Interferon-α/β Inhibits the Apoptosis Induced by Lipopolysaccharide and Interferon-γ in Murine Peritoneal Macrophages

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ABSTRACT

Challenge of elicited peritoneal macrophages with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) was followed by an apoptotic response. These cells expressed cytokine-inducible nitric oxide synthase (iNOS) in response to these stimuli, and the NO released contributed markedly to the apoptotic death, as deduced from the increased viability observed when iNOS activity was inhibited. The antiviral type I IFN (IFN-α/β) downregulated the high levels of NO produced when cells were stimulated with suboptimal doses of LPS and IFN-γ. Moreover, IFN-α/β also decreased cell death in LPS/IFN-γ-activated cells, as determined by the reduction in the content of oligonucleosomal DNA fragments, in the binding of annexin V to the plasma membrane, and in the amount of hypodiploid cells when analyzed by flow cytometry after *in vivo* staining with propidium iodide. Kinetic analysis of the protection exerted by IFN-α/β against the apoptosis induced by treatment with LPS and IFN-γ showed that type I IFNs were very effective when added up to 1 h after IFN-γ/LPS stimulation. Addition of IFN-α/β 4 h after stimulation with IFN-γ/LPS failed completely to prevent apoptosis. This inhibition of apoptosis elicited by IFN-α/β suggests the existence of a mechanism intended to improve macrophage viability in the course of certain viral infections.

INTRODUCTION

INTERFERONS (IFNS) ARE A FAMILY of species-specific polypeptides that mediate several biologic responses.[1] Two classes of IFNs have been described on the basis of receptor engagement, and they are referred to as type I (mainly IFN-α and IFN-β) and type II (IFN-γ).[1,2] These two types of IFN display distinct biologic functions, and they induce the expression of partially overlapping sets of genes,[2] and acting in concert, they can produce controversial effects.[3,4] For example, MHC class II expression is induced by IFN-γ, whereas IFN-α/β inhibited this response.[3] Also, induction of IFN-α/β via viral infection downregulates nitric oxide (NO) production, a process notably potentiated by IFN-γ.[4,5] This antagonism in the modulation of NO synthesis by IFNs might influence the onset of cellular events initiated on expression of the inducible form of NO synthase (iNOS), an activity that is controlled mainly at the transcription level.[6] Regulation of the high-output NO synthesis pathway is especially important for the macrophage, as NO is an effector molecule that participates in the mechanism of host defense operated by this cell.[6,7]

Apoptosis has been revealed as an important process in several physiopathologic conditions.[8] Lipopolysaccharide (LPS)-dependent activation of macrophages and exposure to pharmacologic NO donors are sufficient conditions to induce apoptosis in these cells.[9,10] In murine macrophages, the molecular mechanism controlling this process appears to involve an upregulation of p53,[11] whereas overexpression of Bcl-2 abrogates the NO-dependent apoptosis.[12] This ability of macrophages to suffer apoptosis in response to NO has been interpreted as a mechanism to hinder the development of parasitic strategies by endocyted cells. Moreover, in addition to NO, several concurrent mechanisms seem to favor the occurrence of apoptosis.[11–14] Using quantitative and qualitative methods to evaluate the extent of apoptosis, we show that macrophages activated with IFN-γ and a low dose of LPS display NO-dependent apoptosis and that this process is significantly attenuated on challenge with IFN-α/β. These results suggest that in certain circumstances impairment of macrophage cell death in the course of local viral infection might help to prolong the effectiveness of the macrophage to supply effector molecules intended to abort viral replication.

MATERIALS AND METHODS

Reagents (IFN-α/β and IFN-γ) were from Sigma (Saint Louis, MO), Boehringer (Mannheim, Germany).

Animals and obtaining peritoneal macrophages

Balb/c mice (10 weeks old) were maintained free of pathogens and 4 days before use were injected i.p. with 1 ml of thioglycollate broth. Peritoneal macrophages were prepared as previously described,[15] and cells were seeded at 1 × 10^6/cm^2 in phenol-red-free RPMI 1640 medium containing 10% fetal bovine serum (FBS), and nonadherent cells were removed by extensive washing with medium.

