Effects of Amiodarone-Induced Phospholipidosis on Pulmonary Host Defense Functions in Rats (43979)

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Abstract. **The effect of the induction of pulmonary phospholipidosis by amiodarone on selected pulmonary host defense functions was studied in male Fischer-344 rats. One week of daily amiodarone treatment resulted in a 4.5-fold increase in total phospholipid in alveolar macrophages recovered from the lungs by bronchoalveolar lavage. The presence of the phospholipidosis had no effect on the phagocytosis of heat-killed yeast cells, the induction of luminol-dependent chemiluminescence, or the spontaneous release of interleukin-6 (IL-6) and tumor necrosis factor–α (TNF-α), or spontaneous and LPS-stimulated release of IL-1 by alveolar macrophages *in vitro*. In contrast, the LPS-stimulated release of IL-6 and TNF-α by phospholipidotic alveolar macrophages was enhanced compared with control cells. The pulmonary clearance of *Listeria monocytogenes* following intratracheal administration of the bacteria was not affected by the phospholipidotic condition. It appears that, in the context of the functions studied, the induction of pulmonary phospholipidosis by amiodarone does not impair pulmonary host defense processes in rats, and may actually be associated with the augmentation of some activities.** [P.S.E.B.M. 1996, Vol 211]

Amiodarone is a class III antiarrhythmic agent which is useful in the treatment of supraventricular arrhythmias and for ventricular arrhythmias refractory to conventional antiarrhythmic therapy. Amiodarone is classified as a cationic, amphiphilic drug (CAD) because of its structure: it contains both hydrophobic and hydrophilic domains (1). The use of CADs such as amiodarone by humans, and their administration to laboratory animals, can result in the induction of a phospholipid storage disorder, termed phospholipidosis, which affects primarily the lysosomal compartment of the cell.

While phospholipidosis can develop in a number of tissues, the lungs are particularly susceptible (1, 2). A prominent feature of this disorder is the accumulation of hypertrophic pulmonary macrophages in the alveoli. Biochemically, the cells accumulate polar phospholipids. The storage of this material results in the development of lysosomal lamellar inclusion bodies (1, 3), the ultrastructural hallmark of this disorder.

Biochemical, morphological, and ultrastructural features of CAD-induced pulmonary phospholipidosis have been well characterized (3), but much less is known about the functional or toxicological consequences of this pathological condition, that is, Does the presence of a phospholipidosis result in the alteration of functions of cells or organs involved? Some studies indicate an impairment in cellular function (4–6), while others are consistent with an enhancement (7–11). The purpose of this study was to investigate the effects of amiodarone-induced cellular phospholipidosis on selected features of pulmonary host defense in

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rats. The principal function of pulmonary host defense mechanisms is to clear inhaled particles from the lungs and keep the lungs sterile (12). Alveolar macrophages (AMs) serve as the first line of cellular defense (13). They phagocytize particles and secrete oxidants and mediators that can directly kill microorganisms and that also allow communication with other cells of the immune system. Successful pulmonary host defense is dependent on a functionally integrated system involving multiple factors (14, 15).

In the present study, we have examined the effect of phospholipidosis on selected functions of pulmonary host defense. The phagocytic activity of AMs *in vitro* was studied because of the importance of this activity in assessing AM host defense function. We also investigated the secretion of several substances by AMs *in vitro* because these cells secrete numerous oxidants and cytokines as part of their response to pathogens and particles. To examine the effect of phospholipidosis at the level of the whole organism, we instilled *Listeria monocytogenes* into the lungs and monitored the course of infection.

Materials and Methods

Materials. Amiodarone hydrochloride was a gift of Wyeth-Ayerst Research (Princeton, NJ). *Listeria monocytogenes* was of strain 10403S and serotype I. All other reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO).

Amiodarone Administration. Male Fischer-344 rats (250–300 g) were obtained from Hilltop Laboratories (Scottdale, PA) and given standard lab chow and water *ad libitum*. Amiodarone hydrochloride was suspended in water (50 mg/ml) and administered orally by gavage at a dose of 100 mg/kg daily for 7 days. Control rats received only the water vehicle (2 ml/kg). Since treatment with amiodarone resulted in some weight loss, food was restricted for control rats so that the weight changes were comparable between groups.

