Toxicology and Applied Pharmacology **183,** 66–70 (2002)
doi:10.1006/taap.2002.9469

Insights into the Quantitative Relationship between Sensitization and Challenge for Allergic Contact Dermatitis Reactions

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Received November 6, 2001, accepted January 21, 2002

Insights into the Quantitative Relationship between Sensitization and Challenge for Allergic Contact Dermatitis Reactions. Scott, A. E., Kashon, M. L., Yucesoy, B., Luster, M. I., and Tinkle, S. S. (2002). *Toxicol. Appl. Pharmacol.* **183, 66–70.**

The ability of chemical or pharmaceutical agents to induce allergic contact dermatitis (ACD) is of major health and regulatory concern. As such, tests to identify their sensitizing capacity, such as the guinea pig maximization test and the more recently developed local lymph node assay, are broadly used. Ideally, for risk assessment it is useful to translate results from animal data into establishing safe or no-effect levels for occupational or environmental agents. This, of course, would require consideration of the quantitative relationships between sensitizing and challenge doses as well as other exposure conditions. In the present studies, we modeled two sensitizers, 2,4-dinitrochlorobenzene and squaric acid dibutyl ester, over a large range of concentrations using the LLNA and more traditional tests that measure both sensitization and elicitation responses. Both the sensitization and challenge phases provided similar dose–response curves, demonstrating a threshold followed by a shallow linear increase and eventual plateau at increasing doses. Extending earlier studies by P. S. Friedmann (1994, *Immunotoxicology and Immunopharmacology,* **pp. 589–616, Raven Press, New York) in humans, we observed that the minimum dose required to elicit sensitization or challenge was not static, but rather reflected a "sliding-scale." That is, as the sensitization dose was increased, the concentration required to elicit a challenge response was decreased. Correspondingly, as the challenge dose was increased, the dose required for sensitization was lessened. Taken together, these findings indicate that there is a need to consider dose–response relationships for sensitization and challenge in establishing minimum exposure levels for chemicals that cause ACD.**

Key Words: **allergic contact dermatitis and risk assessment; local lymph node assay and modeling.**

Allergic contact dermatitis (ACD), a cell-mediated immune reaction, occurs in two phases: induction and elicitation. During the induction phase, T cells' sensitization to the antigen occurs subclinically in the draining lymph nodes. The subsequent elicitation phase, initiated by further contact with the antigen and expansion of antigen-specific T cells, is characterized clinically by severe dermal inflammation including erythema and edema. As recently reviewed by Robinson *et al.* (2000), the dose–response relationship for both the induction and elicitation phases of ACD can be influenced by a variety of factors including the application-vehicle system (Heylings *et al.,* 1996; Robinson and Cruze, 1996), the number (Robinson *et al.,* 1991), timing (Bronaugh *et al.,* 1994; Landerson *et al.,* 2001), and duration (McFadden *et al.,* 1998) of exposures, underlying skin irritation (Kligman, 1966), and the use of occlusion (Funk and Maibach, 1994). The guinea pig maximization test (GPMT) is suited for hazard identification, but, as currently performed, provides little information for quantitative risk assessment (Gerberick *et al.,* 2001). The murine local lymph node assay (LLNA) has the unique advantage of providing estimates of relative skin sensitizing potency by using a constant proliferation or stimulation index across studies (i.e., SI 3 values) as an indication of a positive response (Basketter *et al.,* 1999). The LLNA has addressed many of the issues related to dose–response relationships and has allowed for the development of quantitative estimates of relative skin potency, based upon a concentration required to achieve an SI 3, that correlate with no observed effect levels (NOELs) established from human repeat patch tests. A central, but not often addressed, issue relevant in quantitative risk assessment for ACD is consideration of the dynamics between the response relationship for induction/elicitation as a function of their concentrations. For example, does increasing the concentration used for sensitization allow for elicitation to occur at a lower concentration or, alternatively, does increasing the concentration used for elicitation allow for providing sensitization at concentrations below an SI of 3? This variable was initially identified in studies with guinea pigs using the biocide Kathon (Chan *et al.,* 1983) where the authors demonstrated that the number of doses used for induction may be an important factor in deter-

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Abbreviations: LLNA, local lymph node assay; DNCB, 2,4-dinitrochlorobenzene; SADBE, squaric acid dibutyl ester; AOO, acetone-olive oil (4:1); ACD, allergic contact dermatitis; EC, effective concentration that produces a stimulation index (SI) of 3.

