

# Development of a Flow Cytometry Assay for the Identification and Differentiation of Chemicals with the Potential to Elicit Irritation, IgE-Mediated, or T Cell-Mediated Hypersensitivity Responses

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These studies were conducted to investigate the potential use of a flow cytometric analysis method for the identification and differentiation of chemicals with the capacity to induce irritation, IgE- or T cell-mediated hypersensitivity responses. An initial study investigated the ability of equally sensitizing concentrations (determined by local lymph node assay) of IgE-mediated (Toluene Diisocyanate-TDI) and T cell-mediated (Dinitrofluorobenzene-DNFB) allergens to differentially modulate the IgE+B220+ population in the lymph nodes draining the dermal exposure site. Sodium lauryl sulfate (SLS) was also tested as a nonsensitizing irritant control. Female B6C3F1 mice were dermally exposed once daily for 4 consecutive days, with the optimum time point for analysis determined by examining the IgE+B220+ population 8, 10, and 12 days post-initial chemical exposure. At the peak time point, day 10, the IgE+B220+ population was significantly elevated in TDI (41%), while moderately elevated in DNFB (18%) exposed animals when compared to the vehicle (0.8%), and remained unchanged in SLS (2.2%) exposed animals when compared to the ethanol control (2.5%). Experiments in our laboratory and others have demonstrated that the draining lymph node B220+ population becomes significantly elevated following exposure to allergens (IgE- and T cell-mediated), not irritants, allowing for their differentiation. An existing mouse ear swelling assay was used to identify chemical irritants. Therefore, using the end-points of percent ear swelling, percent B220+ cells, and percent IgE+B220+ cells, a combined irritancy/phenotypic analysis assay was developed and tested with tetradecane (irritant), toluene diisocyanate, trimellitic anhydride (IgE-mediated allergens), benzalkonium chloride, dinitrofluorobenzene, oxazolone, and dinitrochlorobenzene (T cell-mediated allergens) over a range of concentrations. Based upon the pattern of response observed, a paradigm was developed for continued evaluation: Irritant exposure will result in significant ear swelling without altering the B220+ or IgE+B220+ populations. Exposure to sensitizers (IgE-mediated or T cell-mediated) will increase the B220+ population and the percent ear swelling will remain unchanged or will significantly increase, depending on the irritancy capacity of the chemical. Both the IgE+B220+ and B220+ populations will become elevated at the same test concentration following exposure to IgE-mediated, hypersensitivity inducing allergens. At its peak, the

percent of IgE+B220+ cells will be equal to the percent of B220+ cells. The B220+ population will increase at a lower test concentration than the IgE+B220+ population, following exposure to T cell-mediated, hypersensitivity inducing allergens. At its peak, the percent of IgE+B220+ cells will reach less than half that of the percent of B220+ cells. The irritancy/phenotypic analysis method may represent a single murine assay able to identify and differentiate chemicals with the capacity to induce irritation, or IgE-mediated or T cell-mediated responses.

**Key Words:** flow cytometry; IgE-mediated response; T cell-mediated response; hypersensitivity; mouse.

Hypersensitivity responses are commonly grouped into 4 dominant classes, Types I through IV (Gell and Coombs, 1963). Type I, immediate hypersensitivity responses occur within minutes of exposure in a previously sensitized individual. They are primarily mediated by IgE antibody, the production of which is largely influenced by the presence of interleukin-4 (IL-4). Examples of IgE-mediated hypersensitivity responses include hay fever, asthma, hives, and urticaria. Type IV, delayed hypersensitivity response, occurs 24 to 72 h following exposure, in a previously sensitized individual. They are primarily cell-mediated responses influenced by the presence of interferon- $\gamma$  (IFN- $\gamma$ ), a cytokine known to inhibit the production of IgE. An example of a T cell-mediated hypersensitivity response is the allergic contact dermatitis caused by poison ivy (Finkelman *et al.*, 1988a,b; Fong and Mosmann, 1989).

Allergic and irritant contact dermatitis accounts for 15% to 20% of all reported occupational diseases. Decreased worker-productivity engendered by occupational skin disease results in an estimated loss of one billion dollars annually. Similarly, approximately 30% of adult asthma may be credited to occupational exposure, with occupational asthma costing an estimated \$400 million yearly (NIOSH 1996). Irritation, Type I (IgE-mediated hypersensitivity responses), and Type IV (T cell-mediated hypersensitivity responses) represent a meaningful portion of these losses. Considerable work has focused on developing methods for the identification of chemicals capable

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of eliciting irritation, IgE-mediated, and T cell-mediated hypersensitivity responses. By identifying these chemical hazards and controlling their workplace levels or release into the environment, the incidence of adverse responses and resultant economic losses should decline.

Many newly developed murine hypersensitivity models have focused attention on the local environment of the lymph nodes draining the site of dermal chemical exposure. Included among these are the cytokine fingerprinting assay and the recently validated local lymph node assay. The local lymph node assay identifies chemical sensitizers by their capacity to induce significant draining lymph-node cell proliferation following dermal exposure, as measured by  $^3\text{H}$ -thymidine incorporation (Kimber *et al.*, 1995). Although effective in identifying potential sensitizers, the local lymph node assay does not differentiate IgE-mediated from T cell-mediated hypersensitivity responses (Kimber *et al.*, 1994).

It is well documented that many of the cytokines present in the local environment during an immune response promote or suppress IgE antibody production, thus affecting the potential occurrence of IgE-mediated hypersensitivity responses (Coleman *et al.*, 1994; Finkelman *et al.*, 1988a, 1988b; Mosmann *et al.*, 1991; Shimoda *et al.*, 1996). It may be possible to identify and differentiate chemicals capable of eliciting IgE-mediated or T cell-mediated hypersensitivity responses by assessing the levels of these cytokines in the draining lymph nodes of animals following dermal chemical exposure (Dearman *et al.* 1995, 1996). Since increased total serum IgE levels have been observed following dermal exposure to chemicals known to cause IgE-mediated responses in humans, investigators have also proposed using the murine total serum IgE ELISA (Enzyme Linked Immunosorbent Assay) to identify IgE inducing allergens (Dearman *et al.*, 1992; Potter and Wederbrand, 1995). This has proven difficult, as the total serum IgE levels of animals exposed to dinitrochlorobenzene (DNCB), a chemical considered not to induce an IgE-mediated response, were significantly elevated when compared to the controls. Additionally, a considerable variation in the total serum IgE levels among individual animals was observed as well as a difference in the IgE response among different strains of mice (Dearman *et al.*, 1992, 1998).

These studies investigate the potential use of a single murine assay as a screening method for the identification and differentiation of chemicals with the capacity to elicit irritation, IgE-mediated, and T cell-mediated hypersensitivity responses. Following dermal chemical exposure, the method relies upon 3 parameters: the percent ear swelling, the percent of IgE+B220+ draining lymph node cells, and the percent of B220+ draining lymph node cells.

## MATERIALS AND METHODS

**Animals.** Pathogen-free female B6C3F1 mice, aged 7 to 14 weeks, were obtained from Taconic Farms (Germantown, NY). Animals were quarantined

for one week prior to use and randomized into dose groups for exposure. Animal care and use were performed following NIH guidelines. Animals were maintained on a 12-h light/dark cycle with the temperature and humidity monitored, ensuring they remained at acceptable levels (18–26°C and 40–70% relative humidity). Food (Agway Rat and Mouse Ration; NIH 07) and tap water were available *ad libitum*.