Flow cytometric analysis

In vivo propidium iodide staining was performed after incubation of the cells with the appropriate ligands in the presence of 0.005% propidium iodide.[16,17] Cells were carefully resuspended and analyzed in a flow cytometer (Becton Dickinson Franklin Lakes, NJ) equipped with a 5-W argon laser, and the forward scatter was plotted against the propidium iodide fluorescence.[16] Cell staining with annexin V was performed following the recommendations of the supplier (Boehringer Mannheim).

Analysis of DNA degradation

Internucleosomal DNA fragmentation was analyzed in agarose gels as follows. Cultured cells (10^6) were washed twice with ice-cold phosphate-buffered saline (PBS), and the plates were filled with 1 ml of 20 mM EDTA, 0.5% Triton X-100, 5 mM Tris-HCl, pH 8.0, and incubated for 15 min at 4°C. Nuclei were removed by centrifugation at 500g for 10 min, and the resulting supernatant was centrifuged at 30,000g for 15 min. The fragmented DNA present in the soluble fraction was precipitated with 70% ethanol plus 2 mM MgSO$_4$, and aliquots were treated for 1 h at 55°C with 0.3 mg/ml of proteinase K. After two extractions with phenol/chloroform, the DNA was resuspended and analyzed in a 2% agarose gel and stained with 0.5 μg/ml ethidium bromide.[10,16] Alternatively, apoptosis was quantified using a cell death ELISA kit based on the detection of mononucleosomes and oligonucleosomes in the cytosol,[10] following the instructions of the manufacturer (Boehringer). The enrichment factor was calculated as the ratio of the activity associated with the treated sample and with the control (untreated).

Measurement of lactate dehydrogenase (LDH) activity

To evaluate the degree of plasma membrane alteration, the release of LDH to the extracellular medium was measured. The medium was aspirated and centrifuged at 15,000g for 10 min, and the LDH activity in the supernatant was assayed in the presence of 0.5 mM pyruvate and 0.15 mM NADH.[18] The activity was expressed in milliunits per milligram of cellular protein after subtracting the contribution of the culture medium.

Nitrite determination

NO release was determined spectrophotometrically by the accumulation of nitrite as described.[15] Nitrite was determined with Griess reagent by adding sulfanilic acid and naphthylene-diamine (1 mM in the assay). The absorbance at 548 nm was compared with a standard of NaNO$_2$.[15]

RNA extraction analysis

Total RNA (2–4 × 10^6 cells) was extracted using the guanidinium thiocyanate method.[19] Equal amounts of RNA were denatured and size-separated by electrophoresis in a 0.9% agarose gel.[10,15] The RNA was transferred to Nytran membranes (NY 13-N, Schleicher & Schüell, Dassel, Germany) with 10 × SSC (SSC is 1.5 mM NaCl, 0.3 mM sodium citrate, pH 7.4) under low vacuum conditions, and the membranes were hybridized with an 817-bp fragment (nucleotides 1–817) from the cDNA of macrophage iNOS labeled with the Rediprime kit (Amersham, Arlington Heights, IL). An 18S ribosomal probe was used to normalize the lane charge.[10,16]

Western blot analysis of iNOS

Cell extracts were prepared after homogenization with 1 mM EDTA, 0.5 mM PMSF, 10 μg/ml leupeptin, and 20 mM Tris-HCl, pH 8.0. Aliquots of 10 μg of protein were submitted to SDS-PAGE (10% gel) and transferred to a PVDF membrane (Amersham). iNOS protein was revealed using a specific antibody (Transduction Laboratories, Exeter, UK) that recognized a protein of 130 kDa.

Statistical analysis

The data shown are the means ± SEM of three or four experiments. Statistical significance was estimated with Student's t-test for unpaired observations. A $p < 0.05$ was considered significant. In studies of Northern and Western blot analysis, linear correlations between increasing amounts of input RNA or protein and signal intensity were observed (correlation coefficients higher than 0.9).