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mining the sensitizing potential of the chemical and that an induction/elicitation concentration dependency existed. This issue is of particular concern in the cosmetic industry where materials can be applied every day for long time periods resulting in a cumulative dose (Johansen *et al.,* 1998) or in the workplace where exposures to potential sensitizers may also occur at low levels over extended periods or, alternatively, at very high concentrations for very short exposure periods (Lushniak, 1995). This relationship also appears applicable in results derived from the LLNA as Kimber and Wisenberger (1991) demonstrated that preexposure to low levels of sensitizers can enhance the LLNA to all but the most potent sensitizers. Friedmann *et al.* (1990, 1994) has addressed a number of these quantitative issues in humans using skin thickness measurements following application of 2,4-dinitrochlorobenzene (DNCB). Although limited concentrations were tested, these studies demonstrated that both the incidence and severity of the allergic response can be affected by the dose of agent used for sensitization and elicitation.

The present investigations were undertaken to further elucidate the quantitative relationships between sensitization and elicitation concentrations and specifically how these variables affect the ability to elicit an ACD response in murine models. This was accomplished by comparing dose–response relationships for sensitization and elicitation over a broad range of concentrations for two established sensitizers, DNCB and squaric acid dibutyl ester (SADBE), using traditional (i.e., sensitization and elicitation) and LLNA protocols. DNCB and SADBE were selected as test agents as these sensitizers are currently being used in companion studies in human populations. Modeling of these experimental data indicated that the doses of antigen required to establish ACD are influenced by the quantitative relationship between the sensitizing and the challenge doses.

MATERIALS AND METHODS

Animals and chemicals. All animal procedures were performed in compliance with AAALAC approved guidelines for the humane treatment of laboratory animals. Six- to eight-week-old C57BL6, female mice were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate for 5–7 days. Animals were maintained in groups of five with controlled lighting on 12-h cycles and provided fresh water and food *ad libitum*. SADBE, DNCB, acetone, and olive oil were purchased from Sigma Chemical Co. (St. Louis, MO).

LLNA. The LLNA was performed with minimal modification of previously described protocols except that C57BL6 mice were employed *in lieu* of CBA (Kimber and Weisenberger, 1989; Scholes *et al.,* 1992; Loveless *et al.,* 1996). Briefly, varying doses of the test chemicals or a vehicle control (AOO; acetone:olive oil in a 4:1 ratio) in 25-μl volumes were applied to equal areas of the dorsum of each ear on days 1, 2, and 3. On day 5, mice were injected via the tail vein with 20 μCi of tritiated thymidine ([^{3}H]TdR; specific activity, 2 Ci/mmol) contained in 0.2 ml of RPMI. Five hours following injection, mice were sacrificed and the draining auricular lymph nodes removed. Lymph nodes from each animal were pooled. Single cell suspensions were prepared, proteins were precipitated with TCA, and incorporation of [^{3}H]TdR was determined. Stimulation indices (SI) for each experimental group were determined as the increase in mean [^{3}H]TdR incorporation in counts per minute (cpm) relative to vehicle controls.

Sensitization and elicitation procedures. Mice were sensitized and challenged by application of the test materials to either the dorsum of the ears or the flank folds. Both exposure routes resulted in comparable responses. For sensitization, mice were treated daily for 3 days with various sensitizing doses of DNCB or SADBE (0.01 to 1.0%) in a volume of 25 μl. An equal volume of the vehicle was used as the control. On days 8, 9, and 10, mice were challenged with 25 μl of 0.125% DNCB on each ear. Ear thickness was measured on day 12 using micrometers. For flank fold challenge, mice were exposed to the test chemical on one fold on day 12 and skin thickness measures were taken on days 14 and 15. An additional study was conducted where mice were challenged with 0.01 and 0.5% concentrations of DNCB on the left and right flank skin, respectively, on days 9, 10, and 11, and flank skin thickness was measured on days 13 and 14.

