Assessment of Cumulative Allergen-Activated Lymph Node Cell Proliferation Using Flow Cytometry

Neil E. Humphreys, Rebecca J. Dearman,1 and Ian Kimber
Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, United Kingdom

Received December 9, 2002; accepted January 24, 2003

The murine local lymph node assay (LLNA) is a method for the prospective identification of chemical contact allergens. The current validated protocol assesses lymphocyte proliferation induced in the draining lymph node as a function of in situ incorporation of radiolabeled thymidine. We have explored the potential utility of an alternative nonradioisotopic marker of cell division, the cytoplasmic dye carboxyfluorescein succinimidyl ester (CFSE). Using this method, the cell phenotype and the number of divisions each cell has undergone can be tracked using flow cytometry. BALB/c strain mice were exposed topically to various concentrations of the contact allergens 2,4-dinitrochlorobenzene (DNCB), oxazolone (ox) or hexyl cinnamic aldehyde (HCA), or to the nonsensitizing skin irritant methyl salicylate (MS). Five days later, lymph node cells (LNC) were labeled with CFSE, cultured for 96 h, then incubated with fluorescent labeled anti-CD4 (T helper) and -CD8 (T cytotoxic) cell antibodies, and proliferating CD4+ and CD8+ cells analyzed by flow cytometry. In LNC populations derived from vehicle-treated animals, less than 1% of either cell population had undergone one cell division or more. Topical exposure to MS (2.5 to 20%) did not increase the frequencies of proliferating cells. Exposure to all three allergens, however, resulted in a marked increase in the percentages of both CD4+ and CD8+ cells undergoing division, with up to 5% and 3% of these cells, respectively, proliferating in response to DNCB and oxazolone, and with lower levels of proliferation stimulated by HCA. These preliminary data suggest that this method may be applied to provide the basis of a nonradioisotopic end point for the LLNA, particularly for the identification of potent contact allergens.

Key Words: allergens; irritants; local lymph node assay; nonradioisotopic; flow cytometry.

Allergic contact dermatitis is an important occupational and environmental health issue (Cronin, 1980; Kimber et al., 2002). In addition to the more traditional guinea pig assays for the assessment of skin sensitization hazard, in recent years an alternative method, the murine local lymph node assay (LLNA), has been accepted as a stand-alone test for the identification of chemicals that have the potential to cause skin sensitization (Basketter et al., 1999a; Dean et al., 2001). This method is based on an understanding that the central event during the induction phase of skin sensitization is the stimulation of lymphocyte proliferative responses in lymph nodes draining the site of topical exposure (Kimber and Dearman, 1991). The LLNA has been extensively validated for its reliability and sensitivity for the prediction of skin sensitization potential in both national and international interlaboratory collaborations, and has been found to correlate well with guinea pig tests and with available experience of sensitization in humans (reviewed in Gerberick et al., 2000). In the standard LLNA, CBA strain mice are exposed topically to chemical on the dorsum of both ears and lymphocyte proliferation measured as a function of in vivo incorporation of radiolabeled thymidine (Gerberick et al., 2000; Kimber and Basketter, 1992).

There has been some interest in the development of alternative end points for the LLNA, including those that might obviate the requirement for radioactive labeling, particularly in certain regions where the use of radioisotopes is regulated strictly. Among the alternative methods that have been examined are the assessment of lymph node activation as a function of changes in lymph node cellularity or lymph node weight (Homey et al., 1998) and analysis of changes in the relative frequencies in lymphocyte subpopulations in lymph nodes draining the site of topical exposure. Although contact hypersensitivity is a T lymphocyte-mediated reaction, it has been demonstrated that the draining lymph nodes of mice exposed topically to contact allergens display an increased frequency of B lymphocytes measured as a function of B220+ cells and IgG/IgM+ cells (Gerberick et al., 1997, 1999, 2002; Manetz and Meade, 1999; Sikorski et al., 1996). It has been proposed, therefore, that analysis of induced changes in the frequency of B220+ cells within draining lymph nodes may provide an adjunct or supplementary read-out for the LLNA (Gerberick et al., 2002). Another alternative end point that has been investigated is the use of a nonradioisotopic analogue of thymidine, bromodeoxyuridine (BrdU), that can be detected using an anti-BrdU antibody (Takeyoshi et al., 2001). Limited experience of this method suggests that it may have some potential for the identification of contact allergens, although the sensitivity with respect to allergen-induced fold changes in BrdU.
incorporation is less than that achieved using radiolabeled thymidine.

