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Comparison of mouse strains using the local lymph node assay

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Abstract

The local lymph node assay (LLNA), as recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), only allows for the use of CBA mice. The objective of these studies was to begin to assess the response of chemical sensitizers in the LLNA across six strains of female mice (C57BL/6, SJL/J, BALB/c, B6C3F1, DBA/2 and CBA). The moderate sensitizer α-hexylcinnamaldehyde (HCA) was chosen as the test chemical, while toluene diisocyanate (TDI) and 2,4-dinitrofluorobenzene (DNFB) were evaluated at single concentrations as positive controls. Draining lymph node cell proliferation following acetone exposure varied across strains. SJL mice had a significantly higher degree of proliferation with 2111 d.p.m./2 nodes. The remaining five strains demonstrated responses which ranged from 345 to 887 dpm/2 nodes. DBA/2, B6C3F1, BALB/c and CBA mice had essentially equal levels of lymph node proliferation following exposure to the three chemicals. While C57BL/6 mice gave similar results as CBA mice following DNFB and HCA adminstration, the LLNA response to TDI was considerably lower. SJL mice provided low stimulation indexes (SI) values for all three chemicals evaluated. Regardless of the level of LLNA response, all six mouse strains identified the sensitization potential of HCA, TDI or DNFB. Based on these studies, DBA/2, B6C3F1 and BALB/c mice are good choices for continued evaluation as additional mouse strains for use in the LLNA.

Keywords: Mouse; Chemical sensitization; Hypersensitivity; LLNA; TDI; HCA; DNFB

1. Introduction

The murine local lymph node assay (LLNA) was described by Kimber et al. (1986) and has been modified and validated over the past decade (Kimber and Weisenberger, 1989; Basketter et al., 1994; Kimber et al., 1994, 1995; Chamberlain and Basketter, 1996; Loveless et al., 1996). It has since been frequently utilized to assess allergic, contact sensitivity potential of chemicals and chemical products. Prior to the LLNA, guinea pig assays were primarily relied upon to evaluate chemical

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sensitization potential. Advantages of the murine LLNA include a quantitative endpoint, a dose response assessment with a reduced number of animals and a shortened assay time.

The LLNA has been approved as an initial screening assay by the Organization for Economic Cooperation and Development (OECD) since 1992. Recently, a Peer Review Panel convened by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), an organization established by 14 Federal regulatory and research agencies with the objective of harmonizing the development, validation and acceptance of toxicological test methods for industry R&D and regulatory authorities, evaluated the usefulness of the LLNA as a stand-alone alternative to guinea pig assays for hazard identification of potential contact sensitizers. In their final report (NIEHS, 1999), the Peer Review Panel endorsed the LLNA to the ICCVAM but outlined several questions regarding the parameters of the assay. Among these was the choice of mouse strain. The standard LLNA protocol allows for the use of CBA mice only; a choice which was made without a systematic evaluation of different mouse strains. Limited LLNA data has been published using different mouse strains (BALB/c and B6C3F1) to evaluate the sensitization potential of chemicals (Kimber and Weisenberger, 1989; Hayes et al., 1998; Hayes and Meade, 1999; Woolhiser et al., 1998). The objective of these studies was to begin to assess the response of chemical sensitizers in the LLNA across six strains of mice which are commonly used in immunotoxicology research.

Draining lymph node proliferation was evaluated following exposures to three concentrations of α-hexylcinnamaldehyde (HCA), a moderate contact sensitizer which is one of the chemicals recommended by the OECD as a positive control for the LLNA. In addition, the strong contact sensitizer 2,4-dinitrofluorobenzene and the potent IgE-mediated sensitizer toluene diisocyanate were evaluated at single, moderate concentrations as positive controls for T cell mediated and IgE-mediated responses.