Statistical analysis. All analyses were performed using SAS (Sas Institute, Cary, NC). The LLNA results were analyzed using nonlinear regression analysis utilizing the natural logarithm of the cpm from the LLNA. A series of nonlinear models was fit and the best fitting model was selected using a likelihood ratio test (Slob, 1999; van Och *et al.,* 2000). The concentration of test chemical required to induce a threefold stimulation of proliferation (EC3) was estimated as follows: Following the determination of the nonlinear regression equation, the vehicle response was established as the number of cpm at the *y* intercept. This value was then multiplied by 3 to obtain a stimulation index representing a threefold increase in cpm (SI 3). The EC3 was determined by extrapolating the value of the SI 3 back to the regression line to determine the concentration of chemical that would induce this threefold increase in cpm. Confidence intervals bounding the estimated EC3 were established using semi-parametric bootstrapping techniques. The semi-parametric bootstrap utilizes nonparametric resampling of residuals from a fitted parametric model (Carpenter and Bithell, 2000). The residuals from the original analysis were resampled and used to generate 500 bootstrap data sets using the parameter estimates from the original analyses. Nonlinear regression analysis was then performed on each bootstrap data set and a new estimate of the EC3 was generated. Ninety percent confidence intervals around the estimated EC3 were determined using the 5th and 95th percentile values from the 500 bootstrap estimates (Carpenter and Bithell, 2000).

Sensitization–challenge experiments were analyzed using analyses of variance. In the case where ear thickness was measured only once following application of the challenge dose, a general linear model analysis of covariance was utilized (PROC GLM), and the prechallenge skin thickness measure was incorporated as a covariate. In the cases where multiple skin thickness measures were taken following application of the challenge dose, mixed model analyses of covariance (PROC MIXED) with repeated measures over time were utilized. Specific mean square error terms were designated to appropriately test fixed effects, and the prechallenge skin thickness measure was used as a covariate.

RESULTS AND DISCUSSION

Several preliminary studies were conducted to help identify quantitative dose–response relationships between sensitization and challenge. Initially, dose–response studies were conducted with DNCB and SADBE in the LLNA to establish the shape of the dose–response curve for sensitization. Figures 1A and 1B show scatter plots along with fitted nonlinear regression lines for the LLNA data using DNCB and SADBE, and their EC3 values are established. The point estimate of the EC3 value for DNCB was 0.057% with a 90% bootstrap confidence interval of 0.0179% for the lower limit, and 0.1077% for the upper limit. The EC3 point estimate for SADBE was 0.13% with a 90% bootstrap confidence interval ranging from 0.0910 to

