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COMPARISON OF MURINE ASSAYS FOR THE IDENTIFICATION OF CHEMICAL SENSITIZERS

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Efforts are under way to develop a phenotypic analysis assay capable of identifying and differentiating chemicals with the potential to induce irritation and IgE-mediated and T-cell–mediated sensitization. These studies compare results obtained with the irritancy/phenotypic analysis assay to those obtained in the mouse ear-swelling test (MEST) and the local lymph node assay (LLNA). Female BALB/c mice (5 animals per group) were exposed topically to the human sensitizers 2,4-dinitrochlorobenzene (DNCB) and potassium dichromate (PDC) as well as the irritant methyl salicylate (MSC), following the protocol for each assay. DNCB was identified as a sensitizer in the LLNA and phenotypic analysis assay at concentrations as low 0.25%. In the MEST, DNCB was considered positive in concentrations as low as 0.01 and 0.05% at the 24- and 48-h measurements of the MEST, respectively. PDC tested positive as a sensitizer in the LLNA and the phenotypic analysis assay at concentrations as low as 0.25 and 0.1%, respectively. In the MEST, PDC was negative at the 24-h time point and tested positive only at the 48-h measurement at the highest concentration tested, 0.5%. Both DNCB and PDC induced an elevation in total serum IgE at the 0.5% concentrations. MSC was negative for sensitizing potential at all concentrations tested in all four assays. Phenotypic analysis of lymph node cells removed from animals dosed with the three chemicals gave results similar to the LLNA, but had the advantage of detecting chemicals with the capacity to induce an increase in the IgE^{+} cell population.

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Historically, regulatory agencies have utilized guinea pig tests for hazard identification of chemical sensitizers. Like the guinea pig assays, the mouse ear-swelling test (MEST) proposed by Gad et al. (1986) also requires the induction and elicitation phases for evaluation but relies on a quantitative measurement of ear swelling as an endpoint as opposed to subjective evaluation of erythema and edema (Buehler, 1965, 1985; Magnusson and Kligman, 1969, 1970). In contrast, the local lymph node assay (LLNA), which has recently undergone peer review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), evaluates only the induction phase of the hypersensitivity response by quantitating [^{3}H]thymidine incorporation into proliferating draining lymph node cells following chemical exposure (Kimber et al., 1991, 1994). As an alternative endpoint to the LLNA, phenotypic analysis of lymph node cells draining the site of chemical exposure has been proposed as a means of identifing chemical sensitizers without the use of radioisotopes (Gerberick et al., 1997; Manetz and Meade, 1999; Sikorski et al., 1996). By combining the endpoints of a murine ear-swelling assay for irritation with phenotypic analysis, a single assay has been proposed for the identification and differentitation of irritants, T-cell–mediated and IgE-mediated sensitizers (Manetz and Meade, 1999). Enzyme-linked immunosorbent assay (ELISA) of total serum IgE has been proposed as a method for identifying chemicals with the potential for inducing IgE-mediated hypersensitivity (Dearman et al., 1991, 1998). Using two well-known human sensitizers, 2,4-dinitrochlorobenzene (DNCB) and potassium dichromate (PDC), and an irritant control, methyl salicylate (MSC), these dose–response studies were conducted to compare results obtained with the irritancy/phenotypic analysis assay, LLNA, MEST, and total IgE ELISA. The chemicals and concentrations used in these experiments were chosen to compare results in this laboratory with those participating in one of the international validation studies of the LLNA (Kimber et al., 1995a).

MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from Charles River and Harlan Laboratories. Upon arrival, the mice were quarantined for 1 week prior to use. Mice were weighed, tail-marked for identification, and assigned to homogeneous weight groups ($n = 5$). All animals were 7–9 weeks of age, weighed 15.5–23.0 g at the start of the study, and were maintained on Agway Rat and Mouse Ration (NIH-07) or Agway Prolab 3500 and tap water ad libitum. Mice were maintained under conditions specified within NIH guidelines. Animal rooms were maintained between 18 and 26°C with 40–70% relative humidity and 12-h light/dark cycles.

Chemicals

1-Chloro-2,4-dinitrobenzene (DNCB; purity 98%), potassium dichromate (PDC; purity 99%), methyl salicylate (MSC), toluene diisocyanate (TDI; purity 99.6%), 2,4-dinitrofluorobenzene (DNFB; purity 99.4%), dimethyl sulfoxide, and acetone were purchased from Sigma Chemical (St. Louis, MO). Acetone (99.8%)/olive oil 4:1 was used as the vehicle for all chemicals except PDC, which was dissolved in dimethyl sulfoxide (DMSO). Concentrations chosen for test articles were based on those used in a LLNA validation study (Kimber et al., 1995a). Choice of DNFB, a strong T-cell sensitzer, and TDI, a potent inducer of IgE, as positive controls was based on historical data obtained in our laboratory. Concentrations of chemicals tested are shown in Table 1.

