



Dose metrics in the acquisition of skin sensitization: Thresholds and importance of dose per unit area

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Abstract

Allergic contact dermatitis is a common occupational and environmental health problem and many hundreds of chemicals have been implicated as skin sensitizers. Sensitization is acquired following topical exposure to a contact allergen and induction of a cutaneous immune response of an appropriate magnitude. For effective assessment and management of human health risks there is a need to appreciate the dose metrics that drive the induction of skin sensitization. The available evidence suggests that under most normal conditions of exposure it is the dose per unit area of chemical that has over-riding impact on the effectiveness of sensitization. The exception to this rule is when the area of the application site drops below a certain critical level. Here we review in detail the evidence which supports dose per unit area as being the critical exposure metric in the induction of skin sensitization, and the mechanistic bases for this relationship.

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1. Introduction

Skin sensitization is acquired following topical exposure of an inherently susceptible individual to an appropriate amount of a contact allergen. An appropriate amount in this context can be described operationally as being that required to induce a cutaneous immune response of the magnitude necessary for a degree of immunological priming (sensitization) that will result in a dermal inflammatory reaction being provoked following subsequent exposure.

The amount of chemical necessary to result in the acquisition of skin sensitization will be determined primarily by two factors: (a) the inherent potency of the contact allergen, and (b) the conditions of exposure (the site, matrix, extent, frequency and duration of exposure). These aspects of skin sensitization have been considered extensively elsewhere, particularly as they relate to risk assessment, and it is not the purpose of this article to review again these variables (Basketter et al., 2002, 2006; Felter et al., 2002, 2003; Gerberick et al., 2001; Kimber et al., 2001; Robinson et al., 2000). Rather, our purpose here is to explore how best to describe the relevant dosimetry for skin sensitization. This is not a trivial question since, in principle at least, there are a number of dose metrics that could be relevant, including for instance that of dose per unit body weight, a descriptor that frequently finds favour in studies of systemic toxicity.

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As a prelude to examination of relevant dosimetry it is helpful to survey briefly the immunobiological bases for the induction of skin sensitization.

2. The immunobiology of skin sensitization

The acquisition of skin sensitization is dependent upon the initiation of a cutaneous immune response of the appropriate quality, and of the necessary magnitude. The central event in sensitization is specific priming of the immune system such that there is a selective clonal expansion of allergen-reactive T lymphocytes. This quantitative increase in the frequency of allergen-specific T lymphocytes equips the immune system with the ability to mount an accelerated and more aggressive secondary immune response following subsequent encounter with the inducing chemical allergen (at the same or a distant skin site) such that a cutaneous inflammatory reaction is provoked. This local inflammatory reaction at the site of exposure is recognized clinically as allergic contact dermatitis. There are available elsewhere detailed reviews of the cellular and molecular mechanisms through which the induction and elicitation of contact allergy are achieved (Dearman and Kimber, 2003; Grabbe and Schwarz, 1998; Kimber et al., 2002; Kimber and Dearman, 2002, 2003).

The need here, however, is for a brief consideration of some of the events induced in the skin and regional lymph nodes that are required for the effective induction of skin sensitization. Of particular importance in this process are believed to be epidermal Langerhans cells (LC), and possibly other cutaneous dendritic cells (DC). The roles of these cells in the initiation of cutaneous immune responses include the recognition and processing of antigen encountered at skin surfaces, and the subsequent transport of that antigen to regional lymph nodes where it is presented to responsive T lymphocytes. In the case of skin sensitization, the inducing chemical allergen must gain access to the viable epidermis where, either directly or indirectly, it associates with native protein and is recognized by LC. These cells process internalized antigen and transport it from the skin, via afferent lymphatics, to draining lymph nodes. Following their mobilization and migration from the skin LC lose the properties of antigen processing cells and acquire instead the characteristics of immunostimulatory DC that are able effectively to present antigen to T lymphocytes in the paracortical regions of lymph nodes. In the absence of effective processing, delivery and presentation of antigen cutaneous immune responses, including skin sensitization, fail to develop. Comprehensive accounts of the roles played by epidermal LC in the acquisition of skin sensitization are available (Cumberbatch et al., 2000, 2003, 2005; Griffiths et al., 2005; Kimber et al., 1998, 2000). It is becoming clear that the biology of LC is extremely complex, as are the biological processes that serve to induce and regulate their mobilization and differentiation. There is also some controversy currently about whether for the effective acquisition of skin sensitization there is a manda-

tory requirement for LC, and evidence that LC can, in different circumstances, display either immunostimulatory or down-regulatory activity (Bennett et al., 2005; Kaplan et al., 2005; Kissenpfennig and Malissen, 2006; Romani et al., 2006). However, under normal conditions it can be assumed that LC play an important, and usually decisive, role in the development of cutaneous immune responses to chemical allergens, and that the behaviour of these cells, and their handling of chemical allergens, is likely to be directly relevant for considerations of thresholds, dose responses and dose metrics in skin sensitization. This will be explored later in this article.