2. Materials and methods

2.1. Animals

Female C57BL/6, SJL/J, BALB/c and CBA mice were purchased from Jackson Laboratories (Bar Harbor, ME). DBA/2 female mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) while female B6C3F1 mice were purchased from Charles Rivers Laboratories in Wilmington, MA. Mice were 8–10 weeks of age upon arrival and were quarantined for 1 week prior to use. Mice were weighed and assigned into homogeneous weight groups ($n = 5$). All mice were provided tap water ad libitum; C57BL/6 mice were fed Purinia NIH R + M/Auto 6F-Ovals 5K67 (NIH31 equivalent lab diet; 6% fat) while all other mouse strains received Agway Prolab Animal Diet (5% fat). Mice were maintained under conditions specified within NIH guidelines (National Research Council, 1996). Animal rooms were maintained between 18 and 26°C and 40–70% relative humidity with light/dark cycles of 12-h intervals. Body weights were recorded at the termination of the study and no statistical differences between groups were noted (data not shown).

2.2. Chemicals

HCA (purity 85%), 2,4-dinitrofluorobenzene (DNFB; purity 99%), toluene diisocyanate (TDI; purity 99.6%) and acetone were all purchased from Sigma–Aldrich (St. Louis, MO). All chemicals were prepared in acetone at the following volume:volume concentrations: 5, 25 and 50% HCA; 0.15% DNFB; 1% TDI.

2.3. LLNA

The LLNA was performed following the method described in the ICCVAM Peer Review Panel report (NIEHS, 1999). On days 1–3, vehicle or test solutions were administered topically to the dorsal surfaces of each ear (25 μl per ear). All exposures were accomplished ± 2 h from the previous day. On day 6, each animal was intravenously injected via one of the lateral tail veins

with 0.2 ml phosphate buffered saline (PBS) containing 20 μCi [^{3}H]thymidine (Dupont NEN; specific activity 2 Ci/mmol). Five hours after [^{3}H]thymidine injection, animals were euthanized by carbon dioxide asphyxiation, and right and left cervical draining lymph nodes located near the bifurcation of the jugular vein were excised and pooled for each animal. Lymph nodes were prepared as single-cell suspensions for each animal by mechanical disaggregation using frosted microscope slides. The samples were washed twice with PBS and precipitated in 5% trichloroacetic acid (TCA) overnight. The following day, cell suspensions were pelleted ($200 \times g$ for 10 min), resuspended in 1 ml TCA, and transferred to 5 ml of scintillation cocktail. Test tubes were rinsed with an additional 1 ml of TCA which was added to the scintillation vials. [^{3}H]thymidine incorporation was determined via beta liquid scintillation counting and dpm for each mouse (dpm/2 nodes) was calculated. Stimulation indexes (SI) for each chemical exposure group were determined by dividing the mean dpm response elicited by a chemical by the mean dpm response following acetone applications.

2.4. Data analysis

These data were analyzed following the recommendations of the ICCVAM Peer Review Panel which suggested three criteria be evaluated when determining chemical sensitization potential: (1) SI above that of vehicle exposed mice; positive responses are defined as $SI \geq 3$; (2) statistical comparisons of dose groups versus vehicle control; and (3) assessment of dose responsive trends. Statistics were conducted using Graph Pad Prism, version 2.01 (San Diego, CA). Lymph node proliferation data from HCA exposed mice were analyzed by one way analysis of variance (ANOVA). When significant differences were detected ($P \leq 0.05$), test groups were compared to the strain, acetone controls using a Dunnett's test. In addition, HCA dose groups were analyzed using a Linear Trend Test to determine dose responsiveness. Lymph node proliferation data from DNFB and TDI exposed mice were individually compared to acetone treated mice of the same strain using an unpaired t-test. Acetone control data were compared across strains using Tukey's multiple comparison test.