A method for the analysis of cell turnover that is gaining some popularity in the fields of immunology and cell biology is tracking cell division using the membrane permeable molecule 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Givan et al., 1999; Lyons, 1999; Parish, 1999). The nonfluorescent highly membrane-permeable diacetate form is taken up readily by cells (Parish, 1999). Once inside the cell, however, intracellular esterases cleave the diacetate groups and the resultant fluorescent moiety, 5,6-carboxyfluorescein succinimidyl ester (CFSE), is retained within the cell. Due to the high reactivity of the succinimidyl group with amines, a pro-cinimidyl ester (CFSE), is retained within the cell. Due to the

between daughter cells in each successive division. Thus, it has

of CFSE-labeled lymphocytes, the marker was divided equally

relevance to the potential application of this technique to

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gone up to 10 successive divisions. Again, the mean

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10 discrete populations can be detected on the basis of decreas-

CFSE label compared with parental cells (Lyons, 1999). Up to

10 discrete populations can be detected on the basis of decreasing

fluorescence intensity, identifying cells that have undergone

up to 10 successive divisions. Again, the mean fluorescence

intensity of the cells has been shown to decrease by 50%

each successive division (Lyons and Parish, 1994). One of the

major advantages of this method is that not only can proliferating cells be identified in complex cell mixtures, but also the use of two or three color immunofluorescence allows immunophenotyping of the dividing cells using monoclonal antibodies conjugated to other fluorescent dyes.

Despite the fact that this technique has been established for some time, it has as yet found little application in toxicology. In the current investigations we have examined the potential utility of this method for the identification of cells proliferating in response to chemical allergens. Dose-response studies have been performed in BALB/c strain mice with two potent contact allergens, oxazolone (ox) and 2,4-dinitrochlorobenzene (DNCB). Additional studies have been performed with hexyl cinnamic aldehyde (HCA), a chemical that is currently recommended as a positive control substance in tests for contact sensitization hazard identification (Steiling et al., 2001), and is described variously as a mild to moderately sensitizing chemical or a weak contact allergen (Basketter et al., 2001; Steiling et al., 2001). The selectivity of the proliferative response has been assessed by analysis of the response to the nonsensitizing skin irritant, methyl salicylate (MS; Kligman, 1966). The frequency of CD4+ and CD8+ proliferating cells within the total intact lymphocyte pool and the percentage of total proliferating lymphocytes have been analyzed. These studies have been conducted according to the standard LLNA exposure protocol; however, BALB/c strain mice, rather than the recommended CBA strain mice, have been used. This strain of mouse has been shown previously to give comparable proliferative responses to those achieved in CBA strain mice using reference contact allergens, including HCA (Woolhiser et al., 2000). The results have been compared with incorporation of radiolabeled thymidine in vitro in cells cultured concurrently.

**MATERIALS AND METHODS**

**Animals.** Young adult (6–12 weeks old) female BALB/c strain mice (Specific Pathogen Free Breeding Unit, Alderley Park, Macclesfield) were used throughout these studies. Mice were housed three to five per cage on flushing metal racks with materials provided for environmental enrichment. Food (SDS PCD pelleted diet; Special Diets Services Ltd, UK) and water were available ad libitum. The ambient temperature was maintained at 21 ± 2°C and relative humidity was 55 ± 10% with a 12-h light/dark cycle. All procedures were approved by the U.K. Home Office and carried out in compliance with the Animals (Scientific Procedures) Act, 1986 under a Home Office granted Project License.

