

Determination of In Vivo Dose Response and Allergen-Specific T Cells in Subjects Contact-Sensitized to Squaric Acid Dibutyl Ester

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Background: Squaric acid dibutyl ester (SADBE) is a known contact sensitizer, but dose-response data are not defined.

Objective: To determine the relationship between sensitization dose and contact hypersensitivity (CHS) response to SADBE in human volunteers. The study also aimed to investigate whether SADBE-reactive blood T cells could be detected using ex vivo mature dendritic cells (DCs) as antigen-presenting cells.

Method: Forty healthy volunteers were sensitized to either 12.5, 25, 50, or 250 µg of SADBE in a 48 µL volume. This was followed by elicitation 2 weeks later with five doses (0, 0.2, 2, 20, and 200 µg in 20 µL). An additional 10 subjects received the elicitation doses without prior sensitization. Blood samples obtained after sensitization were purified into T cells and mature DCs.

Results: A direct relationship between sensitization dose and in vivo CHS response was observed. The SADBE dose that effectively sensitized 50% of the population (ED₅₀) was 22 µg/cm². Significant SADBE-specific T-cell proliferation in vitro was not observed 2 weeks after sensitization but became evident after elicitation.

Conclusion: This study establishes the in vivo dose-response characteristics of immune reactivity to SADBE and antigen-specific T-cell reactivity.

SQUARIC ACID DIBUTYL ESTER (SADBE, Sigma Aldrich, St. Louis, MO) is a known contact sensitizer. It is an industrial stabilizer used in photographic emulsions for its antifog properties.¹ Similar to dinitrochlorobenzene (DNCB), SADBE is used in topical immunotherapy for alopecia areata²⁻⁵ and verrucae.^{4,6} It is also used in research studies involving the induction of T-cell-mediated responses.⁷ Dose-response data exist for DNCB and have contributed to a greater understanding of contact sensitization in humans.⁸ Friedmann and colleagues demonstrated the DNCB dose capable of sensitizing 50% of the population (ED₅₀) to be 16.4 µg/cm². As each allergen is unique, it is equally important to determine the dose-response characteristics of SADBE. A common treatment protocol is to sensitize patients with 2% SADBE in ethanol or acetone and then challenge with

0.1% to 3%.^{3,4,6} However, standardized data based on SADBE dose per area and resulting immune responses are lacking. Such data are essential because studies have clearly demonstrated that dose per unit area and total area of application are important determinants of contact sensitization.^{9,10} We present a method whereby effective sensitization and challenge doses of SADBE are categorized. This was accomplished by using one of four sensitization doses and eliciting contact hypersensitivity with a constant set of challenge doses. This method allowed us to determine the ED₅₀ of SADBE, using specific dose-per-area data. We further determined whether SADBE-specific T-cell proliferation could be measured in vitro with peripheral-blood mononuclear cells (PBMCs) of SADBE-sensitized subjects.

Materials and Methods

All procedures were approved by the Institutional Review Board of University Hospitals of Cleveland.

Subjects and Sensitization

Fifty healthy volunteers (18 females and 32 males) aged from 18 to 60 years (mean, 31 years), without any known prior exposure to SADBE or significant medical or

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dermatologic condition, were enrolled after written informed consent.

Forty of the subjects were randomly assigned to one of four groups that received 12.5, 25, 50, or 250 μg of SADBE. Aliquots of 48 μL of the SADBE (Sigma Aldrich, St. Louis, MO) solution were placed on 1.2 cm filter paper-lined Finn Chambers (Allerderm, Phoenix, AZ), resulting in sensitization doses of 11, 22, 44, and 222 $\mu\text{g}/\text{cm}^2$, respectively. The allergen patch was taped on the lower back or upper buttock area and was removed after 48 hours.

