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Evidence for tumor necrosis factor α as a mediator of the toxicity of a cyclooxygenase inhibitor in Gram-negative sepsis

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Abstract

To investigate the effect of cyclooxygenase inhibition in experimental Gram-negative sepsis, indomethacin was administered to mice at different times (1 or 5 days, or 1 h) before sublethal infection with an intravenous inoculum of *Pseudomonas aeruginosa*. Early indomethacin exposure did not alter the outcome of infection, yet treatment at the time of bacterial challenge resulted in a high mortality rate. Polymerase chain reaction-assisted mRNA amplification in the spleens of infected mice revealed that tumor necrosis factor α (TNF-α) messenger was selectively expressed by the drug-treated and infected mice during the 24 h preceding death. Higher TNF-α levels were found in sera from these mice, whose macrophages produced increased levels of nitric oxide in vitro. Both pentoxifylline, an inhibitor of TNF-α synthesis, and an inhibitor of nitric oxide production improved survival in the indomethacin-treated and infected mice, although no such effect followed the administration of TNF-neutralizing antibodies. These data support the notion that cyclooxygenase inhibitors may exert both positive and negative effects in Gram-negative sepsis, the latter presumably involving overproduction of TNF-α.

Keywords: Indomethacin; Gram-negative sepsis; TMF (tumor necrosis factor); Cyclooxygenase inhibitor; Sepsis

1. Introduction

The ability of tumor necrosis factor α (TNF-α) to induce production of arachidonic acid metabolites, such as prostaglandin E_2 and prostaglandin I_2, may be an important effector function of this proinflammatory cytokine in the pathogenesis of septic and endotoxic shock (Beutler, 1992; Stevens et al., 1993). Accordingly, experimental evidence indicates that cyclooxygenase inhibitors, such as indomethacin or ibuprofen, may prevent hypothermia, changes in blood glucose, acidosis, and lethality in rats given an intravenous injection of human recombinant TNF (Ketelhut et al., 1987). In endotoxic shock of experimental animals, indomethacin does not reduce the levels of circulating TNF-α, but may prevent sickness and death, correlating with a reduction in circulating levels of cyclooxygenase products (Mozes et al., 1991; Johnson and Von Borell, 1994). Evidence, however, for a protective role of indomethacin or other cyclooxygenase inhibitors in live sepsis models is scant (Parant et al., 1980; Horgan et al., 1990; Stevens et al., 1992). Much of the data indicates that cyclooxygenase inhibitors mitigate at most the hemodynamic and metabolic effects of shock, without depressing the endogenous production of proinflammatory cytokines (Mozes et al., 1991; Coran et al., 1992).

Substances that increase cAMP levels, such as prostaglandin E_2 and phosphodiesterase inhibitors, can suppress the production of different cytokines, including TNF-α (Beutler, 1992). Presumably due to inhibition of prostaglandin E_2 synthesis, indomethacin may increase cytokine transcription and synthesis. We have previously shown that exposure of murine macrophages to indomethacin in vitro results in enhanced expression of transcripts specific for granulocyte-macrophage colony-stimulating factor, and that the drug is protective in vivo when administered several days before infection of neutropenic mice with Gram-negative bacteria (Campanile et al., 1993). However, recent in vivo (Sironi et al., 1992; Utsunomiya et al., 1994) and vitro (Griswold et al., 1993; Simpson et al., 1994) evidence indicates that co-exposure to indomethacin may

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strongly enhance TNF-α production in response to lipopolysaccharide or other inflammatory stimuli, and the ability of the drug to inhibit prostaglandin H synthase 1 correlates with its potency to induce TNF-α (Griswold et al., 1993). Therefore, cyclooxygenase inhibitors, in addition to modulation of prostaglandin effector function, may enhance the release of cytokines with either protective or pathogenetic roles, and this could be particularly important in endotoxic and septic shock.

In the present study, we have addressed the issue of the possible balance between beneficial and detrimental effects of cyclooxygenase inhibition in experimental shock. Using a live sepsis model, we have examined the effect of indomethacin administration on the resistance of mice to sublethal challenge with *Pseudomonas aeruginosa*. We found that a vast majority of animals given the drug at the time of infection developed fatal sepsis, which was characterized by an enhanced production of TNF-α. Based on proposed mechanisms of pathogenesis in shock, which call for a major role of TNF (Stevens et al., 1993) and the potent vasodilator nitric oxide (NO) as a mediator of its toxicity (Moncada et al., 1991), we have tested the effects of the administration of inhibitors of TNF-α or NO synthesis on indomethacin-induced lethality in infected mice. Both phosphodiesterase inhibitors (Fletcher et al., 1992; Sekut et al., 1995) and inhibitors of NO synthase (Thiemermann and Vane, 1990; Kilbourn and Griffith, 1992; Teale and Atkinson, 1992; Evans et al., 1994) have indeed been shown by previous studies to improve outcome in experimental sepsis.

2. Materials and methods

2.1. Mice

Hybrid (BALB/cCr × DBA/2Cr)F_1 ($CD2F_1$) mice were purchased from Charles River Breeding Laboratories (Calco, Milan, Italy). Mice of both sexes, ranging in age from 2 to 4 months, were used.

