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Analysis of gene expression induced by irritant and sensitizing chemicals using oligonucleotide arrays

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Abstract

Chemical-induced allergy continues to be an important occupational health problem. Despite decades of investigation, the molecular mechanisms underlying chemical-induced hypersensitivity and irritancy remain unclear because of the complex interplay between properties of different chemicals and the immune system. In this study, gene expression induced by toluene diisocyanate (TDI, a primarily IgE-inducing sensitizer), oxazolone (OXA, a cell-mediated hypersensitivity inducing sensitizer), or nonanoic acid (NA, a non-sensitizing irritant) was investigated using gene arrays. Female BALB/c mice were dermally exposed on the ears once daily for 4 consecutive days. On day 5, the lymph nodes draining the exposure sites were collected and used for RNA extraction and subsequent hybridization to Affymetrix Mu6500 oligonucleotide arrays. Of the 6519 genes on the arrays, there were 44, 13, and 51 genes in the TDI-, OXA-, and NA-exposed samples, respectively, that displayed a minimum of twofold change in expression level relative to the vehicle control. There were 32, 19, and 19 genes that were differentially expressed (with a minimum of twofold change) between TDI and OXA, TDI and NA, OXA and NA, respectively. The differentially expressed genes include immune response-related genes, transcriptional factors, signal transducing molecules, and Expressed Sequence Tags. Based on the gene array results, candidate genes were further evaluated using RT-PCR. There was only about 47% concordance between the gene array and RT-PCR results. Published by Elsevier Science B.V.

Keywords: Gene expression; Oligonucleotide array; Toluene diisocyanate (TDI); Oxazolone (OXA); Nonanoic acid (NA)

1. Introduction

Chemical-induced allergy is a common occupational disease, described as heightened inflammatory processes and associated adverse health effects, which results in significant morbidities. Decades of investigations have advanced our understanding of chemical-induced hypersensitivity in terms of predictive assessment of the sensitizing potential of chemicals [1], and the identification of the role of Langerhans cells [2], keratinocytes, and inflammatory cytokines [3,4] in hypersensitivity responses. It is established that chemicals can preferentially induce certain types of responses. The elicited responses include contact dermatitis (skin hypersensitivity) and asthma (respiratory hypersensitivity) in clinical terms,

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irritant and allergic responses in pathophysiological terms, and Type I (IgE-mediated) and Type IV (cell-mediated) hypersensitivity responses in mechanistic terms. Studies of the pattern of cytokine production in draining lymph nodes have shown that chemical contact allergens preferentially induce a T helper (Th) 1-type cytokine pattern with increased interferon-gamma (IFN-γ) production, and respiratory allergens preferentially induce a Th2-type pattern with high levels of IL-4 and IL-10 production [5]. Chemical irritants have been shown to elicit cutaneous inflammatory responses by activation of innate immune cells in the skin [6], and chemical allergens have been shown to induce specific humoral or cell-mediated immune responses. However, many studies have failed to demonstrate obvious differences pathologically or immunohistologically in cutaneous responses elicited by chemical irritants or allergens [7–9]. Questions concerning the underlying molecular mechanisms of chemical-induced hypersensitivity remain largely unanswered. One important question concerns the preferential elicitation of certain types of responses by chemicals. It is likely that unique interactions between different chemicals and the immune system induce patterns of gene expression that may determine the type of immune responses elicited. Therefore, uncovering the molecular differences induced by different chemicals would give insight into the molecular mechanisms underlying chemical-induced hypersensitivity and aid in future mechanistic studies and predictive assessments.

In these studies, gene array technology was employed to study the differences in gene expression induced by chemicals eliciting different responses. Gene array analysis allows simultaneous detection of gene expression for thousands of characterized and uncharacterized genes using miniaturized high-density array of oligonucleotide probes. Toluene diisocyanate (TDI) was chosen as a primarily IgE-inducing sensitizer, oxazolone (OXA) as a cell-mediated hypersensitivity inducing sensitizer, and nonanoic acid (NA) as a non-sensitizing irritant. Based on the gene array results, candidate genes were further evaluated by RT-PCR. These studies provide preliminary data on differential gene expression from which hypothesis-driven mechanistic studies can be developed.

2. Materials and methods

2.1. Animals

Female BALB/c mice (Jackson Laboratories, Bar Harbor, ME), 6–8 weeks old upon arrival, were quarantined for 1 week prior to the start of experiments. The mice were provided tap water ad libitum and fed Prolab RMH 3500 diet. The animals were maintained under conditions specified within NIH guidelines. Animal rooms were maintained between 18–26°C and 40–70% relative humidity with light/dark cycles of 12-h intervals.

