JOURNAL OF VIROLOGY, June 1989, p. 2785–2789
0022-538X/89/062785-05$02.00/0

Vol. 63, No. 6

Bacterial Lipopolysaccharide and Gamma Interferon Induce Transcription of Beta Interferon mRNA and Interferon Secretion in Murine Macrophages

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Received 26 August 1988/Accepted 27 February 1989

Bacterial lipopolysaccharide (LPS) induces interferon (IFN) secretion and an antiviral state in murine peritoneal macrophages (PM). These cells secrete predominantly IFN-β, as shown by neutralization assays with monoclonal antibodies. Secretion of IFN-β is also induced in PM by IFN-γ. LPS and IFN-γ synergistically stimulated PM to produce IFN in amounts almost comparable to those induced by infection with Newcastle disease virus. Low levels of IFN-β mRNA can be detected in freshly harvested PM by hybridization assays. The accumulation of this mRNA is markedly increased in PM treated with LPS or IFN-γ, and it is further enhanced in the presence of the inhibitor of protein synthesis, cycloheximide. Similar studies were carried out on the RAW 264.7 line of transformed macrophages. These cells are induced to secrete IFN-β by LPS but not by IFN-γ, suggesting that this cytokine may elicit such specific response only in PM. IFN-β mRNA is undetectable in untreated RAW 264.7 cells, and accumulation of this mRNA is induced by LPS but not by IFN-γ. The secretion of IFN induced by these agents in PM and by LPS in RAW 264.7 cells and the corresponding accumulation of IFN-β mRNA are blocked by an inhibitor of protein kinase C, staurosporine. The activity of this kinase is apparently necessary to stimulate accumulation of IFN-β mRNA. The induction of IFN-β by IFN-γ appears to be a characteristic response of PM and may be at least in part responsible for the resistance of these cells to viral infections.

Viruses do not multiply in freshly explanted murine peritoneal macrophages (PM). Therefore, viral dissemination in vivo may be limited in these cells, which have a relevant role in the defense against infections (23). Intraperitoneal injection of anti-IFN-α/β antibodies prior to PM harvesting allows virus multiplication, suggesting that these cells are maintained in an antiviral state by endogenous interferon (IFN) (4). The PM can transfer resistance to viral infections to cocultivated cells (25), but anti-IFN antibodies inhibit this transfer (3, 25). Furthermore, PM cultured for 3 to 5 days become permissive to infecting viruses (25). The loss of virus resistance in these aged PM is correlated with a decrease in the level of IFN-induced 2′-5′-oligoadenylate synthetase activity (25). These observations suggest that freshly harvested PM secrete small amounts of IFN that may not be detected in the culture medium, since IFN is continuously removed by binding to cellular receptors. The PM are presumably induced to produce IFN by unidentified stimuli, but once explanted, they are no longer stimulated and their antiviral state decays.

In this investigation, we have examined some agents that may be responsible for inducing PM to produce IFN. We detected small amounts of IFN-β mRNA in freshly harvested PM. Bacterial lipopolysaccharide (LPS) enhanced accumulation of this mRNA and IFN secretion in PM and RAW 264.7 cells, a line of functional macrophages transformed by the Abelson leukemia virus (26). Treatment with IFN-γ also stimulated PM but not RAW 264.7 cells to accumulate IFN-β mRNA and secrete IFN. This response to LPS and IFN-γ is characteristic of PM and may play a relevant role in stimulating these cells to produce IFN in vivo.

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MATERIALS AND METHODS

Reagents and cells. Murine recombinant IFN-γ (MuIFN-γ) was a gift of Genentech. LPS from *Escherichia coli* serotype O55:B5 L2 637 was purchased from Sigma Chemical Co. Human recombinant IFN-γ was a gift of Biogen. Hybridomas producing rat anti-MuIFN-α (clone 4E-A1) and anti-MuIFN-β (clone 7F-D3) monoclonal antibodies were gifts of Y. Kawade and Y. Watanabe, Kyoto University, Kyoto, Japan. These antibodies were produced and purified as described (3). RAW 264.7 macrophages (26) were cultured in RPMI 1640 medium (MA Bioproducts), and L929 cells were cultured in Dulbecco medium with 10% fetal calf serum. Media were endotoxin-free, as determined by the *Limulus* amoebocyte assay.