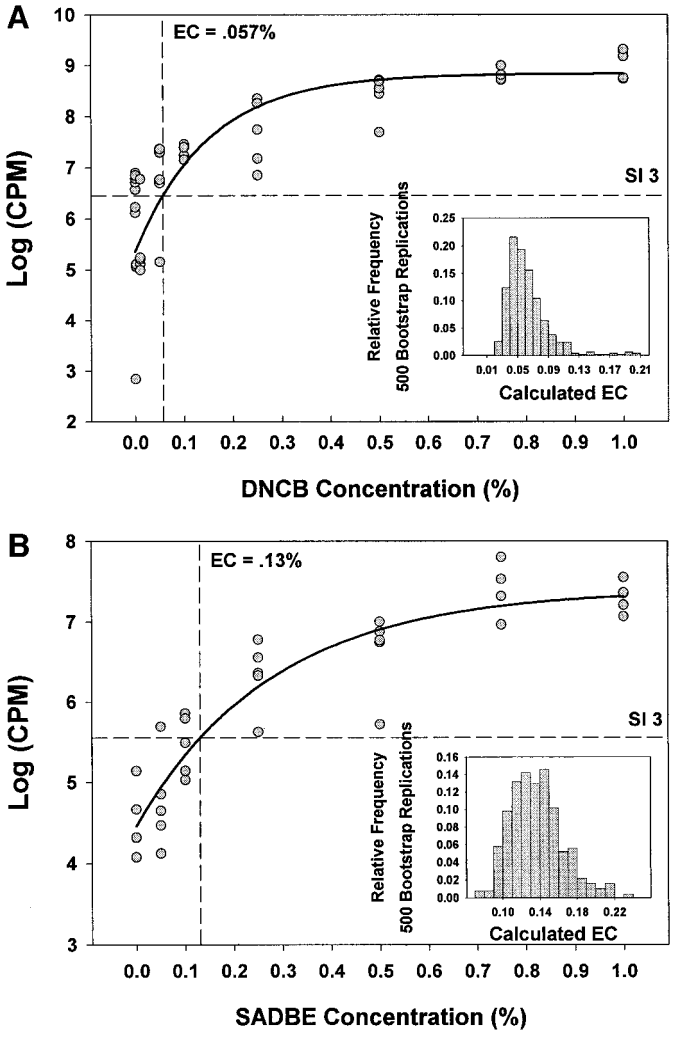

FIG. 1. LLNA results for DNCB and SADBE. Mice ($n = 5$ per group) were treated on the dorsum of each ear with 25 μl of chemical or vehicle daily for 3 days, rested on day 4, injected with [^{3}H]TdR on day 5, and sacrificed 5 h later. The LLNA was performed as described under Materials and Methods. EC3 point estimates and confidence intervals for DNCB are 0.057% with 90% bootstrap CI (0.0179%, 0.1077%). SADBE estimates are 0.13% with 90% bootstrap CI (0.091%, 0.1826%).

0.1826%. The dose–response curve obtained with both chemicals revealed a gradual increase before reaching a plateau at the higher concentrations examined. As shown in Fig. 2, ear thickness in response to a challenge dose of 0.125% DNCB, the highest concentration which failed to induce irritation, increased as a function of the sensitizing concentration. The shape of the dose–response curve was similar whether the sensitization dose was applied to the ear or to the flank. The dose–response curve was linear above sensitization concentrations of 0.1% and showed a clear threshold at concentrations below the EC3. The biological hypotheses for the threshold response for sensitization and elicitation have been recently reviewed (Kimber *et al.*, 1999).

Given that both the LLNA and the sensitization–challenge tests were dose responsive, it was of interest to determine if an interaction existed between the sensitization and challenge doses. Mice were sensitized to concentrations of DNCB by applying the chemical to the dorsum of the ear and challenging on the flank. Skin thickness at the challenge site was recorded at 48 and 72 h following the last challenge (Fig. 3). These data reveal a number of noteworthy quantitative relationships. First, at the 48-h time point, which represents the maximum response, the ability to discriminate differences in the intensity of the skin response, as a function of the challenge dose, was most discernible at sensitization concentrations approximating the EC3 (see Fig. 3A). Further, given a constant challenge dose, the dermal response reached a plateau as a function of the sensitization concentration That is, at sensitization concentrations above the EC3, the vigor of the skin response to a given challenge concentration is independent of the sensitization concentration. Although not as evident, these relationships were also present when skin thickness measurements were taken at the 72-h time point. At this time, the differences in skin thickness associated with challenge doses were minimal and inflammation was present only at the highest challenge concentration tested. This obviously reflects the recovery process, but also indicates that the duration of the inflammatory response is related to the concentration of the challenge.