**Chemicals.** α-HCA (85% pure) was obtained from Aldrich Chemical Co. (Gillingham, Dorset, UK). DNCB (97% pure), 4-ethoxymethylene-2-phenyloxazol-5-one (ox; 80% pure), and methyl salicylate (MS, 99% pure) were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). All chemicals were dissolved in 1:1 v/v acetic:olive oil (AOO) and were prepared freshly, prior to dosing. Chemicals were dispensed in a fume cupboard, with gloves for dermal protection.

**Sensitization regimen.** Animals (n = 3 to 5 per group for chemical; n = 5 per group for vehicle) received 25 μl of various concentrations of chemical in vehicle (AOO), or AOO alone, on the dorsum of both ears, daily for three consecutive days. In some experiments, animals (n = 3) received a single topical application of 25 μl of chemical in vehicle (AOO) on the dorsum of both ears. Protective clothing for licensees handling animals included mask, gloves, and safety spectacles.

**Preparation of draining lymph node cells.** Three or five days after the initiation of exposure, draining auricular lymph nodes were excised into RPMI 1640 (GibcoBRL, Renfrewshire, U.K.) supplemented with 10% heat-inactivat-

ed fetal calf serum (FCS), 2 mM L-glutamine (GibcoBRL), 400 μg/ml streptomycin, and 400 μg/ml ampicillin (Sigma) (RPMI-FCS) and pooled on an experimental group basis. A single cell suspension of lymph node cells (LNC) was prepared under aseptic conditions by mechanical disaggregation through 200-mesh stainless steel gauze. Viable cell counts were performed by exclusion of 0.5% trypan blue and LNC resuspended in phosphate-buffered saline (PBS) at 106 cells/ml.

**CFSE labeling.** Single cell suspensions of LNC in PBS were seeded into Falcon tubes (105 cells/tube). To each 1-ml aliquot of cells, 10 μl of 500 μM CFSE was added in PBS (Cambridge Bioscience, Cambridge, U.K.) was added and cells were incubated at 37°C for 15 min. In order to stop the reaction, 100 μl of FCS was added per ml of cells, the cells washed twice in RPMI-FCS, and resuspended at 107 cells/ml in RPMI-FCS. In some experiments, allergen-

activated LNC were cultured in the presence of 2 μg/ml of the T-cell mitogen concanavalin A (con A; Sigma). Cell preparations were cultured in the dark at 37°C in a humidified atmosphere of 5% CO2 in air at 105 cells/ml for 96 h and harvested by centrifugation for FACs analysis.

