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The Determination of Draining Lymph Node Cell Cytokine mRNA Levels in BALB/c Mice Following Dermal Sodium Lauryl Sulfate, Dinitrofluorobenzene, and Toluene Diisocyanate Exposure

T. Scott Manetz,*,† Denise A. Pettit,‡ and B. Jean Meade†

Department of Pharmacology and Toxicology, Medical College of Virginia/VCU, Richmond, Virginia 23284; †NIOSH/HELD, Morgantown, West Virginia 26505; and ‡Division of Consolidated Laboratory Services, Richmond, Virginia 23219

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Differential modulation has been demonstrated in interleukin-4 (IL-4), IL-10, and interferon gamma (IFN-γ) mRNA and protein secretion patterns of cells isolated from the draining lymph nodes of mice following exposure to T cell and respiratory sensitizers. Using a multiprobe ribonuclease protection assay, the following investigation examined the mRNA expression patterns of multiple cytokines associated with respiratory sensitization for modulation following exposure to chemicals known primarily to induce irritation (sodium lauryl sulfate), respiratory sensitization (toluene diisocyanate), or T cell-mediated hypersensitivity (dinitrofluorobenzene) responses. On days 0 and +5 female BALB/c mice were exposed to either test article or vehicle on the shaven dorsal lumbar region; on days +10 through +12 the mice received test article on the dorsal aspect of each ear. On day +13 animals were euthanized, draining lymph nodes were excised, and mRNA was isolated immediately or following 24 or 48 h of culture in the presence or absence of concanavalin (Con) A. Differential expression of cytokine mRNA was most notable following 24 h incubation with Con A. Modulation of IL-4, -10, and IFN-γ following chemical exposure was consistent with previous studies. In addition, IL-9, -13, and -15 were significantly elevated only following toluene diisocyanate exposure. Further investigations of these cytokines may provide additional insight into the mechanisms of chemically induced respiratory sensitization and provide endpoints for the detection of a chemical's ability to elicit IgE-mediated hypersensitivity responses.

Key Words: **cytokine; IgE; hypersensitivity; interleukin; ribonuclease protection assay; toluene diisocyanate**

Chemical exposure can cause a variety of adverse health effects including IgE-mediated (immediate hypersensitivity) and T cell-mediated (delayed hypersensitivity) responses. IgE-mediated hypersensitivity responses are associated with increased total serum IgE levels, principally involve humoral immunity, and occur within minutes of exposure in sensitized individuals. Conversely, T cell-mediated hypersensitivity responses are inhibitory toward IgE production, predominantly involve cell-mediated immunity, and occur 24 to 72 h following exposure in sensitized individuals.

T cells play a predominant role in the development of IgE and T cell-mediated hypersensitivity responses and two distinct T cell subsets (T helper (Th)1 and Th2) have been identified. T-helper 1 cells promote cell-mediated immunity and are defined by their secretion of interleukin (IL)-2, interferon gamma (IFN-γ), and lymphotoxin (LT). IFN-γ inhibits the proliferation of Th2 cells and favors Th1 cell development (Gajewski and Fitch, 1998; Fong and Mosmann, 1989; Saulnier *et al.*, 1995). IFN-γ has also been shown to inhibit the production of IgE antibody and instead to promote the secretion of IgG_{2a}, an antibody that supports cell-mediated immune responses (Finkelman *et al.*, 1988a). Th2 cells are recognized by their secretion of IL-4, IL-5, IL-6, and IL-10, cytokines that support humoral immune responses. IL-4 is necessary for the production of IgE, and therefore favors the potential for immediate hypersensitivity responses (Finkelman *et al.*, 1988b, 1986). IL-5 synergizes with IL-4 to induce IgE antibody to a variety of antigens (Nagai *et al.*, 1999; Purkerson and Isakson, 1992). The increased proliferation and differentiation of B cells into antibody-secreting plasma cells by IL-6 and IL-10 also encourages a humoral immune response (Kishimoto, 1989; Choe and Choi, 1998). The cell proliferation induced by some Th2 cytokines can be selective. This was demonstrated by the ability of IL-4 and IL-10 to favor Th2 cell proliferation and development over that of Th1 cells (Fernandez-Botran *et al.*, 1988; Bradley *et al.*, 1995; Swain *et al.*, 1990).

Although primarily IgE-mediated (e.g., platinum salts-induced) and T cell-mediated (e.g., dinitrofluorobenzene-induced) disease processes may occur, frequently, and especially in the case of pulmonary responses, multiple mechanisms are involved. The clinical signs and symptoms resulting in asthma may result from numerous disease processes. Even though a strong correlation exists between elevated levels of IgE and increased bronchial reactivity when evaluating responses to

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common areoallergens such as dust mite and pollen, it has been proposed that IgE be seen as a marker for a complex immune response that may include cellular responses and mediators released from mast cells (Platts-Mills, 1992). In cases of asthma resulting from exposure to low-molecular-weight antigens such as the isocyanates, the role of IgE is less clear, with only 10–30% of affected individuals showing elevated IgE levels, and a role for cell-mediated processes has been proposed (Weissman and Lewis, 2000).

Studies into the immunological consequences of dermal exposure to chemical sensitizers have demonstrated the ability of such chemicals to induce cellular proliferation in the lymph nodes draining the exposure site (Kimber *et al.*, 1989). Because many cytokines modulate proliferation and differentiation of lymphoid cells, cytokine profiles in draining lymph nodes have been evaluated as a method of identifying the sensitizing potential of chemicals and of providing a better understanding of the mechanisms of chemically induced allergic responses. Two secretion patterns, closely resembling Th1 and Th2 cytokine secretion profiles, have been observed (Dearman *et al.*, 1997). The profiles induced were dependent on the chemicals' ability to invoke primarily respiratory sensitization or a T cell-mediated hypersensitivity response. Increased levels of IL-2 and IFN-γ were found following T cell-mediated allergen exposure and increased levels of IL-4 and IL-10 were detected following dermal exposure to chemicals with the ability to induce respiratory sensitization. Using the same exposure regimen, similar kinetic profiles were found when comparing cytokine (IL-2, IL-4, IL-10, and IFN-γ) protein levels measured by ELISA, and cytokine mRNA levels determined by RT–PCR. This suggested that the quantification of protein or mRNA levels of cytokines may be useful in evaluating their alteration following dermal exposure to these chemicals (Warbrick *et al.*, 1998; Ryan *et al.*, 1998).

