Optimization of an In Vitro Lymphocyte Blastogenesis Assay for Predictive Assessment of Immunologic Responsiveness to Contact Sensitizers

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Current methods for the predictive and diagnostic assessment of contact sensitization rely on the visual scoring of skin reactions. Predictive animal tests, generally using guinea pigs, require a relatively large number of animals to produce a sufficient database for interpreting skin reaction scores. In vitro assays have the potential of being more quantitative than skin testing and, if so, would require fewer animals. However, although in vitro assays are commonly used to study the cellular immune response to strong contact sensitizers, there has been little effort to validate them for predictive assessment purposes. We have optimized an in vitro lymphocyte blastogenesis assay for detecting the response of mouse lymphocytes to strong contact sensitizers with the eventual objective of applying this assay to moderate and weak sensitizers as well. Lymph node lymphocytes from mice sensitized to the strong contact allergens, dinitrochlorobenzene (DNCB), dinitrofluorobenzene (DNFB), or trinitrochlorobenzene (TNCB), responded (≥12,000 counts per minute (CPM) above background) when cultured with water soluble chemical analogues, di- or trinitrobenzene sulfonic acid (DNBS or TNBS). However, the strong sensitizer, oxazolone (OXAZ), has no water soluble analogue and lymphocytes from mice sensitized to OXAZ responded poorly in vitro (<2000 CPM) to an ethanol-solubilized OXAZ preparation in spite of very strong in vivo sensitization (ear swelling assay). To increase the assay sensitivity, for OXAZ, we modified the antigen presentation conditions by using 1) solubilized antigen-modified adherent spleen cells, 2) dendritic cells from the draining lymph nodes of antigen painted mice, and 3) antigen-modified Langerhans cell-enriched cultured epidermal cells (EC). These approaches increased OXAZ-directed responses to >7000, >20,000, and >100,000 CPM, respectively, under culture conditions optimized for cell density, responder:stimulator cell ratio, culture duration, and responder cell type. Our results represent a first attempt to directly modify cultured epidermal cells with OXAZ and use these cells to stimulate OXAZ-directed blastogenesis in microtiter plate cultures. This optimized assay is now under evaluation for predictive assessment of contact sensitizers relevant to occupational and consumer exposures. J Invest Dermatol 92:860–867, 1989

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urrent animal testing methods for predictive assessment of contact sensitization (CS) are limited to various guinea pig skin sensitization (GPSS) protocols that measure cutaneous erythema and/or edema in suitably induced and challenged animals [1]. These GPSS protocols have been extensively validated over the years and several are accepted by regulatory agencies in the U.S. and abroad [2]. However, the GPSS methods have a number of inherent limitations. Because of the subjective grading of skin reactions, they provide only limited quantitative data on sensitization potential, they do not easily differentiate weak sensitization from irritation reactions, and they require extensive time and relatively large numbers of animals.

The mouse has also been used as an animal model for CS; however, these studies have largely employed only strong sensitizers to investigate the cellular immunologic mechanisms underlying the CS response [3–5]. The mouse studies are more quantitative than the GPSS protocols in that they use an objectively quantifiable ear swelling assay or in situ radioisotope incorporation to measure elicitation of CS in previously induced animals. The ear swelling model

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Abbreviations:
AD: adherent
APC: antigen presenting cells
CPM: counts per minute
CS: contact sensitization
DC: dendritic cells
DNBS: 2,4-dinitrobenzenesulfonic acid (sodium salt)
DNCB: 1-chloro-2,4-dinitrobenzene
DNFB: 1-fluoro-2,4-dinitrobenzene
EC: epidermal cells
GPSS: guinea pig skin sensitization
HBSS: Hank’s balanced salt solution
LC: Langerhans cells
NAD: nonadherent
OXAZ: oxazolone, 4-ethoxymethylene-2-phenyloxazol-5-one
PBS: phosphate-buffered saline
R: S: responder: stimulator
SEM: standard error of the mean
TNBS: 2,4,6-trinitrobenzenesulfonic acid (sodium salt)
TNBC: 1-chloro-2,4,6-trinitrobenzene
TNP: trinitrophenyl
(with various procedural modifications) has recently undergone more rigorous evaluation for predictive assessment of CS to weaker sensitizers and has yielded varied results [6–8].

