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EFFECT OF AMIODARONE ON THE PHOSPHOLIPID AND LAMELLAR BODY CONTENT OF LYMPHOBLASTS *IN VITRO* AND PERIPHERAL BLOOD LYMPHOCYTES *IN VIVO*

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Abstract—Amiodarone is useful for the treatment of ventricular arrhythmias but has been associated with a significant degree of toxicity especially to lung and liver. The drug produces phospholipid accumulation in multilamella lysosomal inclusions in many tissues due to the ability of amiodarone and desethylamiodarone to inhibit phospholipase A. The adverse effects do not correlate with the plasma levels of amiodarone but relate more closely to the cumulative dose. No clear way of predicting amiodarone toxicity has yet emerged. In this report, normal human lymphoblasts in tissue culture were shown by electron microscopy to have dose-dependent increases in multilamellar inclusions when grown with amiodarone at concentrations which are routinely observed in patients receiving the drug. The content of phospholipid also increased but this parameter was not as sensitive as the number of multilamellar inclusions. Finally, lymphocytes from patients treated with amiodarone were examined by electron microscopy and shown to have increased numbers of multilamellar bodies.

Amiodarone is an iodine-containing benzofuran derivative approved in the United States for treatment of life-threatening ventricular arrhythmias. Although its use is associated with potentially serious side-effects, especially on lung and liver, amiodarone is useful because it can suppress arrhythmias which are unresponsive to other drugs and it rarely exacerbates a preexisting arrhythmia or induces a new arrhythmia [1]. Adverse effects are reported in 24–93% of patients but serious side-effects which require discontinuance of the drug occur in 0–26% [2]. The toxic effects generally do not correlate with the plasma levels of amiodarone but seem to be related to cumulative dose.

Amiodarone produces multilamellar inclusions in the lysosomes of many tissues [3–7]. Studies in animals have demonstrated that pulmonary alveolar macrophage and lung tissue phospholipid levels are increased [8, 9], and it is generally believed that the multilamellar inclusions noted in human tissues are accumulations of phospholipids. This conclusion was further strengthened by the observation that amiodarone is one of the most potent inhibitors of lysosomal phospholipase A_1 ever discovered [10]. Desethylamiodarone, the principal metabolite of amiodarone, also inhibits lung lysosomal phospholipase A_1 in a manner equivalent to that of the parent compound [11] and probably plays a role in causing tissue phospholipid accumulation. Amiodarone also inhibits neutral phospholipids A_2 from snake venom [12], but the concentration required to produce 50% inhibition of this enzyme is more than 600 times greater than that needed to inhibit lysosomal phospholipase A_1 to the same degree [10, 11]. It has been suggested that the toxic effects of amiodarone may be related to its ability to cause multilamellar lysosomal inclusions but proof of a link between phospholipid accumulation and the development of organ toxicity is lacking.

Since plasma levels of amiodarone do not allow one to predict the development of clinically significant organ toxicity, we reasoned that the assessment of amiodarone effects on tissue phospholipid levels and multilamellar inclusions might be more useful. In this study, we determined the effect of amiodarone on the content of phospholipid and multilamellar inclusions in cultured lymphoblasts grown in the presence and absence of amiodarone. *In vivo* correlations were sought using peripheral blood lymphocytes from normal subjects and patients receiving amiodarone. We measured lymphocyte phospholipid levels and the number of lamellar inclusions per cell. Our results show that lymphoblasts respond to amiodarone by increasing their phospholipid content and number of multilamellar inclusions at drug concentrations which can be readily attained *in vivo*. A large percentage of patients receiving amiodarone exhibited increased numbers of multilamellar inclusions in their peripheral blood lymphocytes.

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MATERIALS AND METHODS

Lymphoblast experiments. GM130 lymphoblasts

were obtained from N.I.G.M.S. Human Genetic Mutant Cell Repository Institute for Medical Research, Camden, NJ. Cells were grown in 25 cm^2 polystyrene tissue cultured flasks (Corning, Corning Glass Works, Corning, NY) in RPMI 1640 growth medium containing 20% fetal bovine serum and kept at 37° in an atmosphere of 5% CO_2 in air.