2.2. Organism and infection

The origin and characteristics of the *P. aeruginosa* strain used in this study have been described elsewhere (Campanile et al., 1990, 1993, 1994). The organism was routinely cultured in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 18–24 h with constant aeration. For infection, overnight cultures were centrifuged, the soft pellet was resuspended in phosphate-buffered saline (PBS), and 10^{10} cells were injected intravenously. Portions of suitable dilutions were also inoculated onto agar plates for precise enumeration of colony-forming units. All deaths resulting from infection, as proven by clinical signs and histopathological examination, occurred within 3 days of microbial challenge and mostly within the first 24–48 h (mortality was routinely recorded for up to 7 days from challenge). No or minimal mortality occurred as a rule in control mice not given indomethacin and challenged with the same inoculum of bacterial cells. Likewise, no deaths occurred in uninfected mice given indomethacin alone.

2.3. Drugs and reagents

Indomethacin (Liometacen; Chiesi Farmaceutici, Parma, Italy) was dissolved in PBS and administered to mice as a single subcutaneous injection of 4.2, 7, or 12 mg per kg of body weight at different times before microbial challenge. These dosages have been previously shown to up-regulate the production of colony-stimulating factors and the myelopoietic response of neutropenic mice treated with human recombinant interleukin-1β (Campanile et al., 1991, 1992), and to improve survival of *Pseudomonas*-infected mice when the drug was given shortly after myelosuppression (i.e. several days before infection) (Campanile et al., 1993). Prostaglandin E_2 (Sigma, St. Louis, MO, USA) was given as a single intraperitoneal injection in PBS at 2 mg/kg a few minutes apart from indomethacin treatment. Pentoxifylline (Sigma) was administered intraperitoneally in PBS at 30 mg/kg 1 h before infection. In selected experiments mice received a second injection of pentoxifylline 3 h after challenge. N^G-monomethyl-L-arginine (NMLA; Sigma) was given as a single intraperitoneal injection in PBS at 40 mg/kg 0.5 h before infection. In most experiments, negative controls for drug treatments received vehicle alone. Neutralization of endogenous TNF-α was achieved by administering 1 mg/mouse of neutralizing rat anti-mouse TNF-α monoclonal antibody MP6-XT3 (PharMingen, San Diego, CA, USA) intraperitoneally 15 min before infection.

2.4. RNA preparation and detection of cytokine transcripts by polymerase chain reaction (PCR)

These procedures have been previously described in detail (Campanile et al., 1993, 1994). Briefly, 5×10^6 cells (pooled from 2–3 animals) were subjected to RNA extraction by the guanidium thiocyanate-phenol-chloroform procedure. Purified total RNA was incubated with 0.5 μg of oligo(dT) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 h at 42°C after addition of 20 U RNase inhibitors (Boehringer-Mannheim Italia, Milan, Italy), 1.5 mM deoxynucleoside triphosphates, 7.5 U avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) and reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 8 mM $MgCl_2$, 30 mM KCl and 10 mM dithiothreitol, final concentrations) in a final volume of 20 μl. The cDNA was diluted to a total volume of 75 μl with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and frozen at −20°C until use. Amplification of synthesized

cDNA was carried out using interferon-γ (5'-TG AAC GCT ACA CAC TGC ATC TTG G-3' and 5'-CG ACT CCT TTT CCG CTT CCT GAG-3'), interleukin-1α (5'-ATG GCC AAA GTT CCT GAC TTG TTT-3' and 5'-C CTT CAG CAA CAC GGG CTG GTC-3'), interleukin-6 (5'-ATG AAG TTC CTC TCT GCA AGA GAC T-3' and 5'-CA CTA GGT TTG CCG AGT AGA TCT C-3'), TNF-α (5'-ATG AGC ACA GAA AGC ATG ATC CGC-3' and 5'-CC AAA GTA GAC CTG CCC GGA CTC-3') or β-actin specific 5' sense and 3' antisense primers from Clontech Laboratories (Palo Alto, CA, USA). Briefly, 1–5 μl of cDNA was added to a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.0 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 1 μM of each primer, and 0.5 U AmpliTaq polymerase (Perkin-Elmer, Hayward, CA, USA). Each 20 μl sample was overlayed with 25 μl mineral oil (Sigma) and incubated in a DNA Thermal Cycler 480 (Perkin-Elmer) for a total of 30 cycles: 1 min at 94°C, 1 min at 67°C or 60°C (for interferon-γ and β-actin), and 1 min at 72°C. The amplified DNA size, as compared to a positive control (Clontech Laboratories), was 460 bp for interferon-γ, 625 bp for interleukin-1α, 638 bp for interleukin-6, 692 bp for TNF-α, and 540 bp for β-actin. The β-actin primers were used as a control for both reverse transcription and the PCR itself, and also for comparing the amount of products from samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment. Data are representative of three different experiments. Under the employed conditions, control samples from naive mice showed, as a rule, no background cytokine mRNA levels, so that the magnitude of the response to infection and/or treatment could be easily demonstrated.

2.5. Macrophage cultures and production of TNF-α in vitro

Monolayers of plastic-adherent macrophages, obtained by standard techniques from peritoneal exudate cells of naive or infected mice, were exposed overnight to viable *Pseudomonas* cells (10^5/ml) or 1 μg/ml lipopolysaccharide (Sigma), with or without the addition of 10^{-6} M indomethacin. Supernatants were then assayed for TNF content.

2.6. TNF assay

TNF bioactivity in supernatants and sera was measured as cytotoxic activity to WEHI 164 clone 13 murine fibrosarcoma cells (Espevik and Nissen-Meyer, 1986), obtained through the courtesy of P. van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). The assay was performed as described, in the presence of LiCl to optimize sensitivity to TNF-mediated cytotoxicity (Beyaert et al., 1989; Traversari et al., 1992) and using a tetrazolium-based colorimetric assay to estimate mortality of WEHI cells (Campanile et al., 1993). The specificity of the assay was determined by incubating samples with the neutralizing rat anti-mouse TNF-α monoclonal antibody, MP6-XT3. TNF titers were expressed as pg/ml, calculated by reference to a standard curve constructed with known amounts of recombinant TNF-α (Genzyme, Cambridge, MA, USA). Data are the means ± S.E. of triplicate determinations. For measurements of circulating TNF, data are the means for 4 individual mice, each assayed in triplicate.

