

The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans

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Summary

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None declared.

Like all physiological systems, the human immune system exhibits dose–response relationships in its reactions. The strength of sensitization is related to the potency of the immunogen and the dose that reaches the immune system. In skin, as sensitizing dose per unit area ($\mu\text{g cm}^{-2}$) is increased on a log scale, there is a sigmoid dose–response curve for subsequent reactivity. Similarly, the response to elicitation shows a classical sigmoid response to increasing challenge dose, with the dose per unit area again being the determinant. There is a clear inverse correlation between the strength of sensitization and the subsequent dose of antigen to which an individual will respond. This is reflected in the different challenge systems used to diagnose the existence of allergic contact sensitization to a given allergen. The occluded patch test aims to use the highest concentration possible to detect the weakest degrees of allergy, whereas the repeated open application test uses much lower concentrations similar to those encountered in real life, applied repeatedly but without occlusion, to assess clinical relevance. Many authors have attempted to use the lowest concentrations to which rare, highly sensitized individuals can react to define the concentrations which might be free of risk in terms of inducing allergic sensitization. However, it is clear that the dose–response relationships for induction of sensitivity by repeated low-dose exposures must be carefully defined in future studies. This article reviews the dose–response relationships of human contact sensitization.

In the field of contact allergy one of the central questions is: why does the human immune system develop unwanted immune hypersensitivity to environmental chemicals (xenobiotics) that results in apparently valueless skin inflammation? Secondary questions relate to quantitative aspects of the immune system and its responses: for example, how low an exposure to an immunogen can the immune system detect and respond to? The human immune system functions mainly to protect us from harm by pathogens. In order to fulfil this function it has to detect and respond to foreign substances appropriately – that is, it must generate an effective response when the foreign substance is perceived as dangerous, but the system is programmed not to respond to nondangerous substances. The immune system mounts two major types of response – the ‘innate’ response and the ‘adaptive’ or acquired response.

Innate responses are designed primarily to generate rapid defences to danger signals without identifying the activating stimulus in great detail. The agents that are perceived as dangerous are mainly microbes and their proteins, lipids, carbo-

hydrates and nucleic acid components. These microbial constituents are recognized by a diverse set of so-called pattern recognition receptors which include toll-like receptors and scavenger receptors.

Adaptive responses are designed to establish highly specific immunological memory of the relevant antigen/immunogen but they are slow to be initiated as they involve the proliferation of clones of T lymphocytes specifically able to recognize the antigen. In order for the immune system to ‘invest’ in the complex process of waking up naïve T cells and causing them to proliferate, activate B cells to make antibody and establish immunological memory, the immune system must sense that it is important and necessary so to do. In fact, the alerting signals that say ‘this is important’ are the danger signals that activate the innate responses. The cross-talk between the cellular components of the innate and adaptive immune systems is crucial in determining whether T cells are activated and the nature of the effector mechanisms that are generated.

The induction of a new immune response involves the collection/uptake by professional antigen-presenting dendritic

cells (DCs) of foreign material which they process and associate with the major histocompatibility molecules, HLA class I and II. The DCs ‘present’ the major histocompatibility complex (MHC)-associated immunogen to T lymphocytes so that the cell(s) bearing the highly specific cognate receptor for the immunogen can recognize it. In order to be activated to proliferate, the T cell must receive additional signals from the DC via costimulatory molecules and cytokines.

The cell types that respond most energetically to ‘danger’ are macrophages and DCs but most cell types can also participate in innate defensive responses. Cells respond by upregulating all the molecules that might be required for defensive action. However, in the case of the DCs, because they have been alerted to the stimulus being dangerous and hence important, there is also a maturation and augmented expression of key immunostimulatory cytokines including interleukin (IL)-1 β , IL-12 and type 1 interferons as well as costimulatory molecules including CD40, CD80 and CD86. These highly activated, ‘alerted’ DCs are able to present components of the perturbing agent/organism to naïve T lymphocytes with a set of very powerful activation signals that initiate proliferation and clonal expansion – resulting in an active immune response. If the DCs present molecules to T cells without having been alerted to potential danger, various outcomes are possible: the T cells will not proliferate and might even undergo apoptosis – resulting in absence of immune reactivity to that ‘antigen’. Alternatively, a different type of activation may occur in which T cells can act as regulatory T cells, preventing effector T cells from being generated or from responding to the ‘antigen’, thus establishing a form of active immunological tolerance. In mice and humans contact hypersensitivity responses involve activation of T cells of both CD4+ Th1 and CD8+ Tc1 types.^{1–3}

Once clones of memory/effector T cells have been generated they are available to react much more rapidly should their specific target antigen be re-encountered. Importantly, memory cells and cytotoxic T cells can be activated by many cell types capable of showing/presenting the antigen in association with MHC class II or I, respectively. Thus, macrophages and B cells which express MHC class II but which do not express all the costimulatory molecules or even the key cytokines IL-1 β or IL-12 are very good at presenting antigens to memory T cells.

Factors contributing to sensitizing efficiency

The ultimate determinants of whether a chemical induces sensitization are the resultant of a range of host-related factors that influence susceptibility, which may be systemic or local, the intrinsic sensitizing potency of the chemical and the dose that reaches the cells of the immune system.

