Microarray-Based In Vitro Test System for the Discrimination of Contact Allergens and Irritants: Identification of Potential Marker Genes

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BACKGROUND: Animal tests have been used to characterize the potential of chemicals to produce allergic contact dermatitis, but this approach is increasingly a matter of public and political concern. Our aim was to develop and validate an alternative in vitro test that can identify contact allergens.

METHODS: We developed a targeted microarray containing oligonucleotide probes for 66 immune-relevant genes and analyzed gene expression in monocyte-derived dendritic cells (Mo-DCs) treated with 1 irritant (SDS) and 2 prominent contact allergens, nickel and Bandrowski’s base (BB), which is the oxidation product of the most important hair dye allergen, p-phenylenediamine.

RESULTS: Comparing RNA amounts in chemically treated and solvent-treated cells, we identified significant changes in the expression of 21 genes and 10 genes after exposure of immature DCs (iDCs) to nickel and BB, respectively, but not after exposure to SDS. Eight genes were differentially expressed after application of both nickel and BB. Real-time PCR was used to confirm the results for selected genes.

CONCLUSION: We propose a microarray-based in vitro test that might allow the identification of contact allergens. Independently from donor variability, several immune-relevant genes were up- or downregulated after the application of the tested sensitizers to iDCs, therefore presenting potential marker genes. While reducing the number of laboratory animals used, this test would also enable reliable analysis of chemicals using a human system.

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Despite ongoing efforts to establish in vitro testing strategies, in vivo animal tests are still used to assess the sensitizing potential of chemicals leading to allergic contact dermatitis. Most in vitro attempts have explored whether phenotypic or functional changes of antigen-presenting cells can serve as a surrogate for in vivo hazard identification. Dendritic-like cells cultivated from bone marrow, cord blood CD34+ progenitor cells, or CD14+ peripheral monocytes are used instead of antigen-presenting cells of the skin (Langerhans cells), which are difficult to isolate in sufficient numbers.

Different markers of dendritic cell (DC)3 matura- tion have been found to indicate the process of sensitization by contact allergens. Aiba et al. (1) reported that sensitizers increase the expression of HLA-DR, CD54, and CD86 on monocyte-derived DCs (Mo-DCs). Likewise, Coutant et al. (2) found augmented expression of HLA-DR, CD54, CD40, and CD86. In studies by our group (3–5), we observed CD86 as the most reliable marker for sensitizers, but it nevertheless depended on interdonor variation.

Besides the differential expression of surface molecules, the production of specific cytokines has been related to the maturation process of immature DCs (iDCs). The contact sensitizers 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,4-dinitrofluorobenzene (DNFB), NiSO4, and K2Cr2O7 induced upregulation of IL-1β mRNA in Mo-DCs (6). Pichowski (7) confirmed this effect using strong sensitizers, but also found that the upregulation was donor dependent. In further gene expression studies in Mo-DCs, cells were exposed to the allergen dinitrobenzene sulfonic acid (DNBS), and gene expression was analyzed with an Affymetrix U95Av2 Gene Chip (8).
Verheyen et al. (9) observed upregulation of mRNA in CD34-derived DCs for IL1β, IL6, IL8, CCL2, CCL3, CCL4, and CCL3L1 by sensitizers. Investigations on the secretion of cytokines by Mo-DCs demonstrated the induction of IL8 as a parameter identifying contact allergens (10).

In the present work, we used a targeted microarray to show the principal functionality of a microarray-based test system to study the allergen-induced maturation process of Mo-DCs and to discriminate between contact sensitizers and irritants. We primarily selected genes that had already been shown to be involved in DC maturation or antigen-presenting processes. Furthermore, to produce a microarray suitable for toxicological studies, we included in the chip design several genes that had been used in immunotoxicological testing strategies in our laboratory (see Supplemental Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue3) (see, e.g., (10, 4–18)).

iDCs cultured from monocytes from different human blood donors were treated with 2 known contact sensitizers, NiSO4 and Bandrowski’s base (BB), and the irritant SDS. Using flow cytometry and the immune toxicity chip, we compared gene expression in these cells to gene expression in solvent-treated cells. We identified several immune-relevant genes that were differentially expressed after allergen treatment but not after application of SDS. Real-time PCR was used to confirm the results for 9 of those genes.