2.7. Nitrite determination

Monolayers of plastic-adherent macrophages were obtained from peritoneal exudate cells of naive or 2-h infected mice, treated or not with the different drugs. Adherent cells were gently scraped off and resuspended in RPMI 1640 medium containing 10% fetal calf serum, L-glutamine (2 mM), 2-mercaptoethanol (50 μM), and antibiotics. 5×10^5 cells/0.1 ml/well were plated in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA, USA) and incubated overnight (at 37°C in 5% CO_2) in the presence of 100 U recombinant interferon-γ (Genzyme) and 10 ng/ml lipopolysaccharide (Sigma). Fifty μl of supernatant was removed from each well and kept at −20°C for determination of nitrite content. Nitrite concentration, a measure of NO synthesis, was assayed by a standard Griess reaction adapted to microplates, as described previously (Romani et al., 1994). The Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 1 N HCl) and naphthylethylene diamine dihydrochloride (0.15% in H_2O). A volume of 50 μl of reagent was mixed with 50 μl of supernatant and incubated for 30 min in the dark. Absorbance of the chromophore formed was measured at 540 nm in an automated microplate reader. Nitrite was quantitated using $NaNO_2$ as a standard. The data represent the means ± S.E. of quadruplicate determinations and are expressed as μM $NO_2^-/10^6$ macrophages.

2.8. Statistical analysis

In the in vivo infection experiments, each experimental group consisted of 6–8 animals and mortality data were analyzed using the Mann-Whitney *U*-test. Student's *t*-test was used for the results of the in vitro determinations. Each experiment was performed 3–6 times.

3. Results

3.1. Effect of indomethacin administered at the time of Pseudomonas challenge

To clarify the effect of indomethacin treatment on resistance of mice to Gram-negative infection, the animals received the drug at 4.2, 7, or 12 mg/kg 1 h or 1 or 5 days before sublethal challenge with *P. aeruginosa*, and were then examined for course and outcome of disease. Fig. 1 shows that the administration of any dosage of the drug 1 or 5 days before challenge had no effect on resistance to microbial challenge. In contrast, 7 or 12 mg/kg of indomethacin given 1 h before challenge significantly worsened the survival of the treated mice ($P < 0.01$), with nearly 95% mortality at the highest drug dosage. Course of disease, clinical signs, and histopathologic examination all suggested the occurrence of septic shock similar to that found in mice given lethal intravenous inocula of the bacterium (Campanile et al., 1994). In an attempt to clarify the mechanisms of indomethacin toxicity in sublethally infected mice, 12 mg/kg of indomethacin was administered 1 h before infection in all subsequent experiments.

3.2. Cytokine gene expression in indomethacin-treated and infected mice

We evaluated the qualitative expression of genes coding for proinflammatory cytokines in *Pseudomonas*-infected mice treated or not with indomethacin. At 2, 4, 8, or 24 h of infection, we measured mRNA for interferon-γ, interleukin-1, interleukin-6, and TNF-α in the spleens of drug-treated and untreated mice. Uninfected control (naive) mice and mice given the drug in the absence of infection were also assayed (Fig. 2). No significant levels of mRNA coding for any cytokine were expressed by uninfected mice, regardless of drug treatment. Transcripts specific for interleukin-1, interleukin-6, and interferon-γ were readily detected at 2, 4, 8, and 24 h after infection, with no apparent differences between indomethacin-treated and untreated mice. In contrast, TNF-α mRNA was virtually undetectable in the spleens from the latter mice under the conditions of PCR amplification, but was continuously expressed by animals receiving indomethacin at the time of infection. In several independent experiments, maximal and comparable expression of TNF mRNA was observed at 2 and 4 h after *Pseudomonas* challenge.

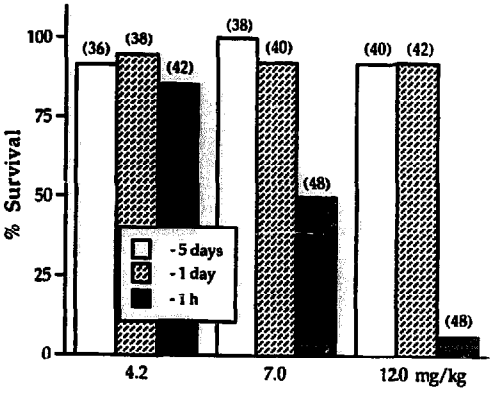

Fig. 1. Effect of indomethacin administration on survival of mice infected with a sublethal inoculum of *P. aeruginosa*. The animals received single injections of 3 different indomethacin dosages 1 or 5 days or 1 h before intravenous challenge with the bacterium. Compiled data from 6 experiments, each consisting of 6–8 animals per group per experiment. Shown in parentheses are total numbers of animals tested per group. The overall survival rate of infected controls not receiving indomethacin (or receiving vehicle alone) was 92.9%.