Susceptibility to sensitization

Age, sex and genetic factors such as HLA haplotype can all influence whether and to what degree an individual reacts to a

chemical/xenobiotic. These factors have been reviewed in detail elsewhere⁴ and space does not permit further review here. Local factors which render subjects susceptible to sensitization include the stratum corneum barrier, xenobiotic metabolism in skin, and concomitant stimuli (e.g. exposure to ultraviolet radiation); see below.

Sensitizing potency of chemicals

Having considered the general processes underlying recognition of foreign, microbe-derived antigens, the question arises as to what is the basis for the immune system recognizing and responding to small xenobiotic molecules such as contact sensitizers. The T-cell receptor is designed to recognize the structure of a peptide of nine to 15 amino acids lying in the groove of the MHC class I or II molecule. In order to be ‘seen’ by the T cell, small molecules must bind to proteins, hence acting as haptens. Hapten-conjugated peptides can then be associated with the MHC groove, making them recognizable by T cells with the appropriate cognate receptor. Some chemicals are intrinsically reactive, while others may be rendered reactive through the actions of drug-metabolizing and detoxifying enzymes. It is thought that the more protein-reactive a chemical the more potent it is as an immunogen/sensitizer. So some chemicals including dinitrochlorobenzene (DNCB), diphenylcyclopropenone, oxazolone and squaric acid dibutyl ester are very potent immunogens, inducing contact sensitization in 100% of normal humans. Much work has gone into devising assays that can predict the sensitizing potency of different chemicals. Thus, tests in animals include the guinea pig maximization test, the Buehler test and the mouse ear swelling test.⁵ Some of these experimental systems involve administering the chemical with an adjuvant which gives the ‘danger’ signal and hence maximizes the chance of the immune system responding. Implicit in this is that the chemical on its own may not be able to induce an immune response but requires the ‘danger signals’ from the adjuvant. Kligman developed a modified human ‘maximization’ test which involved pretreating a skin area with 5% sodium lauryl sulphate (SLS), a detergent with irritant properties. The agent under test was then applied to the area under occlusion for 48 h.⁶ The local lymph node assay (LLNA), which involves quantification of proliferation of lymphoid cells in lymph nodes draining sites of topical application of putative sensitizers, has become widely adopted as a standard mouse-based screening test.^{7,8}

Many chemicals found in everyday life are potential sensitizers; thus Kligman showed 100% sensitization of human volunteers by *para*-phenylenediamine (PPD) using high concentrations of this substance (1 g of 10% PPD in petroleum jelly to give 711 $\mu\text{g cm}^{-2}$ compared with up to 6% in hair dyes).⁹ Indeed, in the LLNA it is more potent than DNCB.¹⁰ However, the doses in which most chemicals are encountered in the normal environment are either too low to activate a ‘positive’ immune response manifesting as allergic hypersensitivity, or they may even induce immunological

tolerance, probably mediated by regulatory T cells. As indicated above, whether an active hypersensitivity or a state of nonreactivity is induced may be a reflection of how the chemical interacts with the innate immune system to produce danger signals. Most potent sensitizers are also irritants and there is evidence that it is the irritant properties that exert the 'adjuvant' effect of stimulating the innate immune response, hence potentiating the activation of the adaptive/acquired immune response. For example, Allenby and Basketter showed that pretreatment with an irritant, SLS, potentiated allergic responses to nickel.¹¹ However, SLS is also a surfactant and the possibility that it simply compromised the epidermal barrier and facilitated absorption of nickel is a possible alternative explanation. This 'danger signal' effect resulting from the irritant properties of sensitizers is a major area of interest but to date the relevant mechanisms are obscure.

Factors determining the dose that the immune system encounters

Many factors affect the dose (number of molecules) of a chemical that reaches the viable layers of the epidermis in which the DCs are found. These include the concentration (quantity per unit area) applied to the surface, the duration for which it is applied, the molecular weight of the compound (those larger than 500 Da do not penetrate into normal skin), the intactness of the stratum corneum barrier, occlusion,¹² the solubility characteristics (lipid-soluble molecules penetrate much better than water-soluble ones), the vehicle and the partition coefficient of the molecule in relation to the constituents of the vehicle and the stratum corneum. Additional factors may include metabolic processing of the compound which may either reduce its effective concentration or, alternatively, might generate an intermediate compound which is the actual sensitizer. These factors will all contribute at every encounter with a chemical – both during the initial sensitizing exposure and also at subsequent elicitation/challenge exposure(s). Indeed, the diagnostic occluded patch test challenge deliberately maximizes the exposure dose by using high concentrations applied for 48 h under occlusion. Additional factors may come into play when there is repeated exposure to a compound either through daily use or in a repeated challenge. These will be discussed below.

