The toxic effects of tumor necrosis factor in vivo and their prevention by cyclooxygenase inhibitors

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ABSTRACT Tumor necrosis factor (TNF) is a macrophage product under active study as an anticancer drug. However, this agent can be very toxic and has been implicated in the pathogenesis of endotoxic shock. After intravenous injection of human recombinant TNF (4 μg/g), growing rats showed an unusual constellation of physiological responses, and all died within 2-4 hr. In 1 hr, TNF caused a sharp fall (2.5°C) in body temperature and a large increase in plasma prostaglandin E2 levels. Blood glucose initially increased, but then a profound hypoglycemia developed by 2 hr. The TNF-treated animals also showed diarrhea, cyanosis, and a severe metabolic acidosis. A single injection of the cyclooxygenase inhibitors indomethacin or ibuprofen before the TNF treatment completely prevented the rapid killing and reduced eventual lethality by 70%. These agents blocked prostaglandin E2 production and prevented the hypothermia, changes in blood glucose, acidosis, and other symptoms. Since similar physiological changes have been reported after endotoxin injection, our data support the suggestion that TNF production is a critical factor in the development of septic shock. These findings also indicate that increased production of prostaglandins or thromboxanes is important in endotoxic shock and argue that cyclooxygenase inhibitors should be useful in its therapy. Indomethacin did not block the cytotoxic effects of TNF in vitro on several transformed cell lines (HeLa, Me 180, or L929). Therefore, combined use of TNF with a cyclooxygenase inhibitor may allow safer administration of high doses of this polypeptide to cancer patients.

Abbreviations: TNF, tumor necrosis factor; PGE2, prostaglandin E2; DHK-PGE2, 13,14-dihydro-15-keto-PGE2.

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reflected changes in PGE₂ release into plasma (32). Arterial blood from the abdominal aorta was collected under ether anesthesia in heparinized syringes prior to CO₂ analysis and blood pH measurements. The rectal temperature was measured with an electronic thermometer (model 497A, Yellow Springs Instrument). All results are expressed as means ± SEM, and the statistical significance of changes seen were evaluated with the unpaired Student’s t test.

RESULTS

Lethal Effects of TNF and Protection by Cyclooxygenase Inhibitors. Intravenous injection of human recombinant TNF (4 μg/g) into the young growing rats caused death of all the animals within a few hours. In the first hour no deaths occurred, but thereafter, there was a progressive loss of viability (Fig. 1 Upper). In a typical experiment, ~20% of the animals that received TNF died in 2 hr, and by 4 hr all of this group were dead.

Because TNF stimulates PGE₂ production in many cells and because inhibitors of arachidonic acid cyclooxygenase (i.e., prostaglandin synthase) can prevent fever induced with subcutaneous TNF (I.C.K. and A.L.G., unpublished data), we investigated whether such inhibitors could influence the TNF-induced lethality. When indomethacin was injected 2 hr before the TNF, it provided a dramatic protection against the rapid killing by TNF (Fig. 1 Upper). Of the 20 animals that received a single injection of indomethacin prior to the TNF, all were alive 4 hr later, whereas all those receiving TNF alone had died (16/16) (Fig. 1 Upper). About one-quarter of the rats (7/20) receiving the indomethacin plus TNF did succumb during the subsequent 20 hr. However, all others remained alive and appeared to grow normally on subsequent days. Not only did a single indomethacin injection prevent most of the mortality when given before the TNF, it also offered clear protection when injected 1 hr after the TNF (data not shown).

In animals treated with a single injection of ibuprofen, another cyclooxygenase inhibitor (Fig. 2 Upper), a clear protection against TNF was also observed, although this protective effect was smaller than that with indomethacin. About 75% of the ibuprofen-treated animals were still alive 6 hr after the TNF injection, and most of the animals (55%) were alive and appeared healthy after 24 hr. The greater protection afforded by indomethacin may be because this agent can inhibit other processes besides the cyclooxygenase (33) or because it inhibits the cyclooxygenase for longer periods than ibuprofen. In fact, ibuprofen provided a large protection initially, which decreased later (Fig. 2) as prostaglandin production returned to the original level (see below).