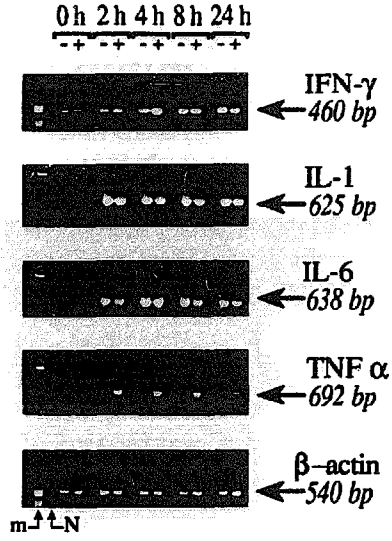

Fig. 2. Interferon-γ (IFN-γ), interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-α mRNA expression in spleen cells from *Pseudomonas*-infected mice revealed by PCR normalized to β-actin. RNA was isolated from control (−) or indomethacin-treated (+) mice either uninfected (time 0) or infected for different times, and the resulting cDNA was used in the PCR with cytokine-specific primers. After amplification, 10 μ2531 of the reaction mix was removed, analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. m, marker track (kb ladder consisting of pBR322 DNA cut with *Hae*III); N, no DNA added to the amplification mix during PCR.

3.3. Induction of TNF-α activity in vitro by indomethacin

To investigate whether indomethacin might actually enhance the synthesis of biologically active TNF-α in response to *Pseudomonas*, peritoneal macrophages were exposed overnight to live bacteria (or lipopolysaccharide) with or without the addition of indomethacin. TNF-α activity was measured in culture supernatants. Table 1 shows that in contrast to lipopolysaccharide, live bacterial cells alone would not induce significant production of TNF-α under the adopted assay conditions. However, in the presence of indomethacin, lipopolysaccharide and *Pseudomonas* resulted in an increased and comparable expression of TNF-α activity. Of interest, indomethacin per se increased TNF-α production in the absence of any stimulus.

Table 1
Effect of indomethacin on production in vitro of TNF-α

Stimulus [a]	TNF-α (pg/ml)	
	Control	Indomethacin
None	0.16 ± 0.09	0.45 ± 0.23 [b]
Lipopolysaccharide	0.41 ± 0.07 [b]	3.51 ± 0.69 [c]
P. aeruginosa	0.15 ± 0.07	3.90 ± 1.07 [c]

[a] Peritoneal macrophages from naive mice were exposed overnight to medium alone, viable *Pseudomonas* (10^5 cells/ml) or lipopolysaccharide (1 μg/ml), with or without the addition of 10^{-6} M indomethacin, and supernatants were assayed for TNF content.
[b] $P < 0.01$ (lipopolysaccharide or indomethacin vs. no treatment).
[c] $P < 0.01$ (indomethacin-treated vs. untreated cultures).

3.4. Detection of TNF-α in sera of indomethacin-treated and infected mice, and effect of pentoxifylline

We next examined the levels of circulating TNF-α in sera of infected mice either untreated or pretreated with indomethacin, indomethacin plus prostaglandin E_2, or indomethacin and pentoxiphylline, a phosphodiesterase inhibitor. Preliminary experiments had shown that pentoxifylline, when administered at 30–50 mg/kg before infection, can significantly improve survival of mice given a lethal intravenous inoculum of *Pseudomonas*, this effect being reversed in a dose-dependent fashion by co-administration of indomethacin (data not shown). Therefore, mice infected with a sublethal bacterial inoculum were treated with indomethacin either alone or in combination with 2 mg/kg prostaglandin E_2 or 30 mg/kg pentoxifylline, and serum levels of TNF-α were measured at 2-h post-infection (Fig. 3). The results showed that higher cytokine levels were present in indomethacin-treated mice, but co-treatment with prostaglandin E_2 or pentoxifylline resulted in TNF-α levels significantly lower than those of infected mice that did not even receive indomethacin. A kinetic analysis (not included in Fig. 3) showed that the 4-h expression (11.6 ± 2.31 pg/ml) of circulating TNF in indomethacin-treated and infected mice was comparable to that (12.8 ± 1.83) found at 2 h, but that circulating TNF had considerably declined at 8 h (3.75 ± 1.25).

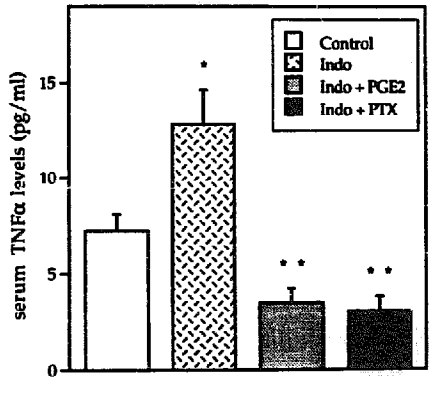

Fig. 3. Levels of TNF-α in sera of *Pseudomonas*-infected mice after treatment with indomethacin (Indo) or Indo + prostaglandin E_2 (PGE_2) or pentoxifylline (PTX). At 2-h post-infection sera from individual mice (4 per group) were assayed in triplicate for TNF-α bioactivity. Results are means ± S.E. * $P < 0.001$ (Indo vs. control); and * * $P < 0.005$ (Indo + PGE_2 or PTX vs. control).

