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# INTERLEUKIN 6 PRODUCTION *IN VITRO*: AN ALTERNATIVE READ-OUT FOR THE LOCAL LYMPH NODE ASSAY

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**Abstract**—The murine local lymph node assay has been developed as an alternative method for the identification of contact allergens. In contrast to guinea pig tests, which rely on visual assessment of challenge-induced dermal reactions, the local lymph node assay measures events occurring during the induction of skin sensitization. Contact allergic potential is measured as a function of hyperplastic responses in draining lymph nodes following systemic administration of [$^3$H]thymidine. We have now examined whether the production *in vitro* of interleukin 6 (IL-6) by draining lymph node cells isolated from sensitized mice provides an alternative endpoint for the local lymph node assay. In comparative experiments, the production of IL-6 by lymph node cells in culture correlated closely with proliferative responses *in vitro*. Only chemicals known to cause contact sensitization elicited measurable (> 150 pg/ml) IL-6 production; non-sensitizing chemicals, including skin irritants, did not. Experience to date suggests that IL-6 production may provide a useful alternative read-out for the identification of chemicals which have a significant skin-sensitizing potential.

## INTRODUCTION

The murine local lymph node assay has been developed as an alternative method for the identification of contact sensitizing chemicals (Kimber and Basketter, 1992). Skin-sensitizing activity is measured as a function of lymphocyte proliferative responses induced in the draining lymph nodes of mice exposed topically to the test material (Kimber *et al.*, 1989; Kimber and Weisenberger, 1989). The local lymph node assay has been the subject of comparisons with guinea pig test methods (Basketter and Scholes, 1992; Kimber *et al.*, 1990) and of interlaboratory validation exercises (Kimber and Basketter, 1992). The conclusion drawn from such studies is that the assay provides a reliable method for the assessment of contact sensitizing potential.

We have questioned now whether a clearer understanding of the central role of cytokines in induction and regulation of immune function provides an opportunity to identify skin-sensitizing chemicals in the local lymph node assay on the basis of molecular, rather than cellular, responses. We have elected in the present study to measure the production by draining lymph node cells (LNC) of interleukin 6 (IL-6), a cytokine known to influence a wide range of immune and inflammatory processes including T-lymphocyte activation and growth (Van Snick, 1990). IL-6 production has been compared with the induction of lymph node cell replicative DNA synthesis.

*Abbreviations*: AOO = acetone–olive oil (4:1); DMF = dimethylformamide; ELISA = enzyme-linked immunosorbent assay: FCS = foetal calf serum; HMDI = dicyclohexylmethane-4,4′-diisocyanate; IL-6 = interleukin 6; LNC = lymph node cells; PBS = phosphate buffered saline; SLS = sodium lauryl sulfate; $^3$H-TdR = [$^3$H]methyl thymidine; TMA = trimellitic anhydride.

## MATERIALS AND METHODS

*Animals and exposure.* Young adult (6–12 wk old) BALB/c strain mice (Barriered Animal Breeding Unit, Alderley Park, Cheshire, UK) were treated topically with non-sensitizing chemicals and with a variety of chemicals known to possess a range of skin-sensitizing potential (see legend to Fig. 1). Exposure concentrations were selected on the basis of either previous experience in this laboratory or data available in the literature. Mice received 25 $\mu$l of the test chemical, or an equal volume of the relevant vehicle alone, on the dorsum of both ears daily for 3 consecutive days.

*Lymph node cell proliferation.* Draining auricular lymph nodes were excised 3 days following the initiation of exposure, pooled for each experimental group and a single cell suspension of LNC prepared under aseptic conditions. Viable cell concentration was adjusted to working values in RPMI-1640 culture medium (Gibco, Paisley, UK) supplemented with 25 mM HEPES, 400 $\mu$g ampicillin/ml, 400 $\mu$g streptomycin/ml and 10% heat-inactivated foetal calf serum (RPMI-FCS). Cells were seeded into 96-well microtitre plates at $4 \times 10^7$ cells/ml and cultured for 24 hr at 37°C in a humidified atmosphere of 5% $CO_2$ in air with 2 $\mu$Ci [$^3$H]methyl thymidine ($^3$H-TdR; sp. act. 2.5 Ci/mmol; Amersham International, Amersham, UK). Culture was terminated by automatic cell

harvesting and $^3$H-TdR incorporation measured by $\beta$-scintillation counting.

