

Several gene programs are induced in ciprofloxacin-treated human lymphocytes as revealed by microarray analysis

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Abstract: Fluoroquinolones have immunomodulatory properties and interfere with cytokine production. The aim of this study was to characterize the extent of the superinduced mRNA levels in activated human lymphocytes incubated with ciprofloxacin (5 and 80 $\mu\text{g/ml}$) using a cytokine gene expression microarray from R&D Systems (Abingdon, UK). Several gene transcripts ($n=104$) were up-regulated in cells treated with ciprofloxacin at 80 $\mu\text{g/ml}$, whereas 98 transcripts were down-regulated out of 847 total genes included on the microarray. The increased mRNAs were distributed between major gene programs, including interleukins (36.5%), signal-transduction molecules (13.5%), adhesion molecules (10.6%), tumor necrosis factor and transforming growth factor- β superfamilies (10.6%), cell-cycle regulators (9.6%), and apoptosis-related molecules (8.7%). To determine the specificity of the microarray, a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), which contained a panel of 12 different cytokine mRNAs, was used. Eleven out of the 12 gene transcripts were up-regulated in the specific RT-PCR, whereas only eight were found to be increased in the microarray. A microarray from Clontech (Hampshire, UK), containing 588 different genes, was also included. Results obtained with this broad-coverage expression array slightly differed compared with the other microarray. We conclude that the fluoroquinolone ciprofloxacin at high concentrations interferes with several gene programs, which is in accordance with a mammalian stress response. From a technical point of view, a discrepancy may exist between data obtained by different microarrays and more specific methods such as quantitative RT-PCR. *J. Leukoc. Biol.* 74: 456–463; 2003.

Key Words: cytokines · fluoroquinolone · RT-PCR

INTRODUCTION

Effects on lymphoid cells have been described for most antibacterial agents that accumulate intracellularly at high concentrations. In recent years, the immunostimulatory effects of fluoroquinolone antibacterial agents have been fully acknowledged. Fluoroquinolones reach concentrations three to 20

times the extracellular concentration in human leucocytes. Effects of fluoroquinolones on the immune system have been thoroughly investigated by us and others (for a review, see ref. [1]). Ciprofloxacin, at moderate and high concentrations (range, 5–80 $\mu\text{g/ml}$), and to a lower degree, other fluoroquinolones, superinduce interleukin (IL)-2 synthesis by mitogen-activated peripheral blood lymphocytes (PBLs) [2, 3]. In addition, the IL-2 receptor density increases in ciprofloxacin-stimulated PBLs. In contrast to the superinduced IL-2 production, the synthesis of IL-1 β and tumor necrosis factor α (TNF- α) by lipopolysaccharide (LPS)-stimulated human monocytes is significantly inhibited by ciprofloxacin at ≥ 5 and 20 $\mu\text{g/ml}$, respectively [4]. Thus, diverse effects on T cells are observed compared with monocytic cells. In human cell systems, the production of human granulocyte macrophage-colony stimulating factor (GM-CSF) and lymphotoxin is also inhibited, albeit at higher drug concentrations (ciprofloxacin, 80 $\mu\text{g/ml}$) [4]. Moreover, in a cell-culture model consisting of mouse splenocytes, a ciprofloxacin-dependent increase of IL-3 and GM-CSF has been observed, reflecting a variation between different species [5].

Experiments with T cell lines and primary T lymphocytes transiently transfected with a plasmid containing the IL-2 promoter and enhancer region fused with bacterial chloramphenicol acetyl transferase (CAT) show ciprofloxacin to enhance IL-2 gene activation [6, 7]. It is interesting that ciprofloxacin induces an earlier and stronger activation of the transcriptional-regulation factors, nuclear factor of activated T cells-1 (NF-AT-1) and activator protein-1 (AP-1) in T cell lines [3]. In primary T cells, ciprofloxacin up-regulates AP-1 only and consequently increases CAT production when the reporter protein is linked to the metallothionein enhancer region, which is mainly governed by AP-1. Under certain in vitro conditions, ciprofloxacin (20–80 $\mu\text{g/ml}$) counteracts the effect of the immunosuppressive agent cyclosporin A, which normally inhibits the phosphatase activity of calcineurin, inhibiting NF-AT-1 activity [6, 8]. Ciprofloxacin has been suggested to interfere with a regulatory pathway common to several cytokines, and analysis of cytokine mRNAs in ciprofloxacin-treated PBLs supports this hypothesis. In addition to enhanced IL-2 mRNA in ciprofloxacin-treated cells, an array of other cytokine

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mRNAs, including interferon- γ (IFN- γ) and IL-4, is increased as compared with control lymphocytes incubated in the absence of ciprofloxacin [6, 7]. In addition to these observations, ciprofloxacin and trovafloxacin at experimental concentrations potentiate IL-8 and E-selectin (CD62E) synthesis in stimulated endothelial cells [9, 10]. Available data thus suggest that ciprofloxacin and other fluoroquinolones promote a program commonly observed in mammalian stress responses.

