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Priming of Human Monocytes for Enhanced Lipopolysaccharide Responses: Expression of Alpha Interferon, Interferon Regulatory Factors, and Tumor Necrosis Factor

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Culture of human monocytes with either granulocyte-macrophage colony-stimulating factor or gamma interferon (IFN-γ) results in a primed state, during which these cells express heightened responses to bacterial lipopolysaccharide (LPS). The production of IFN-α in response to LPS by human monocytes has an absolute requirement for priming. Tumor necrosis factor (TNF) expression is also greatly enhanced in primed monocytes after LPS stimulation, but unlike IFN-α, TNF is readily expressed in unprimed monocytes as well. In an effort to determine the molecular events associated with IFN-α induction in this system, freshly isolated human monocytes were primed by culture with either IFN-γ or granulocyte-macrophage colony-stimulating factor and then treated with LPS; expression of IFN-α subtype 2 (IFN-α_2), IFN regulatory factors (IRFs), and TNF was assessed by Northern (RNA blot) analysis. IRF-1 mRNA is expressed at high levels in monocytes and is regulated by both LPS and priming cytokines, but its expression alone does not correlate with the induction of IFN-α_2 expression. IRF-2 mRNA is expressed in a more gradual manner following LPS stimulation, implying a possible feedback mechanism for inhibiting IFN-α expression. However, nuclear run-on analysis indicates that IFN-α_2 is not transcriptionally modulated in this system, in striking contrast to TNF, which is clearly regulated at the transcriptional level. In addition, IFN-α_2 mRNA accumulation is superinduced when primed monocytes are treated with LPS plus cycloheximide, while TNF mRNA is relatively unaffected. The results demonstrate that priming can affect subsequent LPS-induced gene expression at different levels in human monocytes.

The differentiation of macrophages and their activation for a variety of host defense functions are regulated by the interferons (IFNs) (4). Both IFN-γ and IFN-α/β have been shown to activate macrophages for microbicidal and tumoricidal functions. Macrophages are also efficient producers of IFN-α/β (4). Although several agents (virus, double-stranded RNA, and other polyanionic compounds) act to induce IFN from different cell types, cells of the monocyte/macrophage lineage are uniquely responsive to bacterial lipopolysaccharide (LPS) for the induction of IFN-α/β and a number of other cytokines (4, 18, 22). Murine macrophages isolated by peritoneal lavage or bone marrow culture will produce IFN-α/β in response to LPS (6, 14, 21, 26, 28). In addition, it has been shown that the capacity for IFN production in mice is dependent upon the differentiation state of the cell and is regulated by colony-stimulating factors (7, 21, 30).

We have previously reported that LPS does not induce IFN-α from freshly isolated human peripheral blood monocytes unless these monocytes have been "primed" by being cultured with either granulocyte-macrophage colony-stimulating factor (GM-CSF) or IFN-γ (15). This requirement for priming is LPS specific, since IFN-α is readily induced from fresh monocytes by other stimuli, such as virus and poly(I · C). It is also IFN specific, as LPS readily induces substantial expression of other cytokines such as interleukin-1 and tumor necrosis factor (TNF) from unprimed monocytes.

In studies on IFN induction by viruses or double-stranded RNA, the expression of IFN-α appears to be controlled primarily at the level of transcription of the IFN genes (23, 31). According to currently accepted models, the regulation of IFN-α transcription is primarily mediated by transcription factors which bind to specific sites in the promoters of the IFN genes. Two putative transcription factors which apparently interact with the same site(s) on IFN promoters have been isolated and cloned by Taniguchi and colleagues (8, 12) and are referred to as IFN regulatory factors 1 and 2 (IRF-1 and IRF-2). IRF-1 appears to activate IFN transcription while IRF-2 acts as a repressor when these genes are expressed in transfected cells that do not express endogenous IRFs (8, 13). Recent gene knockout experiments, however, indicate that IRF-1 may not be required for IFN-α expression in vivo (24).

The studies described here were initially undertaken to assess the regulation of IRFs in monocytes primed with GM-CSF or IFN-γ and induced with LPS for IFN-α expression. The expression of TNF was also examined as a model of a strictly regulated cytokine whose expression was not absolutely dependent upon, but was quantitatively affected by, priming. The results indicated that IFN-α, IRFs, and TNF are all differentially regulated in freshly isolated and primed monocytes and that IFN-α and TNF expression is regulated by LPS by different mechanisms.