When the interaction between the sensitization and challenge concentrations was examined at sensitizing concentrations near the established EC3, it was observed that the concentration required to induce sensitization varied depending upon the challenge concentrations. This is demonstrated in Figs. 3A′ and 3B′ when focusing on the EC3 region where concentrations sufficient for sensitization first appear. Even at

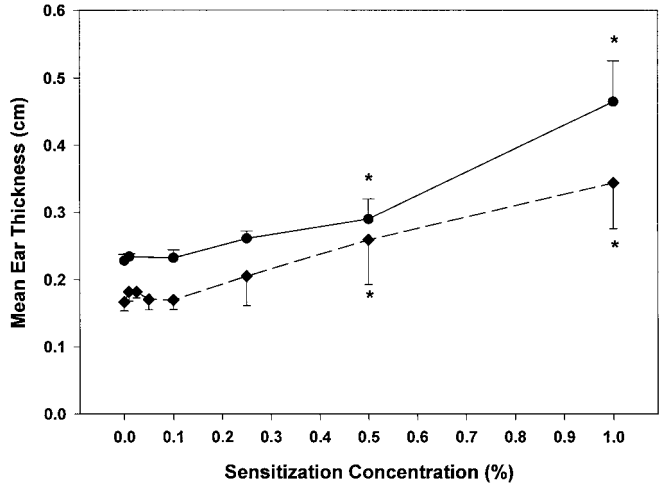

FIG. 2. Dermal response after sensitization and challenge. Mice ($n = 5$ per group) were sensitized on the dorsum of each pinna (●) or on either flank (◆) with 25 μl of DNCB daily for 3 consecutive days with concentrations of sensitizing agent. On days 8, 9, and 10, the mice were challenged on the other pinna with 25 μl of 0.125% DNCB and ear thickness was measured on day 12. Asterisks indicate a significant increase above control levels ($P < 0.05$).

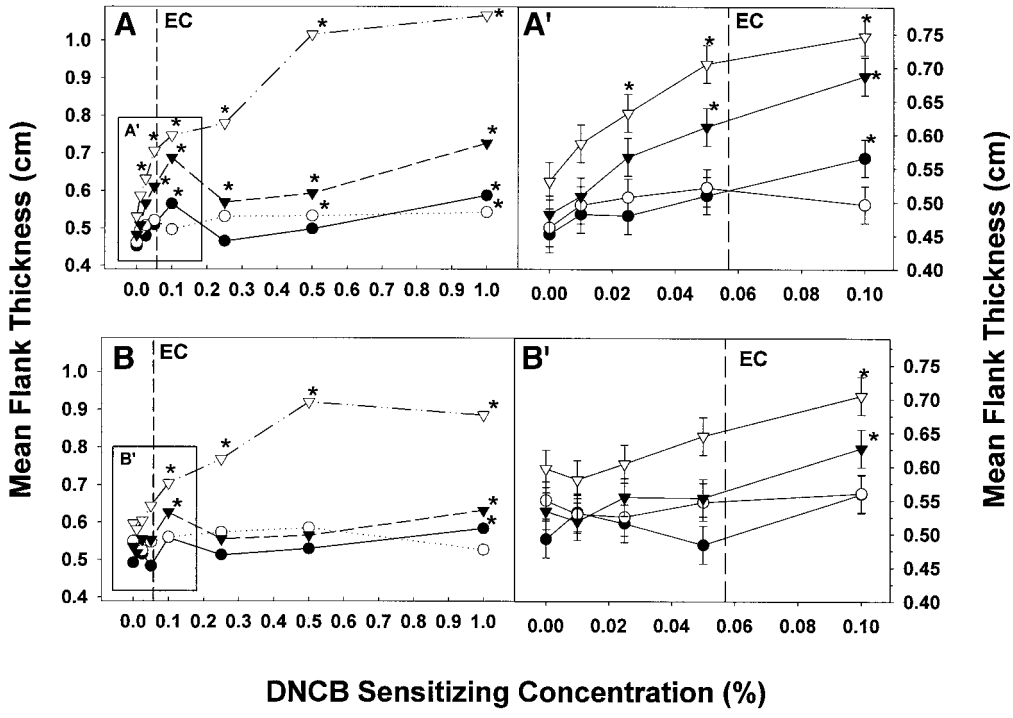

FIG. 3. Quantitative relationship between sensitization and challenge. Mice (5 per group) were sensitized on the dorsum of the pinna as described in Fig. 2. After 12 days, mice were challenged on the flankfold area with 0.01 (●), 0.05 (○), 0.1 (▼), or 0.5% (△) DNCB as indicated under Materials and Methods. Flank fold thickness was measured 48 (A) and 72 (B) h later. A′ and B′ represent enlargements of the indicated areas. Asterisks indicate a significant pairwise difference between the vehicle sensitization dose group at the same concentration of challenge dose.