RESULTS

IFN-α/β inhibited apoptosis induced by IFN-γ and LPS

Incubation for 20 h in the presence of propidium iodide of primary cultures of mice peritoneal macrophages with a low dose of LPS and IFN-γ produced an important accumulation of stained cells in the R2 + R3 quadrants of the flow cytometric spectra (Fig. 1A). Analysis in agarose gels of the DNA from sorted cells corresponding to the R1 and R2 + R3 quadrants confirmed the absence and presence of oligonucleosomes in these selected populations from IFN-γ/LPS-stimulated cells (Fig. 2A).[16] However, challenge of cells with IFN-α/β significantly antagonized the apoptosis induced by IFN-γ/LPS (Fig. 1A). A quantitative analysis of the distribution of cells in the R1 and R2 + R3 panels is shown in Figure 1B. Low doses of LPS induced only minimal apoptosis (20.5% of cells in R2 + R3). However, acting synergistically with IFN-γ, they potentiated the apoptotic death (60% of the cell population), and this process was antagonized by IFN-α/β (41% decrease of cells in R2 + R3). Moreover, the rescue from apoptosis elicited by IFN-α/β in IFN-γ/LPS-activated cells was dependent on the dose of

A
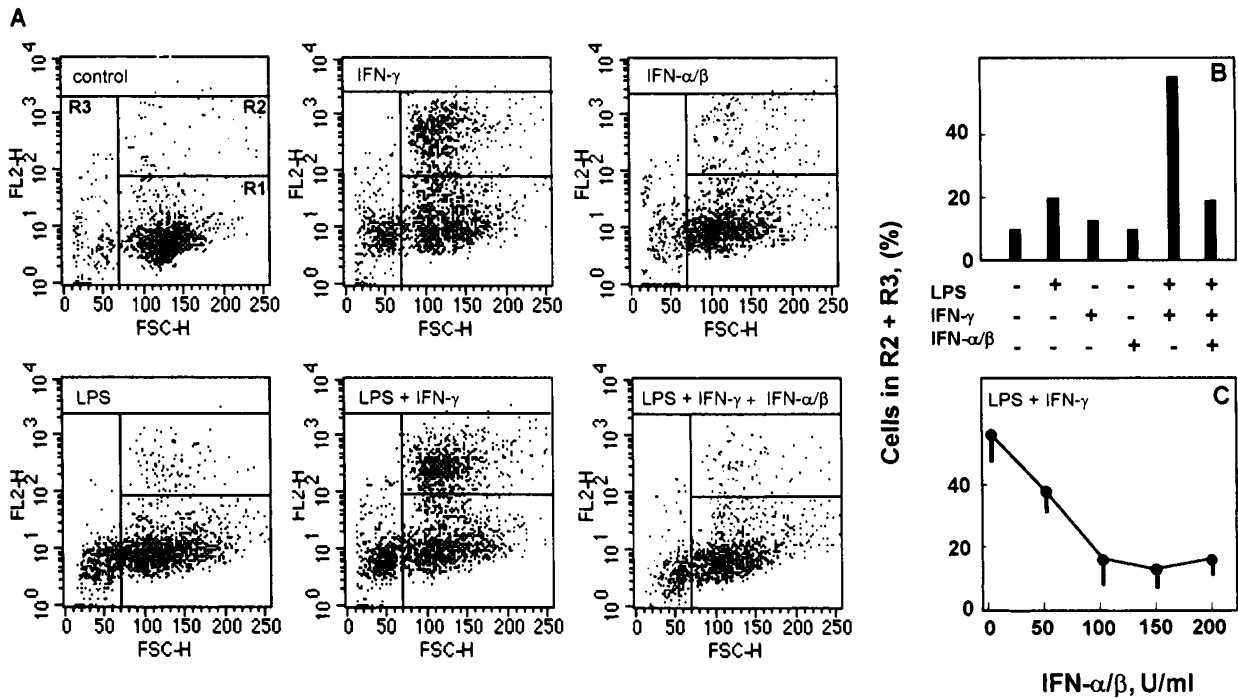
FIG. 1. IFN-α/β protects from IFN-γ/LPS-induced apoptotic death in peritoneal macrophages. Cultures of peritoneal macrophages (1 × 10⁶ cells) were stimulated for 20 h with 100 ng/ml of LPS, 50 U/ml of IFN-γ, 100 U/ml of IFN-α/β, or combinations of these. Apoptosis was followed by flow cytometry after *in vivo* staining with propidium iodide. (A) Apoptotic cells were detected in the R2 + R3 quadrants, whereas viable cells were observed in the R1 quadrant. (B) The percentage of cells in the R2 + R3 quadrants after stimulation with combinations of LPS and IFNs is shown. (C) The dose-dependent protection of IFN-α/β over the IFN-γ/LPS-induced apoptosis is shown. Results show the mean ± SEM of three experiments.
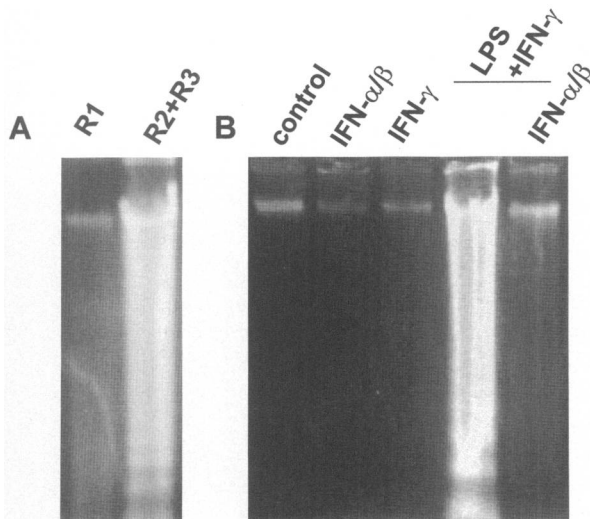
FIG. 2. Analysis on agarose gel electrophoresis of oligonucleosomal DNA. Macrophages were stimulated with IFN-γ/LPS in the presence of propidium iodide as described in Figure 1. After 20 h in culture, 1.5–2 × 10⁶ cells of the respective R1 and R2 + R3 quadrants were sorted, and the fragmented extranuclear DNA was analyzed by electrophoresis in a 1.8% agarose gel (A). Alternatively, cells were incubated for 20 h with the indicated stimuli, and the DNA was analyzed in agarose gels (B). Results show one representative experiment of three.

