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Prevention of Phorbol Myristate Acetate-induced Acute Lung Injury by α-Tocopherol Liposomes

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Phorbol myristate acetate (PMA) is commonly used to produce experimental edema and other tissue injuries in the lung. Lung injuries induced by the administration of PMA has been shown to be mediated mainly by neutrophils. Neutrophils recruited to the lower respiratory tract may damage lung tissues by releasing reactive oxygen species, neutral proteases, and lysosomal enzymes. The present study was conducted to investigate whether α-tocopherol, entrapped in dipalmitoylphosphatidylcholine liposomes and delivered directly to the lung, could counteract some of the PMA-induced lung injuries. Plain liposomes or α-tocopherol containing liposomes (8 mg α-tocopherol/kg body weight.) were intratracheally instilled into the lungs of rats 24 hr prior to PMA exposure (25 μg/kg) and treated rats were killed 3 hr later. Lungs of control animals exposed to PMA developed an increase in lung weight and lipid peroxidation as well as a decrease in lung angiotensin converting enzyme (ACE) and alkaline phosphatase (AKP) activities. PMA treatment also caused an increase in myeloperoxidase (MPO) activity in the lung, suggestive of neutrophil infiltration. Pretreatment of PMA-treated rats with plain liposomes had no effect on PMA-induced injuries. In contrast, pretreatment of rats with liposomal α-tocopherol, 24 hr prior to PMA administration, resulted in a significant elevation of pulmonary α-tocopherol concentration, accompanied by a concomitant reduction in MPO activity and reversal of PMA-induced changes in lung edema, lipid peroxidation, ACE and AKP activities. These results appear to demonstrate that the intratracheal administration of a liposome-associated lipophilic antioxidant, such as α-tocopherol, can significantly ameliorate the toxic effects of reactive oxygen species, putatively released from PMA-stimulated pulmonary target cells and infiltrating neutrophils.

KEYWORDS: Liposome, drug delivery, lung injury, antioxidant, α-tocopherol, phorbol myristate acetate

INTRODUCTION

Acute pulmonary inflammatory reactions have been shown to be associated with many clinical conditions such as endotoxemia, haemorrhagic shock, burns, inhalation injury and various drug and chemical insults (Boyd, 1980; Blennerhassett, 1985; Kehrer and Kacew, 1985; Hammerschmidt and Vercelloti, 1987; Henson and Johnston, 1987; Sibille and Reynolds, 1990; Schleimer et al., 1991; Windsor et al., 1993). The associated lung injuries have been characterized by high permeability pulmonary edema; diffuse intraalveolar haemorrhage; bilateral diffuse pulmonary infiltrations; severe hypoxemia; and decreased pulmonary compliance. The mechanisms of pulmonary injuries observed in these pathological conditions are not known, but considerable experimental and clinical evidence has been reported to implicate the accumulation of neutrophils in the lung as a key contributing factor (Kehrer and Kacew, 1985; Hammerschmidt and Vercelloti, 1987; Sibille and Reynolds, 1990; Schleimer et al., 1991; Windsor et al., 1993).

Neutrophils recruited to the lower respiratory tract have the potential to interact with and perturb the lung's parenchymal cells and extracellular matrix, by releasing not only proteases and lysosomal enzymes, but also reactive oxygen metabolites (Blennerhassett, 1985; Henson and Johnston, 1987; Sibille and Reynolds, 1990; Tate and Repine, 1983). These reactive oxygen metabolites, produced by activated neutrophils, are intended for the destruction of invading pathogens (Babior, 1978; Henson and Johnston, 1987; Sibille and Reynolds, 1990). Under certain circumstances, however, the same reactive species also mediate the modification and

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destruction of normal cell components such as lipids, proteins and nucleic acids, resulting in cell injury and death (Boobis et al., 1989; Farber, 1990; Cochrane, 1991).