Contact Hypersensitivity Assays

Irritancy Assay

A primary irritancy study of the potential sensitizers was performed as described by Hayes et al. (1998) to establish the concentration to be used for challenge in the mouse ear-swelling test. Using a 4-day dosing protocol, pre- and post-treatment ear measurements were obtained to calculate ear swelling. The mean percent ear swelling (± standard error) for each treatment group was calculated and compared to the respective vehicle (VH) for significance and dose response. The minimal irritating concentration (MIC) was defined as the lowest concentration of test material that, after 4 days of exposure, produced a percent ear swelling significantly greater than did vehicle. This concentration was used as the single challenge dose in the MEST.

TABLE 1. Concentrations of Chemicals Tested

		Chemical concentrations						
Chemical	Vehicle	Induction						Challenge (%)
DNCB (w/v)[a]	Acetone[b]	0.01	0.025	0.05	0.1	0.25	0.5	0.5
MSC (v/v)[c]	Acetone[b]	1	2.5	5	10	20	—	20
PDC (w/v)[a]	DMSO	0.025	0.05	0.1	0.25	0.5	—	0.5
DNFB (v/v)[c]	Acetone[b]	0.25	—	—	—	—	—	0.5
TDI (w/v)[a]	Acetone[b]	2.5	—	—	—	—	—	—

Note. DNCB, 1-chloro-2,4-dinitrobenzene; PDC, potassium dichromate; MSC, methyl salicylate; DMSO, dimethyl sulfoxide.

[a]Percent concentration prepared as a weight:volume ratio.

[b]Acetone represents 4:1 acetone/olive oil.

[c]Percent concentration prepared as a volume:volume ratio.

Mouse Ear-Swelling Test

The mouse ear-swelling test (MEST), based on the original procedure described by Gad et al. (1986) was followed, with some minor modifications. Prior to the sensitization period, mice were anesthetized and the dorsal lumbar area of each mouse was shaved. For 3 consecutive days, 50 μL of vehicle, test article, or positive control were applied to the shaven site with a finnpipette (Table 1). Mice were then rested until day 8, when the thickness of the right ear of each mouse was premeasured as described for the irritancy assay. Mice then received a single challenge of 25 μL of vehicle, test article, or 0.5% DNFB divided between the dorsal and ventral surfaces of the right ear pinna. To assess the contact hypersensitivity response, ear measurements were taken 24 and 48 h following challenge. The percent change in ear thickness was calculated as described for the irritancy assay. The mean percent ear swelling for each dose group was compared to the appropriate background control group (induction with the vehicle followed by challenge with the test article MIC, to account for swelling due to irritation) for significance and dose response.

Local Lymph Node Assay

The local lymph node assay (LLNA) was performed as described by Hayes et al. (1998). Radioactivity was counted on a LKB Wallac 1218 beta counter for 5 min and raw data were collected directly from the beta counter. The mean counts per minute (cpm)-background count and dpm for each dose group were calculated and compared to the vehicle for significance and dose response. Dpm values were calculated as the cpm-background count/the efficiency of the beta counter (63.6%).

Irritancy/Phenotypic Analysis

The irritancy/phenotypic analysis assay was performed following the procedure described by Manetz and Meade (1999). Draining lymph nodes were dissociated into single-cell suspensions and stained with 1.0 μg FcBlock (2.4G2, anti FcγII and FcγIII) for 5 min at 4°C followed by staining with RA3-6B2 (PE-labeled anti-B220), R-35-72 (FITC-labeled anti-IgE), and the isotype controls for approximately 45 min in the dark at 4°C. Propidium iodide (PI) was added to the cells for 5 min in the dark at 4°C. The cells were washed and resuspended in staining buffer (PBS, 1.0% bovine serum albumin (BSA), and 0.10% sodium azide) for analysis on a Becton Dickinson FACSVantage flow cytometer. At least 9000 events were collected for each of the samples. Of those events, only viable cells, based on PI staining, were further analyzed by Cellquest (Becton Dickinson). All antibodies were purchased from Pharmingen (San Diego, CA).