3. Results

In these studies, six strains of mice were compared for their response in the LLNA using known human chemical allergens. The LLNA response of each strain to acetone (vehicle), HCA, TDI and DNFB are shown in Table 1. The draining lymph node proliferation following acetone exposure varied across strains. Historically in this laboratory, acetone has not induced an increase in proliferation over naive animals (BALB/c and B6C3F1) and therefore this data may represent basal levels of proliferation for each strains. Acetone exposed SJL mice had a significantly higher degree of proliferation with 2111 dpm/2 nodes as compared to other vehicle treated strains ($P < 0.01$). The remaining five strains demonstrated LLNA responses following acetone which were not statistically different from each other; dpm values ranged from 345 to 887 dpm/2 nodes.

All six mouse strains responded with lymph node proliferation, and stimulation indexes greater than three, following administration of HCA, TDI or DNFB (Tables 1 and 2). HCA (5, 25 and 50%) exposures elicited lymph node proliferation which was dose responsive within each strain tested ($P < 0.01$). The highest SI following HCA exposure occurred in DBA/2 mice (50% HCA induced a SI of 12.0 and 4148 dpm/2 nodes). DBA/2 is the only strain which showed both a $SI > 3$ and statistically significance at all three concentrations of HCA. BALB/c ($SI = 10.9$) and CBA ($SI = 9.4$) mice both demonstrated proliferative responses near 6500 dpm/2 nodes following 50% HCA exposure. While 25% HCA was the lowest concentration to induce a positive response in BALB/c mice based on SI and statistical comparisons, 25% HCA induced a $SI > 3$ but not a statistically significant increase in dpm in CBA mice. This was the only instance in which evaluation by SI and statistical analyses were not consistent. Exposure of B6C3F1 and C57BL/6 mice to 50% HCA resulted in $SI = 8.0$ (3263 dpm) and

SI = 5.1 (4517 dpm) respectively. Significant proliferation was also induced following exposure to 25% HCA in C57BL/6 mice, but not in B6C3F1 mice. Although SJL mice demonstrated the highest degree of lymph node cell proliferation in response to 50% HCA as measured by dpm/2 nodes (8764 dpm/2 nodes), the high degree of proliferation seen in the acetone treated mice resulted in a SI following exposure to 50% HCA which was the lowest of all strains tested (SI = 4.1). According to dpm values, the high to low rank order of the strains following 50% HCA was as follows: SJL > CBA ≥ BALB/c > C57BL/6 > DBA/2 > B6C3F1. Alternatively, the rank order of strains as determined using SI was DBA/2 > BALB/c > CBA > B6C3F1 > C57BL/6 > SJL.

Following 0.15% DNFB exposures, CBA and BALB/c mice demonstrated the largest degree of proliferation with SI values of 35.3 for both strains (24 480 and 20 280 dpm/2 nodes, respectively). B6C3F1 and DBA/2 had the lowest average dpm values (below 13 000 dpm/2 nodes) but demonstrated high stimulation indexes of 30.9 and 32.4. While exposures of C57BL/6 mice to 0.15% DNFB resulted in proliferative responses of approximately 25 870 dpm/2 nodes and an SI = 29.2, SJL mice demonstrated an SI value of 8.0 and 16 790 dpm/2 nodes. Regardless of the level of proliferation for each strain, dose groups exposed to 0.15% DNFB were statistically different from their appropriate strain acetone controls ($P < 0.01$). While the rank order of the strains'

Table 1
Local lymph node assay (LLNA) response (dpm/2 nodes)[a]

