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The Effects of Platelet-Activating Factor (PAF) and a PAF Antagonist (CV-3988) on Smoke Inhalation Injury in an Ovine Model

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The role of platelet-activating factor (PAF) in inhalation injury was studied in sheep, using CV-3988, a PAF antagonist. Following smoke exposure, 15 sheep were divided into three groups of five. Group I animals were untreated; group II animals were treated with CV-3988 before and after exposure; group III animals were treated only after exposure. In group I, platelet and WBC counts were markedly changed within 6 hours ($p < 0.05$); in the treated groups, alteration of cell counts was not obvious. Blood malondialdehyde (MDA) levels were increased significantly only in group I. Blood PGE₂ and TXB₂ levels did not differ among the groups. Mean tissue MDA and mean bronchoalveolar lavage fluid (BALF) MDA concentrations were significantly increased in group I ($p < 0.01$); mean BALF TXB₂ levels were significantly decreased in group I ($p < 0.01$). These data suggest that PAF affects inhalation injury through modulation of lipid oxygenation and that PAF antagonists may offer therapeutic benefit in the management of this injury.

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Smoke inhalation injury is one of the determinants of mortality after major burn injury. The influence of chemical mediators in such injury has not been thoroughly investigated.

Platelet-activating factor (PAF; 1-O-hexadecyl/octadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine) has been reported to be one of the principal chemical mediators in anaphylaxis and several inflammatory responses. In vitro, PAF activates platelets and neutrophils, producing platelet aggregation and enhanced chemotaxis.¹⁻⁵ Platelet-activating factor may also modulate neutrophil-mediated and monocyte-mediated reactions, since the production of lipoxygenase pathway-derived products of arachidonic acid and the generation of oxygen-derived free radicals are evoked in human neutrophils exposed to PAF.^{4,6,7} In vivo, systemic injection of PAF results in acute cardiopulmonary changes, some of which resemble

the alterations observed after smoke inhalation.

The effects of the PAF antagonist CV-3988 (rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolethyl phosphate, BN52021) have been studied in animal models of endotoxin shock and hemorrhagic shock, and following PAF injection. In models of endotoxin shock, PAF antagonists are reported to decrease production of PGE₂ and TXB₂, both arachidonic acid derivatives.⁸⁻¹³

The purpose of the study reported here was to determine the effects of PAF inhibition on lipid oxygenation after inhalation injury, using the PAF antagonist CV-3988.

MATERIALS AND METHODS

Fifteen 1- to 2-year-old, random source male sheep weighing 20-25 kg were used in this experiment. The sheep were maintained in an outdoor covered room and fed commercial chow and water ad libitum. Baseline hematologic data were established 3 weeks before use in the experiment.

The animals were divided into three groups. In group I, five sheep used as controls were exposed to smoke inhalation without any treatment. In group II, five sheep were treated with CV-3988 both before and after smoke exposure. In group III, five sheep received CV-3988 only after smoke exposure.

All sheep were catheterized the day before smoke exposure. Tracheal intubation was done after anesthesia was induced with thiopental. The sheep were ventilated with oxygen and halothane and placed in the supine position. Silastic catheters were placed in a carotid artery for blood sampling and in a femoral vein for infusion. The animals were kept in individual cages throughout the experiment.

Moderate inhalation injury was produced by insufflation of smoke generated by burning ten surgical pads in a metallic

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In conducting the research described in this report, the investigators adhered to the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and with the *Guide for the Care and Use of Laboratory Animals*, National Institutes of Health Publication 86-23.

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canister.¹⁴ Mean postexposure carboxyhemoglobin concentrations were $70.3\% \pm 3.2\%$ in group I, $74.1\% \pm 11.6\%$ in group II, and $68.3\% \pm 2.5\%$ in group III. These differences were not statistically significant.

The CV-3988 was provided by Takeda Chemical Industries, Ltd., Osaka, Japan. Solutions of the inhibitor (5 mg/mL) were prepared by dissolving CV-3988 in saline at 50°C two hours before injection.

