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A Characterization of Amiodarone-Induced Pulmonary Toxicity in F344 Rats and Identification of Surfactant Protein-D as a Potential Biomarker for the Development of the Toxicity[1]

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Amiodarone (AD) is gaining support as a first-line antiarrhythmic drug despite its potentially fatal pulmonary toxicity involving inflammation and fibrosis. The goals of this study were to characterize a rat model of AD-induced pulmonary toxicity (AIPT) and identify a serum biomarker to aid in the diagnosis of the onset of pulmonary toxicity. Male F344 rats were instilled intratracheally with AD (6.25 mg/kg with a 3.125 mg/ml solution) in sterile water or the sterile water vehicle on days 0 and 2, a protocol that led to the development of pulmonary fibrosis on day 28 in the AD-treated animals. Animals were killed on days 3, 5, 6, 7, or 10 and bronchoalveolar lavage (BAL) was performed. Recovery of alveolar macrophages and eosinophils was increased on days 3 and 5, while neutrophil recovery and albumin levels in the first BAL fraction were significantly elevated only on day 3. BAL cells recovered from AD-treated rats at day 3 produced more phorbol myristate acetate-stimulated luminol-dependent chemiluminescence (LDCL) over 20 min than BAL cells from control rats. Experiments using specific inhibitors implicated superoxide and nitric oxide in at least part of the LDCL response. Serum levels of surfactant protein-D (SP-D), a surfactant-associated protein, were increased concurrently with the inflammatory response in the lungs. These findings indicate that this model exhibits transient pulmonary inflammation and damage, with the potential for elevated oxidant production in the lungs and subsequent pulmonary fibrosis. Also, SP-D is proposed as a specific biomarker to monitor the onset of AIPT in this model. © 2000 Academic Press

Key Words: **Amiodarone; amiodarone-induced pulmonary toxicity; surfactant protein-D; inflammation; fibrosis; biomarker.**

Amiodarone (AD), an iodinated benzofuran derivative, is a very effective antiarrhythmic drug and was the most-prescribed antiarrhythmic drug in the United States in 1998 (Connolly, 1999). Although AD had classically been used as a drug of last resort due to its significant toxicities (Mason, 1987; Wilson and Podrid, 1991), its effectiveness against various types of serious arrhythmias has caused a dramatic increase in its use as a first-line therapy (Bauman, 1997). One of the most life-threatening side effects of AD therapy is AD-induced pulmonary toxicity (AIPT) (Martin and Rosenow, 1988a; Reasor and Kacew, 1996). AIPT is characterized by inflammatory cell infiltration into the interstitial and alveolar spaces, potentially progressing to pulmonary fibrosis (Martin and Rosenow, 1988a). As the use of AD increases, the occurrence of its side effects will likely increase accordingly.

Current treatment for AIPT involves a reduction or elimination of the drug dosage, which may leave the patient susceptible to the original arrhythmia. In some cases, corticosteriod therapy is very effective, but this can be accompanied by its own set of undesirable effects (Martin and Rosenow, 1988a). Furthermore, toxicity may recur when the steroid treatment is halted (Joelson *et al.*, 1984).

The etiology of AIPT is unknown (Massey *et al.*, 1995; Reasor and Kacew, 1996). However, several causes have been proposed, including direct toxicity (Martin and Howard, 1985), the involvement of hypersensitivity (Akoun *et al.*, 1984), elevated oxidant involvement (Kennedy *et al.*, 1988), and drug-induced alterations in membrane properties (Honegger *et al.*, 1993). It has also been postulated that the cause is complex and multifactorial, possibly involving several of these mechanisms (Reasor and Kacew, 1996).

Models of AIPT that utilize intratracheal (i.t.) administration of AD have been developed in hamsters (Blake and Reasor,

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1995a; Cantor *et al.*, 1984; Daniels *et al.*, 1989; Leeder *et al.*, 1994; Rafeiro *et al.*, 1994; Wang *et al.*, 1992) and rats (Reinhart *et al.*, 1996; Reinhart and Gairola, 1997) to elucidate the mechanisms by which this disorder develops. Intratracheal instillation of AD leads to an inflammatory reaction in hamsters (Blake and Reasor, 1995a) and a fibrotic reaction in hamsters (Blake and Reasor, 1995b; Cantor *et al.*, 1984) and rats (Reinhart *et al.*, 1996) similar to that observed in humans. Little information exists concerning the inflammatory reaction in rats to i.t. AD. While the hamster has been the animal of choice for many studies of AIPT, the rat model offers a greater potential for studies of the mechanisms that underlie AIPT due to the greater availability of certain molecular reagents and tools. Specifically, antibodies and ELISA kits to specific rat cytokines and signaling molecules are much more widely available than for hamsters. These tools would then permit a more complete characterization of the disorder, including the underlying molecular signaling involved.