Amiodarone, at the indicated concentration, was added to flasks containing 5×10^5 cells and incubated at 37° for the length of the experiment (4 or 7 days). Three replicate cell suspensions were harvested by transferring to a 50-mL conical tube (Falcon) and centrifuging for 5 min at 700 *g*. The pellet was washed with cold buffer containing 0.25 M sucrose and 5 mM Tris buffer (pH 7.4) (ST buffer). This procedure was repeated twice. The supernatants were discarded and the final pellet was resuspended in 2 mL of cold ST buffer. This was transferred to a 16 × 100 mm glass tube. Cell suspensions were kept on ice and homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) three times, each for 15 sec, at a 6.5 setting. Samples were frozen and kept at −70° until assayed for protein and phospholipid. Protein was measured by the method of Lowry *et al.* [13] using bovine serum albumin as a standard. Lipids were extracted by the method of Folch *et al.* [14]. Aliquots of the chloroform extracts were evaporated to dryness in an oven and lipid phosphorus was determined according to the method of Rouser *et al.* [15].

Electron microscopy. Cells for electron microscopy (EM) were harvested and washed thrice with cold phosphate-buffered saline (PBS), pH 7.4, by centrifuging for 10 min at 700 *g*. The buffer was then gently aspirated from the cell pellets and replaced with buffered Karnovsky's fixative (pH 7.4). After 30 min of fixation at room temperature, the pellets were washed 3 times in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 0.1 M cacodylate-buffered 2% osmium tetroxide. Following three buffer washes, the cell pellets were dehydrated through ethanol and propylene oxide and embedded in Epon 812. Thin sections (60 nm) were cut and mounted on unsupported 300 mesh grids and stained in uranyl acetate and lead citrate. Multiple sections were cut from each pellet and examined and photographed with a Zeiss EM 10B electron microscope. Fifty consecutive whole cells were selected from the low power electron micrographs for each drug concentration, and the number of lamellar bodies per cell was determined.

Chemicals. Chloroform and methanol were redistilled before use. Sucrose (Ultrapure) was obtained from Schwarz-Mann, Orangeburg, NY, U.S.A. Trizma base was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Amiodarone was provided by Sanofi Research, Montpelier, France.

RESULTS

Normal human lymphoblasts were grown in RPMI medium containing 20% fetal bovine serum with and without the drug. Amiodarone concentrations of 1, 3.16 and 10 mg/L were chosen to represent normal and high therapeutic levels of drug. After 4 and 7 days in culture the lymphoblasts were harvested and washed, and aliquots were analyzed for protein and phospholipid content. The results are shown in Table 1. The normal phospholipid content of lymphoblasts was 137 nmol/mg cell protein and it was independent of the number of days in culture (data not shown). Amiodarone at 1 mg/L did not cause a statistically significant increase in lymphoblast phospholipid. However, 3.16 and 10 mg/L of amiodarone caused increases of phospholipid of 27 and 31%, respectively, after 4 days of growth. Three additional days of growth did not result in further increases in cell phospholipid.

The ultrastructural appearance of control lymphoblasts and lymphoblasts treated with amiodarone (10 mg/L) for 4 days is shown in Figs. 1 and 2. Normal lymphoblasts had large nuclei and prominent nucleoli with sparse cytoplasm containing numerous mitochondria and elements of endoplasmic reticulum. Multilamellar inclusions were rarely seen. Figure 2 shows a typical lymphoblast with numerous membrane-bound electron dense multilamellar inclusions. Several membrane-bound vesicles were present which contained two or three multilamellar inclusions. No other consistent morphological changes were noted in amiodarone-treated cells. Occasionally, very heavily involved lymphoblasts were encountered having large multicentric lamellar inclusions, such as the cell shown in Fig. 3.