Because TNF stimulates PGE₂ synthesis in several cell types (34) and because of this dramatic protection by the cyclooxygenase inhibitors (Fig. 1 Upper), we attempted to measure body prostaglandin production after TNF injection by determining the levels of the stable metabolite of PGE₂ (DHK-prostaglandin) in plasma. As shown in Table 1, there was a very large (10-fold) increase in the blood content of DHK-prostaglandin within 1 hr after injection, and these high levels were maintained for several hours. Others have noted a similar dramatic increase in this metabolite of PGE₂, as well as of thromboxane B₂ and 6-keto-PGF₁α in endotoxin-treated animals (35, 36). As expected, indomethacin and ibuprofen were found to block completely the large increase in the serum levels of DHK-prostaglandin. For example, in the rats that received indomethacin and TNF, this metabolite of PGE₂ was extremely low (Table 1) for 5 hr after injection of

![Fig. 1](image1.png)

**Fig. 1.** Mortality and changes in body temperature of animals injected intravenously with human recombinant TNF (●) or with indomethacin 2 hr before the TNF (▲). The control animals (○) received only PBS intravenously and the others (●) were injected with indomethacin (i.p.) and PBS 2 hr later. The values for temperature represent the means ± SEM for five to eight animals for each point.

![Fig. 2](image2.png)

**Fig. 2.** Mortality and changes in body temperature in animals injected with TNF intravenously (●) or with ibuprofen (20 mg/kg) 2 hr before the TNF (▲). The control animals (○) received only PBS intravenously and the others (●) were injected with ibuprofen (i.p.) and PBS 2 hr later. The values for body temperature represent the means ± SEM for five to eight animals for each point.
the inhibitor. In those receiving ibuprofen and TNF, DHK-
prostaglandin was also much lower than in the rats receiving only TNF.

Physiological Changes Induced by TNF. A variety of studies were
done to clarify the mechanisms responsible for the rapid killing by TNF and the protection by cyclooxygenase inhibitors. The loss of viability was preceded by a sharp and progressive decrease in body temperature (Figs. 1 Lower and 2 Lower). Within 1 hr after injection, the mean rectal temperature had fallen from 36.8°C to 34.3°C. Between 3 and 4 hr (i.e., just prior to death), their temperature dropped between 29.5°C and 32°C. The dramatic fall in body temperature to 1 hr clearly preceded other toxic symptoms (see below) and thus seems to be a specific physiological effect of TNF rather than a consequence of the loss of viability. This hypothermic effect is particularly noteworthy since at lower intravenous doses, or when the same amount of TNF is injected subcutaneously, TNF induces a fever that can be blocked with cyclooxygenase inhibitors (I.C.K. and A.L.G., unpublished data).

The cyclooxygenase inhibitors also prevented the rapid fall in body temperature and the subsequent progressive hypothermia seen in the animals receiving only TNF. Of those treated with ibuprofen or indomethacin, several showed a slight fall in temperature (0.5°C–1°C), which quickly returned to normal levels (Figs. 1 and 2). If the indomethacin was injected 1 hr after the TNF (when hypothermia was already evident), it caused a rapid increase in body temperature toward normal and it reduced mortality. These findings suggest that some unidentified product of the cyclooxygenase reduces body temperature. This conclusion is quite surprising, since PG2 has generally been associated with induction of fever (37) rather than hypothermia, and inhibitors of prostaglandin synthesis are generally administered to combat fever.

One unusual response noted previously in experimentally induced endotoxin shock is an initial increase in plasma glucose followed by a severe hypoglycemia (28) that may be life threatening. Because of the possible role of TNF in mediating such effects, we measured the blood glucose content at different times after TNF injection. Large biphase changes in blood glucose were found (Fig. 3). Within 1 hr, a clear hyperglycemia developed, followed by a sharp decrease in plasma glucose levels to −30 mg per 100 ml (1.6 mM). By 3 hr after TNF injection, most of the animals were near death, but the blood glucose levels had fallen even further. Such values, if sustained, are too low for survival. The animals receiving indomethacin also did not show significant changes in plasma glucose levels (Fig. 3), in sharp contrast to the large biphase changes in the rats receiving TNF alone. Ibuprofen injection before the TNF also decreased the changes in blood glucose. After 4 hr, the surviving animals that had received

Table 1. Plasma DHK-PGE2 before and after intravenous administration of TNF or saline into rats

<table>
<thead>
<tr>
<th>Injection</th>
<th>DHK-PGE2, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>TNF</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>TNF 2 hr after</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>indomethacin</td>
<td></td>
</tr>
<tr>
<td>Saline 2 hr after</td>
<td>0.35 ± 0.10</td>
</tr>
<tr>
<td>indomethacin</td>
<td></td>
</tr>
<tr>
<td>TNF 2 hr after</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>ibuprofen</td>
<td></td>
</tr>
<tr>
<td>Saline 2 hr after</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>ibuprofen</td>
<td></td>
</tr>
</tbody>
</table>

Initial measurements were made immediately before giving the TNF or saline. *P < 0.001 compared to TNF-treated animals.

TNF and ibuprofen had glucose levels ~40% lower than the untreated control rats. However, this decrease was much less than that in animals treated only with TNF.