Diagnosis of AIPT in patients taking amiodarone is difficult due to the lack of specific symptoms and often to the presence of confounding factors (Martin and Rosenow, 1988a). AIPT is usually diagnosed based on the symptoms of dyspnea as well as radiological findings of diffuse pulmonary infiltrates (Nicholson and Hayward, 1989; Butler and Smathers, 1985; Delany *et al.*, 1993). Patients with concurrent cardiac or respiratory disorders are more likely to go undiagnosed, and AIPT can be fatal in such cases (Pitcher, 1992). A specific marker for the pulmonary response to AD could aid in the early diagnosis of AIPT, possibly before overt toxicity develops. While several serum biomarkers have been proposed for the monitoring of lung diseases, at present none have been identified to detect the onset and progression of AIPT. Serum levels of mucin-1 were elevated in 8 of 10 AD patients with AIPT, but mucin-1 levels were also increased in AD patients that exhibited nonpulmonary toxicities (Devine *et al.*, 1998). Also, elevated serum lactate dehydrogenase (LDH) was found in a patient with AD-induced pneumonitis (Drent *et al.*, 1998). However, LDH is not lung-specific and can be released into the blood in nonpulmonary toxicities.

Recently the serum levels of surfactant protein-D (SP-D) were found to be increased in patients with idiopathic pulmonary fibrosis, interstitial pneumonia with collagen disease, and pulmonary alveolar proteinosis (Honda *et al.*, 1995). Since SP-D is produced in the lungs by alveolar type II cells and is found in the fluid lining the alveoli (Crouch, 1998), it may serve as a more specific marker for lung injury.

Because of the prominent use of AD to treat arrhythmias, there are a large number of patients at risk for developing AIPT. This study was undertaken to characterize a rat model for AIPT with regard to the possible mechanisms involved in its development as well as to examine the potential for serum SP-D to serve as a biomarker for AIPT development in this model.

METHODS

Materials

Amiodarone HCl was a gift from Wyeth-Ayerst Laboratories (Princeton, NJ). All other materials were laboratory-grade purchased commercially.

Laboratory Animals

All studies were performed on specific pathogen-free adult male F344 rats (200–300 g; Hilltop Laboratories, Scottsdale PA). The animals were housed in the university's animal quarters and were allowed at least 1 week to acclimate after arrival from the supplier. Free access to food and water was allowed at all times.

Animal Treatment

Animals were anesthetized with Brevital (sodium methohexital; 40 mg/kg), placed on a slanted board, and suspended from their maxillary incisors as well as being held by an elastic strap across the abdomen. The tongue was held with gauze and a syringe with a ball-tipped 20-gauge feeding needle was inserted transorally into the trachea. Amiodarone (6.25 mg/kg in a 3.125 mg/ml solution) or the vehicle, sterile water, was then administered to the lungs, and the rat was removed from the board and observed until consciousness was regained. The animals received two instillations of AD or vehicle, the first on what is designated day 0 and the second on day 2. Naïve animals were used to obtain the day 0 values presented.

Analysis of Fibrosis

Pulmonary fibrosis was evaluated biochemically and histopathologically 28 days after the first i.t. administration of AD.

Biochemical quantification of hydroxyproline. Hydroxyproline was measured based on the method of Witschi *et al.* (1985). The right-lung lobes of AD-treated and control animals were hydrolyzed in 6 N HCl at 100°C for 72 h to release hydroxyproline. The hydroxyproline was then

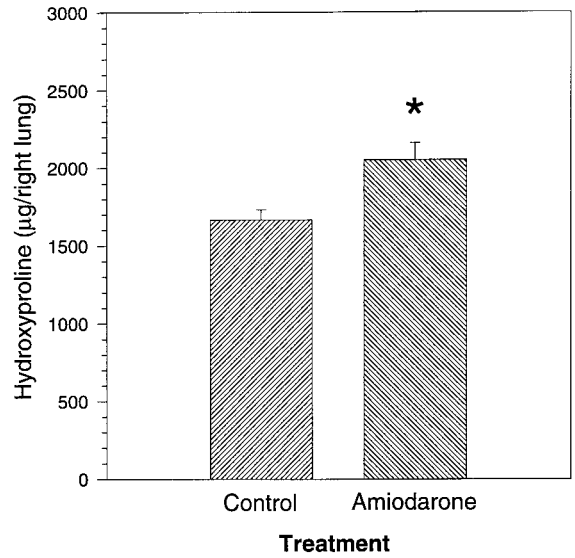

FIG. 1. Effect of AD on right-lung hydroxyproline content. Rats were instilled intratracheally with amiodarone (6.25 mg/kg in a 3.125 mg/ml solution) or an equivalent volume of sterile water (Control) on days 0 and 2. Right lungs were obtained at day 28 and hydroxyproline content was measured as described under Methods. Values are means ± SEM. $N = 8–9$. *Significantly different from control ($p < 0.05$).