Dose–response relationships for induction of sensitization

Use of potent contact sensitizers such as DNCB has taught us much about the quantitative dose–response relationships of the human immune system. Apart from the chemical property of actual or potential reactivity with proteins, it is now clear that the major determinant of how the immune system responds to a sensitizer is the intensity of the sensitization stimulus, that is the dose of sensitizer in relation to the area of exposure – the dose per unit area. The experimental work that underpins this conclusion involved sensitizing groups of

healthy human volunteers with different doses of DNCB on an area of constant size and, in other groups, comparing responses evoked by varying the area of application of the sensitizing dose. Thus, initially, five groups received 62.5, 125, 250, 500 or 1000 µg of DNCB on a circle of forearm skin 3 cm in diameter.¹³ As DNCB remains bound in the skin for some weeks, it is available for recognition as soon as a clone of memory T cells specific for DNCB enters the systemic circulation from the lymph node. The site of initial application of DNCB subsequently becomes inflamed with a contact dermatitis-like reaction – the so-called delayed flare. This is an indicator not only that the individual is now clinically sensitized, but the time at which it occurs is a reflection of the time taken for the clonal expansion of specific T cells that occurs during the generation of clinical sensitization. The proportion of individuals sensitized by increasing doses of DNCB showed a sigmoid log–dose response curve, with 100% being sensitized by 500 µg DNCB and above. There was an inverse relationship between the sensitizing dose and the time taken for the delayed flare to appear – thus it took a mean of 15.5 days to appear for those who received 62.5 or 125 µg but decreased for each group as the sensitizing dose increased, so that those who received 1000 µg showed the delayed flare after only 8 days.¹⁴

Four weeks after sensitization the degree of reactivity was assessed by quantifying responses to challenge with four simultaneous eliciting doses: 3.125, 6.25, 12.5 and 25 µg of DNCB. These challenges were applied to the other forearm on 1-cm paper discs (Al test patches; Epitest, Hyryla, Finland). The patches were left in place for 6 h but the responses were assessed at 48 h both clinically (grade 2 was an erythematous and palpably indurated response) and by thickness which was quantified by use of Harpenden skinfold callipers. The proportion of individuals in each sensitization group that gave grade 2 or stronger (i.e. definitely sensitized) responses followed the same sigmoid curve with log-sensitizing dose as was seen for the delayed flare. Thus the proportions sensitized rose from 8% by 62.5 µg through 62% by 125 µg, 83% by 250 µg to 100% by 500 µg and above. This reveals that there is a normal distribution to the human immune response – some people being low/weak responders, others being high/strong responders. Within each sensitization group the normal distribution was evident – with some people being weakly sensitized or apparently unsensitized, others being moderately reactive and yet others being strongly reactive. The responses (increase in skinfold thickness) plotted against log-challenge dose formed the lower or central portions of sigmoid dose–response curves. When the linear segments of the family of challenge dose–response curves were analysed by a generalized linear interactive model, the slopes of the curves were parallel¹³ (Fig. 1). This revealed that whether individuals were strongly or weakly sensitized, increasing the challenge dose by a given increment (doubling) was associated with a constant factor of augmentation of the response – the slope of the dose–response curve. By extrapolation of the regression lines it could be

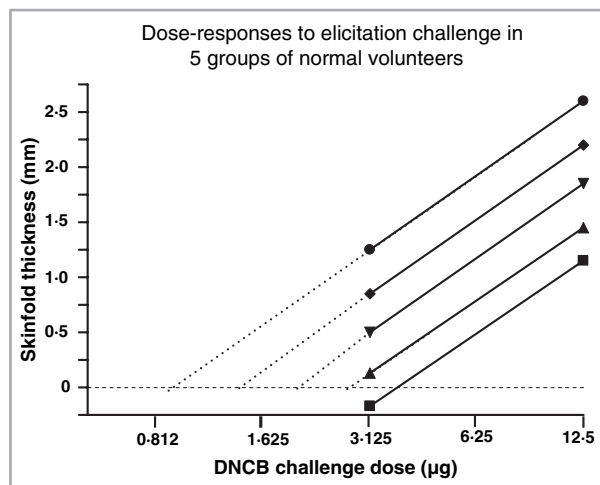


Fig 1. Five groups of healthy volunteers received initial sensitizing doses of 1000 (circles), 500 (diamonds), 250 (inverted triangles), 125 (triangles) or 62.5 µg of dinitrochlorobenzene (DNCB). Four weeks later they were challenged with four doses of DNCB (from 3.125 to 25 µg) and responses measured as thickness with skinfold callipers. The regression lines of the linear portion of the dose-response curve are plotted as solid lines. The calculated extrapolation to $y = 0$ (theoretical threshold elicitation dose) is plotted as dotted lines.

calculated/predicted that the lowest dose (threshold) of DNCB to which each of the groups might respond would be 3.9 µg for the group that received the lowest sensitizing dose and 3.0, 2.0, 1.4 and 0.8 µg, respectively, for the other groups in increasing order of sensitizing dose. These absolute doses applied on 1-cm diameter paper discs convert to 5, 3.8, 2.5, 1.8 and 1 µg cm⁻², respectively.

As mentioned above, one of the important variables determining elicitation responses is the duration of exposure – these DNCB challenge doses were applied only for 6 h, but in pilot work it was established that longer application periods were associated with much stronger responses. So it would be wrong to think that this represents an absolute lowest (threshold) dose which can detect the presence of sensitization. Finally, it was possible to represent the relationship between increasing sensitizing dose and degree of reactivity (strength of sensitization) by plotting on a log scale the sensitizing dose against the elicitation response for each group, represented as the skinfold thickness at the 12.5 µg challenge dose. This showed a log-linear increase in sensitization with increasing sensitizing dose (Fig. 2). The same dose-response relationships were examined in individuals who spontaneously developed three or more unrelated contact allergies (detected in the patch test clinic). These individuals were shown to be ‘high responders’ in that, as a group, they were sensitized to a much greater degree by a given dose of DNCB.¹⁵ This was shown to be a quantitative rather than a qualitative difference, namely, the high-responder end of the normal distribution, as individuals with a single contact sensitivity were intermediate in their sensitizability.

