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A murine local lymph node assay for the identification of contact allergens

Assay development and results of an initial validation study

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Abstract. The development of an alternative predictive test for the identification of contact sensitizing chemicals is described. The method is based upon the fact that, following epicutaneous application, sensitizing chemicals initiate a primary immunological response in the draining lymph node(s) which is characterized by lymphocyte proliferation. Experimental conditions for the measurement in vitro of the induced lymph node cell proliferative response have been optimized. On the basis of the data presented a local lymph node assay was developed in which CBA/Ca strain mice were exposed daily, for 3 consecutive days, to various concentrations of the test chemical, or to vehicle alone, on the dorsum of the ear. Lymph node activation was measured subsequently as a function of increased node weight, the frequency of large pyroninophilic cells and lymphocyte proliferation in the presence or absence of an exogenous source of interleukin 2 (IL-2). The results of a validation study are reported in which 22 well-characterized sensitizing chemicals of varying potency were examined. With the exception of three chemicals where water was used as the application vehicle, positive responses, defined as a substantial increase in lymphocyte proliferative activity, were recorded with all these test materials. Under the conditions employed non-sensitizing chemicals, including non-sensitizing irritant chemicals, failed to influence the immunological status of the draining lymph node. Taken together, the data suggest that the local lymph node assay provides the basis for a rapid and cost-effective alternative to the currently available guinea pig predictive test methods. The local lymph node assay may be of particular value for the evaluation of coloured or irritant chemicals.

Key words: Local lymph node assay – Lymphocyte proliferation – Contact sensitivity predictive testing

Introduction

Currently, the capacity of chemicals to cause allergic contact dermatitis is assessed using guinea pig predictive tests in which reactivity is measured as a function of local erythema induced following topical challenge of sensitized animals (Andersen and Maibach 1985). Of the tests available, the most thoroughly documented and/or widely applied include the Draize test (Draize et al. 1944; Draize 1959; Johnson and Goodwin 1985), the Guinea Pig Maximization test (Magnusson and Kligman 1969, 1970; Wahlberg and Boman 1985), the Occluded Patch test (Buehler 1965, 1985; Ritz and Buehler 1980), the Split Adjuvant technique (Maguire 1973, 1975; Maguire and Cipriano 1985) and the Guinea Pig Optimization test (Maurer et al. 1975, 1980; Maurer 1985). Although such assays have been, and continue to be, of proven value in the toxicological evaluation of skin-sensitizing potential, they are not without limitations (Andersen and Maibach 1985; Oliver et al. 1986). Thus, for instance, the visual assessment of erythema is subjective and may be obscured when irritant or dye chemicals are examined. It is appropriate, therefore, that attempts are made to design reliable alternative predictive test methods.

In the period since the first description of contact hypersensitivity in the mouse (Asherson and Ptak 1968) much has been learnt about the nature and regulation of immune responses to skin-sensitizing chemicals in this species (Asherson et al. 1980; Claman et al. 1980a, b). In recent years murine test methods for the identification of contact allergens have been reported where skin-sensitizing activity is measured as challenge-induced increases in ear thickness (Tanaka 1980; Johnson et al. 1984; Thomson et al. 1984; Gad et al. 1986; Maisey and Miller 1986; Mor et al. 1988). In the present study we describe an alternative strategy for the assessment of sensitizing potential in the mouse, in which activity is measured as a function of immune activation in the lymph node draining the site of chemical application.

The primary immunological response to epicutaneously-applied skin-sensitizing agents is dependent upon the recognition of, and response to, chemical antigens by T lymphocytes (Davies et al. 1969; De Sousa and Parrott 1969; Pritchard and Micklem 1972) and is characterized by hyperplasia in the draining lymph nodes (Oort and Turk 1965; Parrott and de Sousa 1966; De Sousa and Fachet 1972; Asherson et al. 1973; Asherson and Barnes 1973; Kimber et al. 1986a, b). Lymph node activation is reflected by an increase in node weight, the appearance of large pyroninophilic cells and the induction of lymphocyte proliferation.

It was the purpose of this study to examine whether measurement of these parameters of local immune activa-

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tion following topical exposure would provide a reliable correlate of contact sensitizing potential. We describe the conditions under which induced lymph node cell proliferation can be measured in vitro, and report the results of an initial validation exercise in which the activity of more than 20 well-characterized chemicals have been examined in the local lymph node assay.