Table 2 shows the results of an electron microscopic examination of the lymphoblasts grown for 4 or 7 days with various concentrations of amiodarone in the medium. Washed lymphoblasts were examined by electron microscopy and a series of low power photographs was taken from grids prepared from each time and drug concentration. Fifty consecutive cells were examined as described in Materials and Methods and the number of lamellar bodies per cell was determined. The results are given as the percentage of cells having 0, 1, 2, 3, or 4 or more lamellar bodies per cell. Multilamellar inclusions occurred with a measurable frequency in control lymphoblasts grown in RPMI medium with 20% fetal bovine serum, as shown in Table 2; 8 and 10% of cells grown for 4 and 7 days had one inclusion on examination of random sections. No control cells had more than one multilamellar inclusion. At an amiodarone concentration of 1 mg/L, 8% had one inclusion and 6% had two inclusions after 4 days of growth. After 7 days, 12% of the cells had one and 6% had two multilamellar inclusions. At 3.16 mg/L, the percentage of cells having one inclusion increased markedly to 16%; 2 and 4% of cells had 3 or 4 or more inclusions, respectively, after 4 days in culture. These percentages increased further with 7 days in culture as shown in Table 2. At 10 mg amiodarone/L, 22% of cells had one lamellar body while 6% had two and three per cell respectively. Further cell culture at 10 mg/L for 7 days resulted in a remarkable increase in the number of lamellar inclusions: only 12% of cells were free of inclusions; the percentage of cells having 1, 2, or 3 inclusions was 22, 14 and 18%, respectively, and 34% had 4 or more per cell.

A lymphocyte-rich fraction was isolated from

Table 1. Effect of amiodarone on phospholipid content of normal lymphoblasts grown for 4 or 7 days

Days in culture	Phospholipid (nmol/mg cell protein)			
	Amiodarone concentration (mg/L)			
	0	1.0	3.16	10.0
4	137 ± 12 (33)	144 ± 9 (4)	174 ± 24* (8)	180 ± 10* (8)
7	—	138 ± 2 (4)	164 ± 11* (8)	177 ± 6* (8)

Values are means ± SD; the numbers in parentheses are the number of experiments. The 7-day values for amidarone were tested against the 4-day control value, 137 ± 12 (33), because the phospholipid/protein ratio does not change in lymphoblast cultures as a function of days of growth. Similar results are also obtained if one expresses the data in terms of phospholipid per 10^6 cells.

* Significantly different from control, $P < 0.01$ (determined using Student's *t*-test for the difference between unpaired means).