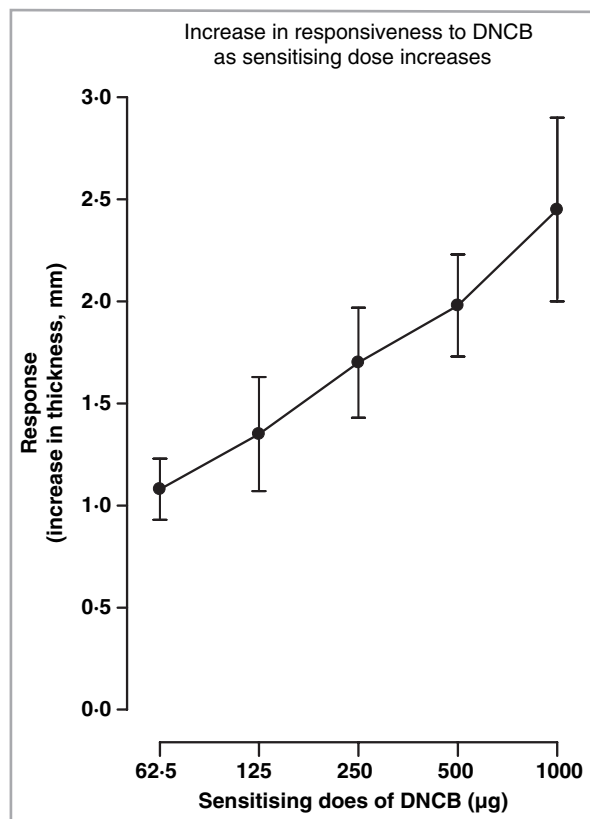


Fig 2. Increase in responsiveness to dinitrochlorobenzene (DNCB) as sensitizing dose increases. Reactivity for each of the five groups shown as response at challenge with 12.5 mg (measured as skinfold thickness) plotted against initial sensitizing dose. Results are shown as mean $\pm$ SEM. Reproduced from Friedmann¹⁴ with permission.

Dose per unit area

The above set of dose-response relationships was the result of applying varying doses of sensitizer to a constant area of skin – 3 cm diameter = 7.1 cm². Therefore the sensitizing doses can also be expressed as the dose per unit area – µg cm⁻² (Table 1). We next examined the effects of varying the area of the sensitizing application while maintaining a constant dose per unit area. Over a wide range of areas – from a circle 4.25 cm in diameter down to 1 cm – if the dose per unit area were kept constant there was no effect of area, even though the total doses varied by up to 10 fold (Table 1). It was only when the area was reduced to 3 mm (by application of a 3-mm diameter paper disc impregnated with DNCB at the desired concentration) that area had an effect and only 26% were sensitized compared with 93% by the same dose per unit area on a 1-cm paper disc. Overall, these results can be interpreted in relation to Langerhans cell (LC) numbers. As the mean density of LCs in forearm skin is about 750 mm⁻², an area 1-cm diameter (0.78 cm²) contains about 59 000 LCs.¹⁶ After application of DNCB up to 20% of LCs migrate down into the dermis¹⁷ – say 6000–12 000 LCs. This number of

Table 1 Relationship between proportions sensitized, area of application, and sensitizing dose of dinitrochlorobenzene (DNCB)

Row	Application site		Sensitizing dose		Number of subjects	Percentage sensitized
	Diameter (cm)	Area (cm ²)	Total (µg)	Concentration (µg cm ⁻²)		
1	3	7.1	1000	142	24	100
2	3	7.1	500	71	40	100
3	3	7.1	250	35.4	30	83
4	3	7.1	125	17.7	30	63
5	3	7.1	62.5	8.8	24	8
6	1.5	1.8	62.5	35.4	7	86
7	2.1	3.5	58	16.4	22	55
8	3	7.1	116	16.4	34	50
9	4.25	14.2	232	16.4	15	66
10	1 cm paper	0.8	30	38	28	93
11	3 mm paper	0.08	3	38	15	26

Data are from several studies with DNCB.^{13,21,23,51} The first five rows are the normal subjects from Figures 1 and 2. Row 6 gives the same dose per unit area as row 3. In rows 7–9 the areas were half and double the standard 7.1 cm² but the dose per unit area was constant. Reproduced from Friedmann⁵² with permission.

LCs is sufficient to sensitize a human fully. Also, for a given number of molecules of DNCB, the potency of sensitization is greater if few LCs present many molecules per cell rather than having the same number of molecules presented by many more LCs at fewer molecules per cell. Similar observations were made by Macatonia *et al.* in mice and the relationship between sensitizing dose and LCs reaching the lymph node defined in quantitative terms.¹⁸