Although many investigators have examined the possible benefit of reactive oxygen metabolite scavengers in several oxidant-induced lung injury models, the therapeutic use of antioxidants in this type of oxidative injury has produced variable results. The inconsistent results among studies may reflect, at least in part, differences in the pharmacokinetic characteristics of the antioxidative agents used. It has been shown that catalase and superoxide dismutase as well as the iron chelating agent, deferoxamine, cannot traverse membrane barriers (Turrens et al., 1984; Padmanabhan et al., 1985; Ward et al., 1988). Glutathione, a small molecular weight compound, is cleared very rapidly from the lung (Jurima-Romet et al., 1990; Suntres and Shek, 1994). α-Tocopherol is too viscous to be delivered in its free form and following solubilization with detergents or emulsifiers, still only a limited amount of the solubilized antioxidant was able to reach the lung (Losowsky, 1972; Gallo-Torres, 1980). More recent studies, however, have shown that the encapsulation of antioxidants within liposomes greatly increases their intracellular delivery to specific target cells and enhance their protective effects against oxidant-induced tissue injury (Turrens et al., 1984; Padmanabhan et al., 1985; Ward et al., 1988; Jurima-Romet et al., 1990; Suntres and Shek, 1994).

Liposomes are artificially prepared phospholipid vesicles enclosing one or more aqueous compartments. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes provide an efficient delivery system because they are biocompatible, biodegradable and relatively non-toxic (Shek and Barber, 1986). Considerable attention has focused on the use of liposomes to deliver drugs, proteins and molecules of potential therapeutic interest to specific sites of action. With respect to oxidant-induced tissue injury, previous studies have demonstrated that the encapsulation of antioxidants in liposomes promote their therapeutic potential against oxidant-induced lung damage, presumably by the ability of liposomes to facilitate the intracellular uptake of macromolecules and to extend the half-lives of the same macromolecules (Kimbelberg and Mayhew, 1978; Poznansky and Juliano, 1984; Shek and Barber, 1986).

It has been noted that a rapid, but transient neutropenia occurs during cellophane-membrane haemodialysis, endotoxemia, pneumococcal bacteraemia, burns, haemorrhagic shock and inhalation toxicity (Boyd, 1980; Blennerhassett, 1985; Kehrer and Kacew, 1985; Hammerschmidt and Vercelloti, 1987; Henson and Johnston, 1987; Sibille and Reynolds, 1990; Schleimer et al., 1991; Windsor et al., 1993). In all these clinical conditions, neutrophils were found to be aggregated and deposited in the pulmonary vasculature during this neutropenic interval. Since the pathophysiologic mechanisms of neutrophil-mediated acute alveolar injuries have been recognized, specific therapy may be attainable, especially if early treatment can be administered before the onset of respiratory failure.

Phorbol myristate acetate (PMA) is a model compound used to study the role of inflammatory cells in the pathogenesis of acute lung injury (Johnson and Ward, 1982; Kerr et al., 1987; Okuda et al., 1992). Morphological studies on the development of PMA-induced lung injury have revealed that the acute-phase injury is partially mediated via neutrophil-derived reactive oxygen species (Johnson and Ward, 1982; Kerr et al., 1987; Okuda et al., 1992). We hypothesized that the appropriate administration of α-tocopherol, a major biological antioxidant capable of sequestering reactive oxygen species and stabilizing biological membranes (Witting, 1980; Niki, 1987; Burton and Ingold, 1989), may prevent or reduce PMA-induced oxidative injuries. Accordingly, the present study was conducted to investigate whether the pretreatment of animals with liposome-associated α-tocopherol could confer a prophylactic effect against neutrophil-dependent lung injury

MATERIALS AND METHODS

Chemicals

Phorbol myristate acetate (PMA) and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or BDH (Toronto, Ont.).

Animals

Male Sprague-Dawley rats (approximate body weight 220–250 g) were purchased from Charles

River Canada, Inc. (St. Constant, Que.). All animals were housed in stainless-steel cages with free access to pelleted purina laboratory chow and tap water. The animals were kept at room temperature (22–24°C) and were exposed to alternate cycles of 12 hr light and darkness. Animals used in this study were cared for and treated in accordance with guidelines recommended by the Canadian Council on Animal Care in the *Guide to the Care and Use of Experimental Animals*.