Materials and methods

Test chemicals. 2,4-dinitrochlorobenzene (DNCB), ethyl-*p*-aminobenzoate (benzocaine), benzylpenicillin (penicillin-G), 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone), 2,4,6-trinitrochlorobenzene (picryl chloride), 2,4-dinitrobenzenesulphenyl chloride, 2,4,5-trichlorophenol, 4-vinylpyridine, *p*-phenylenediamine, phthalic anhydride and nickel sulphate were obtained from Sigma Chemical Co. St Louis, Mo. USA. *p*-nitroso-dimethylaniline, dihydrocoumarin, 2,4-dinitrofluorobenzene (DNFB), cinnamic aldehyde and 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) were obtained from Aldrich Chemical Co. Ltd, Gillingham, UK. Sodium dodecyl sulphate, formaldehyde and potassium dichromate were purchased from BDH Ltd, Poole, Dorset, UK. 2,4-dinitrothiocyanobenzene (DNTB) was obtained from ICN Pharmaceuticals Inc., Plainview, New York, USA. 4-allyl-2-methoxyphenol (eugenol), 2-methoxy-4-propenylphenol (isoeugenol) and trans 3,7-dimethyl-2,6-octadien-1-ol (geraniol) were gifts from D. Basketter, Environmental Safety Laboratory, Unilever Research and Engineering, Bedford, UK. For test development chemicals were dissolved in dimethylformamide (DMF). In the validation study test chemicals were, whenever possible, dissolved in 4:1 acetone:olive oil (AOO). Penicillin-G, nickel sulphate and potassium dichromate were tested as aqueous solutions. Selected literature sources of guinea pig and/or human sensitization test data available for these chemicals are listed in Table 1.

Mice. Young adult (6–8 weeks) BALB/c and CBA/Ca strain mice were used for assay development and the validation study respectively. In one series of comparative experiments C57BL/10J and the outbred strain of Alderley Park mice (Alpk/AP) were used.

Measurement of lymph node cell proliferation. Following the primary application of the test chemical (conventionally in either DMF or AOO) or vehicle alone to the dorsum of the ear, draining (auricular) lymph nodes were excised, pooled for each experimental group and weighed. A single cell suspension of lymph node cells (LNC) was pre-

Table 1. Guinea pig and human sensitization data for the selected test chemicals

Chemical	Sensitization data	
	Guinea pig	Human
cinnamic aldehyde	[a]Prince and Prince 1977	Danneman et al. 1983
4-allyl-2-methoxyphenol (eugenol)	[b]Maurer 1985	Marzulli and Maibach 1980
2-methoxy-4-propenylphenol (isoeugenol)	[a]Klecak et al. 1977	Calnan et al. 1980
trans 3,7-dimethyl-2,6-octadien-1-ol (geraniol)	[a]Klecak et al. 1977	Calnan et al. 1980
2,4-dinitrochlorobenzene (DNCB)	[a]Magnusson and Kligman 1970	Kligman and Epstein 1959
2,4-dinitrothiocyanobenzene (DNTB)	[c]Kimber et al. 1986a	Kimber et al. 1986a
2,4-dinitrofluorobenzene (DNFB)		Garcia-Perez 1978
2,4-dinitrobenzenesulphenyl chloride	Godfrey and Baer 1971	
4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone)	Gad et al. 1986	Gad et al. 1986
2,4,6-trinitrochlorobenzene (pricryl chloride)	[a]Goodwin et al. 1981	Chase 1954
2,4,6-trichloro-1,3,5-triazine (cyanuric chloride)	Stevens 1967	Stevens 1967
p-nitroso-dimethylaniline	[a]Magnusson and Kligman 1970	Kligman and Epstein 1959
2,4,5-trichlorophenol	Stevens 1967	
dihydrocoumarin	[a]Guillot et al. 1983	Malten et al. 1984
phthalic anhydride	Stevens 1967	
4-vinylpyridine	Parker et al. 1985	
p-phenylenediamine	[a]Magnusson and Kligman 1970	Kligman 1966
ethyl-*p*-aminobenzoate (benzocaine)	[a]Magnusson and Kligman 1970	
formaldehyde	[a]Magnusson and Kligman 1970	Marzulli and Maibach 1976
benzylpenicillin (penicillin-G)	[a]Magnusson and Kligman 1970	Kligman 1966
nickel sulphate	[a]Magnusson and Kligman 1970	[a]Magnusson and Kligman 1969
potassium dichromate	[a]Magnusson and Kligman 1970	Cronin 1980

[a] Guinea pig maximization Test; [b] Guinea pig optimisation test; [c] Mouse and rat but not guinea pig data available

pared under asceptic conditions by mechanical disaggregation through sterile 200-mesh stainless steel gauze. Lymphocyte suspensions were washed once in phosphate-buffered saline (PBS; pH 7.2) and resuspended in RPMI-1640 culture medium (Gibco Ltd, Paisley, Scotland) supplemented with 25 mM HEPES, 400 μg/ml ampicillin, 400 μg/ml streptomycin and 10% heat-inactivated (56° C for 30 min) foetal calf serum (RPMI-FCS). Viable cell counts were performed by exclusion of 0.5% trypan blue and the cell concentration adjusted to working values (7.5×10^6 cells/ml) in RPMI-FCS. Lymphocyte suspensions were seeded into 96 well microtitre plates at a concentration of 1.2×10^6 cells/well (five wells per group) and cultured for 24 h at 37° C in a humidified atmosphere of 5% CO_2 in air with 2 μCi ^{3}H-methyl thymidine (specific activity 2.5 Ci/mmol; Amersham International, Amersham, UK). In some experiments the culture was supplemented with a source of interleukin 2 (IL-2). Culture was terminated by automatic cell harvesting and ^{3}HTdR incorporation was determined by β-scintillation counting.

