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Differential Expression of Interferon Regulatory Factor 1 (IRF-1), IRF-2, and Interferon Consensus Sequence Binding Protein Genes in Lipopolysaccharide (LPS)-Responsive and LPS-Hyporesponsive Macrophages

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Macrophages secrete interferon (IFN), as well as other cytokines, following lipopolysaccharide (LPS) stimulation. The interferon regulatory factors (IRFs) comprise a family of DNA-binding proteins that have been implicated in the transcriptional regulation of IFN and certain IFN-inducible genes. We therefore characterized basal and LPS-inducible levels of IRF-1, IRF-2, and interferon consensus sequence binding protein (ICSBP) mRNA in LPS-responsive macrophages and compared the expression of these genes in macrophages that typify two murine models of LPS hyporesponsiveness. In the first model, the LPS-hyporesponsive phenotype of the C3H/HeJ mouse is genetically determined and maps to the *Lps* locus on mouse chromosome 4. In the second model, normally LPS-responsive macrophages acquire a transient LPS-hyporesponsive phenotype following a prior exposure to LPS, a phenomenon referred to as "endotoxin tolerance." Using reverse transcription PCR, we detected basal levels of IRF-1 mRNA in LPS-responsive (Lps^n) macrophages that were approximately 15 times higher than those found in LPS-hyporesponsive (Lps^d) macrophages. Conversely, Lps^d macrophages expressed basal levels of IRF-2 mRNA that were approximately 18 times higher than those expressed in Lps^n macrophages. LPS stimulation resulted in a dose- and time-dependent accumulation of IRF-1, IRF-2, and ICSBP mRNA only in Lps^n macrophages. Cycloheximide inhibited the accumulation of LPS-stimulated IRF-2 and ICSBP mRNA, but not IRF-1 mRNA, thus designating IRF-1 an immediate-early, LPS-inducible gene. Finally, macrophages rendered tolerant to endotoxin expressed elevated but nonmaximal mRNA levels for all three transcription factors that are not reinduced upon secondary challenge with LPS. Thus, the IRFs may represent yet an additional molecular pathway in the complex response to LPS.

The lipopolysaccharide (LPS)-hyporesponsive C3H/HeJ mouse strain differs genetically from the closely related C3H/OuJ LPS-responsive mouse strain at the *Lps* locus on chromosome 4 (48). As a result, macrophages derived from C3H/HeJ mice, which express the Lps^d allele, do not respond to LPS, in vivo or in vitro, to produce cytokines (e.g., interferon [IFN], tumor necrosis factor, interleukin 1, etc.) or other inflammatory mediators (e.g., IP-10, prostaglandin E_2, etc.) that are characteristic of C3H/OuJ (Lps^n) macrophages (reviewed in reference 43). In addition, Lps^d macrophages appear to be functionally less differentiated than Lps^n macrophages, as evidenced by their reduced FcγR capacity, which is reversible by the addition of exogenous IFNs (8, 23). Furthermore, Lps^d macrophages, derived from conventionally reared mice, are permissive for viral replication, while Lps^n macrophages are resistant, a phenotype that is also reversible by prior treatment of Lps^n macrophages with antibodies specific for alpha/beta interferon (IFN-α/β) (45, 46). On the basis of these observations and the findings of Gessani et al. (11) that supernatants from Lps^n macrophages, but not Lps^d macrophages, confer antiviral activity on aged macrophages, the hypothesis has been developed that Lps^n macrophages maintain higher endogenous levels of IFN as a result of their ability to respond to the LPS present in normal gram-negative microbial flora and that these LPS-inducible IFNs underlie the observed phenotypic differences in macrophage function. The principal species of IFN produced in response to LPS has been demonstrated serologically to be IFN-β, although IFN-α and, most recently, IFN-γ species have been detected at both the mRNA and protein levels (2, 10, 16, 44).

Like Lps^d macrophages, Lps^n macrophages preexposed to endotoxin exhibit an LPS-hyporesponsive phenotype upon secondary challenge with LPS (42). In vivo studies of mice rendered endotoxin tolerant revealed significantly diminished levels of circulating IFN and other cytokines upon LPS challenge (18). The common characteristic of low IFN production in these two models of LPS hyporesponsiveness prompted us to examine the molecular phenotypes of these cells with respect to the expression of DNA-binding proteins thought to regulate IFN and IFN-inducible genes.

The transcriptional regulation of IFN and IFN-inducible gene expression has been an area of active research over the past several years that has led to the identification of a novel family of DNA binding proteins, referred to as interferon regulatory factors (IRFs) (27). There are presently four members of the IRF family, IRF-1 (ISGF2), IRF-2 (ISGF1), interferon consensus sequence binding protein (ICSBP), and the γ subunit of interferon gene factor 3 (ISGF3γ), on the basis of the high degree of homology in the DNA binding domains of these proteins (6, 14, 27, 31, 41). IRF-1, IRF-2, and ICSBP bind to the IRF DNA recognition sequence, (G/C)(A)AAA$(N)_{2\text{-}3}$ AAA(G/C)(T/C), that is present in the 5′ flanking regions of the IFN genes and in the IFN-stimulated response elements

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(ISRE) of many IFN-inducible genes (6, 35). In contrast, ISGF3γ binds only weakly to DNA when not complexed with ISGF3α (not an IRF family member) and does not bind to the type I IFN promoter elements recognized by the other IRFs, due to a requirement for additional DNA sequences provided by the ISRE (21, 22, 39). The highly defined function of ISGF3γ in IFN-induced signal transduction has been well described previously (40). Transfection studies have suggested that IRF-1 functions as a transcriptional activator, while IRF-2 and ICSBP serve to repress IRF-1-induced transcription (14, 28). Additionally, it has been suggested that an increase in the ratio of IRF-1 to IRF-2 in the cell may be a critical event in IRF-1-mediated transcription (35). However, the determination of the available IRF-1 and IRF-2 in a cell is undoubtedly more complicated in light of the recent finding that ICSBP forms protein complexes with the other three IRF family members (3). Thus, the dynamic interactions between the individual IRF proteins and their common target DNA sequence may determine the pattern of gene expression that results in a responsive or hyporesponsive phenotype. Therefore, we compared the relative gene expression of IRF-1, IRF-2, and ICSBP in LPS-responsive and -hyporesponsive macrophages.

