An α-Helical Cationic Antimicrobial Peptide Selectively Modulates Macrophage Responses to Lipopolysaccharide and Directly Alters Macrophage Gene Expression[1]

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Certain cationic antimicrobial peptides block the binding of LPS to LPS-binding protein and reduce the ability of LPS to induce the production of inflammatory mediators by macrophages. To gain a more complete understanding of how LPS activates macrophages and how cationic peptides influence this process, we have used gene array technology to profile gene expression patterns in macrophages treated with LPS in the presence or the absence of the insect-derived cationic antimicrobial peptide CEMA (cecropin-melittin hybrid). We found that CEMA selectively blocked LPS-induced gene expression in the RAW 264.7 macrophage cell line. The ability of LPS to induce the expression of >40 genes was strongly inhibited by CEMA, while LPS-induced expression of another 16 genes was relatively unaffected. In addition, CEMA itself induced the expression of a distinct set of 35 genes, including genes involved in cell adhesion and apoptosis. Thus, CEMA, a synthetic α-helical peptide, selectively modulates the transcriptional response of macrophages to LPS and can alter gene expression in macrophages. ***The Journal of Immunology,*** **2000, 165: 3358–3365.**

Sepsis is a condition that results when bacteria or their products enter the bloodstream and cause an overwhelming inflammatory response. Bacterial infections as well as antibiotic treatment cause the release of bacterial cell wall components such as LPS, lipoteichoic acid, and peptidoglycan (1–4). These cell wall components induce sepsis by stimulating the production of IL-1β, IL-6, IL-8, TNF-α, and other proinflammatory cytokines by macrophages. LPS is a potent activator of macrophages and is responsible for sepsis caused by Gram-negative bacteria. The activation of macrophages by LPS is initiated when LPS-binding protein (LBP)[3] transfers LPS to CD14 on the surface of macrophages. LPS-CD14 complexes then signal via Toll-like receptors to activate NF-κB as well as the extracellularly-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases, all of which mediate the production of inflammatory cytokines (5–8).

Interfering with the ability of LPS to bind to macrophages is likely to be an effective mechanism for preventing sepsis (9). We have shown that a variety of cationic antimicrobial peptides bind LPS, block the interaction of LPS with LBP, and suppress the ability of LPS to stimulate the production of inflammatory cytokines by macrophages (10–12). These cationic antimicrobial peptides are a component of the innate host defenses of both vertebrates and invertebrates and are found in all species of life (13). For example, defensins are the most predominant protein species (15% of total protein) in neutrophils. Defensins and other cationic peptides are also found at mucosal and epithelial surfaces and in the gut, lungs, kidneys, and skin. Cationic antimicrobial peptides have broad-spectrum activity against bacteria, fungi, parasites, and viruses. It is becoming increasingly clear that they play an important role in the immune system (14). In addition to their direct antimicrobial activities, they play an important early role in the response to bacterial infections, and in many cases (14) they are induced by the presence of LPS, lipoteichoic acid, and bacteria (14–17). In addition, both naturally occurring cationic peptides as well as synthetic analogues may be useful as therapeutics for suppressing inflammatory responses caused by LPS. For example, CEMA, an α-helical peptide derived from a hybrid of the silk moth cecropin and bee melittin peptides, has been shown to bind LPS, inhibit cytokine production by LPS-stimulated macrophages and macrophage cell lines, and protect mice from lethal endotoxemia (10). Thus, cationic antimicrobial peptides may be a useful tool for preventing sepsis.

To gain a more complete understanding of how LPS activates macrophages and how cationic peptides influence this process, we have used gene array technology to profile gene expression patterns in RAW 264.7 macrophages treated with LPS in the presence or the absence of the cationic antimicrobial peptide CEMA. We found that CEMA selectively inhibited LPS-induced gene expression. For example, while CEMA strongly inhibited LPS-induced expression of a variety of genes, including those encoding the proinflammatory molecules IL-1β, macrophage-inflammatory protein-1α (MIP-1α), MIP-1β, and the CD40 ligand, it had little or no effect on the ability of LPS to induce the expression of ICAM-1, c-*rel*, and several other genes. In addition to selectively inhibiting LPS-induced gene expression, we found that CEMA itself induced

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; MIP, macrophage-inflammatory protein; iNOS, inducible NO synthase; CEMA, cecropin-melittin hybrid.

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the expression of a distinct set of genes. This suggests that natural cationic peptides produced in response to bacterial infections may directly regulate macrophage function in addition to selectively modulating macrophage responses to LPS and directly killing bacteria.

Materials and Methods

Reagents

Salmonella typhimurium LPS was purchased from Sigma (St. Louis, MO). CEMA and LL-37 were synthesized at the Nucleic Acid/Protein Synthesis Unit at the University of British Columbia as described previously (10).

Cytokine production by RAW 264.7 cells

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FCS. RAW 264.7 cells were plated in 24-well dishes at 2.5×10^5 cells/well in the above medium, except that DMEM was phenol red free to prevent interference with the Griess reagent, incubated overnight, and then stimulated with 100 ng/ml *S. typhimurium* LPS alone, 50 μg/ml CEMA alone, or 100 ng/ml *S. typhimurium* LPS and 50 μg/ml CEMA added simultaneously to the cells. The cells were then incubated for 24 h before measurement of IL-1β and NO and for 4 h before MIP-1α measurements. The cultures were assayed for IL-1β by ELISAs (R&D Systems, Minneapolis, MN) that could detect $<$10 pg/ml IL-1β. MIP-1α levels in the supernatant were also measured by ELISA (R&D Systems) that could detect $<$31 pg/ml. These experiments were performed a minimum of three times. MIP-1α, IL-1β, and TNF-α were also measured by ELISA (R&D Systems) in the supernatants of the cells used for RNA isolation (see below).