The purpose of these studies was to investigate the mRNA expression profile of an extended panel of cytokines for modulation in the draining lymph nodes of animals following dermal exposure to respiratory or T cell-mediated sensitizers. The method used examined IL-4, IL-10, and IFN-γ message levels to allow for comparison with the results obtained in previous studies. Additionally, other cytokines whose involvement in chemically induced hypersensitivity reactions are not as well characterized (i.e., IL-5, IL-6, IL-9, IL-13, and IL-15) were measured. These studies used the following chemicals known primarily to induce respiratory sensitization, T cell-mediated hypersensitivity responses, or irritation: toluene diisocyanate (TDI), dinitrofluorobenzene (DNFB), and sodium lauryl sulfate (SLS). Toluene diisocyanate is well known for its ability to induce occupational asthma (Bernstein, 1996; Deschamps *et al.*, 1998; Mapp *et al.*, 1994). Topical TDI exposure leads to increased total serum IgE levels and a Th2 cytokine secretion pattern in mice (Manetz and Meade, 1999; Tee *et al.*, 1998; Son *et al.*, 1998; Dearman *et al.*, 1996). Dinitrofluorobenzene is a chemical known for its ability to cause T cell-mediated contact hypersensitivity responses and a Th1 cytokine secretion profile in mice (Phanuphak *et al.*, 1974; Garcia-Perez, 1974; Dearman *et al.*, 1996). Sodium lauryl sulfate is known for its ability to cause irritation (Lee and Maibach, 1995) and was used as a control.

TABLE 1
Design of the Ribonuclease Protection Assay Studies

Group	Induction (days 0 and 5)	Challenge (days 10–12)
A/A	Acetone	Acetone
A/D	Acetone	0.15% DNFB
D/D	0.15% DNFB	0.15% DNFB
A/T	Acetone	1.5% TDI
T/T	1.5% TDI	1.5% TDI
E/E	30% Ethanol	30% Ethanol
E/S	30% Ethanol	40% SLS
S/S	40% SLS	40% SLS

Note. Induction was via dermal application of the chemical to the shaved dorsal lumbar region, and challenge was via dermal application to the dorsal surface of both ears. Animals were euthanized 1 day following final chemical application and the draining lymph nodes were excised aseptically. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge on the ear.

MATERIALS AND METHODS

Animals. Pathogen-free female BALB/c mice ages 8 to 10 weeks (National Cancer Institute Animal Program, Frederick, MD) were used for these studies. Animals arrived at 4 to 6 weeks of age with body weights between 15 and 23 g. Animals were quarantined for 1 week prior to use and assigned to homogenous weight groups for exposure. Mice, individually identified via tail tattoo, were housed four per cage in plastic cages with sawdust bedding. Animal care and use were performed following NIH guidelines. The animals were maintained on a 12-h light/dark cycle with the ambient air at 18–26°C and 40–70% relative humidity. Food (Agway Rat and Mouse Ration; NIH 07) and tap water were available *ad libitum*.

Chemicals. Toluene 2,4-diisocyanate (purity 99.6%), 2,4-dinitrofluorobenzene (purity $\geq$ 99%), sodium lauryl sulfate (purity $\geq$ 95%), and acetone (purity 99.8%), the vehicle for TDI and DNFB, were purchased from Sigma (St. Louis, MO). Ethanol, the SLS vehicle, was purchased from Fisher Scientific (Pittsburgh, PA) and diluted to 30% with deionized water. The concentrations of TDI (1.5%) and DNFB (0.15%) used are recognized as inducing similar levels of proliferation in the local lymph node assay and 40% SLS is known to be significantly irritating in a mouse ear swelling irritancy assay (Manetz and Meade, 1999; Woolhiser *et al.*, 1998).

Exposure and tissue isolation. A modified version of the exposure method described by Dearman *et al.* (1996) was used with four mice per dose group (Table 1). One day prior to initial chemical exposure, the mice were anesthetized and their dorsal lumbar area was shaved. The mice received 50 μl of the test article or the vehicle to the shaved site on day 0 and 5 days later (day 5). After 5 days of rest, the mice received 25 μl of the chemical to the dorsal surface of each ear, once daily for 3 consecutive days (days 10–12). Animals that received the vehicle on the dorsal lumbar area on days 0 and 5 and the test article on the ear on days 10–12, are referred to as "challenged only." Those who received the test article to both locations on days 0, 5, and 10–12 are referred to as "induced/challenged." Twenty-four hours following the final chemical application, the animals were euthanized (day 13) via CO_2 inhalation. The draining cervical lymph nodes, located at the bifurcation of the jugular

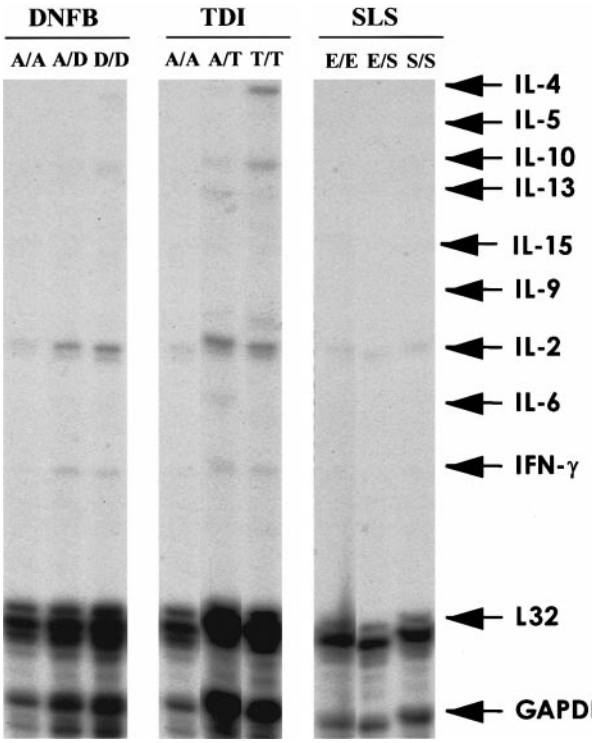

FIG. 1. Ribonuclease protection assay results from mRNA isolated from draining lymph node cells following exposure to DNFB, TDI, or SLS. Animals were exposed as described, and the draining lymph nodes were excised and pooled per group 24 h following final exposure. A representative sample is shown for each exposure condition. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge to the ear.

vein, were aseptically excised and placed into RPMI 1640 growth medium (Sigma) supplemented with 10% fetal calf serum, 25 mM Hepes, 0.2% sodium bicarbonate solution, and 1000 units/ml of penicillin/streptomycin. For the initial time-course study, the lymph nodes of two animals within each dose group were pooled ($N = 2$) in order to allow for a sufficient number of cells. Aliquots of the pooled cells were then taken and the mRNA was isolated immediately or following 24 or 48 h of culture as described below. In those cases in which cell number was ample, cultures were done in duplicate. For the final study, when only a single time point was evaluated (following 24 h of culture), mRNA from individual animals was evaluated.

Lymph node cell preparation and culture. A modified version of the method used by Dearman *et al*. (1996) was used. Lymph nodes were dissociated using the frosted ends of two microscope slides. Viable cell counts were determined using trypan blue exclusion and the cells were centrifuged at 1200 rpm (260*g*) for 10 min. The supernatant was removed and the cells were resuspended in the RPMI to a concentration of 10^7 cells/ml. 5.5×10^6 cells per well were plated into a sterile 48-well plate and incubated at 37°C with 5% CO_2 with or without 2 μg/ml of concanavalin A (Con A) for 24 or 48 h.

