BACTERIAL LIPOPOLYSACCHARIDE (ENDOTOXIN) ENHANCES EXPRESSION AND SECRETION OF β_2 INTERFERON BY HUMAN FIBROBLASTS

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Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria including Escherichia, Salmonella, Shigella, Pseudomonas, and numerous other genera (1, 2). LPS triggers many of the deleterious effects of bacterial infections in animals (1, 2). For example, endotoxemic shock represents the intravascular activation by LPS of the coagulation cascade, the complement system, and the bradykinin system; these events lead to a collapse of the cardiovascular system (1, 2). Host immune mechanisms recognize LPS and respond to its presence by activation of macrophages (3). LPS-activated macrophages secrete a variety of important cytokines such as IL-1, TNF, and lymphotoxin, which play key roles in host defense (4–8). In this article we describe another component in the host response elicited by LPS. We report that the fibroblast, a type of cell present in all body tissues, responds to LPS by secreting IFN-β_2. IFN-β_2 polypeptides have been reported to elicit an antiviral state in fibroblasts, increase immunoglobulin secretion by certain B cell lines, enhance proliferation of plasmacytoma/hybridoma cells, and mediate many of the acute-phase alterations in the secretion of plasma proteins by hepatocytes (9, 10). Taken together, the data obtained suggest that the ubiquitous fibroblast may have a specific role in host defense during the course of Gram-negative bacterial infections.

Human IFN-β_2 comprises a set of at least six proteins of M_r 23,000–30,000, as judged by SDS-PAGE (reviewed in reference 9 and L. T. May et al., manuscript in preparation) and is derived from a gene on human chromosome 7 (11). IFN-β_2 cDNA has been cloned, sequenced, and expressed into biologically active protein in heterologous expression systems (12–16). IFN-β_2 is produced in a variety of tissues comprising fibroblasts, epithelial cells, monocytes, and T cells (9). The IFN-β_2 gene is identical to that for the B cell differentiation factor BSF-2, the hybridoma growth factor HGF, and the hepatocyte-stimulating factor HSF (9, 10). It is evident that IFN-β_2 is engaged in multidirectional communication among different cell types.

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Materials and Methods

Cell Culture. The human foreskin fibroblast strain FS-4 was obtained from Dr. J. Vilček (New York University School of Medicine, New York); procedures for its growth in cell culture and for the IFN-β_2 mRNA induction experiments have been described (15, 17).

RNA Blot-hybridization Analyses. Procedures for isolation of poly(A)$^+$ RNA from FS-4 cells (approximate RNA yield 1 μg per T-175 culture), electrophoresis of the RNA in 1% agarose gels containing 10 mM methylmercury hydroxide, transfer to aminobenzyloxymethyl cellulose paper (ABM paper; Pharmacia Fine Chemicals, Piscataway, NJ), and hybridization with full-length human IFN-β_1 and -β_2 cDNA clones have been described (15, 17).

Assay for Antiviral Activity. These were carried out essentially as described by Sehgal et al. (17). Briefly, FS-4 cells were seeded in 96-well microtiter plates in Eagle's MEM supplemented with 5% (vol/vol) heat-inactivated FCS (56°C for 0.5 h; Gibco, Grand Island, NY) to produce confluent monolayers. 6–8 d later the culture medium was discarded and the monolayers were exposed to 100-μl aliquots of fresh medium containing serial two-fold dilutions of the test samples. After incubation for 24 h, the monolayers were challenged with vesicular stomatitis virus (VSV)[1] (Indiana strain, 10–100 plaque forming units [PFU]/well) such that it produced complete destruction of control FS-4 cell sheets 3–4 d after challenge. 4 d after addition of VSV, the cell monolayers were fixed with ethanol and stained using Gram's crystal violet. Antiviral assays were calibrated using appropriate human IFN-β_1 standards.