Peritoneal macrophages. Male C3H/HeN mice aged 5 to 8 weeks (Charles River Italia, Milan, Italy) were kept pathogen-free with sterile filters and were used within 1 week. PM harvested by washing the peritoneal cavity with RPMI 1640 medium–10% fetal calf serum were seeded in cluster plates or in plastic dishes at 1×10^6 cells per well. After 3 h, nonadherent cells were removed by three washes with medium and the PM were either immediately used or aged for 4 days. Experiments were undertaken when the cells were firmly adherent to the culture wells after vigorous washing. Over 95% of the cells stained for nonspecific esterase and were positive in immunofluorescence studies with a rat monoclonal antibody (F4/80) specific for mouse macrophages, as previously described (4).

Induction and assay of IFN. RAW 264.7 and L929 cells were seeded at 7.5×10^5 and 2×10^5 cells per well,

TABLE 1. Secretion of IFN by cells treated with LPS and MuIFN-γ[a]

Cells	IFN (RU/ml) induced:			
	Posttreatment with:			Postinfection with NDV
	LPS	IFN-γ	LPS + IFN-γ	
Macrophages	104	32	400	1,020
RAW 264.7	32	<2	16	380
L929	<2	<2	<2	526

[a] Freshly harvested PM and other cells were treated for 1 h at 25°C and 1 h at 37°C with 20 μg of LPS per ml, 50 RU of MuIFN-γ, per ml, or a combination of the two. The cells were washed twice and incubated for 15 h in fresh medium at 37°C before the culture supernatant was collected. Cells were infected with NDV, and IFN was titrated as described in Materials and Methods. The average numbers of reference units per milliliter (three separate experiments with standard deviation <15%) are shown. This antiviral activity was completey neutralized by polyclonal antibodies to MuIFN-α/β (data not shown).

respectively, in cluster plates. These cells and PM (10^6 per well) were infected (12) with Newcastle disease virus (NDV) or incubated with 30 μg of LPS per ml and 50 reference units (RU) of IFN-γ per ml for 1 h at 25°C and 2 h at 37°C. The cells were washed twice and incubated for 15 h in fresh medium. The IFN secreted was measured by the cytopathic effect inhibition assay (25), with L929 cells and vesicular stomatitis virus (VSV). The IFN concentration is expressed in reference units per milliliter by comparison with standards obtained from the National Institutes of Health. Neutralization tests of IFN were carried out with monoclonal antibodies, as described by Belardelli et al. (3).

Hybridization assay. Total cellular RNA was extracted as described (7). To synthesize complementary RNA probes, we inserted a 648-base-pair *Bam*HI-*Pst*I fragment of an IFN-β cDNA clone (17) into pGEM-1 (Promega Biotec). The plasmid pGEMmβ3 obtained was linearized with *Eco*RI and transcribed as described (12). The RNA was purified by phenol extraction and electrophoresis on 1% low-melting-point agarose gels. The corresponding gel slice was excised and melted at 65°C. The labeled RNA (~10^6 cpm) was added to 70 to 100 μg of cellular RNA dissolved in a solution containing 20 μl of 80% formamide–0.4 M NaCl–3 mM sodium acetate–1 mM EDTA–20 mM 3-morpholinepropanesulfonic acid buffer (pH 7.0). These reactions were heated for 5 min at 85°C and then for 18 h at 65°C; 0.2 ml of 30 mM sodium acetate–0.1 M NaCl–2 mM zinc sulfate–5% glycerol–1,500 U of S1 nuclease were added for 45 min at 37°C. The samples were extracted with chloroform and fractionated on 5% polyacrylamide gels with Tris-borate buffer (22).

