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# Gene Expression Profiles of Human Small Airway Epithelial Cells Treated with Low Doses of 14- and 16-Membered Macrolides

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**Although long-term treatment with low doses of 14-membered macrolides is widely applied in management of patients with chronic inflammatory diseases, e.g., diffuse panbronchiolitis, chronic bronchitis, or chronic lung damage in newborns, the physiological mechanisms underlying the action of macrolides in these conditions are unclear. To clarify the pathological basis of these diseases and also to aid in the design of novel drugs to treat them, we chose to investigate the molecular target(s) of macrolides. Our experiments involved long-term culture of human small airway epithelial cells (hSAEC) in media containing 14-membered macrolides erythromycin (EM) or clarithromycin (CAM), or a 16-membered macrolide, josamycin (JM), which lacks clinical anti-inflammatory effects. We then analyzed gene expression profiles in the treated cells using a cDNA microarray consisting of 18,432 genes. We identified nine genes whose expression was significantly altered during 22 days of culture with EM, and seven that were altered by CAM in that time. Four of those genes revealed similar behavior in cells treated with either of the 14-membered macrolides, but not JM. The products of these four genes may be candidates for mediating the ability of 14-membered macrolides to suppress chronic inflammation.** 

***Key Words:*** **respiratory system; bronchial epithelial cell; macrolides; erythromycin; anti-inflammatory agents; cDNA microarray.**

Antibiotics belonging to the macrolide family are commonly used to treat acute infections of the respiratory tract, and low doses of erythromycin (EM) are often given on a long-term basis to treat diffuse panbronchiolitis (DPB) (1). The effect of EM in DPB is not considered to reflect antibacterial activity but rather an unknown mechanism that suppresses chronic inflammation because the maximum doses of EM used for DPB are lower than those required to suppress the growth of *Haemophilus influenzae* or *Pseudomonas aeruginosa* (2). Other 14-membered macrolide antibiotics including clarithromycin (CAM), roxithromycin, and azithromycin, are also effective treatments for DPB (3). On the other hand, a 16-membered macrolide, josamycin (JM), does not have this clinical effect even though its basic ring structures and antibacterial activity are similar to those that characterize 14-membered macrolides (3–5). Recent reports have indicated that 14-membered macrolides are also effective against various chronic inflammatory diseases including bronchial asthma, chronic sinusitis, psoriasis vulgaris, and neonatal chronic lung disease (6–9). Although some molecular studies have suggested an inhibitory effect of these compounds on cytokine production in neutrophils, monocytes, and bronchial epithelial cells (4, 5, 10, 11), the precise molecular mechanisms remain unclear.

Advances in cDNA microarray technology have made it feasible to examine expression levels of thousands of genes in a single experiment. Alterations of expression during drug treatment have been investigated by means of cDNA microarrays to clarify drug-response mechanisms, and this approach is potentially useful for identifying molecular targets for novel drugs (12–13). Toward a better understanding of the detailed mechanisms involved in the action of macrolides in airway cells, we have applied expression-profile analysis on cDNA microarrays consisting of 18,432 genes.

We report here an analysis of expression profiles in human small airway epithelial cells that were cultured with one of three macrolides (EM, CAM, or JM) for 22 days, and subsequent identification of four genes whose expression was altered by treatment with

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the 14-membered macrolides but not with the 16-membered JM.

## MATERIALS AND METHODS

*Microarray design and production.* We selected 18,432 independent cDNA sequences from the UniGene database of the National Center for Biotechnology Information. Our cDNA microarray was constructed essentially as described previously (14).

*Cell cultures and macrolides.* Normal human small airway epithelial cells (hSAEC) from a single donor were purchased from BioWhittaker, Inc. (Walkersville, MD). According to the manufacturer's instructions, the cells were cultured in 15-cm Petri dishes (Corning, Inc.) with recommended media (SAGM BulletKit, BioWhittaker). EM (Sigma), CAM (Dainabot Co. Ltd., Tokyo, Japan), and JM (Wako, Osaka, Japan) were each dissolved in methanol to final concentrations of $10^{-6}$ M, representing therapeutic dosage (2). Treatment with each macrolide was started one day after seeding and continued for 22 days.