Assay for Enhancement of B Cell Differentiation. The CESS line of human B lymphoblastoid cells (TIB190; American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 medium (Flow Laboratories, McLean, VA) supplemented with 10% (vol/vol) heat-inactivated FCS (Hazelton Systems, Inc., Aberdeen, MD) and 2 mM glutamine (Gibco). The ability of various dilutions of FS-4 cell culture medium to enhance differentiation of 10^4 CESS cells after 60 h of incubation in 0.2 ml of medium in 96-well microtiter plates (Linbro Chemical Co., Hamden, CT) was determined in triplicate using a reverse plaque-forming cell (PFC) hemolytic assay, which detects the ability of individual differentiated CESS cells to secrete human immunoglobulin IgG (18, 20–22). This assay, which detects lysis of protein A–coupled sheep erythrocytes in the vicinity of each immunoglobulin-secreting CESS cell, has been described in detail elsewhere (18, 20–22). It monitors the differentiation of CESS cells from an IgG-nonsecreting phenotype to an IgG-secreting phenotype.

Reagents. All LPS preparations, cycloheximide, and dexamethasone were purchased from Sigma Chemical Co., St. Louis, MO. The compounds H7 and H8 were purchased from Seikagaku America, St. Petersberg, FL. Recombinant *E. coli*–derived human IL-1α (sp act 3×10^7 U/mg) was a gift from Hoffman-LaRoche, Nutley, NJ, and recombinant *E. coli*–derived human TNF (sp act 4.8×10^7 U/mg) was a gift from The Suntory Institute for Biomedical Research, Osaka, Japan, and, recombinant *E. coli*–derived IFN-β_1 was a gift from Triton Biosciences, Inc., Alameda, CA. Gram's crystal violet was purchased from Fisher Scientific, Orangeburg, NY; α-[^{32}P]dCTP used for nick translation of plasmid DNA was purchased from New England Nuclear, Boston, MA. Autoradiography was performed at −70°C using Kodak XAR-5 film and intensifying screens (Quanta III; DuPont Co., Wilmington, DE).

Results and Discussion

Antiviral Activity of Bacterial LPS in Human Fibroblasts. In the course of experiments to express human IFN-β_2 in *E. coli* we observed that an aqueous extract of *E. coli* could itself induce an antiviral state in human fibroblasts (FS-4 strain) against VSV (Fig. 1). LPS derived from *E. coli* also elicited an antiviral

[1] *Abbreviations used in this paper:* HSF, hepatocyte-stimulating factor; PFC, plaque-forming cell; VSV, vesicular stomatitis virus.