RESULTS

Freshly harvested PM, RAW 264.7, and L929 cells were treated with LPS, MuIFN-γ, or both, washed, and incubated in fresh medium. Both agents induced PM to secrete significant amounts of IFN (Table 1). When added together, LPS and MuIFN-γ synergistically stimulated PM to secrete IFN in amounts almost comparable to those produced after infection with NDV, a standard IFN inducer. LPS also stimulated RAW 264.7 cells to secrete IFN. In response to 20 μg of LPS per ml, these cells secreted less than 1/10 of the IFN induced by NDV (Table 1) and smaller concentrations of LPS were less effective in inducing IFN production (data not shown). However, MuIFN-γ did not stimulate these cells to produce IFN, and L929 cells secreted IFN only in response to NDV (Table 1).

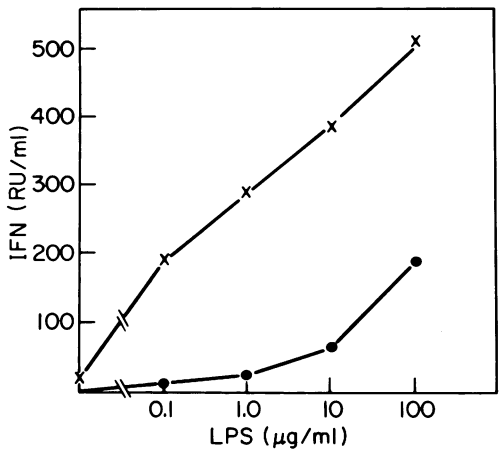

FIG. 1. Production of IFN by freshly harvested PM treated with different concentrations of LPS (●) or LPS and 50 RU of MuIFN-γ (×). Each point represents the average of three experiments with standard deviation <15%.

The synergistic effect of LPS and MuIFN-γ on IFN secretion by PM was further demonstrated by the experiment shown in Fig. 1. Freshly harvested PM were treated with 50 RU of MuIFN-γ per ml and variable amounts of LPS. At concentrations below 1 μg/ml, LPS stimulated IFN production weakly. However, in the presence of IFN-γ, small concentrations of LPS were quite effective in promoting secretion of IFN. In agreement with previous results (3), neutralization experiments with monoclonal antibodies showed that MuIFN-β represented ~90% of the IFN secreted by PM treated with LPS (data not shown).

Further evidence for a specific macrophage response to LPS and MuIFN-γ was obtained by measuring the yield of VSV. PM aged in culture for 4 days were used in these experiments, since freshly harvested PM are naturally resistant to virus infection (4). The PM and RAW 264.7 cells treated with LPS yielded significantly less VSV than did control cells (Table 2). This protection was presumably due to secreted MuIFN-β, since it was abolished when monoclonal antibodies against this IFN were included in the incubations. Treatment with MuIFN-γ was most effective in decreasing VSV yield in PM. This protection was in part due to MuIFN-β secretion by PM and to a lesser extent to Mu IFN-α secretion, as shown by the effect of specific antibodies (Table 2). MuIFN-γ induced an antiviral state in both RAW 264.7 and L929 cells, but it failed to stimulate these cells to secrete MuIFN-β, since neutralizing antibodies had no effect on VSV yield (Table 2). These results show that the antiviral activity of MuIFN-γ on aged PM but not on other cells is in part mediated by secretion of MuIFN-β.

A possible explanation for the antiviral state of freshly harvested PM and for the mechanism of action of LPS and MuIFN-γ was investigated by hybridizing RNA extracted from these cells with a cRNA probe for MuIFN-β mRNA (Fig. 2A). Very small amounts of MuIFN-β mRNA were detected in untreated PM upon prolonged exposure of the autoradiographs. The level of this mRNA was increased in PM treated with the inhibitor of protein synthesis cycloheximide, which stabilizes IFN mRNA (27). This finding shows that PM constitutively transcribe MuIFN-β mRNA at a low rate; a relatively fast turnover of this mRNA may prevent its accumulation. Both LPS and MuIFN-γ induced accumulation of MuIFN-β mRNA in PM (Fig. 2A). This induction was

