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Experience with the Murine Local Lymph Node Assay: Inability to Discriminate between Allergens and Irritants

JOHAN MONTELIUS[1], HELEN WAHLKVIST[1], ANDERS BOMAN[1], PETER FERNSTRÖM[1], LARS GRÅBERGS[1] and JAN E. WAHLBERG[1,2]

[1]*Department of Occupational Dermatology, National Institute of Occupational Health, Solna, and* [2]*Department of Occupational Dermatology, Karolinska Hospital, Stockholm, Sweden*

The murine local lymph node assay is a new predictive test for identifying contact sensitizers. It measures the proliferative response in the lymph nodes during the sensitization phase. In the present study, moderate-to-extreme allergens (from human and guinea pig experience) gave clearly positive results in this assay. However, irritants tested, i.e. sodium dodecyl sulphate (SDS), chloroform/methanol, oxalic acid, triton X-100 and methylsalicylate, also gave positive results, not distinguishable from the results with low-grade/moderate allergens. Two allergens were also tested in the presence of 10% SDS. The effect on proliferation was additive in the first case and synergistic in the second. The local lymph node assay in its present design and with the criteria used for a positive response requires further validation studies and perhaps further development before it can be accepted as an alternative to guinea pig tests for allergenicity. Substances with exclusively irritating properties could falsely be classified as allergens by the method or, alternatively, the allergenicity of chemicals with both allergenic and irritating properties could be overestimated. ***Key words: Predictive testing; Contact allergens; Sodium dodecyl sulphate.***

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J. Montelius, Department of Occupational Dermatology, National Institute of Occupational Health, S-171 84 Solna, Sweden.

Allergic and irritant contact dermatitis are common disorders causing many days of sick-leave each year. To avoid or minimize human exposure to contact allergens it is important to have reliable methods for the identification of contact sensitizers.

The most extensively used species for such predictive tests is the guinea pig (1–6), where results correlate well with the sensitizing properties of chemicals in humans (3). Guinea pig tests are generally accepted by regulatory authorities (7). However, they are quite costly and time-consuming. In addition, it is sometimes hard to discriminate between allergic reactions and irritant reactions. The naked eye and palpation are used for scoring and are considered subjective.

In recent years some alternative methods using mice as test animals have been developed, and two of them, the mouse ear swelling test (MEST) and the murine local lymph node assay (LLNA), have been recommended in the 1992 update of the OECD guidelines for predictive testing (8). These models are suggested for preliminary screening of chemicals: in the case of a positive result the chemical may be classified as a potential sensitizer, while if a negative result is obtained, a guinea pig test is recommended (8).

The LLNA was developed by Kimber et al. (9, 10) and has since then been further elaborated and extensively evaluated in interlaboratory studies (11–13) and comparative studies with guinea pig tests (11–15). It measures the proliferative response in the draining, auricular lymph nodes during the induction phase after epicutaneous application of the chemical to the ear. It is based on the observation that the proliferative activity correlates well with the severity of the elicitation reaction induced by a chemical (16). The proliferation was originally measured as [^{3}H]thymidine incorporation carried out in vitro (9, 10), but later on a technique was developed to measure the proliferation in situ by injecting the [^{3}H]thymidine in the tail vein (17, 18).

The LLNA identifies moderate-to-strong allergens and some weak allergens (10, 15, 19). To enhance sensitivity some modifications have been introduced, including an extension of the assay period from 4 to 5 days and a recommendation that chemicals with equivocal response should be tested in the highest soluble concentration (12, 20). In addition, it is possible to further increase the sensitivity of the assay to less potent allergens by pre-exposure of the test chemical to the shaved flanks of the animals before carrying out the LLNA (21) or by the addition of interleukin 2 to the cell culture when measuring proliferation in vitro (10).

The LLNA was set up in our laboratory in order for us to learn how to perform the assay and to be able to evaluate the method as an alternative predictive test. This paper describes the results obtained with five irritants and eight allergens.

MATERIALS AND METHODS

Animals

Inbred CBA/Ca strain female mice (7–10 weeks) obtained from B&K Universal AB, Sollentuna, Sweden, were used. The mice were allowed to acclimatize for at least 5 days prior to first exposure.