Preparation of liposome-associated α-tocopherol

Liposome-associated α-tocopherol was prepared as previously described by Suntres et al. (1993). Liposomal vesicle size was determined with the use of a Coulter N4SD particle-size analyzer and was found to have a mean diameter of 320 ± 40 nm.

Treatment of animals

α-Tocopherol liposomes (8 mg α-tocopherol/kg body weight) or plain liposomes were intratracheally instilled into the lungs of rats as described by Suntres et al. (1993). Twenty four hours after administration of the liposomal preparations, rats were treated intratracheally with a single dose of PMA (25 μg/kg body weight) to induce pulmonary toxicity. PMA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml and stored in aliquots at −20°C. The frozen PMA was thawed and diluted to 25 μg/ml in isotonic saline, shortly before use. Control animals received an equivalent volume of the vehicle solution.

Experimental design

To investigate whether α-tocopherol instilled intratracheally into the lungs of rats could protect against PMA-induced lung injury, rats pretreated with α-tocopherol liposomes were challenged with a single dose of PMA and killed 3 hours later. The protective effect of liposome-associated α-tocopherol against PMA-induced lung damage was assessed biochemically by measuring the activities of angiotensin converting enzyme (ACE), alkaline phosphatase (AKP), and myeloperoxidase to estimate the magnitude of endothelial cell damage, alveolar type II cell injury, and neutrophil infiltration, respectively. Lipid peroxidation (as measured by the formation of diene conjugates) was used to assess membrane injury.

Tissue preparation

Lungs were removed from animals immediately after decapitation and rinsed with ice-cold saline to remove excess blood. All subsequent steps were carried out at 0 − 4°C. Following rinsing, lungs were quickly weighed and finely minced. Approximately 1 g of lung sample was homogenized with a Brinkmann Polytron in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7.4, to produce a 10% homogenate. The homogenate was centrifuged at 9000 *g* for 10 min in a refrigerated Sorvall RC–5B centrifuge. The post-mitochondrial supernatant was decanted and re-centrifuged at 105,000 *g* for 60 min in a refrigerated Beckman ultracentrifuge to obtain the cytosolic and microsomal fractions. For the measurement of lipid peroxidation, homogenates were prepared as described above except the homogenizing medium contained 3 mM ethylenediaminetetraacetic acid (EDTA).

Enzyme measurements

The activity of angiotensin converting enzyme (ACE) was determined using the Sigma Diagnostic procedure as described by Suntres et al (1992). One unit of ACE activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol furylacrylloylphenylalanine per min at 37°C. Alkaline phosphatase activity was determined as previously described (Boudreau and Nadeau, 1987) and one unit of AKP activity was defined as the amount of enzyme that catalyzed the formation of 1 nmole p-nitrophenol/min at 37°C. The activity of myeloperoxidase in sonicated whole lung homogenates was determined by following the changes in optical density resulting from the decomposition of hydrogen peroxide in the presence of tetramethylbenzidine and was expressed as changes in absorbance measured at 450 nm/min (Mulligan et al., 1991). Protein determinations were estimated by the method of Lowry et al. (1951), using crystalline bovine serum albumin (BSA) as the standard.

Determination of lipid peroxidation

Lung homogenates from treated and control animals were assayed for the presence of diene conjugates as described by Suntres et al. (1992).

Statistical analysis

Data from control, liposome- and liposomal α-tocopherol-treated groups were evaluated by one-way analysis of variance (ANOVA). If the F values were significant, the unpaired two-tailed Student's t test was used to compare the treated and the control groups (Gad and Weil, 1982). The level of significance was accepted at $p<0.05$.