**Thymidine incorporation in vitro.** Parallel cell cultures were established for measurement of proliferation by radiolabeled thymidine incorporation. Cells were seeded in quintuplicate into 96-well, round-bottomed microtiter plates (105 cells/well) and cultured for 96 h at 37°C in a humidified atmosphere of 5% CO2 in air. For the final 24 h of culture, cells were pulsed with 2 μCi [3H]-methyl thymidine (specific activity 2 Ci/mmol Amersham International,
Flow cytometric analyses. Fluorescence staining for CD4+ and CD8+ cells was carried out in 96-well, round-bottomed microtiter plates. Aliquots of 2 × 10^6 cells were incubated with optimal (typically, 1 μg per 10^6 cells) concentrations of Tri-color (TC) conjugated rat monoclonal antiCD4 or CD8 antibodies (anti-CD4 antibody, clone S3.5; anti-CD8 antibody, clone 3B5; Caltag, Burlingame, CA). It was not possible to dual stain proliferating cells for CD4 and CD8 expression as CFSE has such a wide emission spectrum and range of intensities that the use of the FL2 channel is technically very difficult. Cells were incubated for 30 min on ice, washed, and resuspended in FACs buffer to a concentration of 10^7 cells/ml. Cells were fixed in 1% formaldehyde in PBS prior to analysis by flow cytometry. Labeled populations (CFSE labeled cells stained for either CD4 or CD8 expression) were analyzed, using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest Pro software. An intact lymphocyte cell gate was set on the basis of cell size (forward scatter; FSC) and cell granularity (side scatter; SSC) (Chrest et al., 1993). This region was unaltered between samples analyzed on the same day. These events were displayed on an FL1–CFSE versus FL3–CD4/CD8 log dot plot. For each sample, data from 1.5 × 10^5 cells contained within the intact lymphocyte gate were acquired. Regions were drawn to define CD4+ and CD4– and CD8+ and CD8– populations, based on FL3 (TC intensity). Cells proliferating in response to con A (Figures 1A and 1D). The gates derived for mitogen-stimulated LNC underwent, with the majority of cells undergoing one or more division and the number of proliferating cells peaking at 3 cell divisions (approximately 15% of the intact lymphocyte pool). It is important to note that a cell that is identified as, for example, having the appropriate levels of CFSE intensity to have undergone 6 divisions, has previously passed through discrete cell divisions to 1. It is of interest, therefore, to display these data as a function of the cumulative percentage of cells that have passed through each division (Fig. 1C). These calculations reveal that more than 50% of gated cells were induced to proliferate through one cycle of division and approximately 15% of cells have passed through 4 divisions or more. The profile of cell division induced by topical exposure of mice to allergen, in the absence of in vitro restimulation with mitogen, was examined also (Figs. 1D–F; representative experiment). LNC isolated following topical treatment of mice with 0.5% ox (as described above) were labeled with CFSE and cultured for 96 h in the absence of any further stimulation in vitro. After culture with anti-CD4 TC antibody, CFSE and CD4 expression were analyzed by flow cytometry. A proportion of both CD4+ and CD4– cells had been induced to proliferate following exposure of mice to allergen. However, proliferative responses displayed by allergen-activated LNC in the absence of con A were considerably less marked than those observed following restimulation of the same population with mitogen, with a larger percentage of cells retaining high levels of CFSE (not dividing) under the former conditions. The cell populations that had divided in response to allergen exposure in vivo (reduced CFSE intensity) were less discrete than those observed following restimulation with con A (cf. Figs. 1A and 1D). The gates derived for mitogen-stimulated LNC undergoing divisions 1 to 9 were therefore utilized to enumerate the percentages of allergen-activated cells in each successive division (Figs. 1E and 1F). Using these parameters it is apparent that approximately 13% of total LNC (that are CD4+) undergo at least one cycle of division, with peak numbers of proliferating cells (approximately 2.5%) dividing 5 times over the 96-h culture period. Of course, in common with the assessment of
proliferation by thymidine incorporation *in vitro*, this method does not take into account those cells that have already undergone one or more cell divisions *in vivo* prior to lymph node isolation. Interestingly, a subset of cells was observed in the allergen-activated LNC population that expressed a higher intensity of CFSE than did the control undivided (division 0) cells. Based on the FSC/SSC profile of these cells, this subset apparently represents a population of large, nondividing cells.

**FACS profiles of CD4<sup>+</sup> and CD8<sup>+</sup> proliferating cells: Comparisons of ox and MS.** In subsequent experiments, dose-response analyses were conducted to compare the frequency of proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells in lymph nodes isolated following topical exposure of mice to the potent contact allergen ox, or to the nonsensitizing skin irritant MS. In these experiments, and in all subsequent experiments, mice were dosed according to a standard LLNA-type protocol (Kimber and Basketter, 1992), with animals receiving 25 μl of ox (0.01% to 0.25%), or MS (1.25% to 20%) (both delivered in AOO) on the dorsum of both ears, daily, for three consecutive days, with lymph nodes isolated 5 days after the initiation of exposure. Control mice were exposed to vehicle (AOO) alone. Lymph nodes were pooled for each experimental group: a single cell suspension of LNC prepared, labeled with CFSE, and cultured for 96 h in the absence of further restimulation *in vitro*. Aliquots of cells were stained with anti-CD4 and anti-CD8 TC labeled antibodies for the enumeration of CD4<sup>+</sup> and CD8<sup>+</sup> proliferating cells by flow cytometry. Cells in each population have been assigned to cell divisions 1 to 9 using the gates derived for mitogen-activated LNC as described previously (Fig. 1A). FACS profiles for CD4<sup>+</sup> and CD8<sup>+</sup> proliferating cells in ox-stimulated LNC and in those derived following treatment with MS are displayed in Figures 2 and 3, respectively. For ox-treated LNC, a clear dose response effect was observed with respect to increasing percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells passing through cell divisions 1 to 9, compared with the relatively low levels of proliferating cells recorded for LNC isolated from vehicle (AOO)-treated mice (Fig. 2). In contrast, despite exposure to high doses of MS (up to a max-

