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G 619, a dual thromboxane synthase inhibitor and thromboxane A_2 receptor antagonist, inhibits tumor necrosis factor-α biosynthesis

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Abstract

G 619 is 3-carbamyl-(3'-picolyl)-4-methoxy-1-benzamide. The compound is structurally related to picotamide, a previously reported dual thromboxane synthase inhibitor/thromboxane A_2 receptor antagonist, which displays inhibitory activity on tumor necrosis factor-α. The aim of the present work was to study the effect of G 619 on tumor necrosis factor-α synthesis both in vivo and in vitro. Salmonella enteritidis lipopolysaccharide was used to induce tumor necrosis factor-α production. Septic shock was produced in male rats by a single intravenous (i.v.) injection of 20 mg/kg (LD_{90}) of Salmonella enteritidis lipopolysaccharide. Rats were pretreated with G 619 (50 mg/kg, i.v.) or vehicle (1 ml/kg, i.v.) 1 h before endotoxin challenge. Salmonella enteritidis lipopolysaccharide administration dramatically reduced survival rate (0%, 72 h after endotoxin administration), reduced mean arterial blood pressure, increased plasma levels of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ and enhanced serum levels of tumor necrosis factor. Furthermore, endotoxic shock produced characteristic gastric damage, consisting of haemorrhagic infiltrates. Pretreatment with G 619 in vivo significantly protected against Salmonella enteritidis lipopolysaccharide-induced lethality (80% survival rate and 60% survival rate 24 h and 72 h after Salmonella enteritidis lipopolysaccharide injection, respectively), reduced hypotension, decreased plasma thromboxane B_2 and serum tumor necrosis factor-α levels and enhanced blood levels of 6-keto-prostaglandin $F_{1\alpha}$. In rat peritoneal macrophages, G 619 in vitro (25, 50 and 100 μM) significantly blunted ($P < 0.001$) Salmonella enteritidis lipopolysaccharide-stimulated production of tumor necrosis factor-α, whereas it increased 6-keto-prostaglandin $F_{1\alpha}$ and cyclic AMP levels. The present data indicate that G 619 may be useful during disease states characterized by elevated tumor necrosis factor-α levels.

Keywords: G 619; TNF-α (tumor necrosis factor-α); Endotoxin shock; cAMP

1. Introduction

Tumor necrosis factor-α is a pleiotropic cytokine produced mainly by mononuclear phagocytes in response to endotoxin or other products derived from bacteria, viruses or parasites. There has been growing interest regarding this cytokine. It has been demonstrated that tumor necrosis factor-α, besides its important role in the pathogenesis of septic shock (Cerami, 1992; Tracey et al., 1989), is also involved in various other clinical situations. This has been confirmed by a number of studies reported over the last few years showing that tumor necrosis factor-α represents an important mediator of non-septic shock (Squadrito et al., 1992a) as well as of myocardial ischaemia (Squadrito et al., 1993a). Moreover, tumor necrosis factor-α can be found in patients with inflammatory bowel disease (Braegger et al., 1992), chronic heart failure (McMurray et al., 1991), coronary atherosclerosis (Arbustini et al., 1991) and endometriosis (Rock and Markham, 1992). These findings justify the search for new phar-

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macological approaches in order to reduce tumor necrosis factor-α synthesis or production. It has been suggested that eicosanoids may affect the production of tumor necrosis factor: in fact cyclic AMP enhancing eicosanoids (i.e. prostacyclin and prostaglandin E_2) regulate cytokine production (Katakami et al., 1988). More specifically previous findings have indicated that prostaglandins have inhibitory effects on Salmonella enteritidis lipopolysaccharide-stimulated Interleukin-1 and tumor necrosis factor production (Kunkel and Chensue, 1985; Kunkel et al., 1986a,b). Since stimulated macrophages possess a marked capacity for prostaglandin E_2 production, this released prostaglandin could act as an endogenous regulator of macrophage function (Kunkel and Chensue, 1985; Kunkel et al., 1986a,b; Brandwein, 1986). Prostacyclin (PGI_2) has been shown to activate membrane adenylate cyclase, resulting in the elevation of intracellular cyclic adenosine 3′:5′ monophosphate (cAMP) levels, which in turn may inhibit certain macrophage functions (Remold-O'Donnell, 1974; Gemsa et al., 1975; Verghese and Snyderman, 1983) and tumor necrosis factor-α production (Katakami et al., 1988).

