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Studies of the Effect of a Platelet-Activating Factor Antagonist, CL 184,005, in Animal Models of Gram-Negative Bacterial Sepsis

L. W. TORLEY, W. C. PICKETT, M. L. CARROLL, C. A. KOHLER, R. E. SCHAUB, A. WISSNER, S. Q. DEJOY, A. L. ORONSKY, AND S. S. KERWAR*

Oncology and Immunology Research Section, Medical Research Division, Lederle Laboratories, American Cyanamid Company, Pearl River, New York 10965

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The effect of CL 184,005, a potent and specific platelet-activating factor antagonist, has been examined in a variety of animal models relevant to gram-negative bacterial sepsis. Pretreatment of mice with CL 184,005 protected them from the lethal effects of platelet-activating factor. When rats or primates rendered hypotensive with endotoxin were treated with CL 184,005, blood pressure was normalized. Pretreatment of rats with CL 184,005 protected them from the gastrointestinal lesions induced by endotoxin. Pretreatment of rats and mice with CL 184,005 protected them from the lethal effects of endotoxin. Plasma tumor necrosis factor levels in endotoxin-treated mice were lower when the mice were pretreated with CL 184,005. These observations suggest that CL 184,005 may be potentially useful in the treatment of gram-negative bacterial sepsis, and the agent is undergoing clinical evaluation.

Platelet-activating factor (PAF) was first identified in the cell-free supernatant of sensitized rabbit basophils that had been exposed to the sensitizing antigen. When this soluble factor was added to rabbit platelets, it induced their aggregation (4). The structure of PAF and its biological effects have been extensively studied and reviewed (1, 9, 14, 21, 23, 26, 32). PAF is a phospholipid, and a variety of biological effects have been observed when it is administered to animals or added to cells in culture. It has been shown that many of these biological effects are sequelae of the interaction of PAF with its specific receptor present on a wide variety of cells. These effects of PAF suggest that it may be involved in the pathogenesis of inflammatory diseases. Potent and specific PAF antagonists have therefore been synthesized and are currently being evaluated in the treatment of these diseases (2).

Recent studies have shown that PAF also is one of the principal mediators involved in the pathogenesis of gram-negative bacterial sepsis (1, 14). Specifically, the systemic effects of PAF are similar to some of the clinical symptoms associated with gram-negative bacterial sepsis. PAF levels (14) in the circulation are significantly higher in patients with gram-negative bacterial sepsis or in animals treated with *Escherichia coli* lipopolysaccharide (LPS). In vitro studies conducted with macrophages have shown that upon addition of LPS, the synthesis of PAF is induced and PAF can be detected in both the cells and the culture medium. In view of the above observation, it has been suggested that LPS derived from gram-negative bacteria activates macrophages and perhaps other cells in vivo and induces the synthesis and secretion of PAF (14). Additional support for this view is derived from the studies of Terashita et al. (30). In these studies, when rats were pretreated with CV-3988, a PAF antagonist, lethality due to LPS was significantly lowered.

In addition to PAF, tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 have also been implicated in the pathogenesis of gram-negative bacterial sepsis (11, 31, 34). A synergistic interaction between these lymphokines and, additionally, the interaction of these lymphokines with PAF appear to play a significant role in the pathogenesis of gram-negative bacterial sepsis (19).

The present studies describe the biological effects of CL 184,005, a potent and specific PAF antagonist (36), in a variety of models of gram-negative bacterial sepsis. While these models may appear unrelated, they represent the various symptoms associated with this complex disease. Rats, mice, and primates have been used to show that the effect of the agent is not species specific. In addition, the effect of this agent on LPS-induced TNF and IL-6 synthesis is reported. In view of its efficacy in a variety of animal models related to gram-negative bacterial sepsis, this agent is currently undergoing clinical evaluation.

MATERIALS AND METHODS

CL 184,005 (Fig. 1) was synthesized by procedures that have been described previously (36). C-16 PAF was obtained from Calbiochem, La Jolla, Calif., or from Boehringer Mannheim, Indianapolis, Ind., dissolved in methanol, and diluted with phosphate-buffered saline (PBS) or saline to the desired concentration. *E. coli* LPS (0111:B4) was obtained from Sigma Chemical Co., St. Louis, Mo. TNF and IL-6 were obtained from Genzyme, Boston, Mass. Pluronic F 127 liquid (Calbiochem) was provided by the Formulations Section, Lederle Laboratories, Pearl River, N.Y.