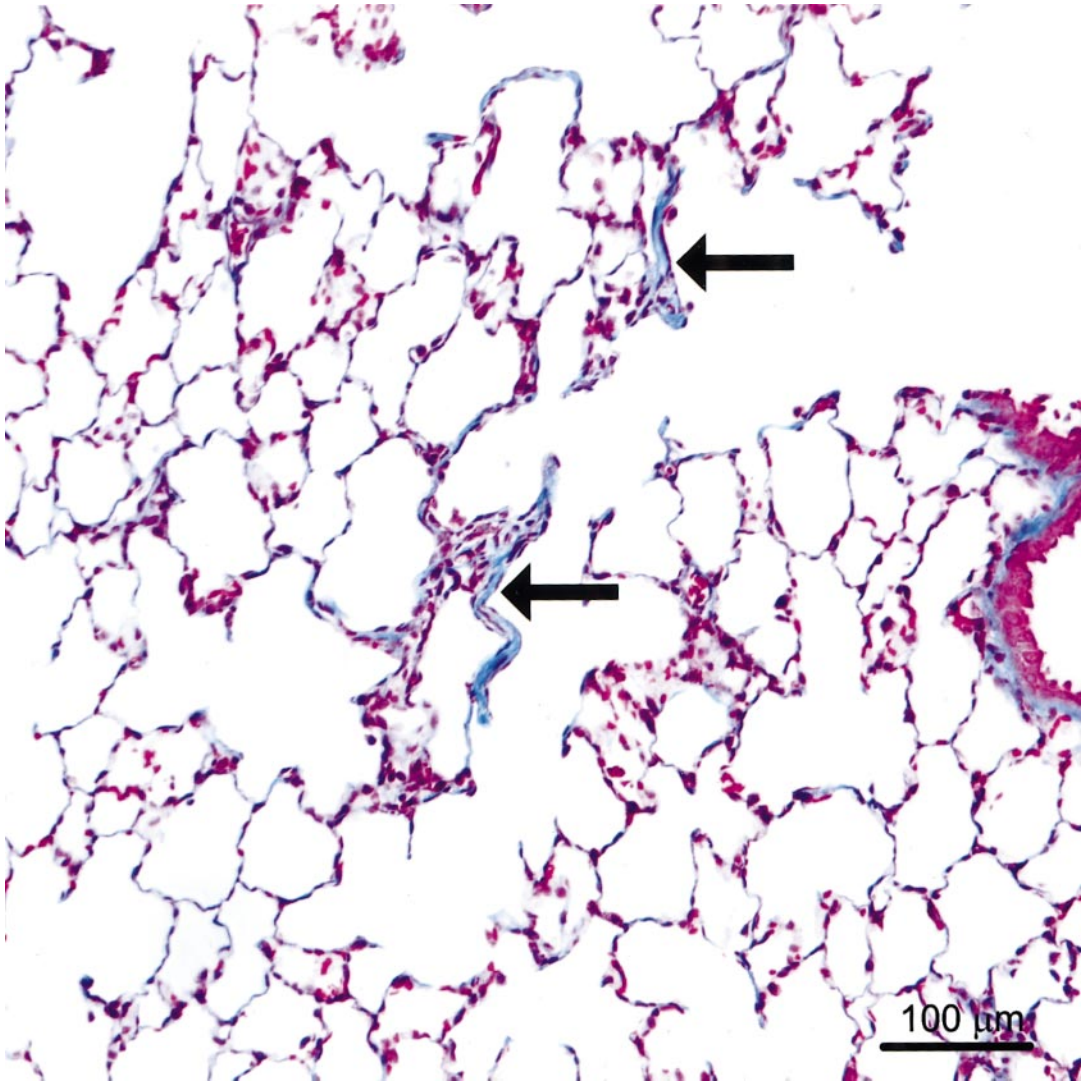

FIG. 2. Effect of AD on left lung histopathology. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2. Left lungs were obtained at day 28, inflated, and fixed, and slides were prepared as described under Methods. All lungs from control animals exhibited normal lung architecture ($N = 5$; data not shown). Lungs from all AD-treated rats displayed minimal to mild, multifocal, interstitial pulmonary fibrosis in all AD-treated rats ($N = 5$). Fibrosis was most frequently observed near alveolar duct regions. A representative field is shown from an AD-treated animal. Arrows indicate areas of septal thickening and collagen deposition evidenced by trichrome staining. Bar indicates 100 μm.

oxidized and converted to a colored product that was measured with a spectrophotometer at 560 nm. *Trans*-hydroxyproline was used as the standard for quantification.

Histopathological evaluation of fibrosis. Fibrosis was characterized histopathologically by fully inflating and fixing the left lung with 10% formalin solution, embedding the tissue in paraffin, and sectioning the tissue for the preparation of microscope slides. Sections of 5 μm were made and stained with either hematoxylin and eosin or trichrome, a stain specific for collagen fibers. Slides were then evaluated using light microscopy.

Bronchoalveolar Lavage

To harvest pulmonary cells for morphologic and functional analysis, bronchoalveolar lavage (BAL) was performed using aliquots of ice-cold calcium-free and magnesium-free phosphate-buffered saline (PBS). The first BAL volume was administered as 2 ml/100 g body wt, and this volume was placed into the lungs for 30 s with light massaging, withdrawn, and again instilled into the lungs for 30 additional seconds. Once withdrawn, this aliquot (designated the first BAL fraction) was kept separate from the rest of the BAL fluid that was recovered. BAL was continued with the individual aliquots pooled until 50 ml of BAL fluid was recovered. The recovered fluid was then centrifuged at 300*g* for 10 min, the supernatant was decanted, and the cells were resuspended. The first BAL fraction was centrifuged separately and the supernatant fluid was frozen at −20°C for subsequent albumin analysis. The cells from this initial fraction were pooled with the rest of the cells recovered from that animal, and the cells were washed two times with PBS. Total BAL cells were counted using an electronic cell counter (Model Z_b; Coulter Electronics, Hialeah, FL). Aliquots of cells were then attached to microscope slides via a cytospin (Shandon Cytospin II; Shandon Inc, Pittsburgh, PA), dried, and stained with an automated slide stainer (Hema-Tek 2000; Bayer Corp., Elkhart, IN)

