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Differential Effects of Tenidap on the Zymosan- and Lipopolysaccharide-Induced Expression of mRNA for Proinflammatory Cytokines in Macrophages

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ABSTRACT. Tenidap is a novel antirheumatic drug that combines cyclooxygenase inhibition with cytokine modulating qualities. We demonstrate here that tenidap inhibits the zymosan-induced expression of both interleukin 1 and tumor necrosis factor α in macrophages, at the mRNA and protein levels. The concentration-dependence of the tenidap-induced inhibition of the expression of mRNA for these proinflammatory cytokines agrees with that of its inhibitory effects on zymosan-induced arachidonate mobilization and changes in phosphoprotein pattern. The effects of tenidap on the lipopolysaccharide-induced expression of these cytokines are more complex. Tenidap inhibits the induction of interleukin 1 by lipopolysaccharide or bacteria, but less potently than the interleukin 1-response induced by zymosan. In contrast, the drug markedly potentiates the lipopolysaccharide-induced expression of tumor necrosis factor α at both the mRNA and protein levels. The latter effect is demonstrated to be due to cyclooxygenase inhibition and is reversed by prostaglandin E_2. BIOCHEM PHARMACOL 52;1: 35–42, 1996.

KEY WORDS. tenidap; interleukin 1; prostaglandin E_2; tumor necrosis factor

Tenidap is a 3-substituted 2-oxindole under clinical investigation as a novel agent for the treatment of rheumatoid arthritis [1]. Tenidap is a powerful inhibitor of the cyclooxygenase pathway of eicosanoid formation. It has also been reported to block the synthesis of 5-lipoxygenase products [2, 3], and has been claimed by some authors to be an inhibitor of this enzyme, but later studies have demonstrated no direct effect of tenidap on the 5-lipoxygenase step in neutrophils [4] and macrophages [5].

Unlike nonsteroidal antiinflammatory drugs, tenidap has disease-modifying properties in rheumatoid arthritis [1]. It has been demonstrated to decrease the acute phase response [1, 6, 7] and had additional effects on leukocytes [8–11]. In macrophages, it is a potent inhibitor of zymosan-induced arachidonate release and changes in the cellular phosphoprotein pattern [5]. There is also growing evidence that tenidap is an inhibitor of the biosynthesis and/or release of proinflammatory cytokines [12–14]. However, a complex series of posttranscriptional events precedes the release of both IL-1† and TNF α. To relate the effects of tenidap on cytokine expression to its other effects on intracellular signal transduction [5], specific oligonucleotide probes for IL-1α, IL-1β, and TNF-α have been used to determine the effect of tenidap on cytokine mRNA expression in macrophages.

The main aim of this study has been to determine if the tenidap-induced inhibition of the production of proinflammatory cytokines is exerted at the mRNA level, and to correlate the results from the cytokine mRNA assay with those from experiments with Western analysis using rabbit antibodies against IL-1β or TNF-α. Furthermore, it was considered important to use different stimuli, not only LPS, but also zymosan and Gram-positive and Gram-negative bacteria, known to utilize different signal transduction pathways [15], to differentiate the actions of tenidap on the induction of proinflammatory cytokines. We also attempted to correlate the effects of tenidap on the induction of macrophage proinflammatory cytokine mRNA to those on other events in intracellular signal transduction, particularly arachidonate release, known to result in these cells from activation of intracellular 85 kDa phospholipase A_2 and changes in cellular protein phosphorylation.

MATERIALS AND METHODS

Materials and Preparation of Macrophages

Resident peritoneal cells were harvested in 4 mL of Medium 199 (Earle's salt, supplemented with 10 mM HEPES, 1% heat-inactivated foetal bovine serum and 20 units/mL

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† *Abbreviations:* IL-1, interleukin 1; LPS, lipopolysaccharide; SSC, sodium sodium chloride citrate; TNF, tumor necrosis factor.

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of heparin) from female outbred NMRI mice (Bommice, Ry, Denmark) by adherence to plastic 25 cm^2 Nunclon culture flasks, using approximately 18×10^6 cells per flask. The cells were incubated in an atmosphere of 5% CO_2 in air, and nonadherent cells were removed 2 hr after plating. To each flask was then added 10 mL of Medium 199 (Flow Laboratories, Irvine, Ayshire, Scotland) containing 10% heat-inactivated foetal bovine serum, and the cells were cultured for 20 hr before being washed with Dulbecco's PBS and equilibrated in 10 mL serum-free Medium 199 before start of the experiment.

