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# The Noninvasive Mouse Ear Swelling Assay

## II. Testing the Contact Sensitizing Potency of Fragrances

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The Noninvasive Mouse Ear Swelling Assay. II. Testing the Contact Sensitizing Potency of Fragrances. THORNE, P. S., HAWK, C., KALISZEWSKI, S. D., AND GUINEY, P. D. (1991). *Fundam. Appl. Toxicol* **17**, 807–820. The noninvasive mouse ear swelling assay (MESA) for contact allergy testing was evaluated using fragrance components and complex fragrance mixtures. The test materials represented weak sensitizers and nonsensitizers. Two versions of the MESA were investigated. Both were noninvasive and utilized only topical abdominal dosing and ear challenge with single applications in BALB/cBy mice. The vit A MESA differed from the regular MESA only in that mice were maintained on a diet with 17-fold higher levels of vitamin A (vit A) acetate beginning 3 weeks prior to induction. Sensitization reactions were determined by measuring the mean increase in ear swelling over baseline at 24, 48 and 72 hr postexposure. Irritation dose–response curves facilitated choosing a high nonirritating challenge dose. Sensitization dose–response curves were developed for cinnamaldehyde (CINN) and a complex fragrance mixture, F-16. From these curves, the SD50 was determined. This value represents the dose which sensitized half the animals and serves to rank the potency of compounds for allergic contact dermatitis and to compare values among different assays. The SD50 for CINN was 21.6% while the $SD50^{vit\ A}$ for F-16 was 26.6%. The other fragrance, isoeugenol (ISOE), and fragrance mixtures, F-07 and F-22, were also found to be weak sensitizers in the MESA and vit A MESA. The results in the MESA for CINN and ISOE were in the range observed with guinea pig test protocols but showed that the MESA was more sensitive than human test protocols. Two of the fragrance mixtures tested in the MESA gave comparable results in the Buehler guinea pig assay. However, the third (F-22) was negative in the Buehler assay and the MESA, but positive in the vit A MESA. The results of this work with weak sensitizers and the companion study (Thorne *et al.*, 1991) with potent sensitizers at low doses illustrate that the noninvasive MESA is as sensitive as many standard guinea pig assays. In addition, it is easier and much less expensive to perform. The vit A MESA has the sensitivity and predictive power needed to test compounds and mixtures for contact sensitizing potency. 

An entire industry has evolved to perform the task of testing consumer products and their ingredients for the potential to induce allergic contact dermatitis (ACD). Therapeutic ingredients, preservatives, vehicles, and fragrances have been the most widely studied compounds, using dozens of assays, predominantly

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in guinea pigs and humans. Manufacturers of consumer products, such as health and beauty aids and home cleaning products, have a particular interest in testing fragrances for their ability to cause contact sensitization. Although there are about 8000 ingredients used in the cosmetics industry, fragrances and biocides stand out as the leading causes of ACD in these products (Eiermann *et al.*, 1982). Since it is common to have 100 million units of a product sold annually, and many millions of people receive daily exposure to these formulations, even weak sensitizers can lead to many cases of ACD. There are many applications for ACD testing, but one of the most important is in the development of product ingredients and formulations that are unlikely to produce immunologic sensitization and dermatitis in people who purchase the end product. ACD test protocols have been developed so as to err on the side of conservatism, with false positive determinations more likely than false negative results. Animal models for delayed-type hypersensitivity have most often employed the guinea pig because this species is tolerant of the restraint and handling associated with this testing, can be tested in a manner similar to human patch testing, and has generally given positive results when tested with known human sensitizers.

Recently however, interest has emerged in developing testing protocols in mice that could replace some or all of the testing currently performed in guinea pigs. Mice are far less expensive than guinea pigs and several promising models exist that yield quantitative data with considerably less effort than the guinea pig protocols. One such method is the mouse ear swelling assay (MESA) which has been tested with several invasive and noninvasive variations. Thus far, the attempts to validate the invasive MESA have met with variable success, as is reviewed in the companion paper (Thorne *et al.*, 1991). We determined that a noninvasive MESA effectively and efficiently identified ACD responses to a known sensitizer administered at low doses that do not elicit positive responses in other test assays. This protocol used topical dosing on the depilated abdomen followed by topical challenge to the ears 5 days later. Ear swelling, as determined with a micrometer, served to quantitate the delayed-type hypersensitivity responses. ACD responses followed linear dose–response curves when presented either as ear thickness increase or as percentage of the mice in each group exhibiting positive responses (percentage responding). The dose–response curve with percentage responding on the ordinate allowed determination of the predicted SD50 which could be used to rank the potency of compounds. This approach was used previously to compare the sensitizing potency of aliphatic and aromatic diisocyanates determined using the noninvasive MESA (Thorne *et al.*, 1987).

Several enhancements of the noninvasive MESA were tested in the companion paper (Thorne *et al.*, 1991). Three different triple dose protocols were not found to increase the sensitivity of the assay. However, supplementing the mouse diet with 255 IU/g vitamin A acetate (vit A) for 3 weeks prior to testing significantly increased the sensitivity of the assay as determined in studies using two dinitrohalobenzenes: dinitrofluorobenzene and dinitrochlorobenzene. In these studies ear swelling responses doubled and dose–response incidence curves were shifted significantly to the left.