cytokine used (Fig. 1C). The protection exerted by IFN-α/β of the apoptosis induced by IFN-γ/LPS was confirmed also by the reduction in the characteristic DNA laddering observed in agarose gels (Fig. 2B). The absence of release of LDH into the culture medium in IFN-γ/LPS-activated cells stimulated with or without IFN-α/β was considered an indication of a negligible necrotic death under these conditions (Table 1).

In addition to evaluation of apoptosis by cell flow cytometry and oligonucleosomal DNA fragmentation, an alternative method to quantify apoptosis was used following exposure of phosphatidylserine residues of the plasma membrane to the ex-

TABLE 1. LACTATE DEHYDROGENASE (LDH) ACTIVITY
IN CULTURE MEDIUM OF ACTIVATED MACROPHAGES[a]

Addition	LDH activity (mU/mg of protein)
None	2.2 ± 0.1[b]
IFN-α/β (100 U/ml)	1.4 ± 0.2
IFN-γ (50 U/ml) + LPS (100 ng/ml)	2.8 ± 0.2
IFN-γ + LPS + IFN-α/β	1.5 ± 0.1

[a]Cultured cells (1 × 10⁶) were stimulated for 20 h with the indicated stimuli, and the LDH activity present in the incubation medium was measured. The total cellular activity was 2.1 U/mg of protein.
[b]Mean ± SEM of three experiments.