Group I animals received only a maintenance volume of lactated Ringer's solution (70 mL/m²/hour) after smoke exposure. Group II animals were pretreated with a bolus injection of CV-3988 (10 mg/kg) 5 minutes before smoke exposure and received a continuous injection of CV-3988 solution (5 mg/kg/hour) and lactated Ringer's solution for 24 hours after smoke exposure. Group III animals received a bolus injection of CV-3988 (10 mg/mL) immediately following smoke exposure and a continuous injection of CV-3988 solution (5 mg/kg/hour) and lactated Ringer's solution for 24 hours after exposure.

Blood samples (1 mL) for peripheral blood cell counts were collected in EDTA before smoke exposure, immediately after smoke exposure, and 0.5, 1, 1.5, 2, 3, 6, 12, 18, and 24 hours after exposure. Two-milliliter samples of blood for malondialdehyde determination were collected into cooled syringes containing 0.2 mL 1% meclofenamate solution on the same schedule, omitting the immediate postexposure sample. Three-milliliter samples of blood for prostaglandin E₂ and thromboxane B₂ determinations were collected into cooled syringes containing 0.2 mL 1% meclofenamate solution before smoke exposure and 2, 6, and 24 hours after exposure. Whole blood samples for malondialdehyde determination and plasma samples for PGE₂ and TXB₂ determination were kept frozen at -40°C until measurement.

Bronchoalveolar lavage was performed after blood sampling at 24 hours. Fifty milliliters of warmed saline was injected into the right lower lobe by way of a fiberoptic bronchoscope; lavage fluid specimens (mean recovery, 60%) were collected into collecting tubes containing 1 mL 1% (wt/vol) meclofenamate solution. After bronchoalveolar lavage, all sheep were killed with pentobarbital. Two-gram specimens of the left lower lobe were taken and homogenized in 5 mL 1% (wt/vol) meclofenamate solution and centrifuged. The supernates were kept frozen at -40°C until measurement.

Concentrations of MDA in samples of blood, bronchoalveolar fluid, and lung tissue were measured by thiobarbituric acid assay: 0.5-mL aliquots of the sample were added to 4 mL trichloroacetic acid—thiobarbituric acid—hydrochloric acid reagent (TCA-TBA-HCl) and to 4 mL TCA-TBA-HCl + butylated hydroxytoluene (BHT). After mixing, the mixtures were heated at 80°C for 30 minutes on a heating block. After the mixtures were cooled to room temperature, a flocculent precipitate was removed by centrifugation at 1,000g for 10 minutes. Absorbance, corrected for reagent blank, was measured at 535 nm. Amounts of MDA were calculated, using the following formula:

$$\text{MDA (mmol/mL sample)} = A_{535\text{nm}} \times (1/156) \times 2$$

where $A_{535\text{nm}}$ = absorbance of sample, corrected for blank and $156 \text{ mmol}^{-1} \text{ cm}^{-1}$ = extinction coefficient of MDA.

The amount of MDA in each sample was then estimated by subtracting the difference between the TCA-TBA-HCl and TCA-TBA-HCl + BHT results from the TCA-TBA-HCl result to correct for MDA production during the assay procedure. This correction was nearly zero in all samples. Measurements of PGE₂ and TXB₂ from peripheral blood and bronchoalveolar lavage fluid were performed by radioimmunoassay.^{8,15-18}

Differences between groups were assessed by one-way analysis of variance at discrete times.

RESULTS

Mean platelet counts were similar in all groups before smoke exposure (Fig. 1). In group I, platelet counts increased during the first 30 minutes after exposure and then decreased sharply, remaining low for the remainder of the period of observation. No obvious changes occurred in group II. In group III, platelet counts rose in the first 30 minutes after exposure, fell sharply at one hour, gradually increased to normal by two hours after exposure, maintained this level for three hours, and then fell to levels equalling group I at 12 and 24 hours after exposure.

Mean WBC counts did not differ significantly before exposure. In group I, WBC counts increased gradually, reaching a maximum (mean 16,000/mm³) between three and 12 hours after exposure (Fig. 2), remaining at a higher level than that in either treatment group. The WBC counts in group III were slightly higher than those in group II. There were significant differences ($p < 0.05$) at three and six hours between group I and both treatment groups. The number of PMNs in peripheral blood was calculated from the number of WBCs and differential counts. The changes in PMN counts in each group paralleled those in the WBC counts (Fig. 3). In group I, the increase in PMNs accounted for the increased WBC count.