Groups	DBA/2	BALB/c	CBA	B6C3F1	C57BL/6	SJL
Acetone	345 ± 54	574 ± 113	694 ± 92	410 ± 25	887 ± 128	2111 ± 307***
5% HCA	1028 ± 135[b,**]	970 ± 169	1766 ± 265	436 ± 236	1267 ± 281	2899 ± 463
25% HCA	2389 ± 454[b,**]	2883 ± 332[b,**]	2878 ± 774[b]	1175 ± 79	2895 ± 497[b,*]	4967 ± 1339
50% HCA	4148 ± 295[b,**]	6279 ± 713[b,**]	6541 ± 2225[b,*]	3263 ± 190[b,**]	4517 ± 873[b,**]	8764 ± 650[b,**]
1% TDI	3250 ± 574**	5644 ± 796**	9526 ± 1678**	5231 ± 674**	2810 ± 299**	10 410 ± 1072**
0.15% DNFB	11 160 ± 1468**	20 280 ± 1294**	24 480 ± 3779**	12 680 ± 985**	25 870 ± 2103**	16 790 ± 1398**

[a] Lymph node cell proliferation following exposure to α-hexylcinnamaldehyde (HCA), toluene diisocyanate (TDI) or 2,4-dinitrofluorobenzene (DNFB). Data are expressed as group means ± standard error, $n = 4$ or 5 mice/group.
[b] LLNA response which was 3-fold greater than acetone controls.
* $P < 0.05$;
** $P < 0.01$ indicate statistical significance from strain acetone control using a Dunnett's test.
*** Statistical difference among strain acetone controls using Tukey's multiple comparison test ($P < 0.01$).

Table 2
Stimulation indexes (SI) following [^{3}H]thymidine uptake in draining lymph nodes[a]

Dose	DBA/2	BALB/c	CBA	B6C3F1	C57BL/6	SJL
5% HCA	3.0	1.7	2.5	1.0	1.4	1.4
25% HCA	6.9	5.0	4.1	2.9	3.3	2.4
50% HCA	12.0	10.9	9.4	8.0	5.1	4.1
1.0% TDI	9.4	9.8	13.7	12.8	3.2	4.9
0.15% DNFB	32.4	35.3	35.3	30.9	29.2	8.0

[a] Local lymph node assay (LLNA) stimulation indexes following exposure to α-hexylcinnamaldehyde (HCA), toluene diisocyanate (TDI) or 2,4-dinitrofluorobenzene (DNFB). Stimulation indexes for each chemical exposure was determined by dividing the mean dpm response towards a chemical by the mean dpm response following acetone applications. $n = 4$ or 5 mice per group.

LLNA response (dpm/2 nodes) following DNFB was C57BL/6 ≥ CBA > BALB/c > SJL > B6C3F1 ≥ DBA/2, the rank order of strains according to SI was CBA = BALB/c ≥ DBA ≥ B6C3F1 ≥ C57BL/6 > SJL.

Following 1.0% TDI exposure CBA (9526 dpm/2 nodes) and B6C3F1 (5231 dpm/2 nodes) mice experienced the highest SI values of 13.7 and 12.8, respectively. DBA/2 and BALB/c mice demonstrated similar SI values of 9.4 and 9.8 following proliferative responses of 3250 and 5644 dpm/2 nodes. The lowest SI values occurred in C57BL/6 and SJL mice. While C57BL/6 mice demonstrated a SI of 3.2 and 2810 dpm/2 nodes following 1% TDI, SJL mice experienced a SI of 4.9 in spite of the highest proliferative response to TDI (10 410 dpm/2 nodes). TDI dose groups within each strain were statistically different from the respective strain acetone controls ($P < 0.01$). The rank order of strains following TDI exposures were different than those following HCA or DNFB exposures; based on dpm values, the highest to lowest order was SJL > CBA > BALB/c ≥ B6C3F1 > DBA > C57BL/6. The rank order based on SI values was CBA > B6C3F1 > BALB/c ≥ DBA/2 > SJL > C57BL/6.