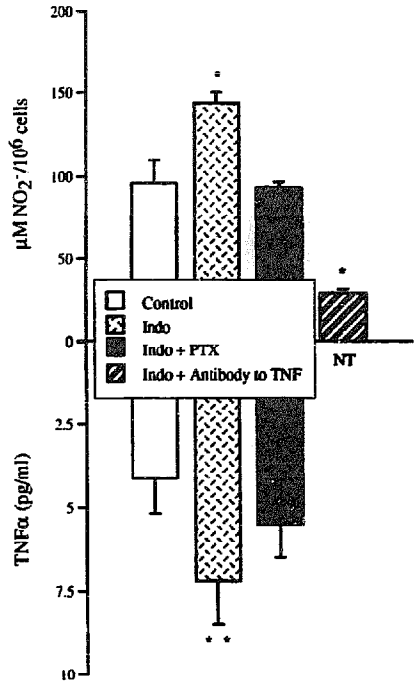

Fig. 4. Upper panel: Production of NO in vitro by macrophages from infected mice treated with indomethacin (Indo) either alone or in combination with pentoxifylline (PTX) or anti-TNF antibody. Cultures were exposed overnight to interferon-γ and lipopolysaccharide prior to assay of their nitrite content. Results (means ± S.E.) are expressed as μM $NO_2^-/10^6$ cells. * $P < 0.005$ (drug treatment vs. control). Lower panel: Macrophage cultures were also assayed for TNF production (pg/ml of supernatant). * * $P < 0.05$ (Indo vs. control). NT, not tested. Controls for NO production included macrophages from infected mice cultured in the absence of interferon-γ and lipopolysaccharide (< 5 μM $NO_2^-/10^6$ cells) as well as interferon-γ + lipopolysaccharide-primed macrophages from naive mice (approximately 50 μM $NO_2^-/10^6$ cells).

3.5. Production of NO in vitro by indomethacin-treated and infected mice, and effect of pentoxifylline or anti-TNF antibody

We also measured the release of NO and TNF-α in vitro by macrophages from infected mice treated with indomethacin either alone or in combination with a single injection of pentoxifylline or anti-TNF antibody. At 2-h post-infection, macrophage cultures were established and exposed overnight to interferon-γ and lipopolysaccharide. Fig. 4 shows that higher levels of NO were released by macrophages from infected mice treated with indomethacin, although co-treatment with pentoxifylline or antibody to TNF completely blocked the enhancing effect of indomethacin or, for anti-TNF, impaired the NO expression of control mice not treated with indomethacin. The pattern of NO production was paralleled by the TNF secretion profile in culture supernatants.

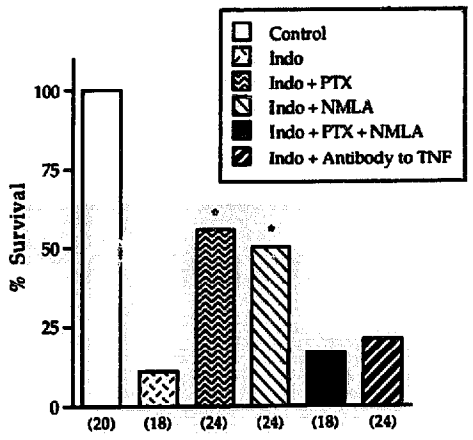

Fig. 5. Effect of pentoxifylline (PTX), NMLA, or anti-TNF antibodies on survival of indomethacin (Indo)-treated and infected mice. Indo-treated animals received 2 injections of PTX (1 h before and 3 h after infection) and/or a single NMLA administration (0.5 h before infection), or anti-TNF antibodies to be challenged intravenously with the bacterium. Compiled data from 3 experiments. Shown in parentheses are total numbers of animals tested per group. * $P < 0.05$ (PTX or NMLA treatment vs. indomethacin alone).

When NO production was measured in macrophage cultures established from naive mice and treated as in the experiment of Table 1, we found that exposure to indomethacin in vitro resulted in a 21.3% increase ($P < 0.05$) in NO release in response to *Pseudomonas*. However, the baseline production of NO induced by bacterial cells was quite limited (12.0 ± 1.6 mM $NO_2^-/10^6$ cells), reflecting the need for externally added interferon-γ to obtain significant NO production by macrophage cultures (see also legend to Fig. 4).

3.6. Effects of pentoxifylline and/or NMLA on indomethacin-induced mortality

Experiments were performed to ascertain whether the administration of pentoxifylline and NMLA (an inhibitor of NO synthase), either separately or in combination, can counteract the adverse effects of indomethacin in sublethally infected mice. Pentoxifylline was given twice at 30 mg/kg, whereas NMLA was given as a single injection at 40 mg/kg, a relatively low dose within the range of doses over which the drug has therapeutic activity (Nava et al., 1992). An additional group of mice was treated with TNF-neutralizing antibodies. We found that either pentoxifylline or NMLA treatment significantly improved survival of the indomethacin-treated and infected mice (Fig. 5). Rather unexpectedly, combined treatment with pentoxifylline and NMLA was without beneficial activity in 2 out of 3 experiments, while in a third, the level of protection afforded was not superior to that of either treatment alone. Although the 2-h expression of circulating TNF-α was greatly reduced by the administration of anti-TNF antibody in the indomethacin-treated and infected mice (from to 14.3 ± 1.31 to 0.35 ± 0.11 pg/ml in one experiment), this anti-TNF strategy did not result in an overall significant protection in 3 different experiments.

3.7. Effects of NMLA on TNF production

Despite the therapeutic activity of early NMLA administration, circulating nitrite could not be measured in indomethacin-treated mice at any time post-infection (data not shown). We examined whether early inhibition of NO synthesis by NMLA might affect *Pseudomonas*-induced TNF production. Mice were treated with indomethacin or indomethacin plus NMLA, as described above. At 2-h post-infection, the levels of circulating TNF were measured in sera and macrophage cultures were established from the same animals. These cultures were exposed overnight to interferon-γ and lipopolysaccharide before measurement of NO and TNF levels in supernatants. We found that NMLA administration decreased the serum levels of TNF-α by an average 10% in 3 independent experiments (from 13.8 ± 1.4 to 12.2 ± 1.2 pg/ml in 1 experiment, $P = 0.41$). The decrease was more evident (> 25%) when the effects of NMLA were examined on TNF-α production in vitro by macrophage cultures. Of interest, the cultures also exhibited a considerable impairment of NO production following exposure of the donor mice to NMLA (Fig. 6).

