difference was found in the latency period until reaction, when area 9 was compared with area 10.

on D3 or D4 and D7. The low concentration side (concentration range A) and the high concentration side (concentration range B) were randomized and the position of the different concentrations and Finn Chambers sizes were also randomized. The study was blinded, so that a nurse applied the patch tests and the principal investigator made note of the readings. The size of the Finn Chambers could not be blinded, because the disc made a mark on the skin when it was removed. The reading scale for the patch test was based on the International Contact Dermatitis Research Group (ICDRG) reading scale, but was further developed in order to recognize even small differences in the allergic reactions. A scale was used, modified from Hindsen and Bruze (12) to include also weaker reactions. The scale was as follows: 0 = no reaction, 1 = few papules with no erythema, no infiltration, 2 = faint erythema with no infiltration or papules,

3 = faint erythema with few papules and no homogenous infiltration. 4 = erythema, homogenous infiltration, 5 = erythema, infiltration and a few papules, 6 = erythema, infiltration and papules, 7 = erythema, infiltration, papules and a few vesicles, 8 = intensive erythema, infiltration and vesicles. The same score was used for the small and large Finn Chambers, because first, the reading scale did not give a countable number of papules but used the term 'few papules/vesicles' and 'papules/vesicles', and secondly there was no problem in discriminating between 'few papules/vesicles' (which was interpreted as a countable number of papules) and 'papules/vesicles' (which was interpreted as an uncountable number of papules). As all the patients were allergic to nickel and all the patients who reacted to the low concentrations also reacted to the high concentrations, we assumed that the reactions with a score from 1 to 3 also were allergic reactions, and these data were therefore also used in the calculations (even though a score below 4 is not considered positive according to ICDRG criteria).

Repeated open application test

Each patient was tested on both arms, 2 areas on each arm, totalling 4 areas. The volar aspects of the forearms were used. On each arm there was 1 small area (3 × 3 cm) and 1 large area (4.5 × 4.5 cm). On the small area, 0.04 ml was applied and on the large area 0.09 ml was applied. The areas were tested with either 1.5% NiSO₄6H₂O or 0.67% NiSO₄6H₂O in aqua with 10% ethanol, these concentrations correspond to the doses shown in Table 2, and the relation to the study design is shown in Fig. 2.

The nickel solutions were delivered in 1 ml syringes, and the patients were instructed to apply the whole content of one syringe on the corresponding area morning and evening, and letting the areas dry spontaneously by evaporation. A test of the 1 ml syringes was performed prior to

Table 1. The dose per area and the total dose applied in the patch test

Area number	Area (cm ²)	Concentration (%)*	Dose per area (µgNi/cm ²) [†]	Total dose (µgNi) [†]	Number of subjects reacting	Score range	Mean score
1	0.50	0.08	6.6	3.3	6/20	0–5	0.5
2	1.13	0.08	6.6	7.5	8/20	0–3	0.5
3	0.50	0.20	15.0	7.5	10/20	0–5	0.8
4	1.13	0.20	15.0	17.0	15/20	0–7	2.1
5	0.50	0.80	66.4	33.2	17/20	0–7	3.9
6	1.13	0.80	66.4	75.0	18/20	0–8	4.6
7	0.50	1.90	150.0	75.0	19/20	0–8	5.0
8	1.13	1.90	150.0	169.5	19/20	0–8	5.3

*The percentage refers to NiSO₄6H₂O (aqueous nickel sulphate).

[†]The µg refers to Ni (nickel).

Table 2. The dose per area and the total dose per application, in the ROAT

Reference for Fig. 2	Area (cm ²)	Concentration (%) [*]	Dose per area (µgNi/cm ²) [†]	Total dose (µgNi) [†]	Number of subjects reacting	Number of days to reaction (range)	Number of days to reaction (mean)
9	9.00	0.67	6.64	59.76	17/17	2–14	5.1
10	20.25	0.67	6.64	135.00	17/17	2–14	4.3
11	9.00	1.50	15.00	135.00	17/17	2–14	4.6
12	20.25	1.50	15.00	305.10	17/17	2–14	4.1

^{*}The percentage refers to NiSO₄6H₂O (aqueous nickel sulphate).

[†]The µg refers to Ni (nickel).

the initiation of the study, and showed a mean volume of 0.0400 ml (lowest value: 0.0359 ml, highest value: 0.0462 ml) and 0.0901 ml (lowest value: 0.0830 ml, highest value: 0.0930 ml), respectively, when a test sample of 20 was performed for each volume. Furthermore, subjects were supplied with new syringes each week, and were instructed to keep the syringes in a refrigerator; on the tip of each syringe there was a plug in order to minimize evaporation. The areas and syringes were coded with a colour in order to blind the doses for the investigator, and in order to make it easier for the patients to use the right syringes on the right areas. The positions of the different concentrations were randomized. The size of the area could obviously not be blinded, because the investigator could see the differences on the area sizes. The reactions were read on D2, D3 or D4, D7, D14 and D21 routinely and additionally if a reaction occurred. The principal investigator noted the reading of the reactions on all the patients according to a reading scale previously developed (13). In case of a positive reaction (according to this scale), the exposure to that area was terminated. On the large area, the required number of papules and vesicles was doubled compared to the small area in order to give the same score. In case of a score of 5 or above (based on the involved area, erythema, infiltration, number of papules and number of vesicles), the exposure to that area was terminated, and if no reaction occurred, or in case of a score below 5, the exposure was terminated on D21.