Materials and Methods

DESIGN AND PRODUCTION OF THE IMMUNE TOXICITY CHIP
Sixty-six immune-relevant genes, 7 housekeeping genes, and 8 negative controls and external spike controls (see Supplemental Table 1 in the online Data Supplement) were spotted on ARChip Epoxy glass slides (Austrian Research Centers GmbH, ARC) using an Omnimgrid Arrayer (GeneMachines). Gene-specific (Austrian Research Centers GmbH, ARC) using an Omnimgrid Arrayer (GeneMachines) were spotted on ARChip Epoxy glass slides controls (see Supplemental Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue3) (see, e.g., (10, 4–18)).

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FLOW CYTOMETRIC ANALYSIS
Cells were harvested and 300 μL of the culture was diluted in 2 mL PBS, 5 g/L BSA, and centrifuged (10 min, 300g). We incubated the cell pellets with 10 μL monoclonal antibody for 30 min using the following antibodies: fluorescein isothiocyanate (FITC) antihuman CD1a, phycoerythrin (PE) antihuman CD86, and anti-HLA-DR FITC (BD Pharmingen). After incubation, cells were washed again and finally diluted in 0.5 mL PBS. We performed flow cytometric analysis on a Coulter Epics XL-MCL (Beckman Coulter Inc.) with EXPo32™ ADC XL 3 Color v1.1C, Expo32v1.2 Analysis v1.1C (Beckman Coulter Inc.). DCs were defined by light scatter, dead cells were gated out, and fluorescence histograms were evaluated after the method of Overton (20).

To determine cytotoxicity, we performed staining with propidium iodide (PI) (Sigma-Aldrich). We incubated 500 μL of the culture with 10 μL PI (0.2 g/L in PBS) for 5 min at 4 °C and measured the percentage of PI-positive cells by use of flow cytometry. Viability was expressed as 100% – cytotoxicity (%).

RNA EXTRACTION
Cells were harvested by centrifugation (10 min, 300g, 4 °C), and cell pellets were resuspended in Trizol (Invitrogen). We isolated total RNA using Trizol as described by the manufacturer followed by an RNeasy minicolumn cleanup (Qiagen). RNA pellets were resuspended in 100 μL RNase-free water, and 350 μL buffer RLT (Qiagen) and 250 μL ethanol were added.
We applied samples to RNeasy minicolumns and performed RNA cleanup according to the manufacturer’s protocol. RNA was eluted with 3× 30 μL of RNase-free water.

LABELING AND HYBRIDIZATION
We analyzed RNA expression levels from chemically treated DCs and from DCs exposed to solvent control on 1 microarray using the 3DNA Array 350-kit (Genisphere) as described by the manufacturer. For each RNA, we performed 2 technical replicates (dye swaps).

Before hybridization, the chip surface was blocked using 50 mmol/L ethanolamine, 0.2 mol/L Tris, pH 9, and 1 g/L SDS for 30 min. After washing the slides in 4× SSC and 1 g/L SDS, the chips were blow-dried and ready for hybridization.

Only 2 μg of total RNA was reverse-transcribed using dt primer from the kit, which incorporates a capture sequence at the 5’ end of the cDNA. The unlabeled cDNA was hybridized to the array overnight at 60 °C using vial 12 hybridization buffer (3DNA Array 350-kit). Subsequently, hybridized cDNA was detected using fluorescently labeled 3DNA dendrimer molecules (3DNA Array 350-kit) with a sequence complementary to the capture sequence of the cDNA. The dendrimer molecules were incubated on the array for 4 h at 60 °C using vial 6 hybridization buffer (3DNA Array 350-kit). Every dendrimer contains on average 375 fluorescent dyes, resulting in a strong signal enhancement.

DATA ANALYSIS
We scanned the slides by use of a GenePix 4000A scanner (Molecular Devices) with adjusted settings (equal for both wavelengths) avoiding saturated signals. Raw image data were extracted using GenePix 3.0 software (Molecular Devices).