RESULTS

Effects of α-Tocopherol Liposomes on PMA-induced Changes in the Lung

Lung weight

Intratracheal instillation of PMA into the lungs of anesthetized rats resulted in a significant increase in wet lung weight (Fig. 1). Pretreatment of animals with liposomes alone did not significantly alter the PMA-induced changes in lung weight. In contrast, pretreatment of animals with α-tocopherol liposomes, 24 hr prior to PMA administration, abolished the PMA-induced effect.

ACE and AKP activities

It has been demonstrated that angiotensin converting enzyme (ACE) and alkaline phosphatase (AKP) activities could serve as indicators of endothelial and type II epithelial cell injury, respectively (Hollinger et al., 1980; Boudreau and Nadeau, 1987). Therefore, the protective effect of α-tocopherol liposomes against PMA-induced endothelial cell and type II epithelial cell damage was also examined in this study. As shown in Fig. 2A, PMA instillation produced a significant decrease (27%) in lung ACE activity compared to that of saline-pretreated animals without PMA challenge. Pretreatment of animals with plain liposomes, 24 hr prior to PMA challenge, failed to alter the PMA-induced decrease in ACE activity. In contrast, pretreatment of rats with α-tocopherol liposomes was found to provide a practically complete protection of the lung against the same PMA challenge. With

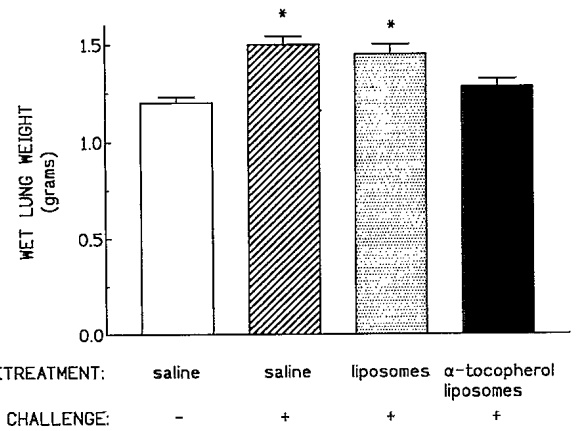

FIGURE 1. Effect of liposome and α-tocopherol-liposome pretreatment on PMA-induced changes in wet lung weight. Animals were instilled intratracheally with either saline, plain liposomes or α-tocopherol liposomes and 24 hr later, they were challenged intratracheally with PMA at a dose of 25 μg/kg body weight. Control animals were also pretreated intratracheally with saline but were not challenged with PMA. Each point represents the mean ± SEM of 5 animals and each asterisk indicates a significant difference ($p<0.05$) between wet lung weights and the corresponding mean level in control animals pretreated with saline but not challenged with PMA.