MATERIALS AND METHODS

Monocytes. Human monocytes were isolated by countercurrent centrifugal elutriation of single-donor peripheral blood leukocyte preparations obtained from the National Institutes of Health apheresis unit, as described previously

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(10). More than 95% of the cells were monocytes, as assessed by Giemsa and nonspecific esterase staining. All media and reagents were free of detectable endotoxin, as measured by the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass.) (19). Monocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), L-glutamine, and gentamicin (GIBCO, Grand Island, N.Y.) after seeding into polystyrene tissue culture plates (Costar, Cambridge, Mass.).

IFN assay. Monocytes were cultured in the presence of either IFN-γ (generously provided by Genentech, Inc., South San Francisco, Calif.) or GM-CSF (generously provided by Schering Corporation, Kenilworth, N.J.). LPS was from *Escherichia coli* O128:B12 phenol extract (Sigma Chemical Company, St. Louis, Mo.). Recombinant human IFN-αB was kindly provided by Ciba-Geigy, Basel, Switzerland. Supernatants were analyzed for IFN activity by a cytopathic effect reduction assay (32). Madin-Darby bovine kidney cells were incubated with supernatant dilutions and subsequently challenged with vesicular stomatitis virus. One unit of IFN activity is defined as the concentration required to reduce cytopathic effect by 50%.

RNA analysis. For Northern (RNA blot) analysis, RNA was isolated by the acid phenol-guanidine isothiocyanate method (3) (RNAzol; TelTest, Friendswood, Tex.), or polyadenylated [poly(A)$^+$] RNA was prepared directly from cell lysates over oligo(dT)-cellulose columns (FastTrack; InVitrogen Corp., San Diego, Calif.). RNA samples were separated by electrophoresis over 1% agarose gels prepared with formaldehyde and ethidium bromide. The gels were blotted onto nylon filters (Bethesda Research Laboratories) overnight and UV cross-linked to immobilize the RNA. Filters were prehybridized in a 50% formamide buffer (Nylohybe; Digene, Silver Spring, Md.). Probes were labeled to high specific activity (>10^9 cpm/μg) by random primer reactions (Prime-It; Stratagene, La Jolla, Calif.), combined with salmon sperm DNA (Digene), and added to prehybridized filters after boiling. Filters were hybridized overnight, washed under high-stringency conditions in 0.2× SSC–0.1% sodium dodecyl sulfate (SDS) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 63°C, and subjected to autoradiography. For IFN-α, filters were washed under lower stringency (0.2× SSC, 45°C) to facilitate hybridization signals to multiple IFN-α mRNAs with imperfect homology to the IFN-α subtype 2 (IFN-α_2) probe.

The probe for IFN-α_2 was an *Eco*RI-*Xho*I insert from a cDNA isolated from a Sendai virus-induced Namalwa library cloned into pBluescript SK(−) with the Uni-ZAP XR cloning system (Stratagene). The probe for TNF was a *Pst*I insert from plasmid pE4 (ATCC 39894; American Type Culture Collection, Rockville, Md.). The probes for IRF-1 and IRF-2 were *Hin*dIII-*Bam*HI and *Xba*I inserts from plasmids pUChIRF-1 and pHIRF4S-51 generously provided by T. Taniguchi, Osaka University, Osaka, Japan. The probe for the GAPDH (glyceraldehyde phosphate dehydrogenase) gene was a *Pst*I-*Xba*I insert from plasmid pHcGAP (ATCC 57090).

Nuclear run-ons. Monocytes were stimulated with the required stimuli, harvested after 45 to 60 min, and prepared for nuclear transcription assays by a method modified from that of Celano et al. (2). Briefly, the cells were lysed in a 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.3 M sucrose. Nuclei were washed in the same buffer and suspended directly in a reaction buffer containing deoxynucleoside triphosphates and 200 μCi of [^{32}P]dUTP. Transcription was allowed to proceed for 30 min, after which the nuclei were treated sequentially with DNase I and proteinase K. RNA was extracted by the method of Chomczynski and Sacchi (3) (RNAzol; TelTest) and reextracted by additional phenol-chloroform treatments. Incorporation of ^{32}P was assessed by scintillation counting, and the samples were incubated for 36 to 48 h with nylon filters that had been previously bound with the appropriate cDNAs. Filters were washed at temperatures of up to 45°C with 0.1× SSC–0.1% SDS and subjected to autoradiography.