The findings herein provide evidence at the molecular level that macrophages from LPS-responsive C3H/OuJ (Lps^n) mice express higher basal levels of IRF-1 and IFN-β mRNA than macrophages derived from LPS-hyporesponsive C3H/HeJ (Lps^d) mice. Conversely, basal levels of IRF-2 mRNA are higher in Lps^d macrophages. In addition, LPS modulates steady-state levels of IRF-1, IRF-2, and ICSBP mRNA in Lps^n but not in Lps^d macrophages. Finally, Lps^n macrophages rendered endotoxin tolerant in vitro, express elevated levels of all three IRFs, and, like Lps^d macrophages, fail to respond to LPS challenge to increase the gene expression of IRF-1, IRF-2, or ICSBP. These findings indicate that IRF gene expression exhibited by normal macrophages is phenotypically distinct from that observed in two separate models of LPS hyporesponsiveness and suggest the possibility that the IRF family of nuclear transactivating factors is involved in the response to LPS.

(This paper represents work carried out by Sheila A. Barber in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Reagents. Phenol-water-extracted *Escherichia coli* K235 LPS was prepared by the method of McIntire et al. (26). Natural murine IFN-α was purchased from Lee Biomolecular (San Diego, Calif.). Cycloheximide was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used at 5 μg/ml, a concentration shown previously to inhibit protein synthesis in these cells (7, 29).

Macrophage isolation and cell culture conditions. C3H/OuJ and C3H/HeJ mice (female, 5 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, Maine), maintained in a laminar flow facility under 12-h alternating light-dark cycles, and fed standard laboratory chow and acid water ad libitum. Research was conducted according to the principles set forth in "Guide for the Care and Use of Laboratory Animals" (19a). Peritoneal exudate macrophages were obtained by peritoneal lavage with sterile saline 4 days after intraperitoneal injection of 3 ml of sterile thioglycolate broth (3%). Cells were washed, resuspended in RPMI 1640 medium supplemented with 2 mM glutamine–100 U of penicillin per ml–100 mg of streptomycin per ml–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–0.3% sodium bicarbonate–2% heat-inactivated fetal calf serum, and added to six-well tissue culture plates (Falcon, Lincoln Park, N.J.) at ~4.5×10^6 cells per well in 2.5 ml. The plates were incubated at 37°C and 6% CO_2 for at least 4 h but no longer than 18 h before the nonadherent cells were removed by washing and the adherent cells remaining were treated as indicated.

Induction of endotoxin tolerance in vitro. The protocol for establishing in vitro endotoxin tolerance has been described previously (42). Briefly, peritoneal exudate cells are cultured as described above for 4 to 6 h, after which nonadherent cells are washed from the plate. Medium or 100 ng of LPS per ml in medium is added to the remaining adherent cells, and the cells are incubated for 19 h. At this time, the plates are washed twice with excess medium, and the cells are recultured in medium alone or medium containing 100 ng of LPS per ml.

Isolation of total cellular RNA. At the indicated time points, cells were washed once with sterile phosphate-buffered saline at room temperature and then lysed with RNAzolB (1 ml per well; Tel-Test, Inc., Friendswood, Tex.). Total cellular RNA was extracted according to the manufacturer's instructions and quantified by spectrophotometric analysis.

TABLE 1. Primer sequences

Factor	Primer	Sequence
IRF-1 (5′→3′)	Sense primer	CAGAGGAAAGAGAGAAAGTCC
	Antisense primer	CACACGGTGACAGTGCTGG
	Probe	GGACTCAGCAGCTCTACCCTA
IRF-2 (5′→3′)	Sense primer	CAGTTGAGTCATCTTTGGGGC
	Antisense primer	TGGTCATCACTCTCAGTGG
	Probe	TTCTCCTGAGTATGCGGTCC
IFN-β (5′→3′)	Sense primer	AGATCAACCTCACCTACAGG
	Antisense primer	TGGAGTTCATCCAGGAGAC
	Probe	CCATCCAAGAGATGCTCCAG
GAPDH (5′→3′)	Sense primer	CCATGGAGAAGGCTGGGG
	Antisense primer	CAAAGTTGTCATGGATGACC
	Probe	CTAAGCATGTGGTGGTGCA
ICSBP (5′→3′)	Sense primer	GATCAAGGAACCTTCTGTGG
	Antisense primer	GAAGCTGATGACCATCTGGG
	Probe	ATGAGTACATGGGTATGACC

Oligonucleotide sequences. The primer sequences for IRF-1, IRF-2, IFN-β, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were kindly provided by Carl Dieffenbach. The primer sets for IRF-1 and IRF-2 correspond to the cDNA regions that exhibit the least IRF sequence homology (Table 1).