There have not been many other studies attempting to quantify induction of contact sensitization in humans. Cardin *et al.* examined the sensitizing potency of Kathon [methylchloroisothiazolinone/methylisothiazolinone (MCI/MI)], applying the compound to normal volunteers in a range of concentrations from 5 to 20 p.p.m. (= 0.0005–0.002%).¹⁹ However, the Kathon was applied on occluded patches at actual doses per unit area of 1.14 or 2.9 µg cm⁻² depending on which patch system was used. Moreover, repeated patch applications were made thrice weekly for nine applications. Although the authors reported the results as ‘20 p.p.m. sensitized 2/45 volunteers’ it is clear that a cumulative dose of 20 µg cm⁻² was applied under occlusion over the 3 weeks. In a large American study 495 individuals were patch tested with a mix of MCI/MI at 250 p.p.m., which is equivalent to 0.025%. The material was applied on Finn chamber patches (presumably 7 mm) which were occluded for 48 h. While 13 individuals were already allergic, three of 495 were sensitized *de novo* by this concentration.²⁰ Although no indication was given of the quantity of MCI/MI applied to each patch it is possible to estimate the exposure. The normal quantity of allergen applied to a 7-mm Finn chamber is about 5–10 mg of white soft paraffin (WSP) (see below), or 10–15 µL of a solution. Ten microlitres of a 0.025% solution contains 2.5 µg of MCI/MI which, on a 7-mm Finn chamber, gives a dose per unit area of 6.4 µg cm⁻².

Factors affecting the responses to elicitation challenge

Factors affecting the response to elicitation challenges include the strength of sensitization, whether the immune response to this antigen has stabilized or is still maturing, the effective dose received by the immune system, local tissue factors such as body site and other chemical perturbing influences including irritancy and whether the challenge is encountered as a single or repeated contact.

Strength of sensitization

The principle factor that determines responses to elicitation challenges with contact sensitizers is the strength of sensitization – whether the individual is sensitized to a weak or a strong degree. This has been dealt with above.

Boosting effect of repeated challenge

During the early weeks and possibly months following initiation of sensitivity, repeated challenges with the sensitizer can boost the strength of sensitization and hence the reactivity. We showed that individuals who received the 50% effective sensitizing dose of DNCB (116 µg applied to a 3-cm diameter circle), who failed to give positive responses to the first elicitation challenge, gave very strong positive responses when exposed to a second challenge some time later.²¹ This indicated that the first challenge had actually boosted the degree of sensitization from a subclinical level to a strong degree of clinical allergic sensitivity. However, when people have been sensitized for a long time, as in the case of nickel sensitivity, repeated challenges are reproducibly the same and there is no sign that repeated or continued exposures augment

reactivity.²² The effect of repeated exposures to low concentrations of potential sensitizers such as those in personal products/cosmetics has yet to be clearly defined, but there is clearly the possibility that repeated exposures will induce and boost sensitization.

The dose of antigen that penetrates the epidermis

This is determined by the same variables as operated for the sensitizing dose – the stratum corneum permeability barrier, the vehicle, and the solubility and partition coefficient of the compound. In the same way as for the induction of sensitization, the dose per unit area is a crucial determinant of response to elicitation challenge. Thus, when a series of DNCB doses, as used by the author for the normal dose–response relationship studies above, was applied to 1-cm diameter paper discs (Al test) of area 0.78 cm², absolute doses of 3.125, 6.25, 12.5 and 25 µg were actually 3.98, 7.96, 15.91 and 31.83 µg cm⁻². This yielded dose-related responses, with all four challenges eliciting positive responses in strongly sensitized individuals, and the responses, measured as increase in thickness, showed a linear relationship with log of challenge dose^{13,15,23} (Fig. 1). In those studies, the DNCB challenges were applied for only 6 h for two reasons: DNCB is a strong irritant and if patch tests on DNCB-naïve individuals are left on for longer, then doses of 15.9 µg cm⁻² and above induce irritant responses which are erythematous but lack substantial oedema (thickness). Secondly, longer-duration applications evoked significantly stronger responses which, at those concentrations, may blister and from which it is impossible to obtain meaningful calliper readings. There are data showing that as duration of application of elicitation challenges increases, there is a reciprocal drop in the concentrations required to elicit positive responses. This was clearly shown for PPD.^{24,25} Similarly, it has been shown for nickel that 48-h application of elicitation challenges evokes a higher frequency of positive responses than 24-h application.²⁶ This observation reflects the fact that longer periods of application result in greater effective doses penetrating through the stratum corneum.

The crucial point about the above is that the DNCB was applied in an acetone solution which, when applied directly on to skin, evaporates and deposits all the DNCB on to the skin, or, when applied to a paper disc, the relative lipid solubility of DNCB ensures that it partitions out of the paper and into the stratum corneum. However, when DNCB or another lipid-soluble molecule is applied in a semisolid base such as WSP, the position is quite different. This was examined by applying different total quantities of nickel sulphate in WSP on 7-mm Finn chambers in nickel-allergic individuals.²² Thus, replicate chambers loaded with 2.5, 5, 10 and 15 mg of ointment containing 5% nickel sulphate were applied to the back in duplicate and the positive responses assessed at 48 h. Responses at chambers loaded with 2.5 mg were smaller and usually one grade weaker than those for chambers loaded with 5 mg. However, there were no differences in