Malondialdehyde levels were significantly increased in group I one hour after exposure, and reached a maximum (mean: 15.5 nmol/mL) at two hours (Fig. 4), remaining elevated through the remainder of the study. No significant increase in MDA concentrations occurred in the treated groups. The differences between group I and the treated groups were significant ($p < 0.05$) from two hours after exposure until the end of the experiment.

The relationship between MDA level and the platelet, WBC, and PMN counts was analyzed by multiple regression analysis. The equation is:

$$\text{MDA} = 0.44 \times \text{WBC} + 0.23 \times \text{PMN} - 0.12 \times \text{Plt} \quad (p < 0.0001)$$

where the MDA concentration is in nanomoles per milliliter and the WBC/PMN/PLT are expressed as counts/mm³ × 10⁻³.

Plasma PGE₂ concentrations were slightly increased in group I only at six hours after smoke exposure (Fig. 5); in the treated groups, PGE₂ levels remained normal. At 24 hours, all PGE₂ concentrations were near normal. The TXB₂ levels did not change during the first six hours of the experiment. They were slightly increased in group I at 24 hours. There were no statistically significant differences in plasma PGE₂ or TXB₂ concentrations between groups at any time.

The MDA level in lung tissue was highest in group I ($84.2 \pm 15.0 \text{ nmol/g}$, Mean ± SD) (Table 1). It was lower in group II ($37.8 \pm 8.9 \text{ nmol/g}$) than in group III ($53.3 \pm 12.1 \text{ nmol/g}$). The differences between group I and the