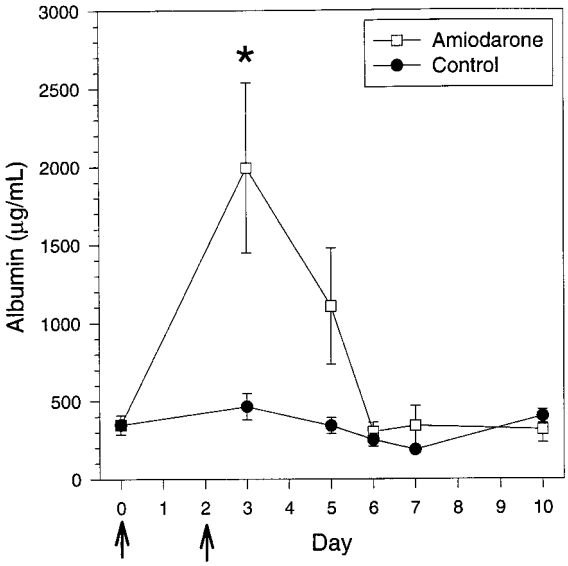

FIG. 3. Effect of AD on albumin levels in the first BAL fraction. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2. Rats were subjected to BAL at various time points and albumin in the first BAL fraction (2 ml/100 g) was assayed as described under Methods. Naïve values are represented at day 0 and arrows indicate intratracheal treatments. Values are means ± SEM. $N = 5$–9. *Significantly different from control ($p < 0.05$).

using Wright–Giemsa stain. Finally, the cells were typed and counted using a light microscope. At least 400 cells were counted per slide. The percentage of each cell type as well as the total number of each cell type recovered from the BAL was calculated.

Serum Preparation

At the time of lavage, blood was drawn from each animal directly from the heart using a 21-gauge needle and syringe. The blood was allowed to clot for at least 1 h and the serum was prepared by centrifugation and frozen at −20°C for subsequent surfactant protein analysis.

Analysis of Albumin

The integrity of the alveolar–capillary barrier was evaluated by measuring the amount of albumin, a protein from the blood, in the first BAL fraction that was recovered from the lungs. Albumin in the BAL fluid was determined according to a Sigma Diagnostics method utilizing the reaction of albumin with bromcresol green. The reaction product was then measured with a spectrophotometer at 628 nm and quantified against known concentrations of bovine serum albumin.

Luminol-Dependent Chemiluminescence (LDCL)

To measure the release of oxidants from BAL cells, LDCL was performed as described by Antonini *et al.* (1994) on a Berthold LB9505C Luminometer. Rats treated with AD or sterile water vehicle underwent BAL, and the BAL cells were counted as described above. Luminol, used as a bystander to produce CL when oxidized, was dissolved in DMSO and then diluted in PBS (pH 7.4). The final luminol concentration in the assay cuvette was 10^{-5} M. Phorbol myristate acetate (PMA) at a final concentration of 2×10^{-6} M was used to activate the oxidative burst. An unstimulated baseline LDCL was obtained by omitting PMA. To selectively inhibit oxidant species and to determine the nature of the LDCL produced, the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mM final concentration) and superoxide dismutase (SOD; 0.2 mg/ml final concentration), a superoxide-scavenging enzyme, were added alone and combined to designated replicates. Samples containing inhibitors were preincubated 5 min prior to PMA addition and subsequent LDCL measurement. The final reaction volume for all samples was 500 µl and the reactions were measured at 37°C for 20 min. Integral CL counts were determined by KINB software for the Berthold 9505C Luminometer.

Surfactant Protein-D (SP-D) Levels

Levels of SP-D were assayed in the sera of naïve, AD-, and vehicle-treated rats at various time points following treatment. Serum SP-D levels were assayed with a sandwich ELISA technique. Rat SP-D, which was purified from lavage fluid from rats 28 days after silica instillation according to the method of Shimizu *et al.* (1992), was used as a standard for the assay. Polyclonal anti-rat SP-D rabbit IgG (10 µg/ml in 0.1 M sodium bicarbonate) was prepared by the method of Shimizu *et al.* (1992) and bound to wells in microtiter plates (Immulon I plates; Dynatech Laboratories, Alexandria, VA) at room temperature overnight. The wells were then incubated with a 5% (w/v) solution of nonfat dry milk in PBS (blocking buffer) to block nonspecific binding. After washing, 100 µl containing purified rat SP-D (0–20 ng) for standards or appropriately diluted serum samples were added to each well. Plates were then incubated for 90 min at 37°C and then washed with 20% blocking buffer, 1% Triton X-100 (v/v) in PBS (antibody buffer). After washing, 200 µl of anti-SP-D antibody-conjugated horseradish peroxidase (30 µg/ml in antibody buffer) was added to the wells and the plates were incubated for 90 min at 37°C. After further washing with 1% Triton X-100 in PBS, 200 µl of the color-developing agent (0.1% *o*-phenylenediamine, 0.015% hydrogen peroxide in 0.1 M citrate buffer, pH 4.6) was added. The reaction was carried out for 5 min at room temperature in a darkened room and was stopped by the addition of 100 µl of 1 M sulfuric acid. The absorbance at OD_{490} was recorded with a Microplate Autoreader EL-311s (BIO-TEC Instruments Inc., Winooski, VT).

Statistics. All data are presented as means ± SEM. Hydroxyproline values were analyzed using Student's *t* test between mean values; all other data were analyzed using a two-way ANOVA followed by Tukey's protected *t* post-hoc test. Statistical significance was established when $p < 0.05$.