Whole blood assay

Blood from three donors was collected by venipuncture into tubes (Becton Dickinson, Franklin Lakes, NJ) containing 14.3 USP units of heparin/ml blood. Whole blood was stimulated with 100 ng/ml LPS in the presence or the absence of peptide (50 μg/ml) in polypropylene tubes at 37°C for 6 h. The samples were centrifuged for 10 min at $2000 \times g$ to separate the plasma and were stored at -20°C until analyzed for IL-1β levels by ELISA (R&D Systems).

NO production

The RAW cells were cultured as described above, and the amount of NO in the supernatant was estimated from the accumulation of the stable NO metabolite nitrite with Griess reagent (Molecular Probes, Eugene, OR). Briefly 150-μl samples or standards and 130 μl of water were added to wells of a 96-well plate in duplicate. The Griess reagent (20 μl) was added to each well, the plate was incubated at room temperature for 30 min, and the OD_{450} was read with a spectrophotometer.

RNA isolation

RAW 264.7 cells were plated in 150-mm tissue culture dishes at 5.6×10^6 cells/dish, incubated overnight, and then stimulated with or without 100 ng/ml LPS in the presence or the absence of 50 μg/ml CEMA for 4 h. After stimulation, the supernatant was removed for the measurement of cytokine production, and the cells were washed once with diethyl pyrocarbonate-treated PBS, then detached from the dish using a cell scraper. Total RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MD). The RNA pellet was resuspended in RNase-free water containing RNase inhibitor (Ambion, Austin, TX). The RNA was treated with DNase I (Clontech, Palo Alto, CA) for 1 h at 37°C. After adding termination mix (0.1 M EDTA (pH 8.0) and 1 mg/ml glycogen), the samples were extracted once with phenol/chloroform/isoamyl alcohol (25/24/1) and once with chloroform. The RNA was then precipitated by adding 2.5 vol of 100% ethanol and 0.1 vol of sodium acetate, pH 5.2. The RNA was resuspended in RNase-free water with RNase inhibitor (Ambion) and was stored at -70°C. The quality of the RNA was assessed by gel electrophoresis on a 1% agarose gel. Lack of genomic DNA contamination was assessed by using the isolated RNA as a template for PCR amplification with β-actin-specific primers (5′-GTCCCTGTATGCCTCTGGTC-3′ and 5′-GATGT CACGCACGATTTCC-3′). Agarose gel electrophoresis and ethidium bromide staining confirmed the absence of an amplicon after 35 cycles.

Mouse cDNA expression arrays

Atlas cDNA expression arrays (no. 7741-1), which consist of 588 selected mouse cDNAs spotted in duplicate on positively charged membranes, were purchased from Clontech. Details of the arrays and the methodology used can be found on the Clontech website: www.clontech.com. Briefly, ^{32}P-radiolabeled cDNA probes were prepared from 5 μg of total RNA using the Moloney murine leukemia virus reverse transcriptase and pooled primers specific for the 588 genes. The ^{32}P-labeled cDNA probes were separated from unincorporated nucleotides using ChromaSpin columns, and 1×10^6 cpm/ml of denatured probe in 5 ml of hybridization solution was used for hybridization. The gene array filters were prehybridized with ExpressHyb containing 0.5 mg/ml sheared salmon sperm DNA (Ambion) before incubating overnight at 71°C with the denatured cDNA probes in a hybridization oven at 5 rpm. The filters were washed extensively at low and high stringency conditions recommended by Clontech and then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 3 days at 4°C. The image was captured using a Molecular Dynamics PSI PhosphorImager. The hybridization signals were analyzed using AtlasImage 1.0 Image Analysis software (Clontech) and Excel (Microsoft, Redmond, WA). The intensities for each spot were corrected for background

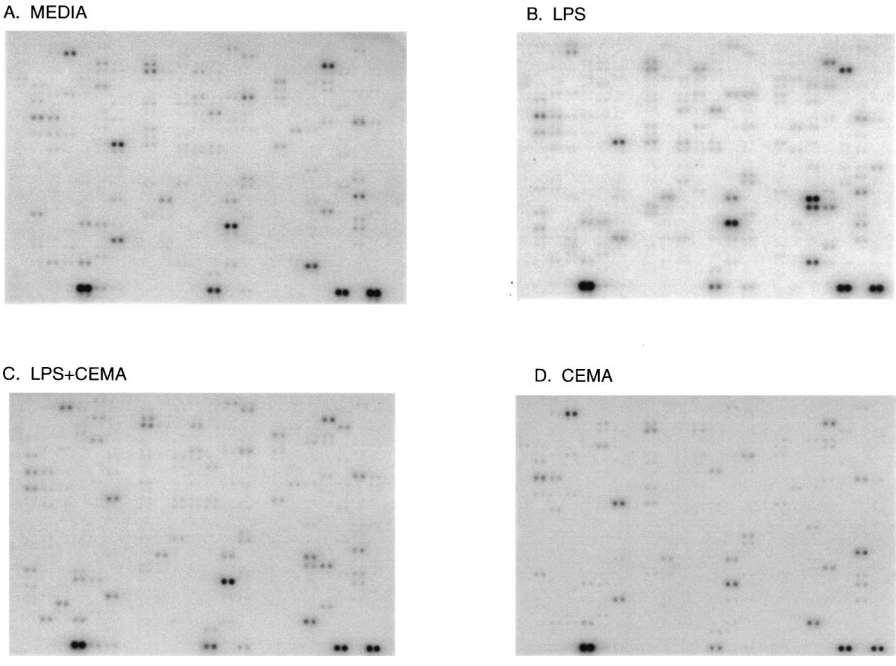

FIGURE 1. Effect of CEMA on LPS-induced gene expression in RAW 264.7 cells. RAW 264.7 cells were stimulated with medium alone for 4 h (*A*), 100 ng/ml *S. typhimurium* LPS (*B*), 100 ng/ml *S. typhimurium* LPS and 50 μg/ml CEMA (*C*), or 50 μg/ml CEMA (*D*). The RNA was isolated from the cells with TRIzol, treated with DNase, and used to make ^{32}P-labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a PhosphorImager and Clontech Atlas software. These data are representative of two or three experiments.