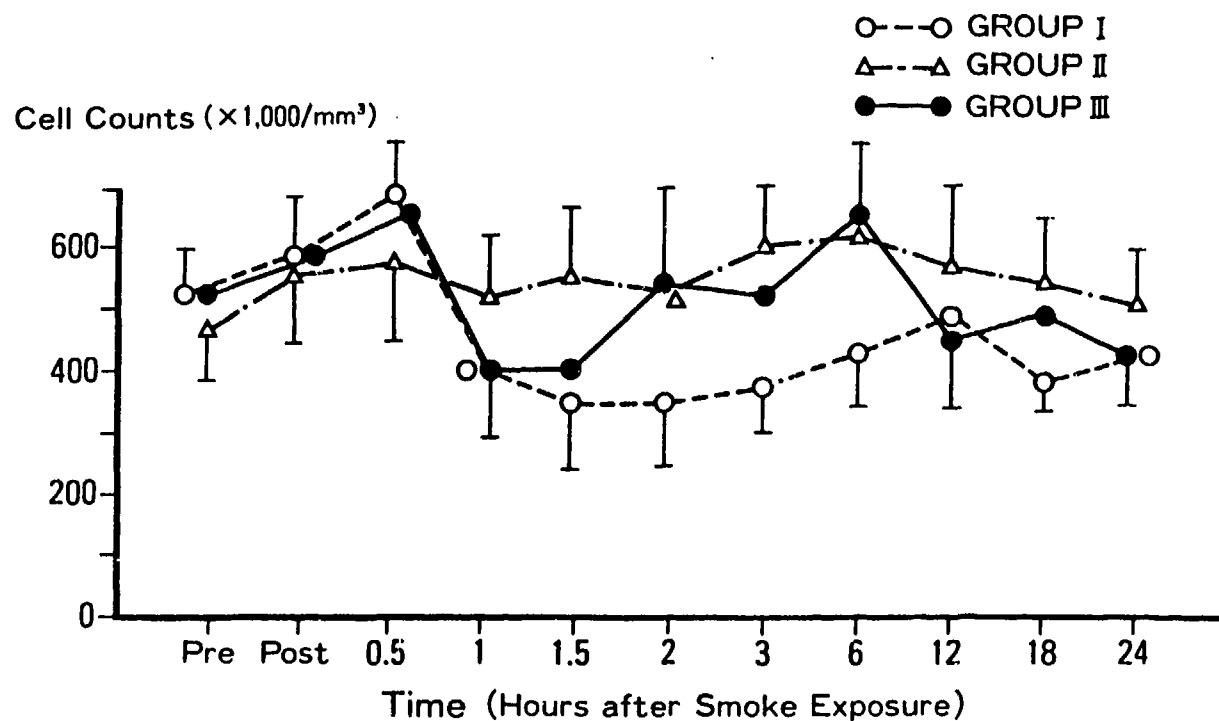
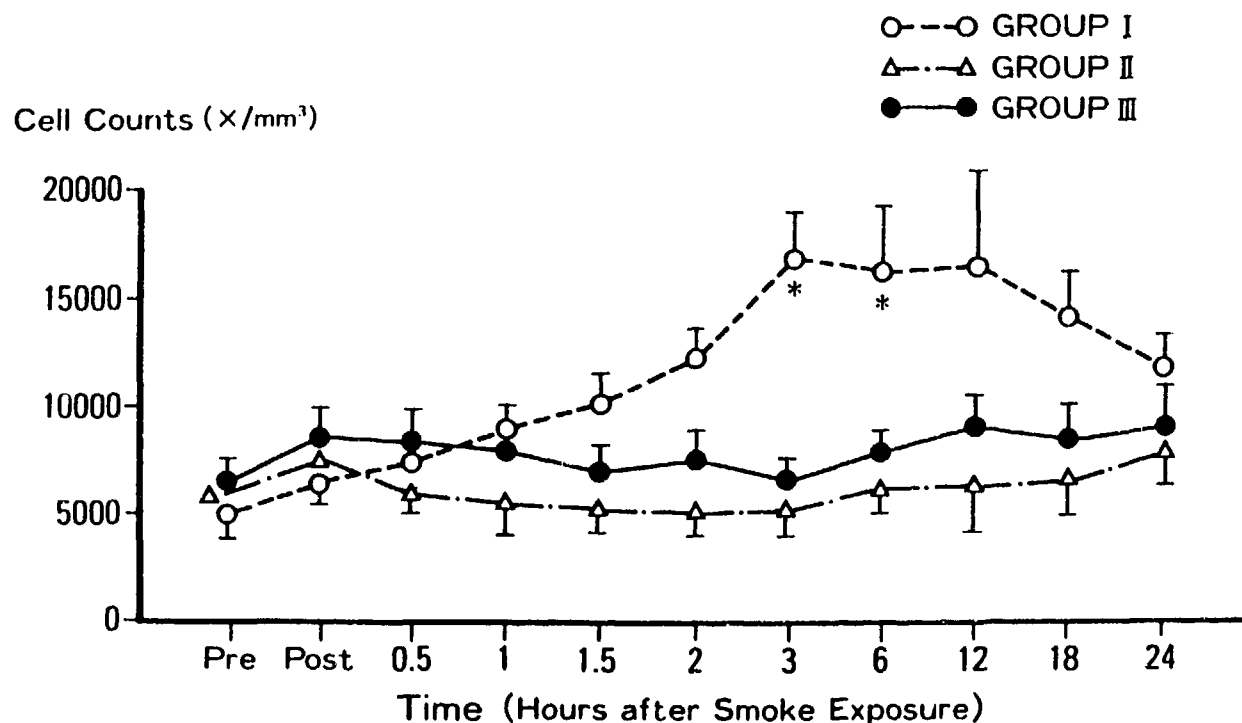


FIG. 1. Platelet counts in peripheral blood.

FIG. 2. White blood cell counts in peripheral blood; * $p < 0.05$, group I vs. groups II and III.

treated groups were statistically significant ($p < 0.01$). Mean MDA concentrations in BALF paralleled those in lung tissue. The lowest value was observed in group II (1.55 ± 0.19 nmol/mL), and the highest was 2.30 ± 0.31 nmol/mL in group I. Here, too, the differences between group I and the treated groups were statistically significant ($p < 0.01$). There were no significant differences in PGE₂ concentrations in BALF; the lowest mean value

(89.0 ± 16.4 pg/mL) occurred in group II. The TXB₂ concentration in BALF was lowest in group I (82.8 ± 5.4 pg/mL); in the treated groups, TXB₂ concentrations were significantly ($p < 0.01$) increased (136.0 ± 15.0 pg/mL, group II; 168.8 ± 4.5 pg/mL, group III).