RNA isolation. The freshly isolated lymph node cells (5.5×10^6) or the cell cultures were centrifuged as before and the supernatant was removed and discarded. The cells were resuspended in 1 ml of Trizol (Gibco, Grand Island, NY) with RNA isolated according to the manufacturer's instructions. The RNA was vacuum dried (1 h), resuspended in 8 μl of hybridization buffer (PharMingen, San Diego, CA), and stored at -70° C until analysis.

Probe synthesis. A mck-1 probe template set and an *In Vitro* Transcription kit (PharMingen) were used according to the manufacturer's instructions. ^{32}P-labeled complementary RNA probes were generated to the following mouse cytokines: IL-2, -4, -5, -6, -9, -10, -13, -15, IFN-γ, and two mouse control mRNA products (L32 and GAPDH).

Ribonuclease protection assay. For the analysis of draining lymph node cytokine mRNA, the Riboquant Multi-Probe RNase Protection Assay Kit (PharMingen) was employed according to the manufacturer's instructions. The previously isolated RNA samples in the hybridization buffer were incubated with the ^{32}P-labeled mck-1 template set probes for 16 h at 56°C to allow for hybridization. Nonhybridized RNA was destroyed by adding RNase A to the samples. Hybridized RNA was isolated following proteinase K digestion by phenol–chloroform and isoamyl alcohol extraction and ethanol precipitation. The hybrids were then heat denatured and separated on a 5% denaturing polyacrylamide gel. The gels were dried and exposed with Biomax-MR film (Kodak) for 24 to 144 h. For quantification, densitometry analysis was performed using a Molecular Dynamics Computing Densitometer. Analysis was performed using Image Quant Version 3.3 Software for the IBM. For each band, central areas of identical size were analyzed within and between gels for each individual cytokine, thus minimizing the potential for variation of analysis between gels. Overall background was subtracted from each densitometric value. Data presented are the average percentage expression relative to L32 and GAPDH as determined using the following equation:

$$(\text{cytokine/L32} + \text{cytokine/GAPDH})/2 \times 100.$$

For statistical analysis, Cochran's *t* test was used because the sample number per dose group varied (Cochran and Cox, 1975).

RESULTS

Initial studies evaluated mRNA isolated from draining lymph node cells of chemically exposed mice 24 h following the last chemical exposure (Fig. 1; Table 2). With the exception

TABLE 2
Densitometry Analysis of Freshly Isolated Lymph Node Cells

Gp	IFN-γ	IL-10	IL-4
A/A ($N = 1$)	0.42	0.27	0.23
A/D ($N = 3$)	0.81 (0.18)	0.41 (0.12)	0.07 (0.04)
D/D ($N = 1$)	0.51	0.75	0.39
A/T ($N = 2$)	1.06 (0.02)	0.31 (0.31)	0.28 (0.28)
T/T ($N = 2$)	0.80 (0.55)	1.00 (1.00)	2.84 (1.97)
E/E ($N = 1$)	1.97	—	—
E/S ($N = 3$)	0.28 (0.28)	0.3 (0.17)	0.07 (0.07)
S/S ($N = 1$)	2.23	0.49	—

Note. Volumetric densitometry analysis of the cytokine mRNA levels from the samples in Fig. 1. Data presented are the average of their percentage expression compared to the GAPDH and L32 control RNAs. Values in parentheses are the standard error of replicate samples. —, data in which the value was zero or lower after background subtraction. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge on the ear.

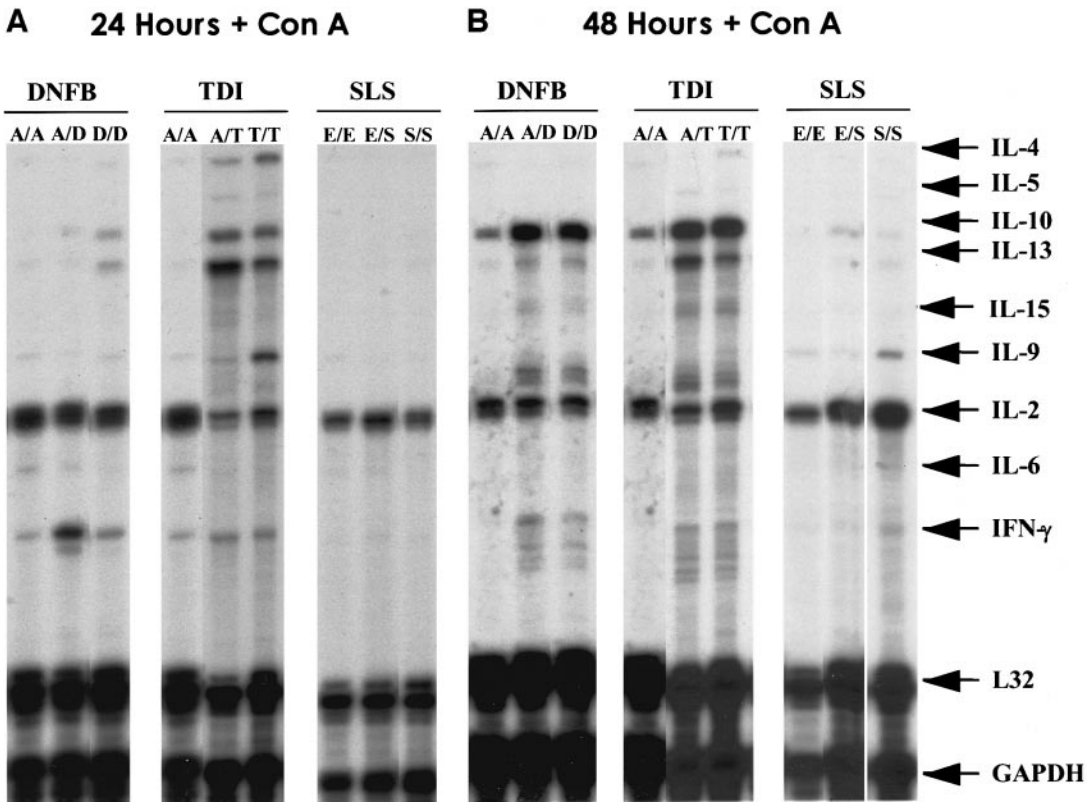

FIG. 2. Cytokine profile of draining lymph node cells following dermal allergen exposure and 24 (A) or 48 h (B) of incubation with Con A (2 μg). Cells isolated from the experiment in Fig. 1 were then cultured in the presence of Con A for the given amount of time, prior to RNA isolation. A representative sample is shown for each exposure condition. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge on the ear.

of an elevation (12-fold) in IL-4 expression following induction and challenge exposure to TDI, minimal alterations in cytokine expression following exposure to the three chemicals were observed in comparison to controls. Interleukin-10 levels were increased 3- to 4-fold over controls in animals that were induced and challenged with DNFB or TDI. Interferon gamma expression was slightly elevated (2- to 3-fold increase) in animals challenged only with DNFB or TDI and induced/challenged with TDI.