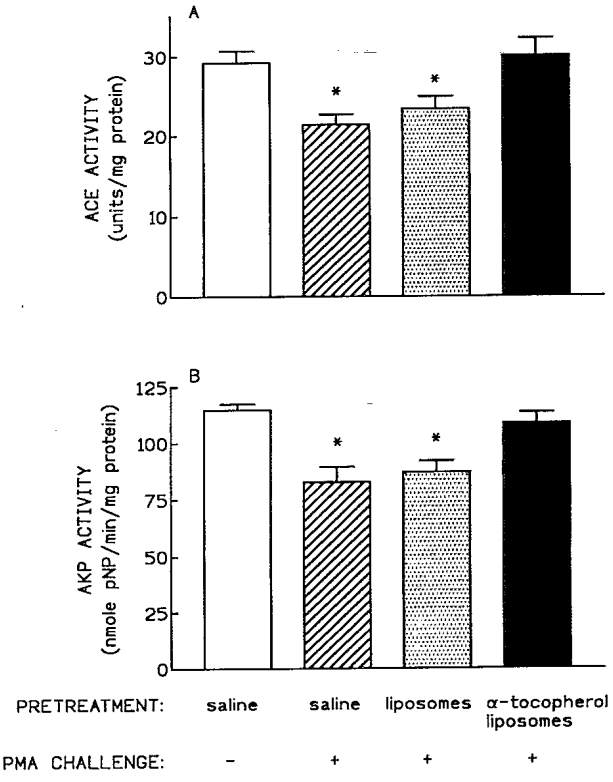

FIGURE 2. Effect of liposome and α-tocopherol-liposome pretreatment on PMA-induced changes in angiotensin converting enzyme [ACE] (panel A) and alkaline phosphatase [AKP] (panel B) activities. Animals were instilled intratracheally with either saline, plain liposomes or α-tocopherol liposomes and 24 hr later, they were challenged intratracheally with PMA at a dose of 25 μg/kg body weight. Control animals were also pretreated intratracheally with saline but were not challenged with PMA. Each point represents the mean ± SEM of 5 animals and each asterisk indicates a significant difference ($p<0.05$) between ACE or AKP activities and the corresponding mean level in control animals pretreated with saline but not challenged with PMA.

respect to the effect of PMA on AKP activity, PMA treatment produced a significant decrease (28%) in AKP activity among saline-pretreated animals. Similarly, a comparable decrease in AKP activity was also observed in liposome-pretreated animals. On the other hand, the AKP activity of animals pretreated with α-tocopherol liposomes was not significantly different from that of animals without PMA challenge.

Lipid peroxidation

PMA-activated neutrophils generate reactive oxygen species that can oxidatively degrade polyunsaturated membrane lipids, in a process known as lipid peroxidation (Girotti, 1985). Since peroxidation of membrane lipids is known to be a major mechanism of oxidant-induced tissue injury, the extent of lipid peroxidation in the lung homogenates of control and treated rats was also measured in the present study. It can be seen from Fig. 3 that the intratracheal instillation of PMA resulted in a 59% increase in lipid peroxidation, as measured by the formation of diene conjugates. Pretreatment of rats with plain liposomes 24 hr prior to PMA challenge did not significantly alter the PMA-induced membrane lipid peroxidation. On the other hand, pretreatment of rats with α-tocopherol liposomes prevented the PMA-induced membrane lipid peroxidation.

Myeloperoxidase levels

The PMA-induced lung injury has been shown to be associated with the infiltration and activation of polymorphonuclear neutrophils, known to contribute to PMA-induced lung damage via oxidative stress-mediated mechanisms (Johnson and Ward, 1982; Kerr et al., 1987; Okuda et al., 1992). In the present study, the infiltration of neutrophils in the lungs of PMA-treated animals was assessed by measuring the activity of myeloperoxidase, an enzyme localized predominantly in neutrophils. As shown in Fig. 4, the myeloperoxidase activity in control animals was significantly elevated (115%) following PMA instillation, suggestive of neutrophil infiltration in the lung. A very similar increase in myeloperoxidase activity was also observed in PMA-challenged rats pretreated with plain liposomes. In contrast, no significant change in lung myeloperoxidase activity was observed among animals pretreated with α-tocopherol liposomes.

DISCUSSION

The present study demonstrated that the lung injury induced by PMA in rats can be ameliorated by

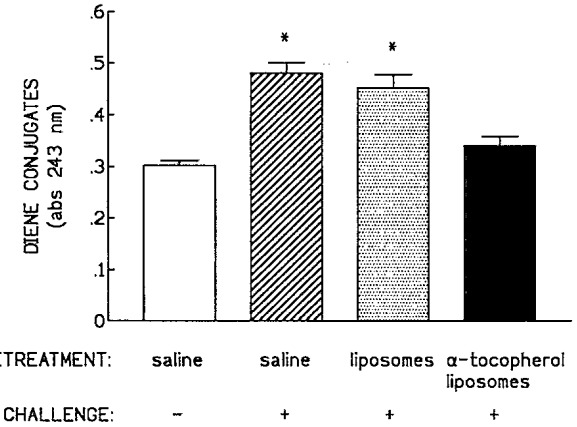

FIGURE 3. Effect of liposome and α-tocopherol-liposome pretreatment on PMA-induced changes in diene conjugates. Animals were instilled intratracheally with either saline, plain liposomes or α-tocopherol liposomes and 24 hr later, they were challenged intratracheally with PMA at a dose of 25 μg/kg body weight. Control animals were also pretreated intratracheally with saline but were not challenged with PMA. Each point represents the mean ± SEM of 5 animals and each asterisk indicates a significant difference ($p<0.05$) between the diene conjugate level and the corresponding mean level in control animals pretreated with saline but not challenged with PMA.