Collection of Alveolar Macrophages. Rats were anesthetized with sodium pentobarbital and killed by exsanguination. AMs were recovered from the lungs by bronchoalveolar lavage using sterile warm, calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS), pH 7.4, as described previously (16). Cells were counted on a hemacytometer and were differentiated using Wright-Giemsa SureStain (Fisher Scientific). Greater than 95% of the cells were large mononuclear cells consistent with AMs. Cells were suspended in a small volume of HBSS (0.5–1.0 ml) and either used in the functional assays the same day as collected or stored at −20°C for total phospholipid assay at a later time.

Phospholipid Assay. Phospholipid was extracted from AMs with chloroform/methanol (2/1, v/v) as described by Folch *et al.* (17). Total phospholipid content of AMs was determined using the method of Ames and Dubin (18) as reported previously (16).

Phagocytosis. To assess phagocytosis of yeast particles, AMs were suspended in RPMI media containing 20% fetal bovine serum. Cells (1×10^6) were plated in 6-well plates and allowed to incubate for 2 days at 37°C in 95% air/5% CO_2. Shorter incubation times resulted in low and inconsistent phagocytosis of the yeast. Yeast (*Saccharomyces cerevisiae*) cells (Sigma) were suspended in HBSS and added to a 0.1% Congo Red solution in a glass tube. The preparation was boiled for 30 min to kill the yeast and allow the dye to be taken up. To each well, 5×10^7 colored yeast were added and the cells/yeast incubated for 2 hr. The cells were removed from culture with 0.25% trypsin and gentle scraping, and washed twice with saline to remove noningested yeast. Cells were placed on microscope slides and stained with Wright-Giemsa stain. Two hundred cells were observed with the light microscope, and the percentage of cells containing colored yeast was calculated.

Luminol-Dependent Chemiluminescence. Luminol-dependent chemiluminescence (LDCL) of alveolar macrophages was performed as described by Antonini *et al.* (19) on resting cells or using phorbol myristate acetate (PMA) (0.5 μg/ml final concentration) and unopsinozed zymosan (2 mg/ml final concentration) as stimulants. Chemiluminescence is a measure of light production by resting or stimulated cells and represents the release of oxidant species. Luminol is used as an amplifier to enhance detection of the light. Data are presented as stimulated counts above spontaneous release (nonstimulated or resting condition).

Clearance of *L. monocytogenes*. After 7 days of amiodarone or water treatment, *L. monocytogenes* (5000 viable bacteria suspended in 0.5 ml of sterile saline) were instilled intratracheally on Day 0 using a 20-gauge ball-tipped feeding needle attached to a 1-ml syringe. Daily administration of amiodarone was continued in order to maintain the phospholipidotic condition, and the rats were killed and lungs removed at Day 3, 7, and 10. Lungs were chopped into small pieces using a McIlwain tissue chopper, homogenized for 30 sec in 5 ml of sterile water using a Brinkmann polytron, diluted with water, and cultured quantitatively on brain heart infusion agar 37°C. Colony forming units were counted after an overnight incubation.

Cytokine Secretion. AMs were incubated at a concentration of 1×10^6 cells in Dulbecco's modified Eagle's medium without serum in the presence and absence of LPS (1 μg/ml) for 18 hr at 37°C in a humidified tissue culture chamber containing 5% CO_2. After incubation, the cell-free supernatants were collected by centrifugation and aliquots assayed for interleu-

kin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor–α (TNF-α). IL-1 activity in supernatants was measured using [^{3}H]thymidine incorporation into D10(N4)M cells as described previously (20).