The primer sequences for ICSBP were chosen by using the computer-driven program Primer Detective (Clontech Laboratories, Inc., Palo Alto, Calif.) in conjunction with the published cDNA sequences obtained from GenBank. The primer sequences selected correspond to the cDNA region exhibiting the least homology with the other IRF family members. In addition, the candidate primers were compared by computer alignment (Genetics Computer Group Gap Program) with IRF-1 and IRF-2 cDNA sequences to reduce the possibility of primer cross-reactivity. The predicted amplification product spans 3 introns.

Reverse transcription (RT)-PCR. cDNA synthesis from 1-μg aliquots of total cellular RNA was carried out as described previously (10), except that 100 ng of IRF-1, IRF-2, ICSBP, IFN-β, or GAPDH specific antisense primers was used instead of the random hexamers. Three microliters of IRF-1, IRF-2, ICSBP, IFN-β, or GAPDH cDNA was added to tubes containing 100 ng of specific sense and antisense oligonucleotide primers (see below), *Taq* buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100), 3 mM $MgCl_2$, 200 μg of dXTP, and 1 U of Amplitaq (Cetus, Emeryville, Calif.) DNA polymerase. PCR cycling was performed in an automated DNA Thermal Cycler Model 480 (Perkin-Elmer Cetus Instrumentation, Norwalk, Conn.), with each cycle consisting of 1 min of denaturing at 94°C, 1 min of annealing at 54 to 57°C, and 1 min of extension at 72°C, following an initial 3-min incubation at 94°C. A 25-μl sample of amplification product was electrophoresed in Tris-borate-EDTA through a 2% agarose gel containing 0.3 μg of ethidium bromide per ml, after which the gel was denatured (1.5 M NaCl, 0.5 N NaOH) for 20 min and neutralized (0.5 M Tris-HCl, 1.5 M NaCl) for 10 min, and the DNA was transferred to Nytran (Schleicher & Schuell, Keene, N.H.) by capillary action according to standard Southern blotting protocol (34). The membranes were UV-cross-linked in a Stratalinker 1800 (Stratagene, La Jolla, Calif.), vacuum baked at 60°C for 1 h, and hybridized to a specific internal oligonucleotide probe. The probes were labeled and detected using the enhanced chemiluminescence (ECL) protocol according to the manufacturer's instructions (Amersham, Arlington Heights, Ill.). The Kodak X-Omat AR 5 film used to detect the light emission was subjected to densitometric analysis on a Datacopy GS Plus scanner (Xerox Imaging Systems, Sunnyvale, Calif.), after which the individual band signals were measured using the National Institutes of Health Image 1.42b16 program. In order to determine optimal PCR conditions, the input RNA was held constant and PCR amplification products were sampled over a range of cycle numbers. A plot of cycle number versus PCR signal was used to select the optimal cycle number within the linear range of signal detection. In addition, serial twofold dilutions of input RNA were analyzed by RT-PCR at the optimal cycle number, which established a linear relationship between input RNA and PCR signal, thus demonstrating the ability of this technique to detect at least twofold differences of initial mRNA copy number. In order to quantify changes in IRF family mRNA levels, serial twofold dilutions of PCR amplification products (known to be positive for the gene of interest) were subjected to ECL analysis for each experiment, in order to relate changes in product concentration to changes in ECL signal. Linear regression of each standard curve generated an equation of the line to be used in the calculation of relative gene expression in the samples of interest. In addition, each sample was normalized to the expression of the housekeeping gene GAPDH, which was not modulated by any of the treatments used. The *y* axis labeled "Relative Gene Expression," used in Fig. 1 and 4, refers to this normalized value.

Probes used for Northern (RNA) blot analysis. The IRF-1 and IRF-2 DNA probes were prepared with the TA Cloning System Version 1.3 of Invitrogen Corporation (San Diego, Calif.) with the following specifications or modifications. The RT-PCR was performed as outlined above except that the extension time for the final cycle was increased to 7 min. Single DNA bands of the correct molecular weight were excised from the gel and subjected to Gene Clean (Bio 101, Inc., La Jolla, Calif.) purification followed by spectrophotometric analysis of yield. Approximately 140 to 160 ng of purified IRF-1 or IRF-2 PCR-derived DNA was precipitated with 75 ng of cloning vector (provided) in 0.3 M NaOAc (pH 7 to 7.5) and 3 volumes of ice-cold EtOH overnight at 4°C. The DNA was collected by centrifugation, washed with 75% ethanol, and resuspended in 9 μl of sterile water to which 1 μl of ligation buffer (provided) and 1 μl of T4 DNA ligase (provided) were added and then incubated overnight at 12°C. Following the transformation procedure provided, white colonies were replica plated onto Whatman nitrocellulose filters overlying antibiotic selective media and incubated overnight at 37°C. The filters were removed, denatured (above), neutralized (above), washed with sterile water, and probed with specific internal oligonucleotides that were end labeled with [γ-^{32}P]dATP by using T4 polynucleotide kinase (Gibco, Grand Island, N.Y.). Plasmids from the transformants were subsequently extracted with Circle Prep (Bio 101) and subjected to restriction enzyme digestion, electrophoresis, and oligonucleotide probe hybridization to confirm the integrity and specificity of the clones. Each clone was sequenced by using the Applied Biosystems Taq Dye-deoxy Terminator Cycle Sequencing protocol with the following specifications. Isolation of the plasmids carrying the IRF-1 or IRF-2 cDNA was carried out with the Qiagen Plasmid Mini Kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions. The vector-specific primers used were SP6 and T7. Cycling was done on a DNA Thermal Cycler Model 480 (Perkin-Elmer Cetus Instrumentation). Extension products were separated from unincorporated dye-deoxynucleosides by using Centri-Sep columns (Princeton Separations Inc., Adelphia, N.J.). Termination products were separated by agarose gel electrophoresis, and the corresponding sequences were determined with an Applied Biosystems Model 373A automated DNA sequencer. The IRF-1 clone contains nucleotides 357 to 564 of the published cDNA sequence (27). The IRF-2 clone contains nucleotides 421 to 635 of the published cDNA sequence with a 6-bp deletion of nucleotides 531 to 536 (14). The cDNA probe used in the detection of ICSBP mRNA was a 1-kb *Eco*RI insert from plasmid 131A (6). The β-actin cDNA probe was a 0.75-kb *Eco*RI insert (38).