**FIG. 1.** Mitogen-induced proliferative responses measured using CFSE incorporation, comparisons with allergen. Mice (*n* = 3) were exposed to 0.5% ox in AOO vehicle on the dorsum of both ears. Three days later, draining auricular lymph nodes were excised, and a single cell suspension prepared and labeled with CFSE. Cells were cultured in the presence (A–C) or absence (D–F) of 2 μg/ml Con A for 96 h. Following staining with anti-CD4 TC labeled antibody, 1.5 × 10<sup>5</sup> intact lymphocytes were analyzed by flow cytometry. These events are displayed on an FL1–CFSE versus FL3–CD4 log dot plot (A, D). The horizontal line divides CD4<sup>+</sup> from CD4<sup>−</sup> populations, which are illustrated by vertical arrows. The horizontal arrow represents decreasing CFSE intensity. The boxes numbered 0 through 9 define proliferating cells through divisions 0 to 9. Data were expressed as percentage of cells per division (B, E) or as a summation of the percentage of cells that had passed through each division (C, F).
FIG. 2. Ox-induced proliferative responses measured using CFSE incorporation. Mice ($n = 3–5$) were exposed to 0.01 to 0.25% ox in AOO vehicle, or AOO vehicle alone on the dorsum of both ears, daily for three consecutive days. Five days after the initiation of exposure, draining auricular lymph nodes were excised, and a single cell suspension prepared and labeled with CFSE. Cells were cultured for 96 h and stained with anti-CD4 or anti-CD8 TC labeled antibody. $1.5 \times 10^5$ intact lymphocytes were analyzed by flow cytometry. These events are displayed on an FL1–CFSE versus FL3–CD4 or CD8 log dot plot. The horizontal line divides CD4+ from CD4– or CD8+ from CD8– populations illustrated by the vertical arrows. The horizontal arrow represents decreasing CFSE intensity. The boxes numbered 0 through 9 define proliferating cells through divisions 0 to 9.