Rabbit platelet-rich plasma (PRP), washed rabbit platelets, or human PRP was prepared by standard procedures. Briefly, 120 to 150 ml of blood was collected in citrate by cardiac puncture from conscious male New Zealand White rabbits. It was centrifuged at 800 rpm (Beckman J-6M/E centrifuge) for 10 min (room temperature) to collect PRP. Platelets were also collected by centrifugation of PRP at 2,800 rpm for 10 min. The platelet pellet was suspended in calcium and albumin-free normal Tyrode buffer, pH 6.3. The suspension containing platelets was centrifuged as above

* Corresponding author.

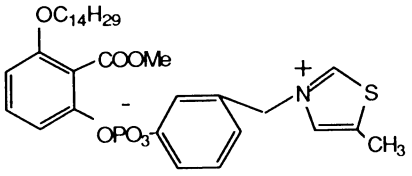

FIG. 1. The structure of CL 184,005.

and washed in the same buffer. After centrifugation, the platelets were suspended in Tyrode buffer, pH 7.4, containing calcium. Human PRP was prepared as described above from blood collected from normal volunteers. The concentration of platelets in human or rabbit PRP was determined by using a Coulter Counter. The platelet concentration was adjusted to 3.5×10^8 to 5×10^8/ml by the addition of either platelet-poor plasma or Tyrode buffer.

The effect of CL 184,005 on PAF-induced platelet aggregation was measured as follows. Briefly, the incubation mixture (0.5 ml) contained 0.4 ml of PRP (containing platelets at a concentration of 3.5×10^8 to 5.0×10^8/ml) and various concentrations of CL 184,005 (dissolved in methanol and diluted with PBS or saline to the desired concentration). After 5 min at 37°C, L-PAF (Calbiochem) was added at concentrations ranging from 5×10^9 to 10^7 M. In some studies, PRP was replaced with a 0.4-ml suspension of washed platelets in Tyrode buffer (pH 7.4) (3.5×10^8 to 5.0×10^8/ml). Platelet aggregation was monitored for 5 min in an aggregometer that was coupled to a dual-channel recording unit. Platelet aggregation was analyzed by a digitizing method (22). Appropriate controls were included for each assay.

In some studies, 7×10^7 washed rabbit platelets were incubated at room temperature for 30 min in 1 ml of Tyrode buffer (pH 7.4) containing ^{3}H-PAF (2.5 nM) and various amounts of CL 184,005. Total binding and nonspecific binding of labeled PAF (the latter was determined by measuring binding in the presence of excess unlabeled L-PAF, 10 to 100 μM) was determined by filtration of the reaction mixture through Whatman GF/C filters, followed by radiometric analysis. Specific binding was calculated as the difference between total and nonspecific binding.

Inhibition of PAF-induced lethality in mice by CL 184,005. Female ICR (20- to 25-g; Charles River, Wilmington, Mass.) mice were used in these studies. The mice were treated with various doses of CL 184,005 (0.25 to 1 mg/kg of body weight, administered intraperitoneally). In all of the in vivo studies, CL 184,005 was dissolved in saline by sonication. One hour later, the mice were treated intravenously with PAF at a dose of 50 μg/kg. Mortality was determined at 2 h post-PAF treatment.

Effect of CL 184,005 on LPS-induced hypotension in rats and primates. Male Wistar rats (Charles River) weighing 225 to 350 g were used in these studies. The rats were anesthetized with a single dose of sodium pentobarbital administered intraperitoneally at a dose of 35 mg/kg. The tissue in the neck area was dissected, and a PE-50 cannula was inserted into the left carotid artery. Mean arterial pressure was measured through this cannula with Statham transducers that were coupled to a Grass Model 7 polygraph. A cannula was inserted into the trachea, and ventilation was maintained by using a small animal ventilator (Harvard Apparatus). Thirty minutes later, when the blood pressure stabilized, the rats were given an intravenous dose of LPS (15 mg/kg in PBS). Five minutes after LPS treatment (when hypotension was stabilized), the rats were injected intravenously with various doses of CL 184,005 or saline. The mean arterial pressure was continuously monitored during the entire course of the experiment.