**Chemicals and antibodies.** Toluene 2,4-diisocyanate (TDI), 2,4-dinitrofluorobenzene (DNFB), sodium lauryl sulfate (SLS), n-tetradecane (Tet), benzalkonium chloride (BkCl), 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone-Oxaz), acetone, the vehicle unless otherwise stated, and 2,4-dinitrochlorobenzene (DNCB), were purchased from Sigma (St. Louis, MO). Trimellitic anhydride (TMA) was from Aldrich Chemical (Milwaukee, WI). Ethanol (ETOH), the SLS vehicle, was from Fisher Scientific (Pittsburgh, PA) and diluted to 30% with deionized water. The RA3-6B2 (anti-B220), R-35-72 (anti-IgE), 2.4G2 (anti Fc $\gamma$ II and Fc $\gamma$ III i.e., Fc Block<sup>R</sup>), and B3B4 (anti-CD23) monoclonal antibodies and isotype controls with FITC or PE label were purchased from Pharmingen (San Diego, CA).

**Local lymph node assay.** The LLNA procedure was a modification of the method developed by Kimber *et al.*, (1989) and later described by Hayes *et al.*, (1998). Five mice per dose group received 12.5  $\mu\text{l}$  of the appropriate test chemical concentration or vehicle to the dorsal and ventral surface of each ear once daily for three consecutive days. Mice were rested on day four. On day five, the mice were injected intravenously (iv) via the lateral tail vein with 0.2 ml (20  $\mu\text{Ci}$ ) of  $^3\text{H}$ -thymidine (specific activity of 0.2  $\mu\text{Ci}/\text{ml}$ ). Five h later the mice were sacrificed by CO<sub>2</sub> inhalation. The left and right draining cervical lymph nodes of each animal, located at the bifurcation of the jugular vein, were excised and placed into 4 ml cold phosphate-buffered saline (PBS). A single cell suspension was generated by dissociating the lymph nodes between the frosted ends of two microscope slides. Cells were washed twice with 10 ml PBS and precipitated in 3 ml 5% trichloroacetic acid (TCA) overnight at 4°C. Following resuspension in 1 ml TCA, the cells were transferred to 5 ml of scintillation cocktail.  $^3\text{H}$ -thymidine incorporation was determined with an LKB Wallace 1218 Beta Counter. Samples were counted for 5 min each and the counts per min (CPM) for each animal adjusted for beta counting efficiency (63.6%) to generate disintegrations per min (DPM) values. Mean DPM–background were calculated for each dose group.

**Exposure and tissue isolation.** For the phenotyping assay, the irritancy-assay dosing method described by Hayes *et al.*, (1998) was used. Briefly, mice were exposed by applying 12.5  $\mu\text{l}$  of the appropriate chemical concentration to the dorsal and ventral surfaces of each ear, once daily for 4 consecutive days. Following sacrifice via CO<sub>2</sub> inhalation, the draining cervical lymph nodes located at the bifurcation of the jugular vein, and, when appropriate, the popliteal lymph nodes, perihilar lymph nodes, and spleens were excised and placed into PBS. In early studies, the lymph nodes from each anatomical location for each dose group were pooled, and phenotypic analysis was performed on three replicate samples taken from the pooled lymph node cells unless otherwise indicated. In later studies, as the method was refined, analyses were performed on right and left lymph nodes pooled from individual animals. Analyses of the spleens were performed on three replicate samples taken from spleen cell suspensions from individual animals.

**Phenotypic analysis.** Phenotyping was performed using flow cytometry. Briefly, the lymphoid organs in PBS, were dissociated using the frosted ends of two microscope slides. Cell counts were determined on a Coulter Counter (ZBI or Z2 model), and approximately  $1 \times 10^6$  cells per sample were added to the wells of a 96-well plate. A volume of 100  $\mu\text{l}$  was used on all remaining steps. The cells were washed with a PBS staining buffer containing 1.0% bovine serum albumin (BSA), and 0.10% sodium azide and then incubated with 1.0  $\mu\text{g}$  of Fc Block<sup>R</sup> for 5 min at 4°C. The appropriate antibodies, at a 1.0  $\mu\text{g}$  dilution in staining buffer were incubated with the samples for approximately 45 min in the dark at 4°C. After washing, cells were incubated with 10  $\mu\text{g}$  of propidium iodide (PI) in the dark at 4°C for five min. Cells were washed, resuspended in staining buffer, and analyzed on a Becton Dickinson FACScan or FACSVantage flow cytometer using a PI viability gate. A PBS sample sheath lacking chelators was used, as the interaction between soluble IgE and

surface CD23 is calcium-dependent (Richards and Katz, 1990). Ten thousand viable events were collected for each sample. Computer analysis was performed on gated lymph-node cell populations. For measurement of surface IgE-expressing cells, a modified acid-stripping method of Kumagai *et al.*, (1975) was used. Briefly, an aliquot of cells was incubated in a solution of 0.005M KCl, 0.09M NaCl, 0.05M sodium acetate, and 1.0% newborn calf serum (NCS) for approximately 1 min. Following addition of 10 ml 1 mM PBS/HEPES, the cells were centrifuged, the supernate was removed, and the cells were resuspended in the appropriate volume of staining buffer. The mRE2-1 cell line, a Chinese hamster ovary (CHO) cell line transfected with the murine CD23 gene, was kindly provided by Dr. Daniel Conrad (Medical College of Virginia/Virginia Commonwealth University) and used as one of the acid-stripping controls. The cells were maintained as described by Cho *et al.*, (1997). mRE2-1 cells were incubated with 5 or 10  $\mu\text{g}$  of murine IgE antibody per million cells for one h at 4°C and stained for phenotypic analysis prior to or following acid stripping.

**ELISA.** Following sacrifice, blood samples obtained via cardiac puncture were placed in 10 × 75 mm culture tubes, allowed to clot for 1 h, and centrifuged at approximately 3000 rpm (1650 × g) for 20 min. The serum fractions were removed and stored in microtest tubes at -30°C until analyzed. Quantification of serum total IgE levels was achieved using a modified ELISA based on the assay described by Keegan *et al.*, (1991). Dynatech Immulon-2 microtiter plates were coated with 10.0  $\mu\text{g}/\text{ml}$  of B1E3 anti-mouse IgE antibody in PBS overnight at 4°C. The blocking buffer was a PBS, 2.0% NCS, 10 mM HEPES solution. After blocking at 37°C for one h, serial dilutions of standard (mouse IgE anti-DNP) beginning at 500 ng/ml and samples beginning at a 1:10 dilution in blocking buffer were incubated on the plate for two h at 37°C. After washing, R1E4 anti-mouse IgE secondary antibody conjugated to biotin was added to the plate at a 10  $\mu\text{g}/\text{ml}$  concentration in blocking buffer for two h at 37°C. After washing, the plates were then incubated with Streptavidin-Alkaline Phosphatase at 1  $\mu\text{g}/\text{ml}$  in blocking buffer for one h at 37°C. Finally, alkaline phosphatase substrate was added and the plates were read on a Beckman Vmax model plate reader at 405–650 nm (for intrawell correction) approximately 30 min later. The data were analyzed using the IBM Softmax Pro 1.2.0 program. Test sera antibody values were calculated by comparison with known amounts of standard. A standard curve was generated using linear values from the Log/Log analysis (values allowing for a slope close to 1.0). The hybridomas used to generate the capture, secondary, and IgE standard antibodies for the mouse IgE ELISA were also kindly donated by Dr. Daniel Conrad, and were purified using an abbreviated method of Liu *et al.*, (1980).