Northern blot analysis. A 10-μg aliquot of total cellular RNA was denatured by heating at 90°C for 5 min in 1.5× loading buffer (4), quickly cooled on ice, and then electrophoresed in 1× MOPS (morpholinepropanesulfonic acid) through a 1.5% agarose gel containing 0.66 M formaldehyde and 0.3 μg of ethidium bromide per ml. Following photography, the gel was rinsed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the RNA was transferred to Nytran (Schleicher and Schuell) overnight by capillary action. The blots were then UV cross-linked and stored in prehybridization fluid (4) at −20°C until probed. A total of 50 to 100 ng of the double-stranded probes was labeled using [α-^{32}P]dCTP with a random primer oligolabeling kit (Pharmacia, Piscataway, N.J.). Phosphor screens were exposed to probed blots, and the band intensity was quantified with Phosphorimager Model 400A and Image Quant 3.0 software (Molecular Dynamics Inc., Sunnyvale, Calif.). Kodak X-Omat AR 5 film was also exposed to the probed blots and was scanned as described above.

RESULTS

Differential basal expression of IRF-1, IRF-2, and IFN-β steady-state mRNA levels in *Lps^n* versus *Lps^d* macrophages. To compare IFN-β and IRF family gene expression in macrophages that exhibit the genetically determined, LPS-responsive or -hyporesponsive phenotypes, total cellular RNA was extracted from peritoneal exudate macrophages derived from C3H/OuJ (*Lps^n*) or C3H/HeJ (*Lps^d*) mice. RNA preparations were subjected to RT-PCR and ECL signal detection with primers and probes specific for IRF-1, IRF-2, ICSBP, IFN-β, or GAPDH mRNA sequences. Detection of IFN-β (Fig. 1A) and IRF-1 (Fig. 1B) mRNA in *Lps^n* macrophages required fewer cycles of amplification than those required to detect IFN-β and IRF-1 mRNA in *Lps^d* macrophages. Comparison of relative gene expression at the first cycle number where both samples have detectable IFN-β and IRF-1 (cycles 33 and 16, respectively) reveals ~15- to 22-fold higher levels of steady-state IFN-β and IRF-1 mRNA in *Lps^n* macrophages than in *Lps^d* macrophages. In contrast, mRNA specific for IRF-2 (Fig. 1C) is detected in *Lps^d* macrophages after fewer cycles of amplification (cycle 16) than those required to detect IRF-2 mRNA in *Lps^n* macrophages (cycle 17). Comparison of relative gene expression at the first common cycle number (cycle 17) demonstrates that *Lps^d* macrophages contain ~18-fold higher levels of steady-state IRF-2 mRNA than *Lps^n* macrophages. There was no consistent differential expression of ICSBP mRNA between the two strains (Fig. 1D).

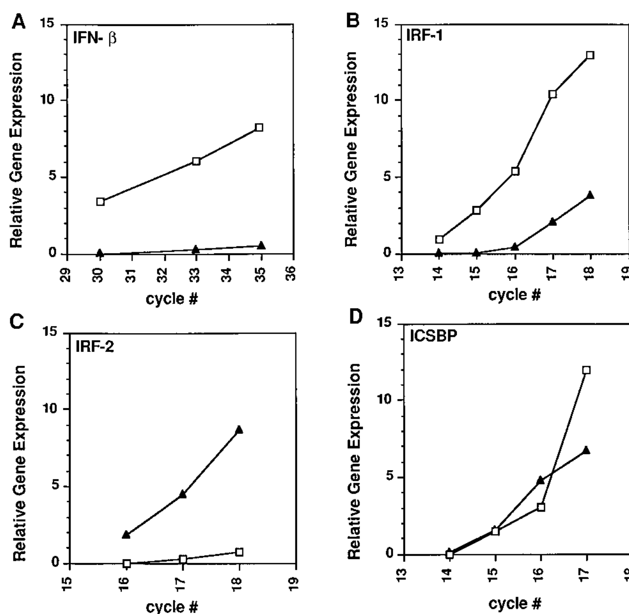


FIG. 1. Basal-level expression of IFN-β, IRF-1, IRF-2, and ICSBP mRNA in C3H/OuJ and C3H/HeJ macrophages. Peritoneal exudate macrophages from C3H/OuJ (□) and C3H/HeJ (▲) mice were cultured as described in Materials and Methods. RNA extracted from untreated macrophages was subjected to RT-PCR over the indicated cycle numbers. Basal levels of IFN-β, IRF-1, IRF-2, and ICSBP mRNA are shown in panels A, B, C, and D, respectively. The data have been normalized to GAPDH gene expression ("Relative Gene Expression"), as described in Materials and Methods, and are derived from a single experiment that is representative of at least three independent experiments.