TABLE 2. ANNEXIN V BINDING DECREASES IN IFN-γ/LPS-ACTIVATED CELLS TREATED WITH IFN-α/β[a]

Addition	Time 6 h	Time 20 h
None	9 ± 1[b]	10 ± 1
IFN-α/β (100 U/ml)	10 ± 1	9 ± 1
IFN-γ (50 U/ml) + LPS (100 ng/ml)	32 ± 2*	40 ± 3*
IFN-γ + LPS + IFN-α/β	19 ± 2*,**	25 ± 3*,**

[a]Macrophages were incubated with the indicated stimuli for 6 or 20 h. The amount of phosphatidylserine exposed to the extracellular medium was evaluated cytofluorometrically by the shift of fluorescence.
[b]Accumulation of annexin V-positive cells (mean ± SEM of three experiments).
*$p < 0.01$ versus control conditions.
**$p < 0.01$ versus IFN-γ/LPS-treated cells.

tracellular medium. Quantitative determination of annexin V binding to membrane phospholipids confirmed that macrophages were effectively protected by IFN-α/β in the course of the apoptosis induced by treatment with IFN-γ/LPS. This reduction in the exposure of phosphatidylserine residues was observed both at 6 h and 20 h on challenge with LPS and IFNs (Table 2).

Synthesis of NO by macrophages treated with IFN-γ/LPS correlated with induction of apoptosis and was antagonized by IFN-α/β

The apoptosis induced by IFN-γ/LPS correlated with an increase of NO synthesis, which was attenuated when IFN-α/β was included as stimulus (Table 3). To address the relevance

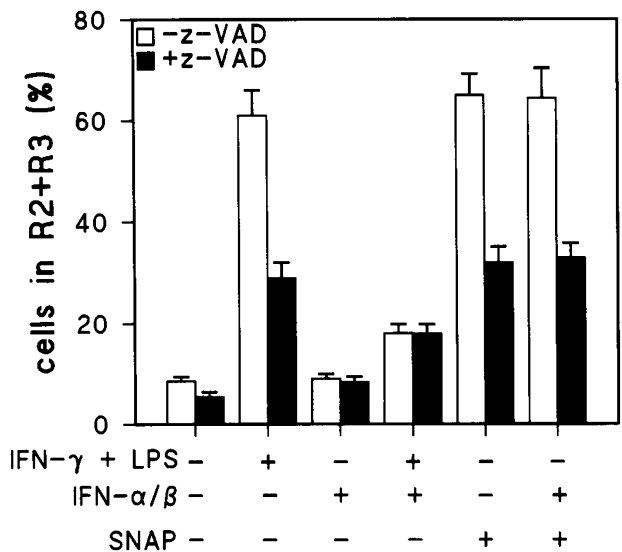FIG. 3. IFN-α/β failed to decrease the NO-donor apoptosis in macrophages. Cultured cells were treated with 50 μM of z-VAD for 30 min before stimulation with 100 ng/ml of LPS, 50 U/ml of IFN-γ, 100 U/ml of IFN-α/β, or 100 μM SNAP. After 20 h in culture, the number of cells present in the R2 + R3 panels was determined by flow cytometry. Results show the mean ± SEM of three experiments.

of NO in the apoptosis observed in this experimental model, NO synthesis was decreased by using an iNOS inhibitor, such as L-NMA, or by using a culture medium deficient in arginine. As Table 3 shows, in the presence of L-NMA(N-Methyl-L-Arginine), apoptosis was significantly impaired (70% and 27% decrease in IFN-γ/LPS and IFN-γ/LPS plus IFN-α/β-stimulated cells, respectively). When the stimulation was performed using arginine-free RPMI-1640 medium, similar results were obtained, although the extent of protection was lower than with

TABLE 3. EFFECT OF IFN-α/β ON NO SYNTHESIS AND APOPTOSIS IN ACTIVATED MACROPHAGES[a]