Cynomologus monkeys weighing 2 to 4 kg were used. Ketamine (10 mg/kg) was given intramuscularly for initial restraint, and xylocaine was infused into the groin area for prevention of pain or discomfort. Additional ketamine, 20 mg/kg, was administered intravenously to induce anesthesia. Following anesthesia, the groin was shaved and the femoral vein and artery were cannulated to blood pressure sensor equipment. A constant infusion of PBS (1 ml/kg/h) was maintained throughout the experiment. Systolic and diastolic blood pressure was continuously monitored. After an equilibration period that lasted 30 min, a bolus dose of LPS (15 mg/kg) was administered through the femoral vein cannula. After the blood pressure had fallen to 20 to 50 mm Hg, a bolus dose of CL 184,005 (0.25 to 2 mg/kg) was administered intravenously over a 1-min period. The saline infusion was replaced by an infusion of PBS (1 ml/kg/h) containing 0.25 to 2 mg of CL 184,005 per kg. At the end of the experiment, the monkeys were sacrificed by an overdose (100 mg/kg, administered intravenously) of pentobarbital.

Effect of CL 184,005 on LPS-induced intestinal permeability and leakage in rats. Male Wistar rats (Charles River) weighing 160 to 225 g were used for these studies. The rats were starved for 18 h before the initiation of these experiments. Access to water was available. The rats were treated with various doses of CL 184,005 or saline administered intraperitoneally. Thirty minutes later, the rats were treated intravenously with LPS (dissolved in PBS containing 1% Evans blue dye) at a dose of 25 mg/kg. One hour after LPS challenge, the rats were sacrificed, and the duodenum and the initial section of the jejunum (6 to 7 cm) were excised. This internal segment of the intestine was washed with 2 ml of saline, and the fluid exudate was collected in centrifuge tubes. The fluid was subjection to sonication (to release any dye that was loosely bound to proteins), and the fluid was clarified by centrifugation. The A_{600} of the exudate was measured. To determine nonspecific diffusion of dye from the circulation into the duodenum and jejunum, normal rats were treated intravenously with LPS-free saline containing 1% dye. These control studies were conducted for each study group. The amount of dye in the gastrointestinal segment was ascertained as described above and was reported for each study group.

Results not reported here showed that the change in the intestinal vascular permeability and the extent of intestinal necrosis were proportional to the absorbance of the intestinal exudate (visual examination of the gastrointestinal tract) and to the amount of LPS administered.

Effect on circulating TNF and IL-6 levels in mice. Male BALB/c mice (Charles River) weighing approximately 20 to 25 g were injected intraperitoneally with saline or CL 184,005. One hour later, the mice were administered LPS (50 mg/kg, administered intraperitoneally). One hour after the LPS injection, blood was collected, with EDTA as the anticoagulant. The samples were kept on ice, and the plasma was isolated by centrifugation. TNF in the plasma was determined by an enzyme-linked immunosorbent assay (ELISA) (16). Pure recombinant murine TNF obtained from Genzyme was used as a standard in each ELISA. In a similar study, the plasma was also assayed for IL-6 by using a 7TD1 hybridoma that requires IL-6 for growth (33).

Effects on LPS-induced lethality in mice and rats. Male

BALB/c mice (Charles River) weighing approximately 20 to 25 g were used in these studies. Since this model is subject to experimental variation, large numbers of mice were used in evaluating the effects of CL 184,005. The mice were treated intraperitoneally with CL 184,005 (10 to 40 mg/kg) or saline. Two hours later, the mice were challenged intraperitoneally with LPS (50 mg/kg), a dose sufficient to kill 80 to 90% of the control mice in 24 h. A second equivalent dose of CL 184,005 was administered at 2 h post-LPS challenge. Mortality was monitored for 24 h.

Pluronic F 127 liquid is a nonionic polyoxyethylene-polypropylene copolymer that sensitizes mice and rats to the deleterious effects of LPS. Like the D-galactosamine model (17), significantly lower doses of LPS are required to induce lethality (25). Since this is a relatively new model of LPS-induced lethality and since a different animal species was used, it was of interest to determine the effects of CL 184,005 in this model. Rats were pretreated with various doses of CL 184,005 administered intraperitoneally. Sixty minutes later, LPS (1 mg/kg) was administered intraperitoneally in 0.2 ml of Pluronic F 127 liquid. Mortality was monitored for up to 48 h post-LPS treatment.

