FUNDAMENTAL AND APPLIED TOXICOLOGY 17, 790–806 (1991)

The Noninvasive Mouse Ear Swelling Assay

I. Refinements for Detecting Weak Contact Sensitizers

PETER S. THORNE,*,[1] CHERYL HAWK,* SUSAN D. KALISZEWSKI,* AND PATRICK D. GUINEY†

** Department of Preventive Medicine and Environmental Health, College of Medicine, University of Iowa, Iowa City, Iowa 52242; and †S. C. Johnson & Son, Inc., Racine, Wisconsin*

Received January 23, 1991; accepted June 4, 1991

The Noninvasive Mouse Ear Swelling Assay. I. Refinements for Detecting Weak Contact Sensitizers. THORNE, P. S., HAWK, C., KALISZEWSKI, S. D., AND GUINEY, P. D. (1991). *Fundam. Appl. Toxicol.* **17**, 790–806. The noninvasive mouse ear swelling assay (MESA) is a model for delayed-type hypersensitivity that holds promise as a testing protocol for allergic contact dermatitis (ACD). The MESA employs only topical sensitization on the abdomen and does not use injections, adjuvants, anesthesia, occlusion, or disruption of the stratum corneum. Five days after induction, the ears are challenged topically and ear swelling measurements taken at 24, 48, and 72 hr indicate the extent of ACD. In this study, refinements of the assay were explored in BALB/cBy mice using dinitrofluorobenzene (DNFB) and dinitrochlorobenzene (DNCB). A complete dose–response curve was developed for DNFB and the dose which sensitized half the mice in a group (SD50, 0.001%, w/v) was used to test noninvasive enhancement protocols. Several triple-dose protocols tested produced no increase in responsiveness and daily dosing showed a trend toward tolerance induction yielding 20% positive responses. Dietary vitamin A supplementation produced a dramatic enhancement of the responses: ear thickness increase was doubled and the SD50 sensitized 94 to 100% of the mice in the vitamin A groups. We conclude that the MESA allowed identification of ACD potency for known sensitizers at very low concentrations which do not produce ACD with other techniques. The importance of dose–response studies for avoiding the high-dose reduced-response region was also shown. Based on the observation that the vitamin A-augmented MESA was considerably more sensitive than with regular feed, a companion study (P. S. Thorne, C. Hawk, S. D. Kaliszewski, P. D. Guiney, *Fundam. Appl. Tox.* **17**, 807–820, 1991) presents tests of the enhancements to the MESA developed in this work, using weak sensitizers and complex mixtures.

Allergic contact dermatitis (ACD) is responsible for nearly half of all occupational dermatoses and about one-fifth of all reported workplace disease. Thus, ACD represents a significant health hazard for workers. From the standpoint of consumers, a sizable portion of dermatological problems result from the use of cosmetics, toiletries, and topical medicaments (Fregert, 1986). Often it is not the active ingredients of these products that are sensitizers but the preservatives, fragrances, colorings, or vehicles (Menné and Christopherson, 1985). Ingredient degradation can produce new sensitizing chemicals that were not present in the original product. A pivotal part of the process of bringing a dermally applied product to market is the testing of the formulation and

Portions of this work were presented at the 29th Annual Meeting of the Society of Toxicology, 14 February 1990, Miami Beach, FL, and at the 11th Annual Meeting of the American College of Toxicology, 31 October 1990, Orlando, FL.

[1] To whom correspondence should be addressed at University of Iowa, AMRF-Oakdale Campus, Iowa City, IA 52242.

0272-0590/91 $3.00

its ingredients for their dermal sensitizing potency. Methodology for this has been developed using, primarily, guinea pigs in an attempt to predict the sensitizing potency of these products in humans. This endeavor has been somewhat successful but when one compares animal test results to human results one generally finds many false positives and some, but fewer, false negatives. These findings of more false positives than false negatives indicate a conservative approach that has evolved largely by design. For example, using the Freund's complete adjuvant test (FCAT), Klecak and co-workers tested 32 fragrance materials recognized as human sensitizers and 21 that had been found through common use to be nonsensitizers (Klecak *et al.*, 1977; Klecak, 1987). In the FCAT, 12 of the 32 sensitizers (38%) were identified as false negatives while 12 of the 21 nonsensitizers (57%) yielded false positive results. Using the FCAT for 268 compounds, Klecak reported agreement with the human-repeated insult patch test on 157 substances and obtained 106 false positives (40%). Although it is best to err on the side of safety, excessive false positives increase the costs of producing marketable products and keep potentially useful products from being marketed.

What one would most like is a simple animal model for ACD that would be predictive of the most sensitive humans. Such a model could potentially allow prediction of the results from a testing trial with a panel of as many as 1000 human subjects through tests with a relatively small number of rodents. Further, if this were done in a dose–response fashion, this would provide information much better than that obtained from a single dose trial. It is also desirable to have an animal model in which the mechanisms are the same as those in the human in terms of percutaneous absorption and distribution, hapten/immunogen processing and presentation, and development of a delayed-type hypersensitivity response.

In recent years considerable effort has been expended toward improving rodent assays for determination of dermal sensitizing potency of industrial chemicals, skin care products, preservatives, fragrance components, and mixtures. In particular, there has been new interest in adaptations of the mouse ear swelling assay (MESA) methodology in which mice previously dosed with the potential sensitizer are administered a challenge dose on the ear, whereupon the ear thickness is monitored to detect the swelling that occurs in conjunction with a delayed-type hypersensitivity response. The advantages of the MESA over guinea pig assays are that (1) mice are less expensive to purchase and maintain than other species, (2) the MESA yields quantitative results through objective measurements which have less propensity for bias and are more amenable to statistical analysis, (3) the MESA can be easily performed by minimally trained technicians, (4) the immunogenetics of the mouse are better known than those of other rodents and there are a large assortment of mouse immunochemicals, (5) inbred strains are readily available thereby allowing syngeneic transfer experiments, and (6) extensive data on percutaneous absorption have been published for mouse skin.

Mouse Ear Swelling Assays

To our knowledge, the published history of the ear swelling assay goes back to 1967 with the work of Frenkel, in which the ears of hamsters were injected with *Besnoitia* as a test for hypersensitivity following infection of these animals with the same organism by subcutaneous injection (Frenkel, 1967). Ear thickness was measured with a caliper at 4, 24, and 48 hr. The following year this method was adapted for the CBA mouse, substituting a topical challenge for the injection and an engineer's micrometer for the caliper (Asherson and Ptak, 1968). Since that time the MESA in many different forms has been used to study the mechanisms of delayed hypersensitivity (Bäck and Larsen, 1982; Ptak *et al.*, 1985; Streilein, 1985; Sy *et al.*, 1977), T-lymphocyte function (Dieli *et al.*, 1985), the immunobiology of dermal and epidermal dendritic cells (Bergstresser *et al.*, 1985; Roberts *et al.*, 1985;

Toews *et al.*, 1980), effects of ultraviolet irradiation on epidermal cell function (Harriott-Smith and Halliday, 1988; Jun *et al.*, 1988; Orita, 1987), phototoxicity (Gerberick and Ryan, 1989), and for testing potential contact sensitizers (Stadler and Karol, 1985; Maisey and Miller, 1986; Gad *et al.*, 1986; Thorne *et al.*, 1986; Stephens *et al.*, 1987; Cornacoff *et al.*, 1988; Descotes, 1988; Hignet *et al.*, 1989; Dunn *et al.*, 1990). It is this latter area which appears to be the least well established. The MESA has not been shown to convincingly predict sensitizing potency for any but the moderate to potent contact sensitizers and attempts to duplicate some of the published works have been unsuccessful.

Since Landsteiner and Jacobs began using guinea pigs as an ACD model in 1935, attempts have been made to augment the sensitivity of the mouse and guinea pig models for contact allergen testing. Approaches include (1) methods to increase the delivered dose, (2) methods to augment the induction of the immune response, and (3) methods to enhance the elicitation of the response. Examples of each of these augmentation approaches are given in Table 1. Most notable are multiple dosing, abrasion or tape stripping, patching, injection dosing, and the use of adjuvants.