Tenidap, provided by Pfizer Inc. (Sandwich, U.K.), was added in 2–20 μL of a DMSO solution. Zymosan (from *Saccharomyces cerevisiae*), LPS (from *Salmonella minnesota*), and 4β-phorbol 12-myristate 13-acetate were purchased from Sigma (St. Louis, MO, U.S.A.). The calcium ionophore A23187 was purchased from Boehringer (Mannheim, Germany) and dissolved in DMSO. Bacteria were obtained as described [15] and added as a suspension in Ca^{2+}/Mg^{2+}-free PBS.

In the majority of experiments, tenidap was added 15 min before the cell stimulant. In a few instances, overnight pretreatment with the drug was performed to allow a certain build-up of a steady-state concentration intracellularly. After 90 min of incubation, with both tenidap and the cell stimulant present, the culture medium was removed and the cells washed with PBS.

Assessment of Cytokine mRNA Expression

Preparation of total mRNA was performed according to Chomzynski and Sacchi [16]. The cells were lysed in a solution containing guadinium thiocyanate (4 M), sodium citrate (25 mM), 2-mercaptoethanol (0.1 M) and sarcosyl (0.5%, w/v). The lysate was transferred to a polypropylene tube, and sodium acetate (2.0 M), water-saturated phenol, and a chloroform-isoamyl alcohol mixture (49:1 w/v) were added, in that order. The sample was mixed and centrifuged, and the upper liquid phase was transferred to a fresh tube containing isopropanol. The mixture was incubated for at least 24 hr at −20°C to precipitate the RNA, which was then pelleted by centrifugation. The pellet was resuspended in the guanidine isothiocyanate-containing solution detailed above, reprecipitated, washed with ethanol, dried, and dissolved in 0.5% SDS.

Total RNA was separated on a formaldehyde-1.2% agarose gel and blotted onto a nylon membrane by 20 × SSC (1 × SSC = 0.15 M NaCl and 0.05 M sodium citrate, pH 7.0) by capillary transfer. The blots were stained with ethidium bromide, baked in a vacuum oven for 2 hr and prehybridized for 12–16 hr at 42°C in 50% formamide, 1% SDS, 5 × SSC, 1 × Denhart's solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrrolidone) and 0.5 mg/mL denaturated salmon sperm DNA and 50 mM sodium phosphate buffer, pH 7.4. Synthetic oligonucleotide probes (Clontech Laboratories Inc, Palo Alto, CA, U.S.A.) with the following sequences were used; TNF-α: 5′-GCC GTT GGC CAG GAG GGC GTT GGC GCG CTG-3′, IL-1α: 5′-CTC TTC TTC AGA ATC TTC CCG TTG CTT GAC-3′, IL-1β: 5′-AGC TTT CAG CTC ATA TGG GTC CGA CAG CAC-3′, β-Actin: 5′-GGG TGT TGA AGG TCT CAA ACA TGA TCT GGG-3′. The probes were radiolabeled by 5′-labeling, using $\gamma[^{32}P]$-ATP, to a specific activity of approximately 10^9 cpm/μg DNA.

Hybridization was carried out at 42°C for 12–16 hr with denatured probe. After hybridization, blots were washed 4 times in 0.2 × SSC for 60 min followed by 4 additional washes at 55°C with 2 × SSC. The blots were dried and exposed to Hyperfilm TM-MP (Amersham, Little Chalfont, U.K.) at −30°C using an intensifying screen. Autoradiographs of the blots were then scanned by video densitometry. In some experiments, a Fujix Bas 2000 phosphoimaging system was used instead. To compare the amounts of mRNA in each sample, a specific oligonucleotide probe for β-actin was used, and hybridization and autoradiography performed as above. OD values were determined for each sample and normalized to values obtained for β-actin expression on the same blot.

Western Blot Analysis of IL-1β and TNF-α

Equal aliquots of whole cell extracts prepared in Laemmli sample buffer were boiled for 5 min and subjected to SDS-PAGE (12% acrylamide). Harvested culture medium was supplemented with bovine serum albumin and adjusted to 6% trichloracetic acid followed by incubation at 4°C and centrifugation at 12,000 × *g* for 30 min. The precipitate was suspended in sample buffer and treated as described for the cell extract. Gels were then equilibrated in transfer buffer and the samples transferred to a nitrocellulose membrane. The membrane was blocked with 1% gelatin followed by incubation with rabbit antibodies against either IL-1β or TNF-α (Genzyme Diagnostics, Cambridge, MA, U.S.A.). Bound antibodies were detected with ^{125}I-labeled goat antirabbit antibodies (0.5×10^6 cpm/mL). Blots were then dried and analyzed by autoradiography or by digital imaging (Fujix Bas 2000, Fuji Ltd, Tokyo, Japan). Extensive washing of membranes preceded each incubation step.

