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Cholera toxin induced novel genes in human lymphocytes and monocytes

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Abstract

Cholera toxin (CT) is well known as an inducer of the accumulation of cellular cAMP through the ADP-ribosylation of the Gs protein by CT. CT is also one of the most powerful mucosal adjuvants. However, the molecular mechanisms of the CT adjuvanticity are not well understood. Here, the transcriptional responses of cultured human lymphocytes and monocytes in response to CT were analyzed using differential display-PCR. The full complement of cellular mRNA was examined by high resolution polyarylamide gel electrophoresis and sequence analyses of the PCR products of 240 primer sets. Over 100 genes with altered expression were initially identified. The expressions of 65 of these genes were further analyzed and confirmed using custom glass cDNA arrays, RT-PCR and real-time PCR. Immunomodulatory genes such as *CD2*, *HIF1*, *CXCL2*, *L-plastin*, *LILR* and *IFI30* were affected by CT. In addition, 14 novel genes with previously unknown functions were found to be CT induced. These CT induced gene expression alterations provide more insight in the mechanisms of CT actions. The CT induced gene expressions alterations could contribute to the CT adjuvanticity.

Keywords: Cholera toxin; Novel genes; mRNA; Differential display; Adjuvant; VEGF; Interleukins; cAMP; cDNA arrays

1. Introduction

Cholera toxin (CT) is the causative agent of cholera. CT consists of a catalytic subunit A and a pentameric receptor binding subunit B bound by a disulfide bond (Lencer and Tsai, 2003; Spangler, 1992; Zhang et al., 1995). The subunit B binds specifically to ganglioside GM_1 receptors on the cell surface. The subunit A catalyzes the ADP-ribosylation of the Gsα subunit of heterotrimeric Gs protein and causes a continuous activation of adenylyl cyclase and results the accumulation of cellular cAMP. The increase in the cellular cAMP continuously activates ion channels and results the imbalance of cellular ion concentrations that lead to the loss of water.

Cholera toxin is not only a virulent factor of the bacterial infection but also a potent mucosal adjuvant (Elson, 1989; Freytag and Clements, 2005). Mucosal immunity functions as the first line of immunological defense against invading pathogens through innate and acquired immunity. There is currently great interest in developing mucosal vaccines against microbial pathogens (Nass, 2002). Recent progress in the mucosal vaccine delivery and adjuvants has generated a number of mucosal vaccines against bacterial and viral infections (Holmgren et al., 2005). However, the mechanisms leading to the innate and adaptive immune responses by the bacterial virulent factors are yet to be elucidated. Administration of antigens with CT results in the abrogation of oral tolerance, the induction of local and systemic humoral immunity and the induction of cytotoxic T cell responses (Snider, 1995). Part of the strong adjuvant activity is evidently due to the stimulation of the development of Th2 cells by CT through the suppressed expression of the counter-regulatory cytokine

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IL12 that reduces the development of Th1 cells (Braun et al., 1999).

The unique structure and activity features in CT that produce the powerful adjuvanticity of CT are not well characterized (Martin et al., 2001). One approach to better understand the mechanisms of action of CT induced responses is to analyze the CT induced gene expression alterations (Belcher et al., 2000). CT evidently induces a number of inflammatory genes in epithelial cells such as *IL1*, *IL6*, *IL8*, *IL-8*, *GRO*, and *MCP-1* (Rodriguez et al., 2001; Soriani et al., 2002). Using cDNA arrays, we showed that CT indeed induced appreciable changes of expression of a number of genes and particularly immunomodulatory genes in human lymphocytes and monocytes (Royaee et al., 2006a,b). These include *IL8*, *IL1β*, *IL6*, *G-CSF*, *GRO2*, *GCP-2*, *interferon-γ*, and *TNFα*. The proteins encoded by these genes stimulate the activation, the proliferation, the migration, and development of T cells, B cells, macrophages, and dendritic cells. The induction of immune stimulatory genes and repression of immuno-inhibitory genes likely contribute to the observed adjuvanticity of CT.