**Ear measurements.** As an indicator of irritancy, the ears of each mouse were measured prior to initial chemical application and post-measured 24 h following final chemical application using a modified Mitutoyo engineer's micrometer (Hayes *et al.*, 1998). To determine the 24 h. percent change in ear swelling the following equation was used: (mean thickness of ear 24 h after chemical application / mean thickness of premeasured ear × 100) - 100.

**Statistics.** All test groups were statistically compared to the appropriate control using the JMP 3.2.2 software for the PC. Homogeneity of data was determined using Bartlett's test (Bartlett, 1937). Homogeneous data were analyzed for significance using Dunnett's test and nonhomogeneous data were analyzed using the Tukey-Kramer test (Dunnett, 1955; Tukey, 1977). For the ELISA data, the animal values were transformed using a natural logarithmic transformation, allowing for parametric analysis via Dunnett's (Dearman *et al.*, 1998). Data with unequal sample numbers were analyzed using the Cochran *t*-test (Cochran and Cox, 1975). Comparison of individual dose groups to control were performed using Student's *t*-test.

## RESULTS

### LLNA Results

Initial studies used known IgE-mediated (TDI) and T cell-mediated (DNFB) hypersensitivity inducing allergens (Butcher

**TABLE 1**  
**Proliferative Response Following DNFB and TDI Exposure**

Dose group	DPM	Fold increase
Acetone	13.1 ± 4.6	
DNFB		
0.01%	39.3 ± 9.9	3.0
0.05%	472.1 ± 57.3	35.9
0.10%	1107.3 ± 168.7**	84.3
0.15%	1837.0 ± 223.3**	139.8
Acetone	7.1 ± 1.2	
TDI		
0.5%	597.5 ± 96.5**	83.8
1.0%	911.0 ± 35.3**	127.8
2.5%	866.3 ± 42.9**	121.5

*Note.* Female B6C3F1 mice (*N*, 5 per dose group) were exposed once daily for 3 consecutive days by receiving 12.5  $\mu\text{l}$  of test article or vehicle to the dorsal and ventral surfaces of each ear. On the 5th day, animals were injected with  $^3\text{H}$ -thymidine. Five h later the animals were sacrificed via CO<sub>2</sub> inhalation and the draining cervical lymph nodes excised. The level of cellular proliferation, a measure of sensitization, was determined by quantifying of the amount of  $^3\text{H}$ -thymidine incorporation into the draining lymph nodes by beta scintillation counting. Values presented are the dose group mean DPM ± SE.

\* Represents a *p* < 0.05 as compared to the respective acetone control using Dunnett's.

\*\* Represents a *p* < 0.01 as compared to the respective acetone control using Dunnett's.

*et al.*, 1976; Garcia-Perez, 1978). The ability of TDI and DNFB to induce sensitization following dermal exposure was examined in the LLNA (Table 1). A dose responsive increase in proliferation was observed in DNFB exposed animals becoming significant at the 0.1% DNFB concentration (1107 mean DPM), reaching a maximum of 1837 mean DPM following 0.15% DNFB exposure compared to 13 mean DPM for the control. At all concentrations tested, the proliferative response induced by TDI exposure was significant, reaching a maximum level of 911 mean DPM in the 1.0% TDI exposure group compared to a 7 mean DPM for the control. The fold increase was approximately 140 and 120 for the 0.15% DNFB and 2.5% TDI exposed animals, and was not significantly different when compared via Student's *t*-test, suggesting that these two concentrations are equally sensitizing.

### Development of the IgE+ Draining Lymph Node B Cell Phenotype

Initial phenotypic analysis studies showed that an increased IgE+ B cell population developed in the draining lymph nodes of animals 8 days following initiation of a 10-day dermal exposure regimen with the known IgE inducing allergens TMA and TDI (data not shown). Based on this data, a time-course study was performed to determine the optimum time point for analysis of the draining lymph node IgE+ B cell population in animals exposed to xenobiotics, using the 4-day irritancy assay dosing schedule described by Hayes *et al.* (1998). This dosing



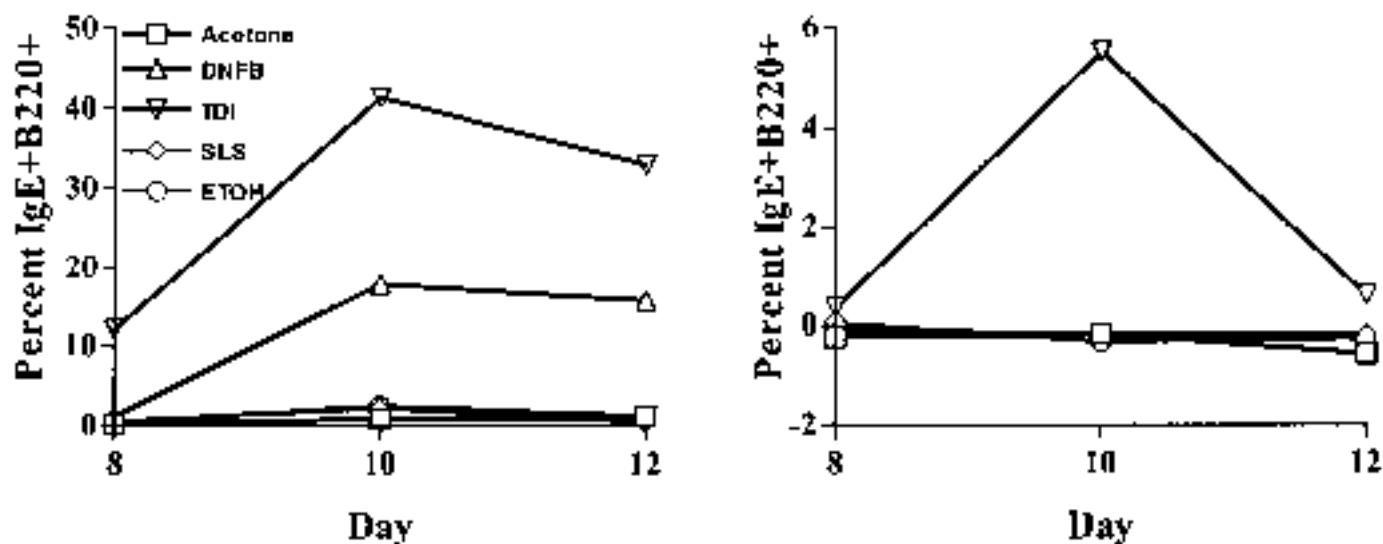


FIG. 1. Time course flow cytometric analysis of the IgE+B220+ population in the draining lymph nodes of female B6C3F1 mice following dermal 2.5% TDI, 0.15% DNFB, or 40% SLS exposure. Animals were exposed once daily for 4 consecutive days. The draining lymph nodes were removed and pooled for each dose group at each time point. At days 8 and 12, there were 4 animals per dose group, except for the acetone and ETOH groups, where there were 8 animals. At day 10, there were 10 animals for each dose group, except for the acetone group where there were 20 animals. Values presented are the average of 3 replicate samples taken from the pooled lymph nodes. (Left) Percent of IgE+ B cells prior to acid stripping. (Right) Percent of IgE+ B cells following acid stripping.