Interleukin 2. Interleukin 2 (IL-2)-rich supernatants were derived from 12-O-tetradecanoylphorbol-13-acetate (TPA)-stimulated cultures of the EL-4 murine thymoma line.

Nylon wool column fractionation. In some experiments T lymphocyte-enriched populations of draining LNC were prepared by passage through nylon wool columns according to a modification of the technique described by Julius et al. (1973). Briefly, 5 ml syringes were packed with 600 mg scrubbed nylon fibre (Travenol Laboratories Ltd, Norfolk, UK) and autoclaved at 121° C for 15 min. Sterile nylon wool columns were flushed with RPMI-FCS and incubated at 22° C for 30 min prior to addition of the LNC suspension. Lymph node cells (10^7 per column) were slowly applied to the nylon in 2.5 ml RPMI-FCS and the column incubated for 45 min at 37° C. Nylon wool non-adherent (T lymphocyte-enriched) populations eluted by gently washing the column with 10 ml RPMI-FCS were washed with PBS and resuspended in growth medium.

Measurement of pyroninophilic cells. Cytocentrifuge preparations were made of LNC suspensions, air-dried and fixed with methanol for 5 min. Slides were stained for 75 min with pyronin and counter-stained with methyl green (Sigma Chemical Co.) (0.16% pyronin, 0.34% methyl green in 0.1 M acetate buffer, pH 6.4). Stained slides were washed in water and the frequency of pyroninophilic lymphocytes measured by inspection of at least 300 cells by oil-immersion microscopy.

Results

Topical exposure of mice to the contact sensitizing chemical 2,4-dinitrochlorobenzene (DNCB) caused a time-dependent induction of proliferation in the draining (auricular) lymph node, which in the experiment recorded in Table 2 was maximal, or nearly maximal, within 3 days of application. Compared with data derived from naive LNC, there was no equivalent induction of lymphocyte proliferation in contralateral auricular nodes draining the site of vehicle (DMF) application (Table 2). To prevent cross-contamination, in subsequent experiments mice were exposed to either the test chemical or vehicle alone on the dorsum of both ears and housed separately.

Table 2. Kinetics of induction of LNC proliferation following exposure to DNCB

Time following exposure (Days)	LNC proliferation ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}	
	DMF	5% DNCB
1	0.48 ± 0.03	0.65 ± 0.06
2	0.42 ± 1.12	2.04 ± 0.31
3	0.54 ± 0.46	4.45 ± 0.64
4	0.47 ± 0.82	4.29 ± 0.71
5	0.34 ± 0.61	5.21 ± 0.17

Groups of mice ($n = 4$) received 25 μl 5% DNCB on the dorsum of the right ear and 25 μl DMF on the dorsum of the left ear at various periods prior to assay. Auricular lymph nodes draining the site of DNCB application and contralateral lymph nodes were isolated and the spontaneous proliferation of single cell suspensions of LNC measured. The spontaneous proliferative activity of auricular lymph node cells isolated from naive mice was also measured and recorded as 0.51 ± 0.05 cpm

Table 3. The influence of exposure to DNCB and DNTB on the draining lymph node. Measurement of increases in lymph node weight, the frequency of pyroninophilic cells and proliferative activity

Expt	Chemical	Mean lymph node weight (mg)	Frequency of pyronin-positive cells (%)	LNC proliferation ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}
1	DMF	1.4	< 1	0.52 ± 0.07
	DNCB	5.4	5.7	5.01 ± 0.12
	DNTB	5.2	6.0	4.72 ± 0.32
2	DMF	1.3	< 1	0.61 ± 0.10
	DNCB	6.0	7.1	5.92 ± 0.29
	DNTB	6.4	5.8	6.58 ± 0.17

Groups of mice ($n = 4$) received 25 μl 5% DNCB, 5% DNTB or an equal volume of vehicle (DMF) alone on the dorsum of both ears. Three days later draining auricular nodes were removed, weighed and pooled for each group. Single cell suspensions of LNC were prepared and proliferative activity recorded. The frequency of pyroninophilic LNC was assessed by examination of cytocentrifuge smears

Further examination of DNCB and a second skin-sensitizing dinitrobenzene derivative, 2,4-dinitrothiocyanobenzene [DNTB] (Kimber et al. 1986a), revealed that the induction of LNC proliferation recorded 3 days following application was accompanied by an increase in lymph node weight and the appearance of pyroninophilic cells (Table 3).