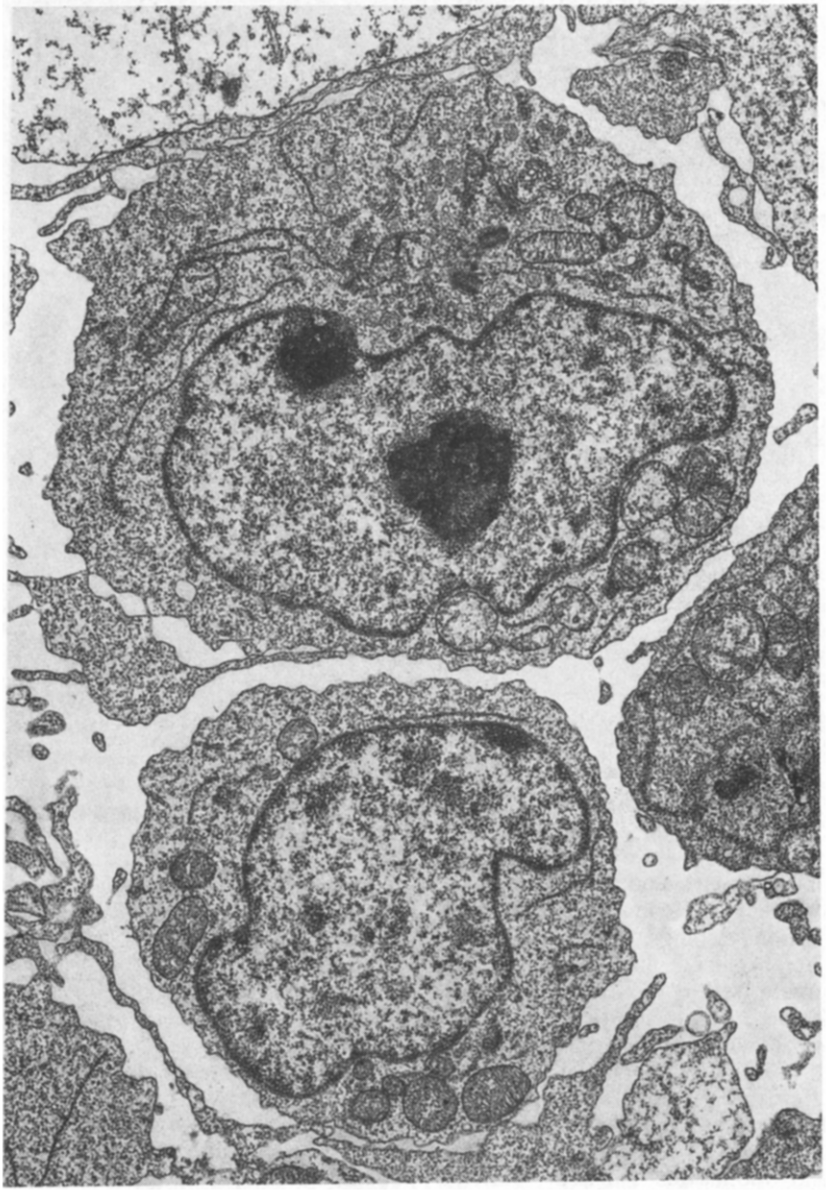

Fig. 1. Electron micrograph of control lymphoblasts incubated in normal culture medium for 4 days. Magnification 9375×.

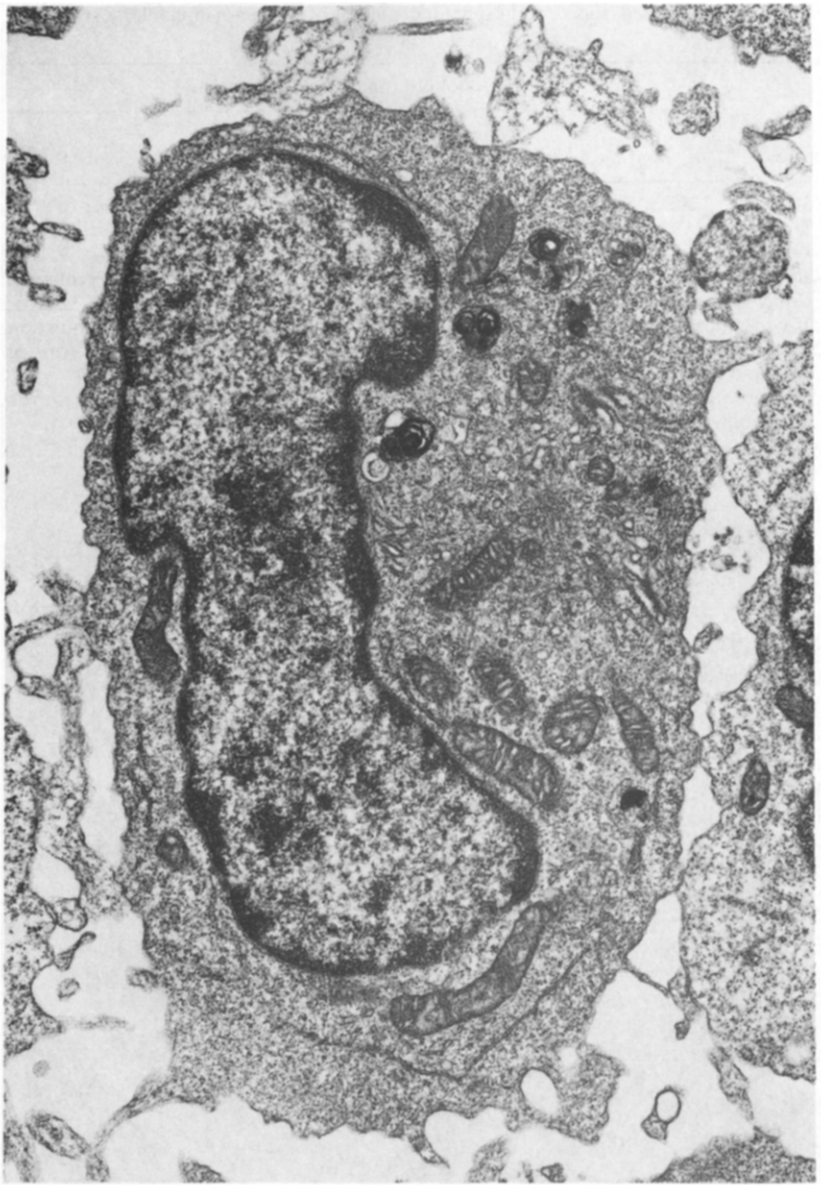

Fig. 2. Ultrastructure of a lymphoblast treated with amiodarone (10 mg/L) for 4 days. Final magnification 16,000×.