Statistical Methods

In all statistical testing, a one-sided, paired-comparisons version of Student's *t*-test was used.

RESULTS

Initially, it was ascertained that tenidap (1–20 μM), under the time and culture conditions used in the cytokine assays, did not affect total cell protein levels in the macrophages.

Control cells did not express mRNA for either IL-1α, IL-1β, or TNF-α, nor did tenidap, in itself, induce expression of mRNA for any of these cytokines. Cells stimulated with phorbol diester expressed minute amounts of IL-1β mRNA and no TNF-α mRNA. The calcium ionophore A23187 caused expression of small amounts of IL-1β and IL-1α, but no recognizable amounts of TNF-α mRNA.

Zymosan caused expression of IL-1α and IL-1β mRNA, and also a low, but clearly detectable, level of TNF-α. It was possible to detect IL-1β mRNA, as well as smaller amounts of IL-1α and TNF-α mRNA, after 30 min of stimulation with zymosan, with increasing amounts of cytokine mRNA being detected after 60 and 90 min of stimulation with this agent (not shown). There seemed to be a plateau after 90 min of incubation, and no further accumulation of mRNA for these cytokines occurred after 2, 3, or 4 hr of stimulation with zymosan. In Western blot analysis, the zymosan-induced TNF-α response was too weak to be reliably detected. No mature IL-1β could be detected in the medium, but the intracellular proIL-1β was clearly seen after zymosan stimulation.

LPS caused expression of all 3 proinflammatory cytokine mRNAs, with a higher level of expression of IL-1β than TNF-α mRNA. Both IL-1β and TNF-α mRNA could be distinctly detected after 30 min of stimulation with LPS; the levels of mRNA for these cytokines increased at a parallel rate to reach a plateau after approximately 90 min. No further accumulation of cytokine mRNA occurred after LPS stimulation for 2 or 4 hr. Similar kinetics have been published [15] regarding the induction of IL-1β and TNF-α by bacteria. Mature, secreted TNF-α, as well as very small amounts of cell-associated, immunoreactive TNF-α, could be detected after LPS stimulation. Again, no mature IL-1β could be detected in the medium, but cell-associated proIL-1β could clearly be detected by Western blot analysis after LPS stimulation.

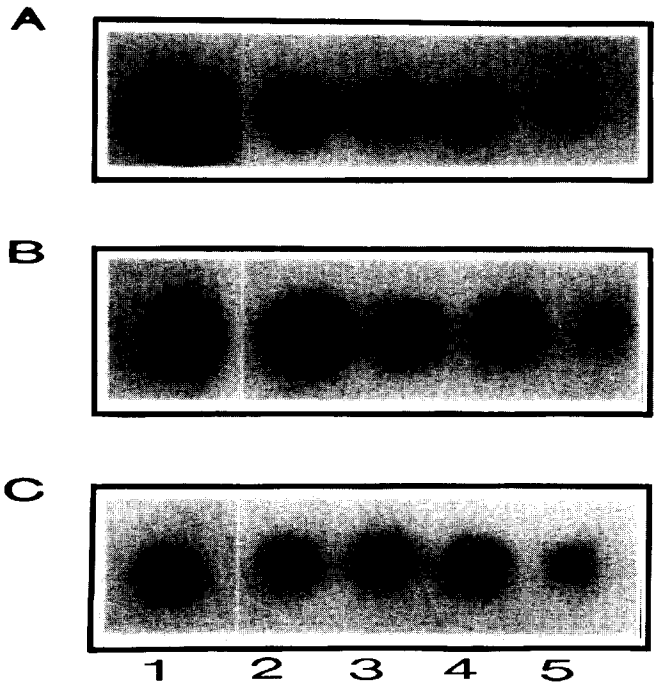

FIG. 1. Effect of tenidap on the zymosan-induced expression of IL-1β (A) and TNF-α (B) mRNA. Total β-actin mRNA is shown in (C). Lane 1: zymosan (300 μg/mL), Lanes 2–5: 0.1, 1, 5, and 20 μM tenidap, respectively, added 15 min before zymosan. Incubation time was 90 min.