The goal of the present study was to screen for mRNAs and gene groups that are affected by the fluoroquinolone ciprofloxacin using two different commercial microarrays compared with conventional Northern blots and Quantikine[®] mRNA analysis. Out of 847 genes investigated in ciprofloxacin-treated cells, a vast number of different up-regulated and down-regulated genes were found comprising eight major gene groups. To analyze the specificity of the microarrays, a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR; Taqman[®]) detecting 12 different cytokine mRNAs was included in our study. It is interesting that 11 out of the 12 gene transcripts were up-regulated in the specific RT-PCR, whereas only eight mRNAs were increased in one of the microarrays. The microarray is a powerful technique for screening purposes, but further analyses have to be done in detail to quantify specific gene transcripts in fluoroquinolone-treated mammalian cells.

MATERIALS AND METHODS

Reagents

Preservative-free ciprofloxacin was kindly provided by Bayer (Wuppertal, Germany). Phytohemagglutinin (PHA; Wellcome, Dartford, UK) was dissolved in RPMI-1640 medium (Life Technologies, Paisley, Scotland).

Cells

Human PBLs from healthy donors were isolated from buffy coats with citrate by centrifugation on a step gradient of Ficoll-Isopaque (Lymphoprep; Amersham Pharmacia Biotech, Uppsala, Sweden) [2]. A total of 10×10^6 cells were cultured at 37°C in a humidified 5% CO₂ atmosphere for 18 h with or without ciprofloxacin at the indicated concentrations in T25 culture flasks (Nunc, Roskilde, Denmark). Culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum and gentamicin (12 μ g/ml; Schering-Plough, Berlin, Germany). PHA (Sigma Chemical Co., St. Louis, MO) at 1 μ g/ml was added at initiation of cultures. To analyze the quality of cell growth, measuring [methyl-³H] thymidine incorporation (5 μ Ci/well; Amersham Pharmacia Biotech) after 72 and 96 h, as described previously [11], assessed proliferation.

RNA isolation, Northern blots, and cDNA probes

RNA was isolated from lymphocytes using a total RNA isolation kit (BD Biosciences, Erembodegem, Belgium), following the manufacturer's instructions. Only RNA with absorbance ratios [optical density (OD)₂₆₀/OD₂₈₀] above 1.5 was used for further analysis. To examine RNA integrity and possible DNA contamination, 0.5 μ g RNA was subjected to 1% agarose gel electrophoresis. For Northern blots, 15 μ g RNA was loaded onto 1% agarose formaldehyde gels and blotted to Hybond-N⁺ nylon filters (Amersham Pharmacia Biotech) overnight. After blotting, filters were hybridized at 42°C for 24–36 h in a buffer containing dextran sulfate, 20 \times saline sodium citrate (SSC; 1 \times SSC=0.15 M NaCl/0.015 M sodium citrate), deionized formamide, and cDNA probes. The hybridization was followed by high-stringency washes in 1 \times SSC, 0.1% sodium dodecyl sulfate (SDS), at room temperature and then in 0.1 \times SSC,

0.1% SDS, at 55°C. Thereafter, the filters were exposed to phosphorimager screens for 18 h at room temperature. Imaging screens were scanned in a personal molecular imager FX (Biorad, Sundbyberg, Sweden) at 50 mm pixel size for high resolution. To manufacture probes, IL-2, TNF- α , and triosephosphate isomerase (TPI) cDNA [7] were radiolabeled with α -[³³P]-dCTP (370 Mbq/ml, 10 μ Ci/ml; Amersham Pharmacia Biotech) using a Rediprime II random prime labeling kit (Amersham Pharmacia Biotech), as described by the manufacturer. Free nucleotides were separated on spin columns (Costar, Cambridge, MA) containing Sephadex G-50 fine (Amersham Pharmacia Biotech).

Quantikine[®] IL-2 mRNA enzyme-linked immunosorbent assay (ELISA)

Total RNA (2 μ g) was hybridized with IL-2-specific biotin-labeled capture oligonucleotide probes (R&D Systems, Abingdon, UK) and digoxigenin-labeled detection probes coated onto a microplate. Antidigoxigenin alkaline phosphatase conjugate, substrate solution, and wash buffer were added as described by the manufacturer.

Human cytokine expression array and data analysis

In experiments with R&D System's microarray (human cytokine expression array containing 847 genes), total RNA (2 μ g) was amplified in two steps, which involved annealing human cytokine-specific primers (R&D Systems), α -[³³P]-dCTP (370 Mbq/ml, 10 μ Ci/ml; Amersham Pharmacia Biotech) and a RT reaction, according to the manufacturer's recommendations. The unincorporated radioactive nucleotides were removed using Sephadex G-25 spin columns. The estimated percentage of incorporation was calculated by $I/(I+U) \times 100$, measuring the amount of radioactivity in the spin column (U) and in the eluate (I) after centrifugation. Typically, an incorporation efficiency of 55–65% was achieved. The radioactively labeled cDNAs were hybridized in 5 \times saline sodium phosphate EDTA buffer (SSPE), 2% SDS, 5 \times Denhardt's reagent to the human cytokine expression arrays (R&D Systems) at 65°C for 12–18 h. Arrays were washed twice in 0.5 \times SSPE, 1% SDS, at room temperature, twice at 65°C, and then at 65°C in 0.1 \times SSPE, 1% SDS. Resulting filters were exposed to phosphorimager screens for 18 h at room temperature followed by scanning.