In order to fully validate the noninvasive MESA and its modifications, it was necessary to go beyond tests using moderate and potent sensitizers at very low doses and perform tests using weak sensitizers and mixtures of weak sensitizers. In this paper we present the results of these tests using two fragrance constituents, cinnamaldehyde (CINN) and isoeugenol (ISOE); and three complex fragrance mixtures, F-16, F-07, and F-22. CINN and ISOE were selected because they are present at levels up to 10% in many fragrance formulations and because they are known to induce ACD in a small number of people. The specific fragrance formulations were chosen because they have been studied in the Buehler guinea pig assay

or in human test panels. F-16 and F-07 contained greater than 10% CINN while F-22 had less than 1% CINN and ISOE. The main hypothesis tested in this work was that the noninvasive MESA with the vit A enhancement (the vit A MESA) is an effective, quantitative assay for ACD that is of comparable or greater sensitivity than the Buehler guinea pig assay. Using weak sensitizers and mixtures, and nonsensitizing compounds, we set out to test this hypothesis.

## METHODS

*Animals.* Male BALB/cBy mice, 6 to 8 weeks old (The Jackson Laboratory, Animal Resources, Bar Harbor, ME), were used for all the experiments. All animals were housed and fed as in the previous experiments (Thorne *et al.*, 1991). Mice received either a regular diet (Formulab Chow No. 5008, Purina Mills, Inc., Richmond, IN) or a vit A-supplemented diet (Special Mix 5751-A made from Formulab Chow No. 5008, Purina Mills, Inc.) modified to contain an additional 255 IU/g feed of vit A acetate.

*Chemicals* The test chemicals and their suppliers were as follows: ISOE, 99%, (mixture of *cis* and *trans* 2-methoxy-4-propenylphenol) [97-54-1] (Aldrich Chemical Co., Inc., Milwaukee, WI); CINN, 99+%, (*trans*-3-phenyl-2-propenal) [14371-10-9] (Aldrich Chemical Co., Inc.); and fragrance mixtures: F-16, F-07, and F-22 (S. C. Johnson & Son, Inc., Racine, WI). HPLC grade acetone was obtained from Fisher Scientific (Pittsburgh, PA). All chemicals were stored desiccated at room temperature except for CINN which was kept refrigerated. Depilon Soft Epil was used for depilating the abdomen of the mice (Hamol International, Cologne, FRG).

*Irritation dose–response.* The highest nonirritating dose for each compound was determined by developing an irritation dose–response curve. Groups of five mice were assessed for swelling over time after receiving various concentrations of test compound in 40 μl applications to the ear. The ear thickness increase (ETI) for each mouse was then determined by averaging the swelling measured at 24, 48, and 72 hr. The mean and standard deviation ETI responses for each group of animals were computed and an upper confidence bound was determined by summing the mean and twice the standard deviation. The challenge dose for each compound was selected on the basis of the upper confidence bound as described in the companion paper (Thorne *et al.*, 1991).

*Sensitization dosing.* Mice were depilated on the day prior to sensitization and on Day 0 were dosed topically on the abdomen with 100 μl of vehicle containing the desired molar amount of test compound. Details of this procedure can be found in the companion article (Thorne *et al.*, 1991).

*Elicitation challenges.* Determinations of the ear thicknesses of the mice were made prior to challenge using an Öditest micrometer equipped with 0.5-cm-diameter pads (Model D-1000, Dyer Co., Inc., Lancaster, PA). Triplicate measurements were taken on the anterior lateral aspect of the ear surface. The mice were then challenged, using a glass-tipped pipette, on their left ear with vehicle only and on their right ear with the previously determined challenge dose of the test compound dissolved in 40 μl of the vehicle. Twenty microliters of the challenge solution was delivered to each side of the ear. The extent of ear swelling was assessed by comparison of the mean thickness of the ear at 24, 48, and 72 hr following challenge with the prechallenge value. Significant ear swelling was defined as an ETI exceeding the upper confidence bound defined in the irritation dose–response studies.

*The vit A MESA.* For the vit A supplementation protocol, mice were placed on the vit A-enhanced diet for 21 days prior to challenge and were maintained on this diet throughout the experiment. In every other way these groups were treated identically to groups on the regular feed. Body weight was carefully monitored in these groups to ensure normal weight gain.

*Data analysis.* Statistical analyses were performed using PC SAS (Version 6.03), UNIX|STAT (G. Perlman, Wang Institute, Tyngsboro, MA), or Paradox 3.0 utilities.

## RESULTS

The efficacy and utility of the MESA were demonstrated in the studies reported in the companion paper (Thorne *et al.*, 1991) using potent sensitizers at low doses. In this work we investigated very weak putative sensitizers administered at high doses. Table 1 lists the test compounds studied and characteristics of their composition. CINN and ISOE were single compounds with greater than 99% purity, while the other three test substances were mixtures of more than 60 ingredients. F-16 contained 41.0% eugenol, 29.4% CINN, 1.1% ISOE, and was composed almost entirely of fragrances. F-07 had a mineral oil base and 15.9% eugenol with an equal amount of CINN. No isoeugenol was detected using gas chromatography/mass spectrometry. F-22 contained no detectable CINN or ISOE, only 0.1% eugenol, and was 43.6% phenyl ethanol. In addition to CINN and ISOE, eugenol content was of interest because it has been shown to be a sensitizer (Goncalo *et al.*, 1988) and is