Elicitation

Two weeks later, the initial sensitization site was evaluated with the following clinical scoring system: 1 = no reaction; 2 = mild macular erythema; 3 = moderate erythema, occasionally with papules; 4 = strong erythematous reaction, including edematous vesicular changes; 5 = extreme or spreading erythema, including bullae or ulcerative changes. Baseline measurement of skinfold thickness (SFT) over five areas on the upper inner arm contralateral to the sensitization site was obtained via a micrometer (Mitutuyo, Tokyo, Japan). Each site was measured thrice and averaged. Elicitation was then performed with 0, 0.2, 2, 20, and 200 μg of SADBE. Aliquots of 20 μL of the respective solution were applied on 8 mm Finn Chambers, which were kept in place for 6 hours. Forty-eight hours later, the sites were evaluated with the clinical scoring system and by repeat measurement of SFT. Although both techniques were recorded to assess response, evaluation of SFT proved to be a more consistent measurement than erythema because our study population included subjects with Fitzpatrick types IV and V skin. The sum of the millimeter increase at each site was calculated to give an overall score for each subject. An additional 10 subjects served as an irritancy control group and underwent elicitation without prior sensitization. A response was considered positive, or the subject successfully sensitized, if the total millimeter increase in SFT was at least two standard deviations greater than the mean increase in SFT of the irritancy control group.

SADBE-Specific T-Cell Proliferation Assay

Two weeks after sensitization, 180 cc of blood were obtained from the subjects immediately before elicitation. In some volunteers, the blood draw was repeated after elicitation. T-cell/dendritic-cell cocultures with PBMCs were used. Dendritic cells (DCs) were generated from the

polystyrene adherent mononuclear cell population obtained from Ficoll separation and were cultured for 9 days in the presence of maturation cytokines (granulocyte-macrophage colony-stimulating factor, 10 ng/mL; interleukin (IL)-4, 10 ng/mL; IL-1 β , 10 ng/mL; IL-6, 20 ng/mL; tumor necrosis factor- α , 40 ng/mL; prostaglandin E_2 , 1 $\mu\text{g}/\text{mL}$). Autologous CD4^+ and CD8^+ T cells were positively selected from the nonadherent mononuclear cell fraction with Dynabeads (Invitrogen, Carlsbad, CA) CD4^+ and CD8^+ isolation kits. Twenty-four hours after isolation, T cells were frozen and stored in liquid nitrogen until day 8. On day 9, mature DCs were harvested and incubated with Roswell Park Memorial Institute (RPMI) medium alone, 1 mM SADBE, or 1 mM DNBS for 60 minutes at 37°C and washed with medium. Cocultures were plated at a responder to a stimulator ratio of 50:1 (10^5 T-cells to 2,000 DCs) and incubated at 37°C in 5% carbon dioxide for 5 days. In the final 18 to 20 hours of culture, 1 uCi of [^3H]-thymidine was added to each well. The cells were then harvested, and T-cell proliferation was measured by thymidine incorporation via liquid scintillation counting.

Analysis

The contact hypersensitivity (CHS) response of each sensitization group was expressed as mean $\pm$ standard error (SE), and the dose capable of sensitizing 50% of a population was determined. A stimulation ratio was calculated to assess the in vitro antigen-specific proliferative response for each peripheral blood sample, using the following formula:

$$\frac{\text{Mean CPM of T-cell + SADBE - treated mDC cultures}}{\text{Mean CPM of T-cell + mDC (untreated) cultures}}$$

(mDC=mature dendritic cells).

Results

Contact Hypersensitivity Response

Our data demonstrate that there is a direct relationship between sensitization dose of SADBE and CHS response (Fig 1). Using 11, 22, 44, and 222 $\mu\text{g}/\text{cm}^2$, we observed an increase in the average CHS response with increasing sensitizing dose. The mean increase in SFT ranged from less than a millimeter in the 11 $\mu\text{g}/\text{cm}^2$ sensitization group to nearly 6 mm in the 222 $\mu\text{g}/\text{cm}^2$ group. Increased pruritus and vesiculation were more evident with the two higher doses (44 and 222 $\mu\text{g}/\text{cm}^2$) than with the two lower doses. The irritancy control group showed a mean SFT