Signal intensities of each array were analyzed using the ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech). The average background on every scanning was subtracted from all signals. Increased levels of gene expression were defined as a ratio (ciprofloxacin-treated cells/untreated control) of 2.0 or above, and decreased mRNA levels were defined as a ratio of 0.6 or below in \geq two donors out of three analyzed.

In addition to the R&D microarray, a microarray from Clontech (Atlas human cDNA expression array, Hampshire, UK) was included in our study. This array comprises 588 different genes. To produce α -[³³P]-labeled cDNA probes, total RNA (2 μ g) was used with cDNA synthesis (CDS) primer mix (Clontech), according to the manufacturer's protocol. The probes were thereafter purified using chromatography. Hybridization to the membranes was performed overnight at 68°C in ExpressHyb solution (Clontech). After hybridization, the membranes were washed four times in prewarmed 2 \times SSC, 1% SDS, at 68°C. Subsequently, a wash with prewarmed 0.1 \times SSC, 0.5% SDS, at 68°C was done. Finally, the membranes were washed in 2 \times SSC at room temperature. The arrays were consequently exposed to phosphorimager screens for 18 h at room temperature, scanned, and analyzed using the ImageQuant software.

Taqman[®] cytokine gene expression real-time RT-PCR

Total RNA (2 μ g/100 μ l reaction) from each donor was used as template in a two-step RT-PCR. As recommended by the manufacturer, the reaction mix prepared for the first step included MultiScribe RT and random hexamers (Applied Biosystems, Foster City, CA). The resulting first-strand cDNAs were transferred to the Microamp Optical 96-well reaction plate, in which each sample was in duplicate. Subsequently, the cDNA was amplified, and results were calculated according to the protocol supplied by the manufacturer.

RESULTS AND DISCUSSION

Effects of ciprofloxacin on thymidine incorporation and lymphocyte cell growth

To assure that the PBLs were properly stimulated and responded to the ciprofloxacin treatment, [³H]-thymidine incorporation was assessed after 48 and 72 h of PHA activation. Ciprofloxacin at 5 μg/ml increased the thymidine incorporation up to threefold as compared with drug-free controls (data not shown). In contrast, 80 μg/ml ciprofloxacin inhibited thymidine uptake.

Quinolones at >20 μg/ml inhibit mammalian cell growth by 30–35%, resulting in a ceased cell-cycle progression through the S phase [12]. PBLs do not, however, undergo apoptosis, and cell numbers are unchanged in cell cultures treated with ciprofloxacin at high concentrations when compared with onset of cultures. Despite that the cell growth apparently is inhibited by quinolones, a two- to threefold increased, tritiated thymidine incorporation is observed in PHA-stimulated human PBLs exposed to quinolones between 1.56 and 6.25 μg/ml. A similar increase has also been observed in ex vivo-stimulated splenocytes obtained from mice subcutaneously injected with ciprofloxacin for a week [13]. Any clear explanation for the quinolone-dependent, increased thymidine incorporation is not yet available. The up-regulated thymidine uptake is, however,

related to the increased IL-2 synthesis [14]. As the T cell growth factor IL-2 belongs to the “early expressed” set of genes and thus is produced within a few hours upon activation and 2–3 days before initiation of cell division, IL-2 consequently governs cell proliferation. By pulsing PBLs with ciprofloxacin for 16 h followed by extensive washing steps, we have shown that the initially increased IL-2 production in ciprofloxacin-treated cells indeed drives the cell into an enhanced uptake of thymidine compared with untreated cells. A linear relationship thus seems to exist between quinolone-dependent superinduction of the IL-2 synthesis and thymidine incorporation [14].

Ciprofloxacin-dependent enhancement of cytokine mRNA steady-state levels in activated lymphocytes and control of RNA integrity

Ciprofloxacin and other fluoroquinolones have been shown to superinduce cytokine gene expression [1]. The ciprofloxacin-dependent cytokine mRNA increase can be detected as early as after 6 h [3], and transcripts accumulate up to 48 and 72 h in ciprofloxacin-treated cells [2, 7]. IL-2 and TNF-α mRNAs are two of the most increased gene transcripts detected in mitogen-stimulated PBLs incubated in the presence of ciprofloxacin. In parallel to the cytokine mRNAs, amongst others, *c-myc*, *c-jun*, and *Fra-1* mRNAs are also up-regulated. This event occurs 2–3 h earlier as compared with the cytokine gene transcripts. Furthermore, as with