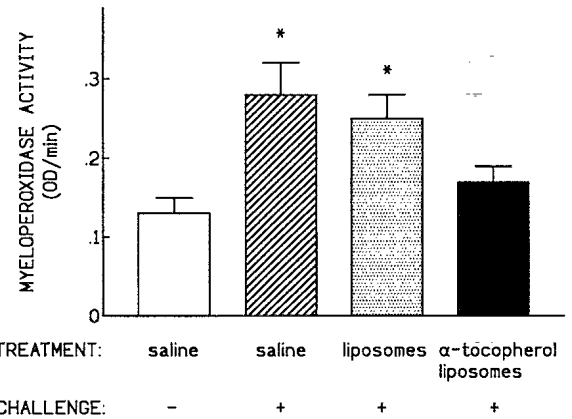

FIGURE 4. Effect of liposome and α-tocopherol-liposome pretreatment on PMA-induced changes in myeloperoxidase activity. Animals were instilled intratracheally with either saline, plain liposomes or α-tocopherol liposomes and 24 hr later, they were challenged intratracheally with PMA at a dose of 25 μg/kg body weight. Control animals were also pretreated intratracheally with saline but were not challenged with PMA. Each point represents the mean ± SEM of 5 animals and each asterisk indicates a significant difference ($p<0.05$) between myeloperoxidase activity and the corresponding mean level in control animals pretreated with saline but not challenged with PMA.

pretreatment with liposome-associated α-tocopherol. The protective effect is evidenced by our observations where the changes in wet lung weight, levels of lipid peroxidation, and activities of enzyme markers (ACE and AKP) were significantly lower in animals pretreated with liposomal α-tocopherol compared to unprotected rats exposed to PMA alone. It is well recognized that PMA is a potent stimulus of neutrophil-derived reactive oxygen species which have been suggested to be responsible for the acute lung injury (Johnson and Ward, 1982; Kerr et al., 1987; Okuda et al., 1992; Rosenbaum and Enket, 1987; Selvaraj et al., 1987; Bautista and Spitzer, 1990) Our present results also strongly indicated that PMA most probably induces lung injury via oxidative stress-mediated mechanisms.

In our study, the abrogation of PMA-induced elevation in myeloperoxidase activity in animals pretreated with liposome-associated α-tocopherol suggested that neutrophil recruitment to the lung was minimal, if any. The lack of significant neutrophil migration to the lung is indicative of little or no pulmonary damage, at least partly attributable to the antioxidant action of α-tocopherol in counteracting the toxic effects of reactive oxygen species. PMA is known to stimulate endothelial cells and macrophages, cells normally present in the lung, to produce reactive oxygen species which result in not only tissue injury, but also the release of prostaglandins, thromboxanes and other neutrophil chemotactic factors (Rosenbaum and Enket, 1987; Selvaraj et al., 1987; Bautista and Spitzer, 1990). The presence of sufficient α-tocopherol in the lung is expected to provide an antagonizing effect on PMA-induced deleterious cascade reactions.