4. Discussion

Toxicology research models which utilize laboratory animals present considerations such as gender, age, species and strain differences. Numerous studies describing biological responses towards xenobiotics which vary due to such variables are presented in the immunotoxicology literature (e.g. Shultz and Bailey, 1975; Brzezińska-Błaszczyk et al., 1980; Gad et al., 1986; Lebrun and Spiegelberg, 1987; Ptak et al., 1988; Herz et al., 1996; Särnstrand et al., 1999). The data reported here are consistent with these observations as six murine strains demonstrated varying levels of lymph node proliferation following exposure to three chemical sensitizers. These studies suggest that the specific combination of strain and antigen may be more important than a strain's Th1/Th2 predominance. It is difficult to establish a Th1/Th2 association between chemical allergen and strain predisposition using these LLNA data. Although C57BL/6 mice are reported to be predisposed to Th1 immune responses (Särnstrand et al., 1999) and as such had the lowest response to the IgE inducing chemical TDI, SJL mice (low IgE responders) gave the highest dpm response to TDI. The other four strains demonstrated high proliferative responses to all three chemicals irrespective of any possible Th1/Th2 predominance.

Kimber and Weisenberger (1989) compared four murine strains in an ex vivo LLNA and reported that CBA mice demonstrated a higher degree of [^{3}H]thymidine uptake than BALB/c mice following 5% DNCB, another potent contact sensitizer. Similarly, HCA, TDI and DNFB elicited larger proliferative responses in CBA mice versus BALB/c mice in these studies; SI values for both strains were equivocal for all three chemicals, however. Dpm values attained from CBA mice following HCA exposures were comparable with those reported previously thereby further strengthening the reproducibility of the murine LLNA between laboratories (Dearman et al., 1998; Loveless et al., 1996). Although the lowest concentration of HCA to induce significant lymph node cell proliferation varied across strains, with a single exception, evaluation using either stimulation indexes or statistical analyses resulted in identical results within a given strain. This is also consistent with the 1996 LLNA validation paper which demonstrated little difference between SI evaluation and statistical analysis of dpm values from individual mice (Loveless et al., 1996).

SJL mice provided low SI values for all three chemicals evaluated. Numerous other investigators have reported low immune responses by SJL mice to a variety of different antigens (Shultz and Bailey, 1975; Watanabe et al., 1976; Yoshimoto et al., 1995). The low SI values following chemical exposures can be largely attributed to the high [^{3}H]thymidine uptake in acetone exposed mice. As mentioned previously, acetone typically does not induce an increase in proliferation over naive animals and therefore this data may represent basal levels of proliferation for SJL mice. Regardless of the lower SI values, SJL mice still identified all three chemicals as sensitizers according to 3-

fold SI values, statistical significance and dose responsive proliferation.

This initial set of studies highlights the importance of mouse strain when developing assay models. While CBA mice were verified as a good selection for the LLNA, the data from these studies suggest DBA/2, B6C3F1 and BALB/c mice are essentially equal to CBA mice when evaluating the sensitizing potential of these three chemicals. While C57BL/6 mice gave similar results as CBA mice following treatment with DNFB and HCA, the LLNA response to TDI was considerably lower than those of DBA/2, B6C3F1, BALB/c and CBA mice. Based on this single series of studies, DBA/2, B6C3F1 and BALB/c mice appear to be reasonable alternative strains for evaluation for use in the LLNA. Additional studies using a broad spectrum of chemical classes will be necessary to validate these findings.

References

Basketter, D.A., Scholes, E.W., Kimber, I., 1994. The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test. Fd. Chem. Toxicol. 32, 543–547.

Brzezińska-Błaszczyk, E., Wyczółkowska, J., Maśliński, C., 1980. Keyhole limpet hemocyanin (KLH)-a potent antigen in reaginic sensitization of mice and rats; a comparison with egg albumin (EA)-induced reaction. Arch. Immunol. Ther. Exp. (Warsz) 28, 533–539.

Chamberlain, M., Basketter, D.A., 1996. The local lymph node assay: status of validation. Fd. Chem. Toxicol. 34, 999–1002.