Autoradiographic examination of draining lymph node sections demonstrated that 3 days following exposure to sensitizing chemicals proliferative activity was restricted to the paracortical (T lymphocyte) region of the lymph node (data not presented). Interleukin 2 (IL-2) is a growth factor necessary for the mitogen- or antigen-driven proliferation of T lymphocytes (Gillis et al. 1980). As the hyperplastic response of LNC 72 h following exposure was apparently attributable exclusively to T lymphocytes,

Table 4. DNCB-induced LNC proliferation. Influence of exogenous interleukin 2 (IL-2) on the measurement of LNC proliferation in vitro

% IL-2	LNC proliferation ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}	
	DMF	5% DNCB
0	0.37 ± 0.08	5.45 ± 0.40
0.5	0.40 ± 0.09	6.67 ± 0.31
1	0.42 ± 0.04	7.45 ± 0.51
5	0.40 ± 0.03	8.72 ± 1.12
10	0.52 ± 0.11	9.04 ± 0.82
20	0.50 ± 0.08	8.54 ± 0.94

Groups of mice ($n = 5$) received 25 µl 5% DNCB in DMF or an equal volume of DMF alone on the dorsum of both ears. Three days later draining auricular lymph nodes were removed and pooled for each experimental group. The spontaneous proliferation of single cell suspensions of LNC was recorded in the absence or presence of various concentrations of interleukin 2 (IL-2)-rich medium

Table 5. DNCB-induced LNC proliferation. Enrichment for T lymphocytes fails to influence ^{3}HTdR incorporation

Expt	LNC proliferation ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}			
	DMF		5% DNCB	
	Unfractionated	Nylon wool non-adherent	Unfractionated	Nylon wool non-adherent
1	0.12 ± 0.05 (0.09 ± 0.02)[a]	0.11 ± 0.01 (0.11 ± 0.01)	5.78 ± 1.07 (9.40 ± 2.02)	4.98 ± 0.27 (10.65 ± 1.16)
2	0.31 ± 0.09 (0.42 ± 0.11)	0.24 ± 0.10 (0.38 ± 0.16)	6.67 ± 0.61 (10.48 ± 0.19)	7.83 ± 0.77 (10.37 ± 1.31)
3	0.26 ± 0.02 (0.34 ± 0.08)	0.22 ± 0.04 (0.41 ± 0.07)	4.15 ± 0.37 (9.02 ± 0.45)	3.49 ± 0.25 (8.18 ± 0.92)

[a] Values in parenthesis represent the mean cpm ± sd recorded in the presence of IL-2

Groups of mice ($n = 4$) received 25 µl of either 5% DNCB in DMF or an equal volume of DMF alone on the dorsum of both ears. Three days following challenge the draining auricular nodes were excised and pooled for each experimental group. The incorporation of ^{3}HTdR was measured in the presence or absence of IL-2 for unfractionated LNC and for LNC populations enriched for T lymphocytes by passage through a nylon wool column. Data represent a summary of three independent experiments

it was appropriate to examine whether inclusion in the assay of an exogenous source of IL-2 would enhance proliferative activity. The data in Table 4 clearly demonstrate that the addition of an IL-2-rich medium causes a concentration-related increase in the proliferative response of LNC isolated from nodes draining the site of DNCB application. Interleukin 2 failed, however, to materially influence the activity of control LNC prepared from DMF-treated mice (Table 4). In subsequent experiments proliferation was recorded in the presence or absence of a 10% concentration of IL-2-rich medium.

T lymphocytes can be enriched from mixed lymphoid cell populations by passage through nylon wool. The proliferative activity of unfractionated draining LNC was compared with that of T cell-enriched populations. The data recorded in Table 5 illustrate that, in three independent experiments, enrichment for T lymphocytes failed to significantly enhance the level of activity recorded.

Table 6. The failure of sodium dodecyl sulphate (SDS) to induce spontaneous LNC proliferation

Treatment	LNC proliferation ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}	
	− IL-2	+ IL-2
5% DNCB	3.52 ± 0.17	5.29 ± 0.38
40% SDS	0.40 ± 0.10	0.42 ± 0.06
20% SDS	0.36 ± 0.04	0.44 ± 0.10
10% SDS	0.43 ± 0.13	0.51 ± 0.02
5% SDS	0.36 ± 0.06	0.56 ± 0.04
DMF	0.38 ± 0.07	0.47 ± 0.05

Groups of mice ($n = 4$) received 25 µl 5% DNCB, various concentrations of SDS or vehicle (DMF) alone on the dorsum of both ears. Three days later draining auricular lymph nodes were excised and pooled for each experimental group. The spontaneous proliferation of single cell suspensions of LNC was recorded in the presence or absence of IL-2

Table 7. A comparison between single and multiple applications on the LNC proliferative response induced by DNCB

Treatment			LNC proliferation on Day 3 ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}	
Day 0	Day 1	Day 2	− IL-2	+ IL-2
DMF	DMF	DMF	0.49 ± 0.04	0.62 ± 0.05
5% DNCB	DMF	DMF	8.04 ± 0.12	11.65 ± 1.02
2% DNCB	DMF	DMF	5.71 ± 0.42	8.44 ± 0.76
1% DNCB	DMF	DMF	4.30 ± 0.53	8.15 ± 0.63
0.75% DNCB	DMF	DMF	3.14 ± 0.15	4.25 ± 0.54
0.25% DNCB	DMF	DMF	1.64 ± 0.09	2.18 ± 0.08
DMF	DMF	0.25% DNCB	0.71 ± 0.08	1.02 ± 0.03
DMF	0.25% DNCB	0.25% DNCB	2.68 ± 0.22	3.71 ± 0.51
0.25% DNCB	0.25% DNCB	0.25% DNCB	5.99 ± 0.42	9.02 ± 1.02