concentrations below that estimated to be the EC3, a successful challenge dose could be obtained provided the challenge dose was high (Figs. 3A and A′). Correspondingly, by increasing the sensitization concentration, one can effectively lower the concentration required to induce a measurable response.

The dose–response interactions between sensitization and challenge concentrations are not unique to a specific exposure paradigm or chemical sensitizer. A similar relationship, as described for DNCB, was observed utilizing SADBE as a sensitizer following a different exposure paradigm (flank fold sensitization–flank fold challenge; Fig. 4). When two different challenge concentrations were compared over a range of sensitization doses, sensitization occurred at lower concentrations in the group given the higher challenge. Further, the shape of the dose–response curve for sensitization to SADBE was similar to that seen for the LLNA (Fig. 1B), in that it was linear at low concentrations and reached a plateau at higher concentrations. This was similar to the results observed with DNCB for the flank fold measurements.

In conclusion, it is well established that the LLNA serves as a reliable means to screen chemicals for their sensitizing potential as it provides an estimate of the minimum sensitizing concentration, defined as the SI 3 value, and a means for potency comparisons between different agents. However, to conduct quantitative risk assessment and allow establishment of NOELs or NOAELs, additional factors need to be considered. One such factor is the need to establish a model that accounts for the influence between the sensitization and the elicitation doses. The ability of high concentrations of antigens to elicit a skin response in animals that are sensitized at low

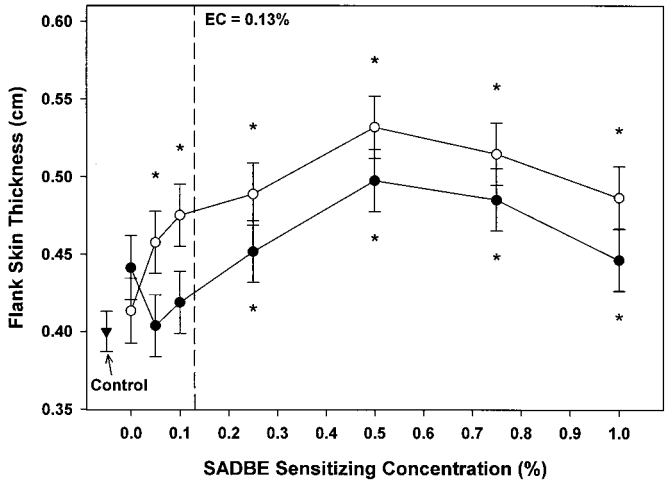

FIG. 4. Skin thickness responses in mice sensitized and challenged with SADBE. Flank skin thickness was determined following sensitization and challenge with SADBE or vehicle control as described in Fig. 3. Mice were challenged with 0.1% (●) SADBE on one flank and 0.5% (○) SADBE on the opposite flank on day 12. Data represent skin thickness 24 h later. Asterisks indicate a significant increase above vehicle controls ($P < 0.05$).

concentrations (i.e., below the SI 3) has interesting applications for quantitative risk assessment since it implies that a "safe exposure level" will be a function not only of the relative potency of the sensitizer but also of the dose–response relationship between sensitization and elicitation. Other factors and variables, such as frequency of exposure and biological half-life, will also need to be considered. Current studies are focused on determining whether similar quantitative relationships exist in humans using a similar experimental design and test agents.

ACKNOWLEDGMENTS

The authors thank Drs. Frank Gerberick and Melanie Flint for their helpful comments.

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