The use of in vitro assays for assessment of CS responses has been primarily limited to the study of strong sensitizers. These assays, which have been used mainly to supplement in vivo data, have included bioassays for lymphokine production [9,10] and the proliferative (blastogenesis) response of lymphocytes from sensitized donors (mice, guinea pigs, and humans) [11–14]. The blastogenesis response has provided additional mechanistic data on the immunobiology of CS and, in clinical studies, has provided some diagnostic utility as well [14]. There have been few reports on the application of in vitro methods for predictive CS assessment. The published literature is either speculative in this regard [15] or describes only preliminary methods development work [16]. However, there remains considerable interest in developing and validating in vitro CS assays to both improve quantitative assessment capabilities and reduce animal use.

Our long-term objective is the predictive assessment of contact sensitization responses by in vitro assay methods. The studies described here represent an initial facet of that objective: the refinement of the in vitro lymphocyte blastogenesis assay to optimize the response of lymphocytes from in vivo sensitized mice to various strong contact sensitizers. We employed sensitizers used routinely for contact sensitization and blastogenesis assays and also included one chemical not readily amenable to cell culture and not directly tested previously for stimulation of the blastogenesis response. Through the approaches described here, we have developed an assay of sufficient sensitivity and specificity to be potentially useful in the routine predictive assessment of contact sensitizers relevant to consumer or occupational exposures.

MATERIALS AND METHODS

Mice Female BALB/c mice (Charles Rivers Laboratories, Wilmington, MA), 2–4 months old, were used for all studies. Animals were acclimated for at least 1 week before entering any study. Three to five animals were used per group and all in vitro assays were conducted with cells from individual mice unless otherwise indicated. Test animals were housed individually and control animals were group housed (3–5/cage). Animals were provided food and water ad libitum.

Test Antigens Test antigens for in vivo sensitization included 1-chloro-2,4-dinitrobenzene (DNCB; Sigma, St. Louis, MO), 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma), 1-chloro-2,4,6-trinitrobenzene (TNB; Polysciences, Warrington, PA), and 4-ethoxy-methylene-2-phenyloxazol-5-one (oxazolone [OXAZ]) (Aldrich, Milwaukee, WI). Test antigens for in vitro studies included 2,4-dinitrobenzene sulfonic acid (DNBS; Sigma), 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma), and OXAZ.

In Vivo Sensitization and Ear Swelling Assay In vivo test antigens (DNCB, DNFB, and OXAZ) were prepared in 4:1 acetone: olive oil for both induction and challenge (see figure legends for test concentrations). The mice were shaved on the ventral surface, and 25 μl of the test chemical was applied to the shaved surface. Control mice were either left untreated or were treated in the same fashion with 4:1 acetone: olive oil alone. On days 4 to 5 after induction, test and control animals were challenged by applying 5 μl of test antigen solution on each side of one ear and 5 μl of 4:1 acetone: olive oil on each of the other ear. Ear thickness measurements were taken for each ear just prior to challenge using an Oiditest caliper (H. C. Kroplin, FRG). Measurements were repeated 24 h (and sometimes 48 h) after challenge. Ear swelling was calculated by subtracting the pre-challenge value from the post-challenge value and then further subtracting any swelling recorded for the vehicle-challenged ear (never more than 10%) from the swelling recorded for the antigen-challenged ear. Ear swelling values are reported in the figures in mm × 10⁻².

In some studies antigen specificity of the ear swelling response was tested. Here, mice were challenged with the inducing antigen on one ear and an alternative antigen on the other ear using the same procedures described above. In this case, no vehicle challenge was done and ear swelling was recorded for each ear by subtracting pre-challenge from post-challenge ear thickness measurements.

Solubilization of OXAZ for Use In Vitro Although water soluble analogues of DNBC, DNFB, and TNCB exist in the form of DNBS and TNBS, this is not the case for OXAZ. In order to solubilize OXAZ for in vitro use, it was first dissolved in absolute ethanol at 100 times the final desired concentration. This dissolved material was then diluted 1:100 in culture medium (RPMI 1640 with 10% fetal bovine serum, 2 mM glutamine, and 50 μg/ml gentamicin, all from Gibco, Grand Island, NY) or Hank’s balanced salt solution (HBSS; GIBCO). The OXAZ precipitated upon dilution in aqueous medium but redissolved in about 30 min if left standing at 37°C. The redissolved material was then pH adjusted (7.2) and filter sterilized before further use. The 1% residual ethanol in the final preparation had no effect on the in vitro assays.

Preparation of Dendritic Lymph Node Cells In some experiments, lymph node cells from naive or antigen-treated mice were used as antigen-presenting cells. The antigen-treated mice were shaved as described, and 100 μl of antigen was applied to the shaved abdomen. Naive and antigen-treated mice were killed in 10 min after antigen application and the inguinal, brachial, and axillary lymph nodes removed and pooled in culture medium. Cell suspensions were prepared by teasing the nodes into culture medium with forceps. The cells were then counted and treated with 50 μg mitomycin C (Sigma) per 10³ cells in 0.5 ml of medium for 45 min at 37°C (to inhibit cell proliferation). The cells were then washed 3 times, counted, and resuspended at the desired concentration for culture.