Statistical analysis was conducted by using the Stat View II program (Abacus Concepts Inc., Menlo Park, Calif.). All survival data (see Tables 1 to 3) were analyzed by using the chi-square analysis (Pearson method and the likelihood-ratio chi-square, gamma, and Kendall's tau-b tests).

RESULTS

The effects of CL 184,005 on PAF-induced platelet aggregation were ascertained. When human PRP was incubated with 100 nM of PAF, CL 184,005 inhibited platelet aggregation with a 50% inhibitory concentration of 600 nM. When rabbit PRP was incubated with 50 nM of PAF, CL 184,005 inhibited rabbit platelet aggregation with a 50% inhibitory concentration of 510 nM. When washed rabbit platelets were incubated with 5 nM PAF, the agent inhibited platelet aggregation with a 50% inhibitory concentration of 20 nM. These results indicate that CL 184,005 is a potent inhibitor of PAF-dependent platelet aggregation. To determine the specificity of CL 184,005 in these PAF-dependent platelet aggregation assays, rabbit platelets were incubated with type I collagen or ADP in the presence of various concentrations of CL 184,005. At concentrations as high as 50 μM, CL 184,005 did not inhibit platelet aggregation (results not shown). Therefore, the effect of CL 184,005 appears to be specific to PAF-induced aggregation. In results also not shown, CL 184,005 at concentrations as high as 5×10^{-5} M did not induce aggregation of washed rabbit platelets, indicating that the agent was devoid of PAF-like platelet-aggregating activity.

A radiolabeled PAF-binding assay was used to prove that CL 184,005 competed with PAF for the receptor. Results shown in Fig. 2 indicate that CL 184,005 competed with ^{3}H-PAF for specific binding to the PAF receptor on platelets. The marginal nonspecific binding of radiolabeled PAF to platelets was not affected by CL 184,005.

After it was established that CL 184,005 was a potent PAF antagonist in vitro, studies were conducted in vivo to ascertain whether the agent would prevent the lethality induced in mice by intravenously administered PAF. Results shown in Table 1 indicate that CL 184,005 was effective in protecting mice from PAF-induced lethality. Significant protection was observed when the mice were pretreated intraperitoneally with CL 184,005 (0.25 to 1.0 mg/kg). Doses smaller than 0.25 mg/kg were ineffective.

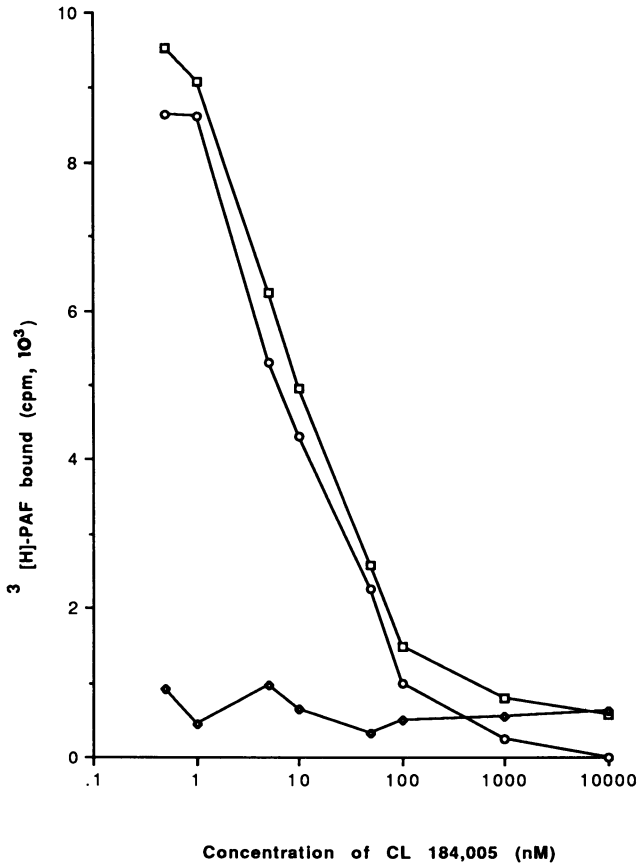

FIG. 2. The effect of various concentrations of CL 184,005 on the binding of radiolabeled PAF to rabbit platelets. □, total binding; ○, specific binding; ◇, nonspecific binding.