TABLE 2. Virus yield of cells treated with LPS or MuIFN-γ and infected with VSV[a]

Cells	Mean $\log_{10}$ VSV yield ± SE in control	Mean $\log_{10}$ VSV yield ± SE after treatment with:				
		LPS	LPS + anti-IFN-β	IFN-γ	IFN-γ + anti-IFN-β	IFN-γ + anti-IFN-α
Aged PM	5.5 ± 0.1	3.5 ± 0.2	4.9 ± 0.3	0.9 ± 0.2	3.3 ± 0.2	1.8 ± 0.3
RAW 264.7	6.0 ± 0.1	2.0 ± 0.3	6.2 ± 0.4	4.7 ± 0.1	4.5 ± 0.1	ND[b]
L929	6.0 ± 0.1	6.3 ± 0.1	6.0 ± 0.1	3.8 ± 0.1	3.7 ± 0.2	ND

[a] The PM were treated after 4 days in culture, and other cells were treated after 1 day with 30 μg of LPS per ml or 50 RU of MuIFN-γ per ml, as described in Table 1. The cells were washed twice and incubated in fresh medium containing, where indicated, monoclonal antibodies neutralizing 10,000 RU of MuIFN-α or 80,000 RU of MuIFN-β. After 18 h, the cells were washed and infected with VSV at multiplicity of 0.05. The virus released in the culture medium after 18 h was titrated on L929 cells, and the $\log_{10}$ VSV yield (mean plus or minus standard error) is shown. Three independent experiments were carried out for each experimental condition.

[b] ND, Not done.

a primary response (not requiring protein synthesis), since MuIFN-β mRNA was superinduced by cycloheximide, particularly in LPS-treated PM. In contrast, MuIFN-γ did not induce transcription of MuIFN-β mRNA in murine RAW 264.7 cells, C127 fibroblasts, and P338D$_1$ monocytic cells. Human IFN-β mRNA, measured by hybridization to a specific probe (12), was not detected in human fibroblasts, A549 lung adenocarcinoma, HEp-2 epithelioid carcinoma, HeLa cervical carcinoma, or SK-MEL-109 melanoma cells treated with human IFN-γ (data not shown).

The lack of protection from VSV infection in untreated RAW 264.7 cells (Table 2) reflected the lack of constitutive expression of IFN mRNA; treatment with LPS induced an antiviral state and transcription of MuIFN-β mRNA (Fig. 2B). This mRNA was transiently induced 4 to 8 h after addition of LPS concentrations as low as 10 ng/ml. The