Differential display-PCR uses carefully selected random primer sets to amplify the full complement of cellular mRNA by polymerase chain reactions (Liang and Pardee, 1992; Martin and Pardee, 1999). High resolution polyacrylamide gel electrophoresis resolves the PCR amplified cDNA. Comparison of the autoradiographic intensities of various cDNA species in the polyacrylamide gels followed by nucleotide sequence analysis identifies differentially expressed mRNA in effector treated cells. Unlike the cDNA array methods, DD-PCR examines cellular mRNA species including both named and unknown novel genes. We undertook the analysis of the CT induced gene expression changes in human lymphocytes and monocytes using DD-PCR. The results revealed a number of CT induced gene expression alterations of several new named and novel genes.

2. Materials and methods

2.1. Materials and cells

CT and CT subunits A and B were obtained from Cal-Biochem, and 1 mg of CT (MW 84 kDa) was reconstituted with 1 ml of water as recommended by the manufacturer and was kept at 4 °C for up to 6 months. Oligodeoxynucleotides were synthesized by Life Technologies. α-[P-33]-ATP was purchased from New England Nuclear. Reagents, buffers, and kits for the reverse transcription and cDNA hybridization were from BD Biosciences Clontech unless specified otherwise. Human monocytes and lymphocytes of peripheral blood mononuclear cells were purified from leukopacks of healthy donors by centrifugation over lymphocyte separation medium (Organaon Tecknika, NC). Monocytes and lymphocytes were then further purified by counter flow centrifugation–elutriation with pyrogen-free, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline as the eluant. The resulting monocytes and lymphocyte preparations had greater than 95% viability. Cells were used immediately. Monocytes and lymphocytes were mixed in the ratio 1:4, which was optimized for the responses to CT and the cell ratio is the same as that in normal blood (Yan et al., 1999). Cells were incubated at a density of 2.5×10^6 ml^{-1} in RPMI 1640 supplemented with glutamine (Biofluids Inc.) and 10% human serum from male AB plasma (Sigma).

Freshly isolated cells at 2.5×10^6 ml^{-1} were incubated with 3 nM CT in cell culture flasks for 16 h at 37 °C under 5% CO_2. The concentration of CT at 3 nM was previously optimized for the gene expression induction in lymphocytes and monocytes (Royaee et al., 2006b). Control cells without CT were kept under the same conditions for the same periods of time.

Isolation of total RNA, cDNA synthesis, labeling and purification of cDNA, RT-PCR, were carried out as previously described (Royaee et al., 2006b).

2.2. Differential display polymerase chain reactions

Reverse transcription and DD-PCR was performed using the Hieroglyph mRNA Profile Kit for Differential Display Analysis (Beckman Coulter Inc.). Briefly 2 μg of total RNA were used in reverse transcription reactions using one of the12 different 31-mer anchored primers (T7-$(dT)_{12}$NG, T7-$(dT)_{12}$NC, T7-$(dT)_{12}$NA, T7-$(dT)_{12}$NT where T7 is the universal T7 primer and N represents any deoxynucleotide except T. The resulting cDNA was subsequently amplified by PCR using one out of 20 different carefully designed 21-mer sense primers in pairs with one of the 12 anchor primers in presence of tag DNA polymerase (PE Biosystem) and 3 μCi of [α-^{33}P]-dATP (NEN). The PCR reactions were performed by first applying a few initial cycles of low stringent annealing followed by cycles of high stringent annealing. Radioactively labeled cDNA fragments were electrophoretically separated on a 6% polyacrylamide long read sequencing gels in 8 M urea at either 1500 V for 2 h or 400 V for 16 h. The gel while attached to the glass backing was put in a heating oven at about 150 °F in front of a fan for 20 min to dry. The gel was repeatedly washed with water and dried until all residual urea was removed. An X-ray film (Kodak Biomax MR) was placed on the dried gel for overnight exposure. The DNA bands with differential intensity on the autoradiogram were cut out of the gel. Each isolated DNA band was mixed with 45 μl of DEPC water and 5 μl of 10× PCR buffer with $MgCl_2$ for prevention of depurination during elution DNA from differential display gel (Frost and Guggenheim, 1999). The mixtures were heated to 92 °C for 5 min. Following elution from the gel slice, the cDNA fragments were reamplified by following the instructions from the Hieroglyph kit. The reamplified DNA products were cloned into pCR®2.1-TOPO® vectors (TOPO TA Cloning kit, Invitrogen), and were subjected to *Eco*RI restriction analysis. Sequence analyses of the inserts were conducted with an ABI Prism 473 sequencer (Perkin-Elmer) using dRhodamine Terminator Cycle Sequencing

Ready Reaction labeling system (PE Applied Biosystems). The sequencing was carried out at the Georgetown University Sequencing Facility.