Table I. *Genes up-regulated by LPS in RAW 264.7 cells and inhibited by CEMA*[a]

Gene[b]	Accession Number	Unstimulated Intensity	Ratio LPS:Unstimulated[c]	Ratio (LPS + CEMA):Unstimulated[d]	Reduction Due to CEMA (%)[e]	Protein/Gene[f]
F4k	M15131	20	105.8	72.2	32	IL-1β
C3f	M83649	20	84.8	59.2	30	Fas
D2I	M20157	20	83.7	47.1	44	Egr-1
F3g	X53798	20	72.7	19.3	73	MIP-2α
E1f	M83312	20	64.5	25.3	61	CD40
B4k	M57422	20	62.1	13.9	78	Tristetraprolin
F7a	L28095	20	59.4	22.1	63	ICE
D1f	U36760	20	51.4	7.5	85	Brain factor 1
D1j	U36340	20	47.8	7.8	84	CACCC box-binding protein BKLF
D1I	M58566	20	46.7	9.0	81	Butyrate response factor 1
F3f	M35590	188	36.8	15.4	58	MIP-1β
D1e	L36435	20	32.9	6.5	80	Basic leucine zipper transcription factor
A1j	U27177	20	31.6	12.9	59	p107
A1k	U36799	20	31.4	7.2	77	p130
C3m	M87039	20	31.0	4.3	86	iNOS
D1h	S68377	20	31.0	9.4	70	Brn-3.2 POU transcription factor
C5e	X72711	20	24.7	14.1	43	Activator-1 140-kDa subunit
F5a	U14332	20	22.2	3.4	85	IL-15
D1d	D26046	20	21.9	4.8	78	AT motif-binding factor
F4d	X14432	20	20.5	11.5	44	Thrombomodulin
B4f	Z48538	20	19.8	8.4	58	Stat5a
C2h	L20331	20	18.4	3.3	82	Adenosine A3 receptor
E3m	X13358	20	18.3	11.4	38	Glucocorticoid receptor form A
D2d	U01036	20	16.5	11.0	34	NF-E2 transcription factor
F3e	X12531	489	15.1	8.3	45	MIP-1α
B5d	U25685	20	12.0	7.0	42	Syk tyrosine-protein kinase
C2n	X65453	20	11.3	3.0	74	CD40 ligand
B7k	M21065	120	8.7	3.6	59	IRF1
C5d	M59378	580	7.1	4.5	37	TNFR-1
B3I	X62700	121	6.7	3.8	44	uPAR1
B3n	U19799	143	6.3	2.6	59	I-κB β
C5c	U37522	151	6.2	2.9	52	TRAIL
B3h	X57349	234	4.5	3.0	33	Transferrin receptor
C3h	U97076	188	4.0	1.7	57	FLIP-L
C5b	X57796	121	3.6	1.5	59	TNF1 (55 kDa)
B3m	U36277	402	3.3	2.0	38	I-κB α
B4d	U06924	858	3.2	2.2	32	Stat1
B4j	D01034	124	3.0	1.7	43	TFIID transcription factor
B4e	U06922	188	2.7	1.1	57	Stat3
C4f	X67914	582	2.6	1.7	36	PD-1
D41	J03168	442	2.2	1.3	43	IRF2

[a] Total RNA was isolated from unstimulated RAW 264.7 cells, and cells treated for 4 h with 100 ng/ml LPS in the presence or absence of 50 μg/ml CEMA. After reverse transcription, ^{32}P-labeled cDNA was used to probe Clontech Atlas gene array filters. Hybridization was analyzed with Atlas Image (Clontech) software. The array experiments were repeated 2–3 times with different RNA preparations and yielded very similar results. The actual changes in the normalized intensities of the housekeeping genes ranged from 0.8 to 1.2-fold, validating the use of these genes for normalization. When the normalized hybridization intensity for a given cDNA was <20, it was assigned a value of 20 (22) to calculate the ratios and relative expression. Genes with a change in relative expression levels >2 and intensities >300 are presented. In general, we had high reproducibility of changes in expression with genes that had intensities of >300. The fold changes from one representative experiment are shown.

[b] The gene classes (given by the first letter of the gene name) include class A, oncogenes, tumor suppressors, and cell cycle regulators; class B, stress response, ion channels, transport, modulators, effectors, and intracellular transducers; class C, apoptosis, DNA synthesis and repair; class D, transcription factors and DNA-binding proteins; class E, receptors (growth, chemokine, IL, IFN, hormone, neurotransmitter), cell surface Ags, and cell adhesion; class F, cell-cell communication (growth factors, cytokines, chemokines, ILs, IFNs, hormones), cytoskeleton, motility, and protein turnover.

[c] The ratio was calculated by dividing the intensities for cells treated with 100 ng/ml LPS by the intensities for unstimulated cells.

[d] The ratio was calculated by dividing the intensities for cells treated with 100 ng/ml LPS and 50 μg/ml CEMA by the intensities for unstimulated cells.

[e] The percent reduction by CEMA of LPS-induced gene expression intensities is represented as the ratio of LPS:unstimulated − (LPS + CEMA):unstimulated divided by the LPS:unstimulated ratio.