2.2. Chemicals and exposures

Toluene diisocyanate (TDI; purity 99.6%), 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, OXA; purity $\geq$ 90%), nonanoic acid (NA; purity $\geq$ 97%), and acetone were purchased from Sigma (St. Louis, MO). Dilutions of all chemicals were prepared in acetone. The concentrations used were: TDI—1.5% (w/v), OXA—1% (w/v), NA—50% (w/v). For TDI and OXA, the concentrations used were based on preliminary data showing that they induced similar quantities of mRNA in the draining lymph nodes (data not shown). The concentration of NA was chosen based on results of previous studies done in this laboratory, showing that it induces significant ear swelling, but low levels of lymph node cell proliferation [10]. The animals were dosed with 25 μl of the test chemicals or the vehicle on the dorsal surface of both ears once daily for 4 consecutive days. On day 5, the animals were sacrificed by CO_2 asphyxiation and the cervical lymph nodes, located at the bifurcation of the jugular vein, were excised and used for RNA isolation.

2.3. RNA extraction

The excised lymph nodes (left and right side) from 10 animals in TDI- and OXA-exposed group were pooled. For the non-sensitizing NA and vehicle, a larger number of animals, 55, were required to obtain sufficient amount of RNA. The lymph nodes were homogenized using a polytron homogenizer (IKA Works, Wilmington, NC). RNA was extracted

using the TRIzol reagent (Life Technologies, Rockville, MD) by following the manufacturer's instruction. Poly(A) + mRNA was isolated from the total RNA using Oligotex mRNA kit (Qiagen, Valencia, CA).

2.4. cDNA synthesis

Double-stranded cDNA was synthesized using the Superscript Choice System (Life Technologies). A T7-$(dT)_{24}$ primer provided by Research Genetics (Huntsville, AL) was used in place of the primers included in the Superscript Choice System. The cDNA was purified using phenol/chloroform extraction and concentrated by ethanol precipitation.

2.5. Synthesis of biotin-labeled cRNA

In vitro transcription was used to produce biotin-labeled cRNA from the cDNA using the Bioarray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). The cRNA was purified using the RNeasy kit (Qiagen). A minimum of 25 μg of labeled cRNA for each treatment sample was prepared and sent to the Research Genetics for subsequent hybridization to gene arrays.

2.6. Target hybridization, probe array scanning and analysis

The fragmentation of cRNA, hybridization of cRNA to Affymetrix GeneChip Mu6500 oligonucleotide arrays (Santa Clara, CA), and scanning were conducted by Research Genetics. Samples were hybridized to the chips for 16–18 h with rotation at 45°C in a hybridization oven. Data were analyzed using Affymetrix GeneChip software. The samples were normalized by Global Scaling. In Global Scaling, each sample is multiplied by a Scaling Factor to make its Average Intensity equal to an arbitrary Target Intensity set by the user. The relative change in mRNA expression levels between two samples was expressed as the "fold change." "Sort score" was calculated by the software taking into account the Average Difference Change in intensity and used to evaluate the significance of difference in expression level of genes between two samples. When two genes display the same fold change, the larger the sort score, the more significant the measured difference in expression level of the gene.

2.7. RT-PCR

Animal exposure to chemicals, draining lymph node isolation, and RNA extraction were performed as described above. To determine the reproducibility of RT-PCR, aliquots of the same mRNA samples used for the gene array analysis and RNA samples from two additional animal experiments following chemical exposures were tested by RT-PCR for selected genes. RT-PCR was performed using ThermoScript RT-PCR System (Life Technologies). First-strand cDNA was synthesized from total RNA using oligo$(dT)_{20}$ primer and ThermoScript reverse transcriptase. PCR was performed by adding 0.5 μg of cDNA to 50 μl of reaction mixture (containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.82 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 μM forward and reverse gene-specific primers, 2 U of Platinum Taq DNA polymerase). All primers were synthesized by Life Technologies. PCR amplification conditions were as follows: pre-heating at 94°C for 2 min, then for each cycle denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min, with final extension at 72°C for 7 min. Preliminary experiments were conducted to determine the optimal number of cycles for each gene so that the PCR products were amplified during the exponential phase of PCR. The number of cycles used were: 40 cycles for IL-4, IL-10 and IFN-γ, 35 cycles for mucin and MP4, 30 cycles for Ly-6 and MIG, and 20 cycles for parotid protein and β-actin. Aliquots of PCR products were separated on 1.5% agarose gel

Table 1
Number of differentially[a] expressed genes between chemical treatments detected by gene array analysis

Comparison	Number of genes
TDI vs. VH	44
OXA vs. VH	13
NA vs. VH	51
TDI vs. OXA	32
TDI vs. NA	19
OXA vs. NA	19

[a]A minimum of twofold change in expression level.

and visualized with UV light after ethidium bromide staining. Densitometric analysis of gel images was performed using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