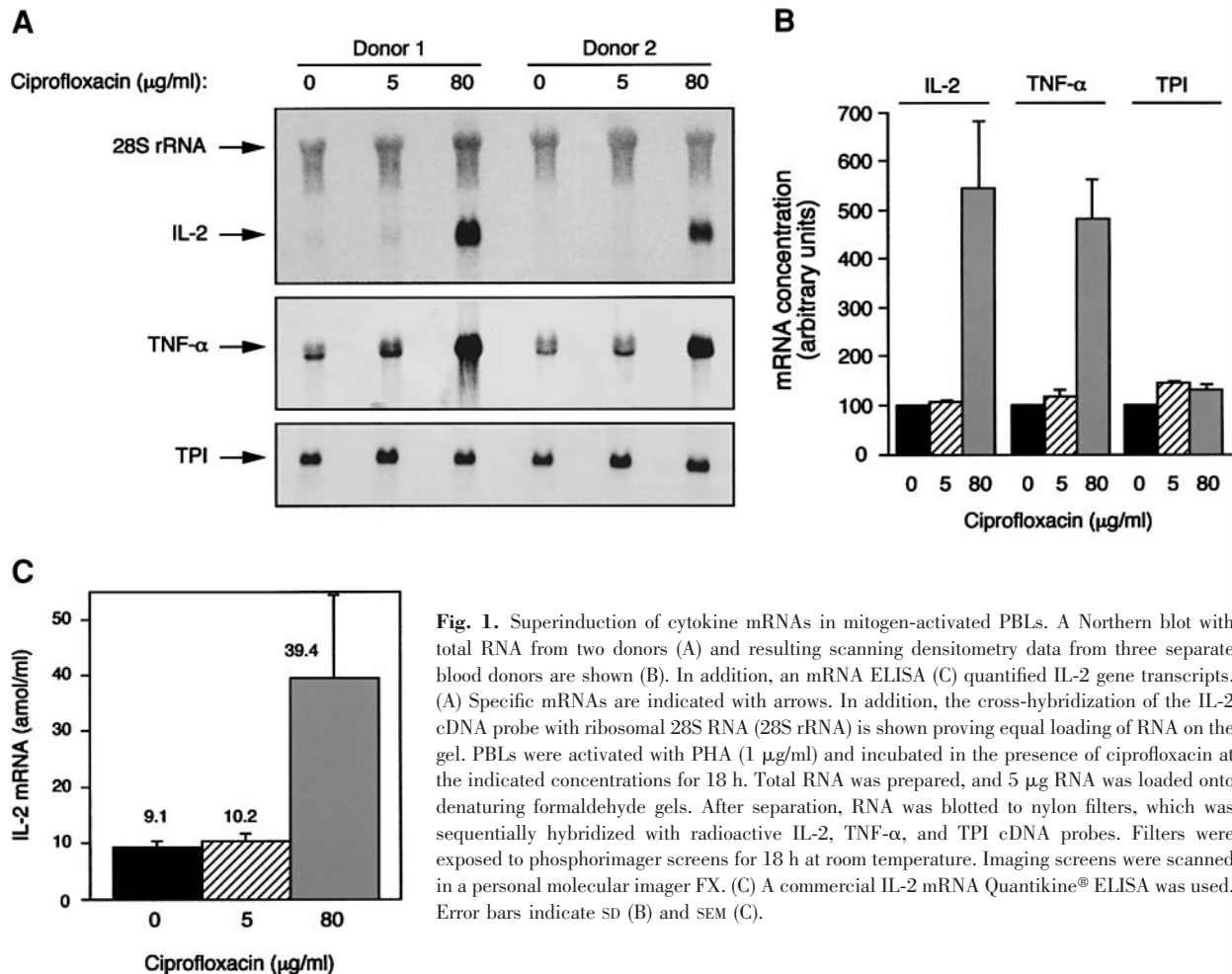


Fig. 1. Superinduction of cytokine mRNAs in mitogen-activated PBLs. A Northern blot with total RNA from two donors (A) and resulting scanning densitometry data from three separate blood donors are shown (B). In addition, an mRNA ELISA (C) quantified IL-2 gene transcripts. (A) Specific mRNAs are indicated with arrows. In addition, the cross-hybridization of the IL-2 cDNA probe with ribosomal 28S RNA (28S rRNA) is shown proving equal loading of RNA on the gel. PBLs were activated with PHA (1 μg/ml) and incubated in the presence of ciprofloxacin at the indicated concentrations for 18 h. Total RNA was prepared, and 5 μg RNA was loaded onto denaturing formaldehyde gels. After separation, RNA was blotted to nylon filters, which was sequentially hybridized with radioactive IL-2, TNF-α, and TPI cDNA probes. Filters were exposed to phosphorimager screens for 18 h at room temperature. Imaging screens were scanned in a personal molecular imager FX. (C) A commercial IL-2 mRNA Quantikine® ELISA was used. Error bars indicate SD (B) and SEM (C).

TABLE 1. Up-regulated Gene Transcripts in the Presence of 80 $\mu\text{g/ml}$ Ciprofloxacin as Determined by a Microarray from R&D Systems. A Microarray from Clontech Is Also Included for Comparison