Chemicals

The allergens used were: 2,4-dinitrofluorobenzene (DNFB), 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole, and ethyl-p-aminobenzoate (benzocaine) purchased from Sigma Chemical Co., St. Louis, MO, USA; p-phenylenediamine (p-PD) from BDH Chemicals, Ltd., Poole, UK; t-cinnamic aldehyde from Heraeus, Karlsruhe, Germany; and hydroxycitronellal from K&K Laboratories, Division of ICN Biomedicals, Inc. Cleveland, Ohio, USA.

The irritants used were: 2-hydroxybenzoic acid methyl ester (methylsalicylate) purchased from Sigma Chemical Co., St. Louis, MO, USA; sodium dodecyl sulfate (SDS), and triton X-100 from Bio-Rad Laboratories, Richmond, CA, USA; and oxalic acid from E. Merck, Darmstadt, Germany.

Phosphate-buffered saline, pH = 7.4 (PBS), was purchased from Sigma Chemical Co., St. Louis, MO, USA. Olive oil was procured from

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Table I. *Allergens tested in the local lymph node assay*

Groups of mice ($n = 4$) received 25 μl of the test chemical dissolved in AOO or DMF on the dorsum of both ears, daily for 3 consecutive days. Control animals were treated in the same way with the vehicle alone. All mice were injected intravenously 5 days after the first treatment, with 250 μl of PBS containing 20 μCi of [^{3}H]thymidine. Five hours later, draining lymph nodes were excised and pooled for each group and thymidine incorporation measured by β-scintillation counting. For further experimental details see Materials and Methods.

Chemical[a]	Vehicle[b]	Concentration[b] (w/v %)	Lymph node weight (mg/node)	[^{3}H]Thymidine incorporation (dpm/node)	SI[c]
DNFB	AOO	Control	2.9	217	–
		0.02	6.8	1390	6.4
		0.10	12.8	6057	28.0
		0.50	13.0	8655	39.9
Oxazolone	AOO	Control	nd[d]	515	–
		0.10	nd	10004	19.4
		0.25	nd	12472	24.2
		0.50	nd	16479	32.0
DNCB	AOO	Control	2.7	316	–
		0.02	3.4	630	2.0
		0.10	5.7	3302	10.5
		0.50	12.3	7268	23.0
p-PD	AOO	Control	2.4	357	–
		0.40	5.0	3714	10.4
		2.00	6.7	5802	16.3
t-Cinnamic aldehyde	DMF	Control	2.8	472	–
		1.0	4.3	2020	4.3
		5.0	6.9	4641	9.8
		25.0	7.8	6025	12.8
2-Mercaptobenzothiazole	DMF	Control	2.9	139	–
		1.0	2.9	412	3.0
		5.0	4.4	1374	9.9
		25.0	5.3	2370	17.1
Hydroxycitronellal	DMF	Control	2.1	240	–
		1.0	2.4	306	1.3
		5.0	2.7	496	2.1
		25.0	3.4	824	3.4
Benzocaine	AOO	Control	2.4	175	–
		1.0	2.4	227	1.3
		5.0	3.0	312	1.8
		25.0	2.2	514	2.9

[a] DNFB, 2,4-dinitrofluorobenzene; Oxazolone, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; DNCB, 2,4-dinitrochlorobenzene; p-PD, para-Phenylenediamine; Benzocaine, ethyl-p-aminobenzoate.

[b] Animals were treated with the chemical dissolved in the vehicle at the concentration indicated. Control animals were treated with vehicle alone. AOO, acetone:olive oil (4:1); DMF, dimethylformamide.

[c] SI = stimulation index (test group value/control group value).

[d] nd = not done.

Aldrich-Chemie, Steinheim, Germany. Ready safe liquid scintillation cocktail was obtained from Beckman Instruments, Inc., Fullerton, CA, USA. [*methyl*-^{3}H] Thymidine (specific activity 2.0 Ci/mmol) was purchased from Amersham International plc, Amersham, UK. The other chemicals i.e. acetone, chloroform, dimethylformamide (DMF), methanol, and methyl ethyl ketone (MEK) were of analytical grade. All chemicals were used as delivered.