Groups of mice ($n = 4$) received 25 µl of various concentrations of DNCB in DMF or DMF alone on the dorsum of both ears on 3 consecutive days. One day following the final application draining auricular lymph nodes were removed and pooled for each experimental group. A single cell suspension was prepared and proliferation measured in the presence or absence of IL-2

As skin irritation is a potential source of difficulty in the currently available predictive test methods for contact sensitization which rely on assessment of erythema and/or induration, we examined whether exposure to a non-sensitizing, irritant chemical would initiate lymph node cell activation. Mice were exposed to increasing concentrations of sodium dodecyl sulphate (SDS) in DMF and proliferative activity recorded 3 days later. Exposure to SDS, even at that concentration (40%) which resulted in local inflammation of the ear as assessed by an increase in ear thickness, failed to induce measurable LNC hyperplasia (Table 6).

In a further attempt to optimize the sensitivity of the assay system we examined whether repeated application of the test chemical to the ear would enhance proliferative responses in the node. The data in Table 7 show that a single application, 3 days prior to assay, of concentrations of

Table 8. LNC proliferation induced by exposure to DNCB. A comparison of responsiveness between four strains of mice

Strain	LNC proliferation ^{3}HTdR incorporation mean cpm ± sd × 10^{-3}			
	DMF		5% DNCB	
	−IL-2	+IL-2	−IL-2	+IL-2
BALB/c	0.42 ± 0.05	0.51 ± 0.08	4.38 ± 0.10	7.41 ± 0.31
CBA/Ca	0.38 ± 0.04	0.48 ± 0.09	7.18 ± 0.68	11.04 ± 1.01
C57BL/10J	0.29 ± 0.04	0.36 ± 0.01	3.62 ± 0.13	5.81 ± 0.37
Alpk/AP	0.75 ± 0.04	0.88 ± 0.05	4.78 ± 0.12	6.97 ± 0.51

Groups of mice ($n = 4$) received 25 µl of either 5% DNCB in DMF or DMF alone on the dorsum of both ears. Three days later draining auricular nodes were removed and pooled for each experimental group. The spontaneous proliferation of single cell suspensions of LNC was measured in the presence or absence of IL-2

DNCB of 0.75% or more resulted in a substantial (>6-fold) increase in both spontaneous and IL-2-augmented proliferation. A single exposure to 0.25% DNCB 72 h before assay resulted in a more modest (>3-fold) elevation of ^{3}HTdR incorporation. As the data reveal, however, repeated exposure to 0.25% DNCB on 3 consecutive days prior to assay resulted in a strong proliferative response which was greater than that achieved following a single application of 0.75% DNCB and equivalent to that recorded with 2% DNCB.

Finally, prior to adoption of a test protocol, we examined strain differences in proliferative responses to DNCB. Of the four strains tested CBA/Ca mice exhibited the most vigorous response 3 days following exposure to 5% DNCB. A representative experiment is summarized in Table 8.

On the basis of the data obtained a protocol was formulated in which CBA/Ca strain mice were exposed to various concentrations of the test chemical or to vehicle alone (usually acetone:olive oil) daily for 3 consecutive days prior to assay. Mean lymph node weight, the frequency of pyronin-positive cells and spontaneous and IL-2-augmented proliferation were recorded. The results of a preliminary trial in which this protocol was employed to examine 22 sensitizing chemicals are recorded in Table 9. The concentrations of chemical selected for testing were determined from data available in the literature and previous experience in this laboratory. The potent sensitizing chemicals examined (such as oxazolone, picryl chloride and dinitrobenzene derivatives) resulted in marked increases in all the parameters measured. However, when less potent agents (trichlorophenol, benzocaine, formaldehyde) were tested it was apparent that proliferative responses provided a more sensitive indicator of lymph node activation than did either changes in node weight or the appearance of pyroninophilic cells. On the basis of LNC proliferation, exposure to all the skin-sensitizing chemicals examined, with the exception of three (penicillin-G, nickel sulphate and potassium dichromate), resulted in measurable changes. There was considerable variation in the level of activity recorded. Thus, exposure to optimal concentrations of some chemicals resulted in 2-fold (geraniol) or 3-fold (eugenol, isoeugenol, trichlorophenol, dihydrocoumarin, benzocaine, formaldehyde) increases in proliferation recorded in the absence of exogenous IL-2. In the presence of IL-2, the increment of enhancement relative to LNC cultures derived from vehicle-treated controls was invariably increased. In all other cases (with the exception of metal salts and penicillin-G) very substantial increases in spontaneous proliferation were observed ranging from 7.6-fold with 10% 4-vinylpyridine to 64.7-fold with 1% oxazolone and 57.4-fold with 10% cyanuric chloride. In most instances addition of exogenous IL-2 resulted in an elevation of proliferation relative to control cultures. When tested, a variety of non-sensitizing chemicals including ethanol, acetone, dimethylsulphoxide, dinitrobenzene, Tween 80 and sodium dodecyl sulphate failed to cause changes in any parameters (data not presented).