2.3. Real-time PCR

Real-time PCR was carried out in triplicate using LightCycler Master SYBR Green I kit (Roche) according to the manufacturer recommendations as previously described (Royaee et al., 2006a). The 340 bp fragment of 18s rRNA (Accession no. M10098) was used as the housekeeping gene using 5′-AATTGACGGAAGGGCACCAC-3′ and 5′-CGGACATCTAAGGGCATCACAG-3′as forward and reverse primers, respectively. Melting-curve data for all the samples were carried out to ensure that same cDNA in CT treated and control samples.

Primer pairs for eight unknown genes induced by CT. The first and second sequences were the sense and antisense primers, respectively, for each cDNA.

Accession no.	Sequence
D86961	CTATTTTCCTGGCTGTGGGA AGTCTATGGCCTTCTGGCAA
AF052146	CCCAGCATGTACCTGGACTT TCACAAGCAGATTGGAGCAC
AB033047	GAACTGGCACAGGAACACCT CTGGGCAGTTCTGGTTTTGT
AB029005	TGGGCTCAGCTCTTACCAGT GGAGTCCGAGTCTTGTCTGC
AB007962	TGTTGTCACTGACTTTGGGC CACAAACTTCCCAAGGAAGGTAA
AL04996	AGCGAATGGATGGATTTGAA AGGATCTCGAGTTTCCAGCA
AL050206	TACATTTTCCCTAAGCGGTG TCGGCTCGTTGAATTCTTTT
NM_020317	AAAGCCTGAACTGTCGGAAA GTACCCCTGAATGGCTCAAA

For CD2, forward primer was 5′-TTCCCAACATCCTC-CTCCAC-3′ and reverse primer was 5′-CGGTCCTCTA-CCATTCTTGC-3′. For HIF-1, the two primers were 5′-AATGATGTAATGCTCCCC-3′ and 5′-TTCTATGACTCC-TTTTCCTG-3′ as forward and reverse primers, respectively.

2.4. Customized cDNA array preparation and data analysis

The differentially expressed transcripts (DETs) or the cDNA clones obtained from DD-PCR were each amplified by PCR in a 100 μl, purified by ethanol-acetate precipitation, and spotted on amine-coated glass microscope slides (Telechem) by a GMS 417 arrayer (affymatrix). The cDNA arrays were then post-processed according to the instruction provided by Telechem and were made ready for hybridization to target cDNAs. The total RNA extracted from the control sample and the cholera toxin treated sample were then reverse transcribed and were subjected to indirect or TSA (Tyramide Signal Amplification or TSA^{TM}) labeling according to the manufacturer instructions (Perkin-Elmer). Hybridization of the targets to probes took place over night under glass cover slip. After washing and staining the cDNA arrays with Cy5 and Cy3 for the treated and the control targets, respectively, an Axon scanner (Axon Inc.) was used to scan the cDNA arrays and the fluorescence intensities were quantified by GenePix software (Axon Inc.).

2.5. ELISA

The IL-6 enzyme-linked immunosorbent assays (ELISA) were conduced using human IL-6 Quantikine ELISA kit from R&D Systems (MN). Briefly the Quantikine system employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 was precoated onto microtiter plates. Standards and various sample dilutions were pipetted into the wells and any IL-6 present was bound by the immobilized antibody. After washing steps, an enzyme-linked polyclonal antibody specific for IL-6 was added to the wells. Following washings to remove any unbound antibody–enzyme reagent, substrate was added to the wells and color developed in proportion to the amount of IL-6 bound in the initial step. The color development was stopped and the intensity of the color was measured by a UV spectrometer (CERES UV 900 HDI, Bio Tek Instruments) at 450 nm.