[f] ICE, IL-1-converting enzyme; IRF, IFN-regulatory factor; TRAIL, TNF-related apoptosis-inducing ligand; uPAR, urokinase plasminogen activator surface receptor (CD87); FLIP, FLICE-like inhibitory protein; PD-1, possible cell death inducer.

levels and normalized for differences in probe labeling using the average values for five genes observed to vary little between our stimulation conditions: β-actin, ubiquitin, ribosomal protein S29, GAPDH, and Ca^{2+}-binding protein. When the normalized hybridization intensity for a given cDNA was <20, it was assigned a value of 20 to calculate the ratios and relative expression (18).

Northern blots

RNA was isolated as described above. Northern blots were performed using the NorthernMax-Gly kit (Ambion). The RNA was separated on glyoxal/DMSO gels and transferred to positively charged membranes (Ambion). The RNA was cross-linked to the filters using UV light, and the filters were then baked at 80°C for 15 min. DNA templates from which probes were produced were generated by PCR using macrophage cDNA and the following pairs of primers: IL-1β, 5′-TCCAGGATGAGGACATGAGC-3′ and 5′-CTTGTGCTCTGCTTGTGAGG-3′; cyclin D1, 5′-CAGCTTAAT GTGCCCTCTCC-3′ and 5′-GGTAATGCCATCATGGTTCC-3′; CD14, 5′-CTGATCTCAGCCCTCTGTCC-3′ and 5′-CAGGAGGATGCAAAT GTTCC-3′; and GAPDH, 5′-AGAACATCATCCCTGCATCC-3′ and 5′-CTGGGATGGAAATTGTGAGG-3′.

Antisense cDNA probes were prepared by incubating 50 ng of the PCR product with antisense primer and modified nucleotides that facilitate repeated stripping of blots (Strip-EZ PCR, Ambion). These single-stranded PCR products were purified using Qiagen spin columns and were biotinylated by incubating them with psoralen-biotin (Ambion) in the presence of 365 nm of UV light. After a prehybridization step, the filters were incubated with biotinylated probes (3 ng in 10 ml of UltraHyb or ZipHyb (Ambion)) at 45°C. Hybridization of the probes to the filter was visualized

Table II. *Genes up-regulated by LPS in RAW 264.7 cells the expression of which is not inhibited by CEMA*[a]

Gene	Accession Number	Unstimulated Intensity	Ratio LPS:Unstimulated	Ratio (LPS + CEMA):Unstimulated	Protein/Gene[b]
A2m	X15842	20	24.4	30.1	c-*rel* protooncogene
A1h	X58876	20	22.3	22.5	Mdm2
D31	D49474	20	17.6	17.5	HMG-box transcription factor
E7I	X52264	20	15.9	17.0	ICAM-1
C5m	D10061	20	14.8	15.6	DNA topoisomerase I
D3d	M74517	20	14.2	18.0	GA-binding protein β2 chain
D5g	M57999	172	4.7	5.0	NF-κB-binding subunit
B2d	U34259	193	3.6	4.3	Golgi 4 transporter
A5e	X13664	283	2.4	2.9	N-*ras* protooncogene
B6a	L02526	722	2.0	2.4	MAPKK1
E5n	X14951	592	2.0	2.2	CD18 β subunit
F3h	U60530	193	1.9	2.1	Mad-related protein 2
A6c	X64713	704	1.8	2.3	Cyclin B1
C5n	D12513	219	1.7	2.9	DNA topoisomerase II
D3m	X53476	994	1.6	2.7	HMG-14 chromosomal protein
E6h	M34510	5970	1.6	1.7	CD14

[a] RNA was isolated from unstimulated RAW 264.7 cells and from RAW 264.7 cells treated for 4 h with 100 ng/ml LPS in the presence or absence of 50 μg/ml CEMA (refer to Table I for details).
[b] MAPKK1, Mitogen-activated protein kinase kinase 1; HMG-14, non histone chromosomal protein.

using the BrightStar nonisotopic detection kit (Ambion), and results were quantitated by densitometry, with GAPDH levels used for normalization.

Results and Discussion

Array studies of endotoxin stimulation of RAW macrophage gene expression and its suppression by the cationic antimicrobial peptide CEMA

We and others have previously shown that many cationic antimicrobial peptides potently inhibit the ability of LPS to stimulate the production of inflammatory cytokines by macrophages (10, 19–24). However, the effects of these peptides on other macrophage functions have not been evaluated in detail. To gain a more complete understanding of how LPS activates macrophages and how cationic peptides inhibit this process, we used gene arrays to profile global patterns of gene expression in the RAW 264.7 murine macrophage cell line. Gene arrays allow high throughput analyses of diverse gene families that permit identification of previously unrecognized effects of LPS and cationic peptides on the host.

We used the RAW macrophage cell line, as it has been used extensively as a model for macrophage responses to endotoxin. However, we confirmed that CEMA inhibited LPS-induced cytokine (IL-6 and TNF-α) production in another macrophage cell line, J774.1, and in elicited mouse peritoneal macrophages (10) as well as in human whole blood. We chose to use CEMA, an α-helical synthetic peptide, since we have shown that it binds to LPS, potently inhibits cytokine production by LPS-stimulated macrophages, and protects mice from lethal endotoxemia (10). In addition to examining the effects of CEMA on LPS-induced gene expression, we asked whether CEMA alone could directly alter RAW 264.7 macrophage gene expression.

RNA was extracted from RAW 264.7 cells that were cultured for 4 h with medium alone, 100 ng/ml *S. typhimurium* LPS, 100 ng/ml LPS plus 50 μg/ml CEMA, or 50 μg/ml CEMA alone. After RT, cDNA probes were hybridized to Clontech Atlas gene array filters. The hybridization of the cDNA probes to each immobilized DNA was visualized by autoradiography and quantified using a PhosphorImager. Representative autoradiographic images of the gene arrays are shown in Fig. 1, and the complete datasets representing the expression levels of all 588 genes in the four different cell populations can be found on our web site (http://www.cmdr.ubc.ca/arraydata1).