the intensity scores of reactions for chambers loaded with 5 mg or more, although the area of the response for chambers loaded with 15 mg was sometimes greater. The probable explanations for these observations are as follows. Firstly, 2.5 mg is not sufficient to cover the chamber so the reaction is smaller. Five and 10 milligrams are contained by the chamber as a 'column' of ointment of different thicknesses or heights. Fifteen milligrams filled the chambers to the brim and even spilled over, producing slightly larger areas of application than the size of the chambers, but the thickness/height of the column of ointment may also have been greater than for the other loading quantities. However, the crucial consideration is: what is happening at the skin surface? A number of molecules of the antigen will diffuse into the stratum corneum, the number being determined by the concentration in the vehicle and the partition coefficients and relative solubilities of the substance in the vehicle and the stratum corneum. The loss of substance from the vehicle into the epidermis will lower the concentration of the substance in the vehicle in a narrow zone where it is in contact with the skin, but a steady state will be reached leaving much of the substance still in solution in the vehicle. So although 5, 10 or 15 mg of a 5% concentration was applied in columns of increasing heights, the same number of molecules penetrate into the epidermis from each column – hence the reactions of equal intensity. Whereas, if all the nickel in 5, 10 or 15 mg had been deposited on to the skin surface in a volatile solvent that left all the solute on the surface, then the effect would have been very different and would have given a dose–response curve. The above observation is of critical importance in the patch test clinic as it shows that as long as an adequate quantity of the ointment containing the antigen is applied, reproducible results will be obtained. The corollary is that workers attempting to demonstrate dose–response relationships should not apply the compound under investigation in a nonvolatile vehicle as, although dose–response curves can be obtained, the actual amounts delivered to the skin surface are uncertain.

Body site

There are clear variations in reactivity at different body sites. Thus forearm skin is significantly less responsive to inflammatory challenges including contact hypersensitivity elicitation with nickel (Fig. 3)²² or ultraviolet-induced erythema.^{27,28} Also, measurement of irritant responses induced by dithranol showed significant site variations between the medial and lateral sides of the forearm.²⁹ When body sites were compared in repeated open application tests (ROATs), the lower arm was less responsive than the upper arm, while back skin was most reactive.³⁰ Importantly, recent exposure to ultraviolet radiation or the use of topical immunosuppressive agents on any body site can reduce responses at that site. These points all mean that performance of challenges on the forearm are less likely to yield positive results compared with other, more reactive sites.

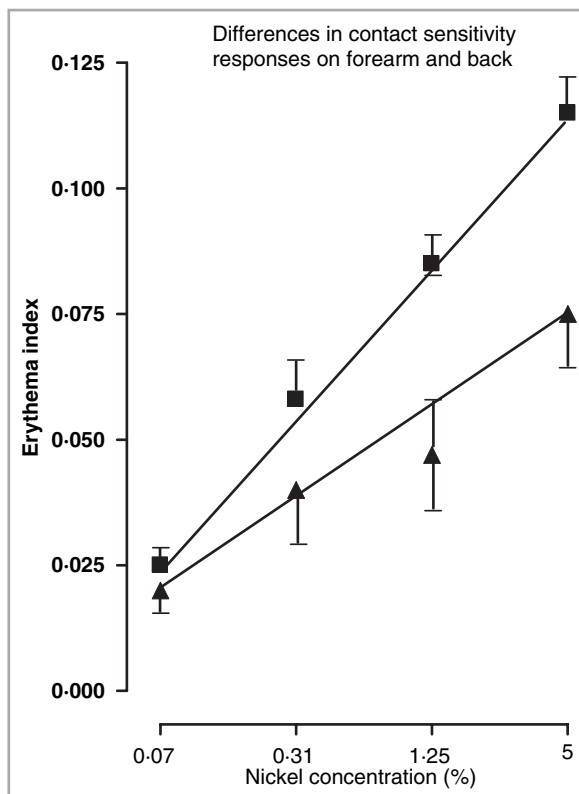


Fig 3. Differences in contact sensitivity responses on forearm and back. Erythema responses as measured with an erythema meter in response to challenge with increasing concentrations of nickel sulphate applied 48 h before. Squares = responses on back; triangles = responses on forearm. Results are shown as mean $\pm$ SEM. Reproduced from Memon and Friedmann²² with permission.

Augmentation of tissue responses either through irritant effects or by the presence of other sensitizers

In proportion to their potency as irritants or sensitizers, xenobiotics applied to the skin activate innate immune responses with induction of a wide range of cytokines and adhesion molecules and activation of DCs, all important for maximizing the efficiency of immune surveillance mechanisms.³¹ This tissue response to chemical perturbation primes the tissues so they can respond to lower concentrations of antigen. Thus, Shuster et al. observed that when a concentration of a contact sensitizer too low to elicit a clinical response was applied to sensitized recipients in combination with a subirritant concentration of an irritant such as SLS, positive patch test responses could be evoked;³² as mentioned above, SLS is not only an irritant but also a detergent and so will be likely to alter the absorption of the sensitizer. Similarly, if two allergens were mixed at concentrations below their elicitation thresholds and applied to individuals allergic to both allergens, the mixture elicited positive responses.³³ This has particular relevance to challenges performed with patients' own products, either in patch tests or in ROATs (see below). Grabbe et al. performed similar experiments in mice, and confirmed the

potentiation by irritancy. One hapten was applied at subeliciting doses in the presence of irritant concentrations of a different hapten to which the mice had not been sensitized,³⁴ thus allowing separation of irritant effects from possible detergent effects.