FIG. 3. MS-induced proliferative responses measured using CFSE incorporation. Mice ($n = 3–5$) were exposed to 1.25% to 20%MS in AOO vehicle or AOO vehicle alone on the dorsum of both ears daily for three consecutive days. Five days after the initiation of exposure, draining auricular lymph nodes were excised, and a single cell suspension was prepared and labeled with CFSE. Cells were cultured for 96 h and stained with anti-CD4 or anti-CD8 TC labeled antibody. $1.5 \times 10^5$ intact lymphocytes were analyzed by flow cytometry. These events are displayed on a FL1–CFSE versus FL3–CD4 or CD8 log dot plot. The horizontal line divides CD4+ from CD4– or CD8+ from CD8– populations, which are illustrated by the vertical arrows. The horizontal arrow represents decreasing CFSE intensity. The boxes numbered 0 through 9 define proliferating cells through divisions 0 to 9.
After 96 h, LNC populations were labeled with anti-CD4 and anti-CD8 TC labeled antibodies for the enumeration of CD4⁺ and CD8⁺ proliferating cells as a fraction of the total intact lymphocyte pool by flow cytometry, as described previously (Figs. 4 and 5). In each of the four experiments, LNC isolated from control (vehicle-treated) animals contained very low levels of proliferating CD4⁺ and CD8⁺ cells. In division one, only 0.58 ± 0.16% and 0.15 ± 0.06% of gated cells were proliferating CD4⁺ and CD8⁺ cells, respectively. In further divisions (2 and greater), 0.23 ± 0.06% and 0.08 ± 0.05% of gated cells were proliferating CD4⁺ and CD8⁺ cells, respectively. Treatment with each of the three allergens, ox, DNCB and HCA, stimulated increased numbers of proliferating CD4⁺ and CD8⁺ cells. In division one or above, approximately 5% and 3% of the intact lymphocyte pool were proliferating CD4⁺ and CD8⁺ cells following topical application of either 0.25% ox or 0.5% HCA or 1.25% MS.
DNCB. Exposure to the rather less potent skin sensitizer HCA induced less marked increases in proliferating cells. Maximal increases were observed with 10% HCA, with 2.5 and 1.2% of gated cells recorded as proliferating CD4$$^+$$/H11001 and CD8$$^+$$/H11001 cells, reaching division one and beyond. At each concentration of ox and DNCB tested, and at the majority of concentrations of HCA examined, marked increases in the number of CD4$$^+$$ cells were recorded in each of cell divisions 1 to 5 compared with vehicle-treated LNC. For CD8$$^+$$ cells, a similar pattern of increases in proliferating cells was observed compared with AOO-exposed controls, with treatment with all doses of ox and HCA and the majority of concentrations of DNCB inducing significant increases in the numbers of cells in divisions 1 to 5. Topical exposure to the nonsensitizing skin irritant MS failed to provoke increased levels of CD4$$^+$$ or CD8$$^+$$ cell turnover. At all concentrations tested (1.25% to 20%), the percentages of CD4$$^+$$ or CD8$$^+$$ cells in each cell division 1 to 9 were very similar to those recorded for vehicle-treated control cells (Fig. 4).

**Enumeration of proliferating cells: Comparisons of CFSE and thymidine incorporation.** Finally, measurement of proliferation as a function of CFSE staining has been compared with the measurement of proliferation in a nonstandard LLNA protocol, with assessment of in vitro incorporation of radiolabeled thymidine rather than incorporation of the radioisotope in vivo (Table 1). In these analyses, CFSE staining is considered as a function of the percentage of cells that have attained at least one division, and in addition to assessment of the percentages of the total intact lymphocyte pool that are prolifer-
ating CD4⁺ and CD8⁺ cells, the total percentage of proliferating cells within the gated population was measured as a function of decreasing CFSE intensity and FSC. These data reveal that all three allergens induced vigorous proliferative responses (measured as a function of thymidine incorporation) at the majority of doses tested. Maximal responses of between 21- and 74-fold increases in proliferation compared with LNC derived from concurrent vehicle-treated controls were recorded. The irritant MS failed to provoke substantial increases in thymidine incorporation, with less than a twofold increase compared with vehicle-treated controls, even at the highest dose tested. Measurement of proliferative responses as a function of CFSE incorporation, particularly for total proliferating cells, was also a relatively sensitive and selective marker for contact allergens. Similar frequencies of CD4⁺, CD8⁺, and total proliferating cells were recorded for all concentrations of the irritant MS as those observed for vehicle-treated controls (between 0.5 and 1%). In contrast, exposure to allergen provoked maximal proliferative responses of between 4 and 10% of the intact lymphocyte pool, with the majority of doses tested inducing 2% or more of cells to proliferate.