Primers used for RT-PCR: mucin apoprotein: 5′-AAGCAACAACTGACTGTTCC-3′ and 5′-TGACTTGTGATGACTTTCACC-3′; parotid protein: 5′-CCATTAAAACCAATGCCCAGAC-3′ and 5′-GAGAGGAGACCCTGAAGAAC-3′; Ly-6: 5′-AGGATGGACACTTCTCACAC-3′ and 5′-ACCCACAATAACTGCTGCC-3′; MIG: 5′-CACTACAAATCCCTCAAAGACC-3′ and 5′-TTCCTTGAACGACGACGAC-3′; MP4: 5′-CTTCCTTTCTCAGCACAACC-3′ and 5′-TCTCCTTTTATTCTCCCAGTCC-3′; β-actin: 5′-TCCGTAAAGACCTCTATGCC-3′ and 5′-TACTCCTGCTTGCTGATCC-3′; IL-4: 5′-TGTCATCCTGCTCTTCTTTCTC-3′ and 5′-GCTCACTCTCTGTGGTGTTC-3′; IL-10: 5′-ACATACTGCTAACCGACTCC-3′ and 5′-ATCACTCTTCACCTGCTCC-3′; and IFN-γ: 5′-CTCTGAGACAATGAACGCTAC-3′ and 5′-TCCTTTTGCCAGTTCCTCC-3′.

Table 2
Genes differentially expressed between TDI and OXA

GenBank accession no.	Gene description	Fold change		
		TDI/VH	OXA/VH	TDI/OXA
Genes expressed higher in TDI				
W77701	EST	1.6	A	2.9
U32332	p13MTCP1	~2	A	2.9
X01697	mRNA fragment for parotid secretory protein	1.2	−2.1	2.7
M93422	adenylyl cyclase Type IV	2.5	−1.1	2.7
AA003990	EST	3.4	1.4	2.4
M29325	L1Md-9 repetitive sequence	2.6	1.1	2.4
W64759	EST	1.8	~ −1.3	2.4
U27177	p107	2.1	1	2.1
U20372	voltage-dependent calcium channel beta-3 subunit	1.6	−1.3	2
X99581	mouse gene for leukocyte-derived seven trans-membrane domain receptor	1.6	−1.2	2
Genes expressed higher in OXA				
U37531	mucin apoprotein	−15.5	−1.1	13.6
X56602	interferon-induced 15-kDa protein	~ −6.6	−1.1	8.6
M16358	major urinary protein IV	−3.3	1.3	4.1
D85904	apg-2	−4.5	1.3	3.6
AA028501	EST	1.8	1.6	3.3
W08033	EST	−2.9	1.1	3.3
X88903	variable light chain	~ −2.4	1	3.1
AA104856	EST	A	~2.9	3.1
X63535	ufo	−1.2	2.3	2.7
M34815	monokine induced by gamma interferon (MIG)	−2.9	−1.1	2.7
X04072	cytotoxic T-lymphocyte-associated gene (CTLA-1)	−3.2	−1.3	2.5
X04097	kidney testosterone-regulated RP-2	A	3.1	2.4
U70859	cationic amino acid transporter (CAT3)	A	~2.4	2.3
AA154337	EST	−1.1	2	2.3
M77497	cytochrome *P*450 naphthalene hydroxylase	−2.5	−1.1	2.3
W15826	EST	−1.6	1.5	2.2
L43567	B-cell receptor	A	~1.2	2.1
AA072239	EST	−1.3	1.5	2.1
D13003	reticulocalbin	−1.5	1.3	2.1
AA123267	EST	−1.5	1.4	2.1
X04653	Ly-6 alloantigen (Ly-6E.1)	−2.3	−1.1	2.1

A indicates that the transcript was absent (undetected) in both samples of a comparison.

2.8. Statistical analysis

Statistical analysis was performed using one-way ANOVA on RT-PCR data for selected genes. When significant difference occurred ($p < 0.05$), the Tukey's Multiple Comparison Test was performed.

3. Results

3.1. Differentially expressed genes between chemical treatments detected by gene array analysis

To identify differences in gene expression induced by different chemicals, the animals were dermally exposed to a chemical that is a documented IgE-inducing sensitizer (TDI), cell-mediated hypersensitivity inducing sensitizer (OXA), non-sensitizing irritant (NA), or control vehicle (acetone). After dosing for 4 days, the local draining lymph nodes were collected and used for mRNA extraction and subsequent hybridization to Affymetrix Mu6500 oligonucleotide arrays. To determine differentially expressed genes, pairwise comparisons were made between chemical treatment samples. The Mu6500 arrays comprise a set of four chips, A–D, and collectively have a total of 6519 mouse genes tiled on chips. Of them, 3286 are known genes and 3233 are Expressed Sequence Tags (ESTs). As summarized in Table 1, of the 6519 genes on the arrays, there were 44 (0.7%), 13 (0.2%), and 51 (0.8%) genes, in the TDI-treated, OXA-treated, and NA-treated samples, respectively, that displayed a minimum of twofold change in expression level over the control vehicle. There were 32 genes (0.5%) between the TDI and OXA samples, 19 genes (0.3%) between the TDI- and NA-treated samples, and 19 genes (0.3%) between the OXA- and NA-treated samples, that dis-