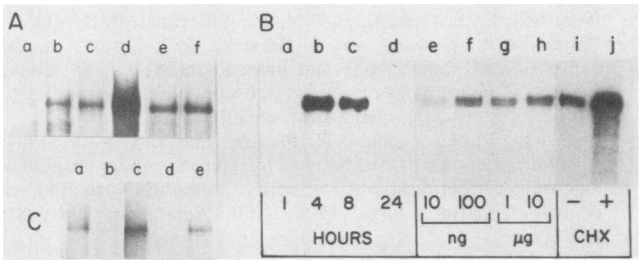

FIG. 2. Analysis of MuIFN-β mRNA in macrophages. Cellular RNA was extracted and hybridized to cRNA transcribed from pGEMmβ13, as described in Materials and Methods. The S1-nuclease-resistant hybrids were fractionated on 5% polyacrylamide gels. The dried gels were autoradiographed for 4 days (A, lanes a and b) or overnight. (A) Electrophoresis of freshly harvested PM. Lanes: a, untreated PM; b, PM treated for 3 h with 5 μg of cycloheximide per ml; c, PM treated with 30 μg of LPS per ml; d, PM treated with LPS and cycloheximide simultaneously; e, PM treated with 50 U of MuIFN-γ per ml; and f, PM treated with MuIFN-γ and cycloheximide. (B) RAW 264.7 cells treated with 10 μg of LPS per ml for the time indicated (lanes a through d), or with different LPS concentrations for 3 h (lanes e through h). RAW 264.7 cells were also treated with 10 μg of LPS plus or minus 50 μg of cycloheximide per ml for 3 h (lanes i and j). (C) Electrophoresis showing inhibition of MuIFN-β mRNA accumulation by staurosporine. PM were incubated for 4 h with 50 RU of MuIFN-γ per ml minus (lane a) or plus (lane b) 3 nM staurosporine. RAW 264.7 cells were incubated either directly with 10 μg of LPS per ml for 3 h (lane c) or after 1 h of pretreatment with 3 nM staurosporine (lane d) or 50 μM *N*-(6-aminohexyl)-chloro-1-naphthalene-sulfonamide (W7) (lane e). These inhibitors were present throughout the treatment with LPS. In some lanes, a double band is seen; this is explained by heterogeneity at the 3′ terminus of cRNA due to premature termination.

hybridization assay used was at least 100-fold more sensitive than the antiviral assay for secreted IFN, which was detected (~2 U/ml) in the medium of RAW 264.7 cells treated with at least 1 μg of LPS per ml. This finding may be explained by the removal of secreted IFN from the medium, due to binding to cellular receptors. Transcription of MuIFN-β mRNA was superinduced by cycloheximide in LPS-treated RAW 264.7 cells (Fig. 2B), but it was not detected in control cells treated with this inhibitor alone (data not shown). This finding indicates that RAW 264.7 cells do not constitutively transcribe MuIFN-β mRNA.

MuIFN-γ increases approximately fivefold the protein kinase C (PKC) activity in murine PM within 3 h (14). Furthermore, LPS primes these cells for an enhanced response to PKC activators (2) and stimulates myristoylation and phosphorylation of a major substrate for this protein kinase (1). These observations suggest that MuIFN-γ and LPS may stimulate accumulation of MuIFN-β mRNA by activating PKC. When PM were treated with MuIFN-γ in the presence of 3 nM staurosporine, an inhibitor of PKC (28), secretion of IFN was inhibited ~80%. In agreement with this finding, accumulation of MuIFN-β mRNA was inhibited in PM treated with MuIFN-γ and staurosporine (Fig. 2C). Secretion of IFN was also inhibited in RAW 264.7 cells treated with this inhibitor and LPS. In this experiment, 3 nM staurosporine was added 1 h before LPS to RAW 264.7 cells that were then infected with VSV as described in Table 2. The antiviral activity of LPS was completely abolished (data not shown), and transcription of MuIFN-β mRNA was drastically inhibited by staurosporine (Fig. 2C).

W7, an inhibitor of calcium-calmodulin-dependent protein kinases (16), and calcium depletion have been reported to block the expression of HLA class II molecules induced by IFN-γ in promyelocytic HL-60 cells (21). These findings suggest that some activities of IFN-γ are mediated by calcium influx and activation of calmodulin-dependent kinases (21). However, W7 had no effect on the stimulation of IFN secretion by LPS in PM and RAW 264.7 cells (data not shown) and did not significantly decrease the accumulation of MuIFN-β mRNA in RAW 264.7 cells (Fig. 2C). This observation suggests that the accumulation of MuIFN-β mRNA induced by LPS is specifically blocked by PKC inhibitors.

DISCUSSION

There is increasing evidence that IFNs are constitutively expressed under some conditions in normal humans (29), rabbits (6), and mice (3, 4, 9–11, 13, 19, 25). The present results demonstrating the presence of small amounts of

MuIFN-β mRNA in PM provide a plausible explanation for the resistance of these cells to virus infection (23) and for the induction of an antiviral state in cocultivated cells (25), since translation of this mRNA may result in IFN secretion. However, we were unable to reproducibly recover IFN in the culture medium of PM (25), in spite of reports that these cells release small amounts of IFN in vitro (9, 24).