Gene group	Gene transcript	Increase ^a (R&D Systems)	Increase ^a (Clontech)
Cytokines and receptors	IL-1 α	10.8 \pm 1.8	2.2 \pm 1.3
	IL-1 β	2.0 \pm 0.7	4.2 \pm 0.2
	IL-2	14.3 \pm 5.6	+++ ^b
	IL-4	0.7 \pm 0.5	+++
	IL-5	+++	+++
	IL-8	3.7 \pm 0.6	4.5 \pm 0.7
	IL-10	3.8 \pm 2.0	4.1 \pm 2.2
	IL-12p35	2.4 \pm 0.1	0.5 \pm 0.4
	IL-12p40	+++	+++
	IL-15	2.2 \pm 0.3	+++
	IFN- γ	8.2 \pm 3.0	4.6 \pm 2.6
	IL-1RI	3.9 \pm 1.9	0.6 \pm 0.4
	IL-2R α	2.9 \pm 0.5	3.3 \pm 0.5
	IL-13R α 1	10.4 \pm 2.5	
	Lymphotactin	4.4 \pm 1.8	
	Gro- α	4.2 \pm 0.7	
	MIP1 α	4.5 \pm 0.9	3.0 \pm 0.3
	MIP3 α	5.0 \pm 0.6	
	CCR-11	+++	
	MIG	8.1 \pm 2.0	+++
Signal transduction	CARDIAK	+++	
	MAPK6	2.4 \pm 0.4	
	DAP kinase	2.4 \pm 0.8	
	NF κ B1	2.1 \pm 0.4	
Adhesion molecules	Bcl-3	2.0 \pm 0.3	
	ALCAM	+++	
	R-cadherin	+++	
	P-selectin	12.2 \pm 6.5	
	SLAM	3.5 \pm 0.2	
TNF superfamily	I-CAM1	2.6 \pm 0.7	
	TNF- α	11.2 \pm 3.0	3.9 \pm 1.4
	TNF- β	6.6 \pm 3.1	2.4 \pm 0.9
	TNFRSF10B/TRAIL R2	8.9 \pm 1.6	5.5 \pm 1.9
Cell-cycle regulators	TNFSF5/CD40L	5.0 \pm 0.8	1.0 \pm 0.3
	cyclin E2	7.3 \pm 1.2	
	cyclin G1	4.7 \pm 2.1	
	c-myc	3.8 \pm 0.8	
Apoptosis-related	MDM2	3.9 \pm 0.6	
	ALOX5	+++	
	Granzyme B	4.6 \pm 1.2	
	Bax- α	4.6 \pm 1.7	
	BAK	3.7 \pm 0.8	
	Bid	2.9 \pm 0.6	
	Bcl-x	1.8 \pm 0.3	

^a The up-regulation was determined as the ratio between drug-treated and control cells. Mean values with SEM from three and two donors (R&D Systems and Clontech, respectively) are given and correspond to fold increase.

^b +++, The increase was not possible to define as a result of undetectable expression in untreated control cells.

Gro- α , Growth-related oncogene- α ; MIP, macrophage-inflammatory protein; CCR, chemokine receptor; MIG, monokine induced by IFN- γ ; CARDIAK, caspase recruitment domain containing kinase; MAPK6, mitogen-activated protein kinase 6; DAP, death-activating protein; ALCAM, activated leukocyte cell-adhesion molecule; SLAM, signaling lymphocytic activation molecule; I-CAM1, intercellular adhesion molecule-1; TNFRSF, TNF receptor superfamily; TRAIL, TNF-related apoptosis-inducing ligand; MDM2, monocyte-derived macrophage-2; ALOX5, 5-lipoxygenase; BAK, bcl-2 homologue antagonist/killer.

the cytokine mRNAs, the immediate early genes are also accumulated in PBLs. In the present study, we chose to stimulate the human PBLs for 18 h to detect an effect of ciprofloxacin on steady-state mRNA levels of immediate-early (e.g., *c-myc*)- and early (e.g., IL-2)-induced genes.

Northern (RNA) blots hybridized with radioactively labeled cDNA probes directed against mRNAs for the early expressed gene transcripts IL-2 and TNF- α , in addition to the house-keeping gene TPI, confirmed previous data. RNA analysis of two donors is shown in **Figure 1A**. An approximately fivefold increase of IL-2 and TNF- α mRNAs was observed at 80 μ g/ml, but not at 5 μ g/ml ciprofloxacin (Fig. 1B). The denaturing gels and subsequent Northern blots proved that the RNA was of high quality, making it suitable for microarray analysis.

Previous studies have revealed that stimulated PBLs in the presence of ciprofloxacin at 5 μ g/ml produce more IL-2 as compared with untreated controls [2]. However, in Northern blots, any significant IL-2 mRNA increase has not been observed at this concentration. Therefore, a sensitive IL-2 mRNA ELISA (Quantikine[®]) was included for comparison. No significant increase at 5 μ g/ml ciprofloxacin was observed, whereas 80 μ g/ml ciprofloxacin superinduced the IL-2 mRNA steady-state levels up to fourfold as compared with the untreated control cultures (Fig. 1C).