TABLE 1

APPROACHES FOR AUGMENTING THE ALLERGIC CONTACT DERMATITIS RESPONSES IN ANIMAL MODELS

Methods for increasing the delivered dose
Injection dosing
Use of occlusive patches
Dermal abrasion or use of tape stripping
Chemical irritation of the site (detergents, surfactants)
Maximal concentrations
Optimize vehicles to enhance absorption
Methods for augmenting the induction of the immune response
Multiple sequential dosing
Use of adjuvants
Multiple routes of administration
Cyclophosphamide
Vitamin A
Removal of quenching substances from mixture
Methods for enhancing the elicitation of the response
Challenge at the highest nonirritating dose or the lowest irritating dose
Challenge by intradermal injection
Challenge by closed patch
Pretreat ears with solvent or vasodilators
Optimize vehicles to enhance absorption

In 1986 studies were performed of the dermal sensitizing potencies and cross-reactivities of four isocyanate compounds using the noninvasive MESA (Thorne *et al.*, 1986, 1987). Isocyanates are recognized as moderate to potent dermal sensitizing agents (Emmett, 1976; Rothe, 1976). These studies demonstrated three regions of effects in plots of ear thickness increase versus log sensitization dose: a no-effect region, a dose–response region, and a high-dose reduced-response region. Incidence data allowed ranking the sensitization potencies of these compounds by determination of the estimated dose to cause 50% sensitization (SD50). This procedure produced the following SD50 from most potent to least potent: 1,6-hexamethylene diisocyanate, 0.088 mg/kg; 4,4′-dicyclohexylmethane diisocyanate, 0.24 mg/kg; 4,4′-diphenylmethane diisocyanate (MDI), 0.73 mg/kg; and toluene diisocyanate (TDI, 80%/20%, 2,4/2,6 isomers), 5.3 mg/kg. Although these studies did not focus on the high-dose reduced-response region the data indicated that the response to MDI was reduced 55% when the dose was increased from 37 to 187 mg/kg and the responses to high doses of TDI were reduced 63 and 75% with 5- and 100-fold increases in dose from 37 mg/kg, suggesting that supraoptimal dosing can lead to false negative determinations. In this work the MESA proved to be an effective method for studying ACD in a manner which mimicked industrial exposures. No injections, abrasion, occlusion, or adjuvants were used and the single sensitizing doses were applied to 12% of the body surface of the mice. This corresponded to a human exposure to the hands and forearms.

Variations of the MESA for use in contact allergy testing have been studied by a number

of investigators in the past five years (Gad *et al.*, 1986; Stephens *et al.*, 1987; Cornacoff *et al.*, 1988; Descotes, 1988; Hignet *et al.*, 1989; Dunn *et al.*, 1990), but no consensus has emerged as to whether there is a mouse method as good as those available in the guinea pig, particularly for weak sensitizers. However, as detailed under Discussion, all of these investigators have utilized invasive approaches in an attempt to augment the responses and in so doing have created an artificial system that may respond less well than the intact animal with noninvasive enhancements to the methods. In these studies, dose–response relationships were not sought and single high concentrations were used for induction. Thus, dosing may have run into the down-regulated response region (Claman *et al.*, 1980; Bergstresser *et al.*, 1985). Further, it is possible that the daily dose regimen induced tolerance among these animals, further lowering the responses. Whatever the reasons for the unreliable data obtained for weak and moderate sensitizers, the conclusion derived from these invasive studies (Cornacoff *et al.*, 1988; Hignet *et al.*, 1989; Dunn *et al.*, 1990) is that the mouse models are unreliable or unsuitable for ACD testing.

Hypervitaminosis A as an Enhancer of ACD Responses

Despite the failures with the MESA described above, successful use of the MESA in our earlier work with isocyanates convinced us of the potential of the noninvasive MESA as a screening assay for ACD. Earlier reports of successful augmentation of ACD responses using vitamin A (vit A) supplementation prompted us to look further into this approach.

There has been a great deal of interest in vit A supplementation and its effects on cell-mediated immunity over the past 2 decades. Vitamin A has been shown to induce epidermal hyperplasia in mice (Conner *et al.*, 1986), guinea pigs (Christophers, 1970), and humans (Plewig and Braun-Falco, 1975), primarily within the stratum spinosum and stratum granulosum by stimulating cell production (Conner and Lowe, 1983). Vitamin A stimulates most cell-mediated immune responses as evidenced by enhanced host-versus-graft reactivity (Jurin and Tannock, 1972; Malkovsky *et al.*, 1983b), specific antitumor immune responses (Medawar and Hunt, 1981; Malkovsky *et al.*, 1983a; Dennert, 1984; Tachibana *et al.*, 1984; Watson *et al.*, 1987), and dermal delayed-type hypersensitivity reactions (Miller *et al.*, 1984). Experimental deficiency of vit A has been shown to decrease delayed-type hypersensitivity responses to DNFB (Smith *et al.*, 1987). It appears that some immune responses are reduced by vit A supplementation. These include primary IgM responses (Barnett, 1983), mitogen-induced lymphocyte proliferation (Bauer and Orfanos, 1981), and phagocytosis of opsonized cells (Rhodes and Oliver, 1980). However, vit A increases the influx of circulating lymphocytes into the draining lymph nodes and increases DNA synthesis within these nodes (Dresser *et al.*, 1970). Increased numbers of macrophages were observed in retinyl palmitate-supplemented mice in the lung (Tachibana *et al.*, 1984) and in the peritoneum (Watson *et al.*, 1987). Katz *et al.* (1987a,b) noted an increase in the overall numbers of accessory cells in the draining lymph nodes and established a correlation between the number of dendritic cells and the degree of increased sensitization in vit A-fed mice compared to controls for two doses of oxazolone. Vitamin A-induced activation of macrophages requires mature T-cells (Watson and Moriguchi, 1989) since vit A-induced inhibition of tumor growth is not observed in thymectomized animals (Patek *et al.*, 1979) and administration of antiserum to T-lymphocytes can reverse enhanced immune responsiveness (Dennert *et al.*, 1979). The chemopreventive and therapeutic efficacy of a synthetic vit A analog, 13-*cis*-retinoic acid, has been demonstrated in a clinical trial (Hong *et al.*, 1990). In this experiment, patients with a primary oral cancer were treated with the vit A analog which significantly reduced the incidence of second regional primary tumors.

The emerging picture is that hypervitaminosis A augments cell-mediated immune responsiveness and that this fact can be exploited therapeutically.

Miller and co-workers investigated the use of vit A-supplemented diet as a means for augmenting the sensitivity of the MESA (Miller *et al.*, 1984). BALB/c mice received either a standard diet or a diet high in vit A acetate for 4 weeks. These investigators found that mice receiving the supplemented diet demonstrated ACD responses to topical challenge doses of oxazolone, a potent sensitizer, that were too low to induce sensitization in the mice fed the standard diet. In a follow-up study Maisey and Miller (1986) evaluated the sensitizing properties of 12 fragrances, four preservatives, and one medicament using the MESA with vit A-supplemented mice and six induction treatments. Parallel experiments with mice fed a regular diet were not performed and the bases for selections of sensitization and challenge doses were not specified. For eight of the compounds comparison was made to the guinea pig maximization test (GPMT). Agreement between the two tests was observed in four of eight cases, with the MESA yielding a positive determination and the GPMT negative in three of the other four cases. It was further noted that two of these latter three compounds gave positive results in human patch testing.

Aims of the Current Study

Two specific aims were identified for the current study. The first aim was to fine tune the noninvasive MESA by developing the complete dose–response of a potent sensitizer, dinitrofluorobenzene, and then characterizing responses to this compound at exceedingly low concentrations. From these low-dose data the SD50 would be determined. The second aim was to use dinitrofluorobenzene at the SD50 to investigate two noninvasive techniques for increasing the sensitivity of this assay: triple dose protocols and hypervitaminosis A. A companion study (Thorne *et al.*, 1991) tested the MESA for its ability to identify weak sensitizers using fragrances and complex fragrance mixtures using these noninvasive enhancements.

METHODS

Animals. Male BALB/cBy mice, 6 to 8 weeks old (The Jackson Laboratory, Animal Resources, Bar Harbor, ME), were used for nearly all the experiments. Some of the DNFB dose–response studies used male BALB/c mice, also 6 to 8 weeks old (Harlan Sprague–Dawley, Inc., Indianapolis, IN), due to the unavailability of BALB/cBy mice following the fire at The Jackson Laboratory. All animals were housed in stable groups of four or five under approved conditions in polypropylene cages with wood shavings bedding and with food and water supplied *ad libitum* in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23). The regular diet for the mice was a laboratory grade chow (Formulab Chow No. 5008, Purina Mills, Inc., Richmond, IN) that comes minimally supplemented with vit A. Carotene content was primarily from alfalfa and was less than 4.5 ppm and vit A acetate or palmitate was added to this feed by the manufacturer to a level of 15 IU/g feed. The mice fed an enhanced vit A diet received Special Mix 5751-A made from Formulab Chow No. 5008 modified to contain an additional 255 IU/g feed of vit A acetate (Purina Mills, Inc.). This feed was stored at 4°C and supplied fresh every other day. Mice were weighed periodically to ensure proper weight gain.