LPS stimulation of *Lps^n* macrophages results in a dose- and time-dependent accumulation of IRF-1, IRF-2, and ICSBP mRNA. The correlation of differential allelic expression at the *Lps* locus with differential basal-level expression of the IRF-1 and IRF-2 genes prompted us to assess the LPS inducibility of these genes in C3H/OuJ macrophages. Total cellular RNA was extracted from C3H/OuJ macrophages at various times after stimulation with LPS. Northern blots were prepared and hybridized to IRF-1-, IRF-2-, ICSBP-, or actin-specific cDNA probes. As shown in Fig. 2, LPS induces the rapid accumulation of IRF-1 mRNA, which reaches maximal levels of 40- to 45-fold over 4 to 6 h. The time-dependent accumulation of LPS-induced IRF-2 mRNA parallels that of ICSBP mRNA, both reaching maximal expression of four- to fivefold over 4 to 6 h. Notably, all three mRNA species were detected above basal levels as late as 28 h after LPS stimulation (data not shown). The dose dependence of LPS-induced IRF family gene expression in *Lps^n* macrophages was determined by extracting total cellular RNA 6 h after stimulation with a range of LPS concentrations. Consistent with previously characterized LPS-inducible genes (19), LPS concentrations as low as 0.1 to 1.0 ng/ml result in IRF-1 and IRF-2 mRNA accumulation, and maximal gene expression occurs after stimulation with 100 ng/ml (data not shown). Interestingly, minimum concentrations of at least 10 ng of LPS per ml are required to induce ICSBP gene expression consistently.

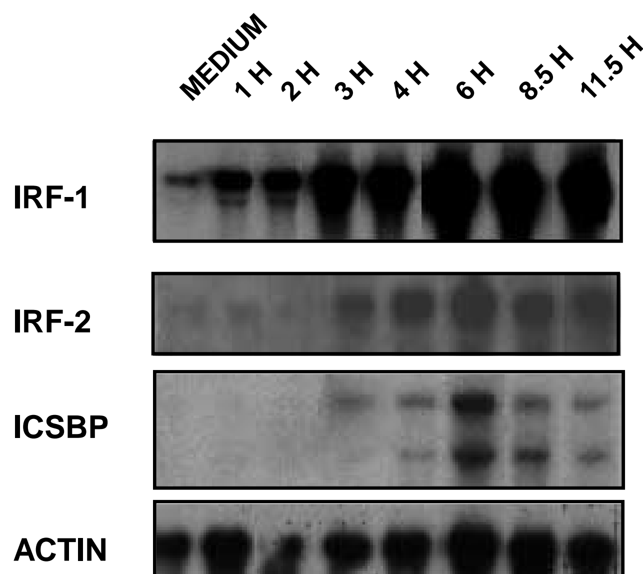
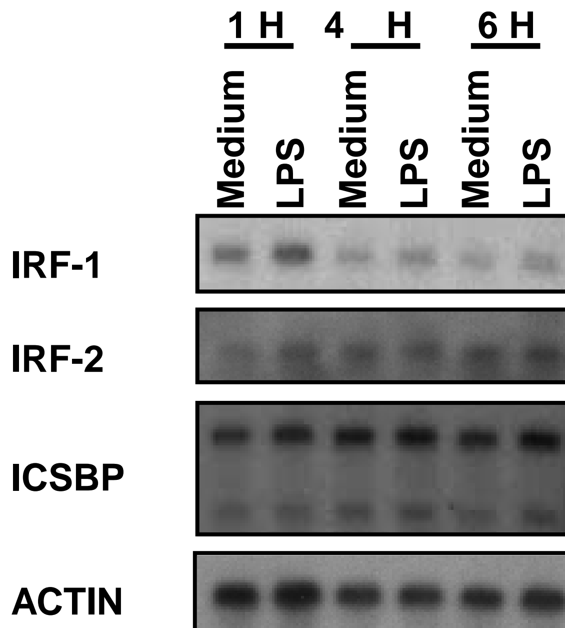


FIG. 2. IRF-1, IRF-2, ICSBP, and β-actin gene expression in C3H/OuJ macrophages treated with LPS. Macrophage cultures were treated with medium or 100 ng of LPS per ml. RNA was extracted at the indicated times after treatment and subjected to Northern blot analysis. The data shown are derived from a single experiment that is representative of at least three independent experiments. The computer-generated images of the scanned Northern blots were reproduced with Aldus FreeHand 3.11 software.



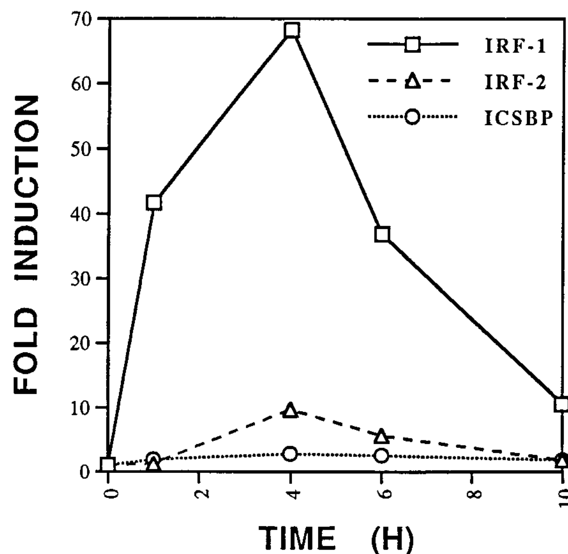


FIG. 3. IRF-1, IRF-2, ICSBP, and β-actin or GAPDH gene expression in C3H/HeJ macrophages treated with LPS or IFN-α. (Top) Macrophages were treated with medium or 100 ng of LPS per ml. RNA was extracted at the indicated times after treatment and subjected to Northern blot analysis. The data shown are derived from a single experiment that is representative of at least three independent experiments. (Bottom) Macrophages were treated with medium or 500 U of IFN-α per ml. RNA was extracted at the indicated times and subjected to RT-PCR analysis for 24 cycles. The data shown have been normalized to GAPDH gene expression, as described in Materials and Methods, and are derived from a single experiment that is representative of at least three independent experiments. The computer-generated images of the scanned Northern blot were reproduced with Aldus FreeHand 3.11 software.