Therefore, in the light of these findings, drugs that shunt eicosanoid metabolism towards prostaglandin production are expected to exert effects on tumor necrosis factor-α synthesis.

G 619 [3-carbamyl-(3′-picolyl)-4-methoxy-1-benzamide] is structurally related to picotamide, a previously reported dual thromboxane synthase inhibitor/thromboxane receptor antagonist (Gresele et al., 1989), that enhances prostacyclin production (Squadrito et al., 1993b).

The present study was, therefore, designed to evaluate the effects of this compound on the pathophysiological events associated with endotoxic shock and on tumor necrosis factor-α production.

2. Materials and methods

2.1. Endotoxin shock procedure

Male Sprague-Dawley rats (200–220 g), fed on a standard diet and with water ad libitum, were used. Environmental conditions were standardized, including a room temperature of 22 ± 2°C and 12-h artificial lighting. Endotoxin shock was induced by administering a single dose of 20 mg/kg of Salmonella enteritidis endotoxin (Boivin preparation, Difco Laboratories). Control rats received an equal volume of vehicle (NaCl).

2.2. Survival evaluation

1 h before endotoxin injection, control rats received a 1 ml/kg 0.9% NaCl i.v. bolus, and treated rats received G 619 (50 mg/kg) as i.v. bolus. Survival rate was evaluated for 72 h after endotoxin administration.

2.3. Arterial blood pressure

A second group of rats was used to monitor blood pressure as described elsewhere (Caputi et al., 1980). Briefly, the animals were anesthetized with urethane (1.3 g/kg) and a cannula (PE 50) was inserted into the left common carotid artery. The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure, monitored for 6 h, was displayed on channels of a polygraph. Arterial blood pressure is reported as mean arterial pressure in mmHg. These rats were subjected to the same experimental protocol as described above.

2.4. Histological examinations

Pretreated shocked and control rats were used for histological examination. The rats were killed 4 h after endotoxin or saline challenge and the gut was removed. The stomach was dissected out and rinsed with saline; representative sections were taken, fixed in 10% formalin, and later stained with haematoxylin and eosin and examined under a light microscope.

2.5. Biological assay for tumor necrosis factor-α activity

Killing of L929 mouse tumor cells was used to measure tumor necrosis factor levels in plasma and in the peritoneal macrophage supernatants on the basis of a standard assay (Ruff and Gifford, 1980). L929 cells in RPMI 1640 medium containing 5% fetal calf serum were seeded at 3×10^4 cells per well in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. Serial 1:2 dilutions of serum (drawn 2 h after endotoxin) and supernatants of peritoneal macrophages (collected 2 h after endotoxin as previously described by Altavilla et al., 1989), were made in the above-described medium containing 1.0 μg of actinomycin D per ml and 100 μl volumes of each dilution were added to the wells. On the next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol), and 0.1 ml of 1% sodium dodecyl sulphate was added to each well to solubilize the stained cells. The absorbance of each well was read at 490 nm with a model BT-100 Microelisa Autoreader. Percentage of cytotoxicity was calculated as $1 - ([\text{A490 of sample}/\text{A490 of control}]) \times 100$. One tumor necrosis factor-α unit was defined as the amount of tumor necrosis factor-α giving 50% cell cytotoxicity. Tumor necrosis factor-α content in the samples was calculated by comparison to a calibration curve made with recombinant murine tumor necrosis factor-α (Nuclear Laser Medicine, Milan,

Italy). To verify if the cytotoxicity tested was due to the presence of tumor necrosis factor-α or to other factors, the assay was repeated after the positive samples were neutralized with a polyclonal rabbit anti-murine tumor necrosis factor antiserum which cross-reacts with rat tumor necrosis factor. A negative assay after this treatment confirmed that the cytotoxic activity was due to tumor necrosis factor. For the in vitro studies macrophages were incubated for 2 h either with Salmonella enteritidis lipopolysaccharide (5 μg/ml) + RPMI (vehicle) or with several doses of G 619 (25, 50 and 100 μM).