Repeated applications

Normal patch tests are designed to detect low degrees of sensitization – i.e. not to miss the presence of weak allergy. They are a highly artificial exposure situation using the maximal nonirritant concentration of a given allergen applied under occlusion, usually for 48 h. In personal products the concentrations of constituents such as preservatives and fragrances are often well below the concentrations used in standard patch tests. Therefore, in efforts to determine whether the actual real-life exposure concentrations are of clinical significance in elicitation of product-related allergic contact dermatitis, researchers have developed challenges designed to mimic real-life use of personal products by repeated application of the product to unoccluded skin – the so-called ROAT. Unfortunately this area of study is confused by two different agendas. The first deals with efforts to determine how best to establish the clinical relevance of allergic sensitivity detected by patch tests. The second and inter-related issue is the question of how strongly individuals are sensitized and what are the lowest concentrations of sensitizers to which they can react. The second agenda attempts to use the lowest levels that highly sensitized individuals can react to – the threshold concentrations for elicitation – to define the safe concentrations for common exposure, presuming that this extrapolates to sensitizing potency.

The ROAT has mainly been used to examine reactivity to fragrances,^{35–39} preservatives^{40–44} and metals,^{11,45,46} although there are reports using ROAT to examine reactivity to other allergens.^{47–49} Because quite varying techniques and approaches have been taken by different investigators, the present review will attempt to distil the main points that have emerged. Thus, individuals are usually selected to receive ROATs on the basis that they have already given positive responses to standard patch test challenge. The agent to be tested is made up in various vehicles including ethanol, water or cream, or the actual product itself is applied. Applications are normally made twice daily to areas of skin between 5 × 5 and 10 × 10 cm, usually on the volar forearm or antecubital fossa, for 7–14 days. As mentioned above, the volar forearm – the most common site of the ROAT application – is the least sensitive site! Application is usually discontinued once positive reactions develop. In some studies, if a given concentration fails to elicit a positive response after 7–14 days the concentration is increased and so on. The overall agreements are that strongly sensitized individuals react more quickly to lower concentrations of the relevant allergen – all of which is completely predictable from the experimental observations on the dose-response relationships of the allergic contact hypersensitivity response.⁵⁰

When expressed as the concentration or percentage that elicits a positive ROAT or patch test, the literature suggests

Table 2 The quantities of contact sensitizer used in repeated open application tests (ROATs) expressed as concentrations and doses per unit area

Reference	Allergen	ROAT conc (%)	Conc (p.p.m.)	Amount applied (μL)	Area (cm ²)	Dose per unit area per application (μg cm ⁻²)	No. of applications	Total dose per unit area (μg cm ⁻²)	% positive responders
Johansen et al. ³⁸	Lyrall	3	30 000	50	9	166.7	28	4667	89
		0.5	5000	50	9	27.8	28	778	61
Farm ⁴⁸	Colophony	20	200 000	5	3.142	318	9	2863	77
		1	10 000	5	3.142	15.9	9	143	31
		0.1	1000	5	3.142	1.6	9	14.3	8
Schnuch et al. ⁴³	MDBGN	0.025	250	500	25	5	28	140	62
		0.01	100	500	25	2	28	56	54
		0.005	50	500	25	1	28	28	33
Zachariae et al. ⁴⁴	MCI/MI	0.00075	7.5	50	9	0.047	56	2.63	56
		0.0002	2	50	9	0.0125	56	0.7	28
Johansen et al. ³⁷	Chloroatranol	0.0025	25	50	9	0.14	28	3.9	100
		0.0005	5	50	9	0.03	28	0.84	92

Data from five studies in which sufficient quantitative data were given to allow these calculations. Many authors report ROAT concentrations as p.p.m. In this table the concentrations, total doses applied and doses per unit area have been calculated to allow comparisons. MDBGN, methyl dibromoglutaronitrile; MCI/MI, methylchloroisothiazolinone/methylisothiazolinone.

that very low concentrations of allergens can elicit positive responses. Thus, for example, when a dilution series of colophony from 20% down to 0.1% was made in acetone/arachis oil and used for occluded patch tests and ROATs, four of 13 colophony-allergic individuals reacted to both challenges down to 1% (Table 2).⁴⁸ The antibacterial agent methyl dibromoglutaronitrile elicited positive ROATs in 13 of 39 allergic individuals at a concentration of 0.005% (50 p.p.m.).⁴³ Chloroatranol, an allergen found in the natural fragrance material 'oak moss absolute', was able to elicit positive ROATs in 12 of 13 oak moss-allergic patients at a concentration of 5 p.p.m. (0.0005%).³⁷ Lyrall [3-cyclohexene-1-carboxaldehyde, 4-(4-hydroxy-4-methyl pentyl)] evoked positive responses in two of 17 allergic patients at 29 p.p.m. and in nine of 18 at 662 p.p.m.³⁸ However, when the details of the test procedure are used to express these concentrations as doses per unit area an interesting picture emerges (Table 2). Johansen et al. have calculated doses in relation to area of application. They showed that applications of 15 μL of allergen at 29 or 662 p.p.m. to 0.8-cm Finn chamber discs gave doses per unit area of 0.9 and 20 μg cm⁻², respectively.³⁸ For the fragrance ingredient chloroatranol, the same group calculated that the most strongly sensitized individuals reacted in ROAT to applications of 0.03 μg cm⁻².³⁷ The individuals most strongly sensitized by DNCB were calculated above to be able to react in occluded patch tests to 1.02 μg cm⁻² although this was only applied for 6 h. Thus, there is a significant caveat relating to the interpretation of ROATs and patch tests which emerges from the failure of many authors to consider the absolute doses applied or the doses per unit area; some examples have been compiled into Table 2 to illustrate the point.