*IL-6 production by LNC.* LNC were seeded into 24-well tissue culture plates and cultured at $4 \times 10^7$ cells/ml for 48 hr as described above. Supernatants were collected, centrifuged at 150 *g* for 5 min and stored at −70°C until analysis. The concentration of IL-6 in supernatants was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Plastic microtitre plates were coated with 2.5 $\mu$g rat monoclonal anti-mouse interleukin 6 (IL-6) antibody/ml (Genzyme, Cambridge, MA, USA) in 0.1 M carbonate buffer (pH 9.6) by overnight incubation at 4°C. The plates were then blocked by treatment for 30 min at 37°C with 5% FCS in phosphate buffered saline (PBS; pH 7.2). Recombinant mouse IL-6 ($1 \times 10^8$ U/mg; Genzyme) diluted in RPMI-FCS and samples of conditioned medium were added to triplicate wells and the plates incubated for 2 hr at room temperature. Plates were then incubated similarly with 8 $\mu$g goat anti-mouse IL-6/ml (British Biotechnology Ltd, Abingdon, UK) and for a further 2 hr at room temperature with a 1:500 dilution of peroxidase-conjugated donkey anti-goat IgG (Serotec, Kidlington, UK) diluted in RPMI-FCS. Enzyme substrate (*o*-phenylene diamine and urea hydrogen peroxide) was added and the reaction terminated by the addition of 0.5 M citric acid after 15 min. Between each incubation the plates were washed with PBS containing 0.05% Tween 20. Optical density at 450 nm was measured using an automated reader (Multiskan, Flow Laboratories, Irvine, UK). The concentration of IL-6 in test samples was calculated from a standard curve derived with recombinant murine IL-6. Results are expressed as IL-6 concentration in pg/ml. The limit of detection of IL-6 was 150 pg/ml.

## RESULTS AND DISCUSSION

The data summarized in Fig. 1 illustrate the results of a study comparing the vigour of lymph node cell proliferative responses with IL-6 production. Under the conditions of exposure used, significant levels of IL-6 production were observed with all the sensitizing chemicals tested, other than 25% cinnamic aldehyde