We found that LPS treatment of RAW 264.7 cells resulted in increased expression of at least 57 genes (Tables I and II column labeled Ratio LPS: unstimulated). These included genes encoding inflammatory cytokines such as IL-1β and IL-15; inducible NO synthase (iNOS); chemokines such as MIP-1α, MIP-1β, and MIP-2α; cell surface proteins such as Fas and CD40; and a variety of transcription factors, including members of the pRb (retinoblastoma) family. Since many of these genes had been previously reported to be LPS-regulated genes (reviewed in Refs. 6–8), it confirmed the validity of our array results. We also identified several

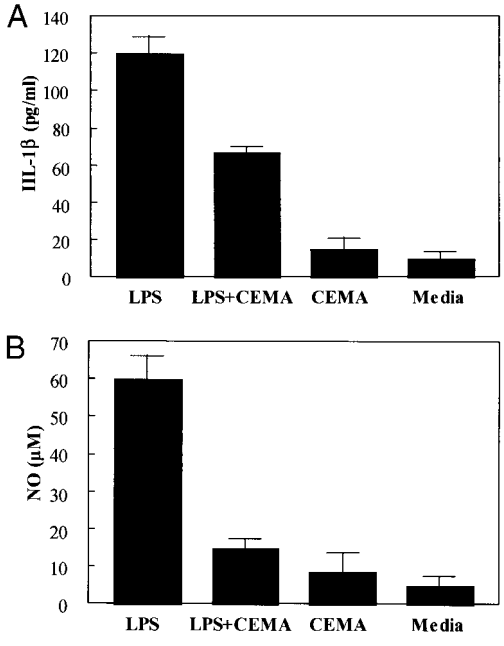

FIGURE 2. Effect of CEMA on LPS-induced production of IL-1β and NO by RAW macrophages. RAW 264.7 cells were stimulated with 100 ng/ml *S. typhimurium* LPS, 100 ng/ml *S. typhimurium* LPS plus 50 μg/ml CEMA, 50 μg/ml CEMA, or medium alone in phenol-red free DMEM an 10% FBS for 24 h. The supernatant was removed and tested for IL-1β by ELISA (*A*) and the amount of NO formed in the supernatant as estimated from the accumulation of the stable NO metabolite nitrite with the Griess reagent (*B*). The data presented are the average of three experiments ± SE.

Table III. *Genes up-regulated by CEMA treatment of RAW 264.7 cells*[a]

Gene	Accession Number	Unstimulated Intensity	Ratio CEMA: Unstimulated	Protein/Gene[b]
F6e	U49739	20	35.4	Unconventional myosin VI
A7e	U09507	20	27.9	p21/Cip1/Waf1; *cdk* inhibitor protein 1
F6c	Y14019	20	24.7	Rab-3b *ras*-related protein
A7f	U10440	20	23.9	p27kip1; G1 cyclin-Cdk inhibitor
A3g	J05205	20	22.7	Jun-D transcription factor
D2I	M20157	20	18.3	Egr-1 transcription factor
A5c	Z50013	20	16.5	H-*ras* protooncogene
E2k	D25540	20	16.2	TGF-β receptor type 1
E7d	X69902	20	15.8	α_4 integrin
A7d	U19597	161	4.0	p19ink4; cdk4 and cdk6 inhibitor
A5f	U15784	176	3.2	Shc-transforming adaptor protein
B1g	M14757	138	3.0	MDR1; multidrug resistance protein
D4I	L03547	156	2.9	Ikaros transcription factor
E2f	M98547	157	2.8	Growth factor receptor
A3a	X87257	123	2.8	*Elk-1 ets*-related protooncogene
C4f	X67914	582	2.7	PD-1 possible cell death inducer
E2j	U36203	148	2.6	*SnoN*; *ski*-related oncogene
F3a	X04480	572	2.5	Insulin-like growth factor-IA
A2g	X51983	147	2.5	c-*ErbA* oncogene
D4m	U25096	207	2.4	Kruppel-like factor LKLF
E2g	U29173	194	2.4	Lymphotoxin receptor
A2k	M16449	181	2.4	c-*myb* protooncogene protein
C6f	D16306	148	2.4	ERCC5 excision repair protein
F3h	U60530	193	2.3	Mad-related protein 2
F6d	X51438	2702	2.2	Vimentin
B4n	U05247	199	2.2	Csk; c-Src-kinase
F7f	X02389	194	2.1	uPAR
A6d	X66032	511	2.0	Cyclin B2
E6j	X07640	494	2.0	MAC-1 α subunit
A2I	J04115	426	2.0	c-Jun protooncogene
A6c	X64713	704	1.9	Cyclin B1
E6e	M27129	1345	1.8	CD44
A6f	S78355	1489	1.7	Cyclin D1

[a] RNA was isolated from unstimulated RAW 264.7 cells and from RAW 264.7 cells treated for 4 h with 50 μg/ml CEMA (refer to Table I for details).
[b] LKLF, Lung Kruppel-like factor; MDRI, multidrug resistance protein; PD-1, possible cell death inducer.

novel LPS-regulated genes, including a winged helix transcription factor called brain factor 1, Brn-3.2 POU transcription factor, PD-1 (possible cell death inducer), and HMG-14 chromosomal protein.