Total IgE ELISA

Following sacrifice of the animals used in the phenotypic analysis assay, blood was collected via cardiac puncture. Serum fractions were stored in microcentrifuge tubes at −80°C until analysis. Total IgE levels were quantified by total serum IgE ELISA as described by Manetz and Meade (1999). Plates were read on a Spectramax Vmax plate reader (Molecular Devices) at 405–650 nm approximately 20 min afterwards. Data were analyzed using the IBM Softmax Pro 1.2.0 program. Test sera antibody values were calculated by comparing their values to the values of known amounts of IgE standard. A standard curve was generated using linear values from the log/log analysis (values allowing for a slope close to 1.0).

Statistical Analysis

Statistical analysis was performed for all test groups using JMP 3.2.2 and Graph Pad Prism for the PC. All test groups were analyzed for homogeneity using the Bartlett's chi-square test (Bartlett, 1937). If determined homogeneous ($p < .05$), the Dunnett's multiple range t test (Dunnett, 1955) was used to compare treatment groups with the control group. If determined nonhomogeneous, data were analyzed using the Tukey–Kramer test or Wilcoxon's rank sum test to compare treatment groups with indicated control groups (Gross and Clark, 1975a; Tukey, 1977). Jonckheere's trend analysis was used to determine dose-responsive effects for irritancy, MEST, and LLNA (Gross and Clark, 1975b). Linear regression analysis was used to determine dose-responsive effects for the irritancy/phenotypic analysis assay. Statistical significance between the positive controls and the vehicle controls in the MEST was determined using a Student's t test. All values are presented as group means ± standard errors.

RESULTS

There were no chemical-related deaths in any of the dose groups. With the exception of the erythema and edema associated with irritancy and contact hypersensitivity responses, all mice appeared clinically normal throughout the study. There were no statistically significant differences in the body weights of the test groups of animals when compared to their respective vehicle controls.

Irritancy Assay

The irritancy assay was used to identify the MIC to be used as the challenge concentration in the MEST. The lowest concentrations of DNCB and PDC demonstrating a significant ($p < .01$) irritation response were

0.5% (data not shown). Trend analysis showed a dose response for the irritancy of these chemicals (DNCB, $p < .01$ and PDC, $p < .05$). No significant irritation was seen in animals exposed to MSC at concentrations as high as 20%, the highest concentration tested; therefore, 20% was used for challenge in the MEST.

Mouse Ear-Swelling Test

Both DNCB and PDC were identified as contact sensitizers in the MEST. Trend analysis of DNCB was found to be significant ($p < .01$) at both the 24-h and the 48-h post measurements, indicating dose dependency. PDC also demonstrated dose dependency at the 24-h ($p < .05$) and the 48-h ($p < .01$) post measurements. Although statistical significance was reached at low concentrations in DNCB-exposed mice, using a greater than 20% increase in ear swelling when compared to control as the criterion for sensitization (Gad et al., 1986), both chemicals were found to be sensitizing at the 48-h post-challenge time point at a concentration of 0.5% (Figures 1 and 2). Induction with concentrations ranging from 1 to 20% MSC followed by challenge with 20% MSC did not produce a significant change in percent ear swelling at 24 or 48 h post challenge as compared to the 20% MSC challenge-only group (data not shown). Based on trend analysis, only the 48-h response of MSC was determined to be dose-dependent ($p < .05$). Although producing statistically significant increases in ear swelling 24 and 48 h post challenge for three studies, the measurements for the positive control groups were highly variable, ranging from 72.9 ± 8.0 to 236.6 ± 30.1 (Table 2).

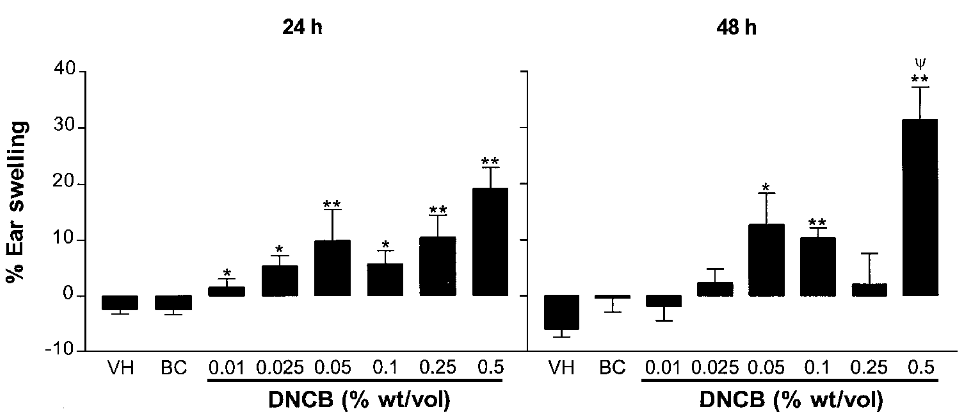

FIGURE 1. MEST response following 1-chloro-2,4-dinitrobenzene (DNCB) exposure. Data are expressed as means ± SE. Dose groups were determined to be nonhomogenous (Bartlett's test) and compared to the BC group (sensitized with vehicle and challenged with 0.5% DNCB) by Dunnett's *t* test, where $^{*}p < .05$ and $^{**}p < .01$. ψ represents those concentrations of chemical that resulted in a 20% increase in ear swelling over BC. Trend analysis was determined significant ($p < .01$) by Jonckheere's test. VH, vehicle; BC, background control.