3. Results

3.1. Differential display-PCR

Human lymphocytes and monocytes were isolated and cultured under identical conditions in the presence and the absence of 3 nM CT for 16 h. Lymphocytes and monocytes were in a ratio of 4 to 1 that was the optimal ratio for the induction of gene expression alterations (Royaee et al., 2006b; Yan et al., 1999). The concentration of CT and the duration of CT exposure were optimized previously for the CT induced gene expression by monitoring the dependence of the gene expression alterations on the exposure time and the CT concentration (Royaee et al., 2006a). The total RNAs isolated from the CT treated cells and control cells were used for DD-PCR analysis. The total RNA was first subjected to independent reverse transcription reactions to synthesize the cDNAs using twelve primers with different combinations of two bases immediately following $(dT)_{12}$. The pool of cDNA from each reverse transcription reaction was amplified in the presence of radioactively labeled [α-P-33] ATP using 20 separate amplification reactions using a set of 20 different designed sense primers for each antisense anchor primer. Each PCR reaction was conducted in duplicate to ensure reproducibility. In total, 240 combinations of PCR amplification reactions were car-

ried out to include the entire complement of statistically possible mRNA sequences as specified by the sense and antisense primers. The products of each amplification reaction from the CT treated and untreated cells using the same set of sense and antisense primers were first analyzed side-by-side by high resolution urea–polyacrylamide gel electrophoresis followed by autoradiography to identify differentially expressed transcripts (DET). A total of 300 DETs were gel excised, extracted, reamplified, and their sizes were determined by agarose gel electrophoresis. Two hundred DETs yielded single discrete bands with sizes comparable to those observed in the high resolution polyacrylamide gels. The reamplified DETs were subcloned and sequenced. The sequence analysis identified 189 DETs.

To confirm the differential display expression patterns observed by DD-PCR, we performed cDNA array analyses for the isolated DETs. We constructed customized cDNA arrays using cDNA probes of the differentially expressed transcripts for named and unknown genes. The construction of the custom cDNA arrays is described in Section 2. The cDNA arrays were hybridized to fluorescently labeled cDNA targets from control and cholera toxin treated cells. The expression patterns of the selected genes were analyzed by Cy3–Cy5 staining and fluorescence intensity measurements. The intensity for each probe was normalized relative to the intensity of housekeeping genes on cDNA arrays. The cDNA array analysis was carried out three times to ensure the consistency of the expression ratios and to confirm the DD-PCR data. The CT affected genes that were identified by DD-PCR and confirmed by cDNA array analysis included 51 named genes and 14 novel genes. Tables 1 and 2 show the named genes and unknown novel genes, respectively, based on the results of cDNA arrays and DD-PCR.

The named genes in Table 1 include several genes that were previously identified in the analysis of CT induced transcriptional responses using cDNA arrays such as the induction of *IL6*, *HIF-1*, *ferritin*, *superoxide dismutase*, and the repression of *IFI30* (Royaee et al., 2005a,b). The proteins encoded by these genes belong to the same four types of proteins that were previously noted, namely genes responded to cAMP, stress, inflammation, and iron accumulation. Several new immunomodulatory genes were found. These included *TNFαIP6*, *GRO2*, *CD2*, *LILRA3*, *MIP3a*, *ILF2*, *CD36*, *HLA-C*, *galectin-8*, and *IFI30*. The finding of the predominance of immunomodulatory genes among CT affected genes using DD-PCR is in accord with the previous results using cDNA microarrays. In addition, CT induced the expression of aquaporin 9, transferrin receptor, and the translation initiation factor IF4b.

3.2. Real-time PCR

Real-time PCR was used to confirm CT responsive genes identified by DD-PCR and cDNA arrays. Specific cDNA primers were first designed for *CD2* and *HIF1* cDNAs. Total RNA from CT treated and untreated cells were used to compare their mRNA concentrations by real-time PCR. All concentrations were determined in the linear range of the real-time PCR. The corresponding melting curves showed distinct thermal transitions and were identical for the amplified cDNA from untreated and CT treated lymphoid cells. Fig. 1A compares the expression ratios of *CD2* and *HIF1* obtained using real-time PCR and cDNA arrays. Thus, real-time PCR confirmed the induction of *CD2* and *HIF1*. The induction of the novel genes was similarly examined. The primers for the eight novel genes shown in Table 2 were designed, synthesized and used to compare the expression of these eight genes in CT treated and control cells. As shown in Fig. 1B, all eight genes showed similar changes using cDNA arrays and real-time PCR. Real-time PCR of these genes confirmed the induction of these novel genes identified by DD-PCR and cDNA arrays.