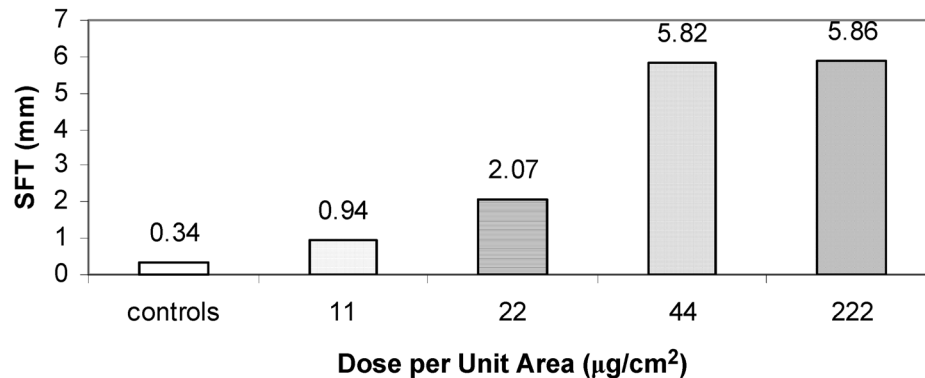


Figure 1. Squaric acid dibutyl ester elicitation reactions. Sensitization doses vs. mean increase in skin fold thickness (SFT).

increase of 0.34 ± 0.18 mm. Hence, an SFT increase of greater than 0.70 mm was considered a positive CHS response. The proportion of subjects within each group who were positive responders increased with increasing

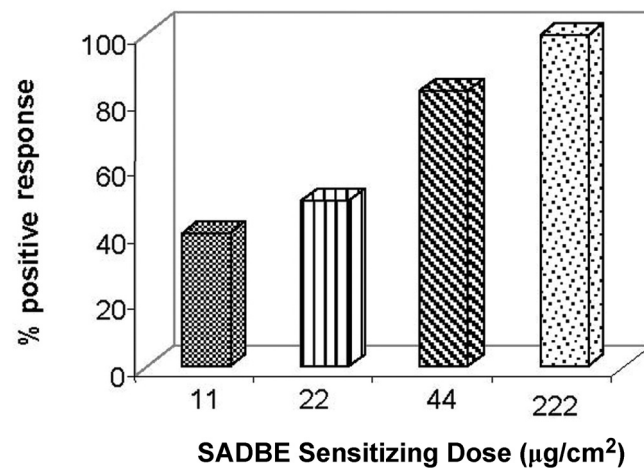


Figure 2. Percentage of subjects successfully sensitized to squaric acid dibutyl ester, increased with increasing sensitization dose.

sensitizing doses of SADBE (Fig 2). The ED_{50} (the dose that successfully sensitized 50% of subjects within a group) was found to be $22 \mu\text{g}/\text{cm}^2$ SADBE. When the CHS response of all positive responders was plotted against elicitation dose, it was found that the mean CHS response also increased with increasing elicitation dose (Fig 3). Likewise, we also observed that the higher the sensitizing dose, the lower the dose necessary to elicit a response upon challenge.

T-Cell Proliferation Assay

In vitro antigen-specific T-cell proliferation assays were initiated, using PBMCs from the highest-dose group, 2 weeks after sensitization; no significant SADBE-specific T-cell response was shown. Since nearly half of the subjects in this group had similar negative results, additional assays in the lower-dose groups were not performed at this time. Another set of T-cell proliferation assays were conducted from PBMCs obtained at later time points after elicitation. Three subjects from the high-dose group ($222 \mu\text{g}/\text{cm}^2$) and