The effects of CL 184,005 on the normalization of blood pressure of both rats and primates rendered hypotensive with LPS were investigated (posttreatment schedule). The mean arterial blood pressure of rats before LPS administration was approximately 100 to 120 mm Hg. Subsequent to LPS treatment, the mean blood pressure decreased to approximately 60 to 80 mm Hg. When, at this time, CL 184,005 was administered intravenously (1 to 10 μg/kg), the mean arterial blood pressure increased in a dose-dependent manner and approached normal values. The results of these studies are expressed as the percentage of recovery from hypotension and shown in Fig. 3. In results not shown, intravenous CL 184,005 (doses of 30 mg/kg) did not affect the

TABLE 1. Effect of CL 184,005 on PAF-induced lethality in mice

Treatment	Dose (mg/kg)	No. of survivors/ no. treated	% Survival
None (controls)	0	17/144	12
CL 184,005	1	27/32[a]	84
	0.5	36/40[a]	90
	0.25	80/106[a]	75
	0.125	48/59[a]	81

[a] Significantly different from control values ($P < 0.05$; chi-square test). Other details of the assay are described in the text.

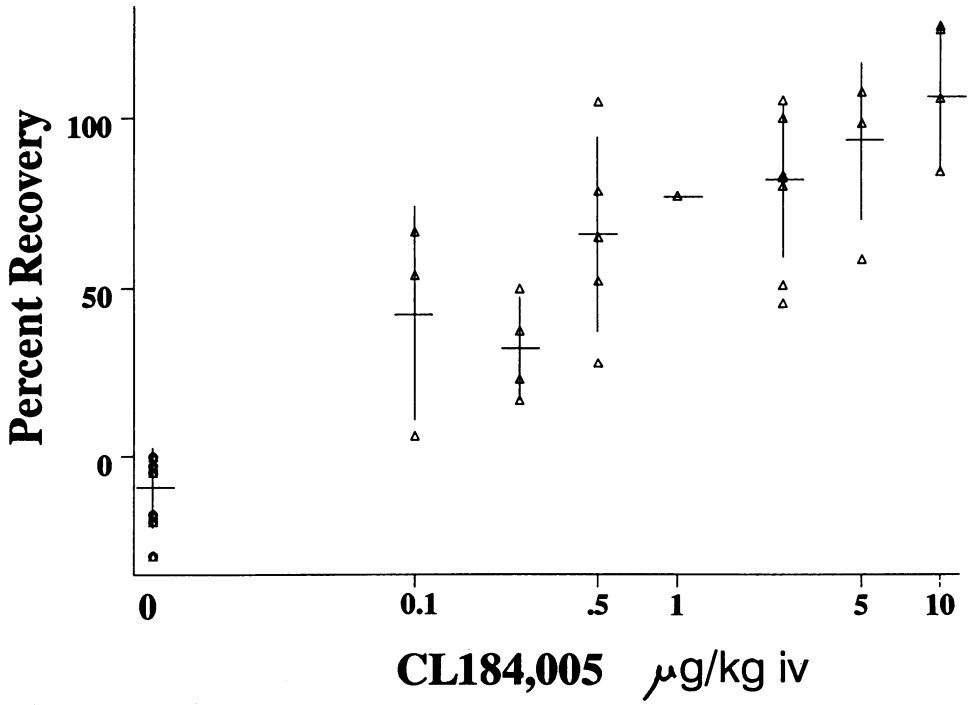

FIG. 3. Effect of CL 184,005 on the recovery from hypotension induced in rats by LPS. Values for blood pressure after administration of CL 184,005 were compared with the initial blood pressure readings to calculate the percent recovery. iv, intravenously.

blood pressure of normal rats. Thus, CL 184,005 does not appear to exhibit the hypotensive effects that have been demonstrated with PAF.