thirty-eight control subjects and twenty-two amiodarone-treated patients by Ficoll–Hypaque gradient centrifugation. After washing with normal saline, the cells were homogenized, and the protein and phospholipid content were determined. The results are shown in Table 3. Lymphocyte isolates from control subjects had 146 nmol lipid phosphorus/mg protein versus 161 in the amiodarone-treated patients; the difference was not statistically significant. The standard deviation of these values was large, probably due to varying percentages of lymphocytes and monocytes isolated. Electron microscopic evaluation of buffy coat lymphocytes showed that 10% of control subjects had inclusions compared with 55% of the amiodarone-treated patients. In controls, 4.6% of cells were affected, having 1.8 inclusions per cell while amiodarone-treated patients had 9.4% of cells affected with 3.4 inclusions per cell. These differences were statistically signifcant.

DISCUSSION

Our studies show that cultured lymphoblast increased their phospholipid content at an amiodarone concentration (3.16 mg/L) which occurred regularly in plasma during therapy. Increasing the amiodarone concentration to 10 mg/L did not

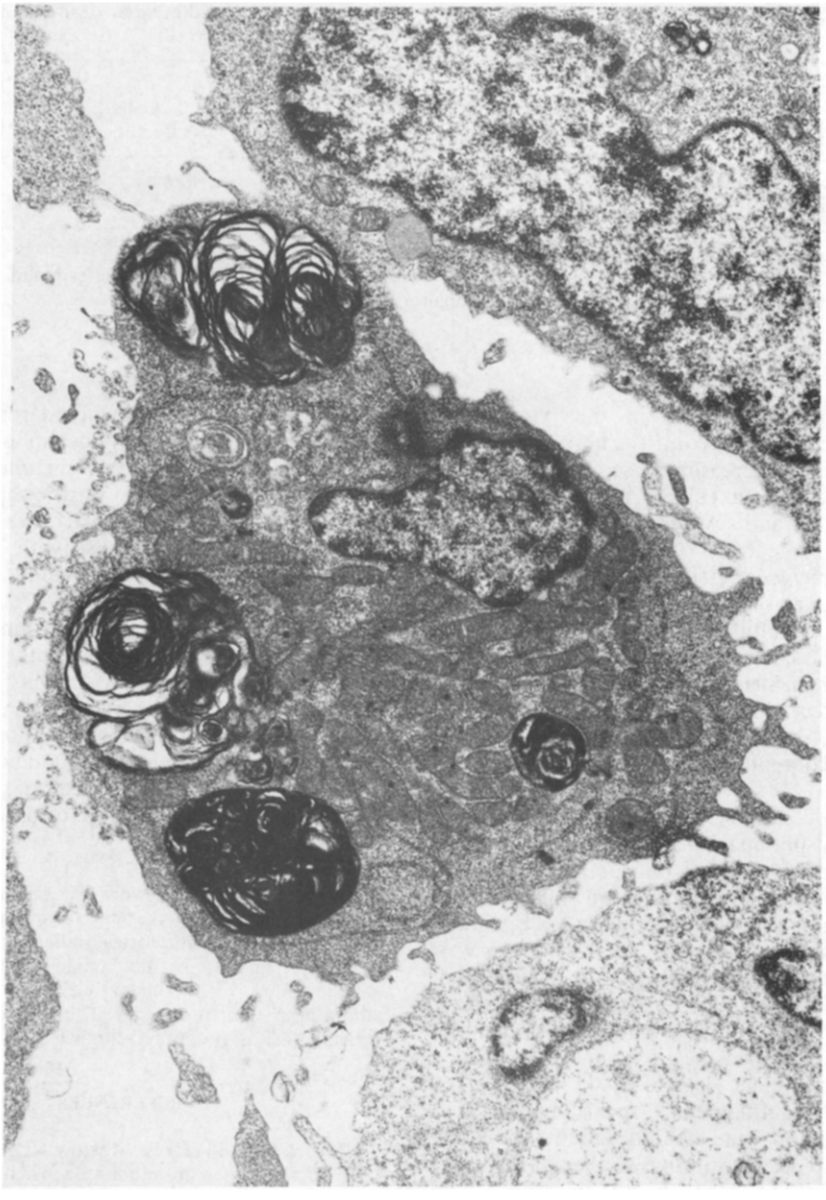

Fig. 3. Lymphoblast treated with amiodarone (10 mg/L) for 4 days and exhibiting very heavy involvement with electron dense multilamellar inclusion. Magnification 13,000×.