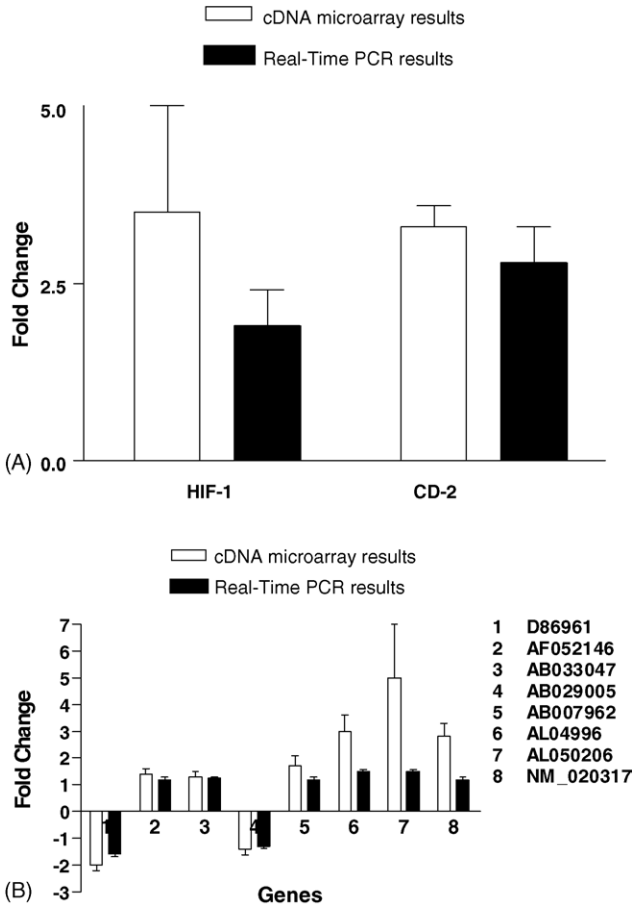

Fig. 1. (A) Expression of CD2 and HIF1 in human lymphocytes and monocytes. The expression ratios of human lymphocytes and monocytes in the presence and absence of 3 nM CT for 16 h were obtained from cDNA arrays and real-time RT-PCT. The primers used for real-time RT-PCR and experimental details for cDNA arrays and real-time RT-PCR are described in Section 2. (B) Expression of genes of unknown functions in human lymphocytes and monocytes. The expression ratios of human lymphocytes and monocytes in the presence and absence of 3 nM CT for 16 h were obtained from cDNA arrays and real-time RT-PCT. The primers used for real-time PCR and experimental details for cDNA arrays and real-time RT-PCR are described in Section 2.