We performed data analysis with the software package Limma for the R computing environment (21). Limma is part of the Bioconductor project at http://www.bioconductor.org (22).

For every hybridization, background intensities were subtracted from the foreground intensities for each spot. Data were normalized with the Lucidea Universal Score Card System using a Loess procedure (21).

For statistical analysis, a linear model was fitted, and the empirical Bayes method (23) was applied. Within-array replicates were integrated in the analysis as described (24). We specified technical replicates (dye swaps) and biological replicates (experiments employing DCs from different blood donors) for each chemical.

We ranked the significance of the differentially expressed genes using the B statistic (23), and we made lists of the top-regulated genes considering the
Table 1. Genes analyzed with quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession number</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL17</td>
<td>NM_002897.2</td>
<td>Hs00171074_m1</td>
</tr>
<tr>
<td>CCR7</td>
<td>NM_001838.2</td>
<td>Hs00171054_m1</td>
</tr>
<tr>
<td>CXCL2</td>
<td>NM_002089.1</td>
<td>Hs00236966_m1</td>
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<td>CXCR4</td>
<td>NM_003467.2</td>
<td>Hs00237052_m1</td>
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<td>IL12B</td>
<td>NM_002187.2</td>
<td>Hs00233688_m1</td>
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<tr>
<td>IL15</td>
<td>NM_172174.1</td>
<td>Hs00542562_m1</td>
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<tr>
<td>IL8</td>
<td>NM_000584.2</td>
<td>Hs00174103_m1</td>
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<td>PGK1</td>
<td>NM_000291.2</td>
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<tr>
<td>PPIA</td>
<td>NM_021130</td>
<td>Hs99999904_m1</td>
</tr>
<tr>
<td>RPLP0</td>
<td>NM_053275.3; NM_001002.3</td>
<td>Hs00999992_m1</td>
</tr>
<tr>
<td>TNFRSF1A</td>
<td>NM_001065.2</td>
<td>Hs00533560_m1</td>
</tr>
</tbody>
</table>

Genes were randomly selected from genes identified as differentially expressed with the immune toxicity chip after exposure to one or both sensitizers (CCL17, CCR7, CXCR4, IL8, TNFRSF1A). TaqMan gene expression assays were used (assay ID).

Results

**DOSE-RESPONSE EXPERIMENTS**

PI staining of DCs exposed to increasing chemical concentrations revealed a decrease in viability for NiSO4- and SDS-treated cells, whereas different concentrations of BB had only minor effects. For further experiments, we chose concentrations resulting in ≥90% viability (300 μmol/L NiSO4, 3 mg/L BB, 10 mg/L SDS). CD86 expression increased with higher concentrations of all chemicals, indicating unspecific upregulation in treated cells, if cytotoxic concentrations are employed (Fig. 1).

**FLOW CYTOMETRIC ANALYSIS OF DCS BEFORE AND AFTER EXPOSURE**

Before exposure, we analyzed expression of the DC marker CD1a using flow cytometry, and >90% of the cells were found to be highly positive (data not shown). After the treatment, we analyzed the effects of chemicals on the expression of 2 maturation markers, CD86 and HLA-DR. We defined the percentage of cells positive for CD86 and HLA-DR by use of the Overton method (Fig. 2) and calculated mean values from all donors.

In microarray experiments analyzing iDCs exposed to NiSO4 for 1, 3, and 5 h and to BB for 4 h, fewer genes were found to be differentially expressed than in cells treated for 24 h (data not shown). Flow cytometric analysis of allergen-treated DCs after 48 h did not show a significant increase of maturation markers compared with 24 h (earlier experiments, data not published). Therefore, iDCs were exposed for 24 h in all experiments.