Ciprofloxacin enhances the induction of several gene programs in mitogen-stimulated lymphocytes as revealed by microarrays

To investigate the possible effect of ciprofloxacin on several genes, a microarray containing a human cytokine expression panel containing 847 different genes was chosen. Total RNA (2 μ g) was amplified using cytokine-specific primers and RT in the presence of α -[³³P]-dCTP, resulting in radioactively labeled cDNA products. The cDNA was hybridized to the arrays followed by washes at high stringency. Finally, filters were exposed to imaging screens that were scanned. When filters were analyzed, and resulting values were adjusted for the

exposure time, several gene products were found to be increased in PBLs incubated with ciprofloxacin at 80 μ g/ml (**Table 1**). For example, the cytokine IL-1 α , IL-2, IL-5, IL-12p40, and IL-13R α 1 mRNAs increased >tenfold in the presence of ciprofloxacin. It is interesting that in addition to cytokine gene transcripts and their respective receptors, mRNAs encoding for signal-transduction molecules, adhesion molecules, and cell-cycle regulators were also increased in PBLs incubated with ciprofloxacin (**Fig. 2A**). Altogether, 12.3% (n=104) of all genes included on the filters were up-regulated by ciprofloxacin as compared with drug-free controls. As expected, several mRNAs encoding for cytokines and their corresponding receptors were increased—36.5% of all up-regulated genes (Table 1). After cytokine mRNAs, the largest groups of up-regulated genes were signal-transduction molecules (13.5%), adhesion molecules (10.6%), proteins belonging to the TNF superfamily (10.6%), cell-cycle regulators (9.6%), and apoptosis-related molecules (8.7%). In contrast to the ciprofloxacin-dependent superinduction of several mRNAs, 11.6% (n=98) of the included gene transcripts were down-regulated in the presence of the antibacterial agent (Fig. 2B and **Table 2**). In another set of experiments, we also incubated PBLs with ciprofloxacin at 5 μ g/ml. In these experiments, mRNA concentrations were unchanged compared with the control cells incubated in the absence of ciprofloxacin (data not shown).

Microarrays are mainly qualitative analyses, and changes of some genes might not be revealed as a result of the set-up of the microarray in question; i.e., different arrays may use various amounts of cDNA and oligonucleotides. The DNA lengths and consequently hybridization temperatures may thus vary. For example, *c-fos*, *JunB*, or IL-4 mRNAs were not up-regulated in ciprofloxacin-treated cells as revealed by R&D's microarray, whereas conventional Northern blots have proven that these mRNAs are equally superinduced as IL-2 mRNA in the presence of ciprofloxacin [3, 7]. To further shed light on the

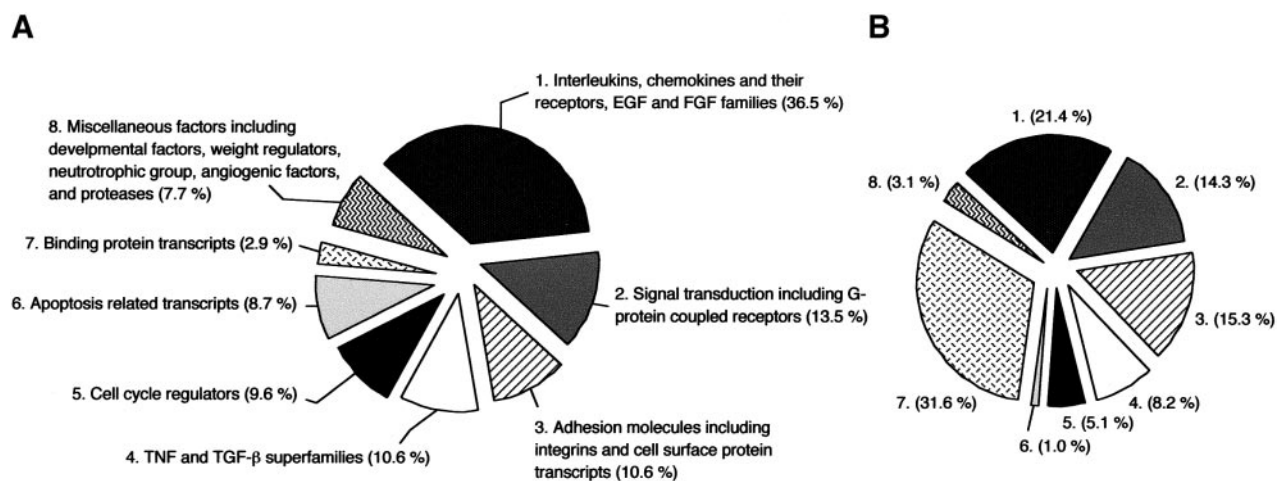


Fig. 2. Microarray analyses indicate that several genetic programs are up- and down-regulated by PBLs incubated with ciprofloxacin. Groups of genes that contain gene transcripts, which are increased (A) or decreased (B), are shown. A total of 847 genes were included on the microarray filters, and 104 and 98 gene transcripts were found to be up- and down-regulated, respectively, in the presence of ciprofloxacin at 80 μ g/ml when compared with drug-free, control PBLs. In (B), the numbering represents the corresponding gene groups that are indicated in (A). Numbers within brackets indicate percentage out of all up (A) or down (B) regulated genes. EGF, Epidermal growth factor; FGF, fibroblast growth factor; TGF- β , transforming growth factor- β .