2.6. Radioimmunoassay of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$

Blood was collected in plastic syringes containing 0.1 ml of indomethacin (4 mg/ml in 0.1 M phosphate buffer, pH 8.0) and 500 U of heparin. The blood was centrifuged (1500 × g for 20 min), and the plasma was collected and frozen at −20°C until extraction. Macrophages were prepared as described previously (Altavilla et al., 1989). For the immunoreactive thromboxane B_2 assay, [^{3}H]thromboxane B_2 was added to a 100-μl plasma sample. The plasma was acidified to pH 3 or 4 with HCl and then extracted with three volumes of ethyl acetate. The ethyl acetate layer was removed and dried under nitrogen. The dried extract was then reconstituted in 0.2 ml phosphate buffer. Thromboxane B_2 was determined as described previously (McCann et al., 1981). The minimum detectable amount was < 8.2 pg/100 μl of plasma. For the immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ (i6-keto-prostaglandin $F_{1\alpha}$) assay, [^{125}I]6-keto-prostaglandin $F_{1\alpha}$ was added to a 100-μl plasma sample. The plasma was extracted, reconstituted, and evaluated as described above. Macrophages were collected and prepared as shown previously (Altavilla et al., 1989). The minimum detectable amount was < 4.1 pg/100 μl of plasma.

2.7. Cellular cAMP content

Monolayers of macrophages (10^6 cells/cm^2) in 24-well tissue culture dishes were prepared as previously described (Altavilla et al., 1989) and stored at −30°C until the assay was done. cAMP extraction was performed as described previously (Kinoshita et al., 1986). In brief, the wells were subjected to freezing and thawing three times and the cells were then removed and transferred to tubes. Following centrifugation for 15 min at 2000 × g, the supernatants were extracted with diethylether and stored at −20°C. cAMP concentrations were measured with a commercially available radioimmunoassay (Amersham). The assay was based on the competition between unlabelled cAMP and a fixed quantity of ^{125}I-labelled cAMP. cAMP may be measured in the range between 0.2–12.8 pmol/l.

2.8. Statistics

The differences between the means of the two groups were evaluated using the analysis of variance followed by Bonferroni's test and were considered significant when $P < 0.05$. For survival data, statistical analysis was performed using Fisher's exact probability test.

3. Results

3.1. Survival

Table 1 shows the ratio of animals surviving in each group to the total number of animals in the group and the percentage survival of each group throughout the

Table 1
Effect of G 619 on survival rate in rats subjected to endotoxin shock

Treatment	Hours after Salmonella enteritidis lipopolysaccharide (Surviving animals)				
	3	6	12	24	72
Sham + vehicle (1 ml/kg)	10/10	10/10	10/10	10/10	10/10
Sham + G 619 (50 mg/kg)	10/10	10/10	10/10	10/10	10/10
LPS + vehicle	10/10	5/10	3/10	2/10	0/10
LPS + G 619 (12.5 mg/kg)	10/10	8/10 [a]	6/10	4/10	2/10
LPS + G 619 (25 mg/kg)	10/10	10/10 [b]	8/10	6/10	3/10
LPS + G 619 (50 mg/kg)	10/10	10/10 [b]	10/10 [b]	8/10 [a]	6/10 [a]

G 619 or vehicle (1 ml/kg) was injected intravenously 1 h before endotoxin challenge. [a] $P < 0.05$; [b] $P < 0.001$.

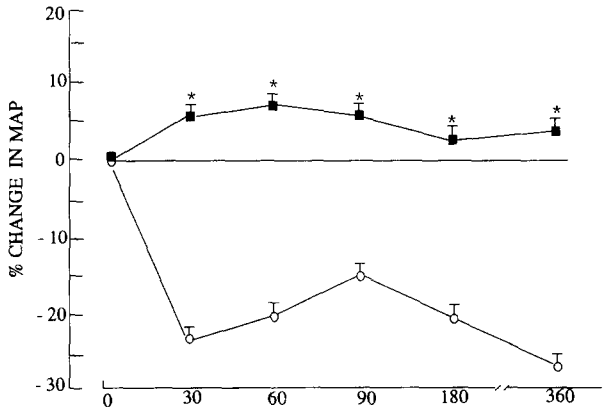


Fig. 1. Effects of G 619 (50 mg/kg, i.v.; closed squares) or vehicle (1 ml/kg, i.v.; open circles) on mean arterial blood pressure in rats subjected to endotoxin shock (Salmonella enteritidis lipopolysaccharide). Each point represents the mean ± S.D. for seven animals. $^{*}P < 0.001$ vs. vehicle.