*RNA preparation, hybridization, and scanning.* Total RNA was extracted from aliquots of each hSAEC culture on days 8, 16, and 22 with Trizol reagent (Life Technologies, Inc.) and then amplified using the Ampliscribe T7 Transcription kit (Epicentre Technologies, Inc.). Preparation of probes, hybridization, and scanning were performed as described previously (14, 15).

*Data analysis.* Signal intensities of Cy3 (untreated control) and Cy5 (treated with macrolides) from the 18,432 spots were quantified and analyzed by substituting backgrounds, using ArrayVision software (Imaging Research Inc., St. Catharines, Ontario, Canada). Subsequently each signal was normalized so that the averaged ratio (Cy3/Cy5) of signals of the 52 housekeeping genes would be 1.0, and the value of root ($Cy3^2 + Cy5^2$) of each signal would be kept throughout the normalization. The 52 housekeeping genes were chosen from the "housekeeping panel" in the web site provided by Brown *et al.* (16). Since the data for spots with intensities below $1 \times 10^5$ rfu (relative fluorescent unit) for both Cy3 and Cy5 signals were unreliable, genes corresponding to those spots were omitted from further analysis.

*RT-PCR.* Each sample of total RNA (10 $\mu$g) was treated with DNase I (Roche) and reversely transcribed to single-stranded cDNAs using oligo$(dT)_{12-18}$ primer with Superscript II reverse transcriptase (Life Technologies, Inc.). Semi-quantitative RT-PCR was carried out for 5 min at 95°C for initial denaturing, followed by 25–35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, in the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA), using the same gene-specific primers as those chosen for constructing the microarray. Quantitative RT-PCR was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Thermal-cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles comprising denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. GAPDH was used for normalization. Statistical comparisons with controls were performed by the Mann–Whitney test. The primers and Taqman probe were as follows: for ZNF205, 5′-CGCTCCTCACTGCCTGGT-3′, 5′-FAM-CCAGATGCCAGTGACTT-TAMRA-3′, 5′-GGGAGAGGTACAAGGCCACA-3′; for interleukin-6 (IL-6), 5′-CTGCAGAAAAAGGCAAA GAATCTAG-3′, 5′-FAM-CCTGACCCAACCACAAA-TAMRA-3′, 5′-GCGCAGAATGA GATGAGTTGTC-3′; for interleukin-8 (IL-8), 5′-TTGGCAGCCTT-CCTGATTTC-3′, 5′-FAM-CAGCTCTGTGTGAAGGT-TAMRA-3′, 5′-TTAGCACTCCTTGGCAAAACTG-3′.

## RESULTS

To examine the effect of treatment with low-dose macrolides on gene expression in hSAEC, we compared expression profiles of 18,432 genes at three different time points (8, 16, and 22 days) in cells treated with one of three macrolides, EM, CAM, or JM, and untreated control cells by means of cDNA microarrays (Fig. 1).

After a normalization procedure (see Methods), we first investigated expression levels of IL-6 and IL-8 at day 22. Quantitative RT-PCR was carried out as shown in Fig. 2. EM did not affect the expression of either IL-6 or IL-8 in hSAEC, whereas both CAM and JM repressed IL-6 expression and CAM repressed IL-8 expression only.

Next, we screened genes with Cy5/Cy3 ratios greater than 2.0 or less than 0.5, and selected 17 and 12 genes whose expression was altered respectively by EM and CAM at two serial time points or at day 22. To confirm the observed changes we performed semi-quantitative RT-PCR, and subsequently measured quantitative RT-PCR for the differently-expressed genes that were known and well-characterized. Significant alterations in expression levels were confirmed in 9 of the 17 and 7 of the 12 selected genes (Tables 1 and 2, respectively). We examined expression of the same genes in cells treated with JM (data not shown).