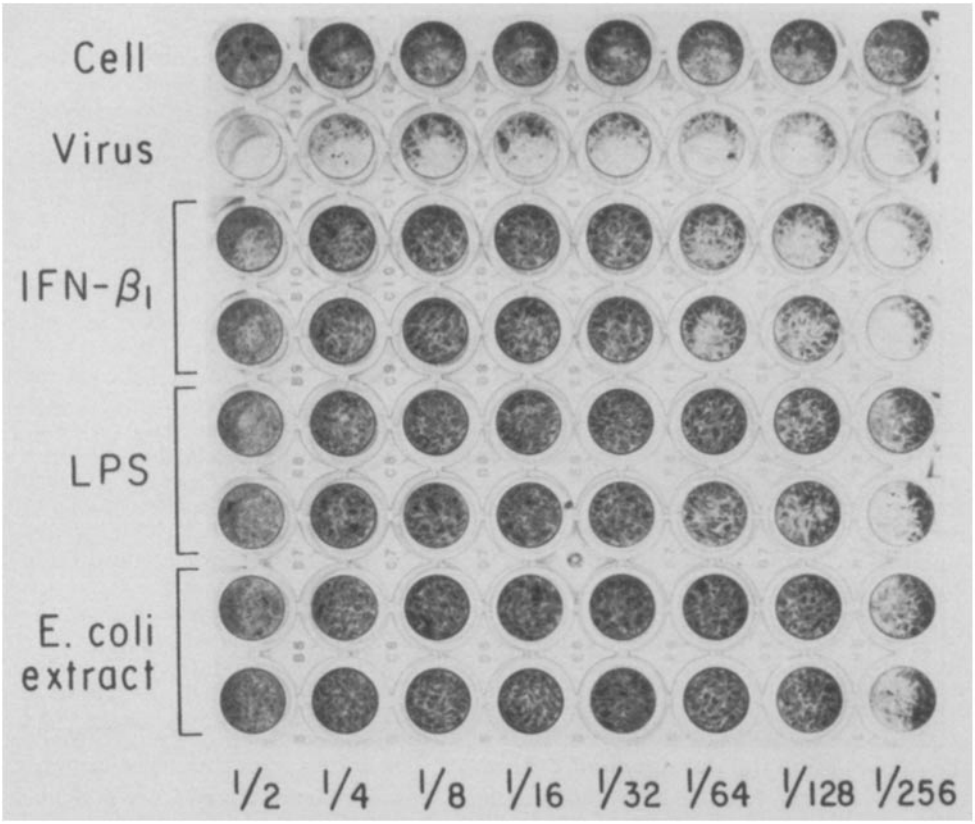

FIGURE 1. Inhibition of the cytopathic effect of VSV in fibroblasts incubated with *E. coli*–derived LPS. FS-4 cells were seeded in 96-well microtiter plates to produce confluent monolayers. 6 d later the culture medium was discarded and the monolayers were exposed to 100 μl aliquots of fresh medium containing serial twofold dilutions (from left to right in the figure) of a recombinant IFN-β_1 preparation (titer 100 IU/ml), a preparation of phenol-extracted LPS derived from *E. coli* serotype 0111:B4 (highest concentration tested was 1 μg/ml), or an extract of *E. coli* strain HB101 (prepared by sedimenting bacteria from a 5 ml log-phase culture, subjecting the pellet to a freeze-thaw cycle, followed by resuspension in 0.5 ml water, sonication, and Millipore filtration [0.22 μm]). After incubation for 24 h, the monolayers were challenged with VSV. 4 d after addition of VSV, the cell monolayers were fixed with ethanol and stained using the crystal violet. Dark-stained monolayers are those that have been protected from virus-mediated cell killing.

state (Fig. 1). This represents a rediscovery of the antiviral effect of LPS in cell culture. Likar et al. (23) reported in 1959 that *E. coli* and *Salmonella typhimurium* endotoxin inhibited poliovirus replication in secondary monkey kidney cells. That a bacterial constituent can have an inhibitory effect on viral multiplication was first demonstrated with the capsular polysaccharide of *Klebsiella pneumoniae* and the pneumonia virus of mice and the mumps virus (24–26). In the 1960s a number of reports documented the antiviral effects of constituents of Gram-negative as well as Gram-positive bacteria in cultures of fibroblasts or epithelial cells (27–32). As the inhibitory substances from the Gram-positive bacteria had a narrow range of antiviral activity (27–29), it appeared unlikely that their action was mediated via the production of endogenous IFN. It is, however, of interest

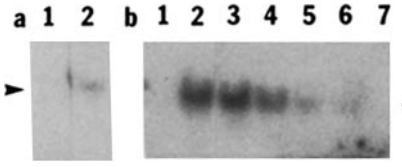

FIGURE 2. Induction of IFN-β_2 mRNA in fibroblasts by an extract of *E. coli* (*a*) or by *E. coli*–derived LPS (*b*). Confluent monolayers of FS-4 cells in T-175 flasks (one flask per group) were exposed for 6–7 h to a 1:10 dilution of a pool of *E. coli* extracts prepared essentially as mentioned in Fig. 1 (sonication was omitted) or to LPS at different concentrations (*E. coli*, serotype 0111:B4). The *E. coli* extract was a pool of samples that displayed antiviral activity at dilutions in the range from 1:2 to 1:8. The levels of IFN-β_2 mRNA were monitored by agarose gel electrophoresis followed by blot hybridization using a full-length human IFN-β_2 cDNA probe. The region of the autoradiogram containing the 1.3 kb IFN-β_2 mRNA is illustrated. *a*: lane *1*, control; lane *2*, *E. coli* extract. *b*: lane *1*, control; lane *2*, LPS, 1 μg/ml; lane *3*, 100 ng/ml; lane *4*, 10 ng/ml; lane *5*, 1 ng/ml; lane *6*, 0.3 ng/ml; lane *7*, 0.1 ng/ml.

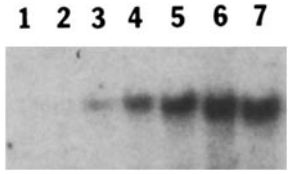

FIGURE 3. Rapid enhancement of IFN-β_2 mRNA levels in fibroblasts by LPS. Confluent monolayers of FS-4 cells in T-175 flasks (one flask per group) were exposed to LPS (*E. coli*, serotype 0111:B4, 5 μg/ml) for the following periods: lane *1*, 0 min; lane *2*, 15 min; lane *3*, 30 min; lane *4*, 45 min; lane *5*, 1 h; lane *6*, 2 h; lane *7*, 3 h. The content of IFN-β_2 mRNA was assayed by blot hybridization.

that actinomycin D inhibited the development of the antiviral state elicited by an *E. coli* extract (32).