In other experiments the antigen-presenting lymph node cells were further processed to recover the dendritic lymph node subpopulation. This procedure was modified from Macatonia et al [17]. Briefly, up to 5 × 10⁷ cells in 8 ml of medium were layered over 3 ml of 14.5% metrizamide (Sigma) and centrifuged for 20 min at 600 × g. The interface cells were recovered, mitomycin C treated, washed, counted, and resuspended for culture as above.

Preparation of Splenic and Epidermal Antigen-Presenting Cells Spleen cell suspensions from normal mice were prepared in culture medium by displacement of cells from the cut spleen capsule by medium injection. The cells were plated in 60 mm tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at 3 × 10⁷ cells/plate. After 1 h incubation (37°C, 5% CO₂), the nonadherent cells were discarded and the adherent cells (AD/spleen) recovered by scraping from the plate.

Epidermal cells were obtained from ear and truncal skin of normal mice as described previously [18]. Briefly, the ears were split with forceps and ear and truncal skin was placed dermal side up on plastic Petri dishes. Subcutaneous fat and cartilage was scraped away with watchmakers forceps and the skins laid dermal side down on a solution of 0.5% trypsin (US Biochemical Co., Cleveland, OH) and incubated (37°C, 5% CO₂) for 30 min. Epidermis was gently scraped off with the dermis and the cells teased apart in HBSS containing 0.025% DNase (Sigma). The cells were then passed through a sterilized nylon filter to remove hair and debris, washed 2 times in HBSS, resuspended in culture medium containing 5 × 10⁻⁴ M 2-mercaptoethanol (Sigma), counted, and incubated in 60 or 100 mm tissue culture dishes at 3 × 10⁷ cells/culture for 2–4 d at 37°C in 5% CO₂. The nonadherent cells were recovered from culture by pipetting and centrifuged over metrizamide as described above for lymph node dendritic cells. Interface cells were recovered, counted, and resuspended in culture medium for further processing. A portion of the AD/spleen and epidermal cells were suspended in HBSS containing 5 mM TNBS or 2 mM solubilized OXAZ and incubated (37°C, 5% CO₂) for 30 min. The cells were then washed and both antigen-treated and nontreated (control) cells were treated with mitomycin C as described above. The cells were then washed, counted, and resuspended in culture medium for addition to cell culture.
Enumeration of Langerhans Cells in Cultured Epidermal Cell (EC) Preparations

In most cases, the proportion of Langerhans cells in EC was determined by morphology; the Langerhans cells could be easily distinguished from other epidermal cells in counting suspensions by the presence of their dendritic processes. The enumeration of dendritic cells in lymph node cell suspensions was also determined by morphology. The accuracy of the morphologic assessment of Langerhans cells was checked in several experiments by indirect immunofluorescent staining of the cell suspensions. Here, \(5 \times 10^7\) EC in phosphate-buffered saline (PBS, Gibco) suspension were incubated in a 1:50 dilution of monoclonal anti-Ia\(^a\) antibody (Becton Dickinson, Mountainview, CA), washed, then incubated in a 1:40 dilution of FITC-labeled anti-IgG (Becton Dickinson), washed again, resuspended at high concentration in 9:1 glycerol:PBS, and counted (at least 500 total cells) with a fluorescence microscope. Control preparations substituted PBS for the primary (anti-Ia\(^a\)) antibody. Fluorescence and morphologic counts of Langerhans cells differed by <10% with the fluorescence counts being slightly higher.