Incorporation of [^{3}H]thymidine in the presence of sample dilutions was compared with that in the presence of dilutions of a standard recombinant IL-1 preparation (10^8 U/mg; Genzyme, Boston, MA), and IL-1 units were calculated by probit analysis. IL-6 activity in supernatants was measured using [^{3}H]thymidine incorporation into B9 cells as described previously (21). The sample dilution curve was related to a standard curve generated with recombinant murine IL-6 (10^8 U/mg; Genzyme), and IL-6 units were calculated by probit analysis. TNF–α was measured in supernatants with a solid phase enzyme-linked immunosorbent assay (ELISA) (Genzyme) employing the multiple antibody sandwich principle (22). Absorbance was read at 450 nm using an automated Bio-Tek microplate reader (Bio-Tek Instruments, Burlington, VT). No cross reactivity was found with IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN-γ, GMCSF or TNF-β.

Statistical Analysis. Data were analyzed using either a Student's *t* test (total phospholipid and yeast phagocytosis) or a two-way analysis of variance (ANOVA) with individual groups compared by the Tukey's protected *t* test (chemiluminescence, Listeria clearance, and cytokine secretion). Statistical significance was set at $P < 0.05$.

Results

Treatment of rats with amiodarone resulted in a phospholipidosis in AMs (1.06 ± 0.11 μmoles lipid phosphorus/10^7 cells, $n = 10$, compared with 0.24 ± 0.03 μmoles lipid phosphorus/10^7 cells for AMs from vehicle-treated rats, $P < 0.05$), $n = 6$ rats for each group.

There was no difference in the phagocytic activity of phospholipidotic AMs toward heat-killed yeast cells *in vitro*, when compared with control cells (Fig. 1).

The presence of a phospholipidosis in AMs did not alter the luminol-dependent chemiluminescence to either PMA, a soluble stimulant, or zymosan, a particulate stimulant (Fig. 2).

The LPS-stimulated secretion of IL-1 by AMs from amiodarone-treated rats was not different from control (Fig. 3). In contrast, phospholipidotic AMs displayed increased secretion of IL-6 (Fig. 4) and TNF-α (Fig. 5) during LPS stimulation *in vitro*. There were no significant differences in spontaneous (non-stimulated) secretion of any of the three cytokines when phospholipidotic AMs were compared with controls.

There was no significant difference in the clearance of intratracheally instilled *L. monocytogenes* from the lungs of amiodarone-treated rats and controls

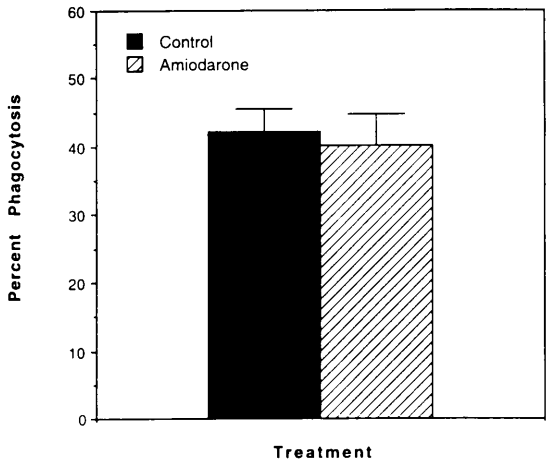

Figure 1. Phagocytosis of yeast particles *in vitro* by alveolar macrophages from control and amiodarone-treated rats. Values are means ± SEM ($n = 5$ rats per group).

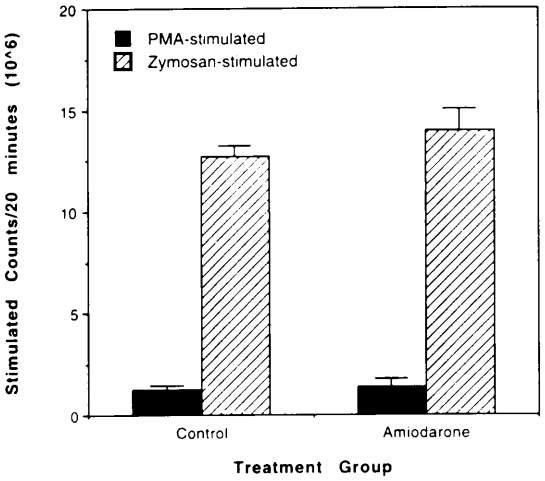

Figure 2. Luminol-dependent chemiluminescence of alveolar macrophages from control and amiodarone-treated rats. Data are expressed as mean stimulated count/20 min (PMA- or zymosan-stimulated counts minus resting counts) ± SEM ($n = 6$). The resting counts/20 min for control cells were 0.300 ± 0.024 × 10^6 and 0.309 ± 0.037 × 10^6 for cells from amiodarone-treated rats.

