

Dichloronitrobenzene: a reappraisal of its skin sensitization potential

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Unlike the closely related chemical dinitrochlorobenzene (DNCB), which is a very strong contact allergen, dichloronitrobenzene (DCNB) has been widely regarded as a non-allergen and, as such, a useful control for its strongly sensitizing counterpart. Nevertheless, it is still an organic chemical species readily capable of penetrating skin and, rather than being regarded as completely inert, it has even been suggested to react with the immune system in such a way that it induces specific tolerance to its chemical structure. We investigated whether DCNB was in reality a non-allergen, or rather merely a weak contact sensitizer. In both a rigorously conducted guinea pig maximization test and in a modified murine local lymph node assay, DCNB was demonstrated to possess weak sensitizing activity. On this basis, DCNB cannot be regarded as inert with respect to contact allergic potential, and is therefore inappropriate as a negative control in studies of skin sensitization.

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In 1935, Landsteiner & Jacobs (1) reported studies in which they had examined in guinea pigs the skin sensitizing activity of a series of chloro- and nitro-substituted benzenes. They demonstrated the essential link between chemical reactivity and skin sensitization, which was later expounded in much greater detail by Dupuis & Benezra (2). Whilst the highly reactive dinitrochlorobenzenes were shown to be potent skin sensitizers, the closely structurally similar chemicals, the dichloronitrobenzenes, were found to be devoid of skin sensitizing activity and, paralleling this, to lack obvious chemical reactivity. In subsequent investigations of contact hypersensitivity, DCNB has been regarded as a valuable negative control, either lacking skin sensitization potential or even behaving as a cause of specific immunological tolerance (3-6). However, the methods available to Landsteiner & Jacobs (1) for the detection of skin sensitizers were less effective than those that have now been in general use for many years (7, 8). Consequently, we have examined again, in both a modified murine local lymph node assay (9) and in the guinea pig maximization test (10), the ability of DCNB to induce skin sensitization.

Materials and Methods

Materials

2,4-dichloronitrobenzene (DCNB) (97% pure) was purchased from Aldrich UK (Gillingham, Dorset, UK). Freund's complete adjuvant was supplied by Difco, Michigan, USA. ³H-Methyl thymidine (product code TRA.310, specific activity 2 Ci/mMol, concentration 1 mCi/ml) was obtained from Amersham International, Amersham, Bucks, UK.

Guinea pig maximization test

The guinea pig maximization test (GPMT) was carried out essentially as originally described (10), but with minor modifications (11). Test concentrations were selected on the basis of preliminary irritation testing. In the main study, test guinea pigs (*n*=20) were treated with 0.5% w/v DCNB in 0.9% aqueous saline mixed as appropriate with Freund's complete adjuvant. The induction of sensitization was boosted 7 days later by an occluded 48 h patch of 1.0% w/v DCNB in acetone.

Control guinea pigs ($n=10$) received identical induction treatment with vehicles and adjuvant alone. Assessment of the degree of skin sensitization was made by challenge of test and control animals 21 days after the initiation of treatment, using 0.1% w/v DNCB in acetone in Finn Chambers (Epitest, Oy, Finland). These patches were removed after 24 h and reactions scored at 48 h and 72 h post-application for the presence of erythema and oedema, using a simple 0–3 scale. Reactions in the test guinea pigs were compared with those in the sham-treated controls. This result was compared with data obtained from a previously conducted GPMT on DNCB which was reported elsewhere (11).

Local lymph node assay

A modified version of the standard local lymph node assay protocol (12) was performed according to the method described by Kimber and Weisenberger (9). BALB/c strain mice (6–8 weeks; Harlan Olac, Bicester, UK) were used throughout. Groups of mice ($n=12$) received 50 μ l of 20% w/v DNCB dissolved in 4:1 v/v acetone: olive oil (AOO), or an equal volume of vehicle alone, on one shaved flank under an occluded patch. Patches were of lint, covered by latex rubber and secured in place with a Poroplast bandage (Scholl UK Ltd, London) and 1 cm tape. Patches were removed after 48 h. 5 days later, mice treated as described above were separated into groups of 3. Groups were then treated on the dorsum of both ears, daily for 3 consecutive days, with 25 μ l of various concentrations of

Table 1. Results obtained with DNCB in the modified local lymph node assay

Group no.	Day 0 patch	Day 5, 6, 7	No. of nodes	Total dpm	dpm/node
1	AOO	AOO	6	520	87
2	AOO	5% DNCB	6	716	119
3	AOO	10% DNCB	6	881	147
4	AOO	20% DNCB	6	1586	264
5	20% DNCB	AOO	6	685	114
6	20% DNCB	5% DNCB	6	1365	228
7	20% DNCB	10% DNCB	6	1272	212
8	20% DNCB	20% DNCB	6	2350	392

DNCB (5%, 10% and 20% w/v) in AOO, or an equal volume of vehicle alone. 3 days following the initiation of exposure, all mice were injected intravenously (via the tail vein) with 250 μ l of phosphate buffered saline containing 20 μ Ci of ^{3} H-methyl thymidine (^{3} HTdR). Mice were sacrificed 5 h later and the draining auricular lymph nodes excised and pooled for each experimental group. A single cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation through 200-mesh stainless steel gauze. Pooled LNC were washed $2\times$ with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18 h. Pellets were resuspended in 1 ml TCA and transferred to 10 ml of scintillation fluid (Optiphase MP; LKB). Incorporation of ^{3} HTdR was measured by β -scintillation counting as disintegrations per minute (dpm) for each experimental group. Results are recorded as stimulation indices relative to vehicle control values.