CL 184,005 was also investigated for its effect on LPS-induced hypotension in the primate. The data shown in Fig. 4 represent the results of single-primate studies; therefore, no statistical analysis was conducted. In a control experiment (Fig. 4A), LPS-induced hypotension was sustained for approximately 400 min without any significant reversal. Results also shown in Fig. 4B and C indicate that when CL

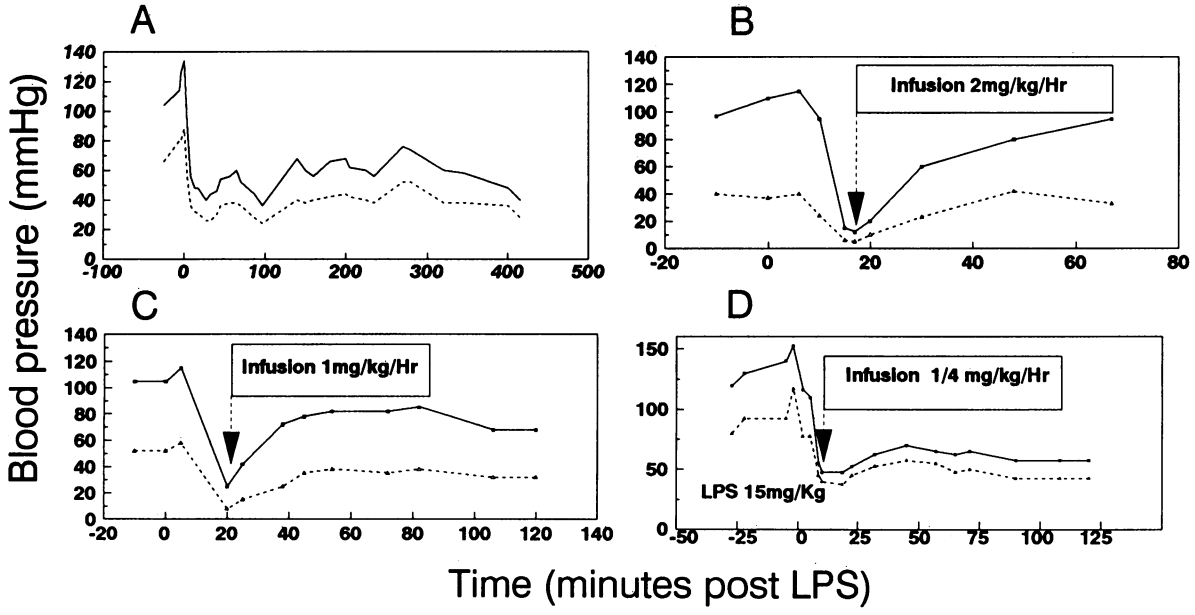

FIG. 4. Effect of CL 184,005 on the recovery from hypotension induced in primates by LPS. (A) Control monkey was treated at time 0 with LPS. After LPS had been administered and the blood pressure had been lowered, CL 184,005 was administered as a bolus of either 2 mg/kg followed by an infusion of 2 mg/kg/h (B), 1 mg/kg followed by an infusion of 1 mg/kg/h (C), or 0.25 mg/kg followed by an infusion of 0.25 mg/kg/h (D). Arrowheads indicate initiation of infusion. In these studies, the volumes of fluid (saline for panel A or CL 184,005 dissolved in saline for panels B to D) administered to all primates were identical. ——, systolic pressure; – – –, diastolic pressure.

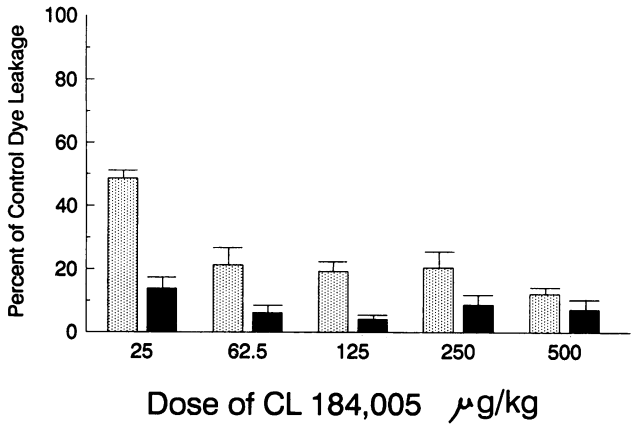

FIG. 5. Effect of CL 184,005 on the gastrointestinal vascular permeability in rats treated with LPS. ▨, rats treated with CL 184,005 at the doses indicated; ■, normal rats treated with LPS-free saline containing Evans blue dye. For each study group, 20 rats were used.