TABLE 1. Tenidap inhibits zymosan-induced cytokine expression

Tenidap (μM)	IL-1β mRNA		TNF-α mRNA	
	n	M ± SEM	*n*	M ± SEM
0.3	6	0.79 ± 0.06	6	0.94 ± 0.05
1	9	0.49 ± 0.09	9	0.75 ± 0.07
5	14	0.44 ± 0.05	14	0.60 ± 0.07
20	12	0.29 ± 0.05	12	0.36 ± 0.07
40	4	0.16 ± 0.03	4	0.22 ± 0.07

Tenidap was added to cell cultures 15 min before zymosan (300 μg/mL). Incubation time was 90 min. After Northern blotting, scanning of autoradiograms or digital imaging and normalization of mRNA load by β-actin probing, results are expressed as a fraction of the IL-1β- or TNF-α mRNA response induced by zymosan alone. Significant ($P < 0.01$) inhibition of IL-1β mRNA expression occurred at 0.3 and 1 μM tenidap, and significant ($P < 0.001$) inhibition at higher concentrations. Significant ($P < 0.01$) inhibition of TNF-α mRNA expression occurred at 1 and 5 μM tenidap, and significant ($P < 0.001$) inhibition at 20 μM.

Tenidap (0.3–40 μM) dose-dependently inhibited the zymosan-induced expression of IL-1α, IL-1β, and TNF-α mRNA. The effect of zymosan-induced TNF-α expression appeared to be somewhat less powerful than that on the expression of IL-1β (Fig. 1), although higher concentrations of tenidap caused significant reduction of TNF-α mRNA (Table 1). Kinetics experiments demonstrated that the tenidap-induced inhibition of IL-1β mRNA could already be detected after 30 min of incubation with zymosan; in general, there was no significant difference in the potency of the inhibition when the incubation time with zymosan was changed. Overnight preincubation with tenidap tended to lower the concentration-dependence of the inhibition somewhat. The zymosan-induced formation of intracellular proIL-1β protein, assessed by Western blot analysis, was also inhibited by tenidap (Fig. 2, lanes 5–8; Table 2), with no great difference in potency to the inhibition at the mRNA level (Table 1).

The A23187-induced expression of IL-1β mRNA was also inhibited by tenidap (Fig. 3). The difference in both cytokine specificity (IL-1 mRNA was expressed in ionophore-stimulated macrophages, and TNF-α mRNA was not), and the amount of cytokine mRNA expressed, suggests that induction by zymosan is not entirely Ca^{2+}-mediated, although zymosan is known to cause a phosphoinositide response in macrophages [5]. Cyclosporin, which inhibits Ca^{2+}-activated protein phosphatase(s), inhibits the induction of both IL-1β mRNA and TNF-α mRNA in response to bacteria [15].

Tenidap also inhibited the LPS-induced expression of IL-1β mRNA (Fig. 4A). As seen in Fig. 4C, equivalent

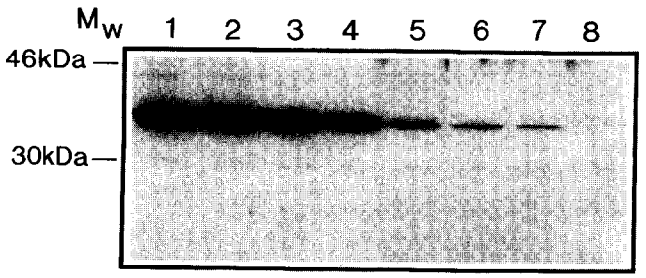

FIG. 2. Effect of tenidap on the formation of proIL-1β in response to lipopolysaccharide (3 μg/mL; Lanes 1–4) and zymosan (100 μg/mL; Lanes 5–8), assessed by Western blotting. Lanes 1 and 5: LPS and zymosan alone, respectively; Lanes 2–4 and 6–8: 1, 5, and 20 μM tenidap added 15 min before LPS or zymosan. Incubation time was 4 hr.