RESULTS

Fibrosis was assessed in two ways in order to ascertain both the extent and pattern of fibrosis. Rats treated on days 0 and 2 with i.t. AD had significantly increased levels of right-lung hydroxyproline compared with control rats on day 28 (Fig. 1). Histopathological evaluation of left-lung sections at day 28 revealed a minimal to mild, multifocal, interstitial pulmonary fibrosis in all AD-treated rats ($N = 5$). Fibrosis was most frequently observed near alveolar duct regions (Fig. 2). Fibrosis was not found in any control animals ($N = 5$).

To analyze the integrity of the alveolar–capillary barrier, albumin was assayed in the first BAL fraction from AD- or vehicle-treated rats as a function of time after treatment (Fig. 3). Albumin was significantly elevated at day 3 in AD-treated rats, consistent with an initial damage and leakage from the blood to the airways soon after AD administration.

Total cell recovery by BAL of AD-treated rats was significantly elevated above controls on both days 3 and 5 (Fig. 4A). Most of the cells recovered were alveolar macrophages, which were also significantly increased on days 3 and 5 following AD treatment (Fig. 4B). A significant increase in the number of polymorphonuclear leukocytes

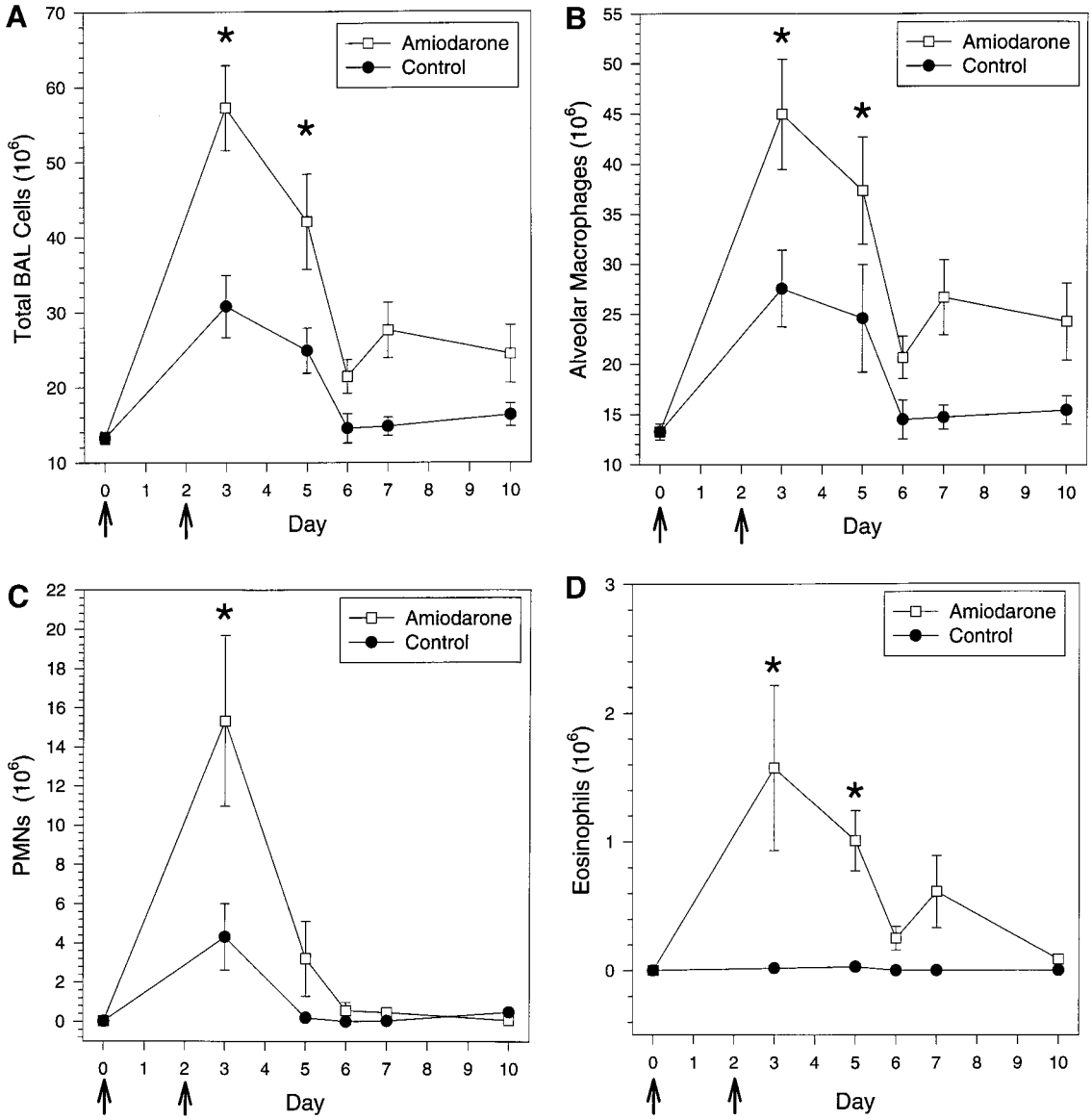

FIG. 4. Effect of AD on the number and type of cells recovered by BAL. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2 and subjected to BAL at various time points following treatment. Total cell number and number of each type of cell recovered in the BAL fluid was determined as described under Methods. (A) Total cell number recovered by BAL; (B) number of alveolar macrophages recovered by BAL; (C) number of PMNs recovered by BAL; and (D) number of eosinophils recovered by BAL. In all panels naïve values are represented at day 0 and arrows indicate intratracheal treatments. Values are means ± SEM. $N = 5–9$. *Significantly different from control ($p < 0.05$).

