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Inflammatory cytokine production in interferon-g-primed mice, challenged with lipopolysaccharide. Inhibition by SK&F 86002 and interleukin-1b-converting enzyme inhibitor

Abstract

Mice challenged with lipopolysaccharide (LPS) produce variable serum levels of pro-inflammatory cytokines, and particularly low levels of interleukin-1b (IL-1b). Interferon-g (IFN-g) has been shown to be an important mediator of bacteria-induced hypersensitivity to LPS in mice. In the present study, we show that mice

pretreated with IFN-g exhibit an enhanced capacity to produce serum IL-1b, IL-1a, tumour necrosis factor (TNF-a) as well as IL-6 in response to LPS. Priming with intraperitoneal (i.p.) injection of 15 mg rat recombinant IFN-g, 18 hours prior to the i.p. LPS (300 mg) challenge resulted in a 4-fold increase in the LPS-stimulated release of IL-1b and a 2- to 7-fold increase in the release of IL-1a, TNF-a, as well as IL-6 into the serum. LPS induced a concentration-dependent increase in the release of IL-1b in isolated peritoneal macrophages from IFN-g-primed mice whereas macrophages from unprimed mice released minute amounts of IL-1b. In addition, nigericin markedly enhanced the release of IL-1b in unprimed mice but not in macrophages from IFN-g primed mice. The cytokine synthesis inhibitor SK&F 86002, administered per os (100 mg/kg), 1 hour prior to LPS challenge, strongly inhibited the rise in serum levels of the four cytokines. Furthermore, treatment with the IL-1b converting enzyme (ICE) specific reversible inhibitor YVAD-CHO resulted in a sharp dose- and time-dependent inhibition of IL-1b secretion in the serum, whereas the other cytokines were not affected. In conclusion, IFN-g priming strongly potentiates the release of proinflammatory cytokines in the serum of mice as compared to LPS stimulation alone, and provides therefore a useful way to test the in vivo potency and selectivity of cytokine synthesis inhibitors.

Full text

INTRODUCTION

Cytokines evoke a complex spectrum of biological responses that are generally thought to regulate host defense. A subset of cytokines, including interleukin-1 (IL-1alpha and IL-1beta) and tumor necrosis factor (TNF-alpha), mediate biological actions consistent with a role as inflammatory mediators [1-3]. The temporary or sustained release of these proinflammatory cytokines is considered to play a critical role in the pathogenesis of acute and chronic inflammation in several diseases such as sepsis, rheumatoid arthritis or inflammatory bowel diseases.

In addition to the critical role of TNF-alpha in endotoxic shock in mice, the determinant role of IL-1 (IL-1alpha and IL-1beta) has been shown by the improved survival in mice injected with lipopolysaccharide (LPS) after pretreatment with the IL-1 receptor antagonist [4] and by the observation that mice deficient in the IL-1beta converting enzyme (ICE, caspase 1) are resistant to high doses of LPS

[5]. In contrast to the high serum levels of TNF-alpha induced by LPS injection in mice, variable and low serum levels of IL-1beta and IL-1alpha are usually observed in response to LPS [6-8]. Higher and more reproducible plasma or serum levels of these cytokines have been obtained in mice sensitised to the action of lipopolysaccharide (LPS) by intraperitoneal injections of killed *Propionibacterium acnes (P. acnes)* [8-11]. It has been suggested that this bacterial sensitisation to LPS is mediated *via* production of IFN-gamma as it does not occur in mice with an impaired capacity to produce IFN-gamma [10], and as overproduction of TNF-alpha and IL-1alpha can be inhibited by IFN-gamma-neutralising antibodies [8, 10]. In addition, mortality and TNF-alpha serum levels in mice challenged with LPS are increased after intraperitoneal or intraveinous injections of murine recombinant IFN-gamma [9, 10]. Accordingly, mice deficient in IFN-gamma receptor show reduced TNF-alpha serum levels upon stimulation with LPS, and are resistant to endotoxic shock [12]. However, normal levels of IL-1alpha and IL-6 are observed in IFN-gamma receptor-deficient mice injected with LPS, suggesting that IFN-gamma may not regulate IL-1alpha and IL-6 production in response to LPS. The first aim of this study was therefore to characterize further the enhancing effect of a pretreatment with IFN-gamma on the production of pro-inflammatory cytokines other than TNF-alpha, namely IL-1alpha, IL-1beta and IL-6 in response to LPS challenge in mice.

Since IL-1 and TNF-alpha are among the principal mediators of immune inflammatory diseases, active research has been carried out to develop agents that can modulate the activity of these proinflammatory cytokines through impaired binding to target cells or inhibition of synthesis. Cytokine blockers include soluble cytokine receptors, cytokine neutralising antibodies and recently developed low-molecular-weight synthetic antagonists. On the other hand, inhibitors of cytokine synthesis interact with the release or the processing of cytokines [13, 14]. Among them, SK&F86002 is the prototype of a novel class of cytokine biosynthesis inhibitors (bicyclic imidazoles) acting on the p38 MAP kinase and exerting broad inhibitory effects on proinflammatory cytokine production [15, 16]. In addition, specific inhibitors of cytokine processing have been described, such as inhibitors of ICE (caspase 1). The second aim of this study was to compare the potency and selectivity of SK&F 86002 and of the ICE inhibitor YVAD-CHO, as inhibitors of LPS-induced cytokine production in mice pretreated with IFN-gamma. The serum levels of IL-1beta, IL-1alpha, TNF-alpha, as well as IL-6 were studied, since the production of these cytokines is reciprocally regulated [1, 12, 17].

MATERIALS AND METHODS

Animals

Balb/c female mice (Iffa Credo, Domaine des Oncins, Lyon, France) weighing 18-22 g at the time of the experiments were used throughout.