**DISCUSSION**

These experiments have demonstrated that proliferative responses induced in draining lymph nodes by topical exposure of mice to skin sensitizers can be measured as a function of CFSE incorporation. The allergens chosen for these investigations were two potent skin sensitizers, ox and DNCB, and a weaker allergen, HCA. Ox is a very potent skin sensitizer in experimental models of contact allergy, although there is currently only anecdotal information regarding its ability to sensitize humans (Loveless et al., 1996). In the standard murine LLNA for contact allergen hazard identification, a chemical is considered positive if, at one or more test concentrations, a stimulation index (SI; ratio of thymidine incorporation in LNC derived from chemical allergen-treated mice compared with concurrent vehicle-treated animals) of three or more is recorded (Gerberick et al., 2000; Kimber and Basketter, 1992). In addition, LLNA dose-response data can be utilized for determining relative skin sensitizing potency of contact allergens as a first step in the risk assessment process. For this purpose, an EC3 value is calculated; this being the amount of chemical required for eliciting an SI of 3, derived by linear interpolation of dose-response data (Basketter et al., 1999b; Kimber and Basketter, 1997). The more potent the chemical, the lower the threshold concentration that induces proliferation, and therefore the lower the derived EC3 value. In an international interlaboratory trial of the LLNA, EC3 values of between 0.0007% and 0.0026% were recorded for ox in standard LLNA conducted in CBA strain mice (Loveless et al., 1996). DNCB, although clearly a very strong contact allergen, is somewhat less potent than is ox, with EC3 values of between 0.03% and 0.06% observed in the same interlaboratory comparisons (Loveless et al., 1996). HCA is considered to be a somewhat weaker contact allergen in humans (Basketter et al., 2001) and is recommended as the positive control of choice for tests for contact sensitization hazard identification (Steiling et al., 2001). Consistent with experience of the skin sensitization potential of HCA in humans, relatively high EC3 values of between 7.0 and 12.2% have been recorded (Dearman et al., 2001).

Measurement of cell division as a function of CFSE incorporation correctly identified all three contact allergens, with marked increases in the percentages of both CD4⁺ and CD8⁺ proliferating T lymphocytes observed in each case when compared with vehicle-treated controls. For DNCB and HCA, the sensitivity of this technique was comparable with that achieved using the standard LLNA protocol, with significant increases in proliferating cells recorded at 0.05% DNCB and 5% HCA; doses close to the EC3 values obtained in the standard LLNA using CBA strain mice. For ox, however, the measurement of cell turnover using CFSE incorporation appeared to be rather less sensitive, with the numbers of proliferating cells returning to background levels (similar to those observed for cells derived from concurrent vehicle-treated control animals) following treatment of mice with 0.01% ox, particularly for CD8⁺ proliferating cells. This concentration of chemical is some orders of magnitude higher than the EC3 value derived using a conventional LLNA (Loveless et al., 1996). The selectivity of this end point was determined using the nonsensitizing skin irritant MS (Kligman, 1966). At all concentrations tested, this chemical failed to stimulate increased cell division measured as a function of CFSE incorporation. In international interlaboratory trials of the standard LLNA, the same application concentrations of MS were also uniformly negative (5 out of 5 laboratories) with respect to failing to induce an SI of 3 or above at any test concentration (Kimber et al., 1995). However, 3 out of 5 laboratories utilized a protocol where individual animals’ lymph nodes were processed, rather than lymph nodes pooled on an experimental group basis, and we were therefore able to conduct statistical analyses on LLNA responses. Two out of these three laboratories found a statistically significant increase in thymidine incorporation at the maximum dose of MS tested (20%); in contrast, there was no evidence for any increase in proliferation measured as a function of CFSE incorporation.