Before turning our attention to an examination of skin sensitization dose metrics it is appropriate to reflect briefly on what is known of dose thresholds as they relate to the induction of skin sensitization and the elicitation of allergic contact dermatitis.

3. Thresholds

An issue about which there is frequently some uncertainty is that of thresholds of exposure. Specifically, the questions are whether there exist thresholds for the induction of skin sensitization in previously (immunologically) naïve subjects, and whether there are thresholds for the elicitation of contact allergic reactions in subjects that are already sensitized. The answer in both cases is yes (Basketter et al., 2002; Boukhman and Maibach, 2001; Kimber et al., 1999), and there are available methods to determine threshold values (Basketter et al., 1997, 2005). Although it is clear that thresholds exist, it is important to acknowledge that these will vary in absolute terms between subjects. Variation among subjects at the induction stage may be due to inter-individual differences in inherent or acquired susceptibility to sensitization and/or exposure conditions. Specifically, differences may result from variations in the extent or duration of exposure, the matrix in which an allergen is experienced, the condition of the skin at the site of contact, and possibly other factors that are largely of unknown cause, but which may be related to, or independent of, the specific contact allergen (Basketter et al., 2002; Friedmann, 1990; Moss et al., 1985). One important determinant of variations between individuals at the elicitation stage of contact allergy is the extent to which sensitization was acquired previously. Thus, there is recognition that, in general terms at least, as the dose of chemical used for sensitization is reduced (and, as a consequence, the degree of sensitization achieved is reduced also), then the higher the dose of the same chemical that will be required to elicit a dermal reaction in the sensitized subject (Friedmann, 1990; Friedmann et al., 1983; Hostynek and Maibach, 2004; Scott et al., 2002).

One other aspect of thresholds is worthy of brief consideration. The acquisition of skin sensitization, and the extent of sensitization achieved, reflects immunological priming. It is theoretically possible therefore that some level of priming might be achieved, but of insufficient vig-

our to result in frank sensitization (such that subsequent challenge would elicit a dermal inflammatory reaction) (Hostynek and Maibach, 2004; Kimber et al., 1999). This appears to be the case in practice also. In studies reported by Friedmann et al. (1990) it was found that subclinical levels of skin sensitization to the contact allergen 2,4-dinitrochlorobenzene (DNCB) could be induced in human volunteers. The approach taken was to expose a panel of normal subjects to a dose of DNCB that, on the basis of previous investigations, was known to result in the sensitization of approximately 50% of naïve individuals. When challenged some time thereafter, and consistent with expectations, half of those in which sensitization had been attempted failed to respond to challenge. When these unresponsive individuals were challenged a second time several weeks later some subjects displayed exaggerated skin reactions compared with control subjects that had previously received the initial challenge regimen alone as a sensitizing stimulus (Friedmann et al., 1990).

4. Dose metrics and the induction of skin sensitization

It is commonly the case that in experimental studies of skin sensitization topical exposure is recorded as a function of the concentration of chemical expressed as percentage weight per volume, or percentage volume per volume. The implicit assumption deriving from this is that a constant amount of chemical allergen would be expected to result in a similar level of sensitization, or frequency of sensitization, irrespective of exposure conditions, and the area of skin over which the chemical was encountered. In fact this is not the case, and the widely reported studies of human skin sensitization conducted by Peter Friedmann and his colleagues in the 1980s have provided strong evidence that the important parameter is the dose of chemical allergen per unit area of skin (Friedmann, 1990, 1996).

The experimental approach used by Friedmann and colleagues was to measure skin sensitization to DNCB among healthy volunteers. In these investigations the effectiveness of sensitization was evaluated as a function of the frequency of subjects displaying skin reactions (determined by measurement of induced increases in skin fold thickness) following challenge. Using this approach it was, for instance, possible to demonstrate that among naïve volunteers (subjects with no history of contact dermatitis or cutaneous inflammatory disease) the proportion of individuals sensitized showed a positive sigmoid relationship with the log of the sensitizing dose (Friedmann and Moss, 1985). Similar investigations were performed to examine the effect of concentration and surface area on the development of sensitization.