Dearman, R.J., Hilton, J., Evans, P., Harvey, P., Basketter, D.A., Kimber, I., 1998. Temporal stability of local lymph node assay responses to hexyl cinnamic aldehyde. J. Appl. Toxicol. 18, 281–284.

Gad, S.C., Dunn, B.J., Dobbs, D.W., Reilly, C., 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.

Hayes, B.B., Meade, B.J., 1999. Contact sensitivity to selected acrylate compounds in B6C3F1 mice: relative potency, cross reactivity, and comparison of test methods. Drug Chem. Toxicol. 22, 491–506.

Hayes, B.B., Gerber, P.C., Griffey, S.S., Meade, B.J., 1998. Contact hypersensitivity to dicyclohexylcarbodiimide and diisopropylcarbodiimide in female B6C3F1 mice. Drug Chem. Toxicol. 21, 195–206.

Herz, U., Lumpp, U., Da Palma, J.C., Enssle, K., Takatsu, K., Schnoy, N., Daser, A., Köttgen, E., Wahn, U., Renz, H., 1996. The relevance of murine animal models to study the development of allergic bronchial asthma. Immunol. Cell Biol. 74, 209–217.

Kimber, I., Weisenberger, C., 1989. A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. Arch. Toxicol. 63, 274–282.

Kimber, I., Dearman, R.J., Scholes, E.W., Basketter, D.A., 1994. The local lymph node assay: developments and applications. Toxicology 93, 13–31.

Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., Ladics, G.S., Loveless, S.E., House, R.V., 1995. An international evaluation of the murine local lymph node assay and comparison of modified procedures. Toxicology 103, 63–73.

Kimber, I., Mitchell, J.A., Griffin, A.C., 1986. Development of a murine local lymph node assay for the determination of sensitizing potential. Food Chem. Toxicol. 24, 585–586.

Lebrun, P., Spiegelberg, H.L., 1987. Concomitant immunoglobulin E and immunoglobuline G1 formation in *Nippostrongylus brasiliensis*-infected mice. J. Immunol. 139, 1459–1465.

Loveless, S.E., Ladics, G.S., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., Scholes, R.V., Scholes, H., Hilton, J., Dearman, R.J., Kimber, I., 1996. Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. Toxicology 108, 141–152.

National Research Council, 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.

NIEHS, 1999. The murine local lymph node assay: a test method for assessing the allergic contact deratitis potential of chemicals/compounds. Research Triangle Park, NC: U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences, NIH Publication No. 99-4494.

Ptak, W., Dobrowolski, Z., Marcinkiewicz, J., Gryglewski, A., 1988. Sex differences in regulation of contact sensitivity reaction in mice. Clin. Immunol. Immunopathol. 47, 289–295.

Särnstrand, B., Jansson, A.-H., Matuseviciene, G., Scheynius, A., Pierrou, S., Bergstrand, H., 1999. N,N′-diacetyl-L-cystine — the disulfide dimer of N-acetylcysteine — is a potent modulator of contact sensitivity/delayed type hypersensitivity reactions in rodents. J. Pharmacol. Exp. Ther. 288, 1174–1184.

Shultz, L.D., Bailey, D.W., 1975. Genetic control of contact sensitivity in mice: effect of H-2 and non H-2 loci. Immunogenetics 1, 570–583.

Watanabe, N., Kojima, S., Ovary, Z., 1976. Suppression of IgE antibody production in SJL mice. J. Exp. Med. 143, 833–845.

Woolhiser, M.R., Hayes, B.B., Meade, B.J., 1998. A combined murine local lymph node and irritancy assay to predict sensitization and irrtancy potential of chemicals. Toxicol. Methods 8, 245–256.

Yoshimoto, T., Bendelac, A., Hu-Li, J., Paul, W.E., 1995. Defective IgE production by SJL mice is linked to the absence of CD4+, NK1.1+ T cells that promptly produce interleukin 4. Proc. Natl. Acad. Sci. 92, 11931–11934.