Table 1
CT induced gene expression alterations of named genes

Gene name	Accession number	Expression ratio[a]	Expression ratio[b]
EGF-like-domain, multiple 5 (EGFL5)	AB011542	+++++	10 ± 2.4
Tumor necrosis factor, alpha-induced protein 6 (TNFAIP6)	NM_007115	++++	10 ± 3
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 SERPINB2	NM_002575	+++	10 ± 2
Dual specificity phosphatase 10 (DUSP10)	AB026436	+++++	9 ± 2
Dehydrogenase/reductase (SDR family) X-linked (DHRSX)	Y16947	++++	9 ± 3
Chemokine (C-X-C motif) ligand 2 (CXCL2)	NM_002089	++++	9 ± 2
Lymphocyte cytosolic protein 1 (L-plastin)	NM_002298	+++	8 ± 3
Superoxide dismutase 1(SOD1)	X02317	++++++	7 ± 2
Zinc finger protein 238 (ZNF238)	NM_006352	++++	7 ± 0.2
Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) (PPBP)	NM_002704	+++++	7 ± 4
Ferritin, heavy polypeptide 1	M11146	+++++	6 ± 0.2
Tissue inhibitor of metalloproteinase 1 (TIMP1)	M59906	+++	5 ± 1.4
Actin binding LIM protein 1 (ABLIM1)	NM_006720	+++	5 ± 3
MAP-kinase activating death domain (MADD)	NM_003682	+++	5 ± 2
U5 snRNP-specific 40 kDa protein (hPrp8-binding) (HPRP8BP)	NM_004814	++	4.5 ± 1
Vacuolar protein sorting 29 (Vps29)	NM_016226	+++	4 ± 1
Component of oligomeric golgi complex 2 (COG2)	NM_007357	++	4 ± 1.5
CD2 antigen (p50)	NM_001767	++++	3.5 ± 1.5
Src-like-adaptor (SLA)	NM_006748	+++++	3 ± 0.5
Transferrin receptor (p90, CD71) (TFRC)	NM_003234	+++	3
Erythrocyte membrane protein band 4.1-like 3 (EPB41L3)	NM_012307	+++	3 ± 0.5
CCR4-NOT transcription complex, subunit 2 (CNOT2)	AF180473	+++	2.6 ± 0.4
Leukocyte immunoglobulin-like receptor, subfamily A (LILRA3)	NM_006865	+++	2.5 ± 0.7
Protein tyrosine phosphatase, receptor type C (PTPRC)	NM_002838	+++	2.5 ± 0.7
BCL2-associated athanogene 5 (BAG5)	NM_004873	++++	2.4 ± 0.6
Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) (TFPI)	NM_006287	++	2.2 ± 0.3
3-Hydroxyisobutyryl-Coenzyme A hydrolase (HIBCH)	U66669	++	2 ± 0.5
Delta sleep inducing peptide, immunoreactor (DSIPI)	NM_004089	++	2 ± 0.5
Chemokine (C-C motif) ligand 20 (CCL20)	U77035	+++	1.8 ± 0.2
Tyrosine 3-monooxygenase (YWHAB)	BC001359	++++	1.8 ± 0.2
Major histocompatibility complex, class I, C (HLA-C)	AJ298116	+++	1.8 ± 0.4
Aquaporin 9 (AQP9)	NM_020980	+++	1.8 ± 0.5
Hypoxia-inducible factor 1 (HIF1A)	U22431	+++	1.8 ± 0.6
DEAD (Asp–Glu–Ala–Asp) box polypeptide 5 (DDX5)	X52104	+++	1.6 ± 0.3
Putative translation initiation factor (SUI1)	AF100737	+++	1.6 ± 0.2
Lectin, galactoside-binding, soluble, 8 (LGALS8)	NM_006499	+++	1.6 ± 0.2
Interleukin enhancer binding factor 2, 45 kDa (ILF2)	NM_004515	+++	1.6 ± 0.5
Interleukin 6 (IL6)	NM_000600	+++++	1.6 ± 1.6
Ninein (GSK3B interacting protein) (NIN)	AF212162	+++	1.4 ± 0.6
ATPase, Ca^{2+} transporting, type 2C, member 1(ATP2C1)	NM_014382	−−−	−1.4 ± 0.3
Eukaryotic translation initiation factor 4B (EIF4B)	NM_001417	−−−−	−1.6 ± 0.2
Leukocyte immunoglobulin-like receptor, subfamily B (LILRB2)	AF004231	−−−	−1.6 ± 0.2
Glucose regulated protein (GRP58)	U42068	−−−	−1.6 ± 0.2
Activated RNA Polymerase II transcription cofactor 4 (PC4)	NM_006713	−−−	−1.6 ± 0.4
Lactate dehydrogenase B (LDHB)	Y00711	−−−	−1.8 ± 0.2
Scavenger receptor class B, member 2 (SCARB2)	NM_005506	−−−	−1.8 ± 0.2
CD48 antigen (B-cell membrane protein) (CD48)	M37766	−−−	−2 ± 0.2
RNA binding motif protein 25 (RBM25)	L40392	−−−	−2 ± 0.2
Inositol polyphosphate-4-phosphatase, type II (INPP4B)	NM_003866	−−−−	−3.3 ± 1
Protein kinase C binding protein 1 (PRKCBP1)	U48251	−−−−	−3.4 ± 1
Interferon, gamma-inducible protein 30 (IFI30)	NM_006332	−−−−	−6.2 ± 0.4

Differentially expressed genes were first identified by DD-PCR and subsequently confirmed using cDNA arrays. The genes are listed in the order of the expression ratios determined by cDNA arrays. '+/−' signs for mRNA expression level referred to the fold difference in the intensity of cDNA bands between the control and the treated by visual inspection in DD-PCR autoradiographs of polyacrylamide gels. Plus signs indicate upregulation and minus signs for downregulation. Custom cDNA arrays were used to determine the expression ratios of these genes in CT treated and control cells. The experiments were repeated three times and the average expression ratios in CT treated and the control cells (minus sign for repression) and the standard deviations are shown above. See Section 2 for experimental details of the DD-PCR and the cDNA arrays.