***Lps^d* macrophages respond to IFN-α but not to LPS to modulate IRF gene expression.** The experimental results depicted in Fig. 1 associated the molecular phenotype of C3H/HeJ macrophages with higher basal levels of IRF-2 mRNA than C3H/OuJ macrophages. To address the possibility that exposure to LPS somehow promotes higher-level expression of transcriptional repressors in *Lps^d* macrophages, we compared IRF-1, IRF-2, and ICSBP mRNA levels in C3H/HeJ macrophages stimulated with 100 ng of LPS per ml. In addition, since many of the functional deficiencies in macrophage differentiation associated with expression of the *Lps^d* allele are reversible by the addition of exogenous IFN (8), we analyzed the pattern of IRF gene expression in *Lps^d* macrophages stimulated with 500 U of IFN-α per ml. Total cellular RNA was extracted at various times over 10 h and subjected to Northern blot or PCR analysis. As anticipated, there was no measurable modulation of IRF-1, IRF-2, or ICSBP mRNA following LPS stimulation in *Lps^d* macrophages (Fig. 3, top), in contrast to *Lps^n* macrophages (Fig. 2). However, *Lps^d* macrophages did respond to IFN-α stimulation (Fig. 3, bottom). IRF-1 mRNA levels increased rapidly, reaching maximal levels of ~70-fold by 4 h. IRF-2 mRNA levels increased later and to a lesser extent (~10-fold), also peaking by 4 h. Consistent with previous findings (6, 32), ICSBP mRNA was not notably modulated by IFN-α.

Cycloheximide does not inhibit the accumulation of LPS-induced IRF-1 mRNA. Since IFN and other LPS-inducible cytokines have been reported to induce IRF family gene expression (6, 9, 14, 27, 31) it was of interest to determine if the induction of these genes was a direct or indirect result of LPS stimulation. To address this point, *Lps^n* macrophages were treated with medium or 100 ng of LPS per ml, in the absence or presence of 5 μg of cycloheximide per ml. Total RNA was extracted after 4.5 h and subjected to RT-PCR. The results, shown in Fig. 4, indicate that LPS, in conjunction with cycloheximide, results in superinduction of IRF-1 mRNA. In contrast, accumulation of LPS-induced IRF-2 and ICSBP mRNA is abolished in the presence of cycloheximide.

LPS stimulation of macrophages rendered endotoxin tolerant does not result in an increase of IRF-1, IRF-2, or ICSBP steady-state mRNA levels. Prior exposure of macrophages to LPS results in a transient state of LPS hyporesponsiveness

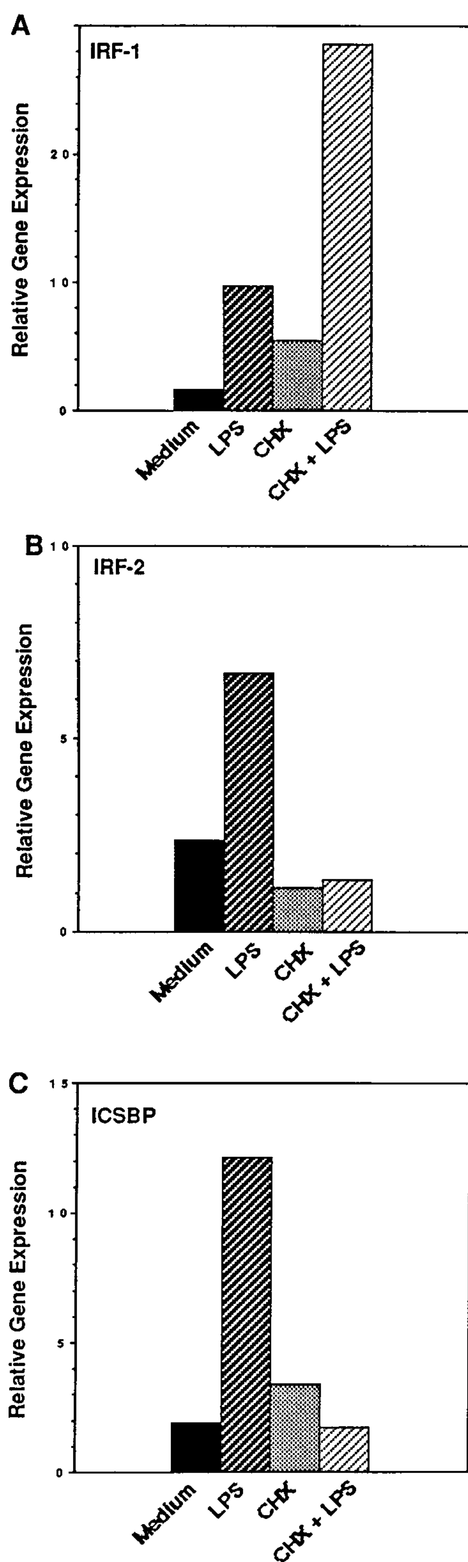


FIG. 4. Effect of cycloheximide (CHX) on LPS-inducible IRF-1, IRF-2, and ICSBP gene expression. C3H/OuJ macrophage cultures were treated with medium, 100 ng of LPS per ml, 5 μg of cycloheximide per ml, or 5 μg of cycloheximide per ml plus 100 ng of LPS per ml. RNA was extracted at 4.5 h after treatment and subjected to RT-PCR for 20 cycles. The data shown are derived from a single experiment that is representative of at least three independent experiments and have been normalized to GAPDH gene expression ("Relative Gene Expression") as described in Materials and Methods.