TABLE 1

COMPOSITION OF THE TEST COMPOUNDS

| Test compound | | Composition |
|---|---|---|
| CINN | 99+% | *trans*-Cinnamaldehyde |
| ISOE | 99% | Isoeugenol (*cis* and *trans*) |
| F-16 | 41.0% | Eugenol |
| | 29.4 | Cinnamaldehyde |
| | 1.1 | Isoeugenol |
| | 6.7 | Caryophyllene |
| | 5.0 | *N*-Amyl salicylate |
| | 4.0 | Benzyl salicylate |
| | 3.0 | Benzyl acetate |
| | 2.4 | Isoamyl salicylate |
| | 2.3 | Methyl cinnamate |
| | 1.1 | Coumarin |
| | 4.0 | > 50 Ingredients < 1% |
| F-07 | 15.9% | Eugenol |
| | 15.6 | Cinnamaldehyde |
| | N.D.[a] | Isoeugenol |
| | 25.2 | Mineral oil |
| | 10.4 | Caryophyllene |
| | 8.7 | Isomenthol |
| | 4.3 | Linalool |
| | 4.2 | *p*-Cymene |
| | 3.3 | $\alpha$-Pinene |
| | 3.0 | $\alpha$-Terpinyl acetate |
| | 2.7 | Limonene |
| | 2.5 | Cineole |
| | 4.2 | > 50 Ingredients < 1% |
| F-22 | 0.1% | Eugenol |
| | N.D. | Cinnamaldehyde |
| | N.D. | Isoeugenol |
| | 43.6 | Phenyl ethanol |
| | 11.9 | *p*-tert-Butyl-$\alpha$-methyl-hydrocinnamaldehyde |
| | 10.9 | Benzyl salicylate |
| | 8.5 | Sesquiterpene (unclassified) |
| | 6.8 | Methyl ionone + methyl isoeugenol |
| | 2.6 | Benzyl acetate |
| | 2.5 | Geraniol |
| | 2.4 | Linalyl acetate |
| | 2.4 | $\alpha$-*N*-Methyl ionone |
| | 8.3 | > 50 Ingredients < 2% |

[a] N.D., not detected.

also claimed to provide quenching of sensitization by CINN (Opdyke, 1976; Opdyke, 1979).

*Determination of the Challenge Concentrations*

The first step in the MESA was to perform irritation dose–response testing to identify the appropriate doses to use for ear challenges on Day 5. Figure 1 illustrates the irritation time-course for CINN, which was typical of those seen with the other four test fragrances. At early timepoints, CINN was quite irritating at concentrations of 10% and higher. Only at the 20% dose was there significant swelling as late as 72 hr postchallenge. The irritation dose–response curve for CINN is shown in the inset to Fig. 1. The data from the irritation dose–response assays for CINN and ISOE are listed in Table 2. The third column lists the mean ETI and the standard deviation for each of the concentrations tested. At 10% CINN the ear swelling was 0.014 ± 0.006 mm, which yielded an upper confidence bound of 0.026 mm. Thus, using a 10% solution for ear challenge meant that the mice needed to meet or exceed an ETI of 0.026 mm for the response to be positive. Similarly, for ISOE, challenge using a concentration of 10% established 0.021 mm as the threshold for positivity. Irritation dose–response data for the fragrance mixtures are shown in Table 3. These three fragrance mixtures were considerably less irritating than the pure fragrance components as demonstrated by the low mean ETI values at 20%: 0.010, 0.004, and 0.005 mm for F-16, F-07, and F-22 as compared to 0.045 mm for CINN and 0.025 mm for ISOE. Although the coefficient of variation about the mean was comparable for CINN, ISOE, F-16, and F-22, the variation for the F-07 irritation data was considerably higher. Since these irritation studies were performed at the same time in precisely the same manner, there was no explanation for this difference. Challenge concentrations selected on the basis of these data were 50% for F-16 and full strength for F-07 and F-22. The challenge dose for F-07 probably should have been 50%, but 100% was mistakenly used. This meant that a very weak positive response could have been called negative because of failure to reach

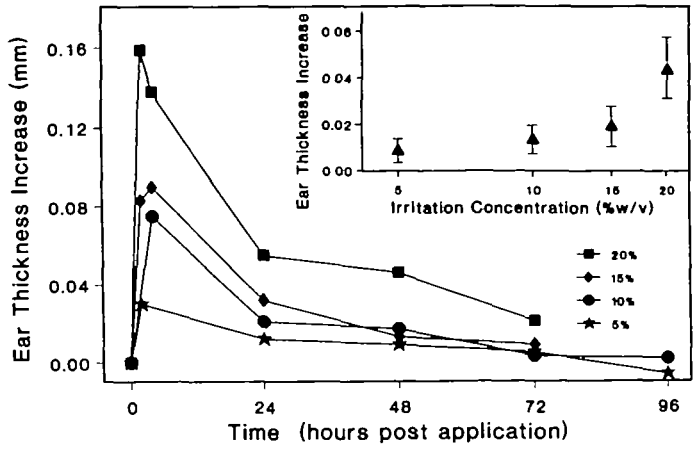


FIG. 1. Irritation response timecourse for cinnamaldehyde with the dose–response curve inset. The ears of mice were dosed with 40 μl of the concentration indicated and the ear thickness increase from the preexposure value was determined. Irritation was severe at 1 and 2 hr postchallenge and dropped sharply between 2 and 24 hr with the curves demonstrating dose–response behavior. At the 48- and 72-hr timepoints 5, 10, and 15% concentrations yielded ear swelling values that were not significantly different. Inset: The group mean irritation data for the average ear thickness increase at 24, 48, and 72 hr are plotted with error bars indicating ±1 SD. Ear swelling was markedly greater at concentrations above 10%. Based upon the upper confidence bounds determined from these data (Table 2), 10% was selected as the challenge concentration for ACD assays.

the upper confidence bound. Since F-07 did produce positive responses using this higher threshold, the inappropriately high challenge dose did not create any problems.