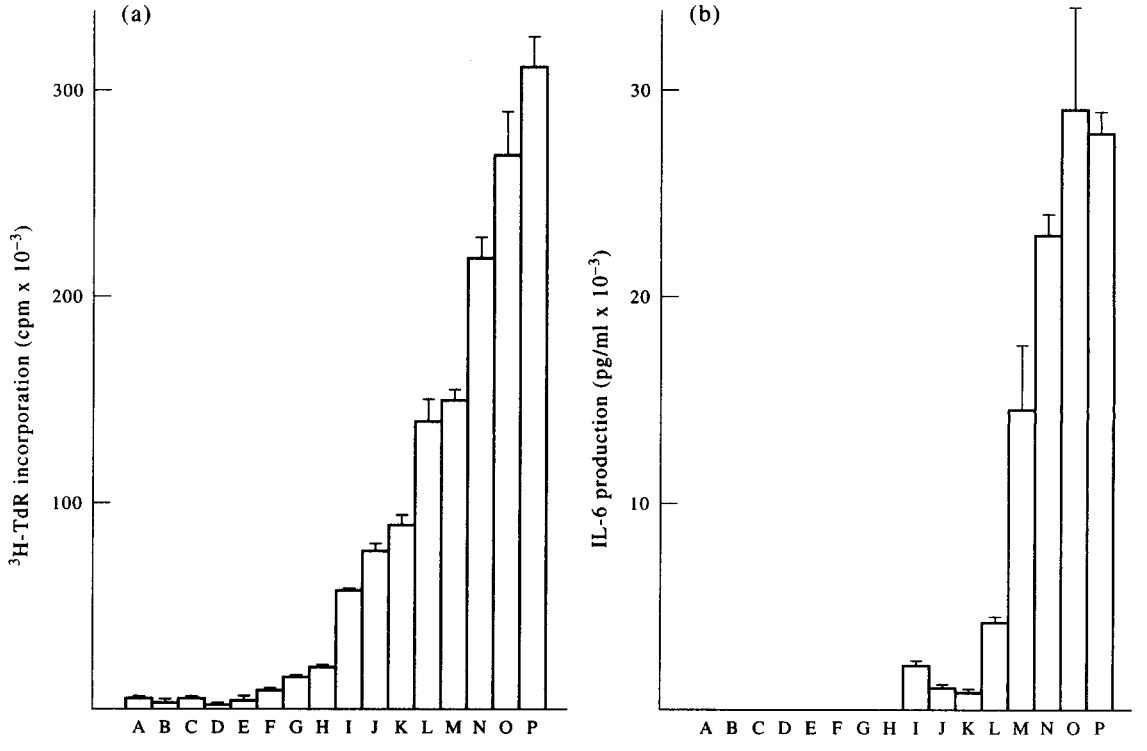


Fig. 1. Induction of lymph node cell (LNC) proliferation (a) and interleukin 6 (IL-6) production (b) by chemicals. Groups of mice were treated with various chemicals as described in Material and Methods, or were untreated. A, naive mice; B, acetone–olive oil (4:1; AOO); C, dimethyl formamide ((DMF); D, ethanol; E, 5% methyl salicylate; F, 25% ethyl-*p*-aminobenzoate (benzocaine); G, 10% sodium lauryl sulfate (SLS); H, 25% cinnamic aldehyde; I, 0.5% 2,4-dinitrochlorobenzene; J, 1% potassium dichromate; K, 50% 2-methoxy-4-propenylphenol (isoeugenol); L, 0.5% diphenylmethane-4,4′-diisocyanate (MDI); M, 0.25% 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone); N, 0.25% 2,4,6-trinitrochlorobenzene (picryl chloride); 0, 10% trimellitic anhydride (TMA); P, 0.5% dicyclohexylmethane-4,4′-diisocyanate (HMDI). With the exception of potassium dichromate and SLS, where DMF was used, the vehicle was AOO. Induced LNC proliferation and IL-6 production were measured as described in the text. Capped vertical bars represent (±) standard deviation. [$^3$H]TdR = [$^3$H]methyl thymidine.

Table 1. Interleukin 6 (IL-6) production by lymph node cells prepared from mice exposed to oxazolone and picryl chloride

| Chemical (%, w/v) | IL-6 (pg/ml) |
|---|---|
| Oxazolone | |
| 0.5 | 3000 |
| 0.25 | 900 |
| 0.1 | <150 |
| 0 | <150 |
| Picryl chloride | |
| 0.5 | 8800 |
| 0.25 | 2100 |
| 0.1 | <150 |
| 0 | <150 |

Vehicle = acetone–olive oil (4:1).

and 25% benzocaine. The strongest responses observed were induced by 0.25% oxazolone, 0.25% picryl chloride, 10% trimellitic anhydride (TMA) and 0.5% dicyclohexylmethane-4,4′-diisocyanate (HMDI), which resulted, respectively, in mean IL-6 concentrations of approximately 15, 23, 29 and 28 ng/ml. Concentrations of IL-6 in supernatants derived from LNC prepared from untreated mice, or mice exposed to non-sensitizing chemicals, including the vehicles acetone–olive oil (AOO) and dimethylformamide (DMF), were below the level of detection.

All the sensitizing chemicals tested caused a significant elevation of $^3$H-TdR incorporation by LNC compared with values obtained with LNC from naive mice and vehicle-treated controls. It is our policy, currently, in the standard local lymph node assay performed with mice of CBA/Ca strain, to classify as sensitizers those chemicals which induce a three-fold or greater increase in isotope incorporation relative to vehicle control values (Kimber and Basketter, 1992). Applying that criterion as an arbitrary measure of activity recorded in the present study, then at the concentrations tested all the sensitizing chemicals examined would be considered to have elicited a positive response. Although, on this basis, sodium lauryl sulfate (SLS), a non-sensitizing skin irritant, failed in these experiments to provoke a positive response relative to vehicle (DMF) values (mean value of $9.11 \times 10^{-3}$ cpm measured with 10% SLS compared with $5.36 \times 10^{-3}$ cpm with DMF), this chemical did induce an increase in proliferative activity. All other non-sensitizing chemicals were without effect.

Dose–response analyses have been performed also and the results of representative experiments with oxazolone and picryl chloride are shown in Table 1. Exposure of mice to concentrations of 0.25% or greater of these chemicals caused substantial IL-6 production by draining LNC. However, treatment with a lower concentration (0.1%) of the same chemicals failed to induce measurable levels of this cytokine. In the same studies the non-sensitizing chemicals SLS and methyl salicylate were examined. At all test concentrations (2.5, 5 and 10% SLS and 5, 10 and 25% methyl salicylate) there was no detectable production of IL-6 by LNC (data not presented).

Taken together, these data demonstrate that the activation of draining lymph nodes during the induction phase of contact sensitization is associated with the production by LNC of IL-6. Moreover, there appears to be a correlation between the vigour of the induced lymphoproliferative response and the synthesis of this cytokine. In the context of the local lymph node assay and the prospective identification of sensitizing chemicals, the measurement of IL-6 offers a number of potential advantages. Evaluation of IL-6 concentration in activated LNC supernatants that can be aliquoted and stored, facilitates inter-laboratory comparisons and longitudinal studies. Furthermore, it is apparent that, using the methods described here, only skin-sensitizing chemicals induce measurable levels (>150 pg/ml) of IL-6. Non-sensitizing chemicals, including those such as SLS, which may in some circumstances provoke low levels of $^3$H-TdR incorporation, fail to induce detectable levels of IL-6. It is clear also, however, that currently IL-6 production is a less sensitive marker of lymph node activation than is LNC proliferation. In the present studies the known skin sensitizer cinnamic aldehyde, which elicits a positive response in the conventional local lymph node assay, failed to induce measurable IL-6 production. Similarly, concentrations of oxazolone and picryl chloride which would undoubtedly yield positive local lymph node assay responses in the context of LNC proliferation did not induce detectable IL-6.

In conclusion, the measurement *in vitro* of IL-6 produced by draining LNC provides a viable, but probably less sensitive, endpoint for the local lymph node assay.

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