**MICROARRAY ANALYSIS AND REAL-TIME PCR**

The sensitizer NiSO4 induced the most prominent augmentation of CD86 and HLA-DR expression in iDCs. Flow cytometry and calculation of the percentage of cells positive for the 2 markers using histogram subtraction showed that on average 80% of the culture expressed CD86 and HLA-DR, compared with the solvent control (Fig. 3). Therefore, NiSO4 clearly induced maturation of the cells. Microarray analysis of the NiSO4-treated cells also indicated strong effects of the sensitizer. Using our criteria (fold induction ≥1.5 or ≤0.6 and B statistic ≥0), 21 genes were found to be differentially expressed compared to sol-
To confirm our microarray results, we analyzed changes in expression of 9 genes by use of real-time PCR (Table 1). The same RNA that was already used for the microarray experiments was used for the PCRs. Fig. 4a shows the fold inductions of the selected genes analyzed with real-time PCR and the microarray results described in Supplemental Table 1 in the online Data Supplement. Except for CCL17 and IL12B p40, all fold inductions measured with real-time PCR were significantly different from 0. Although fold inductions of NiSO₄-treated cells detected by PCR analysis were considerably higher than those measured by microarray analysis, upregulation of all but 1 gene could be confirmed. TNFRSF1A was found to be upregulated with microarray analysis, whereas real-time PCR results indicated its downregulation (Fig. 4a).
The effect of BB on iDCs was lower than the effect of NiSO₄: 56% of the cells were CD86⁺ and 45% were HLA-DR⁺ (Fig. 3). Therefore, BB could induce maturation of approximately half the cells. In accordance with flow cytometry, induction of gene expression with BB was lower than with NiSO₄, as shown by microarray analysis. Compared with solvent control, 10 genes were found to be differentially expressed (see Supplemental Table 1 in the online Data Supplement). For 5 of these genes (IL8, CCL17, CXCR4, TNFRSF1A, and CCR7), we analyzed specificity of the probes with real-time PCR, which confirmed the specificity of the probes for all but 1 gene (TNFRSF1A).

Comparing the gene expression patterns induced by NiSO₄ and BB, we found 8 genes that were differentially expressed in both groups (see the bold data in Supplemental Table 1 in the online Data Supplement). These genes are potential markers for the identification of contact sensitizers.

After exposure of iDCs to SDS, expression of CD86 and HLA-DR was upregulated in only 24% and 23% of the cells, respectively (Fig. 3). Using our criteria, no differentially expressed genes were detected with the immune toxicity chip. We noticed that 27 genes showed weak but significant (B > 0) upregulation of gene expression (see Supplemental Table 1 in the online Data Supplement), but all fold inductions were <1.5.

Real-time PCR was performed for the same genes as for NiSO₄-exposed cells and showed no significant induction of gene expression, confirming the microarray results (Fig. 4b).

**Discussion**

The analysis of chemical-treated DCs revealed clear differences of gene expression patterns between cells treated with sensitizers (NiSO₄, BB) and cells treated with an irritant (SDS). Although a significant increase in RNA levels was observed for several genes after ap-
PCR assay was specific for this gene. Therefore, a probe highly similar to the probe with CDNA FLJ27297 fis, clone TMS04309 (gov/Genes/SeqFinder) revealed unspecific binding of the microarray assay. Differences in sensitivity response. The expression of CCR7 in the induction phase of delayed-type hypersensitivity reactions. The expression of CCR7 was shown to be expressed on T cells (28). CCR8+ T cells are abundant in the skin and rare or absent in peripheral blood, indicating a role in skin-homing of T cells (29). However, Mo-DCs also express CCR8, indicating—like CCR7—its role in DC migration from skin to the lymph node (14).

CXCR4, the membrane receptor for CXCL12, has been shown to play an important role during constitutive basal trafficking of leukocytes (30), and might also contribute to assemble DCs in the lymph nodes (11).

Upregulation of CCR7 and CXCR4 was also found by others. Schoeters and colleagues (31, 32) reported on an upregulation of CCR7 after treatment of CD34+ DCs with several allergens, and this effect was confirmed by Tureci et al. (11) after treatment of Mo-DCs with CD40L.

One group reported the reduction of CXCR4 protein amounts in a fetal skin-derived cell line after treatment with contact sensitizers (33). These results contradict other studies employing Mo-DCs (11, 15) and our own results, where expression of CXCR4 is upregulated upon allergen treatment.

We further found that several chemokines (IL8, CCL17, and CCL22) known to be involved in allergy were upregulated after exposure of iDCs to both sensitizers. NiSO₄ also induced expression of CCL17, whereas BB exposure resulted in upregulation of CCL4.