DISCUSSION

In the present study, group I showed the expected sequence of changes after smoke inhalation. Increased

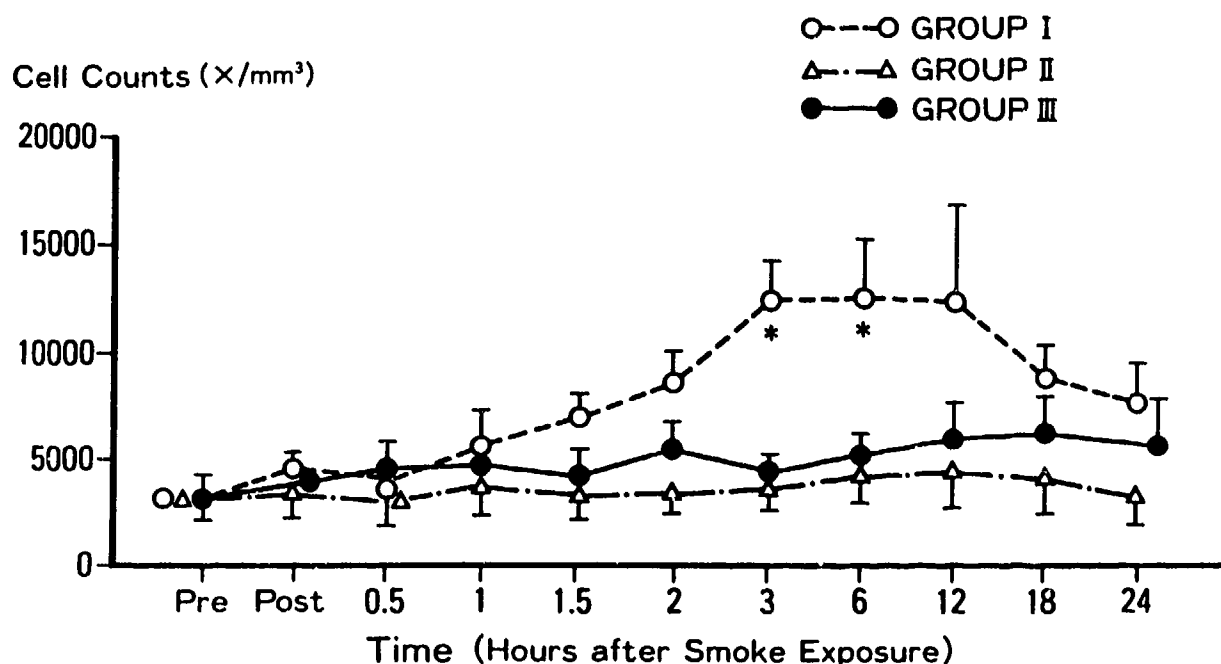


FIG. 3. Polymorphonuclear leukocyte counts in peripheral blood; * $p < 0.05$, group I vs. groups II and III.