Blastogenesis Assay Cultures

Mice were killed after recording the 24 h (and sometimes 48 h) post-challenge ear thickness measurements. Peripheral lymph nodes (axillary, brachial, and inguinal) were pooled from individual mice and cell suspensions prepared by teasing the nodes with forceps into culture medium. In some experiments nodal lymphocytes from individual mice were processed separately and in other experiments all cells within a given test or control group were pooled. In some experiments, an aliquot of the lymph node cells was added to prewashed nylon wool columns (0.6 g nylon wool packed into 10cc syringes, autoclaved, prewashed with medium, and preincubated), the cells washed into the columns (incubated 1 h (37°C, 5% CO\(_2\)), and the nonadherent (NAD) cells eluted by dropwise addition of medium to the top of the columns. The unseparated and/or NAD lymph node lymphocytes are referred to as responder cells in the figures. These cells were plated in quadruplicate in different shaped microtiter plate cultures; round bottom ( #3799, Costar, Cambridge, MA), flat bottom ( #3596, 0.32 cm growth area, Costar), and 1/2 area flat bottom ( #3696, 0.16 cm growth area, Costar) cultures. The cell concentrations and culture plates used are indicated in the figures and figure legends. Sets of quadruplicate cultures then received medium only (control cultures for determining background proliferative activity), various concentrations of water soluble antigen (DNBS, TNBS, solubilized OXAZ) or culture medium, or various numbers of AD/alpha-lymph node, dendritic lymph node, or epidermal antigen-presenting cells (antigen-modified or unmodified). The cultures were then incubated (37°C, 5% CO\(_2\)) for 48-96 h, pulsed with 1 \(\mu Ci/\)culture of \(^3\)H-thymidine, reincubated 24 h, harvested (Skatron Cell Harvester System, Skatron, Sterling, VA), and \(^3\)H-incorporation was determined by liquid scintillation counting.

Blastogenesis was recorded as the change in counts per minute [\(\Delta CPM = \text{antigen or cell stimulated} \times \text{cpm incorporation-background (medium only)}\times \text{cpm incorporation}].\] When cells from individual mice were assayed, the data were presented as mean \(\Delta CPM \pm SEM\) for replicate animals. When pooled cell populations were assayed the data were presented as mean \(\Delta CPM\) for replicate cultures. Standard errors were not always presented for replicate cultures because they were usually <10% of the mean. The standard errors for replicate cultures are presented in Fig 8; however, as an example. Background CPM values are given in the figure legends.

RESULTS

Mouse Ear Swelling Response to Strong Contact Sensitizers

All of the contact sensitizers used in these studies have been shown to produce ear swelling responses upon ear challenge of previously induced animals. Ear swelling responses to dinitrochlorobenzene (DNBC), dinitrofluorobenzene (DNFB), trinitrochlorobenzene (TNBC), and oxazolone (OXAZ) were confirmed in many experiments with typical results shown in Fig 1. DNBC is a potent sensitizer in various guinea pig skin sensitization tests, but has not been extensively studied in mice. It routinely gave the poorest ear swelling response in mice and, in some experiments, produced no ear swelling response in some or all animals. DNFB, TNBC and OXAZ have been widely used in mice and gave ear swelling responses comparable to those reported in the literature; although TNBC was tested at a much lower concentration (1% induction; 0.2% challenge) than typically used (7% induction, 1% challenge) due to excessive primary irritation at the higher concentrations.

Lymphocyte Blastogenesis Response to Solubilized Contact Sensitizers In Vitro

After recording ear swelling responses, test and control (naive or vehicle induced) animals were killed and the lymph nodes draining the induction site (inguinal, axillary, and brachial) were pooled for individual animals and lymphocyte suspensions prepared. Figure 2 shows the blastogenesis responses of lymph node lymphocytes, from the mice presented in Fig 1, to optimal concentrations of water soluble analogues of DNBC,

![Figure 1. Mouse ear swelling response to strong contact sensitizers. Mice were induced/challenged, in 4 separate experiments, with the following materials: DNBC: 3%/1%; DNFB: 1%/0.2%; TNBC: 1%/0.2%; OXAZ: 2%/0.5%. Vehicle induced animals were included as challenge controls. Mice were challenged 4-5 d after induction. Ear thickness measurements were taken before and 24 h after challenge, and the ear swelling was calculated.](image1)

![Figure 2. Blastogenesis response to soluble antigen (DNBS, TNBS) or solubilized OXAZ by lymphocytes from chemical-induced or vehicle-induced mice. Mice were induced and/ or challenged with DNBC, DNFB, TNBC, or OXAZ as described in Fig 1. The ear swelling results are presented in Fig 1. Lymph node lymphocytes were prepared from individual animals and cultured at 4 - 5 \(\times 10^7\) cells/culture in round bottom microtiter plates in medium or medium containing 50 \(\mu g/ml\) OXAZ, 90 \(\mu g/ml\) DNBS, or 270 \(\mu g/ml\) TNBS. DNBS was used as the in vitro antigen for both DNBC and DNFB sensitized mice. Blastogenesis was assessed after 120 h of culture. The mean background (medium only) \(^3\)H incorporation for lymphocytes from antigen induced (and vehicle induced control) animals was as follows: DNBC: 2203 CPM (1581 CPM); DNFB: 7578 CPM (623 CPM); TNBC: 3239 CPM (2224 CPM); OXAZ: 4760 CPM (233 CPM).](image2)
DNFB, or TNCB [i.e., di- or tri-nitrobenzene sulfonic acid (DNBS or TNBS)] or solubilized OXAZ.