*LPS-induced IL-1*alpha*, IL-1*beta*, TNF-*alpha *and IL-6* in vivo

Each batch of LPS (*E. coli* serotype 0111:B4, Sigma, L'Isle d'Abeau, France), was resuspended in pyrogen-free 0.9% saline to 1 mg/ml, aliquoted, stored at - 20° C and thawed immediately before intraperitoneal (i.p.) injection. Control mice were injected i.p. with the same volume of 0.9% saline. Rat recombinant IFN-gamma (RU 53561, 2 x 10^7 anti-viral U/mg, Roussel Uclaf, Romainville, France) was injected i.p. (15 mug) prior to LPS challenge. The cytokine synthesis inhibitors, SK&F 86002 and the tetrapeptide aldehyde Ac-Tyr-Val-Ala-Asp-CHO (YVAD-CHO), were synthesised at Hoechst Roussel (Swindon, U.K.) and administered *per os* and i.p., respectively.

Blood samples were taken by cardiac puncture in mice whose respiration had just stopped after breathing CO_2. With the exception of kinetic studies, mice were bled 1.5-2 hours and 4 hours after LPS injection. Blood samples were immediately placed at 4° C and kept overnight to allow clot formation and retraction. The serum was collected after centrifugation (5,000 g for 5 min) and stored at - 20° C until measurement of cytokine levels by specific enzyme-linked immunosorbent assays (ELISA).

Ex vivo *production of cytokine by LPS-stimulated peritoneal macrophages*

Peritoneal cells were obtained by peritoneal lavage with ice cold RPMI 1640 medium (Gibco Ltd, Paisley, UK) containing heparin (10 IU/ml). Peritoneal lavage was performed in control mice and in mice primed with IFN-gamma. After centrifugation at 2,000 g for 10 min, the cells were resuspended in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum (Biosys, Compiègne, France), 4 mM glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 10 mM HEPES (Sigma), 100 ng/ml penicillin (Gibco) and 100 IU/ml streptomycin (Gibco) and 50 mM mercapto-2-ethanol (Sigma). The cell suspension was adjusted to 1.25 x 10^6 cells/ml and aliquots (500 mul) of cell suspension were placed in each well of a

24-well culture plate (Corning). Cells were allowed to adhere for 2 hours, then non-adherent cells were removed from the wells by gentle rinsing. The remaining adherent cells (macrophages), cultured at 37° C in 5%CO_2/95%CO_2 air, were stimulated with 1 mug/ml LPS (*E. coli* LPS 0111:B4) for 24 hours since, in preliminary experiments, mouse peritoneal macrophages stimulated with LPS for 24 hours released more IL-1beta than after a 6 hours stimulation as previously shown in our laboratory for human monocytes [18]. In some experiments, cells were incubated for an additional 30 min with 10 muM nigericin (Sigma).
Thereafter, the supernatants were harvested, centrifuged at 2,000 g for 10 min and tested for cytokine levels by ELISA.

Specific enzyme-linked immunosorbent assays for cytokines

Specific ELISA were used to determine IL-1beta, IL-1alpha, TNF-alpha and IL-6 levels in mouse macrophage culture supernatant and serum. Flat-bottomed, polystyrene, microtiter plates (Maxisorp, NUNC, Polylabo, France) were coated overnight at 4° C with 50 mul of monoclonal antibodies from Genzyme Corporation (Cambridge, MA, USA): hamster anti-mouse IL-1beta (5 mug/ml), hamster anti-mouse IL-1alpha (0.5 mug/ml), hamster anti-mouse TNF-alpha (2 mug/ml) or rat anti-mouse IL-6 (1 mug/ml) diluted in 0.1 M $NaHCO_3$. The microtiter plates were then washed and blocked with 3% bovine serum albumin (Sigma) in phosphate-buffered saline (Sigma) for 1 hour. Aliquots (50 mul) of diluted samples were added and incubated for 2 hours at 37° C. The plates were then washed 4 times and incubated for 1 hour at 37° C with cytokine-specific rabbit antibodies: 5 mug/ml of rabbit IgG anti-mouse IL-1beta (Innogenetics), or 1/50,000 diluted rabbit IgG anti-mouse IL-1alpha (Genzyme), or 0.4 mug/ml of rabbit IgG anti-mouse TNF-alpha (Genzyme), or 0.5 mug/ml of rabbit IgG anti-mouse IL-6 (Genzyme). After extensive washing, the plates were incubated for 30 min at 37° C with a 1:1,000 dilution of peroxidase-conjugated goat $F(ab')_2$ anti-rabbit IgG (Sigma). After subsequent washings, the plates were incubated with substrate solution (Sigma FAST^{TM} OPD) for 10 min and the reaction was stopped by addition of 100 mul of 4N H_2SO_4 per well. The plates were read using a Dynatech MR5000 plate-reader (absorbance 492 nm) and Biolinx software. The concentrations of cytokines in the samples were calculated by comparison with a standard curve obtained with the respective mouse recombinant cytokines purchased from Genzyme. The limit of detection of the assays for IL-

1beta, IL-1alpha, TNF-alpha and IL-6 was 25 pg/ml. The ELISA for IL-1beta detected only mature IL-1beta [19].

Statistical analysis

Results are expressed as geometric means with 95% confidence intervals (CI). Samples which contained no detectable cytokine were assigned a value of 12.5 pg/ml (half the detection limit of the assays) in order to calculate the group geometric means. Comparison of the results obtained between control, IFN-gamma-treated and compound-treated groups of mice were carried out by analysis of variance excepted where otherwise stated, with P values less than 0.05 were considered significant.