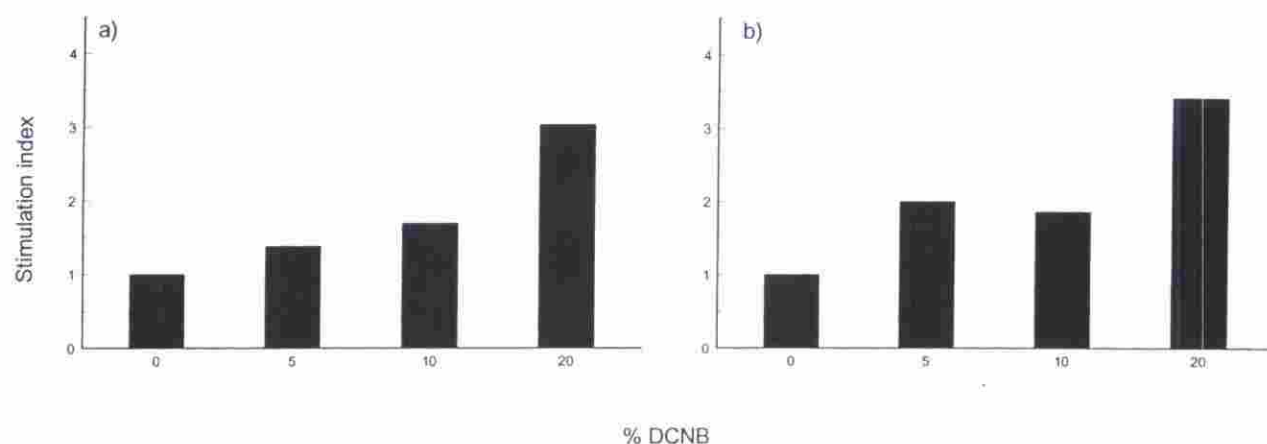


Fig. 1. Groups of mice ($n=12$) were exposed on one shaved flank under an occluded patch to 50 μ l of 20% w/v DNCB in AOO (b), or to an equal volume of vehicle alone (a). Patches were removed after 48 h. 5 days following 1st exposure, mice were separated into groups of 3 and treated on the dorsum of both ears daily, for 3 consecutive days, with 25 μ l of various concentrations of DNCB or with AOO alone. 1 day after the final application, mice were injected with radiolabelled thymidine and the mice sacrificed 5 h later. Draining auricular lymph nodes were isolated, pooled for each experimental group, and processed for β -scintillation counting. Results are expressed as a stimulation index relative to vehicle-treated controls.

Results

It has been reported previously that DNCB is a very potent skin sensitizer in the guinea pig (1), and this has been confirmed at the laboratory where the DCNB guinea pig work has been carried out (11). Considering the present work, in the GPMT, 2 of the 20 guinea pigs sensitized with DCNB displayed clear skin reactions (grade 1 erythema, but no oedema) following 24 h occluded patch challenge at the maximum non-irritant concentration. In contrast, none of the 10 control animals gave any skin reaction in response to an identical challenge. On the basis of the classification scheme originally outlined by Magnusson and Kligman, DCNB would thus be regarded as a mild skin sensitizer (10).

This result was investigated in a 2nd species, the mouse, which has often been used for skin sensitization studies. The detailed results are contained in Table 1. In the modified LLNA that was used in this study, 20% DCNB elicited stimulation indices (compared to the appropriate controls) of 3.43 or 3.05 in mice that had, or had not, been pre-exposed to the same chemical. Furthermore, there was a clear dose response to treatment with increasing concentrations of DCNB. This outcome would be regarded as positive evidence of sensitization using the normal criteria for interpretation of the LLNA (12). The results are presented graphically in Fig. 1.

Discussion

In their pioneering investigations of contact allergenicity, Landsteiner & Jacobs (1) found that, in contrast to 2,4-dinitrochlorobenzene and various dichlorodinitrobenzenes, 3 dichloronitrobenzenes failed to induce sensitization in guinea pigs. In the present study, we have found that, based on the results of 2 independent predictive tests, 2,4-dichloronitrobenzene does have some limited potential to cause contact sensitization. Thus, in both the guinea pig maximization test and a modified local lymph node assay, DCNB provoked weak responses. In the latter assay, when performed according to the standard protocol (12), chemicals are regarded as having the potential to cause skin sensitization if, at 1 or more test concentrations, a stimulation index of 3 greater is induced. In a previous report (9), it was found that pretreatment of mice on the flanks with the test chemical served to enhance local lymph node assay responses to some materials. No significant augmentation was observed here.

Although it is unlikely that DCNB would rep-

resent a risk to man with respect to skin sensitization, these data do suggest that this chemical does not provide an appropriate negative control for use in experimental studies of contact allergy. This does not, however, question the veracity of previous investigations in which DCNB has been regarded as immunologically inert or found to cause tolerance (3-6). It is well-established in human studies that a number of variables may influence the efficiency of skin sensitization, and it has been shown in experimental animals that a single chemical, DNTB (2,4-dinitrothiocyanobenzene), may act as a sensitizer or as an inducer of tolerance in different laboratories (13, 14). The conclusion drawn is that it is unwise to regard DCNB as being immunologically inactive and that its use as a universal negative control in comparative studies of contact allergenicity is inappropriate.

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