The response to these antigens was optimized for various culture conditions including antigen dose (90–270 µg/ml; 0.3–1 mM), culture shape (round), responder cell number (4–5 X 10^6/culture), incubation period (4–5 d), and responder cell type (lymph node vs. splenic lymphocytes). Under such conditions, the responses were specific to sensitized animals; naive or vehicle-induced control mice (including those challenged with antigen 24 h prior to death) showed no blastogenesis response. The in vitro responses in control experiments were optimal from 3 to 7 d after induction and were equivalent regardless of whether or not the sensitized animals were ear challenged (data not shown). For DNCB induced mice, individual animals with little or no ear swelling response still showed lymphocyte blastogenesis responses comparable to animals with measurable ear swelling (data not shown).

In contrast to our observations with DNCB/DNBS, lymphocytes from OXAZ sensitized mice responded poorly to solubilized OXAZ in vitro in spite of the very strong ear swelling response to OXAZ. It was clear from these data that the blastogenesis response to solubilized OXAZ or even the responses to DNBS and TNBS did not approach the sensitivity needed for a predictive assay for weaker sensitizers. Therefore, alternative antigen presentation methods were evaluated to try and improve the assay sensitivity.

### Stimulation of Blastogenesis by Antigen-Modified Adherent Spleen Cells

Plastic adherent spleen cells have commonly been used as a source of antigen-presenting cells [19]. To assess the ability of these cells to stimulate blastogenesis responses to contact sensitizers, adherent spleen cells were incubated with TNBS (5 mM) or solubilized OXAZ (2 mM) to hapten modify the antigen-presenting cells (APC). The APC were then added to cultures of lymphocytes from TNCB sensitized, OXAZ sensitized, or naive mice. The results of a typical experiment are shown in Fig. 3. Lymphocytes from TNCB sensitized mice responded as well to TNP-modified AD/spleen cells as they had to soluble TNBS (see Fig. 2). A low-level autologous response to unmodified AD/spleen cells was detected, but no response was observed with OXAZ-modified AD/spleen cells. Lymph node cells from mice sensitized to OXAZ responded to OXAZ-modified AD/spleen cells three to fourfold better than the prior response to solubilized OXAZ (Fig. 2). They also showed an autologous response to unmodified (or TNP-modified) AD/spleen cells that was about half of the blastogenesis response to the OXAZ-modified AD/spleen cells. However, the OXAZ-modified AD/spleen cells were poor stimulators of autologous reactivity compared to the unmodified AD/spleen cells. In other experiments, adding more stimulator cells (i.e., reducing the responder:stimulator cell ratio) only slightly increased the OXAZ response (e.g., 12,000 CPM above background) and proportionately increased the autologous response to unmodified AD/spleen cells as well (data not shown).

### Blastogenesis Response to Stimulator Cells from the Draining Lymph Nodes of Contact Allergen Treated Mice

It has been reported that epidermal Langerhans cells can bind contact allergens in the epidermis and transport the allergen to the draining lymph nodes where the Langerhans cells then comprise part of the dendritic cell population [20]. Also, lymph node cells from mice treated with oxazolone 24 h earlier have been shown to stimulate OXAZ-specific lymphokine secretion [10] or lymphocyte proliferation [13] in vitro, as well as the adoptive transfer of CS to naive recipients [13]. Based on those findings, lymph node cells from mice painted 24 h earlier on the shaved abdomen with 100 µl oxazolone were used to stimulate a blastogenesis response by responder lymphocytes from OXAZ induced or naive mice (Fig. 4). A very weak response was detected, which peaked at a 1:1 responder:stimulator cell ratio, but was not a definitive OXAZ-directed response because stimulator lymph node cells from unpainted mice were not tested. Responder cells from naive mice showed no proliferative response.