when followed over a period of 10 days after instillation (Fig. 6). With both groups, massive infection developed over the first 3 days after instillation, but by 7 days postinstillation, the number of bacteria in the lungs had been reduced markedly, and by 10 days postinstillation, only a few hundred bacteria remained in the lungs of both groups.

Discussion

Only several studies have addressed the effects of CAD-induced phospholipidosis on AM function, and the results are not consistent (Table I). The conflicting results of our studies and those of Wilson and Lippmann (10), Wilson *et al.* (6), and Ferin's group (4, 5) are unexplained but may result from differences in the degree of phospholipidosis induced by the respective dosing regimens or technical differences in the assays

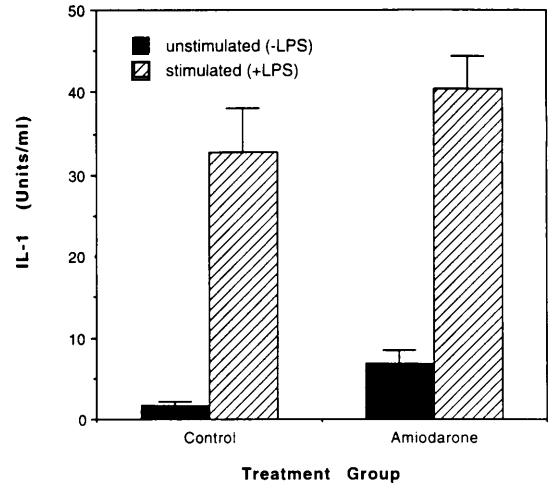

Figure 3. Interleukin-1 secretion by alveolar macrophages *in vitro* in the absence or presence of LPS. Values are means ± SEM (n = 5).

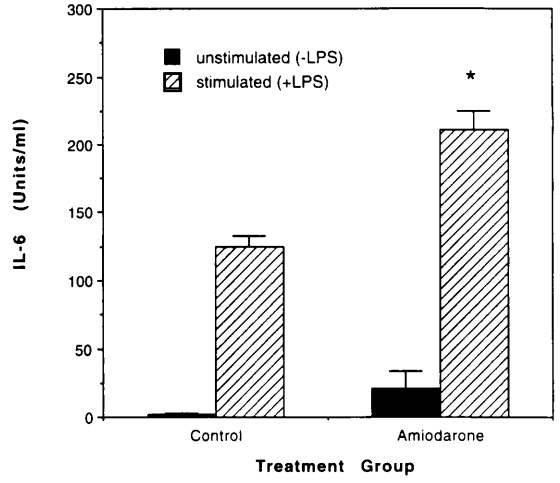

Figure 4. Interleukin-6 secretion by alveolar macrophages *in vitro* in the absence or presence of LPS. Values are means ± SEM (n = 5). $^{*}P < 0.05$ compared with corresponding control.

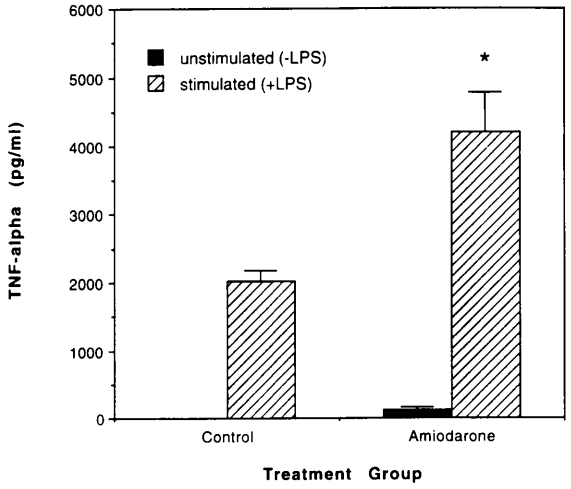

Figure 5. Tumor necrosis factor–α secretion by alveolar macrophages *in vitro* in the absence or presence of LPS. Values are means ± SEM (n = 5). The unstimulated control value is 3.0 ± 0.8 pg/ml. $^{*}P < 0.05$ compared with corresponding control.