amounts of RNA were loaded in each lane as demonstrated after hybridization with a β-actin probe. The tenidap-induced inhibition of LPS-mediated cytokine mRNA induction was less potent than when zymosan was used as a cell stimulant (Table 1 vs Table 3). Again, kinetics experiments showed that inhibition could be clearly detected after 30 min of LPS stimulation, and that the incubation time with LPS did not affect the potency of the tenidap-induced inhibition. Overnight incubation with tenidap, again, tended to lower the concentration-dependence for this tenidap-induced inhibition of cytokine induction. Western blot analysis demonstrated that tenidap also inhibited the LPS-induced formation of intracellular pro-IL-1β protein (Fig. 2, lanes 1–4; Table 4), although less potently than at the mRNA level (Table 3). Surprisingly, tenidap did not inhibit, but potentiated, the LPS-induced expression of TNF-α mRNA (Fig. 4B,C, Table 3). The LPS-induced expression of TNF-α protein was potentiated by both tenidap and indomethacin (Fig. 5). Some higher molecular weight pro-forms of TNF-α were also released by tenidap- or indomethacin-treated cells, probably due to incomplete proteolytic processing. The effect of tenidap was similar to that seen on the expression of mRNA for this cytokine (Table 3 vs Table 4). Cell-associated, immunoreactive TNF-α was barely detectable in the absence of tenidap, but minor amounts of higher M_r TNF-α precursors were detected in

TABLE 2. Tenidap inhibits zymosan-induced production of proIL-1β

Tenidap (μM)	proIL-1β	
	n	M ± SEM
1	4	0.75 ± 0.06
5	4	0.42 ± 0.14
20	4	0.18 ± 0.05

Tenidap was added to cell cultures 15 min before zymosan (300 μg/mL). Incubation time was 4 hr. Results are expressed as a fraction of the proIL-1β response induced by zymosan alone. Significant ($P < 0.05$) inhibition occurred at 1 μM tenidap, and significant ($P < 0.001$) inhibition at higher concentrations.

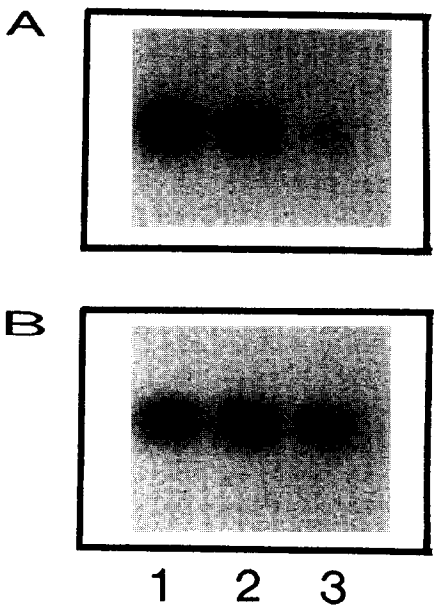

FIG. 3. Effect of tenidap on A23187-induced expression of IL-1β mRNA. Total β-actin mRNA is shown in (B). Lane 1, A23187; Lanes 2–3, 5 and 20 μM tenidap added 15 min before A23187. Incubation time was 90 min.

tenidap-treated cells (not shown), possibly reflecting the increased rate of synthesis.

To further address the question of whether or not there is a causal relationship between cyclooxygenase inhibition and the potentiation of LPS-induced TNF-α expression, we assessed in parallel the dose relationships for these two effects, using tenidap and indomethacin, as well as two additional cyclooxygenase inhibitors, naproxen and ibuprofen. All 4 of these compounds induced a similar potentiating effect on LPS-induced TNF-α expression, and the dose required in each case was that causing severe (>70%) in-

TABLE 3. Effects of tenidap on LPS-induced cytokine expression

Tenidap (μM)	IL-1β mRNA		TNF-α mRNA	
	n	M ± SEM	*n*	M ± SEM
0.1	3	0.94 ± 0.09	2	2.01
0.3	5	0.87 ± 0.20	3	2.30 ± 0.17
1	10	0.77 ± 0.09	8	2.33 ± 0.18
5	7	0.63 ± 0.12	7	2.41 ± 0.16
10	4	0.37 ± 0.07	2	1.98
20	10	0.30 ± 0.07	8	1.79 ± 0.16
40	2	0.22	2	1.44

Tenidap was added to cell cultures 15 min before LPS (3 μg/mL). Incubation time was 90 min. After Northern blotting, scanning of autoradiograms or digital imaging and normalization of mRNA load by β-actin probing, results are expressed as a fraction of the IL-1β- or TNF-α response induced by LPS alone. Significant ($P < 0.05$) inhibition of IL-1β mRNA expression occurred at 1 μM tenidap, significant ($P < 0.01$) inhibition at 5 and 10 μM, and significant ($P < 0.001$) inhibition at 20 μM. There was significant ($P < 0.001$) potentiation of TNF-α mRNA expression at 1, 5, and 20 μM.