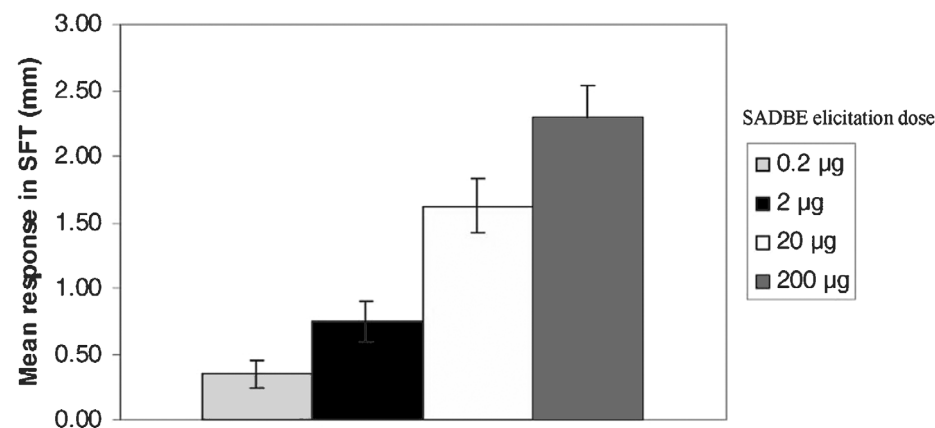


Figure 3. Elicitation response or increase in skinfold thickness (SFT) of all positive responders or subjects who were successfully sensitized, versus squaric acid dibutyl ester (SADBE) elicitation dose.

two subjects from the low-dose group ($11 \mu\text{g}/\text{cm}^2$) demonstrated significant SADBE-specific T-cell response at 10 months after elicitation. To determine if this T-cell response could be observed at a much earlier time point, PBMCs obtained 2 weeks after elicitation from a subject in the $44 \mu\text{g}/\text{cm}^2$ group were tested and likewise showed significant SADBE-specific T-cell proliferative response in vitro. Stimulation ratio data for these subjects are shown in Table 1.

Discussion

Although SADBE is used clinically^{2-6,11} as well as experimentally, this was the first study to demonstrate the in vivo dose-response characteristics of this allergen. Our results demonstrate a direct relationship between sensitization dose of SADBE and degree of CHS response. This was determined by using a model similar to previous in vivo studies on reactivity to DNCB, which is widely used to assess T-cell-mediated immunity.⁹ The SADBE dose capable of sensitizing 50% of a population (ie, the ED_{50}) provides useful information about the sensitizing potential of an allergen. We have shown that the ED_{50} was equivalent to $22 \mu\text{g}/\text{cm}^2$. This is higher than the ED_{50} of DNCB ($16.4 \mu\text{g}/\text{cm}^2$), indicating that SADBE has a relatively lower sensitizing potency. The highest SADBE sensitization dose in this study, $222 \mu\text{g}/\text{cm}^2$, resulted in a sensitization rate of 100%. At present, it is unclear whether clinicians are using doses that are similar or higher than this because doses are often inaccurately expressed in terms of concentration of the SADBE solution instead of actual micrograms per area of application. It must be noted that our study used healthy volunteers, and one could speculate that patients with immune aberrations (eg, atopic patients with recurrent warts) might necessitate higher doses for effective sensitization. In addition, occlusion, which enhances allergen penetration, is not

routinely performed in the clinical setting, which may affect sensitization rates.

Peripheral blood-derived DC and coculture systems were devised as assays to determine the mechanisms of contact sensitization without the need for skin samples, bone marrow aspirates, or cord blood samples.¹²⁻¹⁶ Although there may be significant variability from donor to donor, this is a simple method for obtaining the required cells from peripheral blood and provides a culture system with a sound mechanistic approach for the development of an in vitro method for predictive sensitization testing.¹⁷ In our study, PBMCs obtained before elicitation did not show significant SADBE-specific T-cell proliferation whereas they did reveal significant SADBE-specific T-cell responses after elicitation. These data may suggest that elicitation contributed more significantly to the observed T-cell proliferation than did sensitization dose although further studies with standardized time points and control subjects are needed to validate these results. In addition, the contribution of time alone, without allergen reexposure, to the T-cell response observed in vitro remains speculative.

Conclusion

We have established, for the first time, dose-specific data for effective sensitization with SADBE in healthy human volunteers. Furthermore, we have also demonstrated the feasibility of using an in vitro method to measure antigen-specific T-cell proliferation although its accuracy and validity need further testing.

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Table 1. Summary of Stimulation Ratios*

Sensitizing Dose ($\mu\text{g}/\text{cm}^2$)	Subject No.	Stimulation Ratio
222	7	1.90
	8	3.00
	4	2.50
44	10	4.90
11	2	3.71
	3	3.37

*Obtained from peripheral-blood mononuclear cells of selected subjects from the different sensitization dose groups.

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