Cytokine Profile after 24 h of Culture

During the culture periods, the cells were incubated in the presence or absence of 2 μg/ml Con A, a T cell mitogen known to induce cytokine secretion by allergen-exposed draining lymph node cells (Dearman *et al.*, 1995). In the absence of Con A, overall mRNA expression (cytokine and housekeeping genes) in the 24- and 48-h cultures was too low to allow for characterization (data not shown). Following 24 h of incubation in Con A, the cells isolated from animals exposed to TDI, DNFB, or SLS demonstrated a differential pattern of cytokine mRNA expression (Fig. 2A; Table 3).

When evaluating Th1 cytokines, whose levels are anticipated to be increased following exposure to T cell-mediated sensitizers, the most robust response was observed in IFN-γ levels following challenge only with DNFB (18-fold increase over controls). A 7-fold increase in IFN-γ levels was observed in TDI challenged only or TDI induced/challenged animals. For animals both induced and challenged with DNFB, the level of IFN-γ (3-fold increase compared to control) was lower than that observed in animals exposed to TDI and similar to that seen in animals challenged only with SLS. Interleukin-2 expression was not significantly altered in any of the exposure groups.

More of a differential response was obtained when Th2 cytokines and cytokines more frequently associated with an IgE-mediated response were evaluated. The most robust responses were observed in IL-9, -13, -15, and -10 mRNA expression following exposure to TDI. The mRNA levels for IL-9 were only elevated in TDI-exposed animals, with a 19-fold increase observed in the challenged only group and a 14-fold increase observed in the induced/challenged group compared to the acetone control. Interleukin-15 mRNA expression was increased 116-fold in the TDI-challenged only animals, with minimal alterations in the other exposure groups compared to the controls. TDI-challenged only and induced/challenged animals demonstrated an approximate 60-fold in-

TABLE 3
Densitometry Analysis of 24-h Cultures with Concanavalin A

Gp	IFN-γ	IL-9	IL-15	IL-13	IL-10	IL-5	IL-4
A/A	1.09	0.17	0.35	0.37	0.14	0.10	0.37
(N = 2)	(0.09)	(0.17)	(0.01)	(0.10)	(0.14)	(0.02)	(0.17)
A/D	19.8	0.43	0.12	1.62	0.74	0.19	0.32
(N = 2)	(5.16)	(0.18)	(0.06)	(1.36)	(0.24)	(0.19)	(0.10)
D/D	3.18	0.44	1.64	1.33	1.04	—	0.30
(N = 1)							
A/T	7.88	3.27	40.5	23.5	19.7	1.55	2.67
(N = 1)							
T/T	7.86	2.29	0.21	21.4	12.2	1.42	10.6
(N = 1)							
E/E	1.22	0.37	0.08	0.45	0.18	0.16	0.3
(N = 2)	(0.21)	(0.03)	(0.08)	(0.25)	(0.10)	(0.02)	(0.07)
E/S	4.86	1.29	0.16	1.25	0.86	0.03	0.20
(N = 2)	(2.11)	(0.01)	(0.16)	(0.53)	(0.76)	(0.03)	(0.20)
S/S	1.14	0.47	0.01	0.49	0.38	0.32	0.26
(N = 1)							

Note. Volumetric densitometry analysis of the cytokine mRNA levels from the samples in Fig. 2A. Data presented are the average of their percentage expression compared to the GAPDH and L32 control RNAs. Values in parentheses are the standard error of replicate samples. —, data in which the value was zero or lower after background subtraction. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge on the ear.

crease in IL-13 mRNA levels compared to controls, whereas exposure to DNFB, challenge only, and induction/challenge produced only a 4-fold increase in IL-13. Interleukin-10 mRNA expression was elevated in TDI challenged only (141-fold) and induced/challenged animals (87-fold) compared to the control. Exposure to DNFB or SLS resulted in no more than a 7-fold increase in IL-10. IL-5 mRNA levels were elevated approximately 15-fold in the TDI challenged or induced/challenged animals; and they were relatively unaffected by DNFB or SLS exposure. Increased levels of IL-4 mRNA were observed in TDI-exposed animals, with a 7-fold increase seen in the TDI-challenged only animals and a 29-fold increase in the TDI induced/challenged group.

Cytokine Profile after 48 h of Culture

For the Th1 cytokines, IFN-γ and IL-2, the trends of expression induced by DNFB, TDI, or SLS following 48 h of culture with Con A were similar to those observed after 24 h of culture with an overall decrease in expression (Fig. 2B; Table 4). This pattern was also observed with the Th2 cytokines, IL-9 and IL-5. The differential response in IL-6 expression following DNFB or TDI exposure was more pronounced after 48 h of culture with a 3-fold increase observed following induction or induction/challenge with DNFB and a 13-fold increase following exposure to TDI. IL-15 was expressed to a much greater extent in animals challenged only with TDI (116-fold) compared to animals induced and challenged with TDI (no change) at the 24-h time point. Following 48 h of culture in the presence of Con A, there were no differences in IL-15 expression between these two groups (14-fold increase). The patterns of IL-13 gene expression following TDI or DNFB exposure were similar following 24 and 48 h of culture. IL-4 and IL-10 were differentially expressed in animals exposed to TDI compared to DNFB following 24 h of culture. Following 48 h of culture, no differences in these cytokine profiles were observed related to chemical exposure.

Evaluation of Samples from Individual Animals

Given that the differences in response to T cell-mediated and respiratory sensitizers were most notable following 24 h of culture in Con A, subsequent studies were conducted to further evaluate this time point. In a separate study, animals were exposed to TDI, DNFB, SLS, ethanol, or acetone following the same dosing regimen as shown in Table 1. By analyzing a single time point, lymph node cell number was no longer a limiting factor, allowing for the analysis of samples from individual animals and statistical analysis of the results. Figure 3 shows the reproducibility of cytokine profiles between animals following chemical exposure. These profiles are similar to those seen when pooled samples were examined in Fig. 2A. Densitometry allowed for statistical analysis of data from individual animals, which revealed increased mRNA levels of IL-9, IL-15, IL-13, and IL-10 in animals challenged only with TDI compared to the acetone control group (Table 5). Except for IL-15, the same cytokines were significantly elevated in the TDI induced/challenged animals. Although IL-4 levels were elevated following TDI exposure, the numbers were not statistically significant given the variability in the response.