protocol was chosen because it would permit the incorporation of the irritancy assay parameters into the method. The similarity between this dosing regimen and the 3- and 4-day LLNA dosing protocols would also allow for comparison between chemical test data obtained with the phenotypic analysis assay and the LLNA (Loveless *et al.*, 1996; Woolhiser *et al.*, 1998). Equally sensitizing doses of TDI (2.5%) and DNFB (0.15%) were employed and a 40% SLS nonsensitizing irritant control (Woolhiser *et al.*, 1998) was also included in the study design. The draining lymph node cells were double stained for IgE and B220 and the percentage of IgE+B220+ cells was determined on 8, 10, and 12 days following initial exposure (Fig. 1).

The IgE+ B cell population (Fig. 1, left), was elevated in animals exposed to TDI and to a lesser extent in DNFB-exposed animals, peaking 10 days following initial exposure and appearing to decrease by day 12. At the peak time point, the percent of IgE+B220+ cells for the TDI exposed animals was 41%, approximately a 52-fold increase over the acetone control. The IgE+B220+ population for DNFB exposed animals only reached 18%, a 23-fold increase over the acetone control. This population was unchanged in SLS (2.2%) exposed animals when compared to the ethanol control (2.5%).

The percent of surface IgE expressing B cells, a component of the IgE+ B cell population, was also examined (Fig. 1, right). To accomplish this, surface-bound soluble IgE antibody was removed from the B cell surface by briefly incubating these cells with a mild acid prior to phenotypic analysis. This allowed for the sole detection of surface IgE expressing B cells. A measurable population (5.5%) was present in the lymph nodes of the 2.5% TDI exposed mice. This cell type was

not detected at any of the time points examined in the DNFB and SLS exposed animals. The lack of a measurable surface IgE expressing B cell population in the 0.15% DNFB-exposed animals, coupled with the small size of the population in the TDI exposed animals, suggested that the majority of the IgE+ lymph node B cell population in these dose groups prior to acid stripping was comprised of B cells staining positive for the detection of soluble IgE bound to B cell surface CD23.

As a control to ensure complete removal of surface bound IgE prior to phenotypic analysis, mRE2-1 cells constitutively expressing CD23 were incubated with saturating concentrations of murine IgE antibody. The IgE+CD23+ population was examined prior to and following the acid stripping procedure. As shown in Table 2, when the mRE2-1 cells were incubated with 10  $\mu$ g of IgE, the percent of IgE+CD23+ cells was decreased from approximately 98% to 0.7% following acid stripping, suggesting the acid stripping method efficiently

TABLE 2  
Controls for the Acid Stripping Procedure

Dose group	Pre-acid	Post-acid
5.0 $\mu$ g IgE	94.5 $\pm$ 2.7	0.1 $\pm$ 0.1
10.0 $\mu$ g IgE	97.9 $\pm$ 0.1	0.7 $\pm$ 0.2

Note. mRE2-1 cells were incubated with varying concentrations of IgE and stained for the percent of IgE+CD23+ cells prior to and following acid stripping were determined via flow cytometry. Values represent the mean  $\pm$  SE of three replicate samples taken from the pooled mRE2-1 cells of each IgE dose group.

**TABLE 3**  
Representative Surface Molecule Controls

Marker	Pre-acid	Post-acid
IgM+	18.5 ± 0.3	23.4 ± 1.3
CD23+	44.5 ± 0.8	41.3 ± 0.2
B220+	45.0 ± 0.1	44.7 ± 0.0

*Note.* Percent of surface expressed molecules prior to and following acid stripping in animals following dermal exposure to 2.5% TDI. Values represent the mean ± SE of three replicate samples taken from the pooled draining lymph node cells of the pre or post acid stripping exposure groups.

removed soluble IgE. Also, in a separate experiment, the percent of CD23+ mRE2-1 cells was unchanged by acid stripping (approximately 90% prior to and 88% following acid stripping), suggesting the decreased percent of IgE+CD23+ mRE2-1 cells following acid stripping was not caused by decreased CD23 levels. Likewise, in other acid stripping control experiments using lymph node cells, CD23+, B220+, and IgM+, populations were not altered by the acid stripping procedure, suggesting this method does not affect surface-expressed molecules, including surface IgE (Table 3).

Initially in five repeat experiments, the IgE+B220+ population, following acid stripping, was detected at levels from 0.1% to 8.2% and was present only 10 days following initial exposure. However, additional studies demonstrated a shift in the time course of development of this population (data not shown). This suggested that the measurement of the IgE+B220+ population, following acid stripping, would not represent a reproducible methodology, preventing its use in establishing the phenotypic analysis assay. Therefore, the continued development of the phenotypic analysis methodology was based on the evaluation of the IgE+B220+ population prior to acid stripping (Fig 1, left). The mechanism responsible for the variability of the development of the IgE+ population following acid stripping was not investigated further.

*Correlation of the Development of the IgE+B220+ Phenotype with Total Serum IgE Levels*

Given that the observed increase in IgE+ B cell population appeared to reflect the increased binding of soluble IgE to B cell surface CD23, the total serum IgE levels of the previously phenotyped animals were measured. The total serum IgE levels were found to positively correlate with the development of the IgE+ lymph node B cell population in TDI exposed animals. The total serum IgE levels of the 2.5% TDI exposed animals were slightly increased on day 8, peaked at a significantly increased level at day 10, and remained significantly elevated, though lower, by day 12 when compared to the acetone controls. When examined, the DNFB and SLS exposed animals did not exhibit significantly altered total serum IgE levels. At the peak time point 10 days following initial exposure, total serum IgE levels were 24-fold higher in the TDI (4056 ng/ml) exposed animals, and unchanged in the DNFB (138 ng/ml), and SLS (328 ng/ml) exposed animals when compared to the acetone (167 ng/ml) and ETOH (312 ng/ml) controls (Table 4).