study. Salmonella enteritidis lipopolysaccharide administration significantly reduced survival rate: shocked rats had a 20% survival rate 24 h after endotoxin challenge and 0% survival rate at 72 h after endotoxin challenge (Table 1). G 619 administration (12.5, 25 and 50 mg/kg, i.v.) significantly protected against Salmonella enteritidis lipopolysaccharide-induced lethality. The most effective dose was 50 mg/kg: treated shocked rats had an 80% survival rate 24 h after endotoxin challenge and a 60% survival rate 72 h after Salmonella enteritidis lipopolysaccharide injection (Table 1). Therefore we employed this dose in the functional studies.

3.2. Arterial blood pressure

Animals given endotoxin showed a sharp and profound decrease in mean arterial pressure (Fig. 1). G 619 (50 mg/kg, i.v.), administered 1 h before endotoxin challenge, significantly blunted the sustained decrease in mean arterial pressure (Fig. 1).

3.3. Plasma thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ levels

The time course of the rise in plasma thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ following injection of endotoxin in rats was evaluated (Fig. 2). There was a rapid increase in plasma thromboxane B_2 activity within 30 min after an intravenous bolus dose of endotoxin (Fig. 2) and the levels then began to decline. Plasma levels of 6-keto-prostaglandin $F_{1\alpha}$ increased later (at 60 min following endotoxin) and then remained high (Fig. 2). Administration of G 619 significantly reduced thromboxane B_2 plasma levels (Fig. 2) and enhanced plasma 6-keto-prostaglandin $F_{1\alpha}$ activity (Fig. 2).

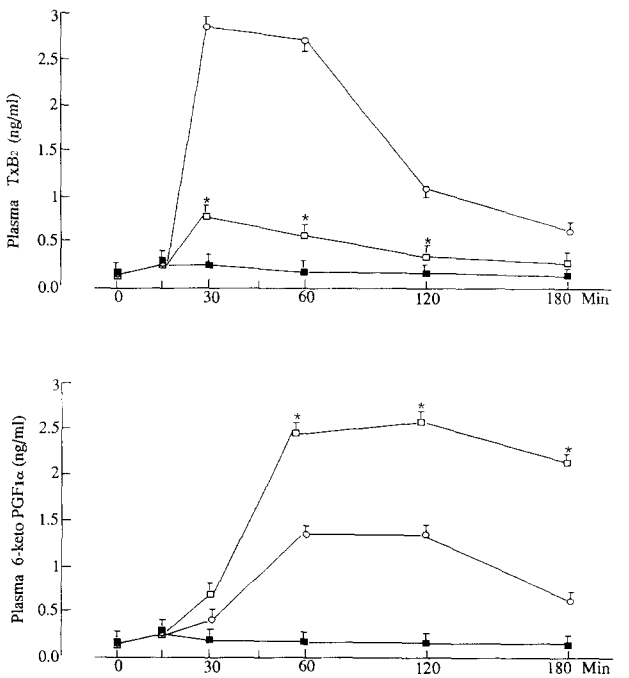


Fig. 2. Effects of G 619 on plasma levels of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ in endotoxin-shocked rats (Salmonella enteritidis lipopolysaccharide). Sham + vehicle (closed squares); Salmonella enteritidis lipopolysaccharide + vehicle (open circles); Salmonella enteritidis lipopolysaccharide + G 619 (open squares). Each point represents the mean ± S.D. for six animals. $^{*}P < 0.001$ vs. Salmonella enteritidis lipopolysaccharide + vehicle.

3.4. Serum tumor necrosis factor-α

Tumor necrosis factor-α was very low (< 5 pg/ml) in the serum of control rats treated with vehicle or G 619 (Fig. 3). In endotoxin-shocked rats, serum tumor necrosis factor-α progressively increased upon endotoxin injection and reached its maximum increase 120 min after endotoxin challenge (Fig. 3). The administra-

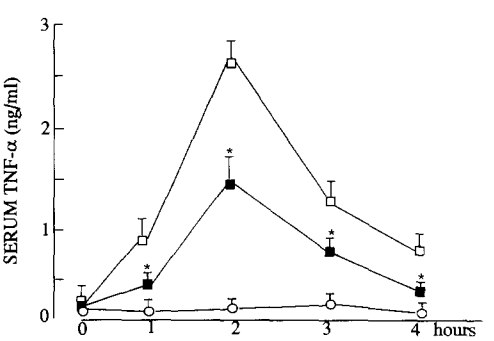


Fig. 3. Effects of G 619 on serum levels of tumor necrosis factor-α in endotoxin-shocked rats. Each point represents the mean ± S.D. for six animals. Sham + vehicle (open circles); Salmonella enteritidis lipopolysaccharide + vehicle (open squares); Salmonella enteritidis lipopolysaccharide + G 619 (closed squares). $^{*}P < 0.001$ vs. Salmonella enteritidis lipopolysaccharide + vehicle.