The combined data showed that expression of four transcripts, three corresponding to ESTs and one to the zinc-finger protein *ZNF205*, were changed by treatment with EM and CAM but not by treatment with JM. Altered expression of these four genes at day 22 was confirmed by RT-PCR, as shown in Fig. 3.

## DISCUSSION

Long-term treatment with low-dose macrolides is an effective therapy for various chronic inflammatory diseases including diffuse panbronchiolitis and chronic bronchitis (1, 4–9). However, the molecular mechanisms involved in the anti-inflammatory action of these compounds are unknown. Several cytokines, including IL-6 and IL-8, have been suggested as candidate mediators because expression of these genes is suppressed in bronchial epithelial cells treated for a few days with 14-membered macrolides (10, 11). We cultured hSAEC for up to 22 days in the present study, and measured the expression levels of these two genes. Neither IL-6 nor IL-8 was affected in hSAEC cultured with EM; on the other hand, CAM and JM both repressed expression of IL-6, and CAM suppressed IL-8. Since JM, a 16-membered macrolide, is reported to have almost no clinical usefulness for DPB (3–5), our data implied that IL-6 and IL-8 were unlikely to be the key molecules involved in the anti-inflammatory function of 14-membered macrolides.

cDNA microarray technology is a potential tool for investigating the mechanism of drug action (12, 13, 17, 18). By analyzing expression profiles in a cDNA mi-