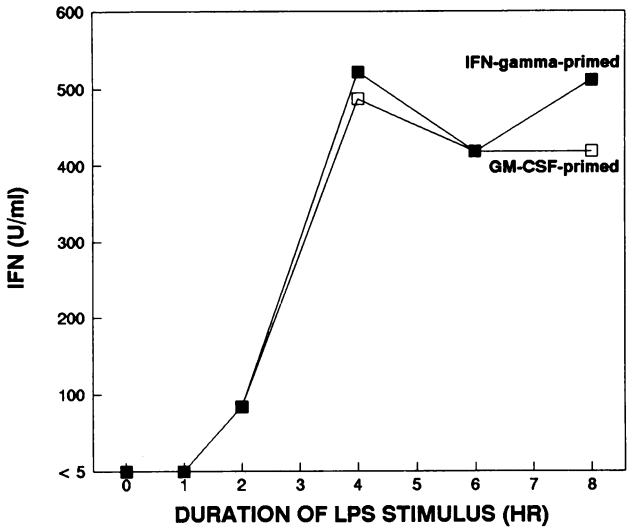

FIG. 1. Time course of IFN-α production in response to LPS. Monocytes from a single donor were cultured for 40 h in 100 ng of IFN-γ (■) or GM-CSF (□) per ml. Cultures were treated subsequently for the indicated times with LPS (1 μg/ml). Supernatants were assayed for IFN activity as described in Materials and Methods.

RESULTS

Time course of IFN-α production by monocytes. Freshly elutriated human monocytes were cultured for 40 h in the presence of either GM-CSF or IFN-γ at 100 ng/ml. LPS (1 μg/ml) was added, and supernatants were harvested at various time points for up to 8 h. IFN activity was detected as early as 2 h following addition of LPS and reached an apparent maximal level by 4 h (Fig. 1). In some experiments, trace levels of IFN were detected as early as 1 h (data not shown). In experiments with monocytes from several different donors, the levels of IFN produced varied from 50 to 500 U/ml, but the kinetics of release were always similar. IFN activity after 24 h was not significantly greater than after 6 to 8 h.

Time course of IFN-α mRNA accumulation. The steady-state expression of mRNA in stimulated monocytes was assessed by isolation of poly(A)$^+$ RNA followed by Northern blotting with a human IFN-α_2 cDNA probe. The kinetics of accumulation was dependent upon the agent used for priming the monocytes. When monocytes were primed with IFN-γ, addition of LPS caused a very rapid and transient expression of IFN-α_2-hybridizing message migrating at approximately 1.4 kb. The hybridizing mRNA was visible by 1 h, reached a peak level at 2 h, and was no longer detectable by 6 h (Fig. 2). When monocytes were primed with GM-

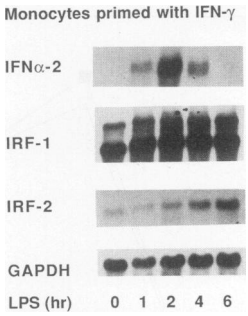

FIG. 2. Northern blot analysis of IFN-γ-primed monocytes treated with LPS. Monocytes from a single donor were cultured with IFN-γ (100 ng/ml) for 40 h, after which they were treated with LPS (1 μg/ml). At the indicated time points, poly(A)$^+$ mRNA was isolated as described in Materials and Methods. The RNA was resolved on a 1% agarose gel (80 × 10^6 cell equivalents per lane) and blotted onto a nylon filter. The filter was hybridized sequentially with the indicated probes and subjected to autoradiography.

CSF, IFN-α_2 message initially accumulated at the same rate, but the levels declined more gradually (Fig. 3); the signal peaked at 1 to 2 h and was still visible at 8 h after a longer (7-day) exposure of the blot.

Time course of IRF expression. The same blots were analyzed for expression of mRNA hybridizing to cDNA probes for IRF-1 and IRF-2 to assess the temporal relationship between IRF expression and induction of IFN. The expression patterns differed depending upon the priming stimulus used. IFN-γ-primed monocytes were already expressing substantial IRF-1 mRNA prior to LPS stimulation, and addition of LPS did not result in significant changes in expression of IRF-1 (Fig. 2). Interestingly, two and sometimes three bands hybridized at high stringency with this probe. The predominant lower band of 2.4 kb and the minor band at about 4.3 kb have been observed previously by others (9). A third, middle band of approximately 3.5 kb appeared to be induced concurrently with the IFN-α_2 mRNA, while the other major bands were stably expressed. Although the 3.5-kb band was present for a longer time than that for IFN-α_2, this band also began to decline by 6 h. This band did not appear for GM-CSF-primed cells, even after prolonged exposure of the autoradiograms from Fig. 3 (see below).