Clearly, the response to whole lymph node stimulator cells was not significantly better than the previous response to solubilized OXAZ itself. However, a recent study demonstrated low-level blastogenesis responses in Terasaki-type hanging drop cultures using a purified dendritic lymph node cell population from OXAZ-painted mice as the antigen-presenting cells [21]. Use of this approach in our larger microtiter plate culture system produced an additional three to fourfold increase in the blastogenesis response over what had been detected with the OXAZ-modified AD/spleen cells (Fig. 5). A dose response was observed that increased with increasing numbers of stimulator cells in the cultures (decreasing responder:stimulator cell ratio). No autologous reactivity was observed in the experiment shown. In repeated experiments similar CPM responses were ob-
Figure 5. Stimulation of the blastogenesis response by dendritic lymph node cells from oxazolone-painted mice. Mice were induced/challenged with 2%/0.5% OXAZ or 1%/0.2% TNCB with naive animals serving as challenge controls. Lymph node lymphocytes from OXAZ sensitized mice (LN-OXAZ), TNCB sensitized mice (LN-TNCB), or naive mice (LN-NAIVE) were then cultured at 4 x 10^6 cells/culture in round bottom microtiter plates containing medium or medium plus stimulator lymph node dendritic cells from responder mice (DC-NAIVE) or mice painted on the shaved abdomen 24 h earlier with 100 µl of 3% OXAZ in 4:1 acetone:olive oil (DC-OXAZ). The stimulator cells were purified from the whole lymph node cell population by centrifugation over 14.5% metrizamide. The recovered interface cells were 1%-2% of the starting population and ~50% dendritic cells by morphology. The stimulator cells were then mitomycin C treated and added to the responder cell cultures at the indicated responder:stimulator cell ratios. Blastogenesis was assessed after 120 h of culture. The mean background (medium only) ^3H incorporation for lymphocytes from antigen induced and naive control animals was as follows: TNCB: 878 CPM; OXAZ: 4464 CPM; NAIVE: 820 CPM.

Figure 6. Stimulation of the blastogenesis response by unmodified and hapten-modified cultured epidermal cells (EC). Mice were induced/challenged with 2%/0.5% OXAZ or 1%/0.2% TNCB with naive animals (no induction or challenge) serving as controls. Lymph node lymphocytes were then cultured at 4 x 10^6 cells/culture in round bottom microtiter plates containing medium or medium plus stimulator EC. The EC were prepared from normal mice, cultured for 3 d, recovered from culture, enriched for Langerhans cell, hapten-modified, and mitomycin C treated. The EC were 9% Langerhans cells as determined by indirect immunofluorescence using monoclonal anti-la antibody. TNBS-modified (EC-TNP), OXAZ-modified (EC-OXAZ), or unmodified EC were then added to the responder cell cultures at the indicated responder:stimulator cell ratio. Blastogenesis was assessed after 96 h of culture. The mean background (medium only) ^3H incorporation for lymphocytes from antigen induced and naive control animals was as follows: TNCB: 4646 CPM; OXAZ: 5514 CPM; NAIVE: 264 CPM.

Effect of Nylon Wool Filtration, Culture Dish Shape, Cell Density, and Responder:Stimulator Cell Ratio on Blastogenesis Response to Hapten-Modified Cultured EC Unseparated and nylon wool nonadherent lymphocytes from OXAZ-sensitized or naive mice were cultured at various cell densities (constant responder:stimulator cell ratio) with OXAZ-modified or unmodified EC. As shown in Fig 7A, the response by unseparated responder cells to OXAZ-modified EC increased dramatically at increasing cell densities, with the result most pronounced in flat bottom cultures. In round bottom cultures, the unseparated responder cells showed a linear increase in response with increasing cell density. In contrast, flat bottom cultures showed a dramatic surge in response between 3.0 and 6.0 x 10^6 cells/culture. The response in 1/2 area flat cultures was also linear, but the dose response curve was more vertical than for the round cultures, such that the response was less than, equal to, and greater than the response in round bottom cultures at 1.5, 3.0, and 6.0 x 10^6 cells/culture, respectively. Nylon wool nonadherent responder cells showed a uniformly better response than the corresponding unseparated lymph node lymphocytes, with responses >100,000 CPM detected in all shaped cultures at the high cell density.

Autologous reactivity to unmodified EC was also detected in these cell cultures. Responder lymphocytes from naive mice responded better to unmodified EC than to OXAZ-modified EC (Fig 7B) as observed previously with AD/spleen antigen-presenting cells. The autologous responses increased with increasing cell density but were much less in flat than in round bottom cultures. Lymphocytes from OXAZ-sensitized mice (Fig 7A), showed autologous responses to unmodified EC that were greater than with responder lymphocytes from naive mice (Fig 7B); however, here the responses were also reduced in flat bottom cultures and at lower cell densities.