[a] Expression ratios obtained by DD-PCR by visual inspection.

[b] Expression ratios determined by custom cDNA arrays from three independent experiments. The average and the standard deviation of the ratios of the expression levels in 3 nM CT treated and control cells at 16 h after exposure are shown.

Table 2
CT induced expression changes of genes with unknown functions

Gene name	Accession number	Expression ratio[a]	Expression ratio[b]	Putative function
DKFZp586F1523	AL050206	+++	5 ± 2	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform
DKFZp564K112	AL049996	+++	3 ± 0.6	HDCMA18P protein
Hypothetical protein dJ465N24.2.1	NM_020317	+++	2.8 ± 0.5	NPD014 protein
KIAA0945 protein	NM_014952	+++	2.5 ± 0.5	Bromo adjacent homology domain containing 1
DKFZp564M1922	AL049950	+++	2 ± 0.6	O-linked *N*-acetylglucosamine (GlcNAc) transferase (UDP-*N*-acetylglucosamine: polypeptide-*N*-acetylglucosaminyl transferase)
A putative protein (BC-2 mRNA)	AF042384	+++	1.7 ± 0.2	Putative breast adenocarcinoma marker (32 kDa)
Chromosome 1 specific transcript KIAA0493	AB007962	+++	1.6 ± 0.5	KIAA0493 protein
Tetratricopeptide repeat domain from the down syndrome region of chromosome 21	D83077	+++	1.5 ± 0.1	Tetratricopeptide repeat domain 3
mRNA for KIAA0942 protein	AB023159	+++	1.5 ± 0.7	Pleckstrin and Sec7 domain containing 3
Clone 24653 mRNA	AF052146	+++	1.4 ± 0.2	CDNA FLJ43660 fis, clone SYNOV4004823
mRNA for KIAA1221 protein	AB033047	+++	1.3 ± 0.2	Zinc finger protein 644
mRNA for KIAA1082 protein	AB029005	−−−	−1.4 ± 0.4	Jumonji domain containing 1B
mRNA for KIAA0206 gene	D86961	−−−	−2 ± 0.2	Lipoma HMGIC fusion partner-like 2
Clone 24487	AF070579	−−−−	−2.5 ± 1	Clone 24487 mRNA sequence

Differentially expressed genes were first identified by DD-PCR and subsequently confirmed using cDNA arrays. '+/−' signs for mRNA expression level referred to the fold difference in the intensity of bands between the control and the treated by visual inspection. A plus sign meant upregulation of a gene, a minus sign meant downregulation of a gene. Custom cDNA arrays were used to determine the expression ratios of these genes in CT treated and control cells. The experiments were repeated three times and the average expression ratios and the standard deviations are shown below. See Section 2 for experimental details of the DD-PCR and the cDNA arrays.

[a] Expression ratios obtained by DD-PCR by visual inspection.

[b] Expression ratios determined by custom cDNA arrays from three independent experiments. The average and the standard deviation of the ratios of the expression levels in 3 nM CT treated and control cells at 16 h after exposure are shown.

3.3. ELISA

IL-6 is known to play key roles in response to infection. The IL-6 levels in the supernatants of cell media from 3 nM CT treated cells for 24 h and control cells under identical conditions were determined by ELISA. The IL-6 concentrations were 424 and 6850 pg/ml in the supernatants of control and CT treated cells, respectively.