(PMNs) recovered by BAL occurred on day 3 (Fig. 4C). Figure 4D shows the recovery of eosinophils was significantly elevated at days 3 and 5.

To assess the level of activation of cells recovered from BAL, PMA-stimulated LDCL was performed. On day 3, cells from AD-treated rats were capable of producing more oxidants when stimulated (Fig. 5). Since the cells are activated on a per cell basis, and significantly more cells are present on days 3 and 5, a potential oxidant burden was calculated by multiplying the LDCL by the total number of BAL cells recovered for each animal. Figure 6 shows that, on days 3 and 5, the potential lung oxidant burden was significantly greater in the AD-treated rats compared to controls.

To assess the oxidant species involved in the LDCL response at day 3, specific inhibitors to various oxidants were utilized. L-NAME is an inhibitor of nitric oxide synthase, leading to reduced NO levels, while SOD is an enzymatic scavenger of superoxide anion. Both inhibitors individually inhibit LDCL of BAL cells from AD-rats (Fig. 7). The combination of L-NAME and SOD inhibited PMA-stimulated LDCL to the level of non-PMA-stimulated cells.

In an attempt to identify a specific serum biomarker for

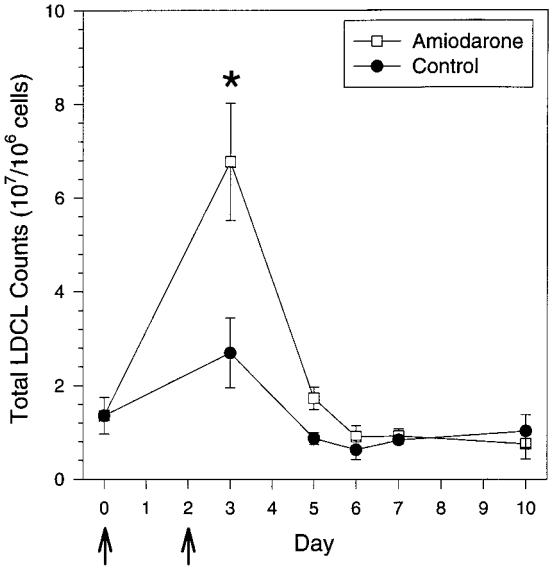

FIG. 5. Effect of AD on total PMA-stimulated LDCL counts over 20 min from BAL cells. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2 and subjected to BAL at various time points following treatment. The recovered cells were subjected to LDCL with PMA stimulation as described under Methods. Naïve values are represented at day 0 and arrows indicate intratracheal treatments. Values are means ± SEM. $N = 5$–9. *Significantly different from control ($p < 0.05$).

AIPT, the levels of SP-D were assayed in the serum of naïve, vehicle-, and AD-treated rats. SP-D levels were elevated in rat serum following AD treatment from day 3 through day 7, during the time that the initial damage and inflammatory reaction were occurring (Fig. 8).

DISCUSSION

A goal of the current study was to develop and characterize a rat model for AIPT that could be used to examine possible mechanisms associated with the development of this disorder. Also, the potential of serum SP-D levels to serve as a biomarker for AD-induced lung damage was examined. Previously, most animal models of AIPT involved the use of hamsters as the test system (Blake and Reasor, 1995a,b; Cantor *et al.*, 1984; Daniels *et al.*, 1989; Leeder *et al.*, 1994; Rafeiro *et al.*, 1994; Wang *et al.*, 1992). Models of AIPT in rats mainly involved oral dosages that did not consistently produce inflammation and fibrosis (Reasor and Kacew, 1996) or took an inordinate amount of time to develop (Carvalho *et al.*, 1996). Only one study (Reinhart *et al.*, 1996) has reported that a single i.t. dose of AD (200 μl of water containing 1.25 mg AD) leads to increased levels of hydroxyproline and abnormal histological findings at 6 weeks in F344 rats. However, when we attempted to replicate the findings of that study, we found that a second AD dose was needed at day 2 to produce a consistent and reproducible elevation of right-lung hydroxyproline levels at 4 weeks. It is unclear why a second AD dose was necessary to add consistency to the previously reported results. One possibility is the time at which the fibrosis was analyzed, although we found no differences between hydroxyproline levels at 4 or 6 weeks following a single i.t. AD dose (6 week data not shown). Another difference in the model systems is the gender of the rats; we used male F344 rats while the previous findings (Reinhart *et al.*, 1996) were reported using female F344 rats.

After the development of fibrosis was confirmed following AD treatment, the initial responses of the lung to the drug treatment were assessed and compared to control animals receiving sterile water, the drug vehicle. Elevated albumin levels in the first BAL fraction of AD-treated rats on day 3 was evidence of damage to the alveolar–capillary barrier, which allowed albumin, a protein from the blood, to accumulate in the alveoli. Therefore, the development of fibrosis in this model was associated with an early and transient damage to the alveolar–capillary barrier.