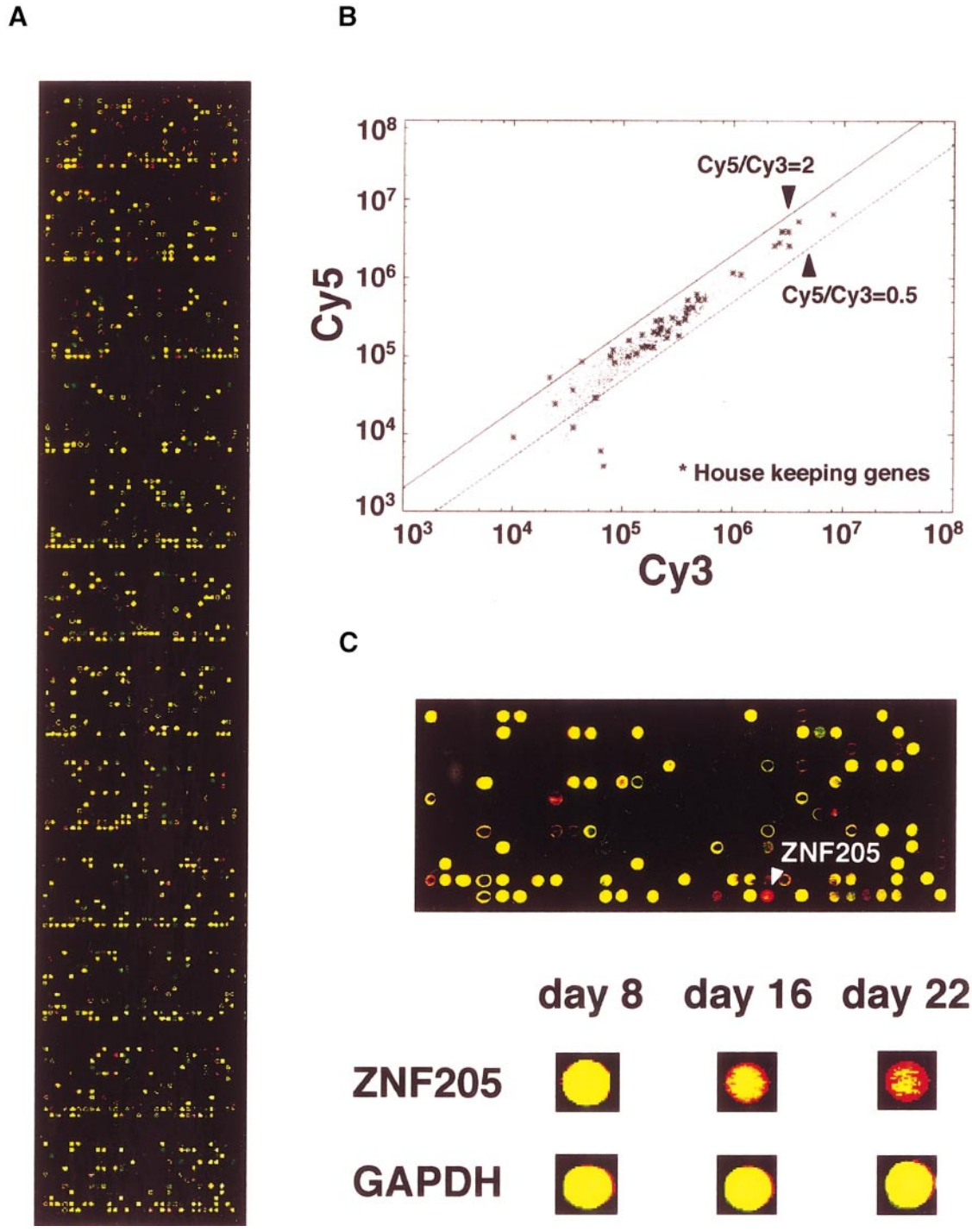


**FIG. 1.** Representative image (A) and scatter plots (B) of cDNA microarray analysis. Treated cells (labeled with Cy5) and control cells (labeled with Cy3) were labeled and hybridized to cDNA microarray. Green color, up-regulated expression; red color, down-regulated expression; yellow, unchanged expression; black, no expression was detected. *ZNF205* gene was gradually down-regulated by treatment with EM during a time course of 22 days (C).

croarray consisting of thousands of cDNA clones, we are able to investigate genome-wide expression of genes to detect those that are influenced by a particular drug. Using cDNA microarray technology we undertook genome-wide screening for the effect of low-dose macrolides on normal hSAEC. Among nearly 20,000 genes examined, most revealed no significant change in expression levels in the presence of macrolides at low concentrations; at most, only nine revealed significant changes in cells treated with EM.

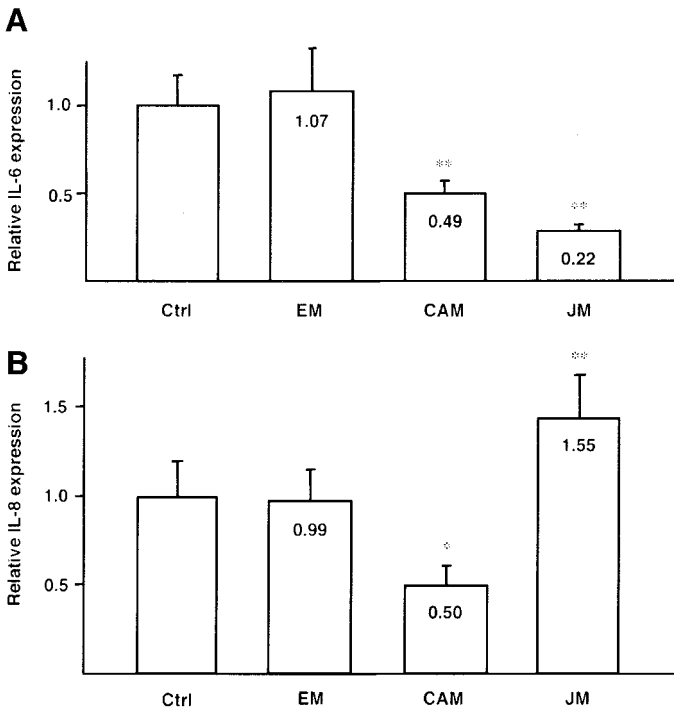


**FIG. 2.** Results of quantitative RT-PCR analyses of IL-6 (A) and IL-8 (B) after treatment of hSAEC with macrolides for 22 days. The results were calculated from nine independent experiments. Results displayed were the averages of these measurements, normalized to 1.0 for the control. $^{**}P < 0.001$; $^{*}P < 0.01$; Error bars: 95% confidence intervals.