When a contact allergen is applied repeatedly to the skin there may be several factors which might allow lower concentrations to elicit positive responses. First, it may be that there

is an accumulation of the compound in the stratum corneum reservoir so the actual/effective concentration ends up significantly higher than that applied to the surface. It is often hard to extract sufficient data from the published reports, but a recent paper by Zachariae et al. expressed both diagnostic patch test and ROATs as dose per unit area and included all the relevant data. First, they showed that standard patch tests with MCI/MI at 0.01% (100 p.p.m.) applied as 3 μg cm⁻² elicited positive responses in all 28 allergic patients tested.⁴⁴ They then performed ROATs at two different concentrations, 2 and 7.5 p.p.m. applied for 4 weeks. Seven of 25 reacted to the first test and all these and an additional seven reacted to the higher dose. Although the daily doses were 0.025 and 0.094 μg cm⁻² the total doses applied over 4 weeks amounted to 0.7 and 2.63 μg cm⁻² (Table 2). If the total dose applied accumulates in the stratum corneum/epidermal reservoir, then these doses are much nearer the dose applied as a diagnostic patch test. Clearly not all of it will be available because of loss by shedding of squames. Second, the tissue priming effects of chemical perturbation (activation of DCs, upregulation of chemokines and adhesion molecules) resulting in locally enhanced immune surveillance may facilitate immune responses to lower concentrations of an antigen. These tissue perturbing effects may be the result of the contact allergen itself or other co-administered substances such as solvents or excipients. Also, the chronic stimulus of repeated applications in the ROAT may well generate stronger innate signals.

Assumptions about elicitation thresholds and exposure levels

One recurrent theme in the literature is the relationship between the concentrations of contact allergens to which sensitized individuals can react (elicitation) and the risks of naïve

individuals becoming sensitized by exposure to such concentrations. It is hard to be precise about the subject because there may be significant differences in the dose/concentration of sensitizer required to induce sensitization by a single exposure compared with that required to sensitize by multiple exposures. Thus, it is generally clear from experimental sensitization as above, that a potent sensitizer such as DNCB given as a single exposure will sensitize more than 50% of people at concentrations as low as $16.4 \mu\text{g cm}^{-2}$. Although a few individuals sensitized by this dose could respond to challenge doses as low as $3.125 \mu\text{g}$ ($4 \mu\text{g cm}^{-2}$), individuals sensitized by 70 or $124 \mu\text{g cm}^{-2}$ could react to challenge with doses as low as $0.8 \mu\text{g}$ (applied on a 7-mm Finn chamber = $1.02 \mu\text{g cm}^{-2}$). These individuals were clinically very strongly allergic and these eliciting doses are not very different from the $2.63 \mu\text{g cm}^{-2}$ to which some of the MCI/MI- or fragrance-allergic individuals appear able to react. The hypothesis that emerges is that repeated doses of low concentrations may sensitize to a very high degree so that sensitized individuals can show elicitation responses at very low antigen doses in the region of a few p.p.m. Apart from the simple consideration of antigen potency, two other factors mentioned above could play an important role in potentiating the sensitizing effect of low concentrations of any agent: (i) repeated exposures will result in accumulation of the chemical in the stratum corneum/epidermal reservoir which would have the same effect as a single application at a much higher dose; and (ii) the tissue perturbation that follows repeated applications of low levels of chemicals would result in activation of innate immune responses including increased expression of cytokines and chemokines together with augmented recruitment of cells of the immune system. This would very likely have significant adjuvant effects on the potency of antigen-presenting DCs to present very low concentrations of the relevant sensitizer. These factors will be inextricably interlinked so distinguishing their contributions would require careful experiments in which the reciprocity of particular doses of sensitizer were compared with regard to activation of innate immune responses. Thus, for example, one application of $100 \mu\text{g}$ could be compared with 10 applications of $10 \mu\text{g}$. These will be factors that operate in real life and not just in artificial or experimental situations.

Concluding observations

The contact hypersensitivity response demonstrates the following characteristics of the dose–response relationships of the human immune system. (i) The magnitude of the immune response (degree of sensitization) is directly proportional to the log of the sensitizing dose, represented by the dose per unit area. (ii) The corollary is that the stronger the degree of sensitization, the lower the challenge dose of the sensitizer to which a sensitized individual will react. This is seen both with standard occluded patch test challenges and with ROATs. It is not surprising that there is a general correlation between the threshold concentrations for elicitation

with either method. (iii) Within any group, the responses exhibit a normal distribution with individuals who may react much more weakly or more strongly than the majority. (iv) The fact that there are individuals who respond to various contact sensitizers, mainly fragrance ingredients and preservatives, at the level of 5–10 p.p.m. indicates that they are sensitized to a high degree. There are two possible explanations for this: firstly, that these are exceptional 'high responders' who are sensitized by chemicals to which most people do not react; secondly, as the relevant sensitizers can induce such strong sensitization when they are normally encountered at low levels in personal products, this indicates that they are actually potent sensitizers. Furthermore, because the exposure concentration is low, it implies that repeated exposures to such low concentrations can still induce high degrees of sensitivity. Therefore future work should concentrate on two main areas: one is defining the dose–response relationships for sensitization by repeated applications of very low doses of antigen. The other is investigation of the effects of different times of application of elicitation challenge under both open and occluded conditions.

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