The total number of cells recovered by BAL from AD-treated rats was significantly elevated on days 3 and 5. Most were alveolar macrophages, which were also significantly elevated in number on days 3 and 5. The number of alveolar macrophages can be increased by recruitment from the surrounding tissue as well as from monocytes recruited from the blood (Sibille and Reynolds, 1990). Stimulated macrophages can play an instrumental role in directing an inflammatory reaction by releasing chemical mediators to direct the recruitment of inflammatory cells as well as releasing oxidants as part

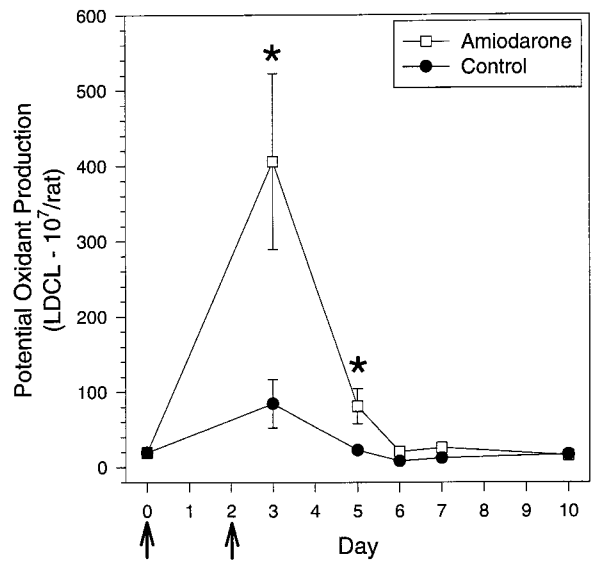

FIG. 6. Effect of AD on potential oxidant production of total BAL cells. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2 and subjected to BAL at various time points following treatment. The recovered cells were subjected to LDCL with PMA stimulation as described under Methods. To quantitate the potential oxidant production from the BAL cells, the number of BAL cells recovered from each animal was multiplied by the per cell LDCL value. Naïve values are represented at day 0 and arrows indicate intratracheal treatments. Values are means ± SEM. $N = 5$–9. *Significantly different from control ($p < 0.05$).

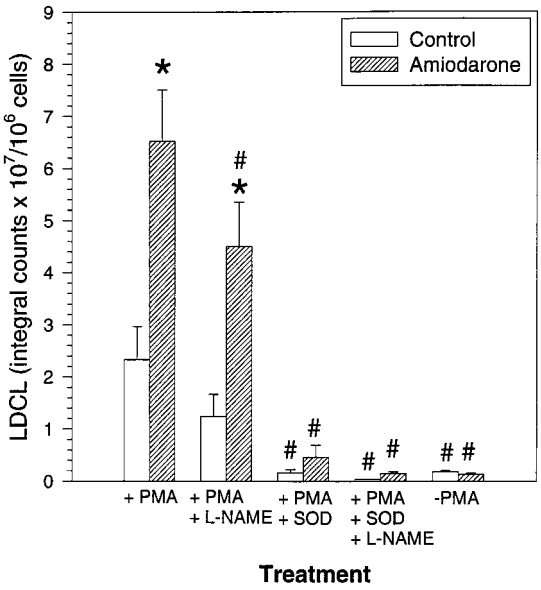

FIG. 7. PMA-stimulated LDCL counts over 20 min from BAL cells on day 3 with specific inhibitors. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2 and subjected to BAL on day 3. Recovered BAL cells were subjected to LDCL with and without PMA stimulation and the nitric oxide synthase inhibitor L-NAME and superoxide scavenger SOD as described under Methods. Values are means ± SEM. $N = 5–9$. *Significantly different from control ($p < 0.05$). #Significantly different from corresponding PMA only value ($p < 0.05$).

of the host-defense system (Laskin and Pendino, 1995). There is evidence that inflammatory cells are recruited to the lungs after AD administration, as the number of PMNs were increased at day 3. PMNs can release a variety of inflammatory mediators as well as oxidant species when recruited and activated. PMNs are recruited by cytokines and chemokines released by macrophages and other pulmonary cells in various types of lung inflammatory reactions (Sibille and Reynolds, 1990).

Eosinophils are another type of inflammatory cells recruited to the lung after AD administration. Eosinophils are more often recruited during inflammatory reactions involving immunological processes, possibly implicating an immunological component to AIPT in this model. Elevated eosinophils have also been reported in a hamster model for AIPT (Blake and Reasor, 1995a) and in the lungs of some human patients with AIPT (Akoun *et al.*, 1991). Eosinophils are generally recruited into the lungs by the cytokine IL-5 released by various lung cells, including activated T lymphocytes. Once recruited, eosinophils can be directed to certain sites within the tissue by the chemokine eotaxin (Hogan *et al.*, 1998). The basis for eosinophil recruitment in this model is not yet clear, but eosinophils can participate in inflammatory reactions by releasing damaging enzymes and oxidant species (Giembycz and Lindsay, 1999).

To assess the capacity for activation of the cells recovered by BAL, BAL cells were subjected to LDCL with and without PMA stimulation. PMA-stimulated BAL cells from AD-treated rats produced significantly more LDCL counts over 20 min than PMA-stimulated cells from control animals at day 3, demonstrating that more oxidants were being produced. This indicates that the BAL cells from AD-treated rats can be activated to produce more oxidant species on a per cell basis on day 3 than BAL cells from control animals. Since the total cell number was also elevated at day 3, the potential total oxidant production was calculated by multiplying the PMA-stimulated LDCL counts, which were on a cell number basis, by the total number of BAL cells recovered. Thus, on day 3, there is a much greater potential for oxidant burden in the lungs of AD-treated animals than in the lungs of control animals. If that potential is realized, elevated cellular oxidant production could therefore account for some of the early damage done to the alveolar–capillary barrier.