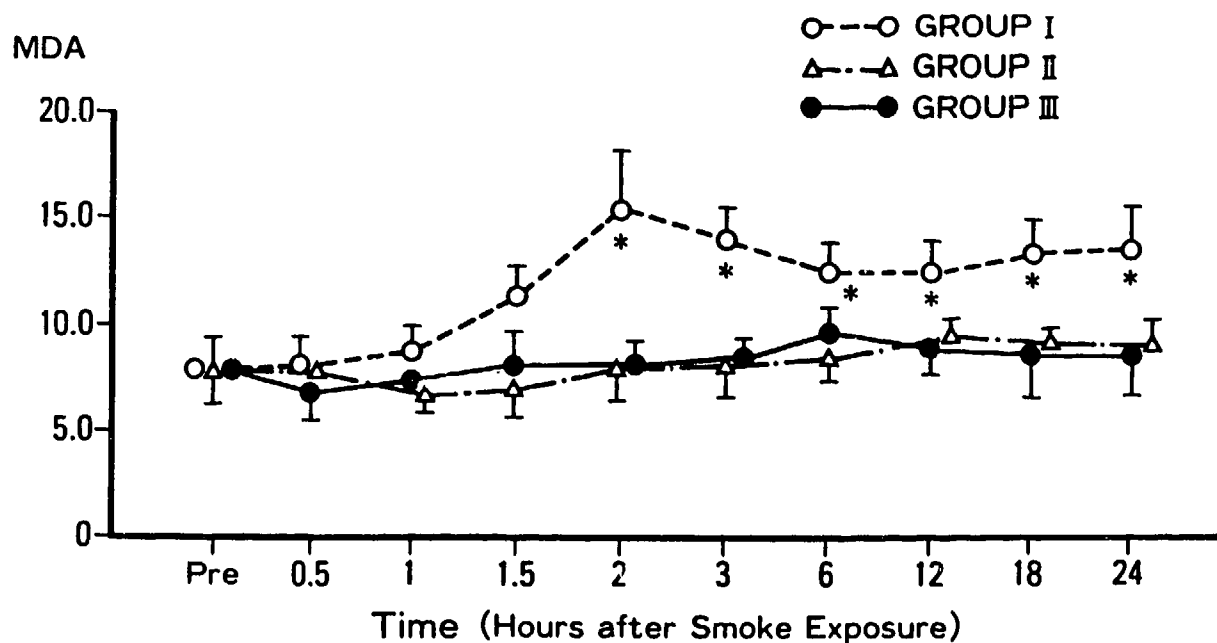


FIG. 4. Blood MDA levels (nmol/mL); * $p < 0.05$, group I vs. groups II and III.

WBC and PMN counts and increased blood MDA levels in these animals indicate an inflammatory process. The early decrease in platelet count is attributable to platelet aggregation induced by smoke exposure. In human adult respiratory distress syndrome, decreased platelet and WBC counts correlate with increases in TXB_2 , PGF_1 and superoxide production and precede a significant rise in neutrophil superoxide production.¹⁹

The PAF structural analog, CV-3988, is an effective inhibitor of PAF-induced platelet sequestration and thrombocytopenia in vivo.²⁰ In group II, a bolus injection of CV-3988 (10 mg/kg) before smoke exposure prevented

the early depletion of platelets observed in groups I and III. It is not clear whether the early recovery of platelet counts in group III was related to treatment. It appears, however, that the postexposure treatment regime did prevent leukocytosis in that group.

A number of techniques (fluorescence, chemiluminescence, conjugated dienes, MDA) have been used to assess lipid peroxidation. Although conjugated dienes tend to correlate with increased lung permeability²¹ and with total lipid content, especially in tissue extracts,²² MDA detected by TBA assay is sufficiently representative to assess serial changes in the present experiment. Demling

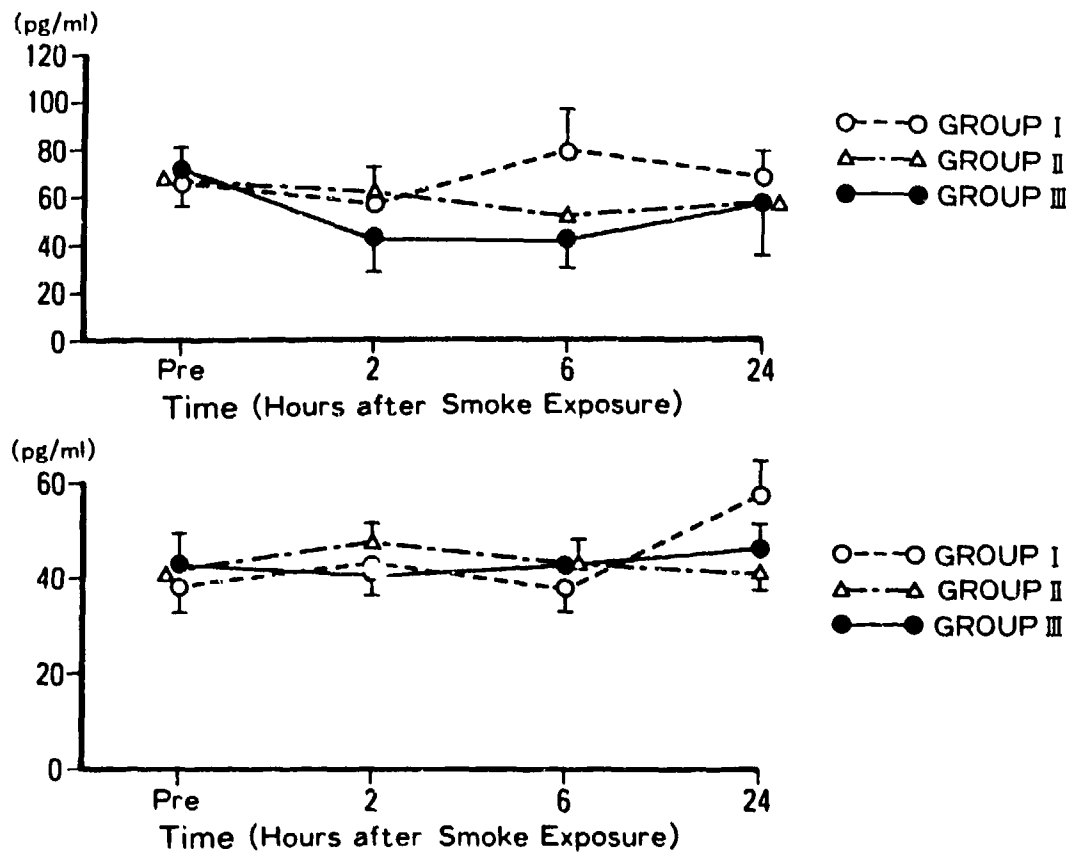
FIG. 5. Plasma PGE₂ and TXB₂ levels.