RESULTS

*Potentiation by IFN-*gamma *of LPS-induced increase in cytokine serum levels*

Our preliminary studies on the time-course of the effects of LPS and on the dose-relationship between endotoxin and cytokine levels in control mice showed that low serum levels were observed for IL-1beta, which reached a plateau between 4 and 8 hours after LPS. IL-1beta was undetectable in the serum after i.p. administration of 10 or 30 mug LPS. Detectable increases in serum IL-1beta, just above the limits of detection (50-100 pg/ml), occurred after 50 mug LPS i.p. injection, and consistent levels of IL-1beta in the range of 150-200 pg/ml were observed 4 hours after 200 to 1,000 mug LPS per mouse (Figure 1). Pretreatment with i.p. IFN-gamma (15 mug, 3×10^5 anti-viral units), 18 hours prior to the LPS challenge, caused an increase in cytokine serum levels for the threshold dose of LPS (50 mug/mouse). The detected peak levels of IL-1beta (4 hours after LPS) were similar to those induced by the high doses of LPS in control mice. Higher cytokine levels were observed with shorter IFN-gamma pretreatment times (*i.e.* 8 or 12 hours), however, the mice were markedly hypothermic and in a state of collapse, hampering mice bleeding for serum cytokine level determination. Therefore, 18 hours pretreatment was studied with higher doses of LPS. In IFN-gamma-sensitised mice, injection of 300 mug of LPS per mouse markedly increased the serum levels of cytokines without inducing near lethal effects. On the other hand, pretreatment with IFN-gamma 42 and 18 hours prior to challenge with 300 mug LPS did not markedly enhance (about one-third increase) the IL-1beta serum levels, 4 hours after LPS, in comparison to

those obtained using a single dose of IFN-gamma 18 hours prior to LPS (data not shown).

The kinetics of the serum responses in control mice challenged with LPS, and in mice primed with IFN-gamma 18 hours prior to challenge with the same dose of LPS (300 mug), are shown in Figure 2. IFN-gamma priming resulted in a rapid increase in IL-1beta levels, starting 2 hours after LPS, which reached 4.7 ng/ml (CI: 4.1-5.3) 4 hours after LPS, corresponding to a 5.5-fold increase *versus* unprimed controls. The peak levels of IL-1beta were in the range of 8 ng/ml, 6 and 7 hours after LPS challenge, corresponding to a 4.4-fold increase *versus* controls. IFN-gamma pretreatment also resulted in an increase in the serum levels of the other cytokines measured. Peak levels of IL-1alpha (2.9 ng/ml, CI: 1.7-3.2) were observed 6 hours after LPS, corresponding to a 7.8-fold increase *versus* controls. Peak levels of TNF-alpha (46.2 ng/ml, CI: 23-91) occurred 2 hours after LPS administration, corresponding to a 2.2-fold increase *versus* controls. Finally, peak levels of IL-6 (536 ng/ml, CI 468-614) occurred 7 hours post-LPS, corresponding to a 4.2-fold increase versus controls. Additional experiments showed that peak levels of TNF-alpha were more readily obtained at 1.5 hours after LPS injection in IFN-gamma-pretreated mice (data not shown). Therefore, these experiments show that pretreatment of mice with a single i.p. injection of IFN-gamma, 18 hours prior to a LPS challenge, induced a sensitisation towards LPS resulting in marked increases of IL-1beta, IL-1alpha and IL-6, in addition to TNF-alpha, in the serum.

Ex vivo *cytokine production by LPS-stimulated peritoneal cells from IFN-*gamma-***sensitised mice***

We next studied the ability of peritoneal adherent cells (macrophages) from IFN-gamma-primed mice to produce cytokines when stimulated *in vitro* with LPS for 24 hours. *In vivo* priming with IFN-gamma did not induce IL-1beta, IL-1alpha or TNF-alpha production by macrophages in the absence of LPS as previously reported for IL-1 production after *in vitro* IFN-gamma-priming of monocytes and macrophages [20-22]. Unprimed peritoneal macrophages stimulated with LPS (1 ng/ml to 20 mug/ml), did not secrete IL-1beta into the culture medium except with 1 mug/ml of LPS, for which low IL-1beta levels were measured (28 pg/ml, CI: 21-37, n = 8). On IFN-gamma-primed macrophages, LPS induced a concentration-dependent release of IL-1beta (Figure 3), and the maximum release was obtained with 1 mug/ml LPS (566 pg/ml, CI: 435-737) corresponding

to a 20-fold increase *versus* controls. IFN-gamma-priming (10 to 15 experiments) also resulted in a 15-fold increase of IL-1alpha (controls: 35 pg/ml (CI: 24-51); primed: 521 pg/ml (CI: 459-590) and a 13-fold increase of TNF-alpha (controls: 716 pg/ml (CI: 483-1061); primed: 9,814 pg/ml (CI: 6419-15003).

The absence of detectable IL-1beta (less than 25 pg/ml), or the weak levels in the supernatants of cells from unprimed mice in response to LPS stimulation, are in agreement with previous observations [6, 19, 23]. Treatment of these LPS-stimulated unprimed macrophages with nigericin, a K^+/H^+ ionophore, resulted in the secretion of mature IL-1beta corresponding to an estimated 5-fold increase in the release of IL-1beta (Table 1). This ionophore has been reported to induce an increase in the activity of ICE [24-26]. In contrast with control mice, addition of nigericin caused no significant increase in IL-1beta in the supernatants of macrophages from IFN-gamma-primed mice (Table 1). Therefore, in the absence of nigericin, peritoneal macrophages from mice primed with IFN-gamma produced and secreted higher levels of IL-1beta in response to LPS than macrophages from control animals treated or not with nigericin.