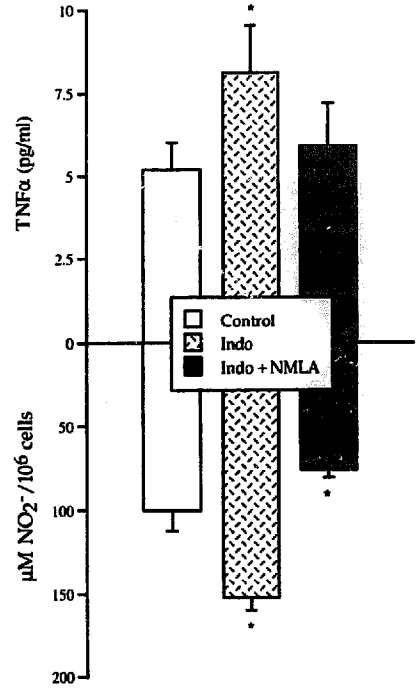

Fig. 6. Upper panel: Production of TNF in vitro by macrophages from infected mice treated with indomethacin (Indo) either alone or in combination with NMLA. Cultures were exposed overnight to interferon-γ and lipopolysaccharide prior to assay of TNF production (pg/ml of supernatant). Lower panel: Macrophage cultures were also assayed for NO release. Results (means ± S.E.) are expressed as μM $NO_2^-/10^6$ cells. * $P < 0.01$ (drug treatment vs. control).

4. Discussion

The present study confirms previous observations in the literature that indomethacin may enhance TNF-α production in vitro and in vivo in response to lipopolysaccharide or other inflammatory stimuli (Sironi et al., 1992; Griswold et al., 1993; Simpson et al., 1994; Utsunomiya et al., 1994). In addition, these data provide evidence for indomethacin-mediated toxicity and TNF-α induction in a live sepsis model, suggesting that TNF-α may contribute to drug-mediated lethality in mice that would otherwise resist systemic challenge with a Gram-negative bacterium.

Of the four different proinflammatory cytokines (interleukin-1, interleukin-6, interferon-γ, and TNF-α) whose message levels were assessed after drug treatment and *Pseudomonas* infection, the pattern of TNF-α mRNA expression appeared to be qualitatively different from that of the other cytokines in indomethacin-treated and untreated mice. Although spleen cells may not be the optimal target for monitoring TNF mRNA, transcripts for this cytokine could not be detected in the untreated mice under the adopted assay conditions, yet these transcripts were continuously expressed by the drug-treated animals. As a result, higher levels of TNF-α were found in the circulation of indomethacin-treated mice, and were also found in supernatants from macrophage cultures exposed to *Pseudomonas* in vitro in the presence of indomethacin. The in vivo effect of the drug on TNF production was reversed by co-administration of prostaglandin E_2 or pentoxifylline, an inhibitor of TNF gene transcription. This demonstrates that the TNF-α-promoting effect of indomethacin was dependent, at least in part, on newly synthesized TNF mRNA.

Pentoxifylline was also capable of inhibiting one functional property of indomethacin-induced TNF-α in vivo, namely the ability to prime peritoneal macrophages for interferon-γ-dependent production of NO in vitro (Oswald et al., 1992). In fact, higher levels of NO were released by macrophages from infected mice treated with indomethacin. The increase in NO production correlated with TNF levels in culture supernatants, and the addition of interferon-γ as co-stimulus was required for the cultures to fully express their NO-producing potential. However, concurrent exposure of donor mice to pentoxifylline or anti-TNF antibody blocked the enhancing effect of the cyclooxygenase inhibitor on NO production, suggesting that the effect was TNF dependent.

Co-injection of pentoxifylline or NMLA with indomethacin resulted in significant and reproducible protection against *Pseudomonas* challenge. Yet, the administration of TNF-neutralizing antibodies resulted in no protection. Similarly ineffective was the combined use of pentoxifylline and NMLA. The protection afforded by the phosphodiesterase inhibitor indicates that overproduction of TNF may be an important mechanism of indomethacin toxicity in our model. However, other anti-inflammatory effects in addition to inhibition of TNF/NO synthesis may have contributed to the beneficial activity of pentoxifylline (Bulut et al., 1993; Sekut et al., 1995). The ineffectiveness of antibody therapy, which nevertheless depleted TNF levels, suggests that a baseline production of TNF may be necessary for the host to cope with infection. An alternative explanation would be that the biologically relevant TNF-α in our model was not readily accessible to antibodies. A critical impairment of the host antimicrobial response, combined with a degree of vasoconstriction detrimental to tissue perfusion (Darville et al., 1993), may also underlie the ineffectiveness of pentoxifylline plus NMLA therapy. Of interest, nitrite production by macrophages from infected mice treated with indomethacin and pentoxifylline was similar to that of mice receiving a sublethal challenge alone, but was greatly impaired in mice on antibody therapy.

Although blockade of the effector function of NO induced by TNF could contribute to the efficacy of NMLA therapy, we were unable to measure circulating nitrite throughout the course of infection. Furthermore, because of its short biological half-life, NMLA would be expected in our model to act primarily via inhibition of the constitutive NO synthase activated by *Pseudomonas*, rather than via inhibition of the inducible NO synthase activated by TNF (Moncada et al., 1991). Because bidirectional influences are known to occur between TNF and NO synthesis (Eigler et al., 1993, 1995), we examined the effect of NMLA treatment on TNF release. Although the inhibition of TNF release was rather limited in vivo, macrophage cultures from mice receiving NMLA were found to produce lower amounts of nitrite and TNF. These data, while confirming the enhancing effect of NO on TNF synthesis (Eigler et al., 1993), strengthen the previous suggestion that NO released by endothelial and vascular smooth muscle cells may exert a paracrine effect on neutrophils and augment the inflammatory response in sepsis by increasing the production of cytokines (Van Dervort et al., 1994). According to this view, the protective effect of NMLA in our model might involve early modulation of the enhancing effect of NO on TNF synthesis.