Chemicals. Test chemicals were as follows: 2,4-dinitrofluorobenzene, DNFB, 99% [70-34-8]; and 2,4-dinitrochlorobenzene, DNCB, 99+% [97-00-7]. Both were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). HPLC grade acetone was obtained from Fisher Scientific (Pittsburgh, PA). The DNFB and DNCB were stored desiccated at room temperature. The material used for depilating the abdomen of the mice, Depilon Soft Epil from Hamol International (Cologne, FRG), was formulated from calcium thioglycolate, lanette wax, and strontium hydroxide.

Irritation dose–response. It is advantageous to use a challenge dose that produces no measurable irritation at 24, 48, and 72 hr. Thus, the highest nonirritating dose was determined by developing an irritation dose–response curve for each compound. Groups of mice (usually five mice/group) received 40 μl applications to the ear of various concentrations of test compound in vehicle. The swelling of each ear was then assessed (as described below) at 1 to 2 hr and at 24, 48, 72, and 96 hr following application and irritation dose–response curves were constructed. 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 sum-

ming the mean and twice the standard deviation. A challenge dose that yielded an upper confidence bound that could be reliably assessed, but still be below the range typically seen with mild irritation, was selected. This upper confidence bound then served to define a positive response in the sensitization studies. In this way, positive responses could be distinguished from irritation responses with 95% certainty.

Sensitization dosing. A summary of the methods for determination of contact sensitization potency is provided in Table 2. On the day prior to the sensitization, the mice were depilated by applying approximately 0.8 g of Depilon and washing it off 5 min later with a soft sponge and warm water. On Day 0 of each experiment, 100 μl of vehicle containing the desired molar amount of test compound was applied, using a glass-tipped pipette, to a 3 by 3-cm area of the depilated abdomen of the mice. Each mouse was held until all the test compound had been absorbed (usually about ½ min).

Elicitation challenges. On Day 5 the thicknesses of both ears of all mice were determined using an Öditest low-tension, spring-loaded micrometer with 0.5-cm-diameter pads (Model D-1000, Dyer Co., Inc., Lancaster, PA). Triplicate measurements of each ear were taken with the micrometer pad on the anterior lateral aspect of the ear surface with the edge of the micrometer pad 2 mm from the outer edge of the ear. These measurements were performed quickly and without the need of anesthesia. Other mouse strains were less tolerant of this but the BALB/cBy mice remained calm during this procedure. The mice were then challenged on their left ear with vehicle only and on their right ear with the previously determined nonirritating dose of the test compound dissolved in 40 μl of the vehicle. The challenge dose was delivered from a glass-tipped pipette while the ear was gently extended with forceps. Half of the challenge solution was delivered to each side of the ear. The extent of mouse ear swelling was then assessed by comparison of the mean thickness of the ear at 24, 48, and 72 hr following challenge with the mean thickness just prior to challenge. Significant ear swelling was defined as an ETI exceeding the upper confidence bound defined in the irritation dose–response studies.

Triple-dose protocols. The protocol for sensitization using multiple dosing followed the same procedure as outlined above, except that mice received the 100 μl topical dosing to the abdomen on each of 3 days and were then challenged 5 days after the final induction dosing.

Vitamin A supplementation assays. 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 all performed using PC SAS (Version 6.03) except for one-way ANOVAs which used UNIX|STAT software (G. Perlman, Wang Institute, Tyngsboro, MA). Simple summary statistics were determined from the database using statistical utilities within Paradox 3.0.

TABLE 2

SUMMARY OF THE MOUSE EAR SWELLING ASSAY

Animals
Inbred BALB/cBy mice, 6 to 8 weeks are used in groups of four or five.
Day 0
Abdomen is depilated and washed, then allowed to dry. Induction dose is applied to abdomen in 100 μl volume.
Day 5
Baseline ear thickness is measured with a spring-loaded, low tension micrometer, three times each ear.
40 μl of test compound diluted to highest nonirritating concentration is applied to the right ear; 40 μl of the vehicle to the left ear.
Ear thickness is measured at 1, 24, 48, 72, and 96 hr postapplication and the ear thickness increase is the mean of 24, 48, and 72 hr values.

RESULTS

The noninvasive mouse ear swelling assay was found to be an effective test system for the study of ACD for potent sensitizers at all doses, including those at which the magnitude of the responses were what one would observe with a weak sensitizer. However, a number of details of the methodology were found to be essential to the success of the MESA. The micrometer used for the determinations was important. Several different micrometers were tested, including one with a digital readout and RS232 output. These devices did not provide the consistency and ease of handling of the modified Öditest which has been widely used. It was important to get the Öditest micrometer adjusted to exert enough pressure to close fully on the ear but not so much that the ear would be unduly squeezed. In addition, having all ear thickness measurements performed by one person increased the reliability of the results. No anesthesia was used in this study, either during dosing or during ear measurements.

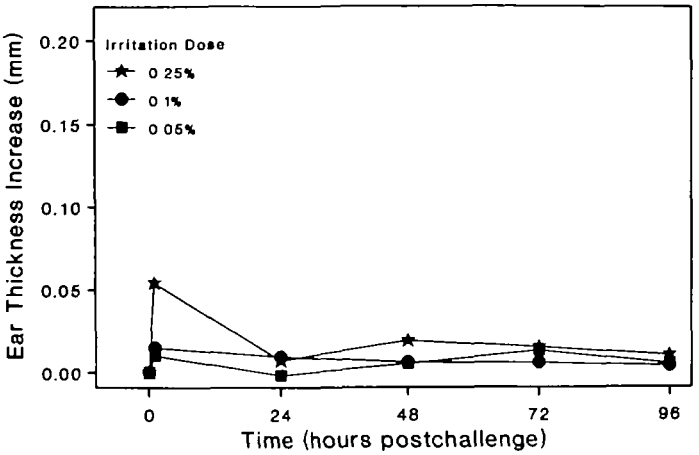

FIG. 1. Irritation response timecourse for DNFB. Mouse ears were dosed with 40 μl of the concentration indicated and the ear thickness increase from the preexposure value was determined over time. On the basis of the upper confidence bound obtained from these data (Table 3), 0.1% was chosen as the challenge concentration for the sensitization studies.

With proper handling, the BALB/cBy and BALB/c mice remained calm while ear measurements were made. Other strains tested (data not reported) did not so willingly submit to the measurements.

Analysis of prechallenge data for all mice in all groups indicated that the thickness of the untreated ears prior to challenge was essentially the same: 0.296 ± 0.010 mm for the left ears and 0.299 ± 0.011 mm for the right ears (coefficient of variation = 3.5%, $N = 252$). Within individual groups of mice the average standard deviation from the mean ear thickness was 0.0066 mm for the left ears and 0.0064 mm for the right ears. Cook's distance regression diagnostic (Kleinbaum *et al.*, 1988) was used to flag outliers in the preexposure data set. Five animals were identified as having had baseline ear thicknesses outside of the established normal limits (i.e., in the upper 5% of the F distribution). Three of these five animals had been noted in the laboratory notebooks as having "red ears" at the time of the preexposure measurement but had been used anyway. On this basis, these three were deleted from the database and the total N dropped from 255 to 252.

For ACD experiments the left ears of the animals were challenged with acetone alone while the right ears received the test compound diluted in acetone. The average ETI for all of the ears dosed with acetone only was 0.001 ± 0.006 mm. This was found to be not significantly different from zero.

Determination of the Dose-Response for DNFB

With an ultimate goal of trying to optimize the MESA to elicit positive responses with weak sensitizers, we chose to first develop a detailed dose-response curve for a potent sensitizer and then use this compound at very low doses and attempt to enhance the responses. To this end we performed an irritation dose-response trial to identify the proper challenge dose and then determined the delayed hypersensitivity responses to DNFB at 12 different sensitization doses in the single dose protocol.