Statistics

The influence of the total dose on the magnitude of the allergic reaction was analysed by comparing the scores from area 1 with 2, 3 with 4, 5 with 6, 7 with 8 (patch test; Table 1, Fig. 1), 9 with 10 and 11 with 12 (ROAT; Table 2, Fig. 2). The areas in this comparison had the same dose per unit area, while the total dose was higher on the large area than on the small area.

The influence of the dose per area on the magnitude of the allergic reaction was analysed, com-

paring area 2 with 3 and 6 with 7 (patch test; Table 1, Fig. 1) and 10 with 11 (ROAT; Table 2, Fig. 2). These areas had the same total dose applied, and the dose per unit area was higher on the small area than on the large area.

Patch test

The paired *t*-test and the Wilcoxon matched pairs signed rank sum test were used to detect any significant differences in the patch test scores on the compared areas within each patient.

McNemar's test of the null hypothesis was used to detect significant differences between any reaction or no reaction (cut point > 0) on the two areas, or a positive versus a negative reaction (cut point > 3).

Repeated open application test

The sign test was used to detect any significant differences in the latency period until a reaction appeared on the two areas, further if the reaction appeared on the same day on the two areas, if the score was higher on one area than the other. Both the latency period and the score when any degree of reaction appeared (cut point > 0) was analysed, and the latency period and score when a positive reaction appeared (cut point > 4) was analysed, based on the morphology (involved area, erythema, infiltration, number of papules and vesicles) from the scale earlier mentioned (13).

The paired *t*-test and the Wilcoxon matched pairs signed rank sum test was also used to see if there were any significant differences in the latency period until any reaction (cut point > 0) and the latency period until a positive reaction (cut point > 4).

Comparison of elicitation doses between the patch test and the ROAT

The paired *t*-test was used to detect any significant differences between the reactivity in the patch test and the ROAT at the same concentration level.

Results

Patch test

All 20 patients completed the study. On the low concentration range, 8 patients reacted to the lowest concentration and 15 patients reacted to the highest concentration. On the high concentration range, 18 patients reacted to the lowest concentration and 19 reacted to the highest concentration (Table 1).

Concentration range A

The strength of the reaction was significantly higher on the large area (mean score 2.1, score range 0–7) than on the small area (mean score 0.8, score range 0–5) ($P = 0.012$, t -test; $P = 0.0088$, Wilcoxon rank sum test) when $15 \mu\text{gNi}/\text{cm}^2$ was applied to both areas (it should be remembered that the total dose was larger on the large area, $17 \mu\text{gNi}$ than on the small area, $7.5 \mu\text{gNi}$). No significant differences were found when testing, if there was any or no reaction (cut point > 0) or if there was a positive or a negative reaction according to ICDRG criteria (cut point > 3) on these two areas.

No significant difference in the score between the small and the large area was found either when $6.6 \mu\text{gNi}/\text{cm}^2$ was applied to the small and large area or when the same total amount of nickel, $7.5 \mu\text{g}$, was applied to the small and large area.

Concentration range B

No significant difference was found when 150 or $66.4 \mu\text{gNi}/\text{cm}^2$ was applied to the small and large area, or when the same total amount of nickel, $75 \mu\text{g}$, was applied to the small and large area.

Repeated open application test

17 of the patients completed the ROAT. 3 subjects were excluded from the ROAT and only participated in the patch test part of the study. 1 was excluded because of eczema on the forearms and the other 2, because they could not co-operate to perform the ROAT. All patients had reactions to all areas. When looking at the days passed until a reaction occurred in the ROAT, it was found that a reaction occurred significantly earlier on the large area (mean days: 4.3, day range: 2–14) than on the small area (mean days: 5.1, day range: 2–14) ($P = 0.043$, t -test; $P = 0.041$ Wilcoxon rank sum test) when $6.64 \mu\text{gNi}/\text{cm}^2$ was applied to the two areas. When looking at the two parameters (i) the latency period until a reaction appeared (cut point > 0) on the two areas and

(ii) the score, when the reaction appeared the same day on the two areas; there were significantly more times, when either the latency period was shorter or the score was higher on the large area ($P = 0.021$) (Remember that the total dose per application was higher on the large area, $135 \mu\text{gNi}$, than on the small area, $59.76 \mu\text{gNi}$). No significant difference was found when looking at the latency period until a positive reaction occurred, or when looking at the score when a positive reaction occurred (cut point > 4).