*Anatomical Variation in the Development of the IgE+B220+ Phenotype*

In subsequent experiments, the IgE+ B cell population was analyzed at the peak time point, 10 days following initial chemical exposure. The anatomical variation in the development of the IgE+ B cell population was examined by comparing the percent of IgE+B220+ cells present in lymphoid organs draining (cervical lymph nodes) or distal to (perihilar lymph nodes, popliteal lymph nodes, and spleen) the dermal exposure site (Fig. 2). No differences were seen in the percentages of IgE+ B cells at different anatomical locations when examining the acetone and naive groups. Although the IgE+ B cell population was elevated in all of the lymphoid organs examined in the 2.5% TDI exposed animals when compared to the acetone control, the population was approximately 2-fold higher in the draining lymph nodes of 2.5% TDI exposed animals when compared to the distal lymphoid organs in these

**TABLE 4**  
Time Course of Total Serum IgE Levels Following Dermal Exposure to Sodium Lauryl Sulfate, Dinitrofluorobenzene, or Toluene Diisocyanate

Chemical	N	Day 8 IgE (ng/ml)		Day 10 IgE (ng/ml)		Day 12 IgE (ng/ml)	
			N		N		N
Acet	8	496 ± 113	19	167 ± 28	7	274 ± 52	
ETOH	8	128 ± 39	8	312 ± 122	2	145 ± 33	
SLS	4	261 ± 143	9	328 ± 114	4	161 ± 65	
DNFB	4	181 ± 17	10	138 ± 20	4	429 ± 116	
TDI	4	926 ± 333	10	4056 ± 951**	4	1606 ± 367**	

*Note.* Serums from individual animals used to generate the data in Figure 1 were analyzed for the amount of total serum IgE by ELISA. Values are the dose-group mean ± SE. Dose groups: acetone, 30% ETOH, 40% SLS, 0.15% DNFB, and 2.5% TDI.

\*\* Represents a  $p < 0.01$  as compared to the acetone group using Cochran's  $t$ -test.

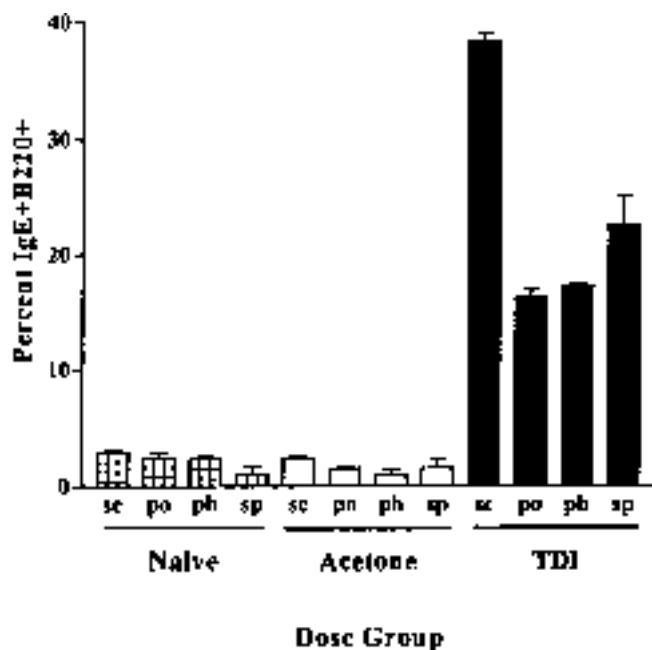


FIG. 2 Comparison of the percent of IgE+ B cells in draining and distal lymphoid organs in naives, or following dermal chemical (2.5% TDI or acetone) exposure.  $N = 10$  for all dose groups. Animals were exposed as described: sc, cervical lymph nodes; po, popliteal lymph nodes; ph, parahilar lymph nodes; and sp, spleens. Data presented are the average of three replicate samples taken from the pooled lymph nodes of each dose group at each anatomical site. The spleen data presented are the average of the individual animal values for each dose group.

same animals. This suggested the draining lymph nodes were the most suitable anatomical site for examination of this population.

#### Expanded Panel of Chemicals

To aid in the differentiation of irritants from sensitizers, the phenotypic analysis method was incorporated into the protocol for an existing mouse ear-swelling irritancy assay. The ability of the combined irritancy/phenotypic analysis assay to identify and differentiate chemicals with the capacity to elicit an irritation, IgE-mediated, or T cell-mediated hypersensitivity response was tested using a panel of well-characterized compounds (Table 5). Known irritants (SLS, Tet) (Lee and Maibach, 1995; Moloney and Teal, 1988), IgE inducing sensitizers (TDI, TMA) (Dearman *et al.*, 1998; Son *et al.*, 1998), a strong T cell-mediated hypersensitivity inducing sensitizer (Oxaz) (De Sousa *et al.*, 1969), and a strong irritant with a weak ability to induce T cell-mediated hypersensitivity (BkCl) (Fuchs *et al.*, 1993; Schnuch *et al.*, 1998) were tested and compared to the controls. Additionally, the increased lymph-node cell number resulting from exposure to sensitizing concentrations of these allergens allowed for the analysis of the lymph nodes of individual animals and the incorporation of statistical analyses into the methodology. However, for each

sample in the acetone, ETOH, and SLS dose groups it was necessary to pool the lymph nodes of two animals in order to obtain the cell number necessary for analysis.

Significantly sensitizing doses of TDI, TMA, Oxaz, and BkCl, as previously determined by a local lymph node assay and significantly irritating concentrations of SLS and Tet, as previously determined by a mouse ear swelling irritancy assay, were employed (Woolhiser *et al.*, 1998). At the concentrations tested, SLS, Tet, BkCl, TMA, and TDI were significantly irritating, as indicated by the increase in 24-h percent ear swelling (Table 5). Similar to previous observations by others, 0.05% Oxaz exposure did not result in significant irritation (Tarayre *et al.*, 1984; Woolhiser *et al.*, 1998).

Previous data from our lab (data not shown) and others (Gerberick *et al.*, 1997; Sikorski *et al.*, 1996) suggested that measuring the percent of B220+ draining lymph node cells in animals following chemical exposure may allow for the differentiation of irritants from T cell-mediated, hypersensitivity inducing sensitizers. Similar results were obtained in this study, with the B220+ population significantly elevated in animals exposed to the sensitizers (TDI, TMA, Oxaz, and BkCl), while unchanged in the irritant (SLS and Tet) exposed animals as compared to the controls (Table 5). Although TDI and TMA have the potential to induce T cell-mediated hypersensitivity responses, they primarily induce IgE-mediated hypersensitivity responses (Dearman *et al.*, 1991; Son *et al.*, 1998; Tee *et al.*, 1998 Thorne *et al.*, 1987). This suggests the measurement of the B220 population may allow for the differ-

TABLE 5

Comparison of Phenotypic Analysis of the Draining Lymph Node Cells in Female B6C3F1 Mice Ten Days Following Exposure to Known Human Irritants, Type I and Type IV Sensitizers

Dose group	24-h % ear swelling <sup>a</sup>	% B220+ <sup>b</sup>	% IgE+B220+ <sup>b</sup>
Acetone	(3.6 ± 1.3)	19.7 ± 0.9	3.8 ± 2.0
30% ETOH	1.8 ± 1.3	16.1 ± 1.3	(0.2 ± 1.0)
40% SLS	53.0 ± 5.7**	22.0 ± 2.0	3.2 ± 1.4
40% Tet	110.0 ± 15.0**	21.4 ± 1.5	2.7 ± 1.4
1.0% BkCl	40.0 ± 5.7**	35.3 ± 1.8**	4.5 ± 2.3
0.05% Oxaz	3.4 ± 2.5	36.8 ± 1.4**	23.1 ± 1.7**
50% TMA	36.0 ± 8.2**	41.5 ± 1.3**	32.5 ± 3.4**
2.5% TDI	190.0 ± 5.5**	41.2 ± 1.1**	36.1 ± 3.6**

Note.  $N = 10$  for all dose groups, except acetone, ETOH, and 40% SLS groups, where  $N$  is 20. For phenotypic analysis, each sample is the average of 3 replicates, with 5 samples for all dose groups. For each sample in the acetone, ETOH, and SLS dose groups, the replicates were taken from the pooled lymph nodes of two animals. For all the other dose groups, the lymph nodes of individual animals were analyzed. The values presented are the dose-group mean ± SE. All dose groups were compared to the acetone vehicle, except for the 40% SLS group, which was compared to the 30% ETOH vehicle.