Condition	L-NMA	Arginine-free medium	SNAP (S-Nitroso-N-Acetyl Penicillamine)	NO release (μM/well)	Apoptosis (% R2 + R3)
None	−	−	−	4.6 ± 0.3[b]	10.2 ± 0.4
IFN-γ + LPS	−	−	−	47.2 ± 5.1	61.4 ± 4.8
IFN-γ + LPS + IFN-α/β	−	−	−	18.5 ± 1.2	19.5 ± 1.7
None	+	−	−	3.9 ± 0.2	12.4 ± 1.1
IFN-γ + LPS	+	−	−	8.5 ± 1.3	18.7 ± 2.0
IFN-γ + LPS + IFN-α/β	+	−	−	5.3 ± 0.9	14.2 ± 1.6
None	−	−	+	0.9 ± 0.1	9.1 ± 0.7
IFN-γ + LPS	−	+	−	2.4 ± 0.5	18.1 ± 1.1
IFN-γ + LPS + IFN-α/β	−	+	−	2.0 ± 0.3	17.5 ± 1.5
None	+	−	+	n.d.[c]	59.5 ± 4.8
IFN-γ + LPS	+	−	+	n.d.	63.7 ± 5.7
IFN-γ + LPS + IFN-α/β	+	−	+	n.d.	60.5 ± 5.1

[a]Macrophages (1 × 10^{6}) were cultured in the presence of propidium iodide and with 100 ng/ml of LPS, 50 U/ml of IFN-γ, 100 U/ml of IFN-α/β, 0.2 mM L-NMA, or 50 μM of SNAP for 20 h, and the amount of nitrite released was measured. After analysis by cytofluorometry, the distribution of cells present in the R2 1 R3 quadrants was considered as indicative of apoptotic cells.
[b]Mean ± SEM of three experiments.
[c]n.d., not determined.
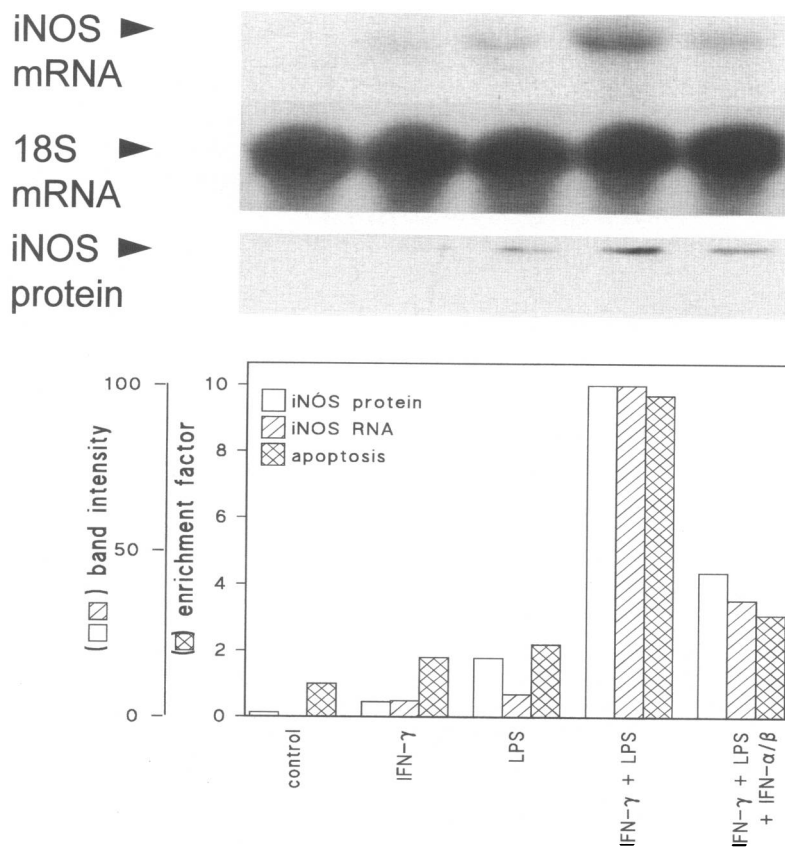