Data for the DNFB irritation study are presented in Fig. 1 and Table 3. Three doses were studied for irritation potency: 0.05, 0.1, and 0.25%. The timecourse plot of the responses (Fig. 1) illustrates that ear swelling was minimal or nonexistent at all time points for all doses except for the 0.25% concentration at 1

TABLE 3

IRRITATION DOSE–RESPONSE RESULTS FOR DNFB

Concentration (% w/v)	Mean ETI[a] (mm, $\bar{x}$ (± SD))	Upper confidence bound (mm, $\bar{x}$ + 2 SD)
0.25	0.013 (0.015)	0.044
0.10	0.007 (0.006)	0.020
0.05	0.006 (0.003)	0.011

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.

hr which displayed slight irritation. As shown in Table 3 the mean ETI for these three concentrations was 0.006, 0.007, and 0.013 mm, respectively, yielding upper confidence bounds of 0.011, 0.020, and 0.044 mm. Since challenge with the vehicle alone yielded a response of 0.001 ± 0.006 mm and an upper confidence bound of 0.013 mm, 0.1% was selected as the challenge concentration. Thus, in the ACD protocol an ETI in excess of 0.020 mm would be regarded as a positive response. In our experience 0.020 mm represents about the lowest ETI value that can be reliably detected in an ACD test with groups of five mice. Had we chosen to challenge with the 0.25% solution we would have unnecessarily reduced the sensitivity of the assay by allowing irritation responses to mask immunologic responses.

The timecourse data for DNFB sensitization dose–response studies are shown in Figs. 2a and 2b. Figure 2a illustrates data for three high doses in BALB/cBy mice with all responses much greater than those of the control group which was administered acetone only (0% DNFB) on Day 0 and then challenged with 0.1% DNFB in the same manner as the other groups. These data display a negative dose–response relationship. Figure 2b illustrates that below 0.1% the responses displayed a positive relationship between dose and ETI and in general had their peak at the 48 hr timepoint (0.005, 0.001, and 0.0001% and some early and late timepoint data were left out for clarity). The data displayed in Fig. 2b were obtained using BALB/c mice due to the unavailability of mice from The Jackson Laboratory at that time. Comparison of the 0.1% data in Figs. 2a and 2b indicates a trend toward greater responsiveness of the BALB/cBy mice, although this was not significant. In these studies, ear thickness was measured at six timepoints. Although it was not necessary to establish the timecourse of the response by making this many determinations, it was of interest to gain as thorough an understanding of the responses as possible. Although the peak response occurred at the 48 hr timepoint, analysis of the data several different ways showed that the greatest sensitivity was obtained when the ear swelling was based upon the mean of the individual responses at 24, 48, and 72 hr postchallenge.

The complete dose–response plot indicating the group means (±1 SD) is shown in Fig. 3. Three regions of effect are delineated: a low-dose no-effect region below 0.0005%, a positive dose–response region from 0.0005 to 0.1%, and a high-dose down-regulated response region above 0.1%. The least-squares regression line fitting the data from 0.0010 to 0.1% yielded a correlation coefficient, r, of 0.82. The data in Fig. 3 are plotted in Fig. 4 as an incidence plot with the percentage of the mice yielding positive responses (ETI > 0.020 mm) plotted against sensitization concentration. The regression line is based on the data from 0.0005 to 0.0025% (r = 0.98), and upon interpolation to the 50% responding point, yielded a predicted SD50 of 0.00104% (0.040 mg/kg). Estimation of the SD50 in the early phase of the study suggested an approximate value of 0.0015%. In the final analysis, this value was found to correspond to the SD63. However, throughout the study 0.0015% was used as the SD50.

Studies of MESA Enhancements Using DNFB at the SD50

To test modifications of the MESA protocol designed to enhance the sensitivity of the assay we chose to utilize DNFB at its SD50, reasoning that if a modification did increase the sen-

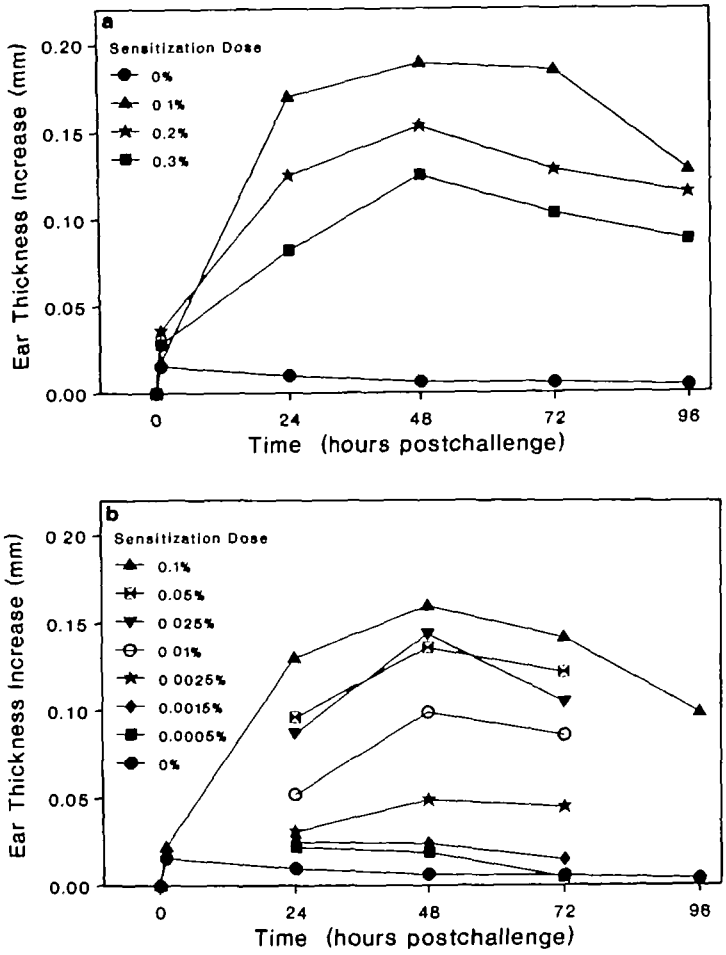

FIG. 2. (a,b) Sensitization dose–response timecourse for DNFB. (a) Responses upon challenge to three high sensitization doses, in addition to the control group (0%) that was given acetone at sensitization. All groups of mice were challenged with 0.1% on Day 5. The data demonstrate that as the dose was increased, the degree of ear swelling fell. Significant hypersensitivity reactions were still apparent at 96 hr postexposure. (b) Swelling following ear challenge of groups of mice sensitized with the lower concentrations listed. A positive dose–response relationship is indicated and the peak response, in most cases, was observed at 48 hr postexposure.

sitivity it would result in higher levels of ear swelling and greater percentages of mice demonstrating positive responses. If suppression of responses were the outcome, then ear swelling would be insignificant and the positive responses would fall significantly since the slope of the regression line in Fig. 4 is quite steep. Although a potent sensitizer dosed at its SD50 is not necessarily equivalent mechanistically to a weak sensitizer administered at high concentrations, this approach did allow us to systematically study the enhancement protocols in a system for which the outcomes were reproducible and well characterized and one for which multitudes of data exist using other animal and human test systems for ACD.

In this phase of the research, two hypotheses were developed: (1) multiple dosing does not enhance responses, but (2) high-dose vit A supplementation does enhance delayed-type hypersensitivity in the mouse. The first hypothesis had not been systematically evaluated

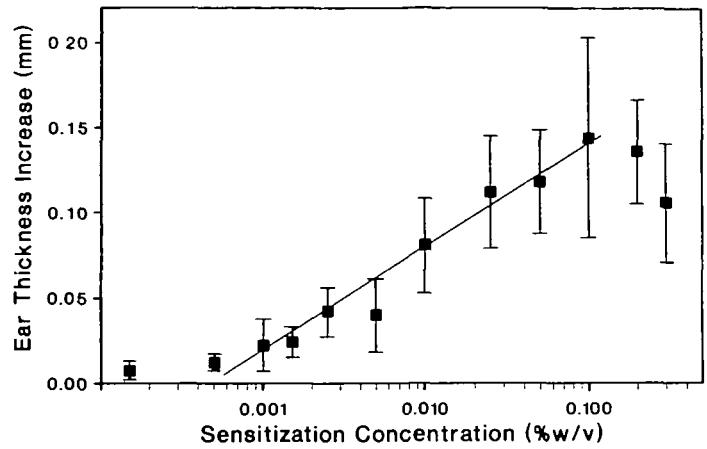

FIG. 3. Sensitization dose–response curve for DNFB. The data indicate a no observed effect level of 0.0004% (0.016 mg/kg), a dose-response range from 0.0005 to 0.1%, and a high-dose reduced-response range above 0.1%. Many "maximization" studies with DNFB have naively used sensitization doses in the reduced-response region. Data points are group means and error bars represent ± 1 SD. The correlation coefficient for the regression was $r = 0.82$.

in the literature in an adjuvant-free mouse assay. However, our experience with isocyanates in the MESA (unpublished data) had demonstrated little enhancement with multiple doses and no success with rechallenges. Other investigators routinely use multiple dose protocols but may not have tested their efficacy. The body of literature demonstrating that vit A stimulates most cell-mediated immune responses and especially the studies of Miller and co-workers (1984) with oxazolone bolstered our confidence in the validity of hypothesis 2.