*Effect of cytokine synthesis inhibitors on LPS-induced cytokine levels in IFN-*gamma-*sensitised mice*

Two types of inhibitors, one described as a broad-range inhibitor (SK&F 86002) and the other one as specific for IL-1beta (YVAD-CHO), have been tested in mice sensitised with IFN-gamma. According to the results of the cytokine kinetic experiments and for convenience, samples to study drug effects on cytokine serum levels were taken at 1.5 or 2 hours post-LPS and at 4 hours post-LPS. SK&F 86002 was administered *per os* at the dose of 100 mg/kg, 1 hour prior to LPS challenge, according to the results previously reported in the literature for inhibition of TNF-alpha production in a murine model of endotoxin shock [15]. As shown in Table 2, this compound significantly inhibited IL-1beta serum content measured 4 hours after LPS challenge. Some inhibition of IL-1beta (34%) was already observed in serum collected 1.5 hours after LPS challenge (Table 2). Significant inhibition of both IL-1alpha and IL-6 levels was observed both at 1.5 and 4 hours after LPS challenge. SK&F 86002 also exerted a major inhibitory effect on the increase in TNF-alpha at 1.5 hours after the LPS challenge, whereas a non-significant effect (50% inhibition, $p = 0.13$) was observed at 4 hours. Therefore, in our model of IFN-gamma-primed mice, the compound SK&F 86002 significantly inhibited the release of all four proinflammatory cytokine studied.

The specific reversible ICE inhibitor, YVAD-CHO [27, 28], was administered i.p. at 50 mg/kg at different time points. As shown in Table 3, YVAD-CHO significantly reduced IL1-beta levels in serum samples taken 4 hours after LPS challenge, when administered together with LPS, as well as 1 to 3 hours after LPS challenge. The inhibitory effect was more pronounced when the compound was administered 2 hours after LPS. Different doses of YVAD-CHO (12.5 to 300 mg/kg) were therefore tested by i.p. injection, either simultaneously or 2 hours after the LPS challenge. As shown in Table 4, YVAD-CHO at 12.5 and 25 mg/kg significantly inhibited the rise in IL-1beta production when injected 2 hours after the LPS challenge, whereas a dose of 100 mg/kg injected simultaneously with LPS is required to obtain the same level of inhibition (50%), the 12,5 and 25 mg doses injected simultaneously with LPS were not effective. For the highest dose (300 mg/kg), a similar reduction in IL-1beta serum levels was observed when YVAD-CHO was administered either simultaneously or 2 hours after LPS.

Administration of YVAD-CHO at 50 mg/kg, 1 or 2 hours after LPS challenge, did not alter IL-1alpha and IL-6 production as measured 4 hours post-LPS whereas, in the same samples, the rise in IL-1beta production was inhibited by 70% (Table 5). In addition, the ICE inhibitor administered at the same time as LPS or 1 hour after LPS did not change the TNF-alpha levels when measured 2 hours after the challenge (Table 5).

DISCUSSION

The role of IFN-gamma as a mediator of the sensitisation towards LPS challenge has been previously illustrated in mice pretreated with killed *P. acnes* [8, 10], which exhibit an enhanced capacity to produce TNF-alpha [8, 10], IL-1alpha [8] and IL-6 [8] in the serum, in response to LPS. The sensitisation to LPS induced by bacteria was markedly reduced after treatment with anti-IFN-gamma monoclonal antibody [8, 10] or in mice with impaired capacity to produce IFN-gamma [10]. In addition, the similar sensitisation to LPS after infection with *Bacillus Calmette-Guerin* was also reduced in mice, with a targeted disruption of the IFN-gamma receptor gene [29]. Further evidence of the role of IFN-gamma has been forwarded in two studies where i.p. administration of recombinant IFN-gamma at similar doses as in the present study (5-15 mug corresponding to 2.5 x 10^4 U - 2 x 10^5 U), increased the production of serum TNF-alpha and the lethality after i.v. or i.p. LPS challenge [9, 10]. In addition, mice deficient in IFN-gamma receptor are resistant to endotoxic shock and show reduced TNF-alpha serum

levels in response to LPS. However, in these mice that lack the IFN-gamma receptor, serum levels of IL-1alpha and IL-6 upon stimulation with LPS, were not decreased with respect to control mice [12]. IFN-gamma production may therefore not regulate IL-1alpha and IL-6 production in the course of a response to LPS. In the present study, we clearly show that pretreatment with i.p. IFN-gamma enhanced the *in vivo* LPS-induced production of serum IL-1beta, IL-1alpha and IL-6 (4 to 7 hours post-LPS), in addition to the production of serum TNF-alpha (1 to 2 hours post-LPS). Therefore, administration of exogenous IFN-gamma (present study) or induction of endogenous IFN-gamma production with injection of bacteria before LPS challenge, potentiate the production of these proinflammatory cytokines even if endogenous IFN-gamma does not appear to regulate IL-1alpha and IL-6 production in the course of an LPS challenge. In addition, this study defines the conditions for a reliable and convenient sensitisation procedure to LPS with IFN-gamma. Indeed, a single i.p. administration of rat recombinant IFN-gamma (15 mug), followed by an LPS challenge 18 hours later induces reproducible increases in serum levels of the four proinflammatory cytokines. The priming effect of IFN-gamma is particularly noticeable for IL-1beta production since very low serum levels of this cytokine are usually obtained after *in vivo* LPS challenge in mice. In contrast, it must be noted that the serum levels of IL-10, an anti-inflammatory cytokine was not altered by IFN-gamma pretreatment (data not shown). This latter result does not support, in our *in vivo* model, the hypothesis that IFN-gamma enhances TNF-alpha production by inhibition of IL-10 synthesis as previously shown in mouse bone marrow-derived macrophages [30].