TABLE 2

IRRITATION DOSE–RESPONSE RESULTS FOR THE FRAGRANCES

| | Concentration % (w/v) | Mean ETI[a] (mm, $\bar{x}$ (± SD) | Upper confidence bound (mm, $\bar{x}$ + 2 SD) |
|---|---|---|---|
| CINN | 20 | 0.045 (0.013) | 0.071 |
| | 15 | 0.020 (0.009) | 0.037 |
| | 10 | 0.014 (0.006) | 0.026 |
| | 5 | 0.009 (0.005) | 0.019 |
| ISOE | 20 | 0.025 (0.007) | 0.040 |
| | 15 | 0.027 (0.011) | 0.050 |
| | 10 | 0.011 (0.005) | 0.021 |
| | 5 | 0.010 (0.005) | 0.020 |

*Note.* SD, Standard deviation.

[a] Ear thickness increase computed by averaging the change from the prechallenge value at 24, 48, and 72 hr postexposure. The value tabulated is the group mean.

### *ACD Testing of the Fragrances and Fragrance Mixtures*

Having established the challenge concentrations, the next step was to test the fragrances for ACD potency using the MESA and the vit A MESA. The sensitization dose–response results for CINN are plotted in Figs. 2 and 3. In Fig. 2, group mean ETIs for mice fed the regular diet or the vit A-supplemented diet are shown. For the regular feed groups there was no dose-related increase in ETI since the slope of the regression line was not significantly different from zero ($F = 3.19, p = 0.097$). A single concentration test of the vit A MESA was performed at 20% sensitization concentration, the approximate SD50. As indicated in Table 4 this group of mice exhibited a significantly higher ETI of 0.063 mm versus 0.022 mm for mice fed the regular diet ($p = 0.013$). Figure 3 shows the data from this experiment plotted as percentage responding versus concentration. This plot indicates incidence and reflects the nature of those demonstrating responses,

TABLE 3

IRRITATION DOSE-RESPONSE RESULTS FOR THE FRAGRANCE MIXTURES

| | Concentration % (w/v) | Mean ETI[a] (mm, $\bar{x}$ (± SD)) | Upper confidence bound (mm, $\bar{x}$ + 2 SD) |
|---|---|---|---|
| F-16 | 100 | 0.035 (0.020) | 0.074 |
| | 50 | 0.015 (0.010) | 0.036 |
| | 20 | 0.010 (0.004) | 0.018 |
| | 10 | 0.006 (0.004) | 0.014 |
| F-07 | 100 | 0.016 (0.015) | 0.045 |
| | 50 | 0.007 (0.012) | 0.031 |
| | 20 | 0.004 (0.006) | 0.016 |
| | 10 | 0.000 (0.007) | 0.014 |
| F-22 | 100 | 0.020 (0.009) | 0.038 |
| | 50 | 0.016 (0.003) | 0.022 |
| | 20 | 0.005 (0.007) | 0.019 |
| | 10 | 0.008 (0.005) | 0.017 |

*Note.* SD, Standard deviation.

[a] Ear thickness increase (see footnote to Table 2).

whereas Fig. 2 indicated the group mean values which included the responders and nonresponders. The regular feed group challenged in the normal way on Day 5 yielded a dose-related response with a correlation coefficient of 0.98 and a predicted SD50 of 21.6%. Three groups tested in the same way, except that they were challenged on Day 3, demonstrated no positive responses at any of the three doses. At 20% CINN, 100% of the group fed the high vit A diet demonstrated positive responses versus 40% positive responses observed in mice on regular feed. These studies with CINN indicated that there was enhancement of ACD in the vit A MESA with a weak sensitizer.

The results for three groups of mice sensitized with ISOE are also listed in Table 4. The two doses tested with mice fed the regular diet, 3 and 10%, both resulted in 100% of the group responding as did the 10% dose in animals supplemented with vit A. Since all mice had positive responses, enhancement of the incidence with vit A could not be determined.

The complex fragrance mixtures were investigated next in the MESA with F-16 receiving the most study. As shown in Table 5, 18 mice were tested in the regular feed MESA at 25% and full strength without any positive responses. There was also no difference in the mean ear swelling observed between these two

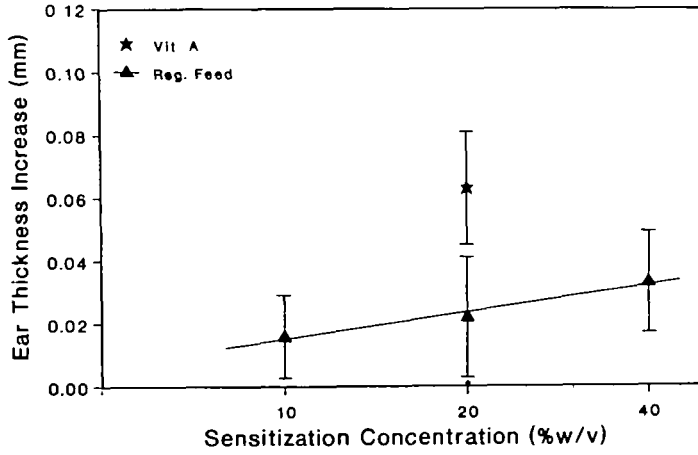


FIG. 2. Sensitization dose-response curve for cinnamaldehyde. The group mean ear thickness increase ±1 SD (error bars) is shown for mice fed with either the regular diet (triangle) or the vit A-supplemented diet (star) and sensitized with the given concentration. Linear least-squares regression yielded a correlation coefficient, $r$ of 0.99, although the regression line was not significantly different from horizontal. At 20% sensitization concentration, the approximate SD50, mice fed the vit A-supplemented diet exhibited a response threefold higher than the mice fed the regular diet, $p = 0.013$.