Induction of IFN-β_2 mRNA by Bacterial LPS in Human Fibroblasts. We observed that the antiviral activity of LPS in FS-4 cells could be completely blocked by an anti-IFN-β antiserum (not shown), thus implicating the induction of endogenous IFN-β by LPS in the development of the antiviral state. Using blot-hybridization methods we determined that extracts of *E. coli* or LPS prepared from *E. coli* enhanced the levels of IFN-β_2 mRNA in FS-4 cells (Fig. 2). It should be emphasized that LPS did not induce detectable levels of IFN-β_1 mRNA (not shown).

LPS enhances IFN-β_2 mRNA levels in FS-4 cells at concentrations as low as 0.3 ng/ml (Fig. 2*B*, lane *6*). The enhancement is near-maximal at an LPS concentration in the range 0.1–1 μg/ml (Fig. 2*B*). The enhancement is rapid; an increase is detected within 30 min after addition of LPS, and is near-maximal by 2–3 h (Fig. 3). The increase is sustained at this high level for at least 20 h thereafter (not shown).

The enhancement of IFN-β_2 gene expression in FS-4 cells by LPS is moderately inhibited by 50 μM H8, a preferential inhibitor of cAMP- and cGMP-dependent protein kinases compared with protein kinase C (33), but is strongly inhibited by 50 μM H7, an inhibitor of protein kinase C as well as of cyclic nucleotide–dependent protein kinases (33) (Fig. 4*A*). These observations are consistent with the possibility that LPS enhances IFN-β_2 gene expression in fibroblasts by activating the protein kinase C signal transduction pathway. Evidence has been obtained in some, but not all studies of the involvement of protein kinase C in the activation of resting murine B lymphocytes by LPS (34–36). It is noteworthy that H7, but not H8, efficiently blocks IFN-β_2 gene expression in fibroblasts treated with IL-1 or TNF (Fig. 4*B*) (17).

LPS increased the levels of IFN-β_2 mRNA in FS-4 cells in the presence of cycloheximide, indicating that new protein synthesis is not required for this

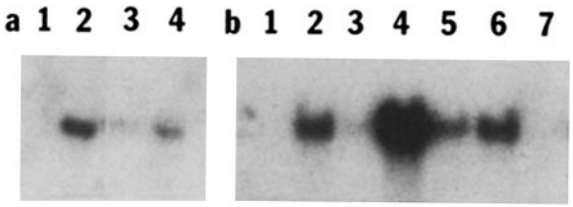

FIGURE 4. Effect of protein kinase inhibitors H7 and H8 on the induction by LPS of IFN-β_2 mRNA in fibroblasts. Confluent monolayers of FS-4 cells (one T-175 flask per group) were incubated for 6 h with LPS (1 μg/ml) alone or together with the protein kinase inhibitors H7 (50 μM) or H8 (50 μM). For comparison, the effect of H7 on the induction of IFN-β_2 by IL-1α (1 ng/ml) and TNF (30 ng/ml) is also illustrated. *a*: lane *1*, control; lane *2*, LPS; lane *3*, LPS and H7; lane *4*, LPS and H8. *b*: lane *1*, control; lane *2*, LPS; lane *3*, LPS and H7; lane *4*, IL-1α; lane *5*, IL-1α and H7; lane *6*, TNF; lane *7*, TNF and H7. The content of IFN-β_2 mRNA was measured by blot hybridization.

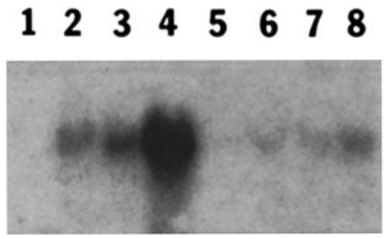

FIGURE 5. Effect of cycloheximide and dexamethasone on the induction by LPS of IFN-β_2 mRNA in fibroblasts. Confluent monolayers of FS-4 cells (one T-175 flask per group) were incubated for 6 h as follows: lane *1*, control; lane *2*, LPS (10 μg/ml); lane *3*, cycloheximide (50 μg/ml); lane *4*, LPS and cycloheximide; lane *5*, dexamethasone, 10^{-6} M; lane *6*, LPS and dexamethasone (10^{-6} M); lane *7*, LPS and dexamethasone (10^{-7} M); lane *8*, LPS and dexamethasone (10^{-8} M). The content of IFN-β_2 was assayed by blot hybridization.