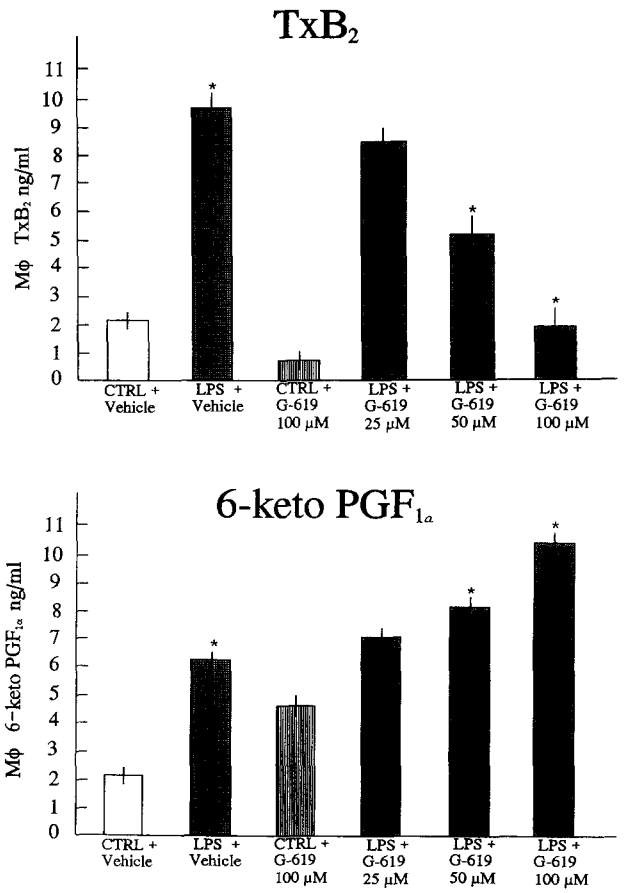


Fig. 4. Effects of G 619 on thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ production by rat peritoneal macrophages stimulated with Salmonella enteritidis lipopolysaccharide. Bar heights represent the mean ± S.D. of six experiments. $^{*}P < 0.01$ vs. Salmonella enteritidis lipopolysaccharide + vehicle or CTRL + vehicle.

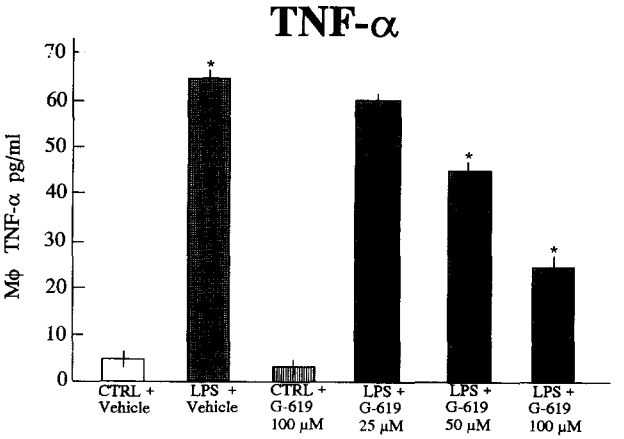


Fig. 5. Effect of G 619 on macrophage tumor necrosis factor-α stimulated with Salmonella enteritidis lipopolysaccharide. $^{*}P < 0.05$ vs. Salmonella enteritidis lipopolysaccharide + vehicle or CTRL (control) + vehicle. Bar heights represent the mean ± S.D. of six experiments. $^{*}P < 0.001$ vs. Salmonella enteritidis lipopolysaccharide + vehicle or CTRL + vehicle.

tion of G 619 (50 mg/kg) reduced the cytokine levels in the serum (Fig. 3).