We then asked whether the binding of CEMA to LPS inhibited all LPS-induced changes in gene expression, or whether it selectively modulated LPS responses. Table I shows that when RAW 264.7 cells were cultured with LPS in the presence or the absence of CEMA, CEMA significantly (30–86%) reduced the ability of LPS to up-regulate the expression of 41 different genes. Interestingly, there was a large variation in the inhibition of LPS-induced gene expression. Notably, CEMA inhibited the LPS-induced up-regulation of many of the inflammation-related genes on the arrays, including IL-1β, IL-15, MIP-1α, and iNOS (Table II). In addition to inhibiting the ability of LPS to increase the levels of cytokine mRNA, we found that CEMA significantly reduced the ability of LPS to induce the expression of a number of genes with other functions. In particular, LPS increased the levels of mRNA for the pRb family retinoblastoma proteins p107 and p130 by >30-fold, and CEMA inhibited these responses by 59% (p107) and 77% (p130). CEMA also decreased the LPS-induced expression of several transcription factors, including basic leucine zipper transcription factor and Brn-3.2 POU transcription factor, by 80 and 70%, respectively. Many previous studies have focused on peptide-mediated inhibition of the proinflammatory genes induced by LPS. This is the first report of an antimicrobial peptide decreasing LPS-stimulated induction of genes other than proinflammatory genes, including genes involved in cell proliferation and apoptosis.

Confirmation of selected array results

To assess the functional significance of these results, we performed ELISAs on culture supernatants from the RAW 264.7 cells. Consistent with the array findings, we found that the levels of the chemokine MIP-1α secreted into the medium were greatly increased by LPS stimulation (cytokine concentrations of 6.3–8 ng/ml compared with <0.2 ng/ml for unstimulated cells) and that CEMA at 50 μg/ml inhibited this response by 46%. Levels of IL-1β (Fig. 2*A*) in the supernatant of RAW macrophages incubated with LPS (100–130 pg/ml) were decreased by 53 $\pm$ 5% (inhibition $\pm$ SE) in the presence of 50 μg/ml CEMA. In whole human blood incubated with LPS and LPS plus 50 μg/ml CEMA for 4–6 h, there was similar inhibition of LPS-induced IL-1β production by CEMA. LPS alone resulted in serum levels of IL-1β ranging from 0.56–0.94 ng/ml, and CEMA inhibited this by 40 $\pm$ 3% (mean inhibition $\pm$ SE). This again is similar to the results with the gene arrays. When the supernatants of the cells used for RNA isolation were tested for the cytokine levels of TNF-α and IL-6, CEMA inhibited the LPS induction of these cytokines by 78 and 86%, respectively, consistent with our previous studies and those with other cell lines and primary macrophages (10, 11).

The gene iNOS encodes the enzyme responsible for inducing the inflammatory mediator, NO. Since the peptide was found to also inhibit LPS-induced iNOS expression, we examined NO levels in the supernatant of the macrophage cells stimulated with LPS and LPS plus CEMA by measuring the accumulation of the stable NO metabolite nitrite with the Griess reagent (Fig. 2*B*). The levels