(42), during which there is a failure of certain LPS-inducible genes to be reinduced upon challenge (19). Since C3H/HeJ LPS-hyporesponsive macrophages exhibited higher basal levels of IRF-2 mRNA than Lps^n macrophages, and since IRF-2 has been shown to exert transcriptional repressor activity (14), Lps^n macrophages rendered endotoxin tolerant in vitro were also assessed for IRF gene expression. Figure 5 illustrates gene expression in endotoxin-tolerant macrophages that were restimulated with either medium or 100 ng of LPS per ml for 1 or 4 h. For each gene examined, the level of restimulated mRNA is shown relative to basal levels (MEDIUM/MEDIUM; macrophages pretreated with medium and challenged with medium) and maximally inducible levels (MEDIUM/LPS; macrophages pretreated with medium and stimulated with LPS for 4 h). At 1 h after restimulation with medium or LPS, levels of IRF-1 and ICSBP mRNA were still near the maximally LPS-inducible levels measured in the experiment. (In three separate experiments, IRF-1 and ICSBP gene expression in LPS/MEDIUM samples was 75 to 95% of that measured in the maximally induced MEDIUM/LPS samples.) In contrast, IRF-2 mRNA was expressed in LPS/MEDIUM samples at only 35 to 50% of maximally LPS-inducible levels. Even after an LPS challenge, these levels fail to be reinduced and continue to diminish over time in culture. As a control, the effect of endotoxin tolerance on the expression of the IP-10 gene was included. Consistent with previous findings (19), the level of IP-10 gene expression at the time of restimulation (LPS/MEDIUM) was very low (about 15% of maximal LPS-induced gene expression) and was not reinduced after subsequent exposure to LPS (LPS/LPS).

DISCUSSION

LPS-hyporesponsive models have been invaluable tools used to elucidate cellular mechanisms involved in macrophage signal transduction, gene expression, differentiation, and activation (5, 24, 33). In the simplest model, LPS stimulation of macrophages begins with signal transduction across the cell membrane that triggers a multitude of intracellular biochemical events, some of which result in the nuclear mobilization or activation of transcription factors that regulate LPS-inducible gene expression. Promoter elements that have been clearly implicated in LPS-mediated transcription include the NF-κB, CK-1, and ISRE motifs (reviewed in reference 13). Ultimately, the cellular LPS-responsive or -hyporesponsive phenotype must reflect a corresponding molecular phenotype directed by transcriptional activators and repressors. The primary goals of our experiments were to characterize the LPS inducibility of the genes that encode the ISRE-binding transcription factors IRF-1, IRF-2, and ICSBP and to compare the expression of these genes in two distinct models of LPS hyporesponsiveness.

Our initial studies focused on a well-characterized, genetically determined model of LPS hyporesponsiveness. From the data provided in Fig. 1, we conclude that Lps^n macrophages express higher basal levels of the transcriptional activator IRF-1 mRNA, while Lps^d macrophages express higher basal levels of the transcriptional repressor IRF-2 mRNA. In addition, we consistently found higher basal levels of IFN-β mRNA in Lps^n macrophages compared with Lps^d macrophages, thus providing molecular evidence to support previous protein neutralization studies in which anti-IFN-α/β antibodies rendered Lps^n macrophages phenotypically like Lps^d macrophages with respect to FcγR expression and virus susceptibility (45, 46). The coincidental elevated expression of IFN-β and IRF-1 mRNA in Lps^n macrophages is not unique to this system (14) and is consistent with models in which IRF-1 is required for the

□ LPS / MEDIUM ▨ LPS / LPS

A

IRF-1

Band Intensity (10^6 pixels)

0 1 2 3 4 5

MAX (MEDIUM / LPS)

BASAL (MEDIUM / MEDIUM)

1 4

B

IRF-2

Band Intensity (10^5 pixels)

0 5 10 15 30

MAX (MEDIUM / LPS)

BASAL (MEDIUM / MEDIUM)

1 4

C

ICSBP

Band Intensity (10^5 pixels)

0 1 2 3 4 5

MAX (MEDIUM / LPS)

BASAL (MEDIUM / MEDIUM)

1 4

D

IP-10

Band Intensity (10^6 pixels)

0 1 8

MAX (MEDIUM / LPS)

BASAL (MEDIUM / MEDIUM)

1 4

Time After LPS Challenge (H)

E ACTIN

FIG. 5. IRF-1, IRF-2, ICSBP, and IP-10 gene expression in macrophages rendered tolerant of LPS. C3H/OuJ macrophages were pretreated with medium or 100 ng of LPS per ml. After 19 h, the cells were washed twice with medium and challenged with either medium or 100 ng of LPS per ml. RNA was extracted at the indicated times and subjected to Northern blot analysis. Treatment codes are expressed as pretreatment/treatment. LPS/MEDIUM and LPS/LPS represent cells rendered tolerant restimulated with medium or LPS, respectively. Basal levels are indicated by a dashed line and represent gene expression in the cells treated with medium at both times (MEDIUM/MEDIUM). The maximal LPS-inducible gene expression (MEDIUM/LPS) measured in the experiment is also indicated by a dashed line. The inset autoradiograms for each gene show the Northern blot results of LPS/MEDIUM, LPS/LPS, LPS/MEDIUM, and LPS/LPS, respectively, from left to right. IRF-1, IRF-2, ICSBP, IP-10, and β-actin gene expression data, from a single experiment that is representative of at least three independent experiments, are shown in panels A, B, C, D, and E, respectively. The computer-generated images of the scanned Northern blots were reproduced with Aldus FreeHand 3.11 software.

transcription of IFN-β mRNA (25), which is then stabilized posttranscriptionally following exposure to LPS (12).