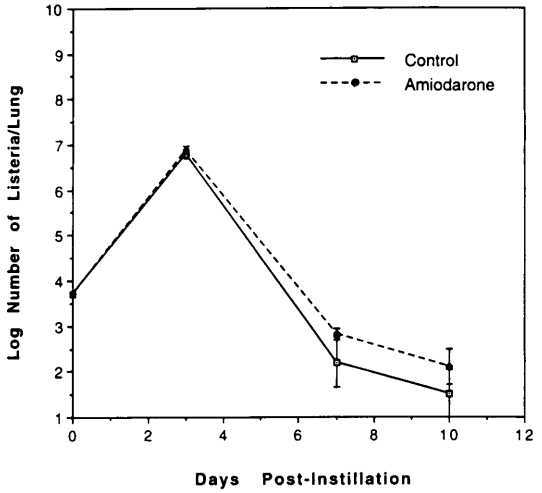

Figure 6. Clearance of *L. monocytogenes* from the lungs of rats following an intratracheal instillation of 5000 bacteria. Values are means ± SEM (n = 5–8 at each time point).

used. Whatever the reasons, it is clear that to generalize the effects of phospholipidosis on AM function is inappropriate.

For the present studies, we utilized amiodarone to induce phospholipidosis in AMs. Amiodarone has been shown repeatedly to induce phospholipidosis in AMs in humans (2). Because of its relevance to human health, we felt amiodarone to be the most appropriate CAD to further examine the effects of phospholipidosis on pulmonary host defense processes.

As reported previously (16), the administration of amiodarone to Fischer-344 rats resulted in the induction of phospholipidosis in AMs. The 4.5-fold higher level of total phospholipid correlates with the development of lamellar inclusion bodies within the cells (16).

To assess the functional integrity of the pulmonary host defense system following induction of phospholipidosis, we examined several functions using both *in vitro* and *in vivo* approaches. The AM is the principal cellular defense system in the lungs, and we found alterations in certain cellular functions, but not others, examined *in vitro* in cells with phospholipidosis.

The phagocytic activity toward heat-killed yeast cells was not altered by drug treatment suggesting that the phospholipidosis did not alter this property.

The oxidant response of cells to either a soluble or particulate stimulant, as assessed by luminol-dependent chemiluminescence, was not affected by the presence of the phospholipidosis. A portion of the chemiluminescence response in this assay is dependent upon peroxynitrite (19). Therefore, by this indirect measure, it appears that the secretion of nitric oxide, an important chemical in both host defense processes (23, 24) and inflammatory responses (25), by the phospholipidotic AMs was not altered.

Table I. Effects of CAD-Induced Phospholipidosis on Alveolar Macrophage (AM) and Whole Lung Pulmonary Defense Functions

CAD	Response	Reference
AM functions studied *in vitro*		
Chlorphentermine	Impaired Fc receptor-mediated and nonspecific receptor-mediated phagocytosis against sheep red blood cells	4
Amiodarone	Impaired phagocytic activity against *Candida albicans*	6
Chlorphentermine or Iprindole	Increased phagocytic activity; increased formation of rosettes with IgG-sensitized sheep erythrocytes; increased killing of *Staphlococcus aureus*	7
Iprindole	Increased zymosan-stimulated release of superoxide anion and hydrogen peroxide	8
Chlorphentermine	Increased zymosan-stimulated chemiluminescence	9
Amiodarone	Increased LPS-stimulated secretion of interleukin-1	10
SK&F 105685	Increased killing of *C. albicans;* increased suppressor cell activity	11
SK&F 106615	Increased suppressor cell activity	11
Whole lung functions		
Chlorphentermine	Reduced clearance of inhaled TiO_2; no effect on the clearance of inhaled ZnO_2	5
Amiodarone	Increased levels of IgG and IgM in bronchoalveolar lavage fluid; no change in anti-ovalbumin antibody in bronchoalveolar lavage of immunized rats	10