Because the peak in TNF production induced by indomethacin was a rather early event (2–4 h) in the course of lethal sepsis, it is possible that TNF-α in our system was acting early, or was even acting as an initiator, in the cascade of endogenous mediators that would direct the inflammatory and metabolic responses eventually leading to severe shock and organ failure. This may help to explain the therapeutic efficacy of maneuvers that were expected to affect TNF synthesis only in the initial phase of the syndrome.

The complex timing of mediator release and balance during sepsis makes it difficult to develop successful therapeutic interventions for this syndrome, and the strategy of inhibiting the host inflammatory response may not be entirely beneficial because immune cells and cytokines have both pathogenetic and protective activities (Natanson

et al., 1994). Because of the aforementioned evidence that cyclooxygenase inhibition may be useful in the therapy of experimental sepsis syndromes (Kettelhut et al., 1987), the present study underlines the possible ambivalent role of prostaglandin E_2 in the synthesis and cellular transduction effects of TNF-α. Whether the balance between these two opposing effects is different in local or systemic pathology involving TNF, is unclear. Given the beneficial effects of cyclooxygenase inhibition in disease states, such as rheumatoid arthritis, where local TNF production contributes to pathology (Arend and Dayer, 1995), it is possible that the role of prostaglandin E_2 in mediating TNF toxicity may be dominant in local disease over its effect on TNF synthesis.

In conclusion, the data of the present study emphasize the potentially dual role of cyclooxygenase inhibition in sepsis, calling attention to the possibility that increased TNF production may result from impaired prostaglandin synthesis. The effect is specific for TNF compared to the uninfluenced synthesis of interleukin-1 (also largely produced by macrophages), thus resembling the selective induction of TNF by NO-releasing agents in human mononuclear cells (Eigler et al., 1993). The described system of live sepsis may represent a sensitive model for estimating the efficacy of anti-TNF specific approaches when evaluating drug candidates for the treatment of sepsis.

References

Arend, W.P. and J.M. Dayer, 1995, Inhibition of the production and effects of interleukin-1 and tumor necrosis factor α in rheumatoid arthritis, Arthritis Rheumatism 38, 151.

Beutler, B., 1992, Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine (Raven Press, New York).

Beyaert, R., B. Vanhaesebroeck, P. Suffys, F. Van Roy and W. Fiers, 1989, Lithium chloride potentiates tumor necrosis factor-mediated cytotoxicity in vitro and in vivo, Proc. Natl. Acad. Sci. USA 86, 9494.

Bulut, V., A. Severn and F.Y. Liew, 1993, Nitric oxide production by murine macrophages is inhibited by prolonged elevation of cyclic AMP, Biochem. Biophys. Res. Commun. 195, 1134.

Campanile, F., L. Binaglia, D. Boraschi, A. Tagliabue, M.C. Fioretti and P. Puccetti, 1990, Antibacterial resistance induced by recombinant interleukin 1 in myelosuppressed mice: effect of treatment schedule and correlation with colony-stimulating activity in the bloodstream, Cell. Immunol. 128, 250.

Campanile, F., L. Binaglia, M.C. Fioretti and P. Puccetti, 1991, Modulation of colony stimulating activity in mice: combined effects of IL-1 and bacterial or indomethacin treatment, Int. J. Immunopharmacol. 13, 955.

Campanile, F., A. Bartocci, L. Binaglia, M.C. Fioretti, E.R. Stanley and P. Puccetti, 1992, Modulation of colony-stimulating activity by interleukin 1 in mice: opposing effects of combined treatment with indomethacin or prostaglandin E_2, Int. J. Immunopharmacol. 14, 655.

Campanile, F., A. Giampietri, U. Grohmann, L. Binaglia, M.C. Fioretti and P. Puccetti, 1993, Protective effect of the cyclooxygenase inhibitor indomethacin against *Pseudomonas aeruginosa* infection in neutropenic mice, Cell. Immunol. 147, 341.

Campanile, F., U. Grohmann, A. Giampietri, L. Belladonna, M.C. Fioretti and P. Puccetti, 1994, Cytokine expression in experimental Gram-negative infection, Fund. Clin. Immunol. 2, 97.

Coran, A.G., R.A. Drongowski, J.J. Paik and D.G. Remick, 1992, Ibuprofen intervention in canine septic shock: reduction of pathophysiology without decreased cytokines. J. Surg. Res. 53, 272.

Darville, T., B. Giroir and R. Jacobs, 1993, The systemic inflammatory response syndrome (SIRS): immunology and potential immunotherapy, Infection 21, 279.

Eigler, A., B. Sinha and S. Endres, 1993, Nitric oxide-releasing agents enhance cytokine-induced tumor necrosis factor synthesis in human mononuclear cells, Biochem. Biophys. Res. Commun. 196, 494.

Eigler, A., J. Moeller and S. Endres, 1995, Exogenous and endogenous nitric oxide attenuates tumor necrosis factor synthesis in the murine macrophage cell line RAW 264.7, J. Immunol. 154, 4048.