Table 2. Effect of amiodarone on lamellar body content of cultured lymphoblasts grown for 4 or 7 days*

Number of lamellar bodies per cell	% Cells having the indicated number of lamellar bodies per cell							
	Amiodarone concentration (mg/L)							
	0		1.0		3.16		10.0	
	4 days	7 days	4 days	7 days	4 days	7 days	4 days	7 days
0	92	90	86	82	74	64	66	12
1	8	10	8	12	16	16	22	22
2	0	0	6	6	4	8	6	14
3	0	0	0	0	2	6	6	18
4 or more	0	0	0	0	4	6	0	34

* Fifty consecutive cells were analyzed.

Table 3. Effect of amiodarone treatment on the phospholipid content and number of multilamellar inclusions in human lymphocytes and mononuclear cells

Treatment group	Phospholipid (nmol/mg protein)	% Patients with inclusions	% Cells affected	Number of inclusions per cell
Control subjects (38)	146 ± 57	10	4.6 ± 3.8	1.8 ± 1.0
Amiodarone-treated (22)	161 ± 69	55	9.4 ± 4.6*	3.4 ± 2.9†

Values are means ± SD, numbers in parentheses are the number of subjects.
*,† Significantly different from control subjects: *$P < 0.02$ and †$P < 0.01$ (determined using Student's t-test for the difference between unpaired means).

produce a further increase in the lymphoblast phospholipid content. In contrast, fibroblasts grown in the presence of increasing doses of drug showed progressive increases in cellular phospholipid levels (Hostetler KY and Aldern KA, unpublished observation).

Electron microscopy is more sensitive than phospholipid analysis in detecting small increases in the numbers of multilamellar inclusions. As shown in Table 2, amiodarone at 1 mg/L caused an increase in the number of inclusions but had no statistically significant effect on the phospholipid content of the cells. At 3.16 and 10 mg/L both increased phospholipid content and multilamellar body counts were noted.

A variety of cationic amphiphilic drugs inhibit lysosomal phospholipase A_1 resulting in an intralysosomal accumulation of phospholipids as intralysosomal multilamellar bodies in many tissues including lung, liver, spleen, retina, muscle, kidney, peripheral nerve and others [6]. Lymphocytes were first shown to have multilamellar inclusions in patients with rhematoid arthritis or systemic lupus erythematosus who were receiving chloroquine [17]. Multilamellar inclusions occur in many cell types in amiodarone treatment [1, 3–6]. Dake *et al.* [7] first demonstrated multilamellar inclusions in lymphocytes of amiodarone-treated patients. Shaikh *et al.* [12] attempted quantitative studies of inclusions in buffy coat white cells in which they compared the serum concentration of amiodarone and the number of inclusion bodies. They found no correlation between the number of inclusions and the serum level or cumulative dose of amiodarone. Our studies of lymphocytes from amiodarone-treated patients showed much lower numbers of inclusions per cell (Table 3).

It is possible that the peripheral blood lymphocyte responses to amiodarone could be used to predict liver and lung toxicity. The patients selected for this study were not exhibiting signs and symptoms of amiodarone toxicity. To attempt to predict amiodarone toxicity, it would be necessary to measure prospectively the serum level of drug, lymphocyte phospholipid levels and multilamellar inclusion counts, as well as biochemical, physiological and radiographic measures of hepatic and pulmonary function. However, even if correlations were noted between lymphocyte phospholipid responses and lung and liver toxicity, a definitive pathophysiological link would still not be established because studies in rats treated chronically with amiodarone suggest that development of pulmonary phospholipidosis does not necessarily lead to pulmonary function abnormalities or interstitial fibrosis [18]. Furthermore, Liu *et al.* [19] obtained bronchoalveolar lavage fluid from amiodarone-treated patients with and without pulmonary toxicity and examined macrophage ultrastructure. They found no correlation between the number of alveolar macrophage inclusion bodies and the clinical status of the patients. These workers concluded that the pulmonary toxicity of amiodarone is at least partly idiosyncratic [19]. Although it is tempting to ascribe organ toxicities to the effects of amiodarone on phospholipid metabolism, there is currently little direct evidence to support a link between the phospholipid inclusions and lung and liver toxicity.

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