Table 3
Genes differentially expressed between TDI and NA

GenBank accession no.	Gene description	Fold change		
		TDI/VH	NA/VH	TDI/NA
Genes expressed higher in TDI				
L40156	surfactant protein D (sftp4)	1.4	A	2.3
X01697	mRNA fragment for parotid secretory protein	1.2	−1.7	2.1
U10092	Ly-49F-GE	~1.6	A	2
X92397	mRNA for Norrie disease gene product	~1.7	A	2
AA168855	EST	~1.7	A	2
AA120171	EST	1.6	A	2
Genes expressed higher in NA				
D85904	apg-2	−4.5	1.1	4.8
W08033	EST	−2.9	1	2.9
X58438	MP4	~1.6	~1.6	2.5
AA119665	EST	−1.9	1.3	2.5
U74359	mothers-against-dpp-related-1 (Madr1)	~ −1.5	1.8	2.5
AA124052	EST	−1.3	1.8	2.4
W07963	EST	−2	1.4	2.3
AA118062	EST	~ −1.8	1.3	2.2
M31419	204 interferon-activatable protein	A	1.4	2.2
D13695	ST2L	−1.1	1.9	2.1
D13003	reticulocalbin	−1.5	1.3	2.1
D76446	TAK1	−1.3	−1.4	2.1
AA154337	EST	−1.1	1.8	2

played a minimum of twofold change in expression level. Of the 32 differentially expressed genes between TDI and OXA (Table 2), five genes in TDI and four genes in OXA were up-regulated, and seven genes in TDI and one in OXA were down-regulated when compared to the vehicle, and the remaining number of genes were not significantly ($<$ 2-fold change) different from the vehicle in expression levels, but were significantly ($\geq$ 2-fold change) different from each other (TDI vs. OXA) due to the opposite direction (up or down) of change in expression level relative to the vehicle. Of the 19 differentially expressed genes between TDI and NA (Table 3), one gene was down-regulated in TDI compared to the vehicle, and the remaining were only different from each other. Of the 19 differentially expressed genes between OXA and NA (Table 4), one gene in OXA and six genes in NA were up-regulated, four genes in NA were down-regulated compared to the vehicle and the remaining were only different from each other. Included in the lists of differentially expressed genes were genes that have or may have a role in immune response (e.g., CTLA-1, interferon-induced 15-kDa protein, monokine induced by gamma interferon, and Ly-49F-GE), and genes that may be involved in signal transduction (e.g., NF-κB, TGF-beta-activated kinase, and ufo). There was also a significant number of genes whose functional significance needs further investigation, for example, genes encoding salivary proteins, major urinary proteins, and kidney testosterone-regulated RP2 protein. Some uncharacterized ESTs were also on the lists. Table 5 lists all the cytokine genes examined by the gene array. Surprisingly, the gene array did not detect most of the cytokine genes, particularly some of the major cytokines that are involved in hypersensitivity responses, such as IL-4, IL-5, IL-10, and IFN-γ.

Table 4
Genes differentially expressed between OXA and NA

GenBank accession no.	Gene description	Fold change		
		OXA/VH	NA/VH	OXA/NA
Genes expressed higher in OXA				
M33975	salivary protein 2	1	A	~ 23.8
U37531	mucin apoprotein	−1.1	−12.9	11.3
AA028501	EST	4.3	1.5	3.6
W15826	EST	1.5	−1.9	2.6
M16358	major urinary protein IV	1.3	−2	2.6
M77497	cytochrome *P*450 naphthalene hydroxylase	−1.1	−2.4	2.2
X04072	cytotoxic T-lymphocyte-associated gene (CTLA-1)	−1.3	−2.7	2.1
U46068	von Ebner minor salivary gland protein	1.5	A	2
Genes expressed higher in NA				
AA061797	EST	A	~ 1.8	3.1
AA125097	EST	−1.3	2	2.8
AA080068	EST	A	A	2.5
M29325	L1Md-9 repetitive sequence	1.1	2.6	2.5
X61600	beta-enolase	−1	2.4	2.5
W65178	EST	A	~ 1.6	2.1
X54149	MyD118	1.3	2.8	2.1
L28118	NF-κB (p105)	−1.2	−1.1	2.1
AA003990	EST	1.4	2.8	2
AA162205	EST	1.1	2.3	2
U20372	voltage-dependent calcium channel beta-3 subunit	−1.3 −1.3	1.6	2