In vitro priming of monocyte/macrophage with IFN-gamma potentiates their capacity to produce cytokines (IL-1beta, TNF-alpha) after exposure to LPS [20, 21, 31, 32]. IFN-gamma markedly increased the LPS-induced mRNA levels for TNF-alpha [32] and stimulation with LPS and IFN-gamma have been also shown to result in increased transcription initiation of TNF-alpha gene and processivity of the transcripts [33]. These results explain the enhancing effect of IFN-gamma on TNF-alpha production induced by LPS. IL-1beta is synthesised as an inactive cytoplasmic 31-kDa precursor (pro-IL-1beta), and extracellular release of its mature form of 17-kDa requires a proteolytic cleavage performed by ICE [27, 28]. Studies with mice deficient in ICE provided evidence that ICE activity is the rate-limiting step in regulating the mature IL-1beta release from LPS-stimulated mouse macrophages [5, 35]. LPS and IFN-gamma induced a moderate upregulation of the expression of ICE mRNA in mouse macrophages but the

possibility that this increase in ICE mRNA leads to an increase in ICE activity was not, however, studied [36]. LPS is a very weak inducer of mature IL-1beta-release from unprimed mouse macrophages [6, 19, 34, present study], suggesting that LPS weakly increased ICE activity in these cells. In contrast, LPS induced a potent release of IL-1beta from mouse macrophages primed *in vivo* with IFN-gamma (present study) or with *P. acnes* [28]. Since the *in vivo*, enhanced capacity to produce cytokines in mice treated with *P. acnes* has been shown to be mediated by IFN-gamma [8, 10], these results suggest therefore that IFN-gamma may play a critical role in ICE activity in mouse macrophages as recently shown in human erythroid progenitor cells [37].

The processing of ICE precursor (termed p45) is not yet fully understood. Several agents promote *in vitro*, the post-translational maturation and release of IL-1beta, including potassium ionophores such as nigericin and ATP [23]. The activity of all of these agents appears to be linked to their common ability to activate potassium efflux from the IL-1beta-producing cells [23-25]. It has been recently demonstrated that nigericin-induced release of IL-1beta is dependent upon activation of p45 ICE processing [26]. The mechanism by which reduced intracellular potassium ion concentration triggers maturation of IL-1beta has been recently shown to involve activation of the calcium-independent phospholipase A_2 ($iPLA_2$) in human monocytes. In this cell type, $iPLA_2$ plays a pivotal role in regulating the cleavage of pro-IL-1beta by ICE, whereas the two calcium-dependent PLA_2, the secretory PLA_2 and the cytosolic PLA_2 did not support IL-1beta processing [38]. In line with these results, we found that nigericin induced a 5-fold increase in the release of IL-1beta from unprimed LPS-stimulated macrophages. In sharp contrast, nigericin did not significantly increase the release of IL-1beta from IFN-gamma-primed macrophages, suggesting that IFN-gamma can mimick the effect of nigericin. Since IFN-gamma induces the expression of potassium currents, particularly a strong appearance of outward potassium currents, in isolated microglial cells [39, 40], and since IFN-gamma has not been shown to activate $iPLA_2$ (although it can activate the calcium-dependent PLA_2 [41]), the processing and export of IL-1beta in IFN-gamma-primed macrophages may be therefore tentatively explained, at least in part, by the ability of IFN-gamma to activate potassium efflux and in turn, ICE processing. Such an effect of IFN-gamma on potassium channels remains however, to be demonstrated in isolated macrophages.

SK&F 86002 is a bicyclic imidazole which belongs to the cytokine-suppressive anti-inflammatory class of drugs (CSAIDs). This compound and related compounds of the bicyclic imidazole class, bind with high affinity to p38 MAP kinase near the ATP binding site, thus rendering p38 kinase inactive [15, 16, 42]. The p38 MAP kinase is necessary for the production of endotoxin-induced cytokines and other mediators such as nitric oxide [43]. Stabilization of mRNAs contributes to the strong and rapid induction of genes in the inflammatory response. The p38 MAP kinase pathway is involved in stress/cytokine-induced gene expression by stabilizing mRNAs *via* MAP-kinase activated protein kinase 2 and an AU-rich region-targeted mechanism [44]. In addition, inhibition of the p38 MAP kinase significantly reduced the DNA binding of TATA-binding protein (TBP) to the TATA box, and may therefore regulate NF-kappaB-dependent gene transcription, whereas inhibition of p38 MAP kinase did not alter NF-kappaB activation [45]. Inhibition of p38 kinase activity by the bicyclic imidazole compounds correlates with cytokine inhibition [42]. With respect to the inhibition of IL-1beta production by LPS-activated human monocytes, SK&F 86002 has been shown to affect two post-translational mechanisms, the synthesis of pro-IL-1beta and the release of IL-1beta into the medium [46]. SK&F 86002 and related compounds also decreased *in vitro* synthesis of TNF-alpha and IL-6 as well as IL-1beta [15, 16, 42, 47]. In addition, these compounds have been shown to inhibit the increase in plasma levels of TNF-alpha induced by LPS challenge in mice [15, 47, 48], and the increase in TNF-alpha and IL-1beta tissue levels in the murine air pouch granuloma model [47]. Oral administration of SK&F 86002, 30 or 60 min prior to the injection of LPS in Balb/c mice, resulted in a dose-dependent suppression of serum TNF-alpha levels, the ED_{50} for this compound being 10 mg/kg [15]. In the present study, it is shown that 100 mg SK&F 86002 administered *per os,* 1 hour prior to LPS challenge inhibited peak TNF-alpha levels by 91%. In addition, the increase in serum levels of IL-1beta, IL-1alpha and IL-6 was also inhibited. Thus, we provide the first *in vivo* demonstration of the inhibitory effect of SK&F 86002 on the production of these four inflammatory cytokines including TNF-alpha. The inhibitory effect of SK&F 86002 on cytokine production in response to LPS, cannot be attributed to the inhibition of eicosanoid metabolism by this compound since the production of cytokine in models of sepsis is not altered by specific inhibition of cyclo-oxygenases or 5-lipoxygenase [49, 50], and since SK&F 86002 is, *in vitro*, about 10- to 200-fold more potent in inhibiting the production of cytokines than the production of prostanoids or leukotrienes [15].

