Efficacy of tumor necrosis factor α and eicosanoid inhibitors in experimental models of neonatal sepsis

Giuseppe Mancuso, Vitaliano Cusumano, James A. Cook, Edward Smith, Francesco Squadrito, Giovanna Blandino and Giuseppe Teti

Institutes of *Microbiology and **Pharmacology, Facoltà di Medicina e Chirurgia dell’Università di Messina, Piazza XX Settembre 4, I-92122 Messina, Italy, †Department of Physiology, Medical University of South Carolina, Charleston, South Carolina 29425, USA, ‡Cardiology Therapeutics Research and Development, Mallinckrodt Medical Inc., St. Louis, Missouri 63134, USA, and §Institute of Microbiology, University of Catania, Catania, Italy

(Received 18 February 1994; accepted 3 March 1994)

Abstract: The potential role of tumor necrosis factor α (TNFα) and eicosanoids in the pathogenesis of experimental neonatal sepsis models was investigated. Lethality was induced in neonatal rats by administration of heat killed group B streptococci (GBS, 7 mg kg⁻¹ intracardially) or Salmonella enteritidis endotoxin (0.35 mg kg⁻¹ intracardially). The relative efficacy of six compounds with putative TNFα and eicosanoid inhibitory actions were tested. These were: ibuprofen (3 and 20 mg kg⁻¹), a cyclo-oxgenase inhibitor; CGS85515 (30 mg kg⁻¹), a lipoygenase inhibitor; LY203647 (30 mg kg⁻¹), a leukotriene D₄ receptor antagonist; pentoxifylline (10, 50 and 100 mg kg⁻¹), a TNF inhibitor; cloricromene (2 and 10 mg kg⁻¹), a thromboxane A₂ synthetase inhibitor with TNFα inhibitory actions; and SKF86002 (2.5, 5, 10 and 20 mg kg⁻¹), a dual cyclo-oxgenase/lipoygenase inhibitor with TNFα inhibitory activity. Pentoxifylline, cloricromene and SKF86002, when given intraperitoneally 2 h before challenge, produced reductions respectively, in plasma levels of TNFα at 2.5 h post-injection with killed GBS (P < 0.05). On the contrary, pretreatment with ibuprofen, CGS85515 or LY203647 did not significantly affect TNFα levels. All compounds significantly attenuated the lethality by killed GBS and S. enteritidis endotoxin. These data suggest that TNFα and eicosanoids contribute to the pathogenesis of shock induced by killed GBS and endotoxemia.

Key words: Tumor necrosis factor; Eicosanoid; Group B streptococci; Endotoxin; Neonatal sepsis

Introduction

Tumor necrosis factor α (TNFα), a proinflammatory and vasoactive cytokine, is considered a key mediator in the pathogenesis of septic shock [1]. Infusion of TNFα in experimental animals mimics some features of endotoxin or Gram-negative septic shock including pulmonary hypertension, systemic hypotension, hypoxemia and lung injury [2–4]. Changes similar to those produced by TNFα also occur during natural [5] and experimental [6,7] infections caused by group B streptococcus (GBS), a common neonatal Gram-positive pathogen. Despite specific antibiotics and other aggressive therapeutic interventions, neonatal GBS sepsis is a serious condition associated with a high incidence of mortality [8].

* Corresponding author. Tel: (90) 674318; Fax: (90) 719910.

SSDI 0928-8244(94)00016-M
Although the pathophysiological role of TNFα in intravascular models of Gram-negative sepsis is well established, less is known about Gram-positive septic shock. Moreover, differences between neonatal and adult shock syndromes have not been thoroughly investigated. Recent studies have shown that GBS sepsis produces increased plasma levels of TNFα in piglets [9], mice [10], and suckling rats [11]. In newborn piglets subjected to GBS sepsis, increased plasma levels of thromboxane B₂ (TXB₂) and 6-keto-prostaglandin F₁α have been reported [12,13]. Blockade of cyclooxygenase or thromboxane synthase prevents GBS-induced pulmonary hypertension in piglets, suggesting a pathophysiologic role of TXB₂ [12–14]. In addition to cyclooxygenase products, previous studies have demonstrated that lipooxygenase inhibitors and leukotriene D₄/E₄ (LTD₄/E₄) receptor antagonists can improve survival and/or block certain sequelae of endotoxic shock [12–18]. Since in GBS sepsis TNFα and eicosanoid levels are increased, TNF/lipooxygenase inhibitors may block and attenuate Gram-positive sepsis sequelae in the suckling rat.