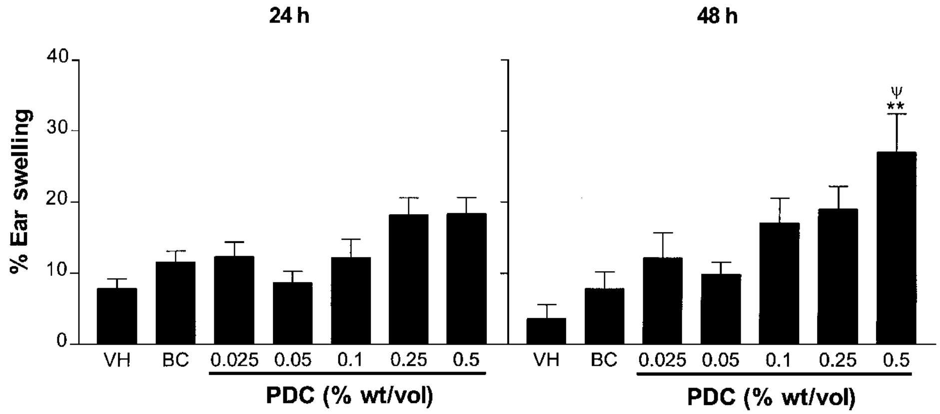

FIGURE 2. MEST response following potassium dichromate (PDC) exposure. Data are expressed as means ± SE. Dose groups were determined to be nonhomogenous (Bartlett's test) and compared to the VC group (sensitized with vehicle and challenged with 0.5% DNCB) by the Dunnett's t test, where $^{*}p < .05$ and $^{**}p < .01$. ψ represents those concentrations of chemical that resulted in a 20% increase in ear swelling over BC. Trend analysis was determined significant ($p < .05$ and $p < .01$, respectively) at the 24- and 48-h time points by Jonckheere's test. VH, vehicle; BC, background control.

Local Lymph Node Assay

In the LLNA, a dose-responsive increase ($p < .01$) in lymph node cell proliferation was seen following exposure to both DNCB (Figure 3) and PDC (Figure 4). A positive response (3-fold increase over control (Kimber et al., 1991) and statistical significance) was observed in animals exposed to concentrations of 0.25% or higher of both chemicals. Animals exposed to MSC demonstrated a slight but dose-responsive increase in

TABLE 2. Positive Controls for the MEST

		Mouse ear swelling test	
Dose group	Challenge[a]	24-h ear swelling[b]	48-h ear swelling[b]
DNCB			
Acetone/OO[c]	0.5% DNFB	9.6 ± 5.7	24.8 ± 6.8**[S]
0.25% DNFB	0.5% DNFB	68.1 ± 11.3**[S]	236.6 ± 30.1**[S]
MSC			
Acetone/OO[c]	0.5% DNFB	17.0 ± 3.3**[S]	19.9 ± 5.0**[S]
0.25% DNFB	0.5% DNFB	59.6 ± 9.4**[S]	72.9 ± 8.0**[S]
PDC			
DMSO	0.5% DNFB	16.5 ± 3.4*[S]	33.5 ± 6.7**[S]
0.25% DNFB	0.5% DNFB	136.8 ± 20.5**[S]	220.5 ± 37.3**[S]

[a]Challenge following induction is only for the MEST.

[b]For the ear swelling, the number of samples was 10. Values are the dose group means ± standard errors.

[c]Acetone represents 4:1 acetone/olive oil.

[S]Statistical difference when compared to the vehicle using the Student's t test: $^{*}p < .05$, $^{**}p < .01$.