In an attempt to identify the oxidant species involved in the LDCL response at day 3, specific inhibitors were utilized. L-NAME was used as an inhibitor of NO production while SOD was used as a scavenger of superoxide anions. L-NAME alone slightly, but significantly, inhibited LDCL production from BAL cells from AD-treated rats. SOD strongly inhibited light production, presumably by scavenging superoxide anions produced by BAL cells. Therefore, much of the light response was dependent on superoxide anion production or reactants produced from it. Even though SOD inhibited more light than L-NAME, L-NAME is a charged molecule that may not penetrate the cell membrane well enough to reach maximally effective inhibitory concentrations in the cytosol. Furthermore,

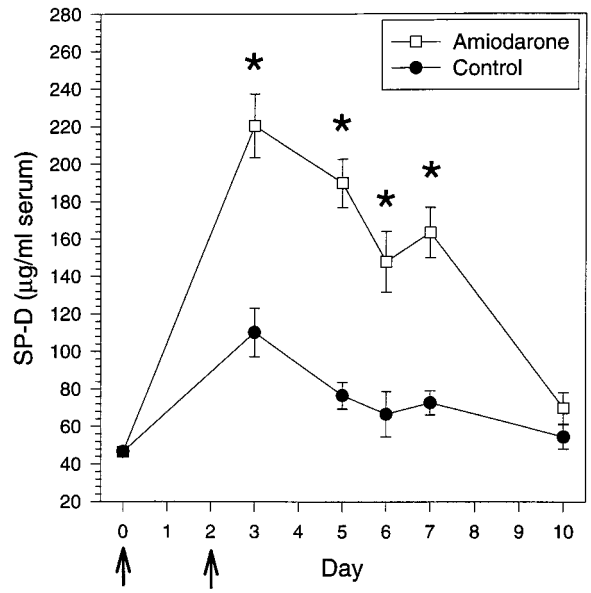

FIG. 8. Effect of AD on SP-D levels in serum. Rats were treated with amiodarone or sterile water (Control) as described in Fig. 1 on days 0 and 2. Serum was collected as various time points and assayed for SP-D as described under Methods. Naïve values are represented at day 0 and arrows indicate intratracheal treatments. Values are means ± SEM. $N = 5–9$. *Significantly different from control ($p < 0.05$).

when SOD and L-NAME were combined, light production from PMA-stimulated BAL cells was inhibited to the level of non-PMA-stimulated cells. Since superoxide anion and NO readily combine to form the powerful oxidant peroxynitrite, it is possible that peroxynitrite production is increased from BAL cells from AD-treated rats. Therefore, AD administration may lead to elevated oxidant stress in the lungs, resulting from elevated production of oxidant species by an increased number of cells in the lungs soon after drug administration. This may cause damage to the lung tissue that, when subsequently repaired, leads to increased collagen deposition and pulmonary fibrosis.

Amiodarone is a member of a class of drugs that cause phospholipidosis, or the abnormal accumulation of phospholipid material in cells and tissues, upon oral administration (Reasor and Kacew, 1996). A study comparing the pulmonary responses of hamsters to either oral or i.t. treatment concluded that lung drug burden and the development of phospholipidosis are not crucial factors in the development of fibrosis in response to AD (Blake and Reasor, 1995b). Oral AD administration led to drug accumulation in lung tissue and significant phospholipidosis without resulting in fibrosis, while i.t. AD treatment produced fibrosis without drug accumulation and phospholipidosis.

The final goal of the study was to identify a biomarker that could easily be measured in the serum and could potentially aid in the diagnosis of AIPT. If applicable to humans, it would be important that this material could be obtained easily and monitored serially from the beginning of AD treatment. Also, it should correlate with the early aspects of the toxicity, such as pulmonary inflammation, so that a proper diagnosis can be made and the patient treated before the onset of irreversible and potentially fatal pulmonary fibrosis. It was found that serum levels of SP-D, a surfactant-associated protein, were elevated during the inflammatory stage of the response and through day 7. Therefore, SP-D levels in humans may serve as a useful marker for the onset of AIPT and may aid in its initial diagnosis, which is often difficult (Martin and Rosenow, 1988b). Currently, diagnosis is made using nonspecific symptoms and chest imaging without specific diagnostic features and can be complicated by the presence of other disorders (Martin and Rosenow, 1988b; Butler and Smathers, 1985; Delany *et al.*, 1993). Substances previously proposed as biomarkers for AIPT may lack pulmonary specificity. SP-D may prove useful as an indicator of the lung-specific damage that occurs early in the development of AIPT. Serum SP-D levels would need to be measured in patients taking amiodarone over time and, if increased, may provide a signal that damage is occurring. Research to characterize changes in serum SP-D levels is required before it could be adopted as a biomarker in humans. However, serum SP-D increases have been correlated with the development of adult respiratory distress syndrome (Greene *et al.*, 1999), pulmonary alveolar proteinosis, idiopathic pulmonary fibrosis, and interstitial pneumonia with collagen vascular diseases in humans (Kuroki *et al.*, 1998). Therefore, there is potential for elevated serum SP-D levels in patients developing AIPT.

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