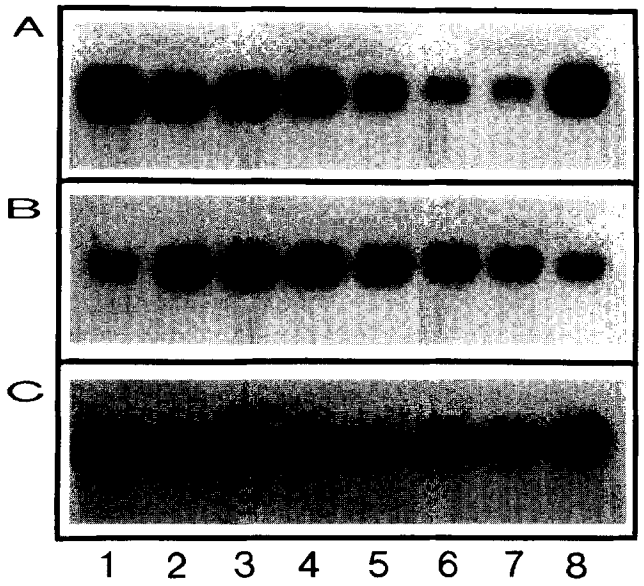

FIG. 4. Effects of tenidap on the LPS-induced expression of IL-1β (A) and TNF-α (B) mRNA. Total β-actin mRNA is shown in (C). Lanes 1 and 8, LPS (3 μg/mL), Lanes 2–7, 0.1, 0.3, 1, 5, 20, and 40 μM tenidap, respectively, added 15 min before LPS. Incubation time was 90 min.

hibition of prostaglandin E_2 formation (not shown). Furthermore, the tenidap-induced potentiation could be reversed by adding nanomolar concentrations of prostaglandin E_2 to the medium (Fig. 6); thus, adding compelling evidence that the LPS-induced formation of TNF-α in resident macrophages is highly sensitive to inhibition by this eicosanoid. Similar results were reported earlier [17, 18], but were then considered unique to immunologically activated, as opposed to resident, macrophages [17]. It should be noted that cyclooxygenase inhibitory concentrations of indomethacin did not affect LPS-induced IL-1β expression, nor zymosan-induced expression of either IL-1β or TNF-α.

These intriguing findings with LPS-stimulated cells

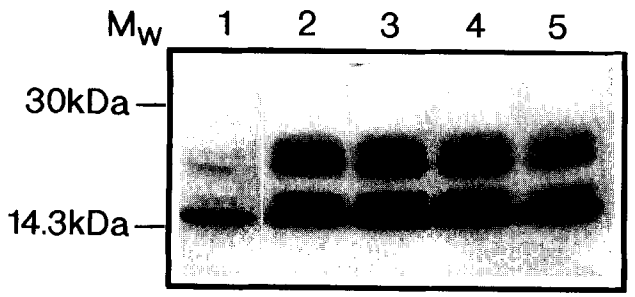

FIG. 5. Effect of tenidap and indomethacin on the formation of immunoreactive TNF-α secreted into the culture medium. The mature 17 kDa TNF-α protein is the lowest band in this figure; the other bands represent higher molecular weight proforms. Macrophages were stimulated with LPS (1 μg/mL) and TNF-α formation was assessed by Western blotting. Lane 1, LPS; Lanes 2 and 3, 1 and 10 μM indomethacin added 15 min before LPS; Lanes 4–5, 1 and 10 μM tenidap added 15 min before LPS. Incubation time was 4 hr.

TABLE 4. Tenidap inhibits LPS-induced proIL-1β production but potentiates TNF-α production

	proIL-1β		TNF-α	
Tenidap (μM)	*n*	M ± SEM	*n*	M ± SEM
1	4	0.98± 0.02	3	2.5 ± 0.21
5	5	0.71 ± 0.11	4	2.5 ± 0.29
20	5	0.49 ± 0.09	4	1.9 ± 0.17
40	2	0.20		

Tenidap was added to cell cultures 15 min before LPS (3 μg/mL). Incubation time was 4 hr. Results are expressed as a fraction of the proIL-1β- or TNF-α response induced by LPS alone. There was significant ($P < 0.05$) inhibition of IL-1β production at 5 μM tenidap, and significant ($P < 0.01$) inhibition at 20 μM. Significant ($P < 0.01$) potentiation of TNF-α production occurred at 5 and 20 μM.