TABLE 1
MDA concentrations in lung tissue and MDA, PGE₂, and TXB₂ concentrations in BALF

Group	Lung Tissue MDA (nmol/g)	BALF		
		MDA (nmol/mL)	PGE ₂ (pg/mL)	TXB ₂ (pg/mL)
I	84.2 ± 15.0	2.30 ± 0.31	118.4 ± 19.7	82.8 ± 5.4
II	37.8 ± 8.9**	1.55 ± 0.19**	89.0 ± 16.4	136.0 ± 15.0**
III	53.3 ± 12.1**	1.67 ± 0.26**	110.6 ± 16.7	168.8 ± 4.5**

** $p < 0.01$ vs. group I.

showed that aortic plasma MDA levels reached their maximum (mean: 8.9 nmol/mL in plasma) at one hour after endotoxemia and then declined with declining permeability in a sheep model.²³ In the present study, MDA levels increased in the control group by one hour after exposure and reached a maximum (mean: 15.5 μmol/mL in whole blood) by two hours after exposure, remaining elevated through the remainder of the study. In contrast, in the treated groups, MDA levels did not increase significantly. The blood MDA concentration was positively correlated with total leukocytes, but negatively correlated with platelet count. Leukocytes interact with platelets in producing peroxidation; serotonin released from platelets increases the cytotoxic effect of PMNs on endothelial cells and enhances the adhesion of

complement-stimulated PMNs to endothelial cells, and activated PMNs appear to recruit platelets.²⁴ A beneficial effect may be exerted by CV-3988 by limiting or reversing the platelet and PMN interactive activation and preventing lipid peroxidation.

In a reported experimental study of smoke inhalation injury, lung lymph 6-keto-PGE₁ alpha concentrations were elevated,²⁵ and arterial TXA₂ levels increased five minutes after exposure, peaked at two hours, and then declined.¹⁶ Mild smoke inhalation injury that did not induce physiologic changes, however, did not cause any increase of plasma eicosanoids (TXB₂ and 6-keto-PGF₁ alpha).²¹ In the present study, smoke exposure was severe enough to produce decreased PaO₂ and to increase lung stiffness.¹⁴ The selection of the sampling times at pre-smoke, 2, 6, and 24 hours after exposure was based on the presence of these physiologic responses and elevated blood MDA concentrations.²⁶ The PGE₂ concentrations were slightly elevated in group I 6 hours after exposure, as were the TXB₂ levels at 24 hours, but these differences were small and not statistically significant in the small number of animals studied.

The elevated tissue MDA level in the control group is similar to that observed in other oxidant injuries.²⁷ In the treated groups, tissue MDA concentrations were significantly lower ($p < 0.01$). The MDA concentrations in BAL fluid reflected a similar trend, but the correlation was not entirely consistent in individual sheep. The

values of PGE₂ and TXB₂ in BALF differ from other reported results, i.e., the mean TXB₂ level was least in the untreated control group, differing significantly from the treatment groups, and the PGE₂ level was least in group II, a nonsignificant difference. The reason for this apparent dissociation between MDA and cyclo-oxygenase products in BALF is not clear.