The data gathered from the *Lps*^{n} and *Lps*^{d} macrophages may be considered in the context of the positive feedback model of IFN-β gene regulation proposed by Harada et al. (14) to describe the differential basal-level gene expression exhibited by LPS-responsive and -hyporesponsive phenotypes. In this model, IFN-β induced by in vivo exposure to LPS may act in an autocrine fashion to increase the basal levels of IRF-1 in *Lps*^{n}, but not *Lps*^{d}, macrophages. In turn, IRF-1 would sustain additional IFN-β steady-state mRNA levels. Such a situation would favor an increased ratio of IRF-1 to IRF-2 in *Lps*^{n} cells and perhaps accounts for some of the observed functional differences between *Lps*^{n} and *Lps*^{d} macrophages. As discussed above, treatment of *Lps*^{d} macrophages with IFN-α normalizes some of the functional differences associated with macrophage differentiation. In this study, we show that IFN-α stimulates *Lps*^{d} macrophages to undergo a rapid transition in IRF molecular phenotype (Fig. 3B). Within 1 h, IRF-1 mRNA levels are increased (~40-fold) in the absence of a corresponding increase in IRF-2 mRNA. Since the relative fold induction of IRF-1 mRNA far exceeds that of IRF-2 mRNA throughout the time course, the phenotype of the IFN-α-treated C3H/HeJ macrophage correlates with an early and sustained increase in the ratio of IRF-1 to IRF-2 gene expression.

We also analyzed IRF family gene expression in *Lps*^{n} macrophages treated with exogenous LPS. Accumulation of IRF-1 mRNA in *Lps*^{n} macrophages is rapidly initiated (an increase in IRF-1 mRNA is detectable within 30 min of LPS stimulation; data not shown), while an increase in IRF-2 and ICSBP mRNA levels is not detected until ~4 h. The lag time prior to the increase in IRF-2 mRNA levels compared with IRF-1 mRNA is consistent with recent data that suggest a role for IRF-2 in the negative feedback regulation of gene expression resulting from potent bioactive stimuli such as LPS (17). The dramatic increase in IRF-1 gene expression (40- to 45-fold) far exceeds that of IRF-2 and ICSBP genes (4- to 6-fold) and may be an important compensatory mechanism since IRF-1 protein has a much shorter half-life, i.e., ~30 min, than that of IRF-2 or ICSBP (half-life ≥8 h [15, 32, 47]). In response to LPS, the accumulation of all three species of mRNA was prolonged, perhaps because of the production of LPS-inducible cytokines such as tumor necrosis factor, interleukin 1, interleukin 6, and IFN-α/β/γ, all of which have been shown to augment IRF-1 and/or IRF-2 gene expression (1, 9, 14, 31, 36). However, IRF-1 mRNA accumulates as a direct consequence of LPS stimulation, as evidenced by its insensitivity to cycloheximide (Fig. 4). Interestingly, cycloheximide alone increases steady-state levels of IRF-1 mRNA that are superinduced in the presence of LPS. This suggests that LPS-inducible IRF-1 gene expression may be regulated, in part, at the level of mRNA stability. Two DNA sequence motifs that may confer mRNA instability have been found in the 3′ noncoding region of the IRF-1 cDNA sequence (27).

Lastly, we analyzed IRF family gene expression in normally LPS-responsive macrophages that have been rendered transiently hyporesponsive to a second exposure of LPS. At the time of LPS challenge, all IRF mRNA levels were still above basal levels. Thus, the pattern of IRF-1 and ICSBP gene expression in this LPS-hyporesponsive model differs from that in the *Lps*^{d} model. Restimulation of macrophages rendered tolerant to endotoxin with LPS failed to reinduce IRF-1, IRF-2, or ICSBP mRNA levels. As reported previously, IP-10 gene expression was very low in endotoxin-tolerant cells and was not reinduced upon LPS restimulation (19). Minimal IP-10 expression, in conjunction with elevated IRF-1 expression, was surprising since LPS-mediated transcription of the IP-10 gene has been correlated with the presence of an ISRE motif in the promoter region (13, 37). One interpretation of this apparent dichotomy is that IRF-1 is not involved in the regulation of the IP-10 gene or two other immediate-early LPS-inducible genes, D3 and IFN-β, whose promoters also contain interferon regulatory elements or ISRE motifs and are also suboptimally expressed in macrophages rendered endotoxin tolerant (reference 19 and data not shown). Alternatively, IRF-1 may play a role in the transcriptional activation of LPS-inducible genes that contain ISRE-IRE motifs but is active only in the presence of additional factors or is somehow inactivated in tolerant cells (3, 14, 28).

To date, the molecular mechanisms that underlie LPS responsiveness are not well defined. The studies described herein characterize the LPS inducibility of IRF-1, IRF-2, and ICSBP genes, thus adding these IRFs to the list of LPS-inducible transcription factors that currently includes c-*fos*, c-*jun*, c-*myc*, and NF-κB (13). The recent finding that macrophages from IRF-1 knockout mice fail to induce iNOS gene expression in response to LPS and IFN-γ strengthens the hypothesis that IRF-1 may play an important role in macrophage responses to LPS (20). Our studies of endotoxin-tolerant macrophages have also revealed a unique, reciprocal situation in which elevated IRF-1 gene expression is coincident with depressed expression of certain ISRE-containing, LPS-inducible genes. Future studies employing IRF knockout mice should elucidate the precise roles of IRF-1, IRF-2, and ICSBP in LPS-induced signaling events.

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