IRF-2 mRNA, also migrating at 2.4 kb, was expressed at low levels in these cells and was induced with a slower, more gradual time course for 4 to 6 h following LPS treatment

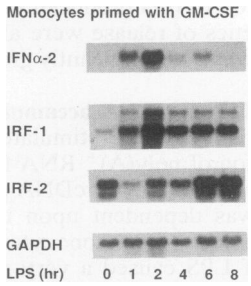

FIG. 3. Northern blot analysis of GM-CSF-primed monocytes treated with LPS. Monocytes were cultured with GM-CSF (100 ng/ml) for 40 h and treated exactly as described in the legend to Fig. 2.

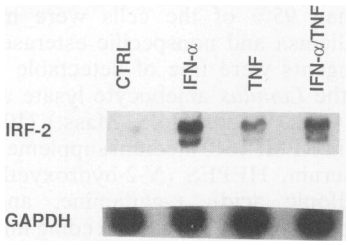

FIG. 4. Regulation of IRF-2 mRNA in IFN-γ-primed monocytes. Monocytes from a single donor were cultured for 40 h in IFN-γ (100 ng/ml) and treated subsequently for 2 h with either IFN-αB (1,000 U/ml), TNF (10 ng/ml), or both. Poly(A)$^+$ mRNA (10^7 cell equivalents per lane) was isolated and analyzed as previously described. CTRL, control (no subsequent treatment).

(Fig. 2). When GM-CSF-primed monocytes were analyzed, IRF-1, expressed at low levels prior to LPS stimulation, appeared to be rapidly induced by LPS (Fig. 3). IRF-1 expression peaked in these cells by 2 h following addition of LPS. In contrast, LPS rapidly and transiently down-modulated the expression of IRF-2, which was significantly expressed prior to LPS addition. IRF-2 mRNA returned after 2 to 4 h and was then even more strongly expressed by 6 to 8 h, similar to its expression in IFN-γ-primed monocytes.

Induction of IRF-2 by monokines. The latent induction of IRF-2 by LPS suggested that this induction might be due to a secondary stimulus elicited by LPS. To examine this possibility, monocytes which had been primed with IFN-γ were stimulated for 2 h with IFN-α, TNF, or both. Figure 4 demonstrates that both IFN-α and TNF induced the expression of IRF-2 from primed cells within 2 h. Stronger induction appeared to occur in response to IFN-α, and the combination of IFN-α and TNF did not elicit more expression than IFN alone.

Expression of IFN-α, IRF, and TNF genes in freshly isolated and primed monocytes. Monocytes were stimulated with LPS for 2 h either directly following isolation or after culture with IFN-γ or GM-CSF for 2 days. Only primed monocytes demonstrated any detectable mRNA for IFN-α_2 following LPS stimulation (Fig. 5, lanes GL and γL). TNF expression, however, was observed in unprimed as well as primed monocytes, although there was substantial enhancement of TNF mRNA accumulation in primed cells. Consistent with the data shown in Fig. 3, IRF-1 mRNA was inducible by LPS in both unprimed and GM-CSF-primed monocytes and was already maximally expressed in IFN-γ-primed monocytes.

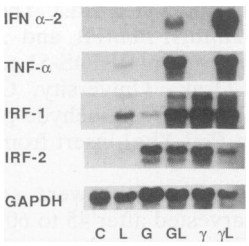

FIG. 5. Monokine and IRF gene expression as a function of priming. Freshly isolated monocytes from a single donor were either not treated (lane C), treated for 2 h with LPS (1 μg/ml) (lane L), or cultured in GM-CSF (lane G) or IFN-γ (lane γ) for 40 h and then treated for 2 h with LPS (lanes GL and γL, respectively). Poly(A)$^+$ mRNA was harvested and hybridized with the indicated probes.

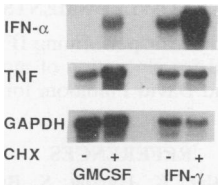

FIG. 6. Effect of CHX on IFN-α induction by LPS. Monocytes were primed by 40 h of culture with either GM-CSF or IFN-γ and subsequently stimulated with LPS (1 μg/ml) in the absence or presence of CHX (10 μg/ml). Poly(A)$^+$ mRNA (80 × 10^6 cell equivalents per lane) was isolated and analyzed with the indicated probes.