To test the first hypothesis, three triple dose protocols were used: a Day 0, 1, and 2 dosing regimen; a Day 0, 2, and 4 regimen; and a Day 0, 3, and 5 regimen. At each of the three dos-

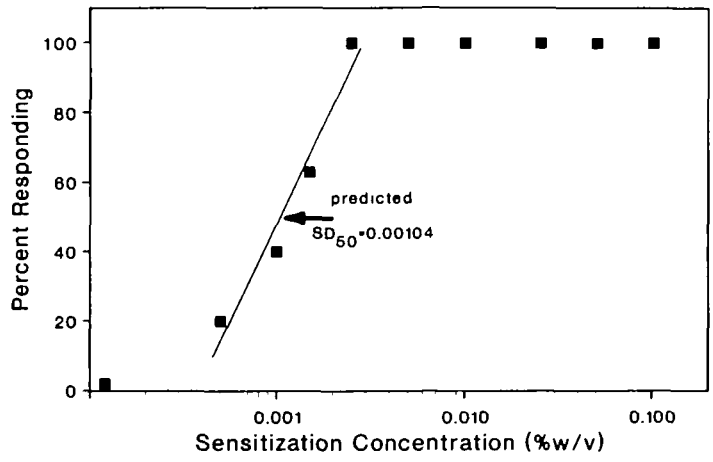

FIG. 4. Sensitization dose–response incidence plot showing the percentage responding in each group of mice versus DNFB sensitization dose. The regression line ($r = 0.98$) yielded a predicted SD50 of 0.0010% (0.040 mg/kg) and an SD100 of 0.0028%. The ear thickness increase (averaged over 24, 48, and 72 hr) required for a positive response to DNFB was 0.020 mm.

ings 100 μl of the SD50 was applied to the abdomen. These results were then compared with single dose groups run in parallel that were dosed just once. All four of these groups were challenged 5 days following the last dose. Table 4 shows that the mean ETI for the Day 0, 3, 5 regimen and the Day 0, 2, 4 regimen were not significantly different than the single dose protocol, with all falling in the range of 0.024 to 0.029 mm. The Day 0, 1, 2 triple dose protocol was somewhat lower. The p values from paired t tests indicated that none of the triple dose approaches produced ear swelling significantly different from the single dose approach. The single dose and nonsequential day, multiple dose protocols all yielded about 60% positive responses. The group of five mice that received doses on three sequential days yielded only 20% positive responses, indicating possible tolerization.

The second hypothesis, that the sensitivity of the MESA might be enhanced by vit A supplementation, was tested also using DNFB at the approximate SD50. Mice of the same age and shipment were divided into two groups: one received the regular feed and the other received the vit A-supplemented formula. Neither the weight gain nor the final weights of animals fed the special diet were significantly different from those of the mice maintained on the regular feed. No changes in physical appearance or behavior were noticed. Although no determinations of actual vit A dose were made, the estimated quantity of vit A consumed was 1000 to 1300 IU/day/mouse. Dosing for both groups began after the mice had been on their respective diets for 3 weeks. In addition, the vit A-supplemented animals were tested in both the single dose and the Day 0, 3, 5 dose protocol to further test hypothesis 1.

The results of the vit A experiments are presented in Fig. 5 and Table 5. One can see from Fig. 5 that the vit A-supplemented group demonstrated the same response timecourse as the regular feed group but had greater ear swelling at all timepoints. Mean ETI values given in Table 5 show that for both the single dose and triple dose protocol the vit A treatment nearly doubled the ear swelling response with high significance ($p < 0.001$). As was seen with the regular feed in Table 4, there was no significant difference in ETI between the single and triple dose protocols for the vit A-supplemented groups. Both the regular feed groups demonstrated about 63% positive responses versus greater than 94% positive responses for the vit A-supplemented groups. The triple dose protocol also failed to enhance the responses in the animals fed the vit A-supplemented diet. On the basis of these data, the multiple dose protocols examined did not enhance the responses, but 3 weeks consuming the vit A-supplemented feed increased the ETI by 80% and resulted in nearly all mice exhibiting positive responses at the SD50.

TABLE 4

TRIPLE DOSE PROTOCOLS COMPARED TO THE SINGLE DOSE PROTOCOL FOR DNFB (ALL DOSES AT THE SD50,[a] 0.0015%)

	Single dose	Days 0, 3, 5	Days 0, 2, 4	Days 0, 1, 2
Mean ETI,[b] mm	0.024	0.029	0.025	0.016
SD,[c] mm	0.009	0.022	0.007	0.010
% Positive	63	62	60	20
N	19	13	5	5
p value[d]	ref.	0.46	0.82	0.08

[a] Sensitization dose that produced significant delayed-type hypersensitivity responses in half the test animals.
[b] Ear thickness increase (See footnote to Table 3).
[c] Standard deviation.
[d] Each triple dose group compared to the single dose group (ref.).

TABLE 5

VITAMIN A SUPPLEMENTATION COMPARED TO THE REGULAR FEED FOR THE SINGLE DOSE PROTOCOL AND A TRIPLE DOSE PROTOCOL (ALL DNFB DOSES AT THE SD50[a] 0.0015%)

	Single dose		Days 0, 3, 5	
	Regular feed	Vitamin A	Regular feed	Vitamin A
Mean ETI,[b] mm	0.024	0.047	0.029	0.047
SD,[c] mm	0.009	0.017	0.022	0.015
% Positive	63	94	62	100
N	19	18	13	9
p value[d]	ref.	<0.001	0.46	<0.001
p value[e]		ref.		0.97

[a] See Table 4 footnote [a].
[b] Ear thickness increase (see footnote to Table 3).
[c] Standard deviation.
[d] Each group compared to the single dose, regular feed group (ref.).
[e] Triple dose, Vitamin A group compared to the single dose, Vitamin A group.

To ensure that these results would hold with another sensitizer at levels other than the SD50, an experiment was conducted in which DNCB was used as the test compound for mice on the regular and special diets. Three doses were tested: a low dose close to the SD50, a higher dose on the dose–response curve, and a very high dose in the down-regulated response region. In this study the single dose protocol was used with five mice per group and the challenge concentration was 0.5%. As shown in Fig. 6 the vit A supplementation produced significantly higher responses in all three regions. These increases over the regular feed groups were 52% for the low dose ($p < 0.05$), 86% for 0.1% ($p < 0.05$), and 280% for the high dose ($p < 0.01$). Thus, vit A supplementation was again shown to increase ACD responses.

The above studies demonstrated that the noninvasive MESA was an effective technique for studying ACD potency for known sensitizers, even at the very low concentrations which do not produce detectable ACD with other techniques. Second, it was shown that dose–response studies are necessary to characterize the responses and avoid the high-dose reduced response region. Third, it was demonstrated that triple dose protocols either had no effect or tended to reduce the ear swelling. Lastly, both the degree of ear swelling and the incidence of positive responses were enhanced by supplementing the diet with vit A for a period of 3 weeks.

DISCUSSION

The overall goal of this study was to validate and refine the MESA with a potent sensitizer and then test several noninvasive enhancements to the method using the same compound at very low doses. Using the strong sensitizer DNFB, we characterized its sensitizing potency across a four order of magnitude range of doses and determined the no-observed effect level, 0.0004% (0.016 mg/kg); the sensitization dose–response range, 0.0005 to 0.1% (0.020 to 4.0 mg/kg); and the high-dose reduced response range, above 0.1% (4.0 mg/kg). This approach also allowed determination of the SD50, 0.040 mg/kg, and showed that DNFB is a more potent sensitizer than all four isocyanates tested previously (Thorne *et al.*, 1986). At the SD50, the mean ear swelling for the group was 0.020 mm and for mice demonstrating positive responses it was 0.037 mm.