In one study naïve subjects were exposed topically to DNCB over a circular administration site of 3 cm diameter (equivalent to a surface area of 7.1 cm²). Subjects were exposed to different amounts of DNCB within that standard application site such that the concentrations of DNCB per unit area of skin received by different groups

of volunteers ranged from 142 µg/cm² to 8.8 µg/cm². As illustrated in Table 1 in those groups that received either 71 µg/cm² or 142 µg/cm² the sensitization rate following subsequent challenge was found to be 100%. However, exposure to lower concentrations of DNCB per unit area of skin were associated with progressively lower rates of sensitization, with 8.8 µg/cm² DNCB resulting in sensitization of only 8% of subjects (Table 1) (White et al., 1986).

Other experiments provided evidence that the observed differences in sensitization rate were not simply a function of the total amount of allergen delivered to the skin. Thus, for instance, as described above and illustrated in Table 1, it was shown that exposure to a total amount of DNCB of 62.5 µg, delivered over an area of 7.1 cm² and resulting in a dose per unit area of 8.8 µg/cm², was associated with a frequency of sensitization of just 8%. However, when the same total amount of DNCB was administered over a smaller surface area (of 1.8 cm²), resulting in an approximately 4-fold increase in the concentration of chemical per unit area (35.4 µg/cm²), then the rate of sensitization was found to be 83% (Friedmann, 1990; White et al., 1986) (Table 1). The interpretation is that the concentration per unit area of skin is of greater importance than the total amount of chemical experienced at the skin surface in driving sensitization.

Further evidence for this conclusion was provided by results deriving from a slightly different experiment with DNCB. In this case groups of volunteers were exposed to different total amounts of DNCB, but at sites of different diameter such that the concentration of DNCB per unit area of skin was the same in each group. Thus, for instance one group received a total of 58 µg of DNCB over an area of 2.1 cm² such as to provide a dose of 16.4 µg/cm². In another group the total amount of DNCB used for exposure was increased to 232 µg, but in this instance was applied over an area of 14.2 cm² to provide again a dose of 16.4 µg/cm². When these groups were challenged subsequently it was found that the rates of sensitization were comparable (Table 1) (Friedmann, 1990; White et al., 1986).

Collectively these data provide compelling evidence that, with DNCB at least, and over a wide range of conditions of exposure, it is the dose of chemical per unit area of skin, rather than total amount of chemical delivered, that is the key metric in terms of the effectiveness with which sensitization is acquired. However, in drawing this conclusion it is necessary to add one important caveat. It is that although the correlation between dose per unit area and the acquisition of skin sensitization appears to hold true over a wide range of exposure conditions (that is over a wide range of dose per unit areas) the relationship is not without limitations. Thus, as discussed previously (Friedmann, 1990) it would be entirely illogical to anticipate that under conditions of a constant dose per unit area the area of application could be reduced to an infinitesimal size without loss of sensitizing potency. In fact, it was found that the relationship between dose per unit area and the

Table 1
Impact of dose per unit area on the acquisition of skin sensitization in human volunteers to 2,4-dinitrochlorobenzene (DNCB)

Group	Application site		Sensitizing dose		No. of subjects	% Sensitized
	Diameter (cm)	Area (cm ²)	Total (μg)	Concentration (μg/cm ²)		
1	3	7.1	62.5	8.8	24	8
2	3	7.1	125	17.7	40	63
3	3	7.1	250	35.4	30	83
4	3	7.1	500	71	30	100
5	3	7.1	1000	142	8	100

Modified from Friedmann (1990) and White et al. (1986).

effectiveness of sensitization does not hold true when very small application sites are considered. Thus, it was shown that exposure of a group of subjects to a total amount of DNCB of 30 μg, delivered over an area of 0.8 cm² (giving a dose per unit area of 38 μg/cm²) resulted in a sensitization rate of approximately 93%. However, in another group of donors where the same dose per unit area was achieved (by applying a total of 3 μg of DNCB over an area of 0.08 cm²) the sensitization rate was found to be only 26% (Rees et al., 1990).

The possible reasons for the failure of dose per unit area to remain the important metric when exposure sites are very small will be considered later.

Persuasive as the evidence deriving from the studies performed with DNCB (and summarized above and in Table 1) is, there has been speculation that DNCB might represent a special case in this regard, or that for some other reason the dose per unit area metric may not apply in some other situations. This appears not to be the case, however, and evidence to support dose per unit area as the most important exposure determinant in driving the induction of skin sensitization derives from studies of human skin sensitization performed some 40 years ago by Albert Kligman (1966).