TABLE 2. Down-regulated Gene Transcripts Detected in Cells Treated with Ciprofloxacin at 80 µg/ml Analyzed with a Microarray from R&D Systems

Gene group	Gene transcript	Decrease ^a
Cytokines and receptors ^b	HCC4	0, 0, 0
	CNTFR-α	0, 0.3, 0
	IL-9Rα	0, 0.7, 0
	S100A1	0, 0.2, 0.1
	SPARC	0.02, 0.3, 0.2
	GAS6	0.1, 0.7, 0
	IFN-γ R1	0.1, 0, 1.0
	IFNAR2	0.7, 0, 0.2
	Fractalkine	1.1, 0.2, 0
	MIP-1δ	0, 0.3, 1.0
	SARP-1	0, 0.3, 0
	M-CSF	1.8, 0.4, 0
IL-16	0.3, 0.4, 1.5	
Signal transduction ^c	GPR65/TDAG8	0, 0.02, 0
	SAP30	0, 1.3, 0
	GPR-17	0.1, 0.7, 0
	MAP2K6	0, 0.3, 0.6
	CHEK1	1.2, 0.4, 0.3
	MD-2	0.1, 0.01, 2.0
Adhesion molecules ^d	Integrinβ3	0, 0, 0
	cad-11	0, 0.9, 0
	CD34	0, 1.1, 0
	CD64	0, 0, 1.0
	Integrinβ7	0.2, 0.1, 1.0
TNF superfamily ^e	CD4	0.3, 0.2, 0.3
	PLAB	0, 1.0, 0
	ALK1	0, 0.8, 0
	TNFRSF14/LIGHT	0.2, 0.5, 0
Cell-cycle regulators ^f	TNFRSF1B	0.4, 0.3, 2.0
	CDK5	0, 1.3, 0
	CDKN2A	1.5, 0.2, 0
	CDK10	0.1, 0.4, 1.2
Apoptosis-related ^g	CDKN2D	0.4, 0.2, 1.2
	A1	0, 0.1, 0.4

^a The down-regulation was determined as the ratio between drug-treated and control cells. Values for all three donors are shown. Zero indicates that no intensity was detected with RNA isolated from ciprofloxacin-treated PBLs, whereas a specific gene induction was found in the corresponding control cells incubated in the absence of ciprofloxacin. ^bHCC4, Hemofiltrate CC Chemokine; CNTFR, Ciliary Neurotrophic Factor Receptor; S100A1, S100 calcium-binding protein A; SPARC, Secreted Protein Acidic and Rich in Cysteine; GAS, Growth-arrest-specific protein; SARP, Secreted Apoptosis Related Protein; M-CSF, Macrophage Colony Stimulating Factor. ^cGPR65/TDAG8, G protein coupled Receptor 65; SAP30, sin-3-associated polypeptide 30; CHEK1, Checkpoint, *S. pombe* homolog; MD-2, MD-2 protein. ^dCD, Cluster of Differentiation. ^ePLAB, Prostate Differentiation Factor; ALK, Activin Receptor-like Kinase. ^fCDK, Cyclin Dependent Kinase. ^gA1, adenosine A1 receptor.

ciprofloxacin-dependent up-regulation of lymphocyte genes, a microarray from Clontech was also included in our study. The Clontech broad-coverage expression array consists of 588 different genes. As can be seen on the selected gene transcripts included in Table 1, IL-4 mRNA steady-state levels were strongly up-regulated. In contrast to results obtained with the

R&D cytokine microarray, IL-1α and IL-12p40 mRNAs were unchanged in PBLs treated with ciprofloxacin. Thus, a comparison between the two different microarrays from R&D and Clontech reveals that they are complementary and also suggests that a certain degree of criticism is required regarding data obtained.

A discrepancy exists between the increased monokine mRNA concentrations observed in PBLs incubated with ciprofloxacin in vitro and in animal models. It has been demonstrated that fluoroquinolones (trovafloxacin and tosufloxacin) protect mice from LPS-dependent mortality when animals are injected with lethal doses of LPS [15]. IL-6 and TNF-α serum concentrations are significantly reduced in fluoroquinolone-treated animals compared with drug-free controls. In parallel, several studies have shown that fluoroquinolones inhibit monokine production by LPS-activated monocytic cells, albeit at drug concentrations higher than the ones achieved in serum [1]. In contrast, ciprofloxacin stimulates bone marrow (BM) regeneration in transplanted and sublethally irradiated mice by up-regulating the IL-3 and GM-CSF synthesis [5, 16]. The treated mice demonstrated a higher number of white blood cells and myeloid progenitor cells in BM and spleen on days 4 and 8 post-irradiation as compared with saline-treated animals. Despite promising results in mouse models, only one successful study exists on this phenomenon in human subjects [17]. In the group “adhesion molecules”, mRNAs, amongst others, vascular cell adhesion molecule 1 and P-selectin (CD62P), were up-regulated (Table 1), whereas CD34 and various integrin mRNAs were inhibited (Table 2). It has been shown that more E-selectin (CD62E) mRNA and protein can be found in cultures with ciprofloxacin- and also trovafloxacin-treated human umbilical venule cells [10]. It is interesting that P- and E-selectin are induced by similar transcriptional-regulation factors, and these two genes are mainly governed by NF-κB. In primary T lymphocytes, however, any effect of ciprofloxacin on phosphorylated NF-κB has not been found [3], but ciprofloxacin seems to mainly interfere with proteins included in the AP-1 complex.