One of the advantages of this flow cytometric method for the measurement of cell turnover is that the phenotype of proliferating cells can be assessed simultaneously. Interestingly, all three contact allergens stimulated increased cell division in both the CD4⁺ and the CD8⁺ T-lymphocyte populations. These data are consistent with previous experience in which the relative contributions of CD4⁺ and CD8⁺ cells to ox-induced proliferative responses were assessed using negative selection (complement depletion) (Kimber et al., 1991). In those experiments, in vitro incorporation of radiolabeled thymidine was measured following depletion of CD4⁺ or CD8⁺
cell populations, revealing that both populations contributed to the proliferative response induced by ox. The fact that in the current experiments fewer proliferating CD8\(^+\) cells were recorded than CD4\(^+\) cells is presumably a reflection of the fact that in resting and in allergen-activated lymph nodes derived from BALB/c strain mice, approximately two thirds of the total T-lymphocyte population are CD4\(^+\) cells (Dearman et al., 1996). Regardless of the fact that more proliferating CD4\(^+\) T cells than CD8\(^+\) T cells were recorded, and considering the current debate as to phenotype of the cellular effectors of contact allergy (Kimber and Dearman, 2002), these data clearly demonstrate that both cell populations are dividing in response to both potent and relatively weak allergens. It is of interest that for DNCB- and HCA-stimulated LNC, the total percentage of proliferating cells within the intact lymphocyte pool was approximately equivalent to the summation of the proliferating CD4\(^+\) and CD8\(^+\) cells, suggesting that these cells accounted for the majority of proliferating cells within the draining lymph node. These data suggest therefore that the reported increases in the frequency of B cells (B220\(^+/\)IgG\(^+/\)IgM\(^-\)) observed in the draining lymph node following topical exposure to similar concentrations of these chemicals (0.25% DNCB and 50% HCA) may be due largely to the preferential accumulation of B lymphocytes within the allergen-activated lymph node, rather than B-cell division (Gerberick et al., 2002). However, independent experiments in which the numbers of proliferating B220\(^+\) cells in resting lymph nodes are compared with those found in allergen-activated lymph nodes are required to formally demonstrate this hypothesis. Following longer exposure protocols where there is the opportunity for secondary follicles and germinal centers to develop, then undoubtedly B lymphocytes will contribute significantly to overall LNC turnover (Kimber et al., 1991).

With respect to the possible utility of this end point in the LLNA, it is likely that the most sensitive (and relatively simple) configuration would be the assessment of the percentage of proliferating cells in the total intact lymphocyte pool. This would obviate the requirement for immunophenotyping, which, although potentially a powerful tool for exploring mechanistic aspects of skin sensitization, adds considerably to the complexity of the method. Clearly there is a need for cell culture and for relatively sophisticated analytical techniques for the successful application of this method. Furthermore, LNC proliferation using the end point of CFSE incorporation must be assessed in the freshly isolated viable cell pool; thus, there is no opportunity for longitudinal comparisons and batch analyses, as there is when the end point is the production of a soluble factor by cultured draining LNC, such as various cytokines including interleukin (IL) 2, interferon \(\gamma\) or IL-12 (Dearman et al., 1999; Hatao et al., 1995).

In common with other methods that rely upon measurement of ex vivo parameters of lymph node activation, the assessment of cell turnover as a function of CFSE incorporation is of somewhat lesser sensitivity compared with measurement of thymidine incorporation in vivo. Thus, exposure to potent contact allergens such as DNCB provoked a maximal three-fold increase in the frequency of B220\(^+\) cells (Gerberick et al., 2002). A somewhat larger dynamic range was recorded for the end point of CFSE expression; thus, maximal increases in lymphocyte division of between 12 and 20 fold were observed for DNCB and ox, respectively. In a standard LLNA with thymidine incorporation, maximal SIs in excess of 75 have been recorded for potent allergens including DNCB (Loveless et al., 1996). One of the reasons why this latter method yields more vigorous responses is because activity is assessed on a whole lymph node basis. Thus, not only is the fact that there is an increase in the frequency of proliferating cells taken into account, but also the fact that there is a marked increase in total lymph node cellularity following exposure to allergen as described above. In some methods where in vitro parameters of lymph node activation, such as incorporation of radiolabeled thymidine, are assessed, a factor for increases in total lymph node cellularity is included in the calculation of the SI (de Jong et al., 2002). If this approach is used, however, it is very important to ensure that the total cell yields of LNC populations are measured accurately and consistently in both allergen-treated and -untreated groups.

In summary, these data demonstrate that it is possible to measure allergen-induced lymphocyte proliferation by flow cytometry using the stable cytosolic fluorescent dye CFSE. This method can be used also to provide information as to the phenotype of the proliferating LNC, although in the context of a potential supplementary end point for the LLNA, assessment of the total percentage of proliferating cells within the intact lymphocyte pool probably represents the most sensitive and relatively simple end point. Further evaluation will be required, to confirm or otherwise, the sensitivity and selectivity of this technique using a range of allergens and nonallergens, although current experience suggests that this method is sufficiently sensitive to identify potent skin sensitizing chemicals.

REFERENCES