The relevant publication by Kligman describes heroic and comprehensive analyses of the various factors that may impact on the development of allergic contact dermatitis. Included in his considerations were the impact of inflammation and various aspects of exposure, including the importance of the area of exposure used for sensitization. For these studies Kligman used male prisoner volunteers and a variety of skin sensitizing chemicals (Kligman, 1966).

In common with the investigations of Friedmann and colleagues with DNCB, one focus of attention in the Kligman studies was the relative importance of dose per unit area and the total delivered dose. In one series of experiments the induction of skin sensitization to 4 contact allergens were examined: ammoniated mercury, monobenzyl ether of hydroquinone, nickel sulfate and neomycin sulfate. Each of the chemicals was applied to groups of volunteers over one of 3 application areas: 0.36 cm², 14 cm², or 56 cm². Although different induction concentrations were used for each chemical, for each individual sensitizer the amount applied per unit area of skin was kept constant by adjustment of the total amount applied. Outcomes were

measured subsequently as the frequency of sensitization among volunteers, judged by challenge-induced erythema.

Using this approach the first important observation was that when application sites with areas of 14 cm² and 56 cm² were compared there was no difference in the rate of sensitization, and this was found to hold true for each of the four sensitizers examined. That is, under these conditions of exposure it is the dose per unit area of chemical, rather than the total delivered amount, that is the important metric for the effectiveness of sensitization.

The second important observation was that, in common with the investigations of Rees et al. (1990) with DNCB, the relationship between dose per unit area and the acquisition of skin sensitization was lost when the area of the site used for sensitization became very small. Thus, in the studies of Kligman, when the application site was reduced to an area of 0.36 cm² (in effect a reduction of some 40-fold in area compared with the application site of 14 cm²) the frequency of sensitization to each of the 4 contact allergens was reduced, despite exposure to the same dose per unit area of skin.

There were two other outcomes of these investigations that are worthy of note. The first of these derives from a comparison with the previous data on the effectiveness of sensitization when chemicals were administered simultaneously over 4 adjacent 14 cm² sites on a single arm (giving a total application area of 56 cm²). It was found that the frequency of sensitization using the 4 separate, but adjacent, applications sites did not differ either from sensitization achieved with a single application site of 14 cm², or with a single site of 56 cm². Again, this was true with each of the 4 contact allergens used. The conclusion drawn is that those 4 sites accomplish no more in driving sensitization than does one site of the same area, or one site of the cumulative area. Again these data support the importance under these conditions of dose per unit area.

The second observation was based upon analysis of sensitization achieved by attempting to induce at a constant dose per unit area through two identical 14 cm² area sites on different arms. The results were very clear. With all 4 contact allergens, induction under these conditions was no more or less effective than was exposure via a single 14 cm² site, or via 4 adjacent 14 cm² sites on the same arm. Of course this result is not unexpected because what in effect has been done is to increase the area of exposure (albeit at two different sites) without impacting the dose

per unit area, and from previous comparisons it was clear that above a minimum application area the important metric is dose per unit area, rather than total area (Kligman, 1966).

Finally, there is one other inference to draw from Kligman's investigations. In separate comparative analyses, and using a different series of contact allergens, it was found that four sequential 48-h exposures to different, but adjacent, sites on one extremity were far more effective than were 4 single 48-h exposures of exactly the same type given sequentially to each of 4 different extremities (arms and legs). The conclusion drawn by Kligman at the time was that "*bombardment of the same node is superior*" ... to "*stimulation of four different nodal systems*" (Kligman, 1966). This is an interpretation that will be considered later.

Taken together, the studies of Kligman provide compelling support for the conclusions drawn from similar investigations with DNCB. Of particular moment is the fact that the importance of dose per unit area as the key metric (under most conditions of exposure) for the acquisition of sensitization is not peculiar to DNCB. Of equal significance is the observation that dose per unit area is not relevant only for very strong contact allergens such as DNCB. Of the 4 contact allergens used by Kligman (and based on sensitizing potency, rather than prevalence) one can be considered a strong sensitizer (monobenzyl ether of hydroquinone), one a moderate sensitizer (ammoniated mercury), and two comparatively weak sensitizers (nickel sulphate and neomycin sulphate).

Before leaving consideration of the data that support dose per unit area as being the important exposure metric for the induction of skin sensitization, it is appropriate to draw attention to some information available from guinea pig studies that support the same conclusion. In their comprehensive book on guinea pig testing for skin sensitization of 1970, Magnusson and Kligman described experiments performed with 3 contact allergens in which different volumes of chemical were applied to guinea pigs over application sites of different areas such that the dose per unit area remained constant (Magnusson and Kligman, 1970). The chemicals examined were DNCB, *p*-phenylenediamine (PPD) and *p*-nitroso-dimethyldimethylaniline (NDMA). The result was that, despite the use of different total exposures to chemical, in the case of all 3 allergens the incidence of sensitization was constant. These guinea pig data are therefore entirely consistent with the view deriving from human studies that, under most conditions of exposure, it is the dose per unit area, rather than the total delivered dose of chemical, that is the key determinant of the effectiveness with which sensitization is acquired.