<sup>a</sup>\*\* Represents a  $p < 0.01$  as compared to the appropriate control using Cochran's t-test.

<sup>b</sup>\*\* Represents a  $p < 0.01$  as compared to the appropriate control using Dunnett's.

TABLE 6  
Irritancy/Phenotypic Analysis Dose-Response Studies with Oxazolone, Benzalkonium Chloride, Dinitrochlorobenzene, and Tetradecane

Dose group	24-h ear swelling	Percent B220+	Percent IgE+B220+	Total IgE (ng/ml)
Experiment #1				
Acetone	2.1 ± 1.6	10.9 ± 0.9	0.0 ± 0.6	68 ± 20
OXAZ				
0.025%	0.6 ± 1.3	21.1 ± 1.3**	5.6 ± 1.5*	52 ± 9
0.050%	2.9 ± 1.0	27.0 ± 0.5**	3.8 ± 0.8	47 ± 8
0.100%	0.4 ± 1.1	29.1 ± 1.9**	5.4 ± 1.7	76 ± 21
0.250%	4.6 ± 2.6	31.6 ± 1.3**	15.5 ± 2.1**	228 ± 63****
BkCl				
0.25%	(1.1 ± 1.1)	13.1 ± 0.5	0.4 ± 0.2	39 ± 6
0.50%	4.9 ± 1.3	16.7 ± 1.8*	2.6 ± 1.8	103 ± 33
1.00%	19.1 ± 3.0****	19.9 ± 2.0**	2.9 ± 1.1	63 ± 12
2.50%	57.9 ± 6.8****	24.3 ± 1.5**	4.1 ± 0.9	44 ± 13
Experiment #2				
Acetone	0.4 ± 1.0	13.2 ± 0.8	1.1 ± 0.3	45 ± 6
DNCB				
0.05%	0.7 ± 1.3	18.8 ± 0.7**	1.5 ± 0.7	53 ± 21
0.10%	1.5 ± 1.3	19.4 ± 1.0**	5.3 ± 1.8	60 ± 11
0.50%	31.4 ± 5.9****	26.0 ± 0.7**	17.0 ± 1.9 <sup>c</sup>	252 ± 51****
1.00%	61.6 ± 10.8****	27.2 ± 1.6**	17.0 ± 2.9 <sup>c</sup>	213 ± 67***
Tet				
10%	6.1 ± 1.4	10.7 ± 1.0	0.9 ± 0.4	59 ± 9
20%	4.1 ± 1.3	12.2 ± 0.8	0.6 ± 0.3	65 ± 21
30%	2.5 ± 2.3	14.2 ± 1.1	3.0 ± 1.0	60 ± 13
50%	40.8 ± 9.2****	13.8 ± 1.7	2.7 ± 0.7	55 ± 15

Note. Values represent the group mean ± SE. For chemical concentrations which were expected to induce significant lymph-node cell proliferation (0.1% OXAZ, 0.25% OXAZ, 1.0% BkCl, 2.5% BkCl, 0.05% DNCB, 0.10% DNCB, 10% Tet, and 20% Tet) there were five animals per group. For vehicle and all other chemical concentrations, in order to reach cell counts required for phenotypic analysis, two animals were pooled for each sample resulting in ten animals per group,  $N = 5$ . For the ear swelling data, both ears were measured for each animal resulting in an  $N$  of 10 or 20. For the phenotypic analysis, each sample value is the average two replicates. Values in parentheses are negative.

\* Represents a  $p < 0.05$  as compared to the acetone group using Dunnett's.

\*\* Represents a  $p < 0.01$  as compared to the acetone group using Dunnett's.

\*\*\* Represents a  $p < 0.05$  as compared to the acetone group using Cochran's  $t$ -test.

\*\*\*\* Represents a  $p < 0.01$  as compared to the acetone group using Cochran's  $t$ -test.

entiation of irritants from sensitizers, regardless of their ability to induce IgE-mediated or T cell-mediated hypersensitivity responses.

When examined, animals exposed to sensitizing concentrations of known IgE inducing chemicals (TMA, TDI) possessed greater than 30% IgE+ B cells in the draining lymph nodes (Table 5). The percent of IgE+ B cells was moderately elevated in Oxaz exposed animals when compared to control. The percent of IgE+ B cells was significantly greater ( $p < 0.05$ ) in the TMA and TDI exposure groups when compared to the Oxaz exposure group.

#### Dose Response Studies

A range of concentrations of Oxaz, BkCl, and Tet similar to those previously examined by others in the murine local lymph node assay (Dearman *et al.*, 1991; Loveless *et al.*, 1996; Woolhiser *et al.*, 1998) were tested in the irritancy/phenotypic

analysis assay (Table 6). DNCB, a well-known T cell-mediated, hypersensitivity inducing allergen was included at relevant sensitizing concentrations (Kligman *et al.*, 1959; Loveless *et al.*, 1996). Exposure to at least one concentration of Tet and BkCl resulted in significant irritation as indicated by the increased percent ear swelling, similar to previous observations (Table 5). Exposure to DNCB also resulted in significant irritation at the tested concentrations, similar to reports by others (Bleumink *et al.*, 1973; Takasu, 1975; Woolhiser *et al.*, 1998). With the exception of Tet exposed animals, which showed an irritant response only at the highest concentration tested, the irritation elicited by exposure to these compounds was dose responsive. The B220+ population, a proposed indicator of sensitization, was dose-responsively increased in animals exposed to the known T cell-mediated, hypersensitivity inducing allergens. The B220+ populations were significantly increased at concentrations of 0.025% Oxaz and 0.05%

TABLE 7  
Irritancy/Phenotypic Analysis Dose Response Studies with Toluene Diisocyanate, Trimellitic Anhydride, and Dinitrofluorobenzene