Macrophages produce IFN in vitro when treated with endotoxin, polysaccharides, and polyanions, which are ineffective in inducing IFN synthesis in other cells (15). These agents may be involved in stimulating IFN production in vivo, but LPS is likely to play a major role, since freshly explanted PM from LPS-hyporesponsive mice do not induce an antiviral state in cocultivated cells (11). These PM are nonpermissive for VSV when first placed in culture, but they become permissive with time (11) and lose resistance to VSV after 2 days in culture (30). Therefore, spontaneous secretion of IFN by PM is correlated with their capacity to produce IFN after stimulation with LPS in vitro (11).

In this investigation, we have examined the effect of LPS and MuIFN-γ on the production of IFN by PM in vitro. LPS, MuIFN-γ, or both promoted MuIFN-β secretion and accumulation of the corresponding mRNA in PM. This response to IFN-γ is apparently specific for PM, since RAW 264.7 cells were stimulated only by LPS. However, the RAW 264.7 cells were quite sensitive to IFN-γ, as shown by the induction of an antiviral state (Table 2).

It should be pointed out that Hughes and Baron (18) have recently reported that a large component of the antiviral activity of MuIFN-γ in L929 cells may be due to production of IFN-α. The present results do not contradict this report, since we have not measured MuIFN-α mRNA, nor have we examined the effect of antibodies neutralizing this IFN on L929 cells treated with MuIFN-γ at low density, as suggested by these authors (18).

Synthesis of new proteins is now required for the response of PM to LPS and MuIFN-γ, since MuIFN-β mRNA is superinduced in the presence of cycloheximide. This primary response may be due to an enhancement of a basal level of transcription or to a stabilization of IFN-β mRNA, both of which would result in accumulation of this mRNA. Nuclear runoff experiments with PM would be needed to prove that LPS enhances the transcription rate. However, LPS induced de novo synthesis of MuIFN-β mRNA in RAW 264.7 cells. This finding leads us to favor the hypothesis that LPS induces transcription of this mRNA also in PM. Furthermore, LPS and MuIFN-γ synergistically stimulated the production of IFN-β in PM and it seems possible that even low concentrations of these agents, possibly in combination with other lymphokines, are responsible for inducing PM to transcribe IFN mRNA in vivo.

It seems unlikely that macrophages are exposed to LPS in the peritoneal cavity, but PM may be stimulated by LPS or by its degradation products in the intestinal lymphatic system and then migrate into the peritoneum. LPS induces transcription of mRNAs for lymphokines, such as tumor necrosis factor and interleukin-1, in macrophages (5). Furthermore, IFN-γ enhances transcription of the mRNAs for tumor necrosis factor and interleukin-1 in PM (8). These lymphokines may synergistically stimulate IFN-β production by autocrine or paracrine mechanisms.

Transcription of the IFN-β gene is controlled by the interaction of protein factors with positive and negative regulatory sequences of its promoter (20). Upon addition of double-stranded RNA, at least one protein is modified and transcription is activated (20). PM apparently regulate the IFN-β gene in additional and unique ways, since LPS and IFN-γ may promote modifications of specific protein factors in PM by unidentified mechanisms. The only clue that we presently have is the observation that an inhibitor of PKC, staurosporine, blocks transcription of MuIFN-β mRNA and secretion of IFN in PM. Experiments with several murine and human cell lines show that staurosporine does not block transcription of IFN-β mRNA induced by double-stranded RNA (unpublished observations). PKC activation may thus be required only for IFN-β mRNA transcription induced by LPS and IFN-γ.

The present findings and previous observations on the antiviral state of PM suggest that these cells regulate IFN production by means of unique mechanisms that presumably evolved to prevent viral dissemination by macrophages which come in contact with different tissues.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-16076 from the National Institutes of Health and by grant 86.02680.44 from the Consiglio Nazionale delle Ricerche.

We thank I. Gresser for helpful suggestions and Y. Kawade and Y. Watanabe for providing the hybridomas producing antibodies to MuIFN-α and MuIFN-β.

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