The present study ascertained if TNFα and eicosanoid inhibitors can increase survival in neonatal GBS sepsis models and if their effects can be correlated with changes in circulating levels of TNFα.

Materials and Methods

Neonatal rats

Neonatal rats (24–48 h old) Sprague Dawley rats were used for these studies. Parental rats were obtained from Charles River Italia (Calco, Italy). Pups from each litter were randomly assigned to control or experimental groups and kept with the mother.

Drugs

Ibuprofen and pentoxyfilleline (Trental) were purchased from Sigma Chimica, Milan, Italy. The following were kind gifts: LY203647, 1-(2-hydroxy-3-propyl)-4-[4(2-[(4H-tetrazol-5-yl)butyl]-2H-tetrazol-5-yl)butoxy]phenyl)ethanone, of Eli Lilly Pharmaceutical Company (Indianapolis, IN); CGS8515, (methyl 2-[(3,4-dihydro-3,4-dioxo-1-naphthalenyl)methylene]benzoate), of Ciba-Geigy Corporation (Summit, NJ); Clorricromene, 8-monochloro-3-beta-diethylaminoethyl-4-methyl-7-ethoxy-carboxyl-methoxy coumarine, of Fidia Research Laboratories, (Abano Terme, Italy); SKF86002, [5-(4-pyridyl)-6-(4-fluorophenyl)-2,3-dihydroimidazol (2,1-b)thiazole], of SmithKline Beecham, (Philadelphia, PA). CGS8515, ibuprofen and LY203647 were dissolved in saline containing 0.1% NaOH. SKF86002, pentoxyfilline and clorricromene were dissolved in normal saline. All drugs were given intraperitoneally (i.p.) isovolumetrically (100 ml) 2 h before challenge. Control animals received drug vehicles.

Killed GBS and endotoxin models

The type III GBS strain H738 [19] was grown in Todd Hewitt broth (Difco, Diagnostic International Distribution, Milan, Italy) to the late stationary phase, washed with H₂O, killed by heating (80°C for 30 min) and lyophilized. Before each experiment, lyophilized bacteria or lipopolysaccharide (LPS) from Salmonella enteritidis (Boivin preparation, Difco) were weighed and resuspended in phosphate buffered saline (pH 7.2, 0.01 M phosphate, 0.15 M NaCl; PBS). Pups were anesthetized with ether and given intracardiac (i.c.) injections of 7 and 0.35 mg kg⁻¹ of killed bacteria or LPS, respectively, in 25 ml. In preliminary experiments it was determined that intracardiac injections were well tolerated by rat pups.

TNFα plasma levels

Heparinized plasma was collected at 1, 2.5 and 5 h after administration of killed GBS (70 mg kg⁻¹) and stored at −70°C until assayed [11]. TNFα was measured in plasma samples by cytotoxicity in L929 murine fibroblasts, as previously described [10,11].

Statistical analysis

Differences in lethality between control and experimental groups were assessed using Fisher’s exact test. Differences in plasma TNFα levels were analyzed by one way analysis of variance and Student-Newman-Keuls test.
Results

Inhibition of plasma TNFα

Heat-killed GBS induced a peak plasma TNFα response at 2.5 h after which the levels subsided to < 30 U ml⁻¹ (Fig. 1). Ibuprofen, a cyclooxygenase inhibitor, CGS85515, a lipoxygenase inhibitor, or LY203647, an LTD₄ receptor antagonist, did not significantly affect TNFα levels (Fig. 1). On the contrary, drugs previously reported to inhibit endotoxin-induced production of TNFα were all effective in decreasing peak TNFα levels (Fig. 1). These drugs included: clorocromene, a coumarine derivative which inhibits thromboxane B₂ synthesis [20]; SKF86002, an inhibitor of both lipooxygenase and cyclooxygenase pathways of arachidonic acid metabolism [21]; pentoxifylline, a methylxantine derivative capable of modulating several leukocyte functions [22,23]. Percent inhibition of peak TNF levels produced by clorocromene, SKF86002 and pentoxifylline were 45, 52 and 61%, respectively.