The presence of phospholipidosis did not affect the spontaneous or LPS-stimulated secretion of IL-1, IL-6, or TNF-α by AMs *in vitro*, and in fact, the LPS-stimulated secretion of IL-6 and TNF-alpha was elevated over that of control AMs. These two cytokines are multifunctional and participate in a variety of aspects of host defense (26–30). Among many functions, IL-6 is involved in T-cell activation, B-cell differentiation, hematopoiesis, and acute phase protein induction (30). TNF-α secreted by AMs is of critical importance for the killing of intracellular pathogens as shown by recent work demonstrating that mice deficient in the 55-kDa TNF receptor are extremely sensitive to *L. monocytogenes* and succumb easily to infection (31, 32).

Cytokines participate in or contribute to a number of disease states in the lungs and other tissues (27, 33). Therefore, the potential for elevated secretion of IL-6 and TNF-α is not without possible adverse effects. TNF-α has been shown to be a proinflammatory agent in the lungs. Following silica exposure, TNF-α secretion by AMs is increased significantly *in vitro* (34). The short-term oral administration of amiodarone at the dose we used does not result in overt pulmonary inflammation. We have reported previously that amiodarone treatment at a higher dose (150 mg/kg) for up to 9 weeks did not result in inflammation as assessed by the presence of neutrophils in bronchoalveolar lavage fluid (16). Wilson *et al.* (35) reported that the oral administration of amiodarone for 6 weeks at a higher dose than we used resulted in an inflammatory response in rats. It is possible that the elevated secretion of certain cytokines *in vitro* may be an indication of the potential for an inflammatory response to develop in the lungs of amiodarone-treated animals. It is of interest that IL-6 is reported to downregulate certain aspects of pulmonary inflammation (33). Therefore, in our *in vitro* system, the elevation of its secretion may represent a cellular response to counteract the effects of elevated TNF-α secretion. It is important to note that the spontaneous secretion of the three cytokines was not different in control and phospholipidotic AMs, suggesting basal secretion of these substances is not influenced by the phospholipidosis. Under any circumstance, the profile of these three and other cytokines would have to be evaluated before one could predict their influence on potential adverse effects of amiodarone-induced phospholipidosis. It would be of interest to compare cytokine levels in bronchoalveolar lavage fluid of control and amiodarone-treated animals to determine whether differences exist in a more physiologically relevant situation. While changes occurred in AMs from the rat, it is not possible at present to extrapolate these results to humans with amiodarone-induced phospholipidosis.

To assess an *in vivo* host defense mechanism, we examined the clearance of *L. monocytogenes* from the lungs following an intratracheal administration. While details of AM participation in resistance to the pulmonary infection by *L. monocytogenes* are not clear, it is well known that at other sites macrophages play a key role in both innate and acquired immunity against this microorganism (36–38). Accordingly, the secretion of various cytokines, including IL-1, IL-6, and TNF-α, have been implicated in resistance to *L. monocytogenes*. The absence of evidence for impaired AM function *in vitro* following induction of phospholipidosis by amiodarone is consistent with our observation in this study that the pulmonary clearance of the bacteria is not impaired in phospholipidotic lungs.

Based on the results of this study, we conclude that the presence of amiodarone-induced phospholipidosis had no significant effect on pulmonary host defense processes in the rat in the context of the functions studied, and in fact, resulted in the enhanced ability of LPS to stimulate the release of certain cytokines *in vitro*. Whether enhanced production of IL-6 and TNF-α *in vitro* could predict the development of subsequent adverse effects is not known and will require further investigation. Considering the discordant results obtained with amiodarone in this and other studies and chlorphentermine and iprindole in previous studies, it is not possible to generalize the effects of CADs on pulmonary host defense. It appears at the present time that phospholipidosis induced by a CAD will have to be examined individually for its effects on pulmonary host defense.

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