Figure 8 further illustrates a direct response comparison between 1/2 area flat bottom cultures in which the responder cell density was held constant (2 x 10^6 cells/culture) and the R:S ratio decreased and cultures in which both responder and stimulator cell density were increased proportionally. Compared to a baseline response at 2 x 10^6 cells/culture and R:S = 10:1, similar increases in response were observed when the R:S ratio was reduced tenfold (to...
Figure 7. Stimulation of the blastogenesis response by unmodified or OXAZ-modified cultured epidermal cells (EC) under various culture shape and cell density conditions. Mice were induced/challenged with 2%/0.5% OXAZ with naive animals (no induction or challenge) serving as controls. Lymph node lymphocytes were pooled for both the test and control groups and aliquots were filtered through nylon wool columns to recover the nonadherent cells. Unseparated (US) and nylon wool nonadherent (NAD) cells were then cultured at 1.5, 3.0, or 6.0 x 10^5 cells/culture in round, flat, or 1/2 area flat bottom microtiter plates containing medium or medium plus stimulator EC. The EC were prepared from normal mice, cultured for 3 d, recovered from culture, enriched for Langerhans cells, OXAZ-modified, and mitomycin C treated. The EC were 20% Langerhans cells as determined by morphology. OXAZ-modified (EC-OXAZ) or unmodified EC were then added to the responder cell cultures at a responder:stimulator cell ratio of 12:1. Blastogenesis was assessed after 72 h of culture. The standard errors of quadruplicate blastogenesis assay cultures were uniformly <10% of the mean CPM values shown. The mean background (medium only) ^H incorporation range (1.5-6.0 x 10^5 cells/culture) for lymphocytes from OXAZ induced and naive control animals in each shape culture was: OXAZ (US): ROUND/651 - 6758 CPM; FLAT/189 - 2172 CPM; 1/2 AREA FLAT/344 - 9978 CPM; OXAZ (NAD): ROUND/709 - 3952 CPM; FLAT/217 - 610 CPM; 1/2 AREA FLAT/424 - 2260 CPM NAIVE (US): ROUND/70 - 643 CPM; FLAT/58 - 265 CPM; 1/2 AREA FLAT/98 - 690 CPM NAIVE (NAD): ROUND/68 - 384 CPM; FLAT/79 - 364 CPM; 1/2 AREA FLAT/131 - 570 CPM.

1:1) by increasing the number of stimulator cells tenfold, or when the total cell density was increased twofold (i.e., 4 x 10^5 responder cells and 4 x 10^4 stimulator cells/culture) maintaining the R:S ratio at 10:1. Autologous reactivity also increased proportionally; however, further increasing the cell density (to 8 x 10^5 responder cells and 8 x 10^4 stimulator cells/culture) produced a greater proportional increase in the autologous response than in the antigen-directed response.

The data from Figs 7 and 8 indicate that the most optimal blastogenesis responses (greatest antigen-directed response with the lowest possible autologous reactivity) favor use of nylon wool nonadherent responder lymphocytes in flat bottom cultures at high cell density and 1/2 area flat bottom cultures at lower cell density. Round cultures produce greater relative autologous activity at all cell densities tested. Within the confines of a limiting number of stimulator epidermal cells, an optimal response would favor use of a moderate increase in total cell density and higher R:S ratio versus a lower cell density and a large increase in stimulator cell number (lower R:S ratio).

DISCUSSION

Contact sensitization (CS) is defined as a delayed or type IV (Gell and Coombs classification) hypersensitivity response. As such, CS is a T-cell mediated immune response induced by T-cell recognition of antigen with the resulting differentiation and proliferation of both antigen-reactive lymphocytes as well as nonspecific lymphocyte proliferation resulting from the secretion of various lymphokines [27]. Numerous studies suggest that CS responses are induced by processing of the allergen in the skin by epidermal Langerhans cells (LC) which then transport the processed antigenic determinant (hapten) to regional lymph nodes. Therein, antigen-specific helper T cells recognize LC-bound antigen (in conjunction with histocompatibility antigen) and are triggered to proliferate and promote the differentiation and dissemination of effector and memory T cells able to elicit a cutaneous response upon their subsequent encounter with the inducing antigen [28-30].

Because proliferation of helper T lymphocytes is an integral part of the induction phase of the CS response, it is reasonable to presume that any chemical capable of inducing a CS response in vivo should also be capable of restimulating lymphocyte proliferative responses in vitro, especially if in vivo primed lymphocytes are used as responder cells. We do not suggest that such proliferation should be considered an in vitro correlate of the in vivo CS response. No in vitro assay can fully duplicate the complex cellular interactions and functions expressed in situ, and it has been well established that nonproliferative effector T lymphocytes from contact sensitized mice can transfer CS responses to naive recipients [31]. However, the antigen-directed in vitro blastogenesis response may still have the potential to identify those chemicals capable of inducing CS responses.
not been previously reported). However, even the responses to DNBS/TNBS (generally 10,000 – 20,000 CPM above background) were considered too low to apply this simple assay to the assessment of weaker contact sensitizers.