Assay

The assay was carried out as recommended by Kimber & Basketter (20). Mice, in groups of four, received a daily topical application of 25 μl of one of three concentrations of the test chemical on the dorsum of both ears. The chemicals were dissolved (w/v) in acetone/olive oil, 4:1 (AOO), DMF or MEK as vehicles (see Tables I and II). All vehicles were approximated to have a density of $\delta = 1.0$. Control mice were treated with an equal volume of the vehicle alone or were untreated. The treatment was repeated for 3 consecutive days. Five days after the first topical application, all mice were injected intravenously through the tail vein with 250 μl PBS containing 20 μCi [^{3}H]thymidine. After 5 h the mice were sacrified using carbon dioxide, and the draining auricular lymph nodes were excised, pooled for each group in a tube containing PBS, and weighed. The average lymph node weight was calculated for each sample. A single-cell suspension of lymph node cells (LNC) was prepared by mechanical disaggregation through 200-mesh stainless-steel gauze. The LNC were rinsed through the gauze with 5 ml PBS into

Table II. *Irritants tested in the local lymph node assay*

Experimental design and [^{3}H]thymidine incorporation were measured as described in Table I except for chloroform/methanol treatment, see footnote [c] below.

Chemical	Experiment	Vehicle[a]	Concentration[a] (w/v %)	Lymph node weight (mg/node)	[^{3}H]Thymidine incorporation (dpm/node)	SI[b]
Sodium dodecyl sulphate	1	DMF	Control	2.5	335	–
			4.0	3.5	1368	4.1
			10.0	5.1	1715	5.1
			25.0	4.7	2245	6.7
	2	DMF	Control	nd[d]	276	–
			5.0	nd	1114	4.0
			10.0	nd	1417	5.1
			25.0	nd	2096	7.6
Chloroform/methanol[c]	1	–	Control	2.1	143	–
			1 day	3.1	482	3.4
			2 days	4.3	1179	8.2
			3 days	4.9	1453	10.1
	2	–	Control	2.6	229	–
			3 days	4.9	1935	8.5
Triton X-100	1	AOO	Control	2.7	391	–
			4.0	3.1	497	1.3
			10.0	3.4	688	1.8
			25.0	4.4	1622	4.1
	2	AOO	Control	2.3	238	–
			4.0	3.0	544	2.3
			10.0	4.2	832	3.5
			25.0	5.4	2131	9.0
Oxalic acid	1	DMF	Control	2.8	258	–
			4.0	2.9	532	2.1
			10.0	4.1	1162	4.5
			25.0	4.0	1077	4.2
	2	DMF	Control	nd	124	–
			5.0	nd	545	4.4
			10.0	nd	553	4.5
			25.0	nd	621	5.0
Methylsalicylate	1	DMF	Control	2.6	228	–
			1.0	2.4	229	1.0
			5.0	3.0	282	1.2
			25.0	3.4	676	3.0
	2	MEK	Control	nd	174	–
			5.0	nd	395	2.3
			10.0	nd	428	2.5
			25.0	nd	1301	7.5

[a] Animals were treated with the chemical solubilized in the vehicle at the concentration indicated. Control animals were treated with vehicle alone. AOO, acetone:olive oil (4:1); DMF, dimethylformamide; MEK, methyl ethyl ketone.

[b] SI = stimulation index (test group value/control group value).

[c] In the case of chloroform/methanol treatment, control animals were untreated and the test animals were treated with chloroform/methanol (2:1) once a day for 1, 2, or 3 days as indicated. [^{3}H]Thymidine incorporation was measured 3, 4, and 5 days after the first treatment, respectively.

[d] nd = not done.

Petri dishes. LNC suspensions were transferred to cone-bottom centrifuge tubes and the Petri dishes were washed with additional 5 ml PBS before centrifugation (120 x g, for 10 min at 20°C). The cells were washed twice (120 x g, for 10 min at 20°C) with 10 ml PBS and precipitated with 3 ml 5% trichloroacetic acid (TCA) for 15 h at 4°C. The following day, the samples were pelleted (450 x g, for 10 min at 20°C), resuspended twice in 0.5 ml TCA and transferred to 10 ml of scintillation fluid. [^{3}H]thymidine incorporation was determined by β-scintillation counting (Beckman LS 6000 TA, USA) and expressed as mean disintegrations per minute/lymph node (dpm/node) for each group. For each test concentration the [^{3}H]thymidine incorporation relative to vehicle-treated controls was derived and recorded as stimulation index. Chemicals can be classified as either "sensitizers" or "not strong sensitizers". For a positive response two criteria must be fulfilled according to Kimber & Basketter (20): 1) At least one concentration of the test chemical must induce an index of a threefold or greater value

Table III. *Time-course of sodium-dodecyl-sulphate-induced proliferation*

Groups of mice ($n = 4$) were treated with DMF or 10% SDS dissolved in DMF (w/v) for 3 consecutive days. Three, 4, 5, or 6 days after the first treatment, all mice were injected with 250 µl of PBS containing 20 µCi of [^{3}H]thymidine, and thymidine incorporation was measured as described in Table I.