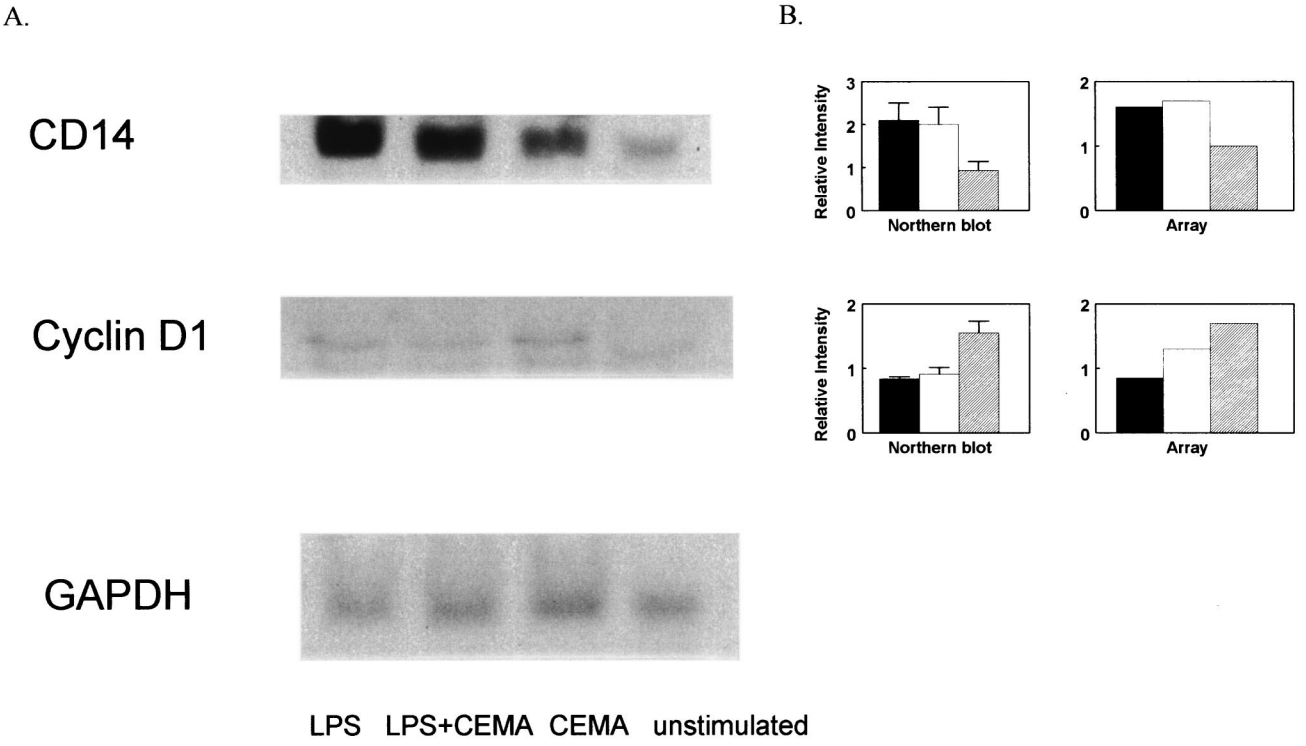

FIGURE 3. Effect of LPS and CEMA on mRNA levels as measured by Northern blot analysis. *A*, RAW 264.7 cells were stimulated with 100 ng/ml *S. typhimurium* LPS, 100 ng/ml *S. typhimurium* LPS plus 50 μg/ml CEMA, 50 μg/ml CEMA, or medium alone for 4 h. Total RNA was prepared for Northern blotting, and the membrane was probed progressively for CD14, cyclin D1, and GAPDH. *B*, The hybridization intensities of the Northern blots were measured with a densitometer and normalized to GAPDH to correct for inconsistencies in loading. The stimulated mRNA level relative to unstimulated cells was calculated as the mean ± SE from three or four experiments. The bar graphs show the ratios of gene expression of LPS-stimulated to unstimulated cells (■), LPS-stimulated, CEMA-treated to unstimulated cells (□), and CEMA-treated to unstimulated cells (▨). A ratio of 1 therefore indicates no stimulation.

of NO were increased in the presence of LPS (0–6.7 to 49.3–71.7 μM) and were inhibited an average of 76 ± 2% by the addition of CEMA (Fig. 2*B*). Similarly, the levels of iNOS on the gene arrays were up-regulated by LPS (31-fold) and inhibited 86% by CEMA. It should be noted that although these results demonstrated the same trends for the transcriptional array and product assays, the measurement of iNOS and IL-1β was performed at 24 h to permit the development of measurable amounts of product, whereas the gene arrays examined transcriptional changes at 4 h.

Evidence for a selective effect of the cationic peptide CEMA in suppressing endotoxin responses

CEMA varied widely in its ability to inhibit LPS-induced gene expression; the transcription of some genes was inhibited by as much as 85% (IL-15), and that of other genes, such as Stat1 and NF-E2 transcription factor, was only partially inhibited (30–40%). Furthermore, CEMA did not block the ability of LPS to increase the expression of 16 other genes (Table II). These genes included several that are strongly up-regulated by LPS such as c-*rel*, *mdm-2*, and ICAM-1. This indicates that the peptide had a selective effect on gene induction by LPS. This was surprising, since we had previously shown that CEMA, like other cationic antimicrobial peptides, binds LPS and inhibits its binding to LBP (12). LBP catalyzes the transfer of LPS to CD14, and the binding of LPS to CD14 is thought to be important for most responses to LPS. Based on this model, one could predict that CEMA would globally suppress responses to LPS. Several explanations are possible for why some LPS responses are not blocked by CEMA. One possibility is that those responses that are not blocked by CEMA do not involve the transfer of LPS to CD14 by LBP. A second explanation is that different responses have different thresholds for induction. Some genes may require a stronger LPS signal to be induced than others. Inhibition of LPS binding to CD14 by CEMA would reduce the ability of LPS to stimulate intracellular signaling reactions. Therefore, genes that require very strong LPS signals to be induced would be inhibited by CEMA, whereas genes that require only small amounts of LPS for signaling may still be induced maximally. A third possibility is that cationic peptides such as CEMA also act directly on macrophages to regulate signaling pathways, and that this differentially affects the ability of LPS to up-regulate the expression of different genes.

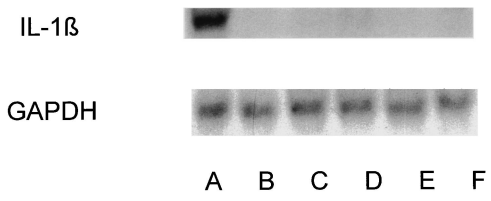

FIGURE 4. Effects of LPS, CEMA, and LL37 on IL-1β mRNA levels as measured by Northern blot analysis. RAW 264.7 cells were stimulated with 100 ng/ml *S. typhimurium* LPS (*lane A*), 100 ng/ml *S. typhimurium* LPS plus 50 μg/ml CEMA (*lane B*), 100 ng/ml *S. typhimurium* LPS plus 50 μg/ml LL37 (*lane C*), 50 μg/ml LL37 (*lane D*), 50 μg/ml CEMA (*lane E*), or medium alone (*lane F*) for 4 h. Total RNA was prepared for Northern blotting, and the membrane was probed for GAPDH and IL-1β.

Direct effect of the peptide CEMA on macrophage transcriptional responses

The possibility that CEMA acts directly on macrophages, as opposed to merely neutralizing LPS, prompted us to determine whether treating RAW 264.7 cells with CEMA alone caused any changes in gene expression. Table III shows that CEMA treatment up-regulated the expression of 35 different genes. The genes most strongly induced by CEMA (by 2- to 35-fold) included ICAM-1, cyclin-dependent kinases inhibitors, the anti-inflammatory cytokine TGF-β type I subunit (TGF-β1) receptor, Jun-D; c-jun related transcription factor, and Egr-1, which controls monocyte development and also appears necessary for the maintenance of macrophage differentiation (Table III). CEMA most notably affected the expression of genes from three families with functions in cell proliferation, apoptosis, and cell adhesion.

The up-regulation by CEMA of genes encoding cell cycle inhibitors suggests that cationic antimicrobial peptides may have anti-mitotic effects on macrophages. CEMA up-regulated the expression of three cell cycle inhibitors, $p21^{Cip1}$, $p27^{kip1}$, and $p19^{ink4}$. This result is analogous to results with lactoferrin, an iron-binding glycoprotein synthesized by epithelial cells and polymorphonuclear cell precursors, that contains an antimicrobial cationic peptide domain called lactoferricin. It was found that lactoferrin treatment of human breast carcinoma cells caused an increase in expression of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (25).