IRF-2 was poorly expressed in unprimed monocytes before or after LPS treatment. IRF-2 was not significantly modulated at the 2-h time point of LPS stimulation in primed monocytes.

Superinduction of IFN-α expression by CHX. Since IRF-1 mRNA was already induced in IFN-γ-primed monocytes but was inducible by LPS in GM-CSF-primed cells, experiments were designed to assess the requirement for protein synthesis for induction of IFN-α_2 mRNA expression under these different priming conditions. IFN-γ- and GM-CSF-primed cells were stimulated with LPS in the presence and absence of cycloheximide (CHX). As has been reported in other IFN induction studies, CHX treatment resulted in superinduction of IFN-α_2 mRNA accumulation in both cases (Fig. 6). Superinduction also had an absolute requirement for priming and LPS. Treatment of freshly isolated monocytes with LPS in the presence of CHX and treatment of primed cells with CHX alone did not induce any detectable IFN-α_2 mRNA expression (data not shown). TNF mRNA accumulation was largely unaffected by CHX treatment, although a slight augmentation was observed in GM-CSF-primed cells.

Transcriptional regulation by LPS. Freshly isolated or IFN-γ-primed monocytes were stimulated with LPS for 45 min, and nuclei were then harvested for analysis of transcription of IFN-α_2, TNF, and GAPDH (Fig. 7). Transcription of TNF was clearly inducible at high levels, while barely detectable transcription of GAPDH and IFN-α_2 was observed under all conditions. TNF transcription was clearly induced by LPS and IFN-γ separately, and IFN-γ-primed monocytes stimulated with LPS also showed a high transcriptional rate for TNF which was not significantly higher than that in unprimed cells, although, in separate experiments, primed cells demonstrate higher TNF transcription than freshly isolated monocytes (data not shown).

DISCUSSION

The control of IFN production by monocytes or macrophages is an important issue for both paracrine activity (inducing antiviral, antiproliferative, or immunomodulatory effects in appropriate target cells) and autocrine activity (modulating the differentiation state of cells of the macrophage lineage) (29). In human monocytes, the inducibility of IFN-α by LPS is strictly regulated by positive and negative signals. First, a priming step is required for LPS induction to occur (15). Priming is stimulated by culture with either IFN-γ or GM-CSF. Macrophage colony-stimulating factor will not provide this priming effect, even though it promotes the survival of monocytes in culture and induces a characteristic morphological response (15). Monocytes cultured in medium with serum alone undergo apoptotic death after 24 to 48 h of culture, as has been described by others (20). After priming, LPS induces a rapid and transient expression of IFN-α. This study was undertaken to further examine the regulation of IFN-α induction by LPS in human monocytes and the potential role of the IRFs in this process. In addition, the absolute priming requirement for IFN-α induction is contrasted with induction of TNF by LPS; TNF expression occurred readily in unprimed cells but was substantially enhanced after priming.

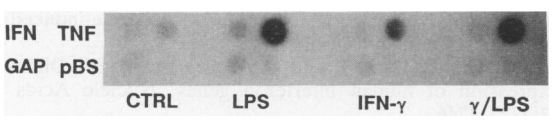

FIG. 7. Transcriptional analysis of monocytes before and after priming with IFN-γ. Freshly isolated (control [CTRL]) or IFN-γ-primed (40 h, IFN-γ) monocytes were used directly or treated with LPS (1 μg/ml) for 45 min (LPS and γ/LPS, respectively), after which nuclei were obtained for transcriptional assays as described in Materials and Methods. IFN, IFN-α_2; GAP, GAPDH, pBS, vector control [pBluescript SK(−)].

The response of the genes examined in this study varied depending on the priming stimulus (IFN-γ or GM-CSF); however, the time course of IFN induction was indistinguishable. In both cases, IFN-α was induced rapidly upon LPS stimulation. Significant IFN activity was detectable by 2 h and reached a maximum in the supernatant by 4 h. The kinetics of IFN secretion in response to LPS was similar to that of other well-characterized monokines, such as TNF and interleukin-1.