Dose group	24-h ear swelling	Percent B220+	Percent IgE+B220+	Total IgE (ng/ml)
Acetone <sup>a</sup>	(2.1 ± 1.1)	12.9 ± 0.5	0.1 ± 0.1	62 ± 5
Acetone <sup>b</sup>	0.1 ± 1.2	12.3 ± 1.4	0.3 ± 0.1	55 ± 15
Acetone <sup>c</sup>	0.3 ± 1.5	17.5 ± 0.8	0.8 ± 0.5	29 ± 2
<b>TDI</b>				
0.1% <sup>a</sup>	(2.4 ± 1.6)	16.4 ± 2.0	1.4 ± 0.8	57 ± 14
0.5% <sup>a</sup>	14.8 ± 3.3**	9.2 ± 2.5**	23.9 ± 3.1**	719 ± 164***
1.00% <sup>b</sup>	94.4 ± 19.3**	32.5 ± 2.2***	7.8 ± 2.3**	2827 ± 888***
2.50% <sup>b</sup>	229.1 ± 16.8**	38.0 ± 1.3***	33.1 ± 1.9**	6500 ± 2361***
<b>DNFB</b>				
0.005% <sup>a</sup>	1.3 ± 2.6	18.7 ± 2.3	0.1 ± 0.1	44 ± 5
0.01% <sup>a</sup>	6.0 ± 4.4	25.0 ± 2.5**	0.8 ± 0.4	54 ± 13
0.05% <sup>a</sup>	3.3 ± 2.1	34.6 ± 0.5**	10.1 ± 1.9**	96 ± 19
0.15% <sup>b</sup>	37.9 ± 4.8**	24.8 ± 1.9***	12.1 ± 0.8**	226 ± 13**
0.30% <sup>b</sup>	160.5 ± 5.9**	29.9 ± 2.2***	15.2 ± 2.0**	150 ± 32*
<b>TMA</b>				
0.1% <sup>a</sup>	1.8 ± 1.6	11.9 ± 1.1	0.8 ± 0.6	58 ± 16
0.5% <sup>a</sup>	7.8 ± 1.7	16.2 ± 0.8	0.4 ± 0.3	68 ± 16
1.0% <sup>c</sup>	1.1 ± 1.2	34.2 ± 0.9***	20.5 ± 1.8***	163 ± 30**
10% <sup>c</sup>	10.8 ± 2.2**	36.8 ± 0.7***	30.5 ± 1.2***	461 ± 96**
25% <sup>c</sup>	7.0 ± 2.6	42.8 ± 1.9A***	35.4 ± 2.4***	698 ± 65**

Note. Data presented is compiled from three studies. Values represent the dose group mean ± SE from analysis of individual animals, 5 animals per group. For the 24-h ear swelling, both ears of each mouse were measured, resulting in an  $N = 10$ . Each phenotypic analysis sample was the average of 2 replicates, with 5 samples per dose group. Values in parentheses are negative.

<sup>a</sup> Experimental groups from Experiment #1.

<sup>b</sup> Experimental groups from Experiment #2.

<sup>c</sup> Experimental groups from Experiment #3.

\* Represents a  $p < 0.05$  as compared to that experiment's acetone group using Tukey-Kramer.

\*\* Represents a  $p < 0.01$  as compared to that experiment's acetone group using Tukey-Kramer.

\*\*\* Represents a  $p < 0.01$  as compared to that experiment's acetone group using Dunnett's.

DNCB, which were also found to be significantly sensitizing in the local lymph node assay (Dearman *et al.*, 1991; Loveless *et al.*, 1996; Woolhiser *et al.*, 1998). Following exposure to 0.5% BkCl, the B220+ population was significantly increased when compared to control. This was not surprising as benzalkonium chloride is reported to be a weak contact sensitizer in humans (Schnuch *et al.*, 1998). Exposure to the irritant Tet, up to concentrations of 40%, did not affect the B220+ population (Table 6).

The percent of IgE+B220+ lymphocytes was not significantly altered following exposure to BkCl or Tet at the concentrations investigated. At concentrations 10-fold higher than required to induce an elevation in the B220+ population, 0.25% Oxaz and 0.5% DNCB exposure induced a moderate elevation in the IgE+B220+ population. These same animals showed a moderate, yet significant increase in total IgE. The total serum IgE levels in the 0.50% DNCB and 0.25% Oxaz exposed animals were maximal at 252 ng/ml and 230 ng/ml, respectively, compared to their respective acetone controls of 45 ng/ml and 68 ng/ml (Table 6).

Additional refinement of the assay methodology (reducing the number of replicates per sample) allowed for phenotypic

analysis of the lymph nodes of individual animals for all of the exposure groups, including the acetone control. TDI, TMA, and DNFB were tested at a range of concentrations including those previously examined by others in the local lymph node assay (Dearman *et al.*, 1991; Maurer *et al.*, 1991; Woolhiser *et al.*, 1998). The data presented in Table 7 are the compilation of three separate experiments, as it was necessary to extend the dose responses for these chemicals in order to obtain no-effect levels for all of the parameters investigated. Exposure to TDI and DNFB resulted in dose-responsive increases in ear swelling, reaching significant levels at 0.15% DNFB (38%) and 0.5% TDI (15%) concentrations compared to acetone controls (0.05% and -2.1%, respectively). The irritancy response to TMA was minimal, with a significant increase only detected in animals exposed to 10% TMA (11%) when compared to the control (0.25%) (Table 7).

The B220+ lymph node cell population became significantly elevated compared to the controls, beginning at concentrations of 0.5% TDI, 0.01% DNFB, and 1.0% TMA. The increase was dose-responsive for all three chemicals. For each primarily IgE inducing allergen, the IgE+B220+ population became significantly elevated at the same chemical concentra-



tion that resulted in a significant increase in the B220+ population (0.5% TDI and 1.0% TMA). The percentage of IgE+B220+ cells approached the percentage of B220+ cells, suggesting the majority of the B cell population was staining IgE-positive. Additionally, the total serum IgE levels of these animals became significantly increased at the 0.5% TDI and 1.0% TMA test concentrations. With animals exposed to DNFB, a T cell-mediated, hypersensitivity inducing allergen, the IgE+B220+ population became significantly elevated at a higher concentration (0.01% DNFB) than was required to significantly increase the B220+ population (0.05% DNFB). At its peak level, the percentage of IgE+B220+ cells was approximately half the value of the percentage of B cells, as indicated by the B220+ population. Following exposure to primarily IgE inducing chemicals (2.5% TDI and 25% TMA), the IgE levels in exposed animals reached 6500 ng/ml and 697 ng/ml, respectively, as compared to the moderate levels induced in animals exposed to 0.15% DNFB (226 ng/ml). The total IgE levels for the acetone controls ranged from 29 ng/ml to 62 ng/ml (Table 7).

## DISCUSSION

The initial goal of these studies was to develop a phenotypic analysis method for the identification of potential IgE-mediated, hypersensitivity inducing allergens by their ability to modulate the presence of IgE+ B cells in the draining lymph nodes of animals following dermal exposure. For the initial development of this methodology, animals were dermally exposed to chemicals known to elicit irritation (SLS), IgE-mediated (TDI), or T cell-mediated (DNFB) hypersensitivity response in humans (Butcher *et al.*, 1976; Garcia-Perez, 1978; Lee, 1995), with the development of the IgE+ draining lymph node B cell population investigated via flow cytometry. Initial studies also examined the draining lymph node CD3, CD4, CD8, and IgM populations, but these markers provided no useful information (data not shown). The presence of an IgE+ B cell population was detected in the draining lymph nodes of animals dermally exposed to TDI and to a lesser extent DNFB. The IgE+ B cell population was determined to be composed of two subpopulations. The smaller subpopulation consisted of surface IgE expressing B cells and was only detected in TDI exposed animals. The larger subpopulation consisted of B cells staining IgE+ by the detection of soluble surface bound IgE and was detected in TDI and at a lower level in DNFB exposed animals. Since equally sensitizing TDI and DNFB concentrations were tested (Table 1), their differential ability to increase the IgE+B220+ population (prior to and following acid stripping) appeared to represent a qualitative difference between the two chemicals.