prompted us to investigate the effects of tenidap on the induction of cytokine mRNA that occurs in response to various bacteria. Both Gram-negative and Gram-positive bacteria were used because the signal transduction pathways employed by them may differ [15]. Tenidap (20 μM) inhibited by 30% the expression of IL-1β mRNA induced by either Gram-negative (*Fusobacterium nucleatum*) or Gram-positive (*Peptostreptococcus anaerobius*) bacteria (not shown). The inhibition was more pronounced (70% at 20 μM concentration) when the stimulus was *Gardnerella vaginalis* (Fig. 7A), which has characteristics of both Gram-positive and Gram-negative species. Again, equivalent amounts of mRNA were loaded in each lane, as evidenced by β-actin probing (Fig. 7C). On the other hand, tenidap slightly potentiated the bacteria-induced expression of TNF-α mRNA (Fig. 7B), irrespective of bacterial species and with a maximum potentiation of 40% at 5 μM concentration. This slight potentiation is consistent with the

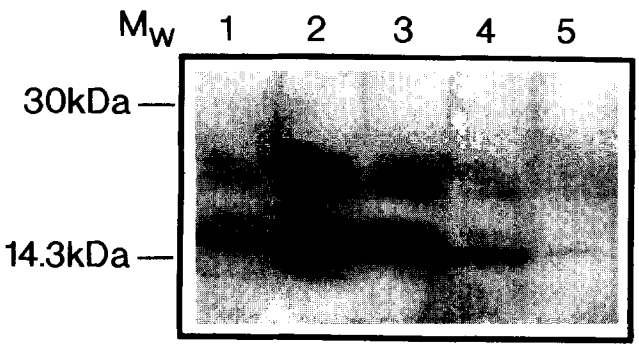

FIG. 6. Effect of tenidap and prostaglandin E_2 on the formation of immunoreactive TNF-α secreted into the culture medium. The mature 17 kDa TNF-α protein is the lowest band in this figure; the other bands represent higher molecular weight proforms. Macrophages were stimulated with LPS (1 μg/mL) and TNF-α formation assessed by Western blotting. Lane 1, LPS; Lane 2, 1 μM tenidap added 15 min before LPS; Lanes 3–5, 1 μM tenidap and 1, 10, and 100 nM prostaglandin E_2 added 15 min before LPS. Incubation time was 4 hr.

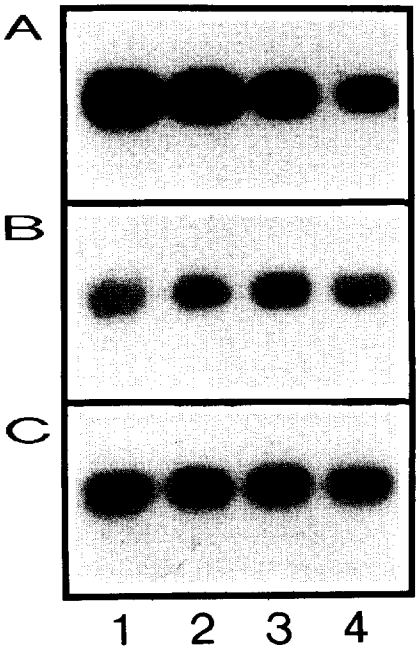

FIG. 7. Effects of tenidap on the *G. vaginalis*-induced expression of IL-1β (A) and TNF-α (B) mRNA. Total β-actin mRNA is shown in (C). Lane 1, bacteria alone; Lanes 2–4, 1, 5, and 20 μM tenidap, respectively, added 15 min before bacteria. Incubation time was 90 min.

much less potent inhibition by prostaglandin E_2 seen upon stimulation with intact bacteria [15], as opposed to LPS (see above).

DISCUSSION

Otterness *et al.* [12] used bioassay to demonstrate that tenidap (3–10 μM) inhibited both zymosan-induced and LPS-induced IL-1 synthesis in mouse macrophages. Another series of experiments, using goat anti-IL-1α and Western blot analysis to assess the levels of the intracellular 34 kDa pro-IL-1α, demonstrated that tenidap potently inhibited the LPS-induced formation of this precursor. This argues against the possibility that tenidap would act only on the proteolytic processing and/or release of the cytokine. Using immunoassay and human peripheral blood monocytes, Sipe *et al.* [13] found evidence that tenidap caused inhibition of LPS-induced biosynthesis of IL-1 and IL-6 as well as TNF-α. However, these results disagree with the findings of Olivera *et al.* [19], using oil-elicited mouse peritoneal macrophages or human monocytes, because these workers found only minimal effects of tenidap on TNF-α production. Furthermore, by stimulating mice with an intraperitoneal injection of LPS, after the animals had been fed tenidap or nonsteroidal antiinflammatory drugs, Griswold *et al.* [20] found that tenidap, indomethacin, naproxen, and ibuprofen all potentiated TNF-α production (100–150% in the case of tenidap).