None of the cytokines assayed were significantly elevated following DNFB induction or induction/challenge. Although

TABLE 4
Densitometry Analysis of 48-h Cultures with Concanavalin A

Gp	IFN-γ	IL-6	IL-2	IL-9	IL-15	IL-13	IL-10	IL-5	IL-4
A/A	0.78	0.20	30.3	0.19	0.31	0.23	3.04	0.10	0.40
(*N* = 2)	(0.38)	(0.20)	(5.12)	(0.19)	(0.13)	(0.23)	(3.04)	(0.01)	(0.29)
A/D	3.52	0.69	24.5	0.46	1.37	2.54	37.0	0.08	0.05
(*N* = 2)	(0.09)	(0.28)	(1.25)	(0.25)	(0.57)	(0.15)	(2.33)	(0.06)	(0.05)
D/D	1.68	0.72	20.8	0.42	0.56	1.42	23.7	0.15	0.19
(*N* = 2)	(0.83)	(0.33)	(0.83)	(0.29)	(0.56)	(1.42)	(12.2)	(0.03)	(0.19)
A/T	3.97	2.61	23.6	1.16	4.33	18.3	40.3	0.52	0.20
(*N* = 1)									
T/T	4.05	2.68	33.8	1.87	4.23	9.14	42.5	0.13	0.6
(*N* = 1)									
E/E	0.81	0.65	25.6	0.97	0.06	—	0.32	0.06	0.13
(*N* = 1)									
E/S	1.88	1.43	49.5	1.1	0.37	1.38	7.06	0.17	0.14
(*N* = 2)	(1.34)	(0.98)	(9.09)	(0.37)	(0.25)	(1.2)	(6.01)	(0.03)	(0.14)
S/S	2.40	1.66	45.7	2.54	0.01	0.72	0.40	0.23	0.09
(*N* = 1)									

Note. Volumetric densitometry analysis of the cytokine mRNA levels from the samples in Fig. 2B. Data presented are the average of their percentage expression compared to the GAPDH and L32 control RNAs. Values in parentheses are the standard error of replicate samples. —, data in which the value was zero or lower after background subtraction. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge to the ear.

the mean expression levels of IFN-γ and IL-2 were elevated several-fold higher than the acetone control, considerable variation in response was observed between animals. Following SLS exposure, no significant differences in the levels of mRNA for any of the cytokines were detected compared to the ethanol control (data not shown).

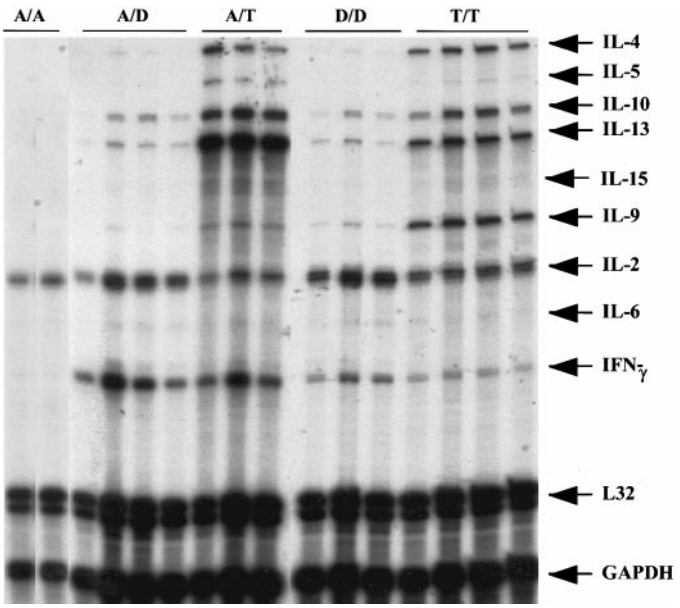

FIG. 3. Evaluation of cytokine mRNA levels for individual animals. Draining lymph node cells were isolated from test article or vehicle-exposed animals and cultured with Con A for 24 h prior to RNA isolation. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge on the ear.

DISCUSSION

Using the ribonuclease protection assay, these studies have evaluated cytokine mRNA production following Con A stimulation by excised draining lymph node cells from mice following dermal exposure to low-molecular-weight chemicals. ConA stimulation of cell lines (Iwai *et al.*, 1992; Harwell *et al.*, 1980) and lymphoid cells from blood (Podwinska *et al.*, 2000; Muret *et al.*, 2000) and lymphoid tissues (Garn *et al.*, 2000; Hallquist *et al.*, 2000; Abrahamsohn *et al.*, 2000; Kang *et al.*, 1999) has been used extensively to investigate the role of cytokines in the pathogenesis of disease. Numerous studies have demonstrated strong correlations between cytokine production following *ex vivo* mitogen stimulation of lymphoid cells and *in vivo* cytokine production or effects. Differential resistance to the intestinal parasite *Trichuris muris* between CBA and B10.BR mice is known to be mediated by differential T helper responses, Th2 and TH1, respectively. Consistent with the ability of the of the CBA mice to eliminate the parasite, lymphocytes recovered from infected CBA mice and stimulated with Con A produced elevated levels of IL-5, whereas lymphocytes recovered from infected B10.BR mice (which are permissive for *T. muris*) produced high levels of INF-γ upon Con A stimulation (Soltys *et al.*, 1999). The opposite response was seen in the case of infection with the causative agent of human granulocytic ehrlichiosis, an obligate intracellular bacterium, in which Th1 responses play an important role early in the infection. C57BL/6 mice demonstrated increased levels of IFN-γ during the early stages of infection with the bacterium, and splenocytes from infected animals demonstrated elevated levels of INF- γ and decreased levels of

TABLE 5
Evaluation of Cytokine mRNA Levels for Individual Animals Following 24 h of Incubation

Group	A/A	A/D	D/D	A/T	T/T
IL-9	0.41 ± 0.21	0.75 ± 0.30	1.50 ± 0.62	10.5 ± 1.56[a]	24.7 ± 3.70[b]
IL-15	0.27 ± 0.16	0.38 ± 0.17	0.51 ± 0.08	12.1 ± 1.57[a]	2.32 ± 0.53
IL-13	0.22 ± 0.22	1.79 ± 0.59	1.87 ± 0.69	58.0 ± 2.89[b]	23.0 ± 3.53[b]
IL-10	0.38 ± 0.29	2.19 ± 0.67	1.84 ± 0.88	26.9 ± 4.18[a]	11.5 ± 3.28[a]

Note. Volumetric densitometry analysis of the cytokine mRNA levels from the samples in Fig. 3. The data presented are the dose group mean ± SE. $N = 4$ for A/D and T/T, $N = 3$ for D/D and A/T, and $N = 2$ for A/A. One sample for the D/D and A/T and two samples for the A/A exposure groups could not be processed due to insufficient RNA isolation. For each cytokine for the individual animals, the densitometric value used was the average of percentage expression compared to both the GAPDH and L32 control RNAs. A, acetone; D, DNFB; T, TDI; E, ethanol; S, SLS. The first letter denotes exposure on the flank; the second letter denotes challenge on the ear.

[a] $p < 0.05$ compared to the acetone group using Cochran's t test.