![Graph](image)

Fig. 1. Plasma TNFα levels in neonatal rats treated with different drugs before challenge with heat-killed GBS. Plasma samples were obtained from individual animals at various times after i.e. challenge with 70 mg kg⁻¹ of GBS. Animals (3 per group) received the following treatments i.p. 2 h before challenge: normal saline (●); ibuprofen, 3 mg kg⁻¹ (○); CGS85515, 30 mg kg⁻¹ (▲); LY203647, 30 mg kg⁻¹ (□); clorocromene 10 mg kg⁻¹ (●); SKF86002, 10 mg kg⁻¹ (▲); pentoxifylline, 10 mg kg⁻¹ (●). * P < 0.05 by one way analysis of variance and Student-Newman-Keuls test. Points and bars represent means ± standard deviations of three observations.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15/35(43) b</td>
<td>25/35(71) c</td>
<td>32/35(91) c</td>
</tr>
<tr>
<td>CGS85515</td>
<td>4/15(27)</td>
<td>5/15(33) c</td>
<td>10/15(67) c</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>3 mg kg⁻¹</td>
<td>5/23(22)</td>
<td>10/23(43) c</td>
</tr>
<tr>
<td></td>
<td>20 mg kg⁻¹</td>
<td>13/17(76) c</td>
<td>13/17(76) c</td>
</tr>
<tr>
<td>LY203647</td>
<td>30 mg kg⁻¹</td>
<td>5/15(33)</td>
<td>8/15(53) c</td>
</tr>
</tbody>
</table>

b, c Rat pups were injected i.p. with vehicle (control) or drugs 2 h before i.e. challenge with heat-killed GBS (7 mg kg⁻¹).

Data are the pooled results of six experiments.

Lethality in sepsis models

The effects of the cyclooxygenase inhibitor ibuprofen, the lipoxygenase inhibitor CGS85515 and LTD₄ receptor antagonist LY203647 are reported in Table 1. All three agents improved survival of rat pups injected i.e. with killed bacteria. Ibuprofen was effective (P < 0.05) at a dose of 3, but not 20, mg kg⁻¹. TNFα inhibitors also improved survival in this model (Table 2). Clorocromene, was effective at doses of both 2 and 10 mg kg⁻¹ after 72 h (P < 0.05). SKF86002 and pentoxifylline enhanced survival at 10 mg kg⁻¹, but not at higher doses. Similar results were obtained with the TNF inhibitors in an endotoxin model with the most effective dose (P < 0.05) being 10 mg kg⁻¹ at 72 h (Table 3).

Discussion

We have previously shown that prophylaxis with anti-TNFα antibodies can significantly prolong survival in a neonatal rat model of GBS sepsis [11]. Many of the pathogenic effects of TNF can be blocked by nonsteroidal antiinflammatory drugs [24,25] and both cyclooxygenase and lipoxygenase products may modulate TNF pro-
duction [17,21]. In the present study the notion that TNF and eicosanoid blocking agents may be beneficial and modify the course of GBS disease was assessed in a neonatal rat model in which lethality was induced by killed bacteria injected intravascularly. Results were compared to shock induced by endotoxin. Our data show that all of the six eicosanoid or TNF inhibitors tested significantly improved survival, but did not offer complete protection.

This is partially at variance with a previous study in which prophylaxis with anti-TNFα antibodies completely prevented lethality in pups injected i.c. with killed GBS (Mancuso and Teti, unpublished observations). This may be explained by the ability of anti-TNFα antibodies to totally abrogate circulating TNFα while only a 45–61% reduction of TNFα levels was observed with TNF inhibitors. In addition, while anti-TNFα antibodies are highly selective, these drugs have multiple actions, some of which may prevent their beneficial effects as TNF-inhibitors.

The protective effect of pentoxifylline observed here is in agreement with findings in neonatal mice infected with Staphylococcus aureus [26]. In the latter study pentoxifylline and its analogs decreased lethality at a dose of 15 mg kg⁻¹, but had opposite effects at higher doses. Similarly, we observed that pentoxifylline was protective at 10 but not 50 mg kg⁻¹ (Tables 2 and 3). Because pentoxifylline was associated with significant systemic hypotension in piglets when given in combination with TNFα [27], it is possible that the lack of protective activity of relatively high pentoxifylline doses are related to its vasodilatory activities.

Shore et al. [28] found that the cyclooxygenase inhibitor indomethacin was effective in improving survival in rats pups injected with live GBS. Similarly, ibuprofen, also a cyclo-oxygenase inhibitor, was protective in our GBS model. In the present study eicosanoid and TNF inhibitors partially protected neonates against endotoxin shock. The effectiveness of these agents was generally lower.