3.5. Macrophage thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$ and tumor necrosis factor-α in vitro

Peritoneal macrophages were collected from normal rats and then incubated in vitro with Salmonella enteritidis lipopolysaccharide alone (50 μg/ml) or together with G 619 (25; 50; 100 μM). Salmonella enteritidis lipopolysaccharide significantly stimulated eicosanoid and tumor necrosis factor-α synthesis (Fig. 4 and Fig. 5). G 619 (50 and 100 μM) blunted Salmonella enteritidis lipopolysaccharide-induced thromboxane B_2 and tumor necrosis factor-α and markedly increased 6-keto-prostaglandin $F_{1\alpha}$.

3.6. Macrophage cyclic AMP levels

Cyclic AMP (cAMP) levels in control macrophages were 2.1 ± 0.2 pmol/10^6 cells (Fig. 6). Incubation of macrophages with Salmonella enteritidis lipopolysaccharide did not increase cAMP content of the macrophages. In contrast, G 619 either alone or in combination with Salmonella enteritidis lipopolysaccharide significantly increased macrophage levels of cAMP (Fig. 6).

3.7. Histological examination

Table 2 summarizes the microscopic alterations observed in endotoxin-shocked rats treated with vehicle or G 619. Endotoxic shock produced characteristic damage of the gastric mucosa consisting of haemorrhagic infiltrates (Table 2, Fig. 7A,B). In contrast,

Table 2
Effect of G 619 on development of gastric hemorrhagic infiltrates in endotoxin-shocked rats

Drugs	No. of animals	Haemorrhagic infiltrates	
Control + vehicle (1 ml/kg)	5	Not present	(5)
Control + G 619 (50 mg/kg)	5	Not present	(5)
LPS + vehicle (1 ml/kg)	7	Heavy	(7)
LPS + G 619 (50 mg/kg)	7	Not present (6); mild	(1)

Hemorrhagic infiltrates were graded as follows: not present, mild and heavy. The stomach was removed 2 h after endotoxin (Salmonella enteritidis lipopolysaccharide) administration. The rats received G 619 or vehicle 1 h before endotoxin injection.

endotoxin shocked rats given G 619 showed only diffuse oedema of the gastric mucosa (Table 2 and Fig. 7A,B,C).

4. Discussion

G 619 (3-carbamyl-(3′-picolyl)-4-methoxy-1-benzamide) is a novel dual thromboxane synthase inhibitor and thromboxane A_2 receptor antagonist (Squadrito et al., 1993b). This compound possesses proved efficacy in intestinal (Squadrito et al., 1992b) and myocardial ischaemia (Squadrito et al., 1993c), but its effect in endotoxin shock had not yet been investigated.

Previous studies have demonstrated that inhibitors of thromboxane A_2 synthase and thromboxane A_2 receptor antagonists improve pathophysiological events associated with endotoxic shock (Feuerstein et al., 1987). In addition elevated plasma and lymph levels of thromboxane B_2 have been consistently found in endotoxin shock (Cook et al., 1980).

Our results concur with these previously reported data: we measured enhanced plasma levels of thromboxane B_2 in rats subjected to endotoxin challenge and, moreover, pretreatment with G 619 succeeded in reducing the plasma levels of thromboxane B_2 and in protecting the rats from the lethality of endotoxin shock. Therefore the salutary activity of G 619 in endotoxin shock may also be ascribed to a reduction in the thromboxane B_2 plasma levels. G 619 also increased the plasma levels of 6-keto-prostaglandin $F_{1\alpha}$. Indeed it has been shown that prostaglandin I_2 exerts protective effects in endotoxin shock (Feuerstein et al., 1987). Therefore the beneficial activity of G 619 in our model of shock might also be, at least in part, due to the enhanced levels of prostaglandin I_2.