[b] $p < 0.01$ compared to the acetone group using Cochran's t test.

IL-4 upon stimulation with ConA (Akkoyunlu and Fikrig, 2000). In a model of autoimmunity, treatment with the protein UK-114 has been demonstrated to dose-dependently inhibit the development of Th1-induced diabetis in NOD mice. *In vitro* Con A stimulation of splenocytes recovered from BALB/c mice following 2 weeks of exposure to UK-114 demonstrated an increase in IL-4 production and a decrease in the production of TNF-α, INF-γ, and IL-2 (Panerai *et al.*, 1999). An evaluation of the serum of patients with Hashimoto's thyroiditis demonstrated an elevation in IL-2, TNF-α, and INF-γ, which correlated with an increase in IL-2 and TNF- α upon ConA stimulation of their blood lymphocytes (Drugarin *et al.*, 2000). The ribonuclease protection assay was chosen for use in these studies due to its ability to simultaneously detect several mRNA species in a single sample. The multiprobe ribonuclease protection assay system incorporates a series of DNA templates into one reaction, which allows comparative analysis of different mRNAs within samples, and, with housekeeping gene probes included, the mRNA levels between samples can also be compared. Like RT–PCR, which would require assaying each mRNA separately, ribonuclease protection assay is a highly sensitive and specific method for detection and quantification of mRNA; however, since there is no amplification of the signal as with RT–PCR, there is less of an opportunity to introduce and amplify experimental error. Therefore, in these studies, combining the use of mitogen driven lymphocytes from chemically exposed animals with the ribonuclease protection assay allowed for the rapid identification of the mRNA of a panel of cytokines from single samples.

Many chemicals have been associated with asthma without a complete understanding of the underlying mechanisms. With knowledge of the mechanisms involved, hazard identification and prevention methods can significantly improve. Most studies related to investigations of cytokine modulation following chemical exposure have focused on the evaluation of the production of IL-4, IL-10, and IFN-γ to determine the dermal sensitization potential of chemicals and to differentiate contact from respiratory sensitizers (Dearman *et al.*, 1999). The RPA patterns of IL-4, IL-10, and IFN-γ mRNA expression observed in these studies following TDI and DNFB exposure were consistent with those previously reported for respiratory and T cell-mediated sensitizers (Dearman *et al.*, 1996, Warbrick *et al.*, 1998). The increased expression of IFN-γ mRNA following DNFB exposure was only observed following induction with DNFB during the sensitization phase. When animals were induced and then challenged with DNFB, the levels of IFN-γ produced following 24 h of culture in Con A were lower than those observed following induction or induction and challenge with TDI.

Expression of mRNA of other Th2 cytokines and cytokines associated with asthmatic responses were also found to be markedly elevated following exposure to TDI, but not to DNFB or SLS. Lymph node cells from TDI-exposed animals expressed elevated levels of IL-9 and IL-13. This profile was reproducible and statistically significant compared with results from the control group. Human studies have demonstrated an increased expression of IL-9 mRNA in the lung tissue in association with asthma (Levitt *et al.*, 1999; Shimbara *et al.*, 2000). Interleukin-9 has the ability to encourage IgE production in mice (Petit-Frere *et al.*, 1993) and to induce the proliferation and differentiation of mast cells, one of the main effector cells in IgE-mediated responses. It has also been shown to have a distinct role in globlet cell hyperplasia and mucus production (Townsend *et al.*, 2000). Studies suggest that IL-9 is largely produced by mast cells following activation (Moeller *et al.*, 1990; Hultner *et al.*, 2000). In these studies IL-9 was found to be upregulated early in mice following induction (challenge-only animals) and to remain elevated in animals following both induction and challenge. IL-13 is a Th2 cell cytokine that shares many of the biological effects of IL-4, including an influence on IgE production (McKenzie *et al.*, 1999). In addition, IL-13 is necessary for the induction of airway hyperresponsiveness and other characteristics of allergic asthma in mice (Grunig *et al.*, 1998; Wills-Karp *et al.*, 1998). A genetic polymorphism of the IL-13 gene found in humans correlated with increased serum IL-13 levels and

asthma (Heinzmann *et al.,* 2000). This suggests a central role for IL-13 in asthma. Like IL-9, IL-13 levels were elevated following both induction and challenge in previously sensitized mice.

Although isocyanate exposure is one of the most frequent causes of occupational asthma, the underlying mechanisms are not completely understood. Pharmacological, irritant, and immunological mechanisms have been proposed (Bernstein, 1996; Deschamps *et al.,* 1998; Mapp *et al.,* 1994). In studies of isocyanate-exposed workers, isocyanate-specific antibodies have been detected in only an average of 15% of symptomatic patients (Deschamps *et al.,* 1998). TDI has been shown to induce high levels of total IgE in mice (Potter and Wederbrand, 1995; Manetz and Meade, 1999; Scheerens *et al.,* 1999 Shimbara *et al.,* 2000) and has also been shown to have the ability to elicit T cell-mediated responses (Kim *et al.,* 1998; Thorne *et al.,* 1987). Interleukin-15 has been demonstrated to be involved in pathways leading to both IgE- and cell-mediated responses. Interleukin-15 promotes T cell proliferation, is involved in natural killer cell development and cytolytic activity, and can induce mast cell growth, therefore promoting IgE-mediated responses (Tagaya *et al.,* 1996). In these studies, IL-15 mRNA was elevated following induction with TDI. In animals both induced and subsequently challenged with TDI, levels of IL-15 expression were not significantly elevated above those in the control. Elevations in IFN-γ, a Th1 cytokine, have also been demonstrated following dermal TDI exposure (Vanderwiel *et al.,* 2000). Consistent with these findings, IFN-γ levels were elevated approximately sevenfold in animals either induced or induced and challenged with TDI.

Dermal exposure to TDI, a potent respiratory sensitizer, induced a distinct cytokine mRNA profile in the lymph nodes draining the exposure site. As is consistent with previous studies, IL-4 and IL-10, cytokines known to support IgE-mediated responses, were elevated following TDI exposure. In addition, these studies demonstrate the elevation of IL-9, IL-13, and IL-15. Though preliminary, these studies offer insight into the mechanisms of respiratory hypersensitivity responses induced by dermal allergen exposure and may offer additional endpoints for methods that screen chemicals for their ability to induce pulmonary hypersensitivity responses.

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REFERENCES

Abrahamsohn, I. A., da Silva, A. P., and Coffman, R. L. (2000). Effects of interleukin-4 an treatment on resistance to *Trypanosoma cruzi*. *Infect. Immun.* **68,** 1975–1979.

Akkoyunlu, M., and Fikrig, E. (2000). Gamma interferon dominates the murine cytokine response to the agent of human granulocytic ehrlichiosis and helps to control the degree of early rickettsemia. *Infect. Immun.* **68(4),** 1827–1833.

Bernstein, J. (1996). Overview of diisocyanate occupational asthma. *Toxicology* **111,** 181–189.