CCL22 and CCL17 are the most abundant chemokines produced by DCs (34). CCL22 is constitutively expressed in iDCs (35), and the upregulation of gene or protein expression during maturation was shown by several groups (16, 17). Its receptor (CCR4) is expressed by recently activated T cells, showing the importance of CCL22 in regulation of DC–T cell contact. Moreover, DCs positive for CCL22 were detected in allergic contact dermatitis in the epidermis and the dermis. The CCL22-positive DCs were mainly mature CD83+ cells. In normal skin, no CCL22 expression was found.

CCL17 is expressed in keratinocytes (36) and DCs (16). As a ligand for CCR4 and CCR8, which are expressed by T cells, it might also be involved in the specific interaction of DCs with T cells.

In humans, CCL17 is upregulated in Mo-DC after lipopolysaccharide (LPS) stimulation, and Hashimoto et al. (16) showed the upregulation of CCL17 in mature DCs.

CCL17 and CCL5 were shown to be involved in a mouse model of allergic contact dermatitis (37). In our study, CCL5 was upregulated after NiSO₄ treatment.

Toebak et al. (10) found higher amounts of CCL17 protein only after application of LPS, NiSO₄, and CrCl₃ to iDCs, whereas other sensitizers did not result in significant upregulation. In their study, IL8 was the only
chemokine significantly upregulated at the protein level in response to all sensitizers analyzed. They proposed a role for IL8 in the onset of Langerhans cell migration in the skin. Moreover, neutrophils induced by IL8 release chemotactic mediators, which attract T cells. Therefore, IL8 might play a role in T cell accumulation via neutrophil attraction.

In our study, the expression of 2 additional cytokines (IL12B, IL15) was upregulated in NiSO4 treated DCs. DC-derived IL15 is required for the functional maturation of DCs (38).

IL12 is required for Th1 cell development, acting in an antagonistic equilibrium with ILA, which favors differentiation of Th2 cells (18). The heterodimeric cytokine is composed of a heavy chain (40 kDa) and a light chain (35 kDa). Both gene products are necessary for the biologically active cytokine; however, transcripts for the light chain were detected in many cell types in low abundance, whereas transcripts for the heavy chain were found only in cells producing biologically active IL12, and their expression was highly regulated (39). Therefore, the upregulation of the p40 gene is a better indicator for the production of active IL12 and it is more easily detected. In line with these facts, only IL12Bp40 was upregulated in our experiments with NiSO4.

Some housekeeping genes were detected as slightly upregulated with the microarray after exposure of iDCs to NiSO4 (PPIA, HPRT1, PGK1) or BB (PPIA). Several studies showed changes in expression of housekeeping genes under certain conditions (40). Therefore, the maturation process of DCs, accompanied by changes in morphology and function, might lead to changes in expression of genes involved in basic processes in the cell. Since all chemicals were applied in concentrations resulting in similar cytotoxicity (Fig. 1), general cytotoxicity can be excluded as reason for the upregulation of these genes.

The microarray assay proved to be very useful to elicit changes in expression of immune-relevant genes after treatment of iDCs with chemical sensitizers and irritants. We will analyze more chemicals to decide whether the currently selected candidate genes are generally able to discriminate between contact sensitizers and irritants. The small-sized panel of probes allows rapid analysis of the results. Our test system could establish a reliable in vitro assay, reducing the number of laboratory animals needed for such testing and offering the advantage of testing using a human system.

**Grant/funding Support:** This work was funded by the Austrian Research Centers GmbH, ARC.

**Financial Disclosures:** None declared.

**Acknowledgments:** We thank all blood donors for giving blood. We are grateful to Rudolf Pichler for spotting the immune toxicity chip and to Markus Mansfeld for performing real-time PCR. Many thanks to Andreas Weinhaeusel for critically reading the manuscript, to Albert Krieger for performing the CGAP Unigene BLAST for TNFRSF1A, to Frank Rasche for support with the PCR analysis, and to Anton Schoeffmann for help with the microarray data analysis in R.

**References**