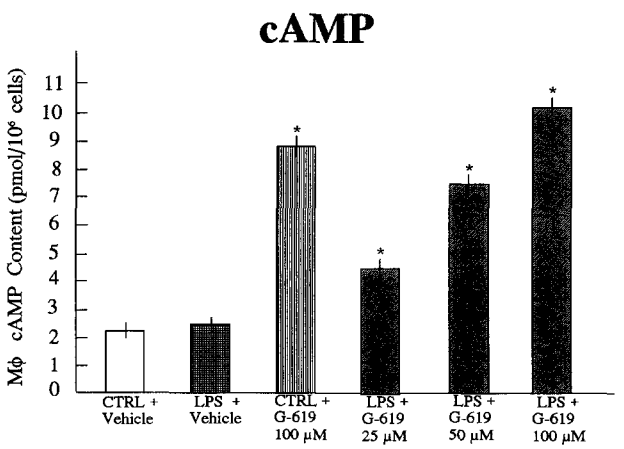


Fig. 6. Effects of G 619 on cyclic AMP (cAMP) levels in control (CTRL) and Salmonella enteritidis lipopolysaccharide-stimulated macrophages. Bar heights represent the mean ± S.D. of six experiments. $^*P < 0.001$ vs. Salmonella enteritidis lipopolysaccharide + vehicle or CTRL + vehicle.

Besides thromboxane A_2, tumor necrosis factor-α plays a role in Salmonella enteritidis lipopolysaccharide-induced shock. Tumor necrosis factor-α, a proinflammatory and vasoactive cytokine (Beutler and Cerami, 1987), is considered a key mediator in the pathogenesis of septic shock.

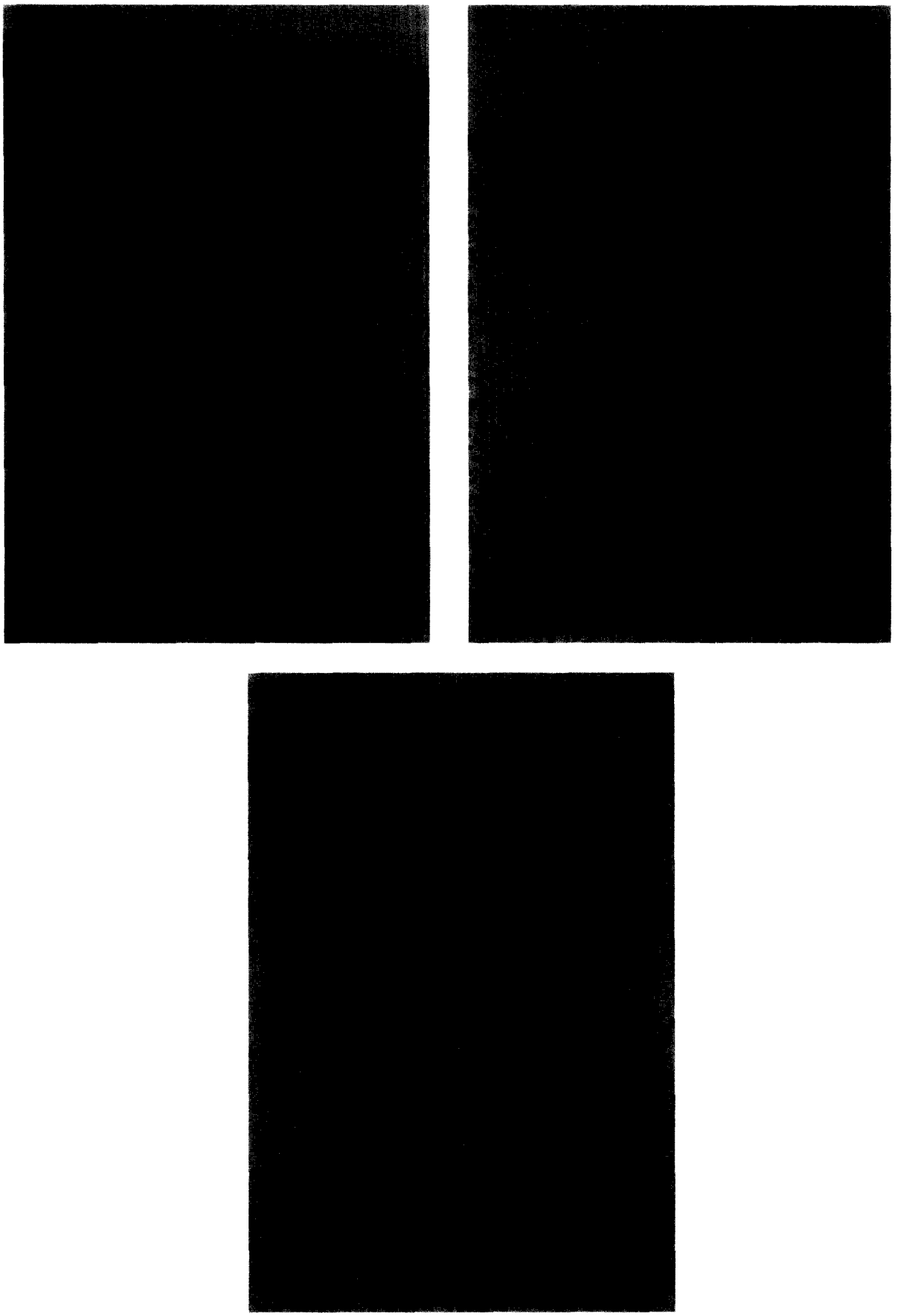
Infusion of tumor necrosis factor-α in experimental animals mimics some features of endotoxin shock including pulmonary hypertension, systemic hypotension, hypoxemia, gastric lesions and lung injury (Johnson et al., 1989; Stephens et al., 1988; Tracey et al., 1987). Because tumor necrosis factor may mediate many of the pathological sequelae of endotoxin shock, methods to inhibit tumor necrosis factor production or its activity are the object of investigation.