FIG. 4. IFN-α/β-attenuated iNOS expression and oligonucleosome release in response to IFN-γ/LPS. Macrophages (1 × 10⁶) were stimulated with 100 ng/ml of LPS, 50 U/ml of IFN-γ, and 100 U/ml of IFN-α/β, and samples were collected for analysis of iNOS mRNA (samples at 6 h) or protein levels and oligonucleosomes in the cytosol (samples at 20 h). The amount of oligonucleosomes was quantified using an antihistone, anti-DNA sandwich ELISA. The amount of iNOS mRNA was determined after normalization for the ribosomal 18S content. iNOS protein was determined in total cell extracts, loading equal amount of protein per lane. Results show one representative experiment of three.
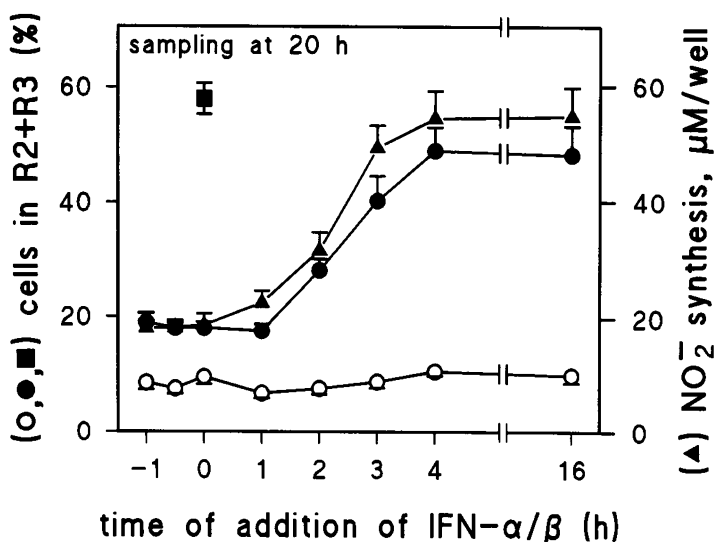
FIG. 5. Time-dependent protection from apoptosis by IFN-α/β in IFN-γ/LPS-activated cells. Macrophages (1 × 10⁶) were activated with (solid symbols) or without (open symbols) 100 ng/ml of LPS and 50 mU/ml of IFN-γ at zero time. IFN-α/β (100 U/ml; ○, ●, ▲) was added at the indicated times. The distribution of cells in the R2 + R3 quadrants in the flow cytometry spectra (○, ●, ■) and the amount of nitrite (▲) present in the culture medium were determined at 20 h with respect to zero time. Results show the means ± SEM of three experiments.