Enhancement studies were conducted at the SD50. This allowed identification of small changes in assay sensitivity in terms of both

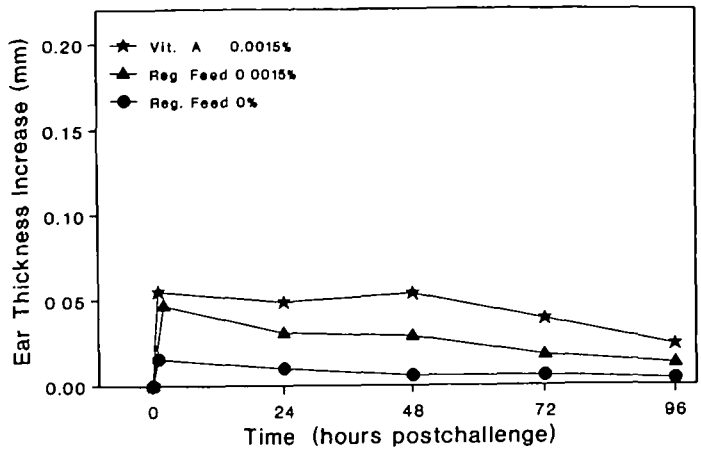

FIG. 5. Low level sensitization response timecourse for mice maintained on the regular feed or on the vit A-supplemented feed for 3 weeks prior to sensitization. The plot compares responses of groups of animals sensitized at the SD50 or to acetone only (regular feed 0%). The vit A supplementation produced responses that were significantly greater than those observed for mice on the regular feed diet at all time points beyond 2 hr.

the ETI and the percentage positive responses. Using this approach, it was found that three different triple dose protocols did not improve the assay but that supplementing the feed with vit A for 3 weeks prior to sensitization significantly increased the sensitivity of the method. In a companion paper (Thorne *et al.*, 1991) we have shown that the enhancement using vit A supplementation also increased the sensitivity of the assay for weak contact sensitizers (fragrances) and mixtures of weak sensitizers (fragrance formulations). Comparison of this method to those in other species with a limited number of fragrance compounds indicates that this method has comparable sensitivity to various guinea pig and human test protocols.

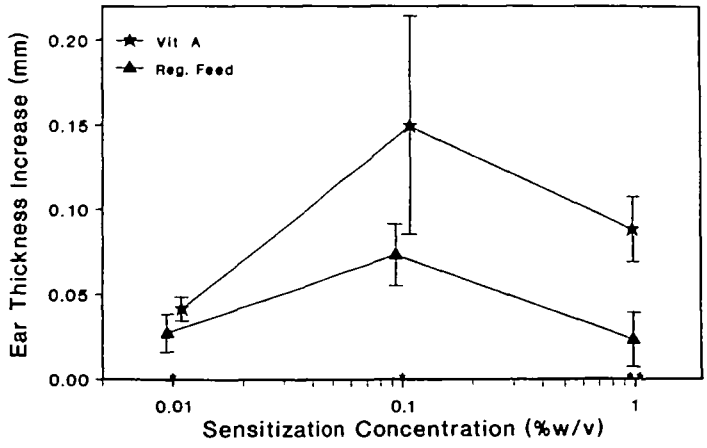

FIG. 6. Sensitization dose–response curve for DNCB comparing mice fed the regular diet with those given the vit A-supplemented diet. The three doses tested were chosen to cover the full range of responses. Error bars indicate ±1 SD (N = 5 mice/group). Differences between the vit A and regular feed groups were significant at 0.01 and 0.1% and highly significant at 1.0%.

Human patch testing with DNCB is nearly always positive with a dose of 100 μg (Hunter *et al.*, 1986). This same dose, delivered as a 0.1% concentration in the MESA, produced delayed-type hypersensitivity in all mice. When guinea pigs were patch tested or injected intradermally with 0.1% DNFB all of the animals demonstrated positive responses (Maurer, 1985). Rather than compare single point data, it would be of interest to compare ACD dose–response curves for DNFB across species and methods. However, few investigators have reported dose–response curves for any contact sensitizers and the data for such a comparison in the case of DNFB are unavailable.

Several other studies have been directed toward developing some form of the MESA as a testing protocol for potential contact sensitizers. An invasive variation of the MESA was used to evaluate the sensitizing properties of 72 industrial chemicals, fragrances, and preservatives, represented by 49 moderate to potent sensitizers and 23 compounds recognized as nonsensitizers (Gad *et al.*, 1986). This assay employed female CF-1 mice which were injected twice intradermally on Day 0 with Freund's complete adjuvant at the induction site. Topical doses of test material were applied to the shaved and tape-stripped abdomens on 4 consecutive days. On Day 7 mice were challenged with 20 μl of test or control solutions to each ear. Ear thickness increase was assessed under ether anesthesia at 24 and 48 hr with the 24 hr values used to calculate response levels. It was reported that using this protocol, 71 of the 72 compounds were correctly identified as sensitizers (48 of 49) or nonsensitizers (23 of 23). DNFB and DNCB were among those sensitizers tested by Gad *et al.* (1986) and found to produce significant swelling. These investigators report 168% swelling at 24 hr for DNFB and 130% for DNCB. The percentage ear swelling was calculated such that *zero* change in ear thickness upon challenge was assigned a value of *100%* ear swelling. These investigators reported fluctuations in baseline ear thickness measurements corresponding to a coefficient of variation of 15.8%. This was considerably higher than our value of 3.5%. The method of Gad *et al.* (1986) required a 20% increase in ear thickness to be judged a positive response ($p \leq 0.001$). In this study a 7% increase (0.020 mm) was accepted for the determination of a positive response ($p \leq 0.05$).

Attempts to independently confirm the results of Gad *et al.* (1986), particularly for weak and moderate sensitizers, have been unsuccessful despite the efforts of several investigators (Stephens *et al.*, 1987; Cornacoff *et al.*, 1988; Hignet *et al.*, 1989; Dunn *et al.*, 1990). Although there were notable problems in some of these studies, it is unclear why Dunn and co-workers did not obtain results similar to those of Gad *et al.* A follow-up study by Gad *et al.* (1987) demonstrated interlaboratory variability among five laboratories for two weak sensitizers. The groups that conducted the work reported by Dunn *et al.* were among those tested by Gad *et al.* In the study by Cornacoff *et al.* (1988) using a modified MESA, the enhancing modifications apparently decreased responsiveness in that DNFB, a model potent sensitizer (Asherson and Ptak, 1968; Sy *et al.*, 1977; Claman *et al.*, 1980; Botham *et al.*, 1987; Fairchild and Moorhead, 1987; Halliday and Muller, 1987; Robertson *et al.*, 1987; Thorne *et al.*, 1990), induced only negligible ear swelling. The problems encountered by Cornacoff *et al.* could have arisen from (1) their use of Rompun and Ketaset anesthesia at sensitization and 1 day prior to challenge, (2) other injections administered to these mice, (3) casting of the mice, (4) two site intradermal injections of Freund's complete adjuvant, or (5) determination of ear swelling in dead mice. These authors stated that MESA results are influenced by edema and individual interpretation. When the mouse ears are challenged with a nonirritating dose there is no edema attributable to irritation. The opposite ear, challenged with vehicle only, acts as a control for identification of edema. Individual interpretation is a problem in guinea pig and human skin testing where results are given subjective grades rather than with the MESA which yields objective parametric values. A

study was conducted by Hignet *et al.* (1989) to compare the mouse ear swelling test to a guinea pig sensitization test. In this work, mice were dosed on the abdomen daily for 3 consecutive days after which they were challenged 6 or 7 days later. Two potent sensitizers were evaluated: DNCB and hydroxylamine hydrochloride. These investigators obtained positive results with DNCB but found that only 10% of the animals were sensitized to HAHCl at 333 mg/ml (33.3%, w/v). Hignet *et al.* (1989) noted difficulty with consistent readings and as a result levels of ear swelling required for a positive response were quite high. The study conducted by Dunn *et al.* (1990) most closely duplicates the methods reported by Gad *et al.*, perhaps because Dunn was a co-author on the 1986 report (Gad *et al.*, 1986). Dunn and coworkers tested three negative control compounds and 7 sensitizers in two test laboratories and 16 other sensitizers in one of two test laboratories. They obtained agreement with the earlier study on only half of the test compounds. Unfortunately, this paper presented only the number of positive responses in each group of animals and provided no ear swelling data for exposed or control animals. Thus, it is difficult to assess the validity of the methods and analyses employed.