Our results show that treatment with YVAD-CHO resulted in an inhibition of the increase in IL-1beta serum levels without significant effects on the levels of IL-1alpha, IL-6 and TNF-alpha. Pharmacokinetic studies in mouse indicated that YVAD-CHO is rapidly cleared from the blood after i.v. or i.p. administrations, since its half-life has been respectively estimated to be 6 and 16 min (data not shown). These observations prescribed the timing of the administration of YVAD-CHO in the *in vivo* experiments designed to assess the inhibitory effect of this ICE inhibitor on IL-1beta production. Production of IL-1beta in the IFN-gamma-sensitised mice occurred mainly during the second and the third hour following LPS injection. Therefore, by administering YVAD-CHO to mice 1 to 3 hours after LPS injection, the inhibitor was present during the window of time when the majority of IL-1beta was produced, resulting in the greatest reductions in the level of IL-1beta measured 4 hours after LPS. A time-related inhibition has been also observed after i.p. administration of this ICE inhibitor in *P. acnes*-sensitised mice challenged with LPS [11]. However, in this latter model, the optimal time of administration of YVAD-CHO was 1 hour after injection of LPS, and the inhibitory effect on plasma IL-1beta levels measured 5 hours after LPS was 62% for a dose of 50 mg/kg. This result could be explained by the earlier increase in serum levels of IL-1beta by about 1 hour in *P. acnes*-sensitised mice with respect to IFN-gamma-sensitised mice. The selective inhibition of IL-1beta production in our model of endotoxic shock is in full agreement with the decrease of IL-1beta levels but not of IL-1alpha, IL-6 and TNF-alpha in tissue chamber fluids, in a subcutaneous murine model of inflammation induced by zymosan, after administration of a different ICE inhibitor (WIN 67694) [19]. In contrast with these data, in ICE-deficient mice, the major defect in IL-1beta release after *in vitro* stimulation with LPS of spleen monocytes [35] or of peritoneal macrophages [5], was associated with a reduction in the release of IL-1alpha [5, 35] whereas only slight reductions were observed for IL-6 and TNF-alpha [35]. In addition, ICE-deficient mice produced no detectable IL-1beta or IL-1alpha *in vivo* after i.p. injection of high doses of LPS [5]. It is interesting to note that ICE may be involved in the processing or release of IL-1alpha [5] and also cleaves the precursor of IFN-gamma-inducing factor (proIGIF) at the authentic processing site, thereby activating IGIF [51]. IGIF (IL-18) stimulates production of IFN-gamma, and ICE-deficient mice sensitised with *P. acnes* and challenged with LPS show a major reduction in serum levels of IGIF and IFN-gamma [51]. In addition, IGIF possesses other proinflammatory properties [52], including stimulation of synthesis of TNF-alpha and IL-1beta [53]. Therefore, the involvement of ICE in the production of IL-1beta, IL-1alpha, IGIF and IFN-gamma may account for the reduction in the production of cytokines other than IL-1beta

(IL-1alpha, TNF-alpha, IL-6), in response to LPS in ICE-deficient mice. In wild type mice, sensitisation to LPS by the induction of endogenous IFN-gamma production by injection of bacteria or administration of exogenous IFN-gamma (present study) before LPS challenge, as well as the incomplete and short inhibition of ICE with the ICE inhibitors in the time-course of LPS challenge, may explain the difference in the response observed in ICE-deficient mice.

CONCLUSION

In conclusion, pretreatment of mice with IFN-gamma caused marked increases in the serum levels of IL-1alpha, IL-1beta, TNF-alpha and IL-6 in response to an LPS challenge, as well as a marked increase in *ex vivo* production of IL-1beta by peritoneal macrophages, characterized by its non-susceptibility to nigericin. We have defined the conditions for a reliable and convenient procedure of sensitisation to LPS. This model of endotoxic shock is suitable for comparing the potency and selectivity of compounds acting through inhibition of cytokine synthesis. In addition, the conservation between human and murine ICE [28, 29] suggests that this model should be appropriate for pharmacodynamic studies of ICE inhibitors.

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Figures

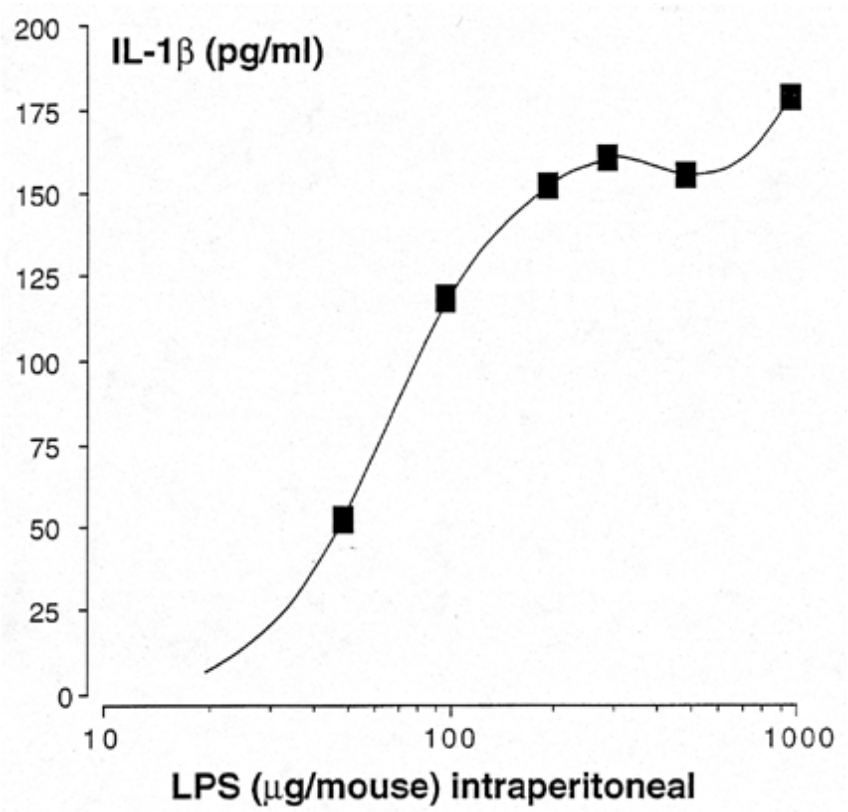


Figure 1

Serum IL-1beta **response to injection of various amounts of LPS into female Balb/c mice.**

Blood was obtained 4 hours after intraperitoneal LPS injection and serum IL-1beta levels were determined by ELISA. Values are the mean values for 2-3 mice.