Activated neutrophils can cause lung injury through the release of reactive oxygen species, neutral proteases and lysosomal enzymes (Boyd, 1980; Blennerhassett, 1985; Kehrer and Kacew, 1985; Hammerschmidt and Vercelloti, 1987; Henson and Johnston, 1987; Sibille and Reynolds, 1990; Windsor et al., 1993). These released products may cause serious damages to the lung such as those observed in this study, namely increased lipid peroxidation and decreased pulmonary enzyme activities due to capillary endothelial cell and alveolar type II cell injuries. It has been proposed in other studies that neutrophil infiltration in the lung is associated with acute lung injury and edema, primarily caused by the release of reactive oxygen species and proteases from invading neutrophils (Boyd, 1980; Blennerhassett, 1985; Kehrer and Kacew, 1985; Hammerschmidt and Vercelloti, 1987; Henson and Johnston, 1987; Sibille and Reynolds, 1990; Windsor et al., 1993). Furthermore, other damaging factors, such as the production of cyclooxygenase products and activation of the complement cascade, may also play a role in the development of pulmonary injury. A number of agents, such as allopurinol, catalase, superoxide dismutase and dimethylthiourea, which can suppress the toxic effects of reactive oxygen species, have been shown to confer a protective effect against oxidant-induced lung injury (Schraufstatter et al., 1984; Kerr et al., 1987; Kuroda et al., 1987; Okuda et al., 1992). In this report, we demonstrated that liposomal α-tocopherol is a highly effective prophylactic agent in counteracting PMA-induced neutrophil infiltration and associated oxidative injuries in the lung.

The ability of liposomal α-tocopherol to prevent PMA-induced lung edema is not well understood at the present time. It has been reported that PMA induces the formation of acute edema only in the presence of neutrophils, capable of producing reactive oxygen species. In support of this argument, PMA does not cause edema in neutropenic animals, in isolated lungs in the absence of neutrophils, or in patients with chronic granulomatous disease, where their neutrophils lack the ability to generate reactive oxygen species (Repine et al., 1974a, 1974b; Shasby et al., 1982). In light of these findings, it is conceivable that PMA-induced edema is primarily caused by neutrophil-dependent oxidative mechanisms. Results of this study suggested that α-tocopherol attenuates PMA-induced lung edema by exerting its antioxidant effects. In support of this suggestion, it has been shown that coinstillation of PMA with other antioxidants, such as catalase and superoxide dismutase, prevents neutrophil accumulation and the accompanying edema in the lung (Shasby et al, 1982; Schraufstatter et al., 1984; Kerr et al., 1987).

The role of neutrophils and neutrophil-derived oxidants in the pathogenesis of acute lung injury has been implicated in many clinical lung disorders. Pulmonary endothelial and/or alveolar cell injury associated with an accumulation of neutrophils in the lung has been observed in animals and humans suffering from burn, sepsis, hemorrhagic shock, and other conditions predisposing to adult respiratory distress syndrome (Boyd, 1980; Tate and Repine, 1983; Blennerhassett, 1985; Kehrer and Kacew, 1985; Hammerschmidt and Vercelloti, 1987; Henson and Johnston, 1987; Sibille and Reynolds, 1990; Schleimer et al., 1991; Windsor et al., 1993). In this report, liposome-associated α-tocopherol has been found highly effective in alleviating PMA-

induced lung injury, presumably by antagonizing reactive oxygen species and preventing the neutrophil accumulation in the lung. This protective effect of liposomal α-tocopherol implicates its possible application as a potential prophylactic agent in alleviating or preventing neutrophil-dependent and oxidant-mediated lung injuries in procedures such as hemodialysis, scheduled surgical operations, and blood reperfusion.

Results from our previous studies have shown that intratracheal administration of our present antioxidant formulation can result in a substantial increase in total and subcellular α-tocopherol concentrations in the lung. Indeed, a 16-fold increase in total pulmonary α-tocopherol content has been observed 24 hr after the administration of α-tocopherol liposomes (Suntres et al., 1993). The same treatment with liposomal α-tocopherol has also been demonstrated to be effective in preventing lung injuries induced by pulmonary oxidants such as paraquat and bleomycin (Suntres et al., 1992; Suntres and Shek, 1993; Shek et al., 1994). It is apparent, therefore, that our α-tocopherol delivery system is potentially useful for the delivery of sufficient antioxidants to the lung, within a relatively short time-period, for ameliorating lung injuries induced by oxidant-mediated mechanisms.

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