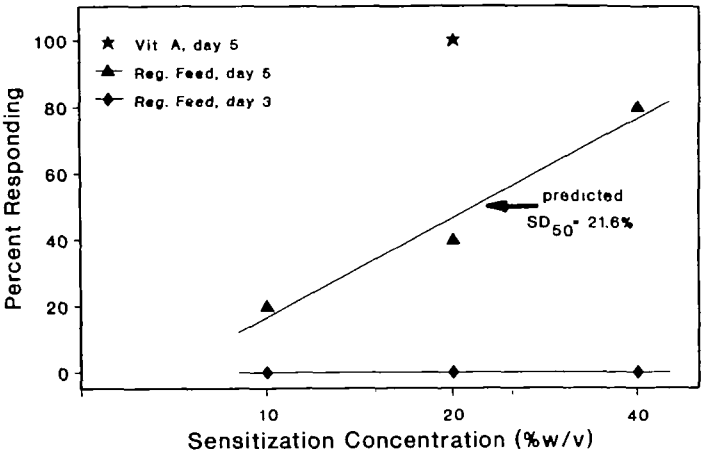


FIG. 3. Sensitization dose–response, percentage responding curve for cinnamaldehyde comparing the mice on regular feed with the vit A-supplemented mice. This incidence plot shows the percentage exhibiting positive responses in each group of mice for three different treatments. The percentage responding for mice fed a regular diet and challenged on Day 5 varied in a dose–response fashion with sensitization concentration. The regression line yielded a predicted SD50 of 21.6% ($r$ = 0.98). None of the mice treated similarly but challenged on Day 3 responded. Mice fed the vit A-supplemented diet and sensitized with 20% cinnamaldehyde demonstrated a 100% response rate as compared to 40% observed with the regular feed.

groups. Using the vit A enhancement, mice were sensitized at 10, 25, and 100% concentrations, with ETIs showing a dose–response effect. This is illustrated in Figs. 4 and 5. Figure 4 demonstrates the regression line ($r$ = 0.97) and significant differences between the two

TABLE 4

MESA AND VITAMIN A MESA RESULTS FOR THE FRAGRANCES

| | Challenge day | Diet | $N$ | Sensitization concentration % (w/v) | Mean ETI[a] (mm, $\bar{x}$ (± SD)) | % Responding |
|---|---|---|---|---|---|---|
| CINN[b] | 5 | Reg feed | 5 | 40 | 0.033 (0.016) | 80 |
| | 5 | Reg feed | 5 | 20 | 0.022 (0.019) | 40 |
| | 5 | Reg feed | 5 | 10 | 0.016 (0.013) | 20 |
| | 5 | Vit A | 4 | 20 | 0.063 (0.018) | 100 |
| | 3 | Reg feed | 5 | 40 | 0.001 (0.003) | 0 |
| | 3 | Reg feed | 5 | 20 | 0.014 (0.007) | 0 |
| | 3 | Reg feed | 4 | 10 | 0.014 (0.006) | 0 |
| ISOE[c] | 5 | Reg feed | 4 | 10 | 0.044 (0.014) | 100 |
| | 5 | Reg feed | 4 | 3 | 0.034 (0.008) | 100 |
| | 5 | Vit A | 5 | 10 | 0.059 (0.018) | 100 |

*Note.* SD, Standard deviation.

[a] Ear thickness increase (see footnote to Table 2).

[b] Positive response ≥ 0.026 mm.

[c] Positive response ≥ 0.021 mm.

TABLE 5

MESA AND VITAMIN A MESA RESULTS FOR THE FRAGRANCE MIXTURES

| | Diet | N | Sensitization concentration % (w/v) | Mean ETI[a] (mm, $\bar{x}$ (± SD)) | % Responding |
|---|---|---|---|---|---|
| F-16[b] | Reg feed | 13 | 100 | 0.014 (0.010) | 0 |
| | Reg feed | 5 | 25 | 0.014 (0.009) | 0 |
| | Vit A | 5 | 100 | 0.080 (0.024) | 100 |
| | Vit A | 5 | 25 | 0.037 (0.017) | 40 |
| | Vit A | 5 | 10 | 0.029 (0.014) | 20 |
| F-07[c] | Reg feed | 7 | 100 | 0.025 (0.023) | 14 |
| | Vit A | 5 | 100 | 0.049 (0.011) | 60 |
| F-22[d] | Reg feed | 9 | 100 | 0.009 (0.011) | 0 |
| | Reg feed | 5 | 100 − 3x | 0.005 (0.003) | 0 |
| | Vit A | 5 | 100 | 0.048 (0.012) | 60 |

*Note.* SD, Standard deviation.
[a] Ear thickness increase (see footnote to Table 2).
[b] Positive response ≥ 0.036 mm.
[c] Positive response ≥ 0.045 mm.
[d] Positive response ≥ 0.038 mm.

treatments at 25% ($p = 0.026$) and at 100% ($p < 0.001$). In Fig. 5, the regression line ($r = 0.99$) illustrates the increase in positive responses from none to 100% of the mice over the range from about a 6% solution to the undiluted fragrance mixture. From this regres-