Table 5
Cytokine expression detected by gene array

Cytokine	Comparison					
	TDI/VH	OXA/VH	NA/VH	TDI/OXA	TDI/NA	OXA/NA
IL-1β	1.1[a]	~ −4.4	1.1	1.8	1.2	~ −5
IL-2	~ −1.6	~ 2.1	1.4	~ −2.1	~ −1.6	1.4
IL-3	1.1	~ −1.1	1.2	1.2	−1	−1.2
IL-4	A	A	A	A	A	A
IL-5	A	A	A	A	A	A
IL-6	A	A	A	A	A	A
IL-7	A	1.6	1.6	~ −1.6	~ −1.4	−1.1
IL-10	A	A	A	A	A	A
IL-12 p35	A	A	A	A	A	A
IL-12 p40	1.5	1.3	1.5	1.1	−1	−1.1
IL-15	1.6	1	1.4	1.6	1.1	−1.4
IFN-α1	A	A	A	A	A	A
IFN-α4	A	A	A	A	A	A
IFN-α5	A	1.2	1.2	~ −1.2	~ −1.7	−1.1
IFN-α8	A	A	A	A	A	A
IFN-β	1.6	A	1.5	1.4	1.1	−1.3
IFN-γ	A	A	A	A	A	A
TNF-β	A	A	A	A	A	A

[a]Numbers indicate fold change in expression level between chemicals in each comparison.
Negative number indicates fold decrease. The sign " ~ " before a number indicates an approximate fold change due to either of the sample in a comparison being at the limit of detection. A indicates that the transcript was absent (undetected) in both samples of a comparison.

3.2. Testing selected genes by RT-PCR

Based on the gene array results, selected differentially expressed genes were further evaluated by RT-PCR (Table 6). The candidate genes were chosen according to the following criteria: (1) a minimum of twofold changes in expression levels between samples; (2) expression levels in chemical treatments differing from those in vehicle treatment; and (3) higher sort score. BALB/c mice were subjected to the same treatments as in the gene array experiment and RNAs were extracted from local draining lymph nodes. RT-PCR was performed using primers specific to each of the selected genes as described in Section 2. RT-PCR results were consistent for the detection of the expression of all the genes tested.

Table 6
Selected genes tested by RT-PCR

GenBank accession no.	Gene description	Comparison	Fold change	Sort score
U37531	mucin apoprotein	TDI vs. OXA	−13.6	20.85
X01697	mRNA fragment for parotid secretory protein	TDI vs. NA	2.1	4.99
X04653	Ly-6 alloantigen	TDI vs. OXA	−2.1	1.34
M34815	monokine induced by gamma interferon (MIG)	TDI vs. OXA	−2.7	1.23
X58438	MP4 gene for a proline-rich protein	TDI vs. NA	−2.5	0.11

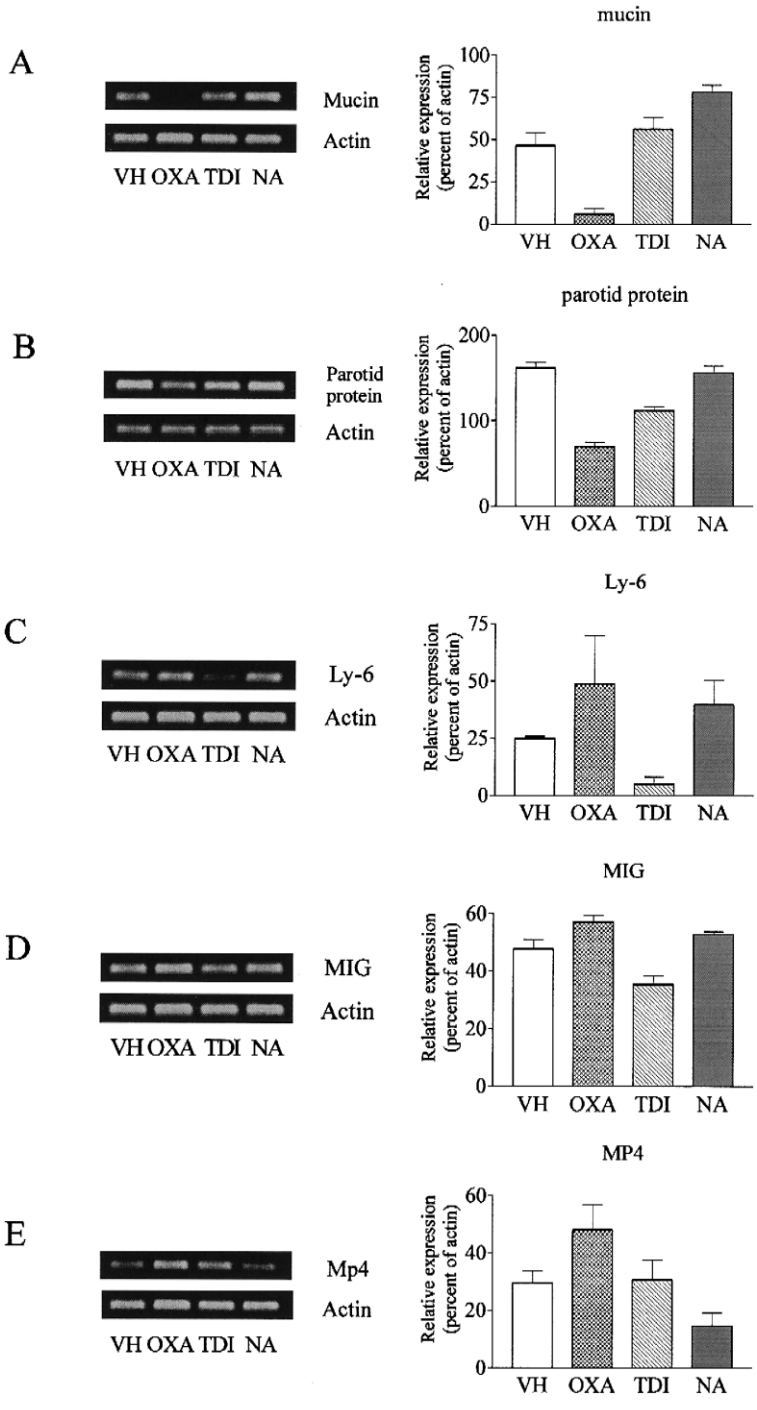
A
Mucin
Actin
VH OXA TDI NA
mucin
Relative expression (percent of actin)
100
75
50
25
0
VH OXA TDI NA
B
Parotid protein
Actin
VH OXA TDI NA
parotid protein
Relative expression (percent of actin)
200
100
0
VH OXA TDI NA
C
Ly-6
Actin
VH OXA TDI NA
Ly-6
Relative expression (percent of actin)
75
50
25
0
VH OXA TDI NA
D
MIG
Actin
VH OXA TDI NA
MIG
Relative expression (percent of actin)
60
40
20
0
VH OXA TDI NA
E
Mp4
Actin
VH OXA TDI NA
MP4
Relative expression (percent of actin)
60
40
20
0
VH OXA TDI NA