Experiment	Treatment[a]	Time-schedule	Lymph node weight (mg/node)	[^{3}H]Thymidine incorporation (dpm/node)	SI[b]
1	DMF	3 days	2.61	317	–
	10% SDS	3 days	5.45	1925	6.1
	10% SDS	4 days	5.65	1943	
	10% SDS	5 days	5.34	1830	
	DMF	6 days	2.90	421	–
	10% SDS	6 days	5.88	2140	5.1
2	DMF	3 days	2.48	357	–
	10% SDS	3 days	4.52	1932	5.4
	DMF	4 days	2.54	388	–
	10% SDS	4 days	5.21	2611	6.7
	DMF	5 days	2.56	377	–
	10% SDS	5 days	5.28	2217	5.9
	DMF	6 days	2.26	334	–
	10% SDS	6 days	5.77	2964	8.9

[a] DMF, dimethylformamid; SDS, sodium dodecyl sulphate.
[b] SI = stimulation index (SDS treated group/DMF treated group).

than that of the vehicle control; and 2) The result must not be incompatible with a biological dose response.

RESULTS

Eight chemicals previously classified as allergens and five chemicals known to cause skin irritation with, to our knowledge, no allergenic properties were tested in the LLNA. The results are shown in Tables I and II.

Allergens

Contact allergens classified as moderate-to-extreme in guinea pig tests all gave clear positive results and showed a dose-response relationship (Table I). DNFB, oxazolone, and DNCB gave stimulation indices between 20 and 40 at the highest concentration tested. t-Cinnamic aldehyde, 2-mercaptobenzothiazole and p-PD gave stimulation indices of 12.8, 17.1, and 16.3 when tested at 25, 25 and 2% concentrations, respectively. p-PD was also tested at 10% concentration, but the animals died during the first days after the first application. At 25% concentration, the less potent skin allergens hydroxycitronellal and benzocaine gave stimulation indices of 3.4 and 2.9, respectively, i.e. on the borderline of being classified as allergens.

Irritants

The results with the five irritants are shown in Table II. SDS and chloroform/methanol (2:1) treatment gave high [^{3}H]thymidine incorporation with stimulation indices for SDS of 6.7 and 7.6 at 25% concentration and 10.1 and 8.5 after 3 days chloroform/methanol treatment (experiments 1 and 2). SDS 10% was tested on eight additional occasions and constantly gave stimulation indices between 4 and 9 (Table III, Fig. 1 and unpublished observations). The other three irritants tested all gave stimulation indices greater than or equal to 3 at the highest concentration tested (Table II). All five irritants demonstrated a clear dose-response relationship and fulfil the criteria for classification as allergens.

The mean weight increase of the lymph nodes shows a good correlation with the increase in proliferation activity for all substances tested, irritants as well as allergens (Tables I and II). However, as earlier demonstrated (10, 20), weight increase is a less sensitive parameter than [^{3}H]thymidine incorporation (Tables I and II).

Time course of SDS-induced proliferation

Normally, a 5-day schedule was used, i.e. radiolabelled thymidine was injected 5 days after the first application of the chemical. To test the time course of the proliferation induced by SDS, the irritant was tested according to a 3-, 4-, 5-, and 6-day schedule. Table III, experiment 1, shows that as early as 3 days after the first application of SDS, maximal proliferative activity was reached, with a stimulation index around 5. When the experiment was repeated (experiment 2), proliferation activity showed a tendency to reach a plateau at day 4, increasing towards the end of the period studied, on day 6.