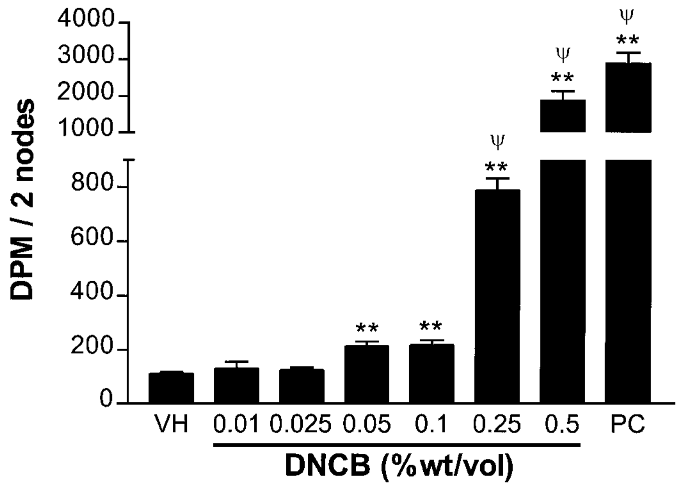

FIGURE 3. LLNA response following 1-chloro-2,4-dinitrobenzene (DNCB) exposure. Data are expressed as group means ± SE. Dose groups were determined to be nonhomogenous (Bartlett's test) and compared to the vehicle group by the Wilcoxon rank test, where $^{**}p < .01$. ψ represents concentrations of chemical that resulted in a 3-fold increase in proliferation over control. Trend analysis was determined significant ($p < .01$) by Jonckheere's test. VH, vehicle; PC, positive control (0.25% DNFB).

lymph node cell proliferation ranging from 85± 18 to 146± 14 dpm, reaching statistical significance following exposure to 20% MSC (146 ± 14 dpm). However, the level of proliferation did not meet the 3-fold increase over control criterion for sensitization (Figure 5). In all LLNAs

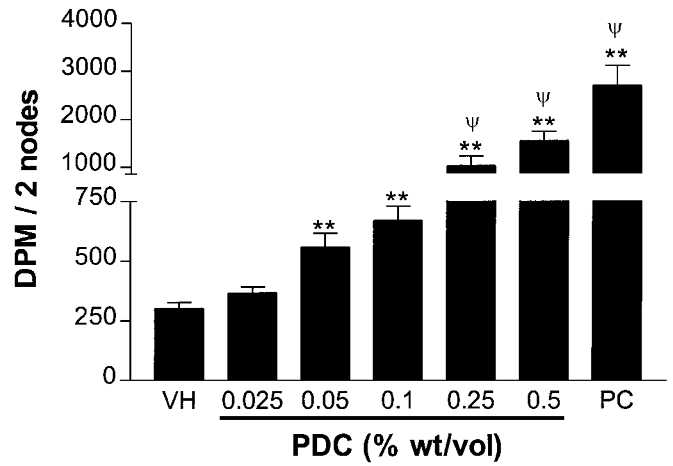

FIGURE 4. LLNA response following potassium dichromate (PDC) exposure. Data are expressed as group means ± standard errors. Dose groups were determined to be nonhomogenous (Bartlett's test) and compared to the vehicle group by the Wilcoxon rank test, where $^{**}p < .01$. ψ represents concentrations of chemical that resulted in a 3-fold increase in proliferation over control. Trend analysis was determined significant ($p < .01$) by Jonckheere's test. VH, vehicle; PC, positive control (0.25% DNFB).

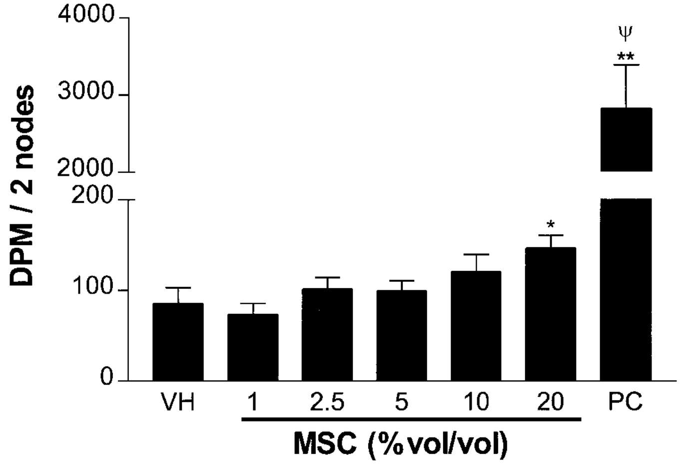

FIGURE 5. LLNA response following methyl salicylate (MSC) exposure. Data are expressed as group means ± standard errors. Dose groups were determined to be nonhomogenous (Bartlett's test) and compared to the vehicle group by the Wilcoxon rank test, where $^{**}p < .01$. ψ represents concentrations of chemical that resulted in a 3-fold increase in proliferation over control. Trend analysis was determined significant ($p < .01$) by Jonckheere's test. VH, vehicle; PC, positive control (0.25% DNFB).

conducted, the positive control, 0.25% DNFB, showed a significant ($p < .01$) proliferative response (>2600 dpm) exceeding a 3-fold increase over control. Results obtained for the positive control groups in the LLNA varied minimally between studies.