Table 7
Agreement between gene array analysis and RT-PCR results

Gene	Gene array	RT-PCR	Concordance
Mucin apoprotein	TDI < VH	TDI ~ VH	−
	OXA ~ VH	OXA < VH	−
	NA < VH	NA > VH	−
	TDI < OXA	TDI > OXA	−
	OXA > NA	OXA < NA	−
	TDI ~ NA	TDI ~ NA	+
Parotid protein	TDI ~ VH	TDA < VH	−
	OXA < VH	OXA < VH	+
	NA ~ VH	NA ~ VH	+
	TDI > OXA	TDI > OXA	+
	OXA ~ NA	OXA < NA	−
	TDI > NA	TDI < NA	−
Ly-6	TDI < VH	TDI ~ VH	−
	OXA ~ VH	OXA ~ VH	+
	NA ~ VH	NA ~ VH	+
	TDI < OXA	TDI ~ OXA	−
	OXA ~ NA	OXA ~ NA	+
	TDI ~ NA	TDI ~ NA	+
MIG	TDI < VH	TDI ~ VH	−
	OXA ~ VH	OXA ~ VH	+
	NA ~ VH	NA ~ VH	+
	TDI < OXA	TDI < OXA	+
	OXA ~ NA	OXA ~ NA	+
	TDI ~ NA	TDI < NA	−
MP4	TDI < VH	TDI ~ VH	−
	OXA < VH	OXA ~ VH	−
	NA ~ VH	NA ~ VH	+
	TDI ~ OXA	TDI ~ OXA	+
	OXA ~ NA	OXA > NA	−
	TDI < NA	TDI ~ NA	−

In RT-PCR analysis, " < " or " > " indicates statistically significant difference between a comparison ($p < 0.05$); " ~ " indicates difference not statistically significant ($p > 0.05$).

Fig. 1 shows representative results of RT-PCR for selected genes. The relative expression in each comparison determined by RT-PCR showed only about 47% concordance with the relative expression determined by gene array analysis (Table 7). For example, gene array analysis showed that expression of the gene encoding mucin apoprotein in the OXA sample was 13.6-fold higher than in the TDI sample and 11.3-fold higher than in the NA sample, and RT-PCR detected a twofold higher expression in the TDI sample and a threefold higher expression in the NA sample than in the OXA sample.