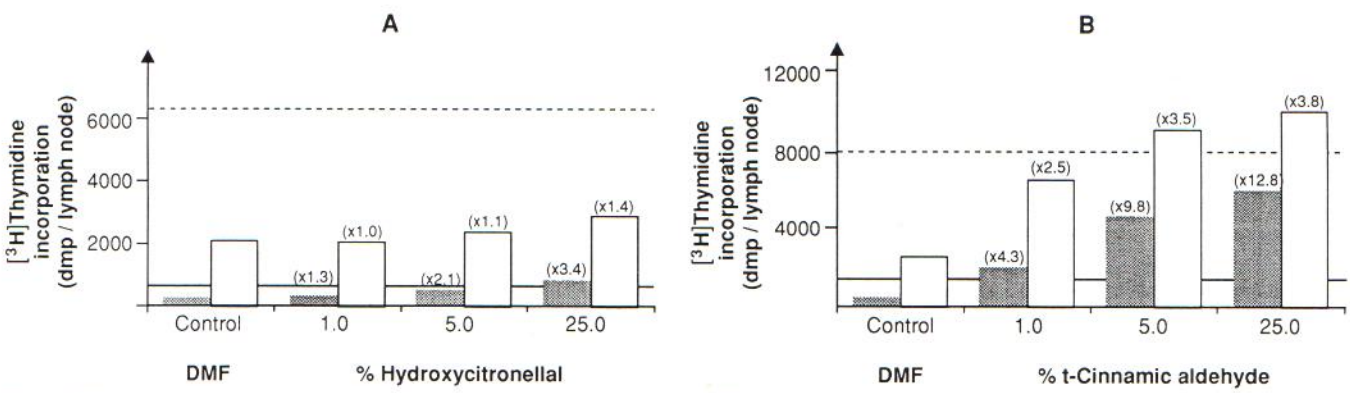

Fig. 1. The effect of a combined treatment with an irritant and an allergen. Groups of mice ($n = 4$) were treated with hydroxycitronellal (A) or t-cinnamaldehyde (B) dissolved in DMF as vehicle at the concentrations indicated, in the absence (■) or in the presence (□) of 10% sodium dodecyl sulphate (SDS) (w/v). Control animals were treated in the same way with the vehicle alone or with the vehicle containing 10% SDS (w/v), respectively. Numbers in brackets show the stimulation indices and dotted and solid horizontal lines show three times the control value with and without SDS, respectively.

Combined treatment with an irritant and an allergen

The effect of treating the mice with allergen and irritant in combination was also studied. Fig. 1A, B shows the proliferative activity caused by hydroxycitronellal and t-cinnamic aldehyde, respectively, in the presence and in the absence of 10% SDS in DMF. When hydroxycitronellal was applied together with 10% SDS there was a parallel shift upwards in the dose-response curve (Fig. 1A). This shift appears to be additive, i.e. the proliferative activity induced by the allergen is added to the activity induced by the irritant. However, when t-cinnamic aldehyde was applied in the same way in the presence of 10% SDS, a similar shift upwards in the dose-response curve was seen, which seems to be synergistic, i.e. the proliferative activity of t-cinnamic aldehyde and SDS in combination is larger than the sum of their activity measured separately (Fig. 1 B). The experiments shown in Fig. 1 have been repeated once with almost identical results.

DISCUSSION

Previously published results using the LLNA (10, 11, 13, 14, 19, 21) with chemicals classified as moderate-to-extreme allergens (3, 4, 13, 14) were essentially confirmed (Table I).

However, the tested irritants without known sensitizing properties, i.e. SDS (3), oxalic acid (22), methylsalicylate (11), the non-ionic surfactant triton X-100 (23), and a mixture of chloroform/methanol (2:1) originally used for lipid extraction (24), gave unexpectedly high proliferation activity (Table II). Stimulation indices greater than or equal to 3 with the irritants nonanoic acid, methylsalicylate and SDS have been reported (25). Furthermore, in an inter-laboratory study (12), one of the tested chemicals, coded 17, was classified as irritant in the guinea pig maximization test and gave SI values of 5.1, 6.2, 3.8, and 11.3, respectively, at 25% concentration, among the four participating laboratories. Our results with SDS also agree with those of a study (26) where 10% SDS induced over three times more proliferative activity than 0.1% SDS with DMF as vehicle. However, the authors did not present the results with vehicle alone.

On the other hand, our results are divergent from some reports that irritants do not induce a significant proliferative response (9–11, 27), e.g. SDS tested in different concentrations and vehicles. One possible reason for this discrepancy could be that SDS preparations of different quality from different manufacturers show a marked variation in their irritating properties and composition (28). In the present study, SDS for electrophoresis from Bio-Rad was used in all the experiments presented (Tables II and III, and Fig. 1). However, in preliminary studies, SDS from another supplier was used (L-4509, Sigma Chemical Co., St. Louis, MO, USA) with similar results, i.e. stimulation indices well above 3 were recorded at 25% concentration (data not presented).