Among those nine genes, filamin A alpha (FLNA) and HSP70/HSP90-organizing protein (HOP), both repressed by EM, have been well characterized and are related to the immune system. Filamin, an actin-binding protein-280, participates in the anchoring of membrane proteins to the actin cytoskeleton, and it interacts with tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) (19). TRAF2 is required for signal transduction from TNFR and related receptors (20), and can also mediate activation of NF-kappa B

**TABLE 1**

EM-Induced Alterations in Gene Expression in hSAEC during a Time Course of 22 Days

| Name | Accession no. | Ratio Day 8 | Ratio Day 16 | Ratio Day 22 |
|---|---|---|---|---|
| ZNF205 | AA595241 | 0.91 | 0.45* | 0.29* |
| FLNA | X53416 | 0.66 | — | 0.46* |
| HOP | M86752 | 0.85 | 0.10* | 0.47* |
| EST | N99344 | 0.47* | 0.36* | 1.18 |
| EST | R37561 | 0.93 | 1.24 | 2.12* |
| EST | AA215685 | 0.75 | 1.24 | 2.94* |
| EST | R42834 | 0.41* | — | 2.44* |
| EST | AI079655 | 1.03 | 1.65 | 2.24* |
| EST | AI033705 | 0.58 | 1.25 | 2.21* |

*Note.* Each ratio was Cy5:Cy3 of its element on the microarray.
* Expression level was up- or down-regulated more than 2.0-fold or less than 0.5-fold, respectively.
— No data were obtained because intensity was below $1 \times 10^5$ rfu for both Cy3 and Cy5 signals.

**TABLE 2**

CAM-Induced Alterations in Gene Expression in hSAEC during a Time Course of 22 Days

| Name | Accession no. | Ratio Day 8 | Ratio Day 16 | Ratio Day 22 |
|---|---|---|---|---|
| ZNF205 | AA595241 | 0.11* | — | 0.12* |
| KIAA0810 | NM025154 | 1.27 | 2.33* | 2.35* |
| EST | AA524754 | 1.24 | 2.51* | 2.19* |
| EST | N95414 | 1.24 | 2.59* | 2.41* |
| EST | AI033705 | 1.05 | 1.07 | 3.29* |
| EST | AI079655 | 1.16 | 1.77 | 3.04* |
| EST | AA215685 | 1.44 | 1.81 | 2.33* |

*Note.* Each ratio was Cy5:Cy3 of its element on the microarray.
* Expression level was up- or down-regulated more than 2.0-fold or less than 0.5-fold, respectively.
— No data were obtained because intensity was below $1 \times 10^5$ rfu for both Cy3 and Cy5 signals.