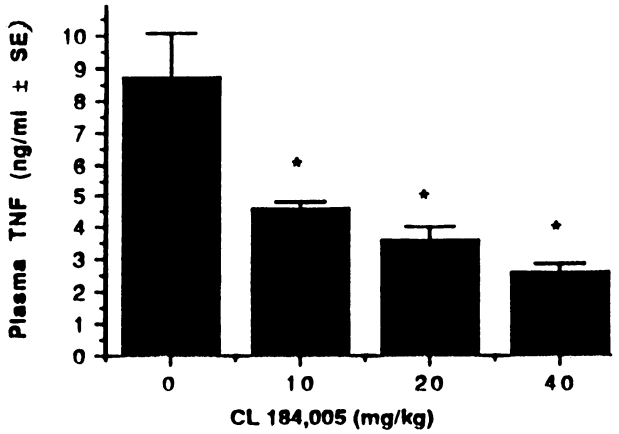

FIG. 6. Effect of pretreatment with CL 184,005 on plasma TNF in mice subsequently administered LPS. ⋆, P of <0.05 compared with control mice treated with LPS alone (Student's t test). For each study group, 10 mice were used.

184,005 was administered as a bolus (1 and 2 mg/kg) followed by an infusion of an equivalent dose (1 to 2 mg/kg/h), blood pressure increased and approached normal values. With a 0.25-mg/kg bolus dose, followed by an equivalent infusion dose, the agent was inactive (Fig. 4D). In other experiments for which the results are not shown, bolus administration of CL 184,005 at 1 and 2 mg/kg without a constant infusion was also efficacious in normalizing the blood pressure.

Treatment of rats with LPS (25 mg/kg, administered intravenously) causes an increase in intestinal vascular permeability. When LPS is administered in Evans blue dye, this lesion can be readily quantitated by a spectrophotometric method. The effect of CL 184,005 on this LPS-induced lesion was measured. When rats were pretreated with various doses of CL 184,005 (intraperitoneally) and then administered LPS, the increase in the intestinal vascular permeability induced by LPS was prevented (Fig. 5). For each treatment group, the optical density of the intestinal fluid was determined and compared with that of animals receiving LPS alone. The data are therefore reported as the percentage of dye leakage detected in rats pretreated with CL 184,005 compared with that in LPS controls. Also reported is the percentage of dye that diffuses into the intestines of normal rats treated with LPS-free saline containing Evans blue. At pretreatment doses ranging from 25 to 500 μg/kg, CL 184,005 significantly prevented LPS-induced increases in gastrointestinal vascular permeability such that at a dose of 500 μg/kg, the amount of dye leaking into the intestine was similar to that detected in normal rats.

A previous study (16) has shown that plasma TNF levels in mice peak at 60 min post-LPS administration. Similarly, IL-6 is also detected in the plasma at 60 min post-LPS treatment. To determine the effect of CL 184,005 on these lymphokines, mice were pretreated with various doses of CL 184,005 (intraperitoneally) and administered LPS intraperitoneally (50 mg/kg). One hour later, these mice were bled, and the amounts of plasma TNF and IL-6 were measured. Pretreatment of mice with CL 184,005 prevented the increase in plasma TNF induced by LPS (Fig. 6). In results not shown, the agent had no effect on plasma IL-6 levels.

Finally, the effects of CL 184,005 on the survival of mice and rats treated with LPS were ascertained. Results shown in Table 2 indicate that pretreatment with CL 184,005 at doses of 10 to 40 mg/kg followed by an equivalent dose post-LPS treatment protected mice from LPS-induced lethality. When mice were treated with a single dose of CL 184,005 and the second dose post-LPS treatment was not administered, the agent was less effective. Pharmacokinetic studies of rodents have shown that the half-life of CL 184,005 is approximately 60 min, which may account for the greater effectiveness of the twice-a-day dosing schedule used.

The results of the effect of CL 184,005 on lethality induced by LPS administered in Pluronic F 127 are shown in Table 3. At a single pretreatment dose ranging from 5 to 20 mg/kg, CL 184,005 induced significant protection from lethality induced by LPS.