5. Interpretation and conclusion

An appreciation of dose metrics in skin sensitization is of course of real importance in developing accurate risk assessments and effective risk management strategies.

The conclusion drawn from the available human and experimental animal data is therefore that under most normal conditions of exposure it is the dose per unit area of chemical, rather than the total delivered dose, that has the over-riding impact on the effectiveness of sensitization. The exception to this rule is, as illustrated with DNCB (Rees et al., 1990), and with other contact allergens (Kligman, 1966), is when the area of the application site drops below a certain critical level.

The interesting question that arises from this conclusion is the mechanistic basis for the importance of dose per unit area. It is likely that even 40 years ago Albert Kligman was correct when he discussed the effects of exposure metrics on the activation of lymph nodes draining the site of encounter with contact allergens (Kligman, 1966). It has been appreciated for many years that cutaneous immune responses, including contact sensitization, are initiated in peripheral lymph nodes draining sites of exposure to antigen (Davies et al., 1969; Oort and Turk, 1965; Wiest and Jung, 1970). More recently, the absolute requirement for draining lymph nodes for the induction of skin sensitization has been shown in lymphotoxin- α -deficient mice that lack peripheral lymph nodes and in which contact hypersensitivity fails to develop (Rennert et al., 2001). Lymph nodes are important because they provide the tissue matrix required to support the cellular and molecular interactions necessary for the effective induction of primary immune responses. Of particular importance in this regard in the context of contact sensitization is the presentation to responsive T lymphocytes of the chemical allergen transported to skin draining lymph nodes by epidermal LC (Cumberbatch et al., 2000, 2003, 2005; Griffiths et al., 2005; Kimber et al., 1998, 2000; Macatonia et al., 1986). As antigen-bearing LC become mobilized and migrate from the epidermis they mature into effective antigen presenting cells—and it is possible that skin sensitization is facilitated also by the transport of antigen to other DC already resident within draining lymph nodes that can similarly serve as immunostimulatory antigen presenting cells. In any event, the likelihood is that there is the need for a certain critical number of antigen-bearing DC reaching and being within draining lymph nodes. This is to provide signals of sufficient magnitude and intensity to overcome the threshold required for the triggering of a primary immune response. Translating this into the dose metric found to be relevant, in most instances, for the acquisition of skin sensitization, the interpretation is that there is a certain critical level of antigen (in this case chemical allergen), and LC availability for transport, required in the area of skin from which there is lymphatic drainage to a single lymph node, or to a single series of lymph nodes. As this critical mass increases then the threshold for effective stimulation is reached and exceeded, and then as the critical mass increases further then so will the vigour of induced immune responses (and the extent to which skin sensitization is acquired). The corollary is that if the same amount

of chemical is distributed over a larger area of skin, where lymphatic drainage is served by several lymph nodes, then the critical mass of available allergen per lymph node will be reduced, and the level of immune activation diminished accordingly.

If this is a correct interpretation then it is necessary to accommodate the observation that the primacy of dose per unit area as the relevant exposure metric holds for most normal conditions of exposure, but becomes irrelevant, or at least less relevant, when the area of the application site falls below a certain level (in practice, a very small area). The suggestion is that under such conditions, where the chemical allergen is available to only a very small area of skin, there will be insufficient numbers of primed LC transporting antigen to the draining lymph node to reach the threshold of activation necessary for an effective immune response.

One final point should be made. A rather different situation may pertain when induction involves repeated application of the same contact allergen at the same skin site, or at different skin sites that drain to a single lymph node, or a single series of lymph nodes. Here the primacy of dose per unit area may be confounded by this second, and potentially important, variable. If in these circumstances repeated exposures at the same site are closely consecutive (such that the first dose has not cleared before the second exposure) then the true dose per unit area is rather more difficult to ascertain, and calculation of the total dose may represent a more pragmatic solution. Although single sequential exposures to separate lymph nodes are still able to induce a cutaneous immune response, the acquisition of skin sensitization will be more vigorous when repeated applications are focused through a single lymph node.

Irrespective of the detailed mechanistic basis for the relevant dose metrics in skin sensitization there is clear evidence that in most normal circumstances it is dose per unit area that will dictate the effectiveness and extent of sensitization achieved.

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