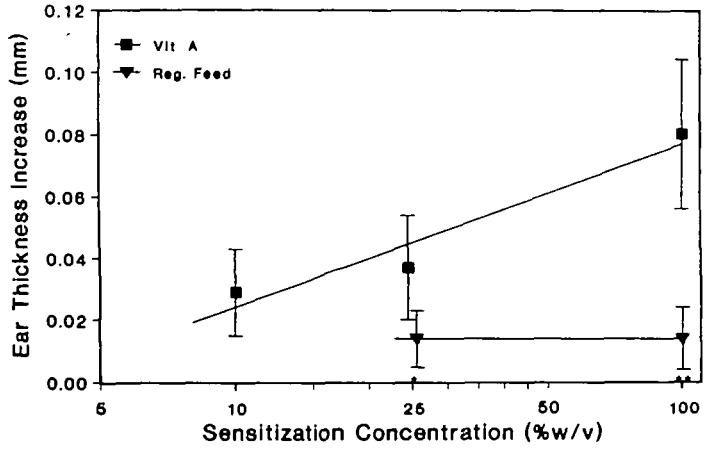


FIG. 4. Sensitization dose–response curve showing ear thickness increase for the complex fragrance mixture, F-16. This dose–response curve illustrates that no positive responses were observed in the mice fed a regular diet at 25 and 100% sensitization concentrations (triangles). Mice fed the vit A-supplemented diet exhibited dose-related responses over the concentration range of 10 to 100% ($r = 0.97$). Differences between these treatments were significant at 25% sensitization concentration ($p = 0.026$) and at full strength ($p < 0.001$).

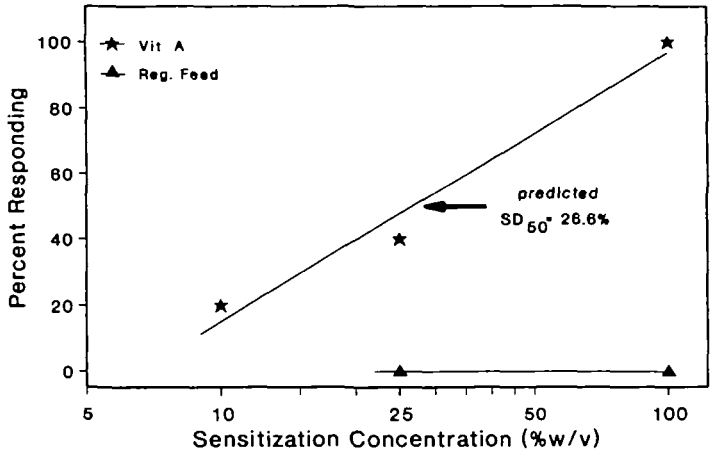


FIG. 5. Sensitization dose–response, percentage responding curve for F-16 comparing the mice on regular feed with the vit A-supplemented mice. The percentage responding data show that the MESA without the vit A enhancement yielded a negative determination for F-16 as a sensitizer and, hence, there was no SD50. With the vit A enhancement, dose-related responses were seen in mice at 10, 25, and 100% concentrations. The predicted $SD50^{vit\ A}$ from the regression line for vit A-supplemented mice was 26.6% ($r = 0.99$). These data demonstrate that the vit A supplementation can increase the sensitivity of the MESA to the extent that a negative determination can become positive.

sion we obtain the value for the predicted $SD50^{vit\ A}$ of 26.6%. In this case, the MESA without the vit A supplementation yielded a negative result, whereas with vit A F-16 was positive for ACD.

One-way analysis of variance of F-16 data demonstrated that the vit A supplementation did not change MESA irritation dose–responses but increased only the sensitization responses. Comparison of the group mean ear swelling due to irritation at the 1-hr timepoint did not differ between the regular feed or vit A groups sensitized with 25% ($p = 0.45$). There was also no difference in the mean ETI between the nonresponders in the 10% vit A group and the unsensitized mice from the irritation dose–response studies fed the regular diet and challenged with the same concentration ($p = 0.20$).

The data for the other fragrance mixtures are shown in Table 5. F-07 produced 1 of 7 responders (14%) in animals fed the regular diet but increased to 60% as a result of vit A enhancement. The mean ETI was doubled with this treatment. F-22 tested negative for ACD in animals dosed with the full strength solution on Day 0 only and in mice dosed with 100% on Days 0, 3, and 5. In the vit A MESA, 60% of the mice had positive responses with F-22. Thus, as with F-16, this compound was converted from negative to positive through the vit A enhancement to the MESA.

The noninvasive MESA was shown to effectively identify as weak sensitizers two fragrance components (CINN and ISOE) and a complex fragrance mixture (F-07). Dietary vit A supplementation increased the responses for these test materials and yielded positive results in two other fragrance mixtures (F-16 and F-22). Tests for acetone-induced ACD in mice, with or without vit A supplementation, yielded negative results. The MESA proved to be a simple and effective protocol for testing fragrance materials for induction of contact sensitization.

## DISCUSSION

In the companion study (Thorne *et al.*, 1991), it was shown that the noninvasive

MESA was sensitive and quantitative and that dietary vit A supplementation increased the sensitivity of the assay significantly when tested with strong sensitizers at very low doses. This second study was designed to determine the effectiveness of this model for identification of ACD caused by weak contact sensitizers, as represented by two fragrances and three complex fragrance mixtures.

Challenge doses were determined by developing an irritation dose–response curve for each compound and then selecting a concentration that produced minimal ear thickness increase at 24, 48, and 72 hr postexposure. The threshold for a positive response was set at two standard deviations above the mean response observed in the irritation study. This ensured with 95% confidence that ETIs were due to ACD rather than irritation. Data indicated that there was no difference in the irritation response between the mice on the regular diet and those fed the vit A-supplemented diet.

The mice treated with CINN in the MESA demonstrated a dose-related response for percentage responding that yielded a predicted SD50 of 21.6%. Exposure at about this dose (20%) sensitized 100% of the mice in the vit A MESA (Fig. 3). With ISOE, all mice were sensitized at 3 and 10% concentrations in the MESA and at 10% in the vit A MESA. F-16 was positive for sensitization in the vit A MESA only and demonstrated dose–response behavior for ETI (Fig. 4) and percentage responding (Fig. 5). The predicted $SD50^{vit\ A}$ was 26.6%. For the other two fragrance mixtures, F-07 and F-22, responses were minimal or zero in the MESA at full strength but were positive in 60% of the mice in the vit A MESA (Table 5).