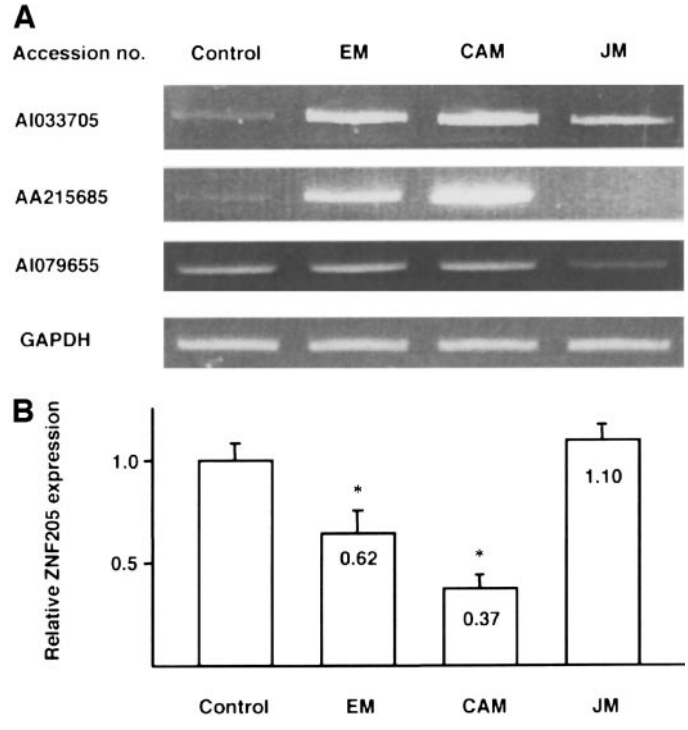


**FIG. 3.** Up- or down-regulation of gene expression by treatment of hSAEC with macrolides for 22 days, as revealed by semi-quantitative RT-PCR for three ESTs (A) and quantitative RT-PCR for *ZNF205* (B). The results of quantitative RT-PCR were calculated from six independent experiments; the results displayed on the histograms were the averages of these measurements, normalized to 1.0 for the control. $^{*}P < 0.01$; Error bars: 95% confidence intervals.

(21). NF-kappa B, a ubiquitously expressed transcription factor, controls the expression of many immune- and inflammatory-response genes (22). TNF failed to activate NF-kappa B in a human melanoma cell line deficient in filamin, and reintroduction of filamin into these cells restored the TNF response (19). Down-regulation of filamin by macrolides might alter the role of TRAF2 by decelerating the signaling of NF-kappaB in inflammatory pathways.

*HOP*, on the other hand, is induced by nitric oxide and bacterial lipopolysaccharide, and mediates association of the molecular chaperons HSP70 and HSP90 (23). Macrolides are one of the adjunctive therapies of psoriasis, a common chronic inflammatory dermatosis (9), and a recent study showed that noncultured psoriatic keratinocytes expressed higher levels of *HOP* than their normal keratinocytes (24). Since we observed down-regulation of the *HOP* gene by EM, the clinical effect of this macrolide on psoriatic keratinocytes may reflect repression of *HOP* expression. Furthermore, although JM is not effective for DPB, our experiments showed repression of IL-6 by this 16-membered macrolide. Therefore the IL genes may not in fact be the mediators of the anti-inflammatory function of 14-membered macrolides, as has been proposed. However, as anti-inflammatory effects are likely to be induced by multiple factors, such genes may play cooperative roles in the process.

We identified four genes, including *ZNF205*, that were altered by both 14-membered macrolides examined (EM and CAM) but not by JM. Although its precise function is unknown, *ZNF205* contains multiple C2H2-type zinc finger motifs, and a Kruppel-associated box at its N-terminus indicates that it might function as a transcription repressor (25). In view of these structural features, altered expression of *ZNF205* could result in disease status. The three other genes up-regulated by EM and CAM but not by JM corresponded to ESTs of an unknown function. Investigation of those genes may help to clarify molecular mechanisms responsible for the anti-inflammatory activity of 14-membered macrolides, and may reveal new molecular targets for development of new drugs to treat chronic inflammatory diseases.

Most drugs exert their pharmacological effects by interacting with specific target-proteins such as receptors, enzymes, or other cellular molecules. Many of the genes encoding these drug targets exhibit sequence polymorphisms, which often alters their sensitivity to specific medications (26–29). Genetic polymorphisms in drug-target molecules already have been linked to differences among individual patients as regards the efficacy or toxicity of certain therapeutic drugs. If polymorphisms are found in the genes whose expression was altered in our study, these sites might serve eventually as clinical markers for predicting sensitivity to therapy.

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