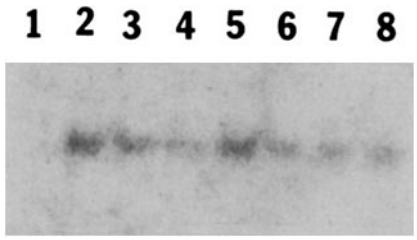

FIGURE 6. Enhancement of IFN-β_2 mRNA levels in fibroblasts by different preparations of bacterial LPS. Confluent monolayers of FS-4 cells (one flask per group) were treated for 6 h with different LPS (5 μg/ml) preparations as follows: lane *1*, control; lanes *2–5*, phenol-extracted LPS from *E. coli* serotype 0111:B4 (lane *2*), serotype 055:B5 (lane *3*), serotype 026:B6 (lane *4*), and *S. typhimurium* (lane *5*); lanes *6–8*, phenol-extracted and chromatographically purified LPS from *E. coli* serotype 0111:B4 (lane *6*), serotype 055:B5 (lane *7*), and serotype 0026:B6 (lane *8*). The content of IFN-β_2 mRNA was assayed by blot hybridization.

induction (Fig. 5, lanes *1–4*). A combination of LPS and cycloheximide "superinduces" the expression of IFN-β_2 mRNA (Fig. 4, lane *4*) (37, 38). Of special interest is the fact that the induction of IFN-β_2 mRNA by LPS is inhibited by dexamethasone (Fig. 5*B*, lanes *6–8*), a glucocorticoid that also inhibits the induction, in FS-4 cells, of IFN-β_2 mRNA by IL-1 and TNF (39). Dexamethasone may affect a common element in the signal transduction pathways used by the three reagents. It has been suggested that dexamethasone inhibits some step proximal to the formation of second messengers through the agonist-induced hydrolysis of inositol phospholipids in LPS-stimulated murine B cells (40).

The enhancement of IFN-β_2 mRNA levels in FS-4 cells is a property of several different LPS preparations. Chromatographically purified LPS derived from three different strains of *E. coli*, as well as LPS derived from a strain of *S. typhimurium* enhance IFN-β_2 mRNA levels (Fig. 6).

B Cell Differentiation Activity of Conditioned Medium from LPS-treated Fibroblast Cultures. One of the immunologic consequences of IFN-β_2 secreted by LPS-treated fibroblasts is illustrated in Table I. FS-4 cells were treated with LPS for 6 h and then incubated in LPS-free medium for another 24 h. The culture

TABLE I

E. coli–derived LPS Stimulates Production of IFN-β_2 by Human Fibroblasts (FS-4 Strain): Enhancement of B Cell (CESS) Differentiation

Additions to CESS cells	PFC/culture when FS-4 cell culture medium was added to CESS cells at dilutions of:		
	1:2	1:8	1:100
A. Culture medium from FS-4 cells treated with:			
Uninduced	112	125	30
LPS (1 μg/ml)	218	277	168
LPS (0.1 μg/ml)	173	292	123
TNF (30 ng/ml) + CHX (50 μg/ml)	147	336	232
	PFC/culture		
B. Controls			
S. aureus Cowan I antigen (0.01% vol/vol)		200	
LPS (1 μg/ml)		66	
None		77*	

Confluent monolayers of FS-4 cells in T-175 flasks containing 10 ml of spent medium were treated for 6 h with LPS at 1 or 0.1 μg/ml or with TNF (30 ng/ml) and cycloheximide (CHX, 50 μg/ml). The cultures were then washed extensively with PBS and incubated in another 10 ml of spent medium per flask. Culture medium was harvested 24 h later, cell debris removed by centrifugation, and appropriate aliquots stored at −70°C. The ability of various dilutions of FS-4 cell culture medium to enhance differentiation of 10^4 CESS cells after 60 h of incubation in 0.2 ml of medium in 96-well microtiter plates was determined in triplicate using a reverse PFC hemolytic assay. As controls, *S. aureus* Cowan I antigen (0.01%, vol/vol; Behring Diagnostics) or *E. coli* LPS (1 μg/ml) was added directly to the CESS cells. Saturating amounts of B cell differentiation factor activity appear to be present in the 1:8 dilution of the medium samples from induced FS-4 cell cultures; at the 1:100 dilution a lesser amount of activity is observed.