Phenotypic Analysis Assay

In the combined irritancy/phenotypic analysis assay, the MIC (at $p < .01$) for DNCB and PDC was 0.25 and 0.5%, respectively (Table 3). DNCB and PDC induced dose responsive increases ($p < 0.01$) in the percentage of $B220^+$ cells from 18.5 ± 1.4 to 33.3 ± 2.6 in DNCB exposed animals and from 28.2 ± 1.9 to 41.0 ± 1.5 in PDC exposed animals with a significant increase over controls observed in mice following exposure to 0.1% for both chemicals. Both DNCB and PDC induced a moderate elevation and statistical significance in the $\%IgE^+$ $B220^+$ cells at higher concentrations (0.25% for both DNCB and PDC). Analysis by linear regression revealed dose-responsive ($p < .01$) effects of DNCB (0.4 ± 0.4 to 23.4 ± 2.6) and PDC (2.0 ± 0.5 to 25.1 ± 1.7) on the percentage of IgE^+B220^+ cells. No significant modulation was seen in the cell surface expression of $B220^+$ or IgE^+B220^+ in animals exposed to concentrations of MSC as high as 20%. For all the endpoints examined (ear swelling, $\%B220^+$, and $\%IgE^+B220^+$ cells), significant responses were observed following exposure to the positive controls, DNFB and TDI.

TABLE 3. Irritancy/Phenotypic Analysis Results Following Dermal Exposure to a Range of DNCB, MSC, and PDC Doses

Dose group	Phenotypic analysis			Total IgE (ng/mL)
	24-h ear swelling[a]	%B220$^+$	%IgE$^+$B220$^+$	
DNCB				
Acetone[b]	(0.8 ± 2.1)	18.5 ± 1.4	0.4 ± 0.4	175 ± 22
0.010%	2.70 ± 2.4	19.6 ± 1.2	0.6 ± 0.3	250 ± 25
0.025%	10.1 ± 2.3	22.9 ± 1.2	0.7 ± 0.1	194 ± 32
0.050%	6.4 ± 3.7	24.3 ± 0.9	1.6 ± 0.7	213 ± 24
0.100%	12.5 ± 4.0$^{*\delta}$	27.8 ± 1.7$^{**\delta}$	4.6 ± 1.1	185 ± 16
0.250%	20.6 ± 3.6$^{**\delta}$	32.0 ± 1.8$^{**\delta}$	16.6 ± 1.9$^{**\lambda}$	490 ± 68
0.500%	40.2 ± 3.7$^{**\delta}$	33.3 ± 2.6$^{**\delta}$	23.4 ± 2.6$^{**\lambda}$	612 ± 79$^{*\delta}$
0.25% DNFB	143.9 ± 17.0$^{**\lambda}$	38.0 ± 3.1$^{**\delta}$	26.3 ± 4.1$^{**\lambda}$	611 ± 198$^{*\delta}$
2.5% TDI	196.8 ± 8.7$^{**\lambda}$	38.9 ± 1.0$^{**\delta}$	33.2 ± 0.9$^{**\lambda}$	1437 ± 169$^{**\delta}$
MSC				
Acetone[b]	4.1 ± 2.8	20.2 ± 0.4	0.8 ± 0.3	330 ± 31
1.0%	3.6 ± 2.9	23.1 ± 1.0	1.0 ± 0.4	298 ± 47
2.5%	5.3 ± 2.0	23.4 ± 1.3	1.3 ± 1.1	209 ± 25
5.0%	1.2 ± 3.0	19.4 ± 0.6	0.5 ± 0.2	285 ± 22
10.0%	3.3 ± 2.0	20.6 ± 1.0	0.1 ± 0.0	82 ± 6
20.0%	(0.1 ± 2.4)	22.7 ± 1.0	1.6 ± 0.8	110 ± 0
0.25% DNFB	160.3 ± 7.7$^{**\delta}$	49.1 ± 1.8$^{**\delta}$	25.5 ± 2.3$^{**\delta}$	926 ± 49$^{**\delta}$
2.5% TDI	141.5 ± 15.2$^{**\delta}$	44.2 ± 1.5$^{**\delta}$	28.3 ± 1.9$^{**\delta}$	1083 ± 41$^{**\delta}$
PDC				
DMSO	22.9 ± 3.4	28.2 ± 1.9	2.0 ± 0.5	458 ± 87
0.025%	29.9 ± 2.3	29.1 ± 1.3	1.1 ± 0.4	462 ± 180
0.050%	34.6 ± 3.3	35.3 ± 2.9	0.6 ± 0.2	345 ± 42
0.100%	31.7 ± 3.3	36.5 ± 1.3$^{*\delta}$	7.6 ± 3.1	272 ± 39
0.250%	40.2 ± 1.9$^{*\lambda}$	41.0 ± 1.5$^{**\delta}$	20.3 ± 1.7$^{**\lambda}$	401 ± 139
0.500%	74.1 ± 6.2$^{**\lambda}$	38.1 ± 3.6$^{*\delta}$	25.1 ± 1.7$^{**\lambda}$	1972 ± 280$^{**\delta}$
Acetone[b]	7.6 ± 1.5	18.5 ± 1.0	2.3 ± 1.0	357 ± 25
0.25% DNFB	175.0 ± 6.6$^{**\lambda}$	47.6 ± 1.8$^{**\delta}$	25.3 ± 1.8$^{**\delta}$	1172 ± 173$^{*\delta}$
2.5% TDI	178.8 ± 7.6$^{**\lambda}$	38.6 ± 1.2$^{**\delta}$	28.3 ± 2.3$^{**\delta}$	2200 ± 190$^{**\delta}$