In Table 6, the MESA data for CINN and ISOE are compared to data obtained in various guinea pig and human assays for contact sensitization. The sensitization concentrations administered are listed along with the method of dosing: epicutaneous application, intradermal injection, with or without Freund's complete adjuvant (FCA). This information is important because intradermal injection bypasses the stratum corneum and thus excludes the important protective role that structure serves (Klecak, 1987). FCA enhances the immune response but also alters the nature of the response. Table 6 illustrates that for CINN, the MESA compared favorably with the invasive mouse ear swelling test reported by Gad *et al.* (1986), while the vit A MESA appeared more sensitive than the Gad method. Results from six different guinea pig protocols illustrate that the vit A MESA data for CINN fell within the range of these assays. Human testing of CINN has generally shown positive results. One study of 53 subjects yielded no positive responses in the modified Draize test (Marzulli and Maibach, 1980) while another study using the human maximization test (Kligman and Epstein, 1975) produced 11 positive responses in 25 subjects. Because of the extreme variability (0 to 44% responding) of the human values, it is difficult to compare these data with the MESA results in a quantitative fashion.

The bottom half of Table 6 provides a comparison of test results for ISOE. The MESA yielded an SD50 that was less than 3%. Klecak *et al.* (1977) developed a dose–response curve using the guinea pig open epicutaneous test that yielded an SD50 of about 5%. As indicated in Table 6, guinea pig tests with ISOE have yielded results that bracket those observed in the MESA. Human testing indicates that the MESA is more sensitive than the modified Draize and the human maximization test or the human repeated insult patch test (HRIPT) performed in normal subjects. Patch testing of 2461 eczematous patients tested in a dermatology clinic yielded 2% responders using a concentration of 5%. Since the MESA sensitized all mice at 3 and 10%, it was more sensitive than tests in this human population biased for dermatological problems.

The specific fragrance formulations, F-16, F-07, and F-22, were selected for testing in the MESA because they had all been studied in the Buehler guinea pig assay (Buehler, 1965; Ritz and Buehler, 1980), and F-22 had also been tested in the HRIPT with a test panel of

TABLE 6

COMPARISON OF THE MOUSE EAR SWELLING ASSAY TO GUINEA PIG AND HUMAN TEST PROTOCOLS

| Compound | Species | Assay[a] | Sensitization concentration[b] | % Positive responses | SD50[c] | Reference |
|---|---|---|---|---|---|---|
| CINN | Mouse | MESA | 40ec | 80 | 21.6 | This work |
| | | | 20ec | 40 | | |
| | | | 10ec | 20 | | |
| | | Vit A MESA | 20ec | 100 | ~5 | This work |
| | | MEST | 10ec, FCA | 30 | | Gad *et al.*, 1986 |
| | Guinea pig | OET | 3ec | positive | | Klecak, 1985 |
| | | GPMT | 25ec, 5id, FCA | positive | | Klecak *et al.*, 1977 |
| | | GPMT | 5ec, 5id, FCA | 100 | | Senma *et al.*, 1978 |
| | | GPMT | 2ec, 2id, FCA | 80 | | Prince and Prince, 1977 |
| | | SAT | 2ec, FCA | 100 | | Prince and Prince, 1977 |
| | | Opt Test | 0.1id, FCA | 100 | | Maurer, 1985 |
| | Human | Patch Test[d] | 2ec | 4 | | Calnan *et al.*, 1980 |
| | | Mod. Draize | 1ec | 0 | | Marzulli and Maibach, 1980 |
| | | Mod. Draize | 1ec | 1.8 | | Marzulli, and Maibach, 1980 |
| | | HMT | 2ec | 44 | | Klingman and Epstein, 1975 |
| | | HMT/HRIPT | 3ec | positive | | Klecak, 1985 |
| ISOE | Mouse | MESA | 10ec | 100 | <3 | This work |
| | | | 3ec | 100 | | |
| | | Vit A MESA | 10ec | 100 | | This work |
| | Guinea pig | OET | 100ec | 100 | ~5 | Klecak *et al.*, 1977 |
| | | | 30ec | 100 | | |
| | | | 10ec | 83 | | |
| | | | 3ec | 33 | | |
| | | GPMT | 25ec, 5id, FCA | positive | | Klecak *et al.*, 1977 |
| | | GPMT | 100ec, 1id, FCA | 100 | | Magnusson and Kligman, 1969 |
| | | Opt Test | 0.1id, FCA | 85 | | Maurer, 1985 |
| | | FCAT | 5id, FCA | 100 | | Tsuchiya *et al.*, 1985 |
| | Human | Patch Test[d] | 5ec | 2 | | Calnan *et al.*, 1980 |
| | | Mod. Draize | 8ec | 12 | | Marzulli and Maibach, 1980 |
| | | HMT/HRIPT | 8ec | 0 | | Klecak, 1985 |

[a] MESA, Noninvasive mouse ear swelling assay; Vit. A MESA, MESA using mice fed Vitamin A-supplemented diet; MEST, invasive mouse ear swelling test; HMT, human maximization test; HRIPT, human repeated insult patch test; OET, open epicutaneous test; GPMT, guinea pig maximization test; SAT, split adjuvant test; Opt Test, optimization test; FCAT, Freund's complete adjuvant test.

[b] ec, epicutaneous; id, intradermal injection; FCA, Freund's complete adjuvant, id.

[c] Predicted SD50 (induction dose to sensitize 50% of the animals).