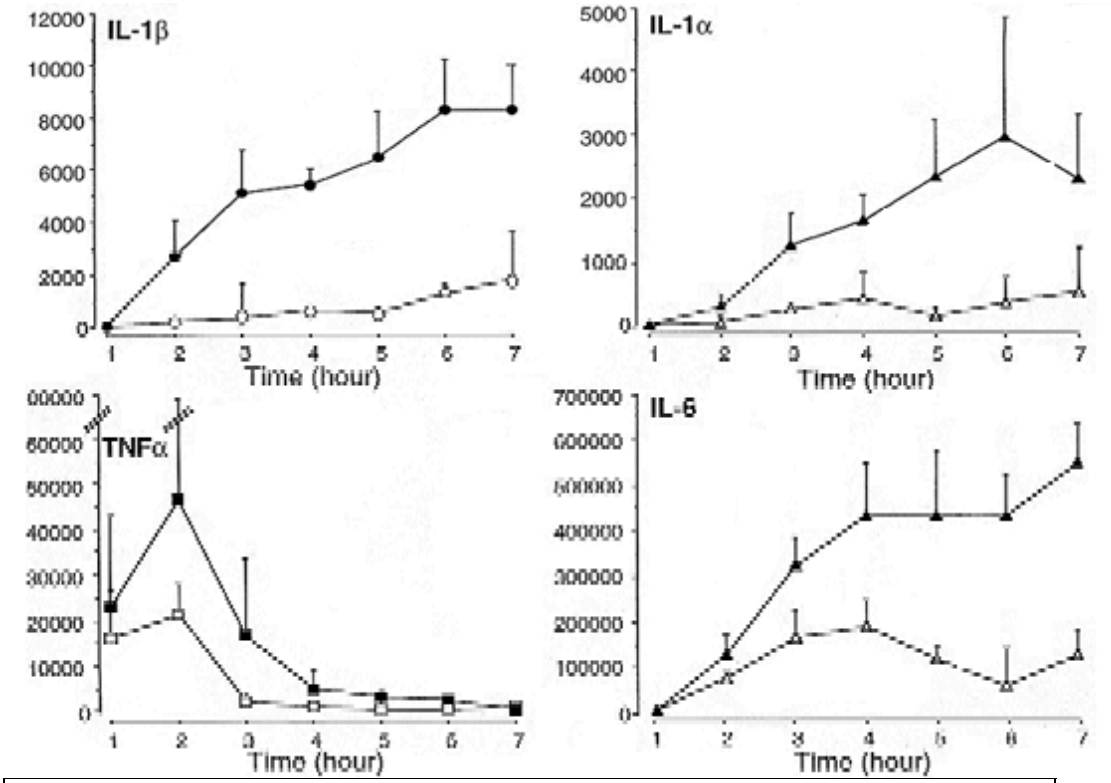


Figure 2

Time course of the effects of intraperitoneal LPS (300 mug/mouse) on the production of IL-1beta, IL-1alpha, TNF-alpha and IL-6 in the serum of non-sensitised mice (open symbols) or in mice sensitised by intraperitoneal injection of rat IFN-gamma (15

mug/mouse, 18 hours prior LPS challenge - closed symbols).

Data are shown as the geometric means with the upper limits of the 95% confidence intervals for groups of 4 non-sensitised mice or 7-8 sensitised mice at all time points.

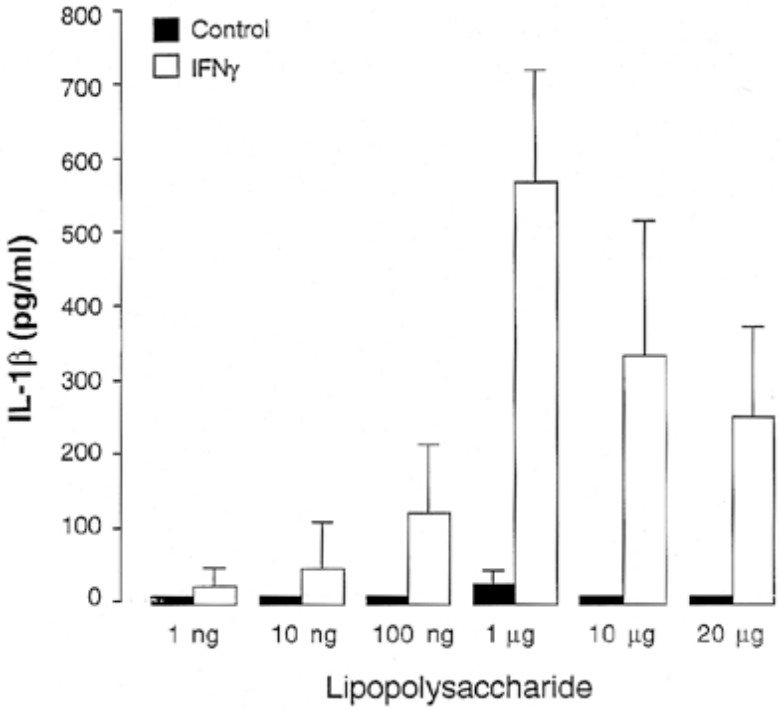


Figure 3

***Ex vivo* LPS-stimulated production of IL-1beta by peritoneal macrophages harvested from non-sensitised mice (closed bars) or from mice sensitised by intraperitoneal injection of rat IFN-gamma (open bars).**

Data are shown as the geometric means with the upper limits of the 95% confidence intervals of 8 experiments for each concentration of LPS.