[a]For the 24-h ear swelling, the number of samples was 10 (2 ears for each mouse). Each phenotypic analysis sample is the average of two replicates, with 5 samples per dose group (combined left and right nodes for individual animals). For each sample the replicates were taken from the lymph nodes of an individual animal. Values are the dose group means ± standard errors.

[b]Acetone represents 4:1 acetone/olive oil.

$^{\delta}$Statistical difference when compared to the vehicle group using Dunnett's *t* test.

$^{\lambda}$Statistical difference when compared to the vehicle using the Tukey-Kramer test.

$^{*}p < .05$. $^{**}p < .01$.

Total Serum IgE ELISA

Total serum IgE levels were significantly elevated in DNCB-exposed (612 ± 79 ng/mL) and PDC-exposed (1972 ± 280 ng/mL) animals at the highest concentration tested, 0.5% (Table 3). Serum IgE levels were unaffected in animals treated with concentrations of MSC as high as 20%. Animals exposed to the positive controls, TDI and DNFB, exhibited significant increases in total serum IgE in all studies, with values ranging from 1083 ± 41 to 2200 ± 190, and 611 ± 198 to 1172 ± 173, respectively.

DISCUSSION

As the immunology of the mouse has become better understood, more mechanistically based methods for detecting chemical sensitizers are being designed. These methods expand on the guinea pig models, which have historically been used for the evaluation of the potential hazard of chemicals as related to hypersensitivity responses. However, unlike the guinea pig models, which rely on the subjective evaluation of the elicitation phase with the visual determination of erythema and edema as the endpoint (Buehler, 1965; Magnusson and Kligman, 1969), these murine assays utilize the more quantitative endpoints of ear swelling (Gad et al., 1986), [^{3}H]thymidine uptake (Kimber and Weisenberger, 1989), phenotypic analysis using cell surface markers (Manetz and Meade, 1999), and detection of IgE by the total serum IgE ELISA (Dearman et al., 1998).

The 4 assays employed in these studies evaluate different endpoints in the hypersensitivity response, but all require the induction of sensitization and the clonal expansion of lymphocyte populations. The LLNA quantitates the incorporation of [^{3}H]thymidine into proliferating draining lymph node cells following chemical exposure (Kimber et al., 1991, 1994, 1995a). Once cells have begun proliferating, the surrounding microenvironment of cytokines and growth factors directs T-cell development toward either a Th1 or a Th2 response (Cumberbatch et al., 1992; Dearman et al., 1995, 1996a, b; Kimber, 1994; Kimber et al., 1995b). Once T cells have been activated, they interact with B cells, leading to the activation and differentiation into antibody (including IgE)-secreting plasma cells. Upon clonal expansion, B cells express high amounts of B220/CD45, and under the influence of IL-4, some cells will begin to express surface IgE. The irritancy/phenotypic analysis assay utilizes flow cytometry to quantify the number of cells expressing B220 and surface or membrane-bound IgE (via CD23) to identify sensitizers and differentiate between T-cell– and IgE-mediated responses (Manetz and Meade, 1999). Once IgE has been secreted by the plasma cells, circulating IgE can be detected using the total serum IgE ELISA (Dearman et al., 1998). Using ear measurements prior to and following chemical challenge, the MEST evaluates the elicitation phase of the hypersensitivity response in already sensitized animals (Gad et al., 1986).