The presence of a small surface IgE expressing B cell population (5.5%) in the draining lymph nodes of TDI exposed animals was consistent with studies examining the development of surface IgE+ B cells in response to parasitic infection.

Previous studies indicated that animals undergoing an IgE response to *Nippostrongylus brasiliensis* infection contained a measurable (2.7%) surface IgE expressing B cell population in the draining mesenteric lymph nodes (Katona *et al.*, 1983). The surface IgE expressing B cell has recently been shown to be necessary for IgE production. Following stimulation with known IgE inducing allergens, animals lacking B cells with the ability to express surface IgE produced negligible amounts of total and antigen-specific IgE (Achatz *et al.*, 1997). With the functional role of this population in mind and the absence of a significant surface IgE expressing B cell population in DNFB exposed animals, measuring this population appeared to be a potential method for identifying chemicals able to elicit IgE-mediated hypersensitivity responses. However, during repeat experiments, a shift in the time course of the development of this population was observed (from day 10 to day 9), which complicated its use in establishing the phenotypic analysis methodology (data not shown). Although not applicable to this methodology, the surface IgE expressing B cell population generated by TDI exposure was greater than the surface IgE expressing B cell population previously detected in animals responding to parasitic infection (Katona *et al.*, 1983). The ability of this method to generate a surface IgE expressing B cell population of this size may be useful for generating sufficient cell numbers to perform functional studies of this cell population.

The majority of the draining lymph node IgE+ B cell population in the TDI and DNFB-exposed animals consisted of B cells staining IgE+ by detection of soluble IgE bound to the B cell surface. Since CD23, the low affinity IgE receptor, is primarily expressed on the B cell surface in the mouse, it seems likely that soluble IgE is binding the B cell via CD23 (Waldschmidt *et al.*, 1988). The percent of CD23+ B cells with bound IgE would depend on the level of soluble IgE present in the draining lymph node environment, as determined by local (draining lymph nodes) and/or systemic (bone marrow and spleen) IgE production. It would be expected that the development of the IgE+B220+ population would positively correlate with total serum IgE levels, as was the case in most allergen-exposed animals (Tables 4, 6, and 7). However, in some cases, the IgE+B220+ draining lymph node cell population was increased following allergen exposure, while the total serum IgE levels were unchanged. In these cases, local, draining lymph node IgE production may be sufficient to provide soluble IgE that binds B cell surface CD23, resulting in an increased IgE+B220+ draining lymph node population without elevating total serum IgE levels (Table 4, 0.15% DNFB; and Table 7, 0.05% DNFB). The reproducible measurement of the IgE+B220+ population prior to acid stripping allowed for its use as an end point in the development of this methodology.

The phenotypic analysis method was incorporated into an established mouse ear-swelling irritancy assay and the potential of this irritancy/phenotypic analysis assay to identify and

TABLE 8

Paradigm for the Identification and Differentiation of Chemicals with the Capacity to Induce Irritation, IgE-mediated, or T cell-mediated Hypersensitivity Responses

Chemical	Ear swelling	Percent B220+	Percent IgE+B220+
Irritant	+	-	-
IgE-mediated	±	+	% IgE+B220+ $\cong$ % B220+ <sup>a</sup>
T cell-mediated	±	+	% IgE+B220+ < 50% B220+ <sup>b</sup>

<sup>a</sup> At its peak, the percentage of IgE+B220+ cells will approximately equal the percentage of B220+ cells. Additionally, the percent of IgE+B220+ cells will become significantly increased at the same test concentration that significantly elevates the percent of B220+ cells.

<sup>b</sup> At its peak, the percent of IgE+B220+ cells will remain below 50% of the percentage of B220+ cells. Furthermore, the concentration of chemical required to significantly increase the IgE+B220+ population will be significantly greater than the concentration necessary to elevate the B220+ population.

differentiate chemicals with the capacity to elicit irritation, IgE-mediated, or T cell-mediated responses was investigated with a well characterized panel of compounds. Irritants, IgE-mediated and T cell-mediated, hypersensitivity inducing sensitizers were identified and discriminated by their differential ability to modify the 3 assay parameters: percent ear swelling, percent B220+ cells, and percent IgE+B220+ cells. Based upon the results of these studies, the following paradigm was developed for further testing (Table 8).

Exposure to known chemical irritants should result in increased ear swelling, without significantly altering the B220+ or IgE+B220+ populations. Sensitizer exposure (IgE- or T cell-mediated) may or may not result in increased ear swelling, depending on the chemical's capacity to induce irritation and will dose-responsively increase the B220+ population. Following exposure to IgE-mediated, hypersensitivity inducing chemicals, the initial increase in the IgE+B220+ population should occur at the same test concentration that initially elevates the B220+ population. Furthermore, the percent of IgE+B220+ cells should approach a level similar to the percent of B220+ cells. T cell-mediated, hypersensitivity inducing allergens may show a moderate elevation in the IgE+B220+ population. However, with T cell-mediated, hypersensitivity inducing allergens, the increase in the IgE+B220+ population should occur at a higher chemical concentration than is necessary to significantly elevate the draining lymph node B220+ population. At its peak, the IgE+B220+ population should only approach approximately 50% of the level of the B220+ population. This difference in the ability of IgE-mediated, hypersensitivity inducing allergens and T cell-mediated, hypersensitivity inducing allergens to modulate the IgE+B220+ population in reference to the B220+ population may serve as a method for differentiating the two responses. As demonstrated, to evaluate these markers

it is essential to test the candidate compounds over a range of concentrations.

Previous comparisons between the available murine assays and guinea pig models for the identification of IgE inducing allergens determined that murine assays were more rapid, cost effective, and easily performed (Briatico-Vangosa *et al.*, 1994). In a single assay, this method examines the potential for increased IgE production similar to the murine total IgE ELISA and identifies sensitizers, as does the local lymph node assay. In addition, the assay may have the potential to differentiate irritants from sensitizers and IgE inducing sensitizers from T cell-mediated, hypersensitivity inducing sensitizers.

In conclusion, using the IgE+B220+ marker, the development of an IgE+ draining lymph node B cell population was observed in animals following exposure to known human IgE inducing allergens. Time course experiments determined the population peaked 10 days following initial chemical exposure. The IgE+ B cell population was determined to be composed of two subpopulations represented by surface IgE expressing B cells and B cells staining IgE+ by detection of soluble IgE bound to the cell surface, presumably via CD23. In most cases, development of the IgE+ B cell population was dose-responsive and correlated with the animals' total serum IgE levels, another known indicator of IgE-mediated hypersensitivity responses. The B220 marker was found to be dose-responsively elevated by both IgE inducing and T cell-mediated allergens and unaffected by irritants. By evaluating ear swelling, as well as the levels of the B220+ and IgE+B220+ populations over a range of test concentrations, the combined irritancy/phenotypic analysis method may be useful for the identification and differentiation of chemicals able to induce irritation, IgE-mediated, or T cell-mediated hypersensitivity responses. These studies have focused on dermal exposure to low molecular weight chemicals, but preliminary data also indicates that a modified form of this method may be applicable to respiratory exposure with high molecular weight proteins.

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