Table 1
IL-1beta secretion by peritoneal macrophages isolated from non-sensitised mice and mice sensitised by an intraperitoneal injection of rat IFN-gamma (15 mug/mouse, 18 hours before macrophage isolation)

	IL-1beta (pg/ml)	
Stimulus	Macrophages non-sensitised mice	Macrophages IFN-gamma sensitised mice
None	12.5[a]	12.5[a]
LPS + medium	24[b] (14-41)	1,004[b] (768-1,313)
LPS + vehicle	24 (15-40)	1,175 (844-1,635)
LPS + nigericin	123[c] (89-171)	1,071 (799-1,437)

Macrophages were stimulated for 24 hours with 1 mug/ml of LPS, supernatants were harvested for quantitation of IL-1beta by an ELISA, specific for mature murine IL-1beta, after an additional 30 min incubation with either culture medium, nigericin vehicle or 10 muM nigericin. Values are the geometric means for 6 to 10 experiments and the 95% confidence intervals are shown in parenthesis.
[a] Samples which contained no detectable IL-1beta were assigned a value of 12.5 pg/ml (half the limit of detection of the ELISA);
[b] $P < 0.01$ *versus* control (without LPS, Mann-Whitney U test);
[c] $P < 0.005$ *versus* LPS + vehicle.

Table 2
Inhibition of LPS-induced serum cytokine levels (pg/ml) in IFN-gamma-sensitised mice by SK&F86002 administered *per os*, 1 hour to prior to LPS, at a dose of 100 mg/kg Serum cytokine levels were measured at 1.5 and 4 hours after LPS challenge

Treatment	Time (hour after LPS)	IL-1beta	IL-1alpha	TNF-alpha	IL-6
Control (n = 10)	1.5	1,289 (813-2,042)	177 (84-377)	14,808 (8,764-25,020)	28,825 (23,598-35,211)
SK&F86002 (n = 10)	1.5	793 (245-2,562)	40* (18-89)	1,344** (800-2,257)	5,476** (3,745-8,005)
Control (n = 10)	4	5,954 (4,794-7,395)	564 (317-1,003)	2,206 (1,205-4,036)	242,959 (192,527-242,959)
SK&F86002 (n = 9)	4	969** (539-1,743)	82* (42-159)	1,148 (741-1,777)	34,348** (29,643-34,348)

Values are the geometric means and the 95% confidence intervals are shown in parenthesis.
* $P < 0.05$, ** $P < 0.001$.

Table 3
Inhibition of LPS-induced IL-1beta production in IFN-gamma-sensitised mice by the ICE inhibitor

Time of YVAD-CHO administration (hour after LPS)	Serum IL-1 beta 4 hours after LPS[a] (pg/ml)	Inhibition (% *versus* control)
Control	2,451 (2,059-2,917)	0
0	1,344[b] (883-1,875)	45
1	705[b] (405-1,225)	71
2	614[b,c] (485-779)	75
3	744[b] (475-1,166)	69

YVAD-CHO was administered by i.p. injection at a dose of 50 mg/kg.
[a] values are the geometric means for nine mice and the 95% confidence intervals are shown in parenthesis;
[b] $P < 0.006$ *versus* control;
[c] $P < 0.007$ *versus* YVAD-CHO administered with LPS.

Table 4
Inhibition of LPS-induced serum IL-1beta production in IFN-gamma-sensitised mice by the ICE inhibitor administered either simultaneously with LPS or 2 hours after LPS

	Administration with LPS		Administration 2 hours after LPS	
YVAD-CHO (mg/kg)	IL-1 beta[a] (pg/ml)	Inhibition (% *versus* control)	IL-1 beta[a] (pg/ml)	Inhibition (% *versus* control)
Control	2,799 (2,322-3,374)	0	3,002 (2,249-4,006)	0
12.5	2,841 (2,436-3,313)	–	1,508[b] (1,079-2,107)	50
25	2,946 (2,246-3,863)	–	1,163[b] (1,005-1,344)	61
50	1,604[b] (1,369-1,879)	43	799[b] (544-1,174)	73
100	1,403[b] (1,178-1,672)	50	838[b] (598-838)	72
150	904[b] (511-1,600)	68	415[b] (243-708)	86
300	368[b] (258-525)	87	450[b] (327-619)	85

YVAD-CHO was administered by i.p. injection.
[a] values measured 4 hours after LPS are the geometric means for seven mice and the 95% confidence intervals are shown in parenthesis;
[b] $P < 0.05$ *versus* control.

Table 5
Inhibition by the ICE inhibitor YVAD-CHO of LPS-induced IL-1beta, IL-1alpha and IL-6 production in serum, as measured 4 hours after LPS challenge, and of LPS-induced TNF-alpha as measured 2 hours after LPS challenge in IFN-gamma-primed mice

YVAD-CHO Administration (h after LPS)	IL-1 beta	IL-1 alpha	IL-6	TNF-alpha
Control (no YVAD-CHO)	3.56 (2.91-4.35)	1.35 (1.06-1.73)	606.72 (443.73-829.59)	22.14 (19.13-25.63)
0	2.53 (1.70-3.75)	1.92 (1.63-2.26)	619.24 (475.95-805.66)	26.30 (22.7-30.41)
1	1.53* (0.95-2.48)	1.67 (1.26-2.21)	527.55 (446.41-623.44)	25.53 (21.21-30.72)
2	1.05* (0.71-1.53)	1.34 (1.06-1.69)	478.49 (402.00-569.54)	–

YVAD-CHO was administered by i.p. injection at a dose of 50 mg/kg.
Values are the geometric means for 8 to 10 mice expressed in ng/ml and the values in parenthesis indicate the 95% confidence intervals;
* $P < 0.005$ *versus* control.

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