[d] Patch testing in 2461 eczematous patients.

191. In addition, these fragrance formulations were chosen because they spanned a range of concentrations of cinnamaldehyde (29.4, 15.6, and <0.1%). CINN was shown in the vit A MESA to yield a mean ETI of 0.063 mm at a concentration of 20% (Table 4). The dose–response curve for F-16 predicted a mean ETI of 0.068 mm at a concentration where the content of cinnamaldehyde was 20% (Fig. 4). Tests with F-07 at full strength, which corresponded to 15.6% cinnamaldehyde, yielded a mean ETI of 0.049 mm (Table 5). Thus, these

TABLE 7

COMPARISON OF THE MOUSE EAR SWELLING ASSAY TO GUINEA PIG AND HUMAN TEST PROTOCOLS

| Assay | Trial | F-16 Dose,[a] mg | F-16 % Positive | F-07 Dose,[a] mg | F-07 % Positive | F-22 Dose,[a] mg | F-22 % Positive |
|---|---|---|---|---|---|---|---|
| MESA[b] | Regular feed ($N$ = 5 to 13) | 100 | 0 | 100 | 14 | 100 | 0 |
| | | 25 | 0 | | | | |
| | Vitamin A ($N$ = 5) | 100 | 100 | 100 | 60 | 100 | 60 |
| | | 25 | 40 | | | | |
| | | 10 | 20 | | | | |
| | Control ($N$ = 5) | 0 | 0 | 0 | 0 | 0 | 0 |
| Buehler guinea pig assay[c] | Test ($N$ = 20) | 45 | 88 | 45 | 63 | 90 | 0 |
| | Control ($N$ = 10) | 0 | 0 | 0 | 0 | 0 | 0 |
| Human repeated insult patch test[d] | Test ($N$ = 191) | — | — | — | — | 12 | 0 |

[a] Total quantity of test fragrance mixture applied during the sensitization regimen.
[b] The noninvasive mouse ear swelling assay described herein.
[c] Six hour contact occlusive patch applied to restrained animals three times over 2 weeks.
[d] Twenty-four hour contact occlusive patches applied nine times over 3 weeks.

data indicated consistency in the magnitude of the response across compounds. In terms of the percentage responding, this same comparison yielded 100% responding with 20% CINN, 83% responding with 20% cinnamaldehyde in F-16, and 60% responding with 15.6% cinnamaldehyde in F-07. The International Fragrance Association advises formulators to balance the amount of cinnamaldehyde with an equal amount of eugenol to produce quenching and hopefully prevent sensitization. For these compounds this technique was unsuccessful. The third fragrance formulation tested, F-22, had less than 0.1% CINN and ISOE and was formulated to have a low potential for sensitization. F-22 produced 60% responses in the vit A MESA. This mixture contained 11.9% of *p-tert*-butyl-$\alpha$-methylhydrocinnamaldehyde, which may induce sensitization, 2.5% geraniol which has been shown to be a weak human sensitizer (Calnan *et al.*, 1980), and lesser quantities of other recognized sensitizers.

Table 7 provides a comparison of the MESA and the vit A MESA with the Buehler guinea pig assay and the HRIPT. The results reported for the latter two tests were performed under contract by Hill Top Laboratories, Inc. (Cincinnati, OH). The comparison in Table 7 lists the cumulative sensitization dose and the percentage positive responses observed in each test. For both the MESA and the Buehler assay none of the control animals met the definition for a positive response. For F-16 and F-07, results from the Buehler assay were quite close to those for the vit A MESA. This was not the case for F-22 which was negative in the guinea pig but positive in the vit A MESA. Since this fragrance mixture was negative in the guinea pig testing, it was subjected to human testing using the HRIPT on the final product formulation. The concentration of the fragrance mixture in this product was 1.3%, therefore the cumulative dose tested was just 12 mg. In the HRIPT none of the 191 subjects responded positively upon induction and challenge with

F-22. It should be recognized that at the 95% confidence level, 15 of every 1000 subjects may be sensitized by a substance that is negative in the HRIPT (Marzulli and Maibach, 1987). At this point in time, the determination for F-22 would appear to be a false positive in the vit A MESA.

The MESA was found to have many advantages over other test protocols. It is much simpler to perform because induction and challenge are performed with one dosing each, and no stripping of the stratum corneum, occlusive patching, anesthesia, or injections are used. The data obtained are parametric and more quantitative than the subjective grading of responses in the guinea pig. Many positive determinations in guinea pig assays are based upon grade 1 and grade 2 responses. However, a study by Fischer and Maibach (1987) showed the accuracy of grade 2 responses to be 80 to 90% and grade 1 responses to be 20%. The MESA is also far less expensive because mice are cheaper to purchase and maintain; the experiment lasts only 8 days for the MESA or 29 days for the vit A MESA as opposed to approximately 45 to 60 days for guinea pig assays. The costs for testing one compound at one concentration in the Buehler guinea pig assay are five times the cost for testing the same compound at four doses in the vit A MESA; this includes labor and overhead.

Mouse ear swelling models have been extremely attractive to dermatotoxicologists but have been criticized for lacking the sensitivity afforded by the more aggressive guinea pig assays. The work presented in this paper and the companion paper (Thorne *et al.*, 1991) has illustrated the sensitivity and simplicity of the noninvasive mouse ear swelling assay with and without enhancement through dietary vit A supplementation. Dose–response experiments with potent sensitizers at very low doses and very weak sensitizers at high doses have shown that the MESA has comparable sensitivity to many of the guinea pig and human test protocols. Given this success and the ease of the method we expect that there will be renewed interest in the mouse ear swelling assay.

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