Espevik, T. and J. Nissen-Meyer, 1986, A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes, J. Immunol. Methods 95, 99.

Evans, T., A. Carpenter, A. Silva and J. Cohen, 1994, Inhibition of nitric oxide synthase in experimental Gram-negative sepsis, J. Infect. Dis. 169, 343.

Fletcher, M.A., T.M. McKenna, E.H. Owens and V.M. Nadkarni, 1992, Effects of in vivo pentoxifylline treatment on survival and ex vivo vascular contractility in a rat lipopolysaccharide shock model, Circ. Shock 36, 74.

Griswold, D.E., L.M. Hillegass, J.J. Breton, K.M. Esser and J.L. Adams, 1993, Differentiation in vivo of classical non-steroidal antiinflammatory drugs from cytokine suppressive antiinflammatory drugs and other pharmacological classes using mouse tumour necrosis factor α production, Drugs Exp. Clin. Res. 19, 243.

Horgan, P.G., J.A. Mannick, D.B. Dubravec and M.L. Rodrick, 1990, Effect of low dose recombinant interleukin 2 plus indomethacin on mortality after sepsis in a murine burn model, Br. J. Surg. 77, 401.

Johnson, R.W. and E. Von Borell, 1994, Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin, J. Anim. Sci. 72, 309.

Kettelhut, I.C., W. Fiers and A.L. Goldgerg, 1987, The toxic effects of tumor necrosis factor in vivo and their prevention by cyclooxygenase inhibitors, Proc. Natl. Acad. Sci. USA 84, 4273.

Kilbourn, R.G. and O.W. Griffith, 1992, Overproduction of nitric oxide in cytokine-mediated and septic shock, J. Natl. Cancer Inst. 84, 827.

Moncada, S., R.M.J. Palmer and E.A. Higgs, 1991, Nitric oxide: physiology, pathophysiology and pharmacology, Pharmacol. Rev. 43, 109.

Mozes, T., F.J. Zijlstra, J.P. Heiligers, C.J. Tak, S. Ben-Efraim, I.L. Bonta and P.R. Saxena, 1991, Sequential release of tumor necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs: protective effects of indomethacin, Br. J. Pharmacol. 104, 691.

Natanson, C., W.D. Hoffman, A.F. Suffredini, P.Q. Eichacker and R.L. Danner, 1994, Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis, Ann. Intern. Med. 120, 771.

Nava, E., R.M.J. Palmer and S. Moncada, 1992, The role of nitric oxide in endotoxic shock: effects of N^G-monomethyl-L-arginine, J. Cardiovasc. Pharmacol. 20 (S12), S132.

Oswald, I.P., T.A. Wynn, A. Sher and S.L. James, 1992, Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation, Proc. Natl. Acad. Sci. USA 89, 8676.

Parant, M., G. Riveau, F. Parant, C.A. Dinarello, S.M. Wolff and L. Chedid, 1980, Effect of indomethacin on increased resistance to bacterial infection and on febrile responses induced by muramyl dipeptide, J. Infect. Dis. 142, 708.

Romani, L., P. Puccetti, A. Mencacci, E. Cenci, R. Spaccapelo, L. Tonnetti, U. Grohmann and F. Bistoni, 1994, Neutralization of IL-10 up-regulates nitric oxide production and protects susceptible mice from challenge with *Candida albicans*, J. Immunol. 152, 3514.

Sekut, L., D. Yarnall, S.A. Stimpson, L.S. Noel, R. Bateman-Fite, R.L. Clark, M.F. Brackeen, J.A. Menius and K.M. Connolly, 1995, Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation, Clin. Exp. Immunol. 100, 125.

Simpson, S.Q., R. Singh and D.E. Bice, 1994, Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-α production by murine macrophages, Am. J. Resp. Cell. Mol. Biol. 10, 284.

Sironi, M., M. Gadina, M. Kankova, F. Riganti, A. Mantovani, M. Zandalasini and P. Ghezzi, 1992, Differential sensitivity of in vivo TNF and IL-6 production to modulation by antiinflammatory drugs in mice, Int. J. Immunopharmacol. 14, 1045.

Stevens, D., A.E. Bryant and S.P. Hackett, 1993, Sepsis syndromes and toxic shock syndromes: concepts in pathogenesis and perspective of future treatment strategies, Curr. Opin. Infect. Dis. 6, 374.

Stevens, M.G., G.W. Pugh and L.B. Tabatai, 1992, Effects of gamma interferon and indomethacin in preventing *Brucella abortus* infections in mice, Infect. Immun. 60, 4407.

Teale, D.M. and A.M. Atkinson, 1992, Inhibition of nitric oxide synthesis improves survival in a murine peritonitis model of sepsis that is not cured by antibiotics alone, J. Antimicrob. Chemother. 30, 839.

Thiemermann, C. and J. Vane, 1990 Inhibition of nitric oxide synthesis reduces the hypothension induced by bacterial lipopolysaccharides in the rat in vivo, Eur. J. Pharmacol. 182, 591.

Traversari, C., P. van der Bruggen, I.F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costesec and T. Boon, 1992, A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E, J. Exp. Med. 176, 1453.

Utsunomiya, I., S. Nagai and S. Oh-ishi, 1994, Differential effects of indomethacin and dexamethasone on cytokine production in carrageenin-induced rat pleurisy, Eur. J. Pharmacol. 252, 213.

Van Dervort, A.L., L. Yan, P.J. Madara, J.P. Cobb, R.A. Wesley, C.C. Corriveau, M.M. Tropea and R.L. Danner, 1994, Nitric oxide regulates endotoxin-induced TNF-α production by human neutrophils, J. Immunol. 152, 4102.