In summary, this study shows that PAF inhibition affects the pathophysiologic changes of smoke inhalation injury. The PAF antagonist, CV-3988, appears beneficial in the treatment of such injury; a possible mechanism of this beneficial effect is prevention or alteration of lipid peroxidation.

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DISCUSSION

DR. JOSEPH C. STOTHERT (Galveston, Texas): I would like to thank the authors for kindly presenting me with a copy of their manuscript several weeks in advance of the meeting. Briefly, this paper examines the effect of a platelet-activating factor antagonist, CV-3988, on a sheep model of smoke inhalation injury. The authors demonstrate an insignificant change in platelet count associated with infusion of the drug, compared with a control group undergoing smoke inhalation alone. A significant rise in the white blood cell count, primarily polymorphonuclear leukocytes, was demonstrated in the control group at three and six hours, compared with the experimental group.

Levels of malondialdehyde, MDA, in blood and lung tissue were noted to be significantly elevated in the smoke group only. There was no significant rise in levels of this indicator of lipid peroxidation in the groups undergoing infusion of CV-3988. Similar results were obtained in the bronchoalveolar lavage fluid, which was examined for presence of MDA. The authors also examined the effects of infusion of CV-3988 on production of the metabolites of the arachidonic acid cascade. Their data demonstrate little difference between control and treatment groups in plasma levels of prostaglandin E₂ and thromboxane B₂. They did find significant elevations in thromboxane B₂ levels in bronchoalveolar lavage fluid in the animals undergoing infusion of CV-3988.

I have several comments and several questions. My first comment is the title of this paper is "The Effects of Platelet-Activating Factor and CV-3988 on Smoke Inhalation Injury in an Ovine Model." At no time do the authors infuse platelet-activating factor into their model system. I feel the title should reflect the study as primarily a study looking at the effects of an antagonist on their model. This study nicely demonstrates some effects seen in peripheral tissues and purported mediators of hemodynamic and cardiovascular changes seen following inhalation injury; however, there are no data in the paper concerning the clinical outcome of the various groups. I feel it

would be of benefit to record the effects of the infusion of the drug, as well as the outcome of the study from the standpoint of the patient.

My questions are as follows. Did you examine any physiologic variables in these animals, such as cardiovascular changes, compliance of the lungs, lung water, or determine wet weight/dry weight ratios of the lungs after death of the animal?

The authors have also performed a controlled experiment using the injury alone, infusion of the drug before injury and continuing after injury, and finally infusing the drug after injury alone. It is vital from a scientific standpoint to include a group where only CV-3988 is infused to determine what effects this drug has on the formation of lipid peroxidation and products of the arachidonic acid pathway. Have you performed these experiments?

Finally, would the authors care to comment on why the metabolite of the arachidonic acid pathway, thromboxane B₂, is increased in the bronchoalveolar lavage fluid in the treatment group, where tissue levels are lower than the control group?

I would like to compliment the authors on the presentation and thank the Association for inviting me to discuss this paper.

DR. CUTHBERT SIMPKINS (Baltimore, Maryland): I think it was a very fine paper with good, clear data, good differences. The only suggestion I would like to offer is that there be a negative control, something that has a similar chemical structure to the antagonist, but is known not to inhibit the action

of platelet-activating factor. I think that might have addressed the question of specificity, and it would have strengthened the assertion that it is platelet-activating factor that you are inhibiting in vivo.

DR. H. IKEUCHI (Closing): We have not assayed PAF directly nor infused PAF, but the title reflects our opinion that the improvement effected by the PAF antagonist is consistent with there being a role for PAF in the pathogenesis of inhalation injury.

Other data obtained from these study animals indicate that in the antagonist-treated groups, matching of air flow and blood flow was better maintained and that the decrease in compliance observed in the control animals did not occur in the treated animals. Morphologic examination of the lungs also revealed lesser accumulation of PMNs in the pulmonary vasculature of the treated animals.

Control animals receiving CV-3988 without smoke exposure were not included in this study. Group II animals received the inhibitor five minutes before smoke exposure; since their immediate postexposure measurements were similar to those in the other groups, we have considered it unlikely that CV-3988 had a significant influence on the monitored variables before injury.

We have not evaluated other compounds of the class to which CV-3988 belongs and are unable to say whether the effects are chemical-structure specific.

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