Some cell proliferation activity in the draining local lymph nodes induced by SDS and other irritants has been suggested to be due to "environmental" antigens being transported to the lymph nodes, or to be attributable to non-antigen-specific lymphocyte proliferation (12, 20). SDS treatment of the skin in humans induces a large increase in the draining lymph of different inflammatory cytokines and cells, including Langerhans' cells (29–32). A similar increase in Langerhans' cells in the draining lymph nodes after SDS treatment occurs in mice (26).

The kinetics of the SDS-induced proliferative activity (Table III) shows that it is a fast event, reaching a maximal or almost maximal level at the first time point studied, i.e. 3 days after the first treatment with SDS. This fast kinetic profile accords well with proliferative responses induced by potent allergens (16) and with the time course of Langerhans' cell accumulation in draining lymph nodes after SDS treatment (26).

It has been proposed that adding SDS to all vehicles used could reduce the background noise sometimes induced by irritation (20). This is in our opinion not possible, since some allergens may act synergistically with the irritant, as shown here for t-cinnamic aldehyde and SDS (Fig. 1B), while others may give an additive response, as shown here for hydroxycitronellal and SDS (Fig. 1A). The reason for this synergistically increased activity seen with t-cinnamic aldehyde could be enhanced penetration of the allergen in the presence of SDS, resulting in a larger effective dose. This effect on absorption seems a less likely explanation of the synergistic increase, since a similar increase with hydroxycitronellal would be expected. Some other mechanism appears to be working. Perhaps the higher irritating properties of t-cinnamic aldehyde compared to those of hydroxycitronellal (T. Fischer, personal communication) cause this difference. This conclusion agrees with the recent report by Cumberbatch et al. (26), who showed that 10% SDS does not enhance the absorption of 0.1% DNCB although SDS increases 0.1%-DNCB-induced proliferation synergistically. Experiments where two irritants are combined are planned.

Hydroxycitronellal and benzocaine, classified as moderate sensitizers (3, 4, 14), both gave low stimulation indices when tested at 25% concentration in AOO (Table I). These results agree well with those of Basketter & Scholes (14), who reported stimulation indices for hydroxycitronellal of 3.6, 5.9, and 8.5 with 25, 50, and 100% test concentrations, respectively. With benzocaine, using different exposure periods, vehicles, concentrations and an optimized protocol, negative results were almost always obtained. Our experience with benzocaine is that, with AOO as vehicle, the response is low and variable, possibly because benzocaine at higher concentrations, e.g. 25%, precipitates on the ears as soon as the acetone evaporates, which could result in a low effective dose. However, preliminary experiments with DMF as vehicle gave a bell-shaped doseresponse curve, with a maximum around 5% concentration, and a stimulation index above 3 (to be published). The decline in stimulation index at higher concentrations in DMF could be due to a toxic or other inhibitory effect, or may be a consequence of the biological activity of benzocaine as a local anaesthetic. Indeed, local anaesthetics have been shown to cause vasoconstriction at low doses and vasodilatation at high doses (33). Dose-dependent changes in the skin clearance of benzocaine could account for the equivocal responses observed with this allergen. However, the effect of local anaesthetics on skin perfusion is complicated and hard to predict (34). Further studies will be required to clarify this possibility.

The control values, untreated or vehicle-treated groups, nor-

mally fell within the interval between 200 and 400 dpm per lymph node, but values down to around 100 and up to around 500 dpm per lymph node were occasionally seen (Tables I, II, and III). In two experiments, values up to around 1000 dpm per lymph node were recorded (not shown). At present we have no explanation of this variation and how it affects the results. It seems that high proliferative activity in the control group results in a relatively lower stimulation index for the tested chemical and vice versa, i.e. a low value in the control group results in a high stimulation index.

The murine local lymph node assay is a convenient method and offers many advantages over guinea pig methods regarding speed, work, cost and an objective end result uninfluenced by the colour of the chemical tested. We feel that it also constitutes a useful model for studying events in the induction phase.

However, the quite strong proliferation induced with irritants, at least in our hands, must be better elucidated before the method can be accepted as a predictive test method for suspected contact allergens. Hopefully, measurement of other parameters may better quantify the sensitization potential of a chemical and better discriminate between allergic and irritant reactions.

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