Discussion

Allergic contact dermatitis is an example of a delayed hypersensitivity reaction and is dependent upon the immunological integrity of the host. For a chemical to induce contact sensitization it must initiate a T lymphocyte-mediated immune response. The data contained within this report suggest that evaluation of the immunological status of the lymph node(s) draining the site of application provides an alternative strategy for the identification of contact allergens.

The primary immune response to contact sensitizing chemicals is characterized by an increase in lymph node cellularity and weight, the appearance of activated pyroninophilic cells and the induction of lymphocyte proliferation (Turk and Stone 1963; Turk 1967; Asherson and Barnes 1973; Kimber et al. 1986a, b). In these, and previous studies (Kimber et al. 1986b), non-sensitizing chemicals were found to cause no significant changes in any of these parameters. A similar approach to the assessment of contact-sensitizing potential in guinea pigs has been described (Bull et al. 1985). In that study the ability of acrylates and certain dinitrobenzene derivatives to induce contact allergy was measured as a function of the appearance of large pyroninophilic cells (LPC) in the paracortical region of draining lymph nodes (Bull et al. 1985). Our experience is that, although with strong skin-sensitizers the frequency of pyronin-positive cells appears to provide an accurate correlate of lymph node activation, it is of more limited value when less potent agents are examined. Similarly, the changes in lymph node weight observed following exposure to less potent sensitizers were relatively modest.

All the chemicals tested in the preliminary validation exercise have been previously shown to possess skin sensitizing potential on the basis of experimental studies, the results of guinea pig predictive assays and/or from human patch test data (Table 1). Although no firm criteria for establishing mathematically what constitutes a positive response in the local lymph node assay have yet been defined, all but three of these chemicals (nickel sulphate, potassium dichromate and penicillin-G) caused a substantial increase in lymphocyte proliferation following epicutaneous application. There is evidence that, under the correct conditions of exposure, mice are capable of mounting contact allergic reactions to both potassium dichromate (Gad et al. 1986; Mor et al. 1988) and nickel sulphate (Gad et al. 1986) and it is arguable that the absence of clear signs of lymph node activation following exposure to these salts (and penicillin-G) in the present study is a consequence of the vehicle chosen (water). Mor et al. (1988)

Table 9. The local lymph node assay. An examination of 22 contact sensitizing chemicals