Conjugating the contact allergen to antigen-presenting cells produced a dramatic increase in assay responsiveness. Adherent spleen cells were only moderately effective antigen-presenting cells (Fig 3); however, cultured epidermal cells enriched for Langerhans cells (LC) by simple density gradient centrifugation were very effective in this assay. The antigen-presenting function of cultured LC has been well documented [18,25,26] as having the greater efficiency of LC at stimulating CS responses relative to other antigen-presenting cell types [14,22]. In our hands, the response of lymphocytes from TNCB sensitized mice to TNP increased three to sevenfold using EC-TNP versus TNBS (Figs 2 and 6, and data not shown). More dramatically, the response of lymphocytes from OXAZ sensitized mice to EC-OXAZ increased 40 – 70-fold relative to the low response of cultures stimulated with solubilized OXAZ alone (compare Figs 2 and 7).

The use of antigen-modified AD/spleen cells and EC introduced the problem of autologous lymphocyte stimulation, a well documented phenomenon likely resulting from lymphocyte recognition of major histocompatibility complex coded antigens of the stimulator cells [32]. We were able to reduce this reactivity by reducing the assay duration, use of flat-bottom culture plates, and use of nylon wool nonadherent responder lymphocytes. The additional selection of appropriate cell density and responder:stimulator cell ratio helped us to maximize the “window of sensitivity” between autologous reactivity and the antigen-directed response (Figs 7 and 8).

All of the blastogenesis responses described here were antigen specific. This is particularly apparent in the response to OXAZ in which lymphocytes from TNCB sensitized mice showed no reactivity to OXAZ-modified adherent spleen cells (Fig 3) or EC-OXAZ (above the autologous response to unmodified EC) (Fig 6). Reactivity to EC-TNP by lymphocytes from naive or OXAZ sensitized mice (Fig 6, and data not shown) could be attributed to a primary anti-TNP response which has been described recently [33]. Increased reactivity to EC-TNP by lymphocytes from OXAZ sensitized mice versus naive control mice was likely due to an increased lymphoid hyperplasia in the regional lymph nodes of OXAZ painted mice and the resulting increase in anti-TNP specific precursor T lymphocytes residing in the lymph nodes [33].

Two of the major difficulties in adopting any in vitro cell culture methodology for assessment of chemical toxicity are the lack of solubility of chemicals of interest in aqueous cell culture medium, and the direct cytotoxicity of the chemical in vitro. One way to circumvent this problem for contact sensitizers is by the use of in vivo haptenated antigen-presenting cells. Langerhans cells have been shown to bind covalent allergens on the skin and transport the chemical to the regional lymph node. This has been demonstrated with ferritin [20] and, more recently, with fluorescein isothiocyanate-[34]. It also likely occurs with OXAZ in view of the ability of lymph node cells from OXAZ painted mice to stimulate OXAZ-specific responses [10,13,21]. We were encouraged by the strong specific blastogenesis response to lymph node dendritic cells from OXAZ painted mice (Fig 5) under our culture conditions. This may provide an alternate methodology for in vitro assay of chemicals whose solubility or cytotoxic properties preclude direct addition to cells in culture.

The studies reported here represent a first attempt to optimize standard blastogenesis assay procedures for eventual predictive assessment of contact sensitizers. The general approach has long been considered to be of considerable potential, but always with the caveat that much work was needed to apply the approach to either diagnostic or predictive assessment of CS [15,35]. Recently, Kimber and co-workers have proposed a blastogenesis assay measuring increased, interleukin-2 potentiated, background proliferation by lymphocytes from the draining lymph nodes of mice treated with strong contact sensitizers [16]. Although their preliminary data is
encouraging, the assay’s reliance on background (nonspecific) proliferation may reduce its sensitivity relative to methods employing direct antigen restimulation in vitro.

Given the data presented here, we believe that we have refined the antigen-stimulated lymphocyte blastogenesis assay to the extent that we can now proceed to validate this method for detecting responses to those chemicals capable of inducing an in vivo CS response. Our ultimate success will depend on two factors: 1) the ability to effectively deliver the chemical to the immune system in vivo, and 2) the appropriate adaptation of our optimized culture system to the chemical and physical properties of the test material in question. By successfully addressing these two factors we hope to validate an important new approach to assessing the skin sensitization potential of chemicals relevant to consumer and occupational exposures and reduce the number of experimental animals required to make skin sensitization safety assessments.

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