### Table 2
Protection by TNF inhibitors against mortality induced by heat-killed GBS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lethality 24 h</th>
<th>Lethality 48 h</th>
<th>Lethality 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23/60(38)</td>
<td>43/60(72)</td>
<td>51/60(85)</td>
</tr>
<tr>
<td>Cloricromene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg kg⁻¹</td>
<td>7/21(33)</td>
<td>11/21(52)</td>
<td>13/21(62)</td>
</tr>
<tr>
<td>10 mg kg⁻¹</td>
<td>6/23(26)</td>
<td>10/23(43)</td>
<td>13/23(57)</td>
</tr>
<tr>
<td>SKF86002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mg kg⁻¹</td>
<td>7/16(44)</td>
<td>9/16(56)</td>
<td>11/16(69)</td>
</tr>
<tr>
<td>5 mg kg⁻¹</td>
<td>6/21(29)</td>
<td>13/21(62)</td>
<td>14/21(67)</td>
</tr>
<tr>
<td>10 mg kg⁻¹</td>
<td>7/28(25)</td>
<td>13/28(46)</td>
<td>15/28(54)</td>
</tr>
<tr>
<td>20 mg kg⁻¹</td>
<td>6/15(40)</td>
<td>10/15(67)</td>
<td>13/15(87)</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg kg⁻¹</td>
<td>4/16(25)</td>
<td>6/16(38)</td>
<td>7/16(44)</td>
</tr>
<tr>
<td>50 mg kg⁻¹</td>
<td>6/15(40)</td>
<td>8/15(53)</td>
<td>12/15(80)</td>
</tr>
</tbody>
</table>

* Rat pups were injected i.p. with vehicle (control) or the indicated drugs 2 h before i.c. challenge with heat-killed GBS (7 mg kg⁻¹). Data are the pooled results of 10 experiments.

* No. of dead/total (%).

* P < 0.05 as compared with control by Fisher’s exact two-tailed test.

* P < 0.01 as compared with control by Fisher’s exact two-tailed test.

a

### Table 3
Protection by TNF inhibitors against endotoxin-induced mortality

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lethality 24 h</th>
<th>Lethality 48 h</th>
<th>Lethality 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21/41(51)</td>
<td>31/41(76)</td>
<td>34/41(83)</td>
</tr>
<tr>
<td>Cloricromene</td>
<td>4/20(20)</td>
<td>8/20(40)</td>
<td>8/20(40)</td>
</tr>
<tr>
<td>10 mg kg⁻¹</td>
<td>6/12(50)</td>
<td>7/12(58)</td>
<td>7/12(58)</td>
</tr>
<tr>
<td>10 mg kg⁻¹</td>
<td>3/11(27)</td>
<td>5/11(45)</td>
<td>5/11(45)</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>3/12(25)</td>
<td>4/12(33)</td>
<td>4/12(33)</td>
</tr>
<tr>
<td>50 mg kg⁻¹</td>
<td>6/13(46)</td>
<td>7/13(54)</td>
<td>12/13(92)</td>
</tr>
</tbody>
</table>

* Rat pups were injected i.p. with vehicle (control) or drugs 2 h before i.c. challenge with S. enteritidis LPS (0.35 mg kg⁻¹). Data are the pooled results of eight experiments.

* No. of dead/total (%).

* P < 0.05 as compared with control by Fisher’s exact two-tailed test.

* P < 0.01 as compared with control by Fisher’s exact two-tailed test.
than that previously observed in adult endotoxin models [15,16,20,21,23]. This may relate to the fact that neonatal mice and rats are more sensitive to lethality induced by endotoxin or killed bacteria compared with adult animals (G. Teti, unpublished observations).

All the drugs tested were protective against both endotoxin and killed GBS in our studies. This may reflect similar pathophysiologic roles of TNF and eicosanoids in sepsis induced by Gram-positive and Gram-negative bacteria. The biologic activity of endotoxin has been largely attributed to the lipid A interaction with macrophages which triggers the release of eicosanoids, TNF and other cytokines. Major components of the cell wall of Gram-positive bacteria such as peptidoglycan [29] and lipoteichoic acid [30] are able to activate macrophage secretory and cellular responses. The ‘generic’ response of the macrophage probably in large contributes to similarities in the systemic inflammatory reaction to the Gram-negative and Gram-positive cell wall components.

Because of the continuing serious problem with GBS sepsis in the neonate other adjunctive therapeutic approaches will be necessitated [8]. In addition to therapy with antibiotics and antibodies directed against GBS, agents which block endogenous inflammatory mediators may offer some promise. Whether TNF and eicosanoid inhibition will prove effective in conjunction with antibiotics or anti-GBS antibodies must await further investigation.

Acknowledgements

This study was supported in part by grants ‘40%’ and ‘60%’ of the Ministero dell’ Università e della Ricerca Scientifica e Tecnologica, and by Target project: ‘Biotechnology and Bioinstrumentation’ of the Consiglio Nazionale delle Ricerche of Italy.

References