By 3 hr after TNF injection, the animals also showed a severe metabolic acidosis. Their arterial pH fell, their Pco2 decreased by ~30%, and their arterial bicarbonate concentration was about one-half that in the controls (Table 2). These changes are similar to those seen in patients with a severe acidosis due to sepsis, diabetes, shock, etc. The rats that received indomethacin before the TNF did not show a metabolic acidosis (Table 2). Their blood bicarbonate levels were 50% higher than in animals that received only TNF, and 3 hr after TNF injection, their arterial Pco2 and pH were indistinguishable from those in control rats. Another symptom that became evident about 1 hr after TNF injection was diarrhea. In the hour before death, the animals appeared very lethargic and showed cyanosis in their extremities. None of these symptoms was evident in the rats that received indomethacin before the TNF. Both diarrhea and cyanosis have also been reported in experimental endotoxin-induced shock (38–40).

Although these various toxic effects resemble ones reported in endotoxin shock (Table 3), they cannot be explained by the very low levels of endotoxin in the TNF (~0.12 ng per mg of protein). To make sure that these effects were not due to contamination by endotoxin, the TNF solution was heated at 70°C for 15 min. This treatment should destroy TNF (1) activity but does not affect endotoxin. When the heated material was injected, it did not cause death of the animals, a decrease in body temperature, or diarrhea. Thus TNF, through a product of the cyclooxygenase enzymes, elicits these toxic responses.

Table 2. Effects of TNF and indomethacin on arterial pH, Pco2, and bicarbonate concentrations

<table>
<thead>
<tr>
<th>Injection</th>
<th>pH</th>
<th>Pco2, mm Hg</th>
<th>HCO3, μmol/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>7.48 ± 0.02</td>
<td>29.9 ± 0.3</td>
<td>21.4 ± 1.1</td>
</tr>
<tr>
<td>TNF</td>
<td>7.33 ± 0.02*</td>
<td>22.2 ± 2.1*</td>
<td>11.1 ± 0.9†</td>
</tr>
<tr>
<td>TNF 2 hr after</td>
<td>7.43 ± 0.02</td>
<td>26.0 ± 1.6</td>
<td>16.7 ± 1.0‡</td>
</tr>
<tr>
<td>indomethacin</td>
<td>7.35 ± 0.06</td>
<td>39.3 ± 5.0</td>
<td>20.7 ± 0.5</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to saline control. †P < 0.01 compared to saline control. ‡P < 0.05 different from TNF group.
**Table 3. Summary of physiological changes evident after injection with high concentration of TNF or endotoxin**

<table>
<thead>
<tr>
<th></th>
<th>TNF (mg/kg)</th>
<th>Endotoxin (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Decreases</td>
<td></td>
</tr>
<tr>
<td>Blood glucose</td>
<td>Increases then decreases</td>
<td>Changes (41)</td>
</tr>
<tr>
<td>Serum PGE$_2$</td>
<td>Large increase*</td>
<td>Increases then decreases sharply*</td>
</tr>
<tr>
<td>Cyanosis</td>
<td>Observed*</td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Observed*</td>
<td></td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>Observed*</td>
<td>Observed (42, 43)</td>
</tr>
</tbody>
</table>

*Cyclooxygenase inhibitors prevent.

**DISCUSSION**

**TNF and the Physiology of Shock.** It seems likely that TNF production plays an important protective role in combating microbial infections and neoplastic disease. Presumably, the benefits to the organism from TNF production must be rather large, since TNF is very toxic at high doses. This ability of TNF to kill rapidly (Figs. 1 and 2) is probably due to multiple physiological actions, which seem to result from a dramatic increase in synthesis of prostaglandin and related metabolites (Table 1). The drop in body temperature (Figs. 1 and 2), the hyperglycemia and subsequent hypoglycemia (Fig. 3), the acidosis (Table 2), diarrhea, and cyanosis could all be prevented by use of cyclooxygenase inhibitors. The crucial product(s) of this enzyme and their exact role in these various responses are completely unclear. However, it is noteworthy that administration to rats of high levels of PGE$_2$ can cause diarrhea (A. Tashjian, personal communication), and prostaglandins have been reported to promote insulin release (44). The apparent requirement for some prostaglandin or thromboxane for the lowering of body temperature is particularly intriguing (Fig. 2) and is without precedent in the literature. Obviously, the cyclooxygenase inhibitors should be very useful tools in clarifying the mechanisms responsible for these unusual pathologic responses.