* Mean of six experiments.

medium obtained from LPS-treated FS-4 cells was then tested for its ability to stimulate differentiation (immunoglobulin secretion) by an Epstein-Barr virus–transformed human B cell line (CESS) (18, 19). It is clear from Table I that LPS-treated FS-4 cells produce a cytokine that increases the number of IgG-secreting CESS cells. Treatment of FS-4 cells with LPS or TNF at maximally effective concentrations yielded culture media that caused comparable increases in the number of IgG-secreting CESS cells (Table I).

Fibroblasts are not the only kinds of cells that secrete IFN-β_2 when exposed to LPS. It is known that human monocytes exposed to LPS secrete a protein called the hepatocyte-stimulating factor (HSF) that alters the expression of plasma protein genes in the liver (41–49). For example, HSF decreases the synthesis of albumin and increases the synthesis of fibrinogen, haptoglobin, α_2-macroglobulin, and C-reactive protein. The alterations in liver gene expression constitute the acute-phase response that occurs in acute bacterial or mycoplasma infections, and tissue damage and is elicited by HSF as well as IL-1 (41–49). However, HSF and IL-1 elicit qualitatively and quantitatively different responses in the expression of plasma protein genes (41–49). It has recently been shown that HSF and IFN-β_2 are the same molecule (10).

The acute-phase response mediated via the action of monocyte-derived IFN-β_2/HSF on the liver leads to systemic effects on the organism. In contrast, the fibroblast-derived IFN-β_2 may primarily give rise to effects of local importance. The ubiquitous fibroblasts play a key role in post-injury repair. LPS at concentrations in the range of 0.1 ng/ml to 5 μg/ml stimulates the proliferation of chick embryo, murine, bovine, and human fibroblasts (including FS-4 cells) (50–53). Production of IFN-β_2 by fibroblasts exposed to LPS is consistent with the hypothesis that IFN-β_2 is part of an homeostatic mechanism that regulates fibroblast proliferation (37, 38). The IFN-β_2 secreted by LPS-stimulated fibroblasts may enhance in situ immunoglobulin secretion by B lymphocytes. Additional evidence for a role of IFN-β_2 in the pathophysiology of acute infections is provided by the findings of strong enhancement of IFN-β_2 gene expression in fibroblasts during virus infections (54 and D. C. Helfgott and P. B. Sehgal, unpublished data).

Summary

The human β_2 interferon (IFN-β_2) gene, a gene that also codes for B cell differentiation factor 2 (BSF-2), plasmacytoma/hybridoma growth factor (HGF), and hepatocyte-stimulating factor (HSF), is expressed in a variety of lymphoid and nonlymphoid tissues. Endotoxin, or bacterial lipopolysaccharide (LPS) preparations derived from the outer membrane of *Escherichia coli* or *Salmonella typhimurium* rapidly elevate IFN-β_2 mRNA level in human skin fibroblasts (FS-4 strain). *E. coli*–derived LPS enhances IFN-β_2 mRNA expression in FS-4 fibroblasts at a concentration as low as 0.3 ng/ml; this response is near-maximal in the range of 0.1–1 μg/ml LPS. The increase in IFN-β_2 mRNA level caused by LPS in FS-4 cells is detected within 30 min after addition of LPS, is sustained for at least 20 h thereafter, appears to involve the protein kinase C signal transduction pathway, does not require new protein synthesis, and is inhibited by dexamethasone in a dose-dependent fashion (in the range 10^{-6}–10^{-8} M). Cultures of LPS-treated FS-4 cells exhibit an antiviral state against vesicular stomatitis virus, which can be prevented by anti-IFN-β antiserum. Medium obtained from LPS-treated FS-4 cell cultures enhances the number of immunoglobulin-secreting cells in cultures of human B-lymphoblastoid (CESS) cells. Thus, LPS may trigger a number of host defense mechanisms in the course of infection due to Gram-negative bacteria by enhancing IFN-β_2 production by the ubiquitous fibroblast.

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