Chemicals that had been used previously in the development of the MEST and in the international validation of the LLNA were evaluated to compare the results obtained using these 4 murine assays (Gad et al., 1986; Kimber et al., 1995a). These chemicals included the human irritant methyl salicylate (Done, 1967; Speer, 1979) and two potent sensitizers, potassium dichromate and 2,4-dinitrochlorobenzene (DNCB) (Basketter and Scholes, 1992; Botham et al., 1989; Heylings et al., 1996; Kimber et al., 1991). Results obtained in the LLNA for these studies are comparable with the results of 5 laboratories participating in the international validation of the LLNA (Kimber et al., 1995a), further supporting the reproducibility of the LLNA among laboratories.

Upon analysis of the results, those assays evaluating the induction phase of contact hypersensitivity were consistent in identifying the concentration of the chemical at which sensitization occurred. DNCB was identified to be sensitizing in the irritancy/phenotypic analysis assay and LLNA at concentrations as low as 0.1 and 0.25%, respectively (Table 3, Figure 3), while PDC was determined to be sensitizing at concentrations as low as 0.25% in both assays (Table 3, Figure 4). MSC was not identified as a sensitizer in any of the 3 assays measuring the induction phase (Table 3, Figure 5). Although both DNCB and PDC are considered primarily T-cell–mediated sensitizers, IgE responses have been seen at higher concentrations (Dearman et al., 1991, 1998). In all cases where an elevation in total serum IgE was detected, the percentage of $IgE^{+}B220^{+}$ cells was elevated in the irritancy/phenotypic analysis assay (Table 3). Consistent with the findings of Manetz and Meade (1999) for these T-cell–mediated sensitizers, the elevation in $IgE^{+}B220^{+}$ cells was seen at concentrations higher than those required to increase B220. The reproducibility of the LLNA and the irritancy/phenotypic analysis assay is illustrated when one compares the positive control values for each of the assays. DNFB induced a consistent proliferative response ranging from 2693 to 2822 dpm in the LLNA (Figures 3–5) and ranging from 37 to 49% $B220^{+}$ and 25 to 26% $IgE^{+}B220^{+}$ cells in the irritancy/phenotypic analysis assay (Table 3). Consistent results were also observed for the IgE-inducing positive control, TDI, in the irritancy/phenotypic analysis assay, with ranges of 38 to 45% $B220^{+}$ and 28 to 33% $IgE^{+}B220^{+}$ cells (Table 3). The Total IgE ELISA, LLNA, and irritancy/phenotypic analysis assay minimize human error by the use of a plate reader to determine optical density values, a beta scinitillation counter to measure cpm, or flow cytometry to quantify cell population percentages, respectively.

In evaluating the elicitation phase in the MEST, much more variability existed in the dose–response relationships than in the LLNA and irritancy/phenotypic analysis assay. Whereas concentrations as low as 0.25% DNCB and PDC were sufficient to induce a positive response in both the LLNA and the irritancy/phenotypic analysis assay, the lowest sensitizing concentration to induce a positive response in the MEST was 0.5% at the 48-h measurement (Figures 1 and 2). The variability of the assay is further demonstrated by comparing results from the positive controls in each study. Values for ear swelling ranged from 60 to 221% (Table 2). Some of the variability in this assay is related to the potential for human error, since a hand-held micrometer is used to obtain ear measurements and reproducible measurements require that the same position on the ear pinna be measured and the tension of the micrometer be consistent from animal to animal.

In addition to exhibiting less variability, as discussed by members of the peer review panel of the LLNA by the ICCVAM, tests evaluating the induction phase of hypersensitivity may be more relevant for hazard identification. These assays focus on the potential for sensitization, which is the prerequisite of allergic hypersensitivity responses and constitutes the hazard. The further development of a secondary immune response (elicitation phase) in a sensitized individual or animal model involves more risk assessment (secondary challenge conditions), which may also account for increased variability in results (NIEHS, 1999).

There has been a progression in the development of models capable of detecting chemicals with the capacity to induce chemical hypersensitivity. The MEST quantitatively evaluates ear swelling to assess the hypersensitivity response to a chemical, but obtaining reproducible results is sometimes difficult (Dunn et al., 1990). The LLNA measures the incorporation of [^{3}H]thymidine during the induction phase using a beta counter for more easily reproducible results, but the use of radioactivity may be problematic because of the need to dispose of radioactive waste. The irritancy/phenotypic analysis assay uses an alternative endpoint to the LLNA by quantifying cellular populations using flow cytometry and suggesting differentiation of sensitizers as T-cell–mediated or IgE-mediated based on cell marker expression (Manetz and Meade, 1999). Absence of radioactivity and the ability to differentiate and characterize chemical irritants and sensitizers makes the irritancy/phenotypic analysis assay an attractive solution to the problem of chemical hazard identification.

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