The present findings provide strong support for the suggestion by Beutler and Cerami that TNF is a critical factor in the pathogenesis of septic shock (19, 20, 27). Virtually all the physiological responses noted here after administering high concentrations of TNF were ones reported previously by one group or another upon endotoxin treatment of animals. For example, large increases in circulating prostaglandins have been reported in a variety of experimental models for shock (36, 45–48), and thromboxanes, PGI$_2$, and PGE$_2$ have also been proposed as important mediators of irreversible shock (35, 36, 45–48). Furthermore, administration of nonsteroidal anti-inflammatory drugs has been reported to improve survival and circulatory function in endotoxemic and hemorrhagic shock (49, 50). Even though these agents seem to reduce the lethal effects of endotoxin and TNF in animals, they have not been used clinically in shock patients (51). The present findings provide a clear rationale for their usefulness in prevention or treatment of acute septic shock (43), since they clearly can block a number of life-threatening sequelae of high-circulating levels of TNF (Table 3). In our studies, a single injection of cyclooxygenase inhibitors provided complete protection against the very rapid killing by TNF, although some of the treated animals (30%) died of unknown causes between 6 and 24 hr. Possibly, repeated administration of indomethacin or ibuprofen would have reduced this mortality further.

Thus even though activated monocytes can release many potent factors into the circulation, such as interleukin 1, overproduction of TNF may itself account for most of the life-threatening symptoms of irreversible shock. One surprising finding with TNF was the large fall in body temperature, since TNF itself is pyrogenic at low doses and since endotoxin elicits interleukin 1 production and fever (52). However, we have found that injection of high doses of *Escherichia coli* endotoxin causes hypothermia (unpublished observations). Thus, the effects of high and low doses of endotoxin parallel the changes observed with different doses of TNF. At low doses, TNF causes fever, as may occur in mild infections; at high levels, it causes hypothermia, such as that associated with septic shock. A dramatic decrease in temperature, similar to that induced with intravenous TNF (Figs. 1 and 2) has also been observed in dogs receiving lethal doses of *E. coli* (41). This phenomenon was attributed to impaired cardiovascular function (43, 53) leading to insufficient supply of substrates for heat production in peripheral tissues (54). Although possible, the rapidity of the fall in temperature with TNF and its reversal by indomethacin suggest a more specific hypothermic mechanism perhaps mediated by an unidentified product of the cyclooxygenase pathways.

In the present studies, a severe metabolic acidosis was evident 3 hr after the TNF injection (Table 1), as is commonly observed in various forms of shock (43). A similar decrease in arterial pH has been reported in endotoxin-treated dogs, which can be prevented with ibuprofen (51) in accord with the present observations. An initial hyperglycemia followed by hypoglycemia has also been observed after administration of endotoxin to rodents (28). Increased secretion of insulin has been suggested as the cause of this hypoglycemia (55). However, in late stages of shock, glucose uptake by peripheral tissues may increase even before a significant increase in serum insulin occurs (56). In cultured myotubes, TNF can stimulate glucose uptake and oxidation (57); if a similar effect occurs in adult tissues, it could contribute to the severe hypoglycemia (Fig. 3) observed after TNF or endotoxin injection.

**Applications of TNF in Cancer Therapy.** Although TNF has real potential as an anticancer drug (1, 7, 20, 58, 59), its toxicity, especially at high doses, may be a major obstacle to its therapeutic use. The present findings suggest that TNF may be administered to patients more safely by injecting it simultaneously with a cyclooxygenase inhibitor. Although indomethacin or ibuprofen can reduce the toxicity of TNF, it is possible that such agents also block the cytotoxic and cytostatic actions of TNF on various tumors. We therefore tested this possibility using several transformed cell lines:

![Fig. 4. Effect of indomethacin on TNF-induced cytotoxicity. HeLa D98/AHZ. Cells were seeded in microtiter plates; after 1 day (when the cells were still subconfluent), indomethacin and TNF were added at increasing concentrations. Three days later, the remaining viable cells were stained with crystal violet (7). The results are expressed as the percentage of surviving cells relative to the control without TNF after correction for the cells present at day 1.](image-url)
HeLa cells, L292 mouse fibroblasts, and M180 cells. The response to TNF was not affected in HeLa (Fig. 4) or M180 cells and was reduced only slightly in L292 cells treated with concentrations of indomethacin (50 μM) that should prevent all prostaglandin synthesis (data not shown). Thus the cytotoxic effects of TNF on these transformed cells seem largely independent of prostaglandin production.

These findings argue that indomethacin may be useful in reducing the toxic effects of TNF without preventing its anti-neoplastic actions. This conclusion will have to be confirmed with various transplanted tumors in vivo, where direct cytotoxicity by TNF may not be the only mechanism contributing to its anti-neoplastic actions (e.g., local necrosis). Nevertheless, the findings in Fig. 4 emphasize that not all TNF effects involve prostanooids and suggest that combined administration of TNF and a cyclooxygenase inhibitor could enhance the therapeutic usefulness of TNF. This attractive possibility, as well as the possible use of such inhibitors in treating septicemia, should be tested systematically.

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