Dexamethasone has been used to decrease tumor necrosis factor-α production, an effect that results largely from a blockade of the translational derepression of tumor necrosis factor mRNA (Beutler et al., 1986; Han et al., 1990). It has also been suggested that certain methylxanthine derivatives and dibutyryl cyclic adenosine monophosphate, may diminish tumor necrosis factor production in vitro (Strieter et al., 1988; Endres et al., 1991). Furthermore, agents capable of reducing the synthesis of this cytokine display protective effects in septic shock (Lechner et al., 1993; Squadrito et al., 1992c).

G 619 was able to reduce the levels of tumor necrosis factor-α and to protect against the pathological sequelae associated with septic shock. This suggests that the protective activity of G 619 may also result from a reduction in the formation and/or release of this inflammatory cytokine.

In vitro studies on peritoneal macrophages showed that G 619 reduces the Salmonella enteritidis lipopolysaccharide-induced enhancement in both thromboxane B_2 and tumor necrosis factor-α and increases 6-keto-prostaglandin $F_{1\alpha}$ and cAMP. In contrast, incubation of macrophages with Salmonella enteritidis lipopolysaccharide did not modify macrophage cAMP. This confirms previous data showing that Salmonella enteritidis lipopolysaccharide interaction with its specific receptor on macrophages induces the production of intracellular signals that do not involve cAMP.

Fig. 7(A). Representative stomach section from a control rat. Normal structure (arrows); original magnification 180×. (B) Representative stomach section from a vehicle-treated endotoxin shocked rat. Hemorrhagic infiltrates (arrows); original magnification 180×. (C) Representative stomach section from endotoxin-shocked rat treated with G 619. Absence of hemorrhagic infiltrates (arrows); original magnification 180×.



It has been shown that tumor necrosis factor-α production can be regulated by changes in intracellular cAMP concentrations and this control is exerted at a post-transcriptional level (Taffet et al., 1989). More specifically it has been suggested that an increase in cellular cAMP may down-regulate the synthesis of tumor necrosis factor-α. Therefore it could be hypothesized that G 619 decreases tumor necrosis factor-α production by inducing the synthesis of this endogenous modulator of tumor necrosis factor-α synthesis. The enhancement in the intracellular cAMP effect could be either direct or, more likely, via an increase in intracellular prostaglandin I_2, which has been shown to increase cAMP. Therefore it could be argued that the beneficial effects of G 619 in septic shock may be a direct consequence of the reduction in the biosynthesis of tumor necrosis factor-α.

Endotoxin shock is also characterized by specific gastric lesions (Wallace and Whittle, 1986).

In keeping with these findings, our endotoxin-shocked rats showed haemorrhagic infiltrates in the mucosa. Treatment with G 619 prevented the development of gastric haemorrhagic infiltrates. The administration of recombinant tumor necrosis factor-α in experimental animals is associated with shock and widespread tissue injury, including marked gastric damage (Tracey et al., 1987). Since G 619 reduced the circulating levels of tumor necrosis factor-α it might be hypothesized that G 619 protects against the gastric alterations by reducing the circulating levels of the inflammatory cytokine.

In conclusion, our data indicate that G 619 may be a useful drug during states characterized by elevated tumor necrosis factor-α levels.

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