Chemical	Test Concentration (%)	LNC proliferation ^{3}HTdR incorporation cpm ± sd × 10^{-3}		Mean Lymph Node Weight (mg)	Frequency of Pyronin – Positive Cells (%)
		−IL-2	+IL-2		
cinnamic aldehyde	0	1.06 ± 0.02	1.67 ± 0.34	1.0	< 1
	1	1.21 ± 0.33	2.24 ± 0.31	1.8	2.3
	2.5	3.14 ± 0.25	6.14 ± 0.66	2.0	5.8
	5	13.27 ± 1.33	20.27 ± 1.61	2.9	6.3
4-allyl-2-methoxyphenol (eugenol)	0	0.67 ± 0.23	0.98 ± 0.16	1.8	< 1
	25	0.92 ± 0.12	1.89 ± 0.30	2.5	3.1
	50	3.31 ± 0.35	6.22 ± 0.59	2.4	8.5
	100	1.99 ± 0.62	4.34 ± 0.37	2.2	2.0
2-methoxy-4-propenylphenol (isoeugenol)	0	0.75 ± 0.09	1.25 ± 0.34	1.7	< 1
	5	1.32 ± 0.23	1.84 ± 0.55	2.0	2.0
	10	3.73 ± 0.64	6.92 ± 1.02	3.5	3.8
	25	2.26 ± 0.51	2.18 ± 0.93	5.3	2.1
trans 3,7-dimethyl-2,6-octadien-1-ol (geraniol)	0	1.13 ± 0.14	ND	1.5	1.3
	12.5	0.97 ± 0.08	0.92 ± 0.10	2.1	ND
	25	1.34 ± 0.18	3.23 ± 0.20	2.7	2.3
	50	2.92 ± 1.47	5.77 ± 0.53	2.8	3.6
2,4-dinitrochlorobenzene (DNCB)	0	0.42 ± 0.06	0.23 ± 0.09	0.9	< 1
	0.5	7.04 ± 0.42	12.95 ± 0.59	4.3	4.8
	1	15.78 ± 1.49	20.75 ± 2.48	7.3	7.2
	2.5	15.55 ± 2.16	28.70 ± 3.97	7.7	6.5
2,4-dinitrothiocyanobenzene (DNTB)	0	1.11 ± 0.10	1.78 ± 0.3	1.2	< 1
	0.5	11.83 ± 5.38	21.27 ± 2.00	5.7	4.0
	1	17.93 ± 0.42	26.19 ± 1.75	5.9	7.3
	2.5	20.04 ± 1.41	32.55 ± 3.72	7.7	7.1
2,4-dinitrofluorobenzene (DNFB)	0	1.48 ± 0.15	2.04 ± 0.13	1.1	< 1
	0.5	15.71 ± 2.10	25.34 ± 3.22	6.1	7.3
	1	18.27 ± 2.07	29.32 ± 2.02	5.4	7.9
2,4-dinitrobenzenesulphenyl chloride	0	2.12 ± 0.87	3.62 ± 0.91	1.0	< 1
	1	40.45 ± 12.71	62.85 ± 7.71	7.5	6.4
	2.5	84.10 ± 5.18	129.15 ± 4.44	9.1	9.3
	5	89.13 ± 11.27	141.32 ± 9.45	11.0	10.0
4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone)	0	1.85 ± 0.20	2.76 ± 0.33	1.2	< 1
	0.25	88.71 ± 4.37	144.59 ± 14.43	5.3	6.4
	0.5	136.92 ± 12.62	150.84 ± 8.97	10.1	10.0
	1	119.65 ± 12.38	167.33 ± 14.21	9.7	11.9
2,4,6-trinitrochlorobenzene (picryl chloride)	0	1.05 ± 0.15	2.01 ± 0.09	1.7	< 1
	0.25	27.62 ± 2.51	46.24 ± 3.71	5.2	6.3
	0.5	45.65 ± 3.98	81.56 ± 5.53	8.2	10.1
	1	78.74 ± 4.40	125.26 ± 0.94	8.7	11.7
2,4,6-trichloro-1,3,5-triazine (cyanurie chloride)	0	0.98 ± 0.27	1.16 ± 0.44	1.0	< 1
	2.5	63.20 ± 3.96	91.16 ± 6.30	7.7	6.2
	5	54.59 ± 2.60	81.06 ± 7.05	7.6	7.2
	10	64.29 ± 1.91	87.03 ± 5.27	6.3	7.1
p-nitroso-dimethylaniline	0	1.12 ± 0.12	0.91 ± 0.19	1.7	1.5
	2.5	35.72 ± 2.18	45.92 ± 1.68	6.7	ND
	5	21.19 ± 2.36	31.03 ± 2.49	5.8	5.6
	10	18.74 ± 2.10	22.80 ± 1.67	5.0	6.6
2,4,5-trichlorophenol	0	0.88 ± 0.24	1.18 ± 0.19	0.7	< 1
	5	2.81 ± 0.60	3.17 ± 0.21	2.4	1.4
	10	3.14 ± 0.20	5.30 ± 0.47	2.3	1.7
dihydrocoumarin	0	1.01 ± 0.14	1.29 ± 0.08	0.9	< 1
	2.5	1.38 ± 0.21	4.84 ± 0.37	1.6	< 1
	5	2.27 ± 0.92	6.60 ± 0.71	1.5	1.4
	10	3.15 ± 0.11	7.58 ± 0.41	1.4	1.8
phthalic anhydride	0	1.02 ± 0.16	1.61 ± 0.21	1.2	< 1
	2.5	29.71 ± 2.13	52.61 ± 4.24	4.7	4.0
	5	54.48 ± 4.76	76.87 ± 5.14	6.2	5.4
	10	43.09 ± 3.73	78.16 ± 1.91	5.5	6.8

Chemical	Test Concentration (%)	LNC proliferation ^{3}HTdR incorporation cpm ± sd × 10^{-3}		Mean Lymph Node Weight (mg)	Frequency of Pyronin – Positive Cells (%)
		−IL-2	+IL-2		
4-vinylpyridine	0	0.92 ± 0.15	1.27 ± 0.09	1.2	<1
	2.5	4.38 ± 0.32	10.41 ± 1.02	2.2	2.2
	5	6.96 ± 0.82	13.45 ± 1.27	3.6	2.7
	10	7.07 ± 0.65	14.86 ± 1.13	2.7	4.2
p-phenylenediamine	0	0.46 ± 0.03	0.92 ± 0.10	1.3	<1
	2.5	5.10 ± 0.94	10.78 ± 1.64	2.8	2.7
	5	8.49 ± 0.61	13.21 ± 1.40	3.0	3.8
	10	8.96 ± 0.46	14.46 ± 0.63	3.9	3.6
ethyl-*p*-aminobenzoate (benzocaine)	0	0.97 ± 0.11	1.15 ± 0.16	0.7	<1
	2.5	1.01 ± 0.09	1.27 ± 0.12	0.9	<1
	5	1.61 ± 0.13	2.02 ± 0.24	1.1	1.5
	10	3.84 ± 0.82	5.10 ± 1.16	0.8	1.2
formaldehyde	0	1.18 ± 0.14	1.32 ± 0.27	1.2	<1
	2.5	0.92 ± 0.10	1.47 ± 0.09	1.0	<1
	5	2.88 ± 0.31	6.79 ± 0.53	2.0	1.7
	10	3.77 ± 0.42	6.42 ± 0.81	1.7	1.4
benzylpenicillin[a] (penicillin-G)	0	0.63 ± 0.19	0.58 ± 0.23	1.1	<1
	5	0.65 ± 0.17	0.56 ± 0.08	1.0	<1
	10	0.48 ± 0.09	0.52 ± 0.09	0.9	<1
	20	0.64 ± 0.23	0.77 ± 0.13	1.2	<1
nickel sulphate[a]	0	1.01 ± 0.21	1.31 ± 0.09	1.3	<1
	10	1.32 ± 0.16	1.38 ± 0.08	1.2	<1
	20	0.92 ± 0.17	1.32 ± 0.21	1.3	<1
	40	1.66 ± 0.07	2.69 ± 0.31	1.6	1.5
potassium dichromate[a]	0	0.97 ± 0.08	1.03 ± 0.11	0.9	<1
	5	0.79 ± 0.10	0.96 ± 0.07	1.3	<1
	10	0.70 ± 0.09	1.01 ± 0.08	1.0	<1
	20	0.99 ± 0.11	1.15 ± 0.12	1.4	<1