The induction of mRNA that hybridized to the IFN-α_2 probe also occurred rapidly. The steady-state accumulation of message differed depending on the priming stimulus. With IFN-γ priming, the IFN-α_2 message appeared rapidly and was only transient; message was undetectable by 6 h. In GM-CSF-primed cells, however, IFN-α_2 message was still detectable by 6 h but disappeared by 8 h. This difference may be due to variable mRNA stability or simply to a longer duration of transcription. The difference did not appear to significantly affect the ultimate quantity of IFN secreted. However, the use of different donor cells makes this difficult to assess.

These results are in contrast to those of previously published studies in which viral induction was used. In those systems, IFN mRNA is present for several hours, even though the reported half-life of the mRNA is very short (23, 31). It is possible that different IFN-α genes whose mRNAs may not hybridize efficiently to the IFN-α_2 probe under the lower-stringency conditions used here are regulated in a different manner. However, since expression of the detectable message exists only under conditions in which IFN activity is detected in the medium, this is unlikely. In addition, the hybridizations were performed under lower-stringency conditions than with the other genes examined, and IFN-α_2 has been found to be one of the predominant IFN genes expressed in many different systems (16).

The most striking difference between the monocytes primed by IFN-γ and by GM-CSF was reflected in the expression of the IRF genes. With IFN-γ priming, IRF-1 was already upregulated and IRF-2 was expressed at low levels. LPS had no significant effect on IRF-1 expression but resulted in a gradual increase in IRF-2 expression. With GM-CSF priming, virtually the opposite situation existed with respect to IRF expression: IRF-1 was expressed only at low levels, while IRF-2 was present at high levels prior to LPS stimulation. Addition of LPS induced high levels of

IRF-1 and downregulated IRF-2 in a rapid manner that was temporally consistent with the induction of IFN-α expression. This reciprocal regulation of the IRF genes by LPS is, to our knowledge, the first description of such antagonistic effects on IRF expression by a single stimulus and may be relevant to other genes under the control of IRFs for their transcription. The induction of IRF-2 message by cytokines which are produced in response to LPS (such as IFN-α and TNF) suggests, but does not prove, the existence of least one mechanism by which negative feedback could control the expression of the genes for these potent bioactive factors. All of the LPS-induced cytokines from monocytes that have been examined are expressed in a rapid and transient manner, consistent with a feedback inhibition mechanism.

The nature of the unique larger mRNA species (4.2-kb band in Fig. 2 and 3 and 3.5-kb band in Fig. 2) hybridizing with the IRF genes is unclear. It is possible that highly homologous mRNA species encoding related transcription factors are expressed in these cells. A more intriguing explanation is that there are some alternatively spliced mRNAs with distinct functions, as have been described for other transcription factors (11, 17).

The IRF data were not consistent with a functional role of IRFs in the induction of IFN-α_2 by LPS, and direct transcriptional analysis suggested that in fact IFN-α_2 is not transcriptionally regulated in this system. This is in contrast to TNF expression, which appears to be highly transcriptionally regulated by LPS. Therefore, additional posttranscriptional events must occur to fully induce IFN-α_2 expression. It is not possible to perform comparative studies of mRNA turnover in fresh versus primed monocytes, since there is no detectable IFN-α_2 mRNA in monocytes treated with LPS alone, even in the presence of CHX.

The accumulation of IFN-α_2 message must occur at the level of mRNA processing, transport, or degradation. Splicing events are not likely to be involved, since IFN-α genes have no introns. The IFN-α genes do possess the AU-rich motifs in their 3′ untranslated regions that may represent a common mRNA-destabilizing sequence found in many inflammation mediator genes, including the TNF gene (1, 27). The effect of CHX treatment demonstrates that protein synthesis is not required for the induction of IFN-α mRNA by LPS with either IFN-γ or GM-CSF as the priming stimulus. This result argues against a functional role for IRF genes which are modulated in response to LPS (in GM-CSF-primed monocytes, Fig. 3). The superinduction by CHX implies the existence of a labile protein that inhibits IFN-α mRNA accumulation even in the presence of LPS (27). Unlike the apparent enhancing effect of CHX on transcription of the IFN-β gene (5, 25), nuclear run-on analysis in monocytes indicated that it has no such effect on IFN-α transcription in the absence or presence of LPS (data not shown).

The nature of the priming phenomenon is still unclear. One intriguing possibility is that primed monocytes express a new surface structure(s) with which LPS can interact and generate some additional intracellular signal(s) resulting in the induction of IFN-α or the enhanced expression of TNF. The results presented here indicate that priming is not the simple consequence of effects on transcription factors but more likely affects both transcriptional and posttranscriptional processes through effects on early LPS signaling pathways.

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