DISCUSSION

The pathogenesis of gram-negative bacterial sepsis involves cytokines, lymphokines, and PAF (6–8, 14, 37). Studies of animal models relevant to gram-negative bacterial sepsis have demonstrated that pretreatment with monoclonal antibodies to TNF, IL-6, or LPS can prolong survival when they are administered before LPS or bacterial challenge (5, 27, 29). Results of recent clinical studies with a human or murine monoclonal immunoglobulin M against LPS indicate a significant improvement in the survival of patients with gram-negative bacterial sepsis (20, 40). A direct

TABLE 2. Effect of various pretreatment doses of CL 184,005 on lethality induced in mice by LPS

Pretreatment[a]	Dose (mg/kg)	No. of survivors/ no. treated	% Survival
None (saline control)	0	105/685	15
CL 184,005	10	55/120[b]	45
	20	97/235[b]	41
	40	190/270[b,c]	70

[a] Mice were pretreated with CL 184,005 at the doses indicated and a second equivalent dose was administered at 60 min post-LPS treatment. Other details of the assay are described in the text.

[b] Significantly different from control values ($P < 0.05$; chi-square test).

[c] Significantly improved survival compared with that of mice treated with 10 and 20 mg of CL 184,005 per kg.

TABLE 3. Effect of various pretreatment doses of CL 184,005 on lethality induced in rats treated with LPS in Pluronic F 127 liquid[a]

Pretreatment	Dose (mg/kg)	No. of survivors/ no. treated	% Survival
None (saline control)	0	63/637	10
CL 184,005	5	41/72[b]	57
	10	112/187[b]	60
	15	126/175[b,c]	72
	20	217/272[b,c]	80

[a] Other details of the assay are described in the text.
[b] Significantly different from control values ($P < 0.05$; chi-square test).
[c] Significantly improved survival compared with that of rats treated with 5 and 10 mg of CL 184,005 per kg.

comparison of the efficacy of the human and murine monoclonal immunoglobulin M cannot be made because of the differences in the clinical protocol and design. Nevertheless, it appears that the human antibody is beneficial to patients with gram-negative bacteremia with refractory shock; the murine antibody is beneficial to those patients who have gram-negative infection but are not in refractory shock (7, 15). Although a large number of patients were evaluated in these clinical studies, questions that are related to the efficacy, safety, and cost-effectiveness of the human monoclonal anti-LPS immunoglobulin M have been recently raised (3, 12, 18, 24, 28, 38, 39).

The role of PAF antagonists in gram-negative bacterial sepsis also has been extensively investigated. These studies (summarized in reference 9) have demonstrated that PAF antagonists can improve survival provided that these antagonists are administered before LPS treatment. These antagonists are inactive when administered post-LPS treatment.

CL 184,005 is a new PAF antagonist, and unlike many others reported (14), it down regulates the synthesis of TNF that is induced in mice by LPS. For this reason, extensive evaluation of CL 184,005 has been conducted for a variety of animal models relevant to gram-negative bacterial sepsis. Different animal species have been used in this evaluation. Results reported in this communication indicate that it (i) can protect mice from PAF-induced lethality, (ii) protects rats from LPS-induced increase in gastrointestinal vascular permeability, (iii) protects rats and mice from lethality induced by LPS, and finally, (iv) reverses the deleterious cardiovascular effects of LPS.

The mechanism by which CL 184,005 decreases plasma TNF in LPS-treated mice has not been investigated. The effect of CL 184,005 may represent a nonspecific effect of this agent on TNF synthesis. However, an earlier study in this laboratory (16) showed that a PAF antagonist such as alprazolam can decrease the amount of plasma TNF in mice treated with LPS. It is possible that agents such as CL 184,005 that bind to the PAF receptor may, by an unknown mechanism, down regulate TNF synthesis. Additional studies are needed to prove this hypothesis. Nevertheless, the dual activity of CL 184,005 (PAF antagonism and a decrease in TNF levels) provides for considerable optimism for its clinical efficacy as a single agent in the treatment of gram-negative bacterial sepsis. In addition, it can be combined with other therapies such as anti-LPS, anti-TNF, or anti-IL-6 or anti-IL-1 receptor antagonists (5, 27, 29, 35) in the treatment of this disease. While additional studies are required, it has been observed that rising TNF levels in the circulation of patients with gram-negative bacterial sepsis are associated with a poor prognosis, whereas stable or decreasing levels of TNF correlate with survival (10, 13). A priori, if this dual activity of CL 184,005 is detected in humans, the outcome of the clinical trials with this agent in gram-negative bacterial sepsis could be very promising.

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