Fig. 1. Results of RT-PCR for selected genes. Female BALB/c mice were dosed with acetone (VH), oxazolone (OXA), toluene diisocyanate (TDI) or nonanoic acid (NA) on the dorsal surface of both ears once daily for 4 consecutive days. On day 5, the draining lymph nodes were collected and used for RNA extraction. First-strand cDNA was synthesized as described in Section 2. PCR was performed using gene-specific primers. Aliquots of PCR products were separated on 1.5% agarose gel and visualized after ethidium bromide staining with UV light. Left panels show representative gel images. Relative expression quantitation was obtained by densitometric analysis of the gel images (right panels). Relative expression of the gene was represented as percent of actin (mean ± S.E.M.). (A) Expression of mucin apoprotein. (B) Expression of parotid protein. (C) Expression of Ly-6 alloantigen. (D) Expression of monokine induced by gamma interferon (MIG). (E) Expression of MP4 proline-rich protein.

RT-PCR was performed to evaluate the expression of three cytokine genes: IL-4, IL-10, and IFN-γ. As shown in Fig. 2, RT-PCR detected the expression of all three cytokines. The expression of IL-4 was higher in the TDI sample than in the OXA sample and barely detectable in the vehicle and NA samples, and the expression of IL-10 and IFN-γ was not much different between samples.

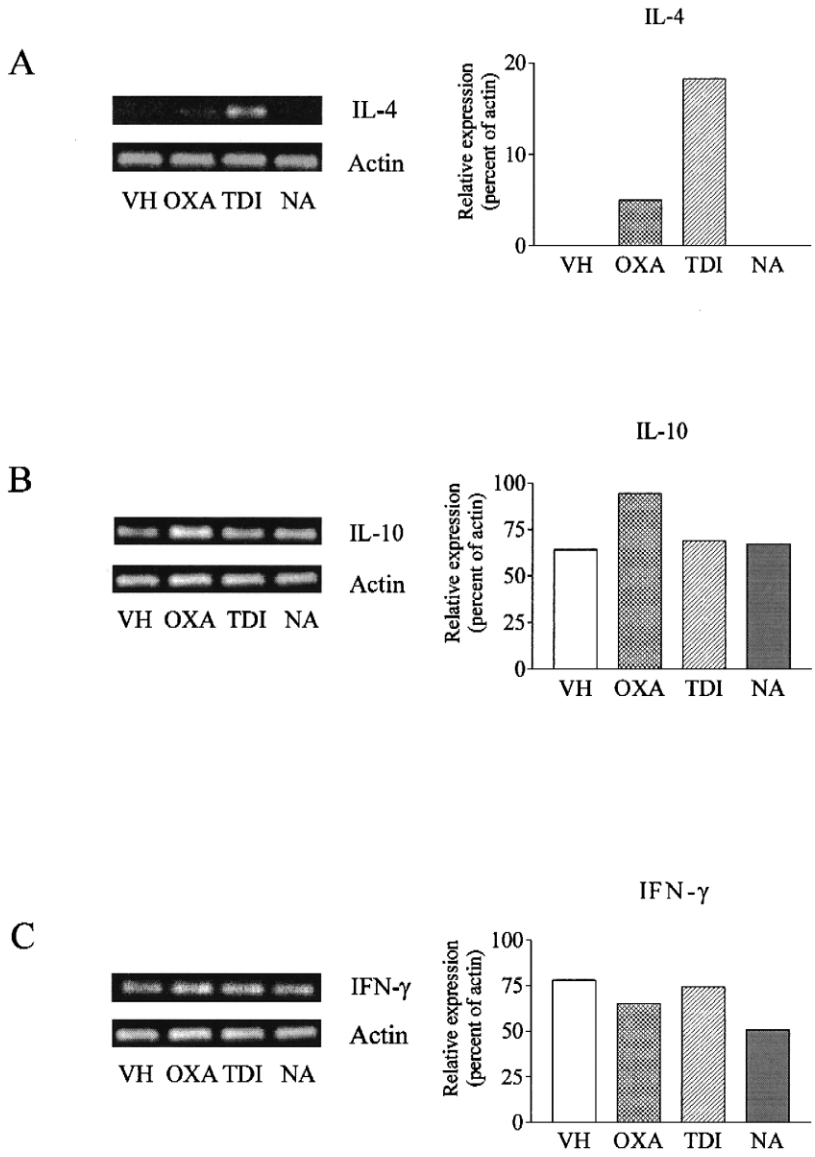

Fig. 2. Cytokine expression detected by RT-PCR. Female BALB/c mice were dosed with acetone (VH), oxazolone (OXA), toluene diisocyanate (TDI) or nonanoic acid (NA) on the dorsal surface of both ears once daily for 4 consecutive days. On day 5, the draining lymph nodes were collected and used for RNA extraction. First-strand cDNA was synthesized as described in Section 2. PCR was performed using gene-specific primers. Aliquots of PCR products were separated on 1.5% agarose gel and visualized after ethidium bromide staining with UV light. Relative quantitation was obtained by densitometric analysis of the gel images. Relative expression of the gene was represented as percent of actin. (A) Expression of IL-4. (B) Expression of IL-10. (C) Expression of IFN-γ.