Bradley, L. M., Yoshimoto, K., and Swain, S. L. (1995). The cytokines IL-4, IFN-gamma, and IL-12 regulate the development of subsets of memory effector helper T cells in vitro. *J. Immunol.* **155,** 1713–1724.

Choe, J., and Choi, Y. S. (1998). IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. *Eur. J. Immunol.* **28,** 508–515.

Cochran, W., and Cox, G. (1975). *Experimental Designs.* Wiley, New York.

Dearman, R. J., and Kimber, I. (1999). Cytokine fingerprinting: characterization of chemical allergens. *Methods* **19,** 56–63.

Dearman, R. J., Basketter, D. A., and Kimber, I. (1995). Differential cytokine production following chronic exposure of mice to chemical respiratory and contact allergens. *Immunology* **86,** 545–550.

Dearman, R. J., Basketter, D. A., and Kimber, I. (1996). Characterization of chemical allergens as a function of divergent cytokine secretion profiles induced in mice. *Toxicol. Appl. Pharmacol.* **138,** 308–316.

Dearman, R. J., Smith, S., Basketter, D. A., and Kimber, I. (1997). Classification of chemical allergens according to cytokine secretion profiles of murine lymph node cells. *J. Appl. Toxicol.* **17,** 53–62.

Deschamps, F., Prevost, A., Lavaud, F., and Kochman, S. (1998). Mechanisms of occupational asthma induced by isocyanates. *Ann. Occup. Hyg.* **42,** 33–36.

Drugarin, D., Negru, S., Koreck, A., Zosin, I., and Cristea, C. (2000). The pattern of a T(H)1 cytokine in autoimmune thyroiditis. *Immunol. Lett.* **71,** 73–77.

Fernandez-Botran, R., Sanders, V. M., Mosmann, T. R., and Vitetta, E. S. (1988). Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *J. Exp. Med.* **168,** 543–558.

Finkelman, F. D., Katona, I. M., Mosmann, T. R., and Coffman, R. L. (1988a). IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J. Immunol.* **140,** 1022–1027.

Finkelman, F. D., Katona, I. M., Urban, J. F., Holmes, J., Ohara, J., Tung, A. S., Sample, J. V., and Paul, W. E. (1988b). IL-4 is required to generate and sustain in vivo IgE responses. *J. Immunol.* **141,** 2335–2341.

Finkelman, F. D., Katona, I. M., Urban, J. F., Snapper, C. M., Ohara, J., and Paul, W. E. (1986). Suppression of in vivo polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1. *Proc. Natl. Acad. Sci. USA* **83,** 9675–9678.

Fong, T. A., and Mosmann, T. R. (1989). The role of IFN-gamma in delayed-type hypersensitivity mediated by Th1 clones. *J. Immunol.* **143,** 2887–2893.

Gajewski, T. F., and Fitch, F. W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* **140,** 4245–4252.

Garcia-Perez, A. (1974). Occupational dermatitis from DNFB with cross sensitivity to DNCB. *J. Immunol.* **112,** 115–123.

Garn, H., Friedetzky, A., Kirchner, A., Jager, R., and Gemsa, D. (2000). Experimental silicosis: A shift to a preferential IFN-gamma-based Th1 response in thoracic lymph nodes. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **278,** 1221–1230.

Grunig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. S. Mohrs, M., Donaldson, D. D., Locksley, R. M., and Corry, D. B. (1998). Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282,** 2261–2263.

Hallquist, N., Hakki, A., Wecker, L., Friedman, H., and Pross, S. (2000). Differential effects of nicotine and ageing on splenocyte proliferation and

the production of Th1- versus Th2-type cytokines. *Proc. Soc. Exp. Biol. Med.* **224**, 141–146.

Harwell, L., Skidmore, B., Marrack, P., and Kappler, J. (1980). Concanavalin A-inducible, interleukin-2-producing T cell hybridoma. *J. Exp. Med.* **152,** 893–904.

Heinzmann, A., Mao, X. Q., Akaiwa, M., Kreomer, R. T., Gao, P. S., Ohshima, K., Umeshita, R., Abe, Y., Braun, S., Yamashita, T., Roberts, M. H., Sugimoto, R., Arima, K., Arinobu, Y., Yu, B., Kruse, S., Enomoto, T., Dake, Y., Kawai, M., Shimazu, S., Sasaki, S., Adra, C. N., Kitaichi, M., Inoue, H., Yamauchi, K., Tomichi, N., Kurimoto, F., Hamasaki, N., Hopkin, J. M., Izuhara, K., Shirakawa, T., and Deichmann, K. A. (2000). Genetic variants of IL-13 signaling and human asthma and atopy. *Hum. Mol. Genet.* **9(4),** 549–559.

Hultner, L., Kolsch, S., Stassen, M., Kaspers, U., Kremer, J. P., Mailhammer, R., Moeller, J., Broszeit, H., and Schmitt, E. (2000). In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9. *J Immunol.* **164(11),** 5556–5563.

Iwai, Y., Bickel, M., Cohen R. B., and Pluznik, D. H. (1992). Concanavalin A-induced granulocyte-macrophage colony-stimulating factor production in a murine T-cell line is post transcriptionally controlled. *Exp. Hematol.* **20,** 271–275.

Kang, L. C., Kerben, M. J., Ugen, K. E., and Specter, S. C. (1999). Modulation of ex vivo cytokine production by spelocytes using in vitro combined therapeutics in murine retroviral model. *DNA Cell Biol.* **18,** 585–592.

Kim, Y. S., Maslinski, W., Zheng, X. X., Stevens, A. C., Li, X. C., Tesch, G. H., Kelley, V. R., and Strom, T. B. (1998). Targeting the IL-15 receptor with an antagonist IL-15 mutant/Fc gamma2a protein blocks delayed-type hypersensitivity. *J. Immunol.* **160,** 5742–5748.

Kimber, I., Hilton, J., and Weisenberger, C. (1989). The murine local lymph node assay for identification of contact allergens: A preliminary evaluation of in situ measurement of lymphocyte proliferation. *Contact Dermatitis* **21,** 215–220.

Kishimoto, T. (1989). The biology of interleukin-6. *Blood* **74,** 1–10.

Lee, C. H., and Maibach, H. I. (1995). The sodium lauryl sulfate model: An overview. *Contact Dermatitis* **33,** 1–7.

Levitt, R. C., McLane, M. P., MacDonald, D., Ferrante, V., Weiss, C., Zhou, T., Holroyd, K. J., and Nicolaides, N. C. (1999). IL-9 pathway in asthma: New therapeutic targets for allergic inflammatory disorders. *J. Allergy Clin. Immunol.* **103,** S485–S491.

Manetz, T. S., and Meade, B. J. (1999). 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. *Toxicol. Sci.* **48,** 206–217.