4. Discussion

CT is well known for the induction of cellular cAMP and cAMP is known to induce gene expression of a number of genes. Relatively little is known on the effect of CT on gene expression. We have thus far carried out the analysis of the CT effects on the gene expression of human lymphocytes and monocytes by three different approaches including cDNA microarrays of 800 selected genes (Royaee et al., 2006b), cDNA arrays of selected regulatory genes (Royaee et al., 2006a), and in the present investigation DD-PCR analysis. The analysis using cDNA microarrays showed that four groups of genes were affected including the cAMP responsive genes, the stress response genes, the immune response genes, and iron regulation genes. Host transcriptional responses to forskolin, a cAMP agonist, and MDL-12, a cAMP antagonist, were compared to the CT induced responses to identify cAMP induced gene expression alterations (Royaee et al., 2006b). The analysis of more immune response genes on nylon cDNA arrays revealed a large number of genes involved in the inflammation were affected. The results are consistent with the CT's powerful adjuvanticity in mucosal immunity. The present analysis using DD-PCR confirmed the previously observed CT induced changes in gene expression and revealed additional involved genes. Although the full complement of cellular mRNA was completely analyzed, relatively few genes were identified due to the limitations of DD-PCR in sensitivity and the modest potency of CT in gene expression alterations.

The most notable effect of CT on the host gene expression based on these analyses is the predominance of the immunomodulatory genes. Thus, far, these included *IL8*, *IL1b*, *IL6*, *IL10*, *G-CSF*, *GRO2*, *GCP2*, *MCP1*, *MIP1*, *MIP3*, and *VEGF* (Royaee et al., 2006a). The protein level of IL-6 was found to increase 15-fold in CT treated cells. Although whether the induction of these genes in human lymphoid cells is necessary and sufficient for CT as a powerful adjuvant is yet to be determined, the proteins encoded by these genes are known to mediate the recruitment, the migration, the activation, the proliferation, and the development of lym-

phocytes. These results resemble some of the CT induced genes in epithelial cells. Human epithelial cells express high levels of cytokines IL-8, GRO, MCP-1, MIP1α, and MIP1β in response to the infection of enteroinvasive microorganisms (Kagnoff and Eckmann, 2001). Enterotoxins induce *IL-6*, *IL-10*, *IL1R*, *IL-1α*, and *IL1β* in epithelial cells (Soriani et al., 2002). Thus, CT is able to induce the inflammatory gene expression in both epithelial and lymphatic cells. Although lymphatic cells are separated by the epithelial cells from the lumen in gut, the lymphatic cells are readily accessible through transcytosis (Lencer et al., 1995). IL1 has been used as substitutes of CT as mucosal adjuvants (Staats and Ennis, 1999). MCP1 and MIP1 have been used as adjuvants in DNA vaccine development (Lu et al., 1999). Thus, the CT induced immunoregulatory genes may contribute to the potent stimulation of the immune response through the combined effects of these cytokines, chemokines, and VEGF. The high level of increase of the VEGF expression induced by CT is consistent with the upregulation of HIF1 (Fig. 1A), which regulates the expression of VEGF (Ferrara et al., 2003). Since the mucosal immunity requires the migrations of various cell types involved in the mucosal immune response, the expression of VEGF could facilitate the cellular development.

DD-PCR provides a powerful method to explore the CT induced gene expression alterations in particular in the identification of novel genes. The Gene Ontology database (Ashburner et al., 2000) provided insights into the putative function of five of the novel genes in Table 2. Based on structural and functional homology with other eukaryotic gene products, gene ontology analysis indicated that novel genes (AL050206, AL049950, AF042384, D83077, and AB033047) that were subject to the CT induction or repression were important regulatory proteins. AL050206 (PPP2R2A) has protein phosphatase type 2A activity. AL049950 (OGT) is involved in the O-linked *N*-acetylgluocosamine transferase activity. AF042384 (BC-2) encodes a putative breast adenocarcinoma marker (32 kDa). D83077 (TTC3) exhibits ubiquitin-protein ligase activity. AB033047 (ZNF644) binds DNA and regulates transcription. While the roles of these gene products in CT action are not at all well established at present, the current finding of these novel genes revealed the involvement of both cell surface receptors and intracellular signaling pathways in the mechanisms of CT actions. Additional putative functions of these novel genes were obtained based on the structural motifs and the protein classifications (Table 2). Although the functions of these genes are yet to be determined, their CT responsiveness suggests that at least some of these genes may be involved in the immunomodulation. Further studies using either knockout or CT mutant treated cells or animals could reveal more direct information on their functions.

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