The effect of CEMA on cell proliferation could also be related to the anti-cancer properties observed with some cationic peptides, including CEMA (26, 27). Such peptides are selectively more toxic toward tumor cells than toward nonmalignant cells, although the mechanism of their activity is not fully understood (26–28). CEMA was shown here to have effects on a number of genes involved in apoptosis. For example, CEMA up-regulated PD-1 (Table III), and CEMA down-regulated the expression of a number of apoptosis-related genes (http://www.cmdr.ubc.ca/arraydata1), including the apoptosis inhibitors BAG-1, Bcl-2 (both with a ratio of CEMA to medium of 0.4), and A20 zinc finger protein (ratio of CEMA to medium of 0.1). These data might help explain the results of a previous study, which found a cecropin-melittin hybrid peptide to have an apoptotic effect on a murine macrophage cell line (29).

Cell migration is controlled by multistep processes that includes chemoattraction, cell-cell adhesion, and, in some cases, transmigration through cell layers (30). It has been reported that two human α-defensin peptides, human neutrophil peptide HNP-1 and -2, have chemotactic activity for murine and human T cells and monocytes (31, 32), while human β-defensins are chemotactic for immature dendritic cells and memory T cells through interaction with CCR6 (33). LL-37, a human neutrophil α-helical peptide (34), has also been suggested to have chemotactic activity for T cells and neutrophils (35), and the porcine peptide, PR-39, has chemotactic activity for neutrophils (36). CEMA up-regulated the expression of the urokinase plasminogen activator receptor, which is widely expressed on different cell types, including hemopoietic cells, and has been shown to involved in cell adhesion, chemotaxis, receptor clustering, and changes in cell shape (37). CEMA also up-regulated a number genes involved in cell adhesion, including ICAM-1, α_6 integrin and MAC-1 (Table II) and, to a lesser extent, α_5 integrin, CD44, and CD45 (data not shown).

There have been a number of reports of the roles of cationic peptides in the immune system (14). It is becoming increasing clear that their effects on innate immunity are wide ranging and much more involved than their antimicrobial activity. This is the first report demonstrating that a cationic peptide, CEMA, has global effects on macrophage gene expression. There have been some reports that demonstrate that cationic peptides permeabilize eukaryote cells (28). Risso et al. found that two antimicrobial peptides, BMAP-27 and -28, permeabilized eukaryote cell membranes and possibly interacted with negatively charged sialyl residues on the membrane, causing Ca^{2+} flux into the cytosol (28). This could be a potential mechanism of how cationic peptides could alter macrophage signaling or gene expression. While the mechanism warrants further investigation, this report clearly shows for the first time that cationic antimicrobial peptides directly influence gene expression in macrophages of a large number of diverse genes.

Confirmation of selected array data by Northern analysis

Although the array data were reproducible, and we had confirmed some of our findings with ELISAs, we also wanted to directly confirm that LPS and CEMA affected mRNA levels similarly to the ways indicated by the gene arrays. We chose to perform Northern blots to analyze the expression of IL-1β, CD14, and cyclin D1, since these genes represent the three different scenarios we had observed. According to the gene array results, IL-1β mRNA levels were strongly up-regulated by LPS, and this response was reduced by CEMA (Table I). Conversely, CD14 mRNA levels were modestly up-regulated by LPS, and this response was not blocked by CEMA (Table II), while cyclin D1 mRNA levels were not induced by LPS, but were modestly up-regulated by CEMA. All these results were confirmed by the Northern blots, and the quantification of these results is shown in Fig. 3. We conclude that the gene arrays successfully identified multiple patterns of gene expression and demonstrated trends similar to those observed by Northern blot analysis. To demonstrate that these results were not confined to the synthetic antimicrobial peptide CEMA, LL-37, a human neutrophil α-helical peptide (34), was tested alongside CEMA and was also found to inhibit LPS-induced gene expression of IL-1β (Fig. 4) and MIP-2α (data not shown) in the RAW macrophages to an extent similar to that observed with CEMA. Furthermore, preliminary studies indicated that LL37 was also able to up-regulate a variety of genes in RAW cells.

In summary, we have used gene arrays to profile global changes in gene expression in macrophages treated with LPS in the absence or the presence of the cationic antimicrobial peptide, CEMA, as well as demonstrated a direct effect of CEMA on RAW macrophages. Two novel findings have resulted from these experiments. First, we found that CEMA selectively inhibited LPS-induced changes in gene expression. While the ability of LPS to induce 41 genes was significantly inhibited by CEMA, the induction of an additional 16 genes was unaffected by CEMA, even though it is known that CEMA interferes with the first step in LPS signaling, the binding of LPS to LBP. It is clear that CEMA has effects other than interference with LPS:LBP binding, since CEMA can suppress endotoxin-stimulated induction of cytokines even when added to RAW macrophages up to 1 h after endotoxin (10). Interestingly, the induction by LPS of inflammatory mediators was significantly inhibited by CEMA, indicating that cationic peptides may selectively down-regulate macrophage inflammatory functions as opposed to other cellular processes. Our second novel finding was that cationic peptides such as CEMA can directly influence macrophage gene expression by either up- or down-regulating the expression of a wide variety of genes, including those that affect cell proliferation, apoptosis, and cell-cell interaction. The mechanism by which such peptides regulate gene expression was not studied here, but we suggest that cationic antimicrobial peptides interact with cell surface receptors and/or can enter cells and directly influence signaling pathways as previously suggested (38). Given the potential use of cationic antimicrobial peptides as

antibacterial agents and anti-inflammatory agents, the effects of these peptides on macrophages and other host cells warrant further investigation.

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