Ciprofloxacin-dependent induction of cytokines as examined by a quantitative RT-PCR (Taqman[®])

The most ultimate quantitative mRNA analysis currently available is real-time PCR using cybergreen-labeled primers. The same total RNA from mitogen-activated PBLs as examined in the microarrays (Fig. 1A) was analyzed with cytokine-specific primers and related to internal controls. Eleven out of 12 cytokine mRNA steady-state levels were increased up to 23-fold in PBL cell cultures incubated with ciprofloxacin at 80 µg/ml (Table 3). It is interesting that the mRNAs encoding for the cytokines IL-1α, IL-8, IL-10, and IL-12p40 were enhanced up to 1.7-fold at 5 µg/ml ciprofloxacin as compared with the drug-free controls. In the R&D Systems microarray, eight of the 12 mRNAs were found to be up-regulated by ciprofloxacin at the higher concentration, whereas in the microarray supplied by Clontech, only six out of the 12 cytokine mRNAs were detected, as increased in ciprofloxacin-treated cells (Table 1).

TABLE 3. Quantitative RT-PCR (Taqman®) of Cytokine mRNAs in PBLs Incubated with Ciprofloxacin at 5 and 80 µg/ml^a

Cytokine mRNA	Ratio (ciprofloxacin-treated cells/control) at a drug concn of ^b	
	5 µg/ml	80 µg/ml
IL-1α	0.5 ± 0.08	14.7 ± 3.6
IL-1β	1.0 ± 0.1	4.2 ± 1.5
IL-2	0.9 ± 0.3	12.8 ± 12.2
IL-4	0.6 ± 0.2	1.5 ± 1.6
IL-5	0.6 ± 0.2	0.3 ± 1.0
IL-8	1.8 ± 0.4	7.2 ± 1.9
IL-10	1.7 ± 0.2	7.6 ± 3.0
IL-12p35	0.9 ± 0.4	1.61 ± 1.9
IL-12p40	1.2 ± 0.4	23.4 ± 8.0
IL-15	1.0 ± 0.1	1.6 ± 0.2
IFN-γ	1.2 ± 0.7	17.1 ± 7.1
TNF-α	0.8 ± 0.4	5.6 ± 0.8

^a Human PBLs were isolated and stimulated for 18 h in the presence of ciprofloxacin at the indicated concentrations. Total PBL RNA obtained from two donors (as shown in Fig. 1A) was first transcribed using random hexamers. Thereafter, first-strand cDNAs were transferred to a Microamp Optical 96 well plate and further analyzed with specific primers, detecting IL-2 and 11 other selected cytokine mRNAs as indicated. All samples were run in duplicates and related to internal controls for each sample. ^b Values obtained for mRNAs in ciprofloxacin-treated PBLs were related to the internal controls supplied by the manufacturer and divided by values obtained for drug-free controls followed by multiplication with a factor 100.

The gene program “cell cycle regulators” is influenced by ciprofloxacin

mRNAs belonging to the gene program “cell cycle regulators” were also increased by ciprofloxacin (Table 1). These superinduced gene transcripts may be related to the observation that ciprofloxacin and other quinolones at >20 µg/ml inhibit cell growth by up to 35%, causing impaired cell-cycle progression [12].

The present study reveals that several gene programs are hyperinduced in ciprofloxacin-treated PBLs. The pattern of up-regulated genes found at the mRNA level in PBLs resembles induction of a bacterial multigene cluster that is designated the SOS (“save our synthesis”) system [18]. In bacteria, quinolones trigger the SOS response upon DNA damage, and the three main proteins, RecA, RecBCD, and LexA, are consequently induced. The physiological effects of the SOS induction include the inhibition of cell division and enhanced DNA repair, resulting in increased survival of a damaged bacterium. Thus, the data in the present study support and further shed light on the fact that a mammalian stress and/or “DNA damage” response are induced by the fluoroquinolone ciprofloxacin.

Is the putative stress response related to a topoisomerase II inhibition in the mammalian cell? Evidence on quinolone interaction with eukaryotic topoisomerases exists in intact cells and with purified enzymes [18, 19]. To investigate this hypothesis, PBLs were treated with the quinolone derivative CP-115,953, which displays a high specificity against mammalian topoisomerase II. It is interesting that CP-115,953 superinduces IL-2 and IFN-γ gene transcription, albeit to a slightly lower degree as compared with ciprofloxacin [20]. For compar-

ison, the topoisomerase II inhibitor etoposide does not potentiate IL-2 transcription, whereas the DNA-modifying drug cisplatin does [21]. The effect of cisplatin on IL-2 transcription is, however, considerably weaker compared with, for example, ciprofloxacin. Etoposide has another mechanism of action than the fluorinated quinolones, and cisplatin only secondarily inhibits topoisomerase II. Thus, the strong IL-2 stimulatory properties of the quinolones are unique and cannot, in the light of data with anticancer drugs, be solely linked to a DNA damage or a general cytotoxic effect.

Concluding remarks

Despite the fact that microarrays are very informative, more specific assays are needed to fully characterize the effects of the widely used quinolone antibiotics on mammalian cells. Several increased but also decreased steady-state mRNAs can be detected in human lymphocytes incubated with the fluoroquinolone ciprofloxacin as revealed by microarray analyses. The results obtained in the present study finally support the hypothesis that ciprofloxacin at high concentrations induces a mammalian stress response resulting in induction of an array of genes.

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