Nevertheless, these attempts to duplicate the findings of Gad *et al.* (1986) illustrate the difficulty in obtaining consistent results with invasive MESA protocols. All of these studies employed multiple dosing on sequential days. Our results bring into question the need for multiple dosing and indicate that dosing on sequential days may tolerize the animals to some degree. The studies by Gad *et al.* (1986) and Dunn *et al.* (1990) used single DNFB sensitization doses that were very high (0.5%). As shown in Fig. 3 this concentration is well within the supraoptimal dose region and would yield artificially low predictions of the sensitizing potency of the compound.

The work reported here served to validate, refine, and enhance the noninvasive MESA. As a first step toward gaining acceptance, a method for ACD testing must be validated using potent, moderate, and weak sensitizers as well as nonsensitizers. Research presented in the companion paper (Thorne *et al.*, 1991) builds on the studies presented here and reports tests of the noninvasive MESA using weak sensitizers and mixtures.

ACKNOWLEDGMENTS

The authors acknowledge the generous support of S. C. Johnson & Son, Inc., who funded the majority of the work described in this manuscript. The University of Iowa Graduate College is acknowledged for their support of S. D. Kaliszewski in the form of a Graduate Research Assistantship. Ms. Yun Ling Hu is acknowledged for her contributions during the pilot phase of this study.

REFERENCES

ASHERSON, G. L., AND PTAK, W. (1968). Contact and delayed hypersensitivity in the mouse. I. Active sensitization and passive transfer. *Immunology* **15**, 405–410.

BÄCK, O., AND LARSEN, A. (1982). Contact sensitivity in mice evaluated by means of ear swelling and a radiometric test. *J. Invest. Dermatol.* **78**, 309–312.

BARNETT, J. (1983). Immunomodulatory effects of 13-*cis*-retinoic acid on the IgG and IgM response of BALB/C mice. *Int. Arch. Allergy Appl. Immunol.* **72**, 227–233.

BAUER, C. E., AND ORFANOS, R. (1981). Trimethylmethoxyphenyl-retinoic acid (Ro10-1670) inhibits mitogen induced DNA synthesis in peripheral blood lymphocytes. *Br. J. Dermatol.* **105**, 19–24.

BERGSTRESSER, P. R., SULLIVAN, S., STREILEIN, J. W., AND TIGELAAR, R. E. (1985). Origin and function of Thy-1+ dendritic epidermal cells in mice. *J. Invest. Dermatol. Suppl.* **85**(1), 855–905.

BOTHAM, P. A., RATTRAY, N. J., WALSH, S. T., AND RILEY, E. J. (1987). Control of the immune response to contact sensitizing chemicals by cutaneous antigen-presenting cells. *Br. J. Dermatol.* **117**, 1–9.

CHRISTOPHERS, E. (1970). Die wanderungskinetik postmitotischer epidermiszellen. Autoradiographische untersuchungen. *Arch. Klin. Exp. Derm.* **236**, 161–172.

CLAMAN, H. N., MILLER, S. D., CONLON, P. J., AND MOORHEAD, J. W. (1980). Control of experimental contact sensitivity. In *Advances in Immunology* (F. J. Dixon and H. G. Kunkel, Eds.), Vol. 30, pp. 121–157. Academic Press, New York.

CONNER, M. J., ASHTON, R. E., AND LOWE, N. J. (1986). A comparative study of the induction of epidermal hyperplasia by natural and synthetic retinoids. *J. Pharmacol. Exp. Ther.* **237**, 31–35.

CONNER, M. J., AND LOWE, N. J. (1983). Induction of ornithine decarboxylase activity and DNA synthesis in hairless mouse epidermis by retinoids. *Cancer Res.* **43**, 5174–5177.

CORNACOFF, J. B., HOUSE, R. V., AND DEAN, J. H. (1988). Comparison of a radioisotopic method and the mouse ear swelling test (MEST) for contact sensitivity to weak sensitizers. *Fundam. Appl. Toxicol.* **10,** 40–44.

DENNERT, G. (1984). Retinoids and the immune system: Immunostimulation by vitamin A. In *The Retinoids* (M. B. Sporn, A. B. Roberts, and D. S. Goodman, Eds.), Vol. 2, pp. 372–390. Academic Press, New York.

DENNERT, G., CROWLEY, C., KOUBA, J., AND LOTAN, R. (1979). Retinoic acid stimulation of the induction of mouse killer T-cells in allogeneic and syngeneic systems. *J. Natl. Cancer Inst.* **62,** 89–94.

DESCOTES, J. (1988). Identification of contact allergens: The mouse ear sensitization assay. *J. Toxicol. Cutaneous Ocul. Toxicol.* **7,** 263–272.

DIELI, F., ROMANO, G. C., ABRIGNANI, S., COLIZZI, V., AND SALERNO, A. (1985). Infection of mice with Newcastle disease virus inhibits the T suppressor afferent cell circuit which regulates contact sensitivity to picryl chloride. *Cell. Immunol.* **94,** 225–230.

DRESSER, D. W., TAUB, R. N., AND KRANTZ, A. R. (1970). The effect of localized injection of adjuvant material on the draining lymph node. II. Circulating lymphocytes. *Immunology* **18,** 663–670.

DUNN, B. J., RUSCH, G. M., SIGLIN, J. C., AND BLASZCAK, D. L. (1990). Variability of a mouse ear swelling test (MEST) in predicting weak and moderate contact sensitization. *Fundam. Appl. Toxicol.* **15,** 242–248.

EMMETT, E. A. (1976). Allergic contact dermatitis in polyurethane plastic moulders. *J. Occup. Med.* **18,** 802–804.

FAIRCHILD, R. L., AND MOORHEAD, J. W. (1987). Soluble factors in tolerance and contact sensitivity to DNFB in Mice. VII. Characterization of a monoclonal, efferent-acting suppressor factor with specificity for DNP/H-2K. *Cell. Immunol.* **105,** 147–160.

FREGERT, S. (1986). Contact allergens and prevention of contact dermatitis. *J. Allergy Clin. Immunol.* **78,** 1071–1072.

FRENKEL, J. K. (1967). Adoptive immunity to intracellular infection. *J. Immunol.* **98,** 1309–1319.

GAD, S. C., DUNN, B. J., DOBBS, D. W., REILLY, C., AND WALSH, R. D. (1986). Development and validation of an alternative dermal sensitization test: The mouse ear swelling test (MEST). *Toxicol. Appl. Pharmacol.* **84,** 93–114.

GAD, S. C., DOBBS, D. W., DUNN, B. J., REILLY, C., WALSH, R. D., AULETTA, C. S., HILE, R. A., REAGAN, E., AND YENSER, B. (1987). Interlaboratory validation test (MEST). In *In Vitro Toxicology: Approaches to Validation.* (A. M. Goldberg, Ed.), pp. 275–292. Liebert, New York.

GERBERICK, G. F., AND RYAN, C. A. (1989). A predictive mouse ear swelling model for investigating topical phototoxicity. *Food Chem. Toxic.* **27,** 813–819.

HALLIDAY, G. M., AND MULLER, H. K. (1987). Sensitization through carcinogen-induced Langerhans cell-deficient skin activates specific long-lived suppressor cells for both cellular and humoral immunity. *Cell. Immunol.* **109,** 206–221.

HARRIOTT-SMITH, T. G., AND HALLIDAY, J. (1988). Suppression of contact hypersensitivity by short-term ultraviolet irradiation. I. Immunosuppression by serum from irradiated mice. *Clin. Exp. Immunol.* **7,** 144–148.

HIGNET, S., DORKO, J. D., KENNAH, H. E., AND BARROW, C. S. (1989). Evaluation of the mouse ear swelling test (MEST) as a replacement for guinea pig sensitization testing. *Toxicologist* **9,** 71.

HONG, W. K., LIPPMAN, S. M., ITRI, L. M., *et al.* (1990). Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.* **323,** 795–801.

HUNTER, J. A. A., CARR, M. M., BOTHAM, P. A., GAWKRODGER, D. J., MCVITTIE, E., ROSS, J. A., AND STEWART, I. C. (1986). Experimental contact dermatitis using 2,4-dinitrochlorobenzene in humans. In *Skin Models* (R. Marks and G. Plewig, Eds.), pp. 140–146. Springer-Verlag, New York.