the iNOS inhibitor (Table 3). These results confirm the important contribution of NO to apoptosis in this system and suggest that the protection exerted by type I IFNs is mediated mainly through the inhibition of NO synthesis. To determine if this is indeed the case, macrophages were treated with an NO donor in the presence of combinations of different IFNs, LPS, and L-NMA. Under these conditions, the same extent of apoptosis was observed regardless of the treatment of the cells (Table 3).

One of the mechanisms by which NO induces apoptosis in peritoneal macrophages involves the activation of caspases.[12,20] When cells incubated with the caspase-1 inhibitor z-VAD were treated with IFN-γ/LPS or the NO-donor SNAP, an important decrease in apoptosis was observed. However, IFN-α/β was more efficient than z-VAD in preventing apoptosis in IFN-γ/LPS-stimulated cells. Moreover, the presence of IFN-α/β did not affect the protection by z-VAD in SNAP-treated cells (Fig. 3). These results support the conclusion that IFN-α/β does not alter the apoptotic pathway triggered by NO in macrophages.

The relevance of NO synthesis in the induction of apoptosis in cultured peritoneal macrophages and the protection exerted by IFN-α/β through a decrease in NO synthesis was further reinforced by the observation of an important parallelism between the iNOS mRNA and protein levels and the accumulation of oligonucleosomal DNA moieties in the cytosol of these cells. As Figure 4 shows, the synergistic effect of IFN-γ and LPS on iNOS expression was significantly antagonized by IFN-α/β. At the protein level, the relative amount of iNOS in cells treated with IFN-γ/LPS in the absence or presence of IFN-α/β correlated with the NO synthesis measured in Table 3. Moreover, when apoptosis was quantified in samples of these cells using an ELISA that determines the amount of mononucleosomal and oligonucleosomal DNA moieties in the cytosol, a good correlation between the levels of iNOS and nucleosomal enrichment was observed (Fig. 4). Furthermore, the antiapoptotic effect of IFN-α/β exhibited a very precise temporal pattern, as maximal protection was observed when type I IFNs were simultaneously added with IFN-γ/LPS (Fig. 5). Lower protection was observed when IFN-α/β was added 1 h after macrophage activation and was completely inefficient with IFN-α/β 4 h after activation (Fig. 5). This temporal dependence of the protection by IFN-α/β paralleled the synthesis of NO in these cells and suggests a close correlation between inhibition of both NOS expression and apoptosis.

DISCUSSION

Our data demonstrate that stimulation of macrophages with IFN-γ and a suboptimal dose of LPS elicited an apoptotic response that was mostly dependent on the synthesis of NO. In this experimental model, simultaneous stimulation in the presence of IFN-α/β protected efficiently against apoptosis. However, IFN-α/β was unable to inhibit the apoptosis elicited by an NO donor, suggesting that this cytokine specifically acts to interfere the IFN-γ/LPS signaling pathway leading to iNOS expression.[4–6] IFN-α/β was unable to potentiate the protection depending on caspase inhibitors when apoptosis was induced with pharmacologic NO donors.

The protective effect of IFN-α/β over the apoptosis induced

by IFN-γ/LPS has been confirmed based on various independent criteria: a shift in the content of propidium iodide-positive cells on in vivo labeling, a decrease in the exposure of phosphatidylserine residues in the plasma membrane, and a reduced release of oligonucleosomes to the cytosol determined both by agarose gel electrophoresis and by detection with a specific anti-DNA and antihistone sandwich immunoassay. The antiapoptotic effect of IFN-α/β can be explained on the basis of a reduced NO synthesis by IFN-γ/LPS-activated cells. Moreover, this capacity of IFN-α/β to inhibit apoptosis is due to the expression of iNOS through a synergism between LPS and IFN-γ,[6–9] a situation distinct from that obtained after stimulation with high doses of LPS, probably involving the engagement of additional signaling pathways sufficient to promote apoptotic death, regardless of NO synthesis.[9,10,21] Indeed, when macrophages were stimulated with a high dose of LPS, concentrations of IFN-α in the range of those used in this work enhanced the apoptotic response.[22]

IFN-α/β induces a potent antiviral activity in addition to other immunomodulatory effects.[1,23] In fact, viral infection may alter the regulation of certain macrophage functions, and an IFN-α/β-dependent downregulation of NO synthesis has been described.[4,5,24] However, the antiviral activity of NO seems to be specific for certain viruses.[24] Moreover, the occurrence of high NO production appears to contribute to the pathogensis in vivo after viral infection.[5,10,25] Interestingly, a reduction in NO synthesis in mice infected with type I herpes simplex virus ameliorated markedly the pneumonia characteristic of this infection.[26] These results stress the beneficial effects of a negative regulation of the NO synthesis pathway, such as occurs during certain viral infections via type I IFNs.[5,26]

The mechanism by which IFN-α/β protects from apoptosis might include other pathways in addition to inhibition of NO synthesis, as transforming growth factor (TGF)-β expression by activated murine macrophages (a potent inducer of apoptosis in these cells) is specifically impaired by IFN-α.[27]

Because IFNs play a central role in the activation of macrophages by inducing the expression of several proteins that in general improve the functional capabilities of macrophages, the antiapoptotic effects of IFN-α/β may contribute to the maintenance of functional activated cells in the course of viral infections.

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