ND = Not determined; [a] = aqueous solution

Groups of CBA/Ca strain mice ($n = 3$) received a daily application for 3 consecutive days of 25 µl of various concentrations of the test chemical in vehicle (except where indicated acetone: olive oil) or an equal volume of vehicle alone on the dorsum of both ears. One day following the final application draining auricular lymph nodes were excised, pooled for each experimental group and weighed. A single cell suspension of LNC was prepared and proliferation measured in the presence or absence of IL-2. The frequency of pyroninophilic LNC was assessed by examination of cytocentrifuge smears

have suggested that topical exposure to potassium dichromate in dimethylsulphoxide, but not in other vehicles such as methanol or Triton, results in contact sensitization. It will be of interest in future studies to explore whether application of penicillin-G and skin-sensitizing metal salts in vehicles other than water will induce local lymph node activation. The tentative conclusion that can be drawn from the studies reported here is that the local lymph node assay provides a sensitive means of identifying contact sensitizing chemicals prepared in a vehicle suitable for open epicutaneous application.

The mouse has been employed in other attempts to develop alternative methods for identifying contact allergens, in which reactivity is measured as challenge-induced increases in ear thickness (Gad et al. 1986; Maisey and Miller 1986). In a comprehensive study by Gad et al. (1986) the mouse ear swelling test (MEST) was optimized and used to examine a variety of skin-sensitizing and non-sensitizing chemicals, including a number of those tested in the present study. Among the chemicals which elicited the strongest responses in the MEST were oxazolone, picryl chloride, DNFB, DNCB, *p*-nitroso-dimethylaniline and *p*-phenylenediamine (Gad et al. 1986). At the concentrations examined, all of these agents caused very substantial (>15-fold) increases in lymphocyte proliferation in the local lymph node assay. Some of the chemicals which resulted in less marked net increases in ear thickness in the MEST, such as eugenol (19%) and formalin (15%) also proved positive in the local lymph node assay (increases in proliferation, in the absence of interleukin 2, of 4.9-fold and 3.2-fold respectively, at optimal test concentrations). Other chemicals examined in this study have also been evaluated in an ear thickness assay described by Maisey and Miller (1986) in which mice were maintained on a diet supplemented with vitamin A. In their study reactions were recorded for individual animals. Thus, for instance, exposure to cinnamic aldehyde resulted in a greater than 50% increase in ear thickness in six of ten mice tested. Dihydrocoumarin and benzocaine caused a greater than 50% increase in two of ten and seven of ten animals, respectively (Maisey and Miller 1986). All of these chemicals proved positive in the local lymph node assay.

Taken together, these data suggest that the murine local lymph node assay described here is capable of detect-

ing at least strong sensitizing chemicals and also compounds such as eugenol and geraniol which exhibit relatively weak and variable responses in predictive guinea pig tests (Klecak et al. 1977; Maurer 1985).

The local lymph node assay has the cost-advantages of a murine test discussed by Gad et al. (1986). Furthermore, like the MEST, the local lymph node assay is objective, rapid, requires less test substance than guinea pig methods and can be used to evaluate coloured materials which can be difficult to assess by currently available predictive tests. In contrast to the MEST, however, the local lymph node assay offers two additional advantages. Firstly, under the conditions employed, irritant non-sensitizing chemicals appear not to influence the immunological status of the draining lymph node. Thus, although further confirmation is required, the local lymph node assay may prove useful for the analysis of irritant chemicals without the need to first establish the maximal non-irritant concentration. Secondly, unlike the MEST described by Gad et al. (1986) and many of the more sensitive guinea pig tests, the local lymph node assay does not rely on the use of adjuvants.

Finally, the data presented demonstrate that the judicious use of interleukin 2 provides a means of increasing the sensitivity of the local lymph node assay through a selective enhancement of stimulated lymph node cell proliferation.

Although a more extensive validation study will clearly be required, there is sufficient information available to conclude that the local lymph node assay provides a rapid and cost-effective method for identifying at least strong sensitizing chemicals and may be of particular value for assessing the sensitizing potential of coloured or irritant test materials.

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