Our results agree with those of Otterness *et al.* [12] that tenidap inhibits the zymosan-induced-IL-1 response, adding that it has a similar, although less pronounced, inhibitory effect on the induction of TNF-α by this stimulus. The dose-response relationship from the Western blot analysis of intracellular pro-IL-1β, with progressive inhibition in the concentration range 3–20 μM tenidap, agrees well with that reported by Otterness *et al.* [12]. The results also strongly suggest that tenidap has differential effects when LPS or Gram-negative bacteria are used as stimulus. Although tenidap inhibits the LPS-induced IL-1 response less potently than zymosan-mediated IL-1 induction, it markedly potentiates the LPS-induced TNF-α response, both at the mRNA and protein levels. This potentiation of the LPS-induced TNF-α is shared with other cyclooxygenase inhibitors, and is due to relief from prostaglandin-mediated inhibition of the TNF-α response. In general, our results indicate that the effect of tenidap on the synthesis of IL-1α, IL-1β, and TNF-α is exerted primarily at the level of transcription, posttranscriptional processing, or mRNA stability, with no, or little, further inhibition on the later steps of translation, proteolytic processing, and release.

Because the intracellular signalling that leads to expression of IL-1α/β and TNF-a mRNA is still largely unknown, it is not possible to pinpoint the site(s) of action of tenidap. It is worth pointing out, however, that earlier studies have demonstrated that similar concentrations (5–20 μM) of tenidap also inhibit zymosan-induced arachidonate release and enhanced phosphorylation of cellular proteins in macrophages responding to zymosan [5] or LPS (not shown). The phorbol-diester-induced phosphorylation pattern is not affected by tenidap, which argues against a direct effect on relevant isoforms of protein kinase C. Furthermore, tenidap in itself induces increased phosphorylation of several proteins, and attenuates the zymosan-induced activation of the phospholipase C process [5]. Whether or not the latter effect has anything to do with tenidap's cytokine modulatory effects is uncertain, however, because protein kinase C activation is not sufficient to cause expression of mRNA for the proinflammatory cytokines, and Ca^{2+}-mediated signalling only leads to a weak mRNA response. It is likely that there is one or more additional signal chain involved in cytokine expression and activated by zymosan, LPS, and bacteria. It is particularly interesting that, although tenidap inhibits both the IL-1 and the TNF-α response induced by zymosan, it has a differential effect, inhibiting IL-1 but potentiating the TNF-α response, when LPS is the stimulus. This argues, in addition, for differences in the signal transduction leading to IL-1α/β and TNF-α expression at the mRNA level.

A possible target for the signalling to TNF-α mRNA expression could be the transcription factor NF-κB, which has been implicated in the LPS-induced induction of TNF-α [21–23]. NF-κB may also play a role in the induction of IL-1 [24, 25]. Another transcription factor subunit that may well be involved in the induction of TNF-α is NFATp, which originally was thought to be specific for T

cells, but now is known to also be present in monocytic cells [26]. The activation and/or nuclear translocation of these transcription factors has been shown to require both phosphorylation and dephosphorylation reactions, which might both be targeted by compounds interfering with the expression of cytokine mRNAs. Tenidap's effects on protein phosphorylation would provide a mechanism for its possible interaction with transcription factors.

The main conclusions of the present paper are that, although clinically attainable concentrations of tenidap (1–5 μM) inhibit the induction of IL-1 and TNF-α by zymosan, there is a differential effect with regard to the response to LPS. Although the LPS-induced IL-1 response is inhibited by tenidap (5–20 μM), the TNF-α response is instead potentiated by tenidap, due to its cyclooxygenase inhibition. The impression of tenidap from clinical and laboratory studies [1, 7, 27] of rheumatoid arthritis is definitely not that this drug potentiates TNF-α production. It does not seem to differ from piroxicam in its effects on serum TNF-α levels in RA patients [28]. One reason for this discrepancy between clinical data and laboratory studies using LPS as a stimulus might be that the response to zymosan is a better model for macrophage activation in chronic inflammatory arthritis than challenge of the cells with bacterial lipopolysaccharide.

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