No significant difference was found when $15 \mu\text{gNi}/\text{cm}^2$ was applied to the small and large areas or when the same total amount of nickel, $135 \mu\text{gNi}$, was applied to the small and large areas.

Comparison of elicitation doses between the patch test and the ROAT

It was found that in the ROAT there were significantly more reactions (100%) to $15 \mu\text{gNi}/\text{cm}^2$ per application and $6.6 \mu\text{gNi}/\text{cm}^2$ per application than in the patch test with the small Finn Chambers, where only 50% had a reaction to $15 \mu\text{gNi}/\text{cm}^2$ ($P = 0.002$) and 30% had a reaction to $6.6 \mu\text{gNi}/\text{cm}^2$ ($P < 0.0001$).

Discussion

In the patch test, there was a significantly higher score on the large area compared to the small area when $15 \mu\text{gNi}/\text{cm}^2$ was applied; this indicates that the strength of the reactions, at this concentration level was influenced by the total dose, which was higher on the large area ($17 \mu\text{gNi}$) than on the small area ($7.5 \mu\text{gNi}$). This was only found in one of the low concentrations. It seems that in the high concentration range (0.8–1.9%) at the patch test, the chosen concentrations were so high that the allergic reaction was triggered to a degree where discrimination of the reactions was not possible. We could not find a relationship between the dose per unit area and the magnitude of the allergic reaction. Perhaps, if the differences in area sizes had been larger, and the concentration had been in between the ones chosen for this study, the results would have been different. It could also be that the critical area for elicitation of nickel allergy lies above 0.5 cm^2 , and that below this critical area more allergen per unit area is needed to elicit an allergic reaction, as is the case with sensitization (3, 4, 8).

In the ROAT, the reaction appeared significantly earlier on the large area than on the small area when $6.64 \mu\text{gNi}/\text{cm}^2$ was applied per application. This indicates that the reactivity was influenced by the total dose per application, which was

higher on the large area (135 µgNi) than on the small area (59.76 µgNi). So, in ROAT, the total dose of nickel seems to influence the days passed until a reaction appears and the magnitude of the allergic reaction. However, this was only found for the low concentration. The fact that the same results were found in both the patch test and the ROAT, support that the total dose has an influence on the allergic reaction at some doses or areas. This was only seen at low concentrations, but it is also at the low concentrations that the development of disease can be prevented, and it is therefore important to be aware of a possible relationship between the total dose and the development of an allergic reaction. There was no relationship between the dose per unit area and the days passed until a reaction appeared or the magnitude of the allergic reaction. Again it would have been interesting to see what would have happened, if there had been a larger difference between the areas. In one study that tested 13 patients with several allergens in a ROAT, no difference was found when the same dose per unit area was applied to 1 and 10 cm², though the reaction appeared later on the small area in some cases (14).

It maybe that the area and thereby the total dose has an influence on the elicitation reaction, when the dose is close to the threshold concentration; this could explain why we only found a significant result at one of the patch test concentrations and at one of the ROAT concentrations.

The patch test score has one weakness, the number of papules and vesicles was not counted; if they had been counted, the score could have been modified so that the large area had to have the double number of papules in order to get the same score as the small area. Furthermore, the probability of a papule to occur on the large area is increased compared to the small area, just because of the larger area. In future studies, when investigating the influence of total dose and dose per unit area it would be wise to develop a patch test score that takes this into account. Alternatively, other measures such as skin thickness or blood flow could be valuable. The ROAT score takes the difference in area size into account.

Results from other clinical studies also indicate that it is not only the dose per unit area that is important for the elicitation of an allergic reaction. In one study, a clear dose–response was seen in relation to a use test with a diazolidinyl-urea-containing cream when looking at the concentration (in percentage) of allergen in the cream, but not when looking at the amount of allergen applied per skin area (15). In another clinical study, testing 4 allergens (fragrance mix, wool wax alcohols, methylchloroisothiazolinone–

methylisothiazolinone and formaldehyde) in large and small Finn Chambers on persons allergic to these allergens, more positive reactions were found to the large Finn Chambers even though the dose per unit area was larger in the small Finn Chambers (16). In a third study on the effect of dose per area on the intensity of response to a cutaneous irritant, it was shown that increasing the area also increased the irritant response (17).

In this study it was found that significantly more subjects had a reaction to the ROAT, to a dose per area (per application), compared to the same doses in the patch test. This could be due to accumulation of allergen in the skin, or a sequential stimulation of the immune system.

If the data from this study can be translated to real-life-exposure situations, it would mean that if a nickel allergic person is exposed to low nickel concentrations on a large skin area, an allergic reaction might develop despite the low concentration. Further experimental elicitation studies are needed to confirm these findings.

In conclusion, the size of the exposed area and therefore the total amount of applied nickel might influence the latency period until a reaction appears and the strength of the elicitation response, at some concentrations, even though the dose per area is the same.

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