4. Discussion

The observation that chemicals preferentially induce differential responses and the difficulty in separating the inflammatory responses induced by irritant and sensitizing chemicals have intrigued dermatologists and immunologists for decades. Finding the answers to this puzzle depends on understanding the molecular mechanisms of chemical-induced hypersensitivity and irritation. Detection of the molecular differences induced by different chemicals will be critical to mechanistic studies and predictive assessment of the sensitizing potential of chemicals.

Gene array analysis provides the most efficient way to simultaneously evaluate the expression of thousands of genes in different samples [11–14]. This study used the gene array technology to detect the differences in gene expression following exposure to chemicals that are known to induce irritancy, IgE-mediated, or cell-mediated hypersensitivity. The gene array study identified a total of 115 genes out of 6519 genes that were significantly different (a minimum of twofold change) in expression levels between chemical treatments. The differentially expressed genes cover a wide spectrum. However, the gene array failed to detect most of the cytokine genes in all the samples. In contrast to gene array, RT-PCR detected the expression of all of the three selected cytokine genes (i.e., IL-4, IL-10, and IFN-γ). RT-PCR is more sensitive than gene array because RT-PCR is an amplification-based method. Therefore, although gene array technology has the advantage over other conventional methods of simultaneous detection of a great number of genes, it may have limitations based on its detection limit. The discrepancy between the gene array and more conventional methods, such as RT-PCR and Northern Blot, observed in this study and others [15] may not be fully accounted for by sensitivity; other factors may contribute to this problem. To resolve the discrepancy further comparative studies are needed.

It is known that cytokines play important roles in the development of hypersensitivity responses. The RT-PCR result of the cytokine detection showed that TDI, a primarily IgE-inducing sensitizer, expressed higher IL-4 level than other samples, which is in line with previous studies [5], and the expression of IL-10 and IFN-γ did not show significant difference among samples. It is known that Type I hypersensitivity is an IgE-mediated humoral response that is largely dependent on Th2 cytokine production, such as IL-4 and IL-5, and Type IV hypersensitivity is a cell-mediated response in which Th1 cytokine production, IFN-γ, plays a critical role. However, using in vivo models of immune responses, there is increasing evidence of the coexpression of Th1 and Th2 cytokines [6,16–18].

Some genes that may have a role in cell-mediated responses, such as the gene for a monokine induced by gamma interferon (MIG) and CTLA-1, the gene encoding one of the important cytolytic enzymes in $CD8^+$ T lymphocyte, were shown by gene array analysis to be down-regulated in the TDI sample. This suggests that following exposure to this IgE-inducing sensitizer, down-regulation of genes involved in cell-mediated responses may be necessary for the development of an IgE-mediated hypersensitivity response.

Gene array also showed that the largest differential expression between OXA and TDI or OXA and NA was observed for the genes encoding salivary protein 2 (23.8-fold higher in OXA than in NA) and mucin apoprotein (11.3-fold higher in OXA than in NA, 13.6-fold higher in OXA than in TDI). However, the RT-PCR showed that the mucin apoprotein level was higher in NA and TDI than in OXA. The salivary proteins are exclusively expressed in salivary glands under normal physiological conditions. The regulation of the expression of these proteins may be altered under pathophysiological conditions, such is the case for the parotid secretory protein, which was found to be expressed outside the salivary glands in diabetic animals [19]. Further studies are needed to define the biological significance of the expression of these salivary proteins in lymph nodes and their roles in chemical-induced hypersensitivity. Mucins comprise a family of highly glycosylated proteins. There are two classes of mucins, epithelium-associated mucins and endothelium/lymphocyte-associated mucins [20]. The former is expressed in the epithelia of the body and plays a role in protecting the mucosal surfaces. The latter is expressed on endothelial cells, including the high endothelial venules of lymph nodes, and different subsets of lymphocytes. The endothelium/lymphocyte mucins are involved in adhesion cascade, and there-

fore participate in the inflammation process and in lymphocyte homing. Abnormal mucin expression has been shown to be associated with a number of pathological conditions, such as cancer, inflammatory bowel disease, and respiratory disease [21–24]. To define the significance of alterations in mucin gene expression following chemical treatments that were observed in this study, further investigation is needed.

Using gene array technology, the expression of thousands of genes in chemical sensitizer- and irritant-treated samples have been systematically evaluated. Numerous differentially expressed genes and a large number of commonly expressed genes were identified following different chemical exposure. The differentially expressed genes include immune response-related genes, transcriptional factors, signal transducing molecules, and Expressed Sequence Tags. These findings will aid in the understanding of the molecular events in chemical-induced hypersensitivity responses, and lay the foundation for future studies into roles of the identified differentially expressed genes. The gene array is a high-throughput technology and provides rapid, parallel analysis of gene expression for a great number of genes. However, like any other technology, the gene array has its own limitations. And based on the amount of discordance observed in these studies, further validation and characterization of gene array data by other methods would be recommended.

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