Mapp, C. E., Saetta, M., Maestrelli, P., Di Stefano, A., Chitano, P., Boschetto, P., Ciaccia, A., and Fabbri, L. (1994). Mechanisms and pathology of occupational asthma. *Eur. Respir. J.* **7,** 544–554.

McKenzie, G. J., Fallon, P. G., Emson, C. L., Grencis, R. K., and McKenzie, A. N. (1999). Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *J. Exp. Med.* **189,** 1565–1572.

Moeller, J., Hultner, L., Schmitt, E., Breuer, M., and Dormer, P. (1990). Purification of MEA, a mast cell growth-enhancing activity, to apparent homogeneity and its partial amino acid sequencing. *J. Immunol.* **144,** 4231–4234.

Muret, J., Marie, C., Fitting C., Payen D., and Cavaillon, J. M. (2000). Ex vivo T-lymphocyte derived cytokine production in SIRS patients is influenced by experimental procedures. *Shock* **13,** 169–174.

Nagai, H., Ueda, Y., Tanaka, H., Hirano, Y., Nakamura, N., Inagaki, N., Takatsu, K., and Kawada, K. (1999). Effect of overproduction of interleukin 5 of dinitrofluorobenzene-induced allergic cutaneous response in mice. *J. Pharmacol. Exp. Ther.* **288,** 43–50.

Panerai, A. E., Sacerdote P., Bianchi, M., Nicoletti, F., Manfredi, B., Gaspani, L., Bartorelli, A., Ceciliani, F., and Ronchi, S. (1999). Chronic administration of UK-114, a multifunctional emerging protein, modulates the Th1/Th2 cytokine pattern and experimental autoimmune diseases. *Ann. NY Acad. Sci.* **876,** 229-235.

Petit-Frere, C., Dugas, B., Braquet, P., and Mencia-Huerta, J. M. (1993). Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine lymphocytes. *Immunology* **79,** 146–151.

Phanuphak, P., Moorhead, J. W., and Claman, H. N. (1974). Tolerance and contact sensitivity to DNFB in mice. I. In vivo detection by ear swelling and correlation with in vitro cell stimulation. *J. Immunol.* **112,** 115–123.

Platts-Mills, T. (1992). Mechanisms of bronchial reactivity: The role of immunoglobulin E. *Am. Rev. Respir. Dis.* **145,** S44–S47.

Podwinska, J., Lusiak, M., Zaba, R., and Bowszyc, J. (2000). The pattern and level of cytokines secreted by Th1 and Th2 lymphocytes of syphelitic patients correlate to the progression of the disease. *FEMS Immunol. Med. Microbiol.* **28,** 1–14.

Potter, D. W., and Wederbrand, K. S. (1995). Total IgE antibody production in BALB/c mice after dermal exposure to chemicals. *Fundam. Appl. Toxicol.* **26,** 127–135.

Purkerson, J. M., and Isakson, P. C. (1992). Interleukin 5 (IL-5) provides a signal that is required in addition to IL-4 for isotype switching to immunoglobulin (Ig) G1 and IgE. *J. Exp. Med.* **175,** 973–982.

Ryan, C. A., Dearman, R. J., Kimber, I., and Gerberick, F. (1998). Inducible interleukin 4 (IL-4) production and mRNA expression following exposure of mice to chemical allergens. *Toxicol. Lett.* **94,** 1–11.

Saulnier, M., Huang, S., Aguet, M., and Ryffel, B. (1995). Role of interferon-gamma in contact hypersensitivity assessed in interferon-gamma receptor-deficient mice. *Toxicology* **102,** 301–312.

Scheerens, H., Buckley, T. L., Muis, T. L., Garssen, J., Dormans, J., Nijkamp, F. P., and Van Loveren, H. (1999). Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. *Am. J. Respir. Crit. Care. Med.* **159,** 1074–1080.

Shimbara, A., Christodoulopoulos, P., Soussi-Gounni, A., Olivenstein, R., Nakamura, Y., Levitt, R. C., Nicolaides, N. C., Holroyd, K. J., Tiscopoulos, A., Lafitte, J.J., Wallaert, B., and Hamid, Q. A. (2000). IL-9 and its receptor in allergic and nonallergic lung disease: Increased expression in asthma. *J. Allergy Clin. Immunol.* **105,** 108–115.

Soltys, J., Goyal, P. K., and Wakelin, D. (1999). Cellular immune responses in mice infected with the intestinal nematode *Trichus muris*. *Exp. Parasitol.* **92,** 40–47.

Son, M., Lee, M., Kim, Y. T., Youn, J. K., and Park, H. (1998). Heterogeneity of IgE response to TDI-HSA conjugates by ELISA in toluene diisocyanate (TDI)-induced occupational asthma (OA) patients. *J. Korean Med. Sci.* **13,** 147–152.

Swain, S. L., Weinberg, A. D., English, M., and Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* **145,** 3796–3806.

Tagaya, Y., Burton, J. D., Miyamoto, Y., and Waldmann, T. A. (1996). Identification of a novel receptor/signal transduction pathway for IL-15/T in mast cells. *EMBO J.* **15,** 4928–4939.

Tee, R. D., Cullinan, P., Welch, J., Burge, P. S., and Newman-Taylor, A. J. (1998). Specific IgE to isocyanates: A useful diagnostic role in occupational asthma. *J. Allergy Clin. Immunol.* **101,** 709–715.

Thorne, P. S., Hillebrand, J. A., Lewis, G. R., and Karol, M. H. (1987). Contact sensitivity by diisocyanates: Potencies and cross-reactivities. *Toxicol. Appl. Pharmacol.* **87,** 155–165.

Townsend, M., Padraic, F., Matthews, D., Smith, P., Jolin, H., and McKenzie, A. (2000). IL 9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity* **13,** 573–583.

Vanderwiel, R. J., De Jong, W. H., Spiekstra, S. W., Van Dijk, M., Fluitman, A., Garssen, J., and Van Loveren, H. (2000). Assessment of preferential T-helper 1 or T-helper 2 induction by low molecular weight compounds using the local lymph node assay in conjunction with RT–PCR and ELISA for interferon-γ and interleukin-4. *Toxicol. Appl. Pharmacol.* **162,** 77–85.

Warbrick, E. V., Dearman, R. J., Basketter, D. A., Gerberick, G. F., Ryan, C. A., and Kimber, I. (1998). Analysis of cytokine mRNA expression following repeated exposure of mice to chemical contact and respiratory allergens. *J. Appl. Toxicol.* **18,** 205–213.

Weissman, D. N., and Lewis, D. M. (2000). Is specific antibody determination diagnostic for asthma attributable to low-molecular-weight agents? *Occup. Med.—State of the Art Rev.* **15,** 385–397.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T. Y., Karp C. L., and Donaldson D. D. (1998). Interleukin-13: Central mediator of allergic asthma. *Science* **282,** 2258–2261.

Woolhiser, M. R., Hayes, B. B, and Meade, B. J. (1998). A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. *Toxicol. Methods* **8,** 245–256.