JUN, B.-D., ROBERTS, L. K., CHO, B.-H., ROBERTSON, B., AND DAYNES, R. A. (1988). Parallel recovery of epidermal antigen-presenting cell activity and contact hypersensitivity responses in mice exposed to ultraviolet irradiation: The role of a prostaglandin-dependent mechanism. *J. Invest. Dermatol.* **90,** 311–316.

JURIN, M., AND TANNOCK, I. F. (1972). Influence of vitamin A on immunological response. *Immunology* **23,** 283–287.

KATZ, D. R., DRZYMALA, M., TURTON, J. A., HICKS, R. M., HUNT, R., PALMER, L., AND MALKOVSKY, M. (1987a). Regulation of accessory cell function by retinoids in murine immune responses. *Br. J. Exp. Pathol.* **68,** 343–350.

KATZ, D. R., MUKHERJEE, S., MAISEY, J., AND MILLER, K. (1987b). Vitamin A acetate as a regulator of accessory cell function in delayed-type hypersensitivity responses. *Int. Arch. Allergy Appl. Immunol.* **82,** 53–56.

KLECAK, G. (1987). Identification of contact allergens: Predictive tests in animals. In *Dermatotoxicology* (F. N. Marzulli and H. I. Maibach, Eds.), 3rd ed., pp. 227–275. Hemisphere, New York.

KLECAK, G., GELEICK, H., AND FREY, J. R. (1977). Screening of fragrance materials for allergenicity in the guinea pig. I. Comparison of four testing methods. *J. Soc. Cosmet. Chem.* **28,** 53–54.

KLEINBAUM, D. G., KUPPER, L. L., AND MULLER, K. E. (1988). *Applied Regression Analysis and Other Multivariable Methods,* 2nd ed., pp. 197–205. PWS-Kent, Boston.

MAISEY, J., AND MILLER, K. (1986). Assessment of the ability of mice fed on vitamin A supplemented diet to respond to a variety of potential contact sensitizers. *Contact Dermatitis* **15,** 17–23.

MALKOVSKY, M., DORE, C., HUNT, R., PALMER, L., CHANDLER, P., AND MEDAWAR, P. B. (1983a). Enhancement of specific antitumor immunity in mice fed a diet enriched in vitamin A acetate. *Proc. Natl. Acad. Sci. USA* **80,** 6322–6326.

MALKOVSKY, M., EDWARDS, A. J., HUNT, R., PALMER, L., AND MEDAWAR, P. B. (1983b). T-cell mediated enhancement of host-versus-graft reactivity in mice fed a diet enriched in vitamin A acetate. *Nature (London)* **302,** 338–340.

MAURER, T. (1985). The optimization test. In *Current Problems in Dermatology* (K. E. Andersen and H. I. Maibach, Eds.), Vol. 14, pp. 114–151. Karger, New York.

MEDAWAR, P. B., AND HUNT, R. (1981). Anti-cancer action of retinoids. *Immunology* **42,** 349–353.

MENNÉ, T., AND CHRISTOPHERSON, J. (1985). Epidemiology of allergic contact sensitization. In *Current Problems in Dermatology* (K. E. Andersen and H. I. Maibach, Eds.), Vol. 14, pp. 1–30. Karger, New York.

MILLER, K., MAISEY, J., AND MALKOVSKY, M. (1984). Enhancement of contact sensitization in mice fed a diet enriched in vitamin A acetate. *Int. Arch. Allergy Appl. Immun.* **75,** 120–125.

National Institutes of Health (1985). *NIH Guide for the Care and Use of Laboratory Animals,* NIH Publ. No. 86-23.

ORITA, M. (1987). Effects of ultraviolet irradiation on surface marker expression by epidermal immunocompetent cells and contact sensitization to dinitrofluorobenzene in mice. *Br. J. Dermatol.* **117,** 721–733.

PATEK, P. Q., COLLINS, J. L., YOGEESWAREN, G., AND DENNERT, G. (1979). Antitumor potential of retinoic acid: Stimulation of immune mediated effectors. *Int. J. Cancer* **24,** 624–628.

PLEWIG, G., AND BRAUN-FALCO, O. (1975). Kinetics of epidermis and adnexa following vitamin A acid in the human. *Acta Derm. Venereol. Suppl.* **74,** 87–98.

PTAK, W., BERTERA, M., PTAK, M., IVERSON, G. M., AND GREEN, D. R. (1985). Suppression and contrasuppression in the induction of contact sensitivity by the administration of cellbound antigen-antibody complexes. *J Immunol.* **135**(4), 2312–2318.

RHODES, J., AND OLIVER, S. (1980). Retinoids as regulators of macrophage function. *Immunology* **40,** 467–472.

ROBERTS, L. K., SPANGRUDE, G. J., DAYNES, R. A., AND KRUEGER, G. G. (1985). Correlation between keratinocyte expression of Ia and the intensity and duration of contact hypersensitivity responses in mice. *J. Immunol.* **135,** 2929–2936.

ROBERTSON, B., GAHRING, L., NEWTON, R., AND DAUNES, R. (1987). *In vivo* administration of interleukin 1 to normal mice depresses their capacity to elicit contact hypersensitivity responses: Prostaglandins are involved in this modification of immune function. *J. Invest. Dermatol.* **88,** 380–387.

ROTHE, A. (1976). Occupational skin diseases caused by polyurethane chemicals. *Berufs-Dermatosen* **24,** 7–24.

SMITH, S. M., LEVY, N. S., AND HAYES, C. E. (1987). Impaired immunity in vitamin A-deficient mice. *J. Nutr.* **117,** 857–865.

STADLER, J. C., AND KAROL, M. H. (1985). Use of dose–response data to compare skin sensitizing abilities of dicyclohexylmethane-4,4′-diisocyanate and picryl chloride in two animal species. *Toxicol. Appl. Pharmacol.* **78,** 445–450.

STEPHENS, T. J., DRAKE, K., RENSKERS, K. J., TEAL, J., PENNEY, D., KAMINSKY, M., GRAY, T., AND NORTHROOT, H. (1987). Preliminary evaluation of the mouse ear swelling test (MEST). *Toxicologist* **7,** 83.

STREILEIN, J. W. (1985). Circuits and signals of the skin-associated lymphoid tissues (SALT). *J. Invest. Dermatol. Suppl.* **85,** 10–13.

SY, M., MILLER, S. D., AND CLAMAN, H. N. (1977). Immune suppression with supra optimal doses of antigen in contact sensitivity. I. Demonstration of suppressor cells and their sensitivity to cyclophosphamide. *J. Immunol.* **119,** 240–244.

TACHIBANA, K., SONE, S., TSUBURA, E., AND KISHINO, Y. (1984). Stimulatory effect of vitamin A on tumoricidal activity of rat alveolar macrophages. *Br. J. Cancer* **49,** 343–348.

THORNE, P. S., HAWK, C., KALISZEWSKI, S. D., AND GUINEY, P. D. (1990). Refinement of a non-invasive mouse model for testing weak contact sensitizers. *Toxicologist* **10,** 151.

THORNE, P. S., HAWK, C., KALISZEWSKI, S. D., AND GUINEY, P. D. (1991). The noninvasive mouse ear swelling assay. II. Testing the contact sensitizing potency of fragrances. *Fundam. Appl. Toxicol.* **17,** 807–820.

THORNE, P. S., HILLEBRAND, J. A., LEWIS, G. R., AND KAROL, M. H. (1986). Experimental contact sensitization by isocyanates. *Toxicologist* **6,** 15.

THORNE, P. S., HILLEBRAND, J. A., LEWIS, G. R., AND KAROL, M. H. (1987). Contact sensitivity by diisocyanates: Potencies and cross-reactivities. *Toxicol. Appl. Pharmacol.* **87,** 155–165.

TOEWS, G., BERGSTRESSER, P. R., AND STREILEIN, J. W. (1980). Epidermal langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* **124,** 445–453.

WATSON, R. R., AND MORIGUCHI, S. (1989). Effect of retinyl palmitate and 13-cis retinoic acid on immune functions in immunodeficient, nude mice. *Life Sci.* **44,** 387–395.

WATSON, R. R., MORIGUCHI, S., AND GENSLER, H. L. (1987). Effect of dietary retinyl palmitate and selenium on tumoricidal capacity of macrophages in mice undergoing tumor promotion. *Cancer Lett.* **36,** 181–187.