Amiodarone-Associated Pulmonary Fibrosis
Evidence of an Immunologically Mediated Mechanism

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A case of pulmonary fibrosis attributed to amiodarone was studied immunologically and morphologically. A specific antibody of the IgG class was identified in the serum of this patient which reacted with the patient’s own lung tissue. The immunoglobulin did not react with normal lung tissue nor was there evidence of reaction with lung tissue from patients with pulmonary fibrosis but without a history of amiodarone therapy. The patient probably developed a humoral antibody response to a lung-amiodarone complex with the amiodarone acting as a hapten which bound in vivo to the lung tissue. The subsequent antibody-antigen reaction stimulated a proliferation of pulmonary fibroblasts and probably enhanced the fibro-collagen deposition in the lung.

Amiodarone hydrochloride is a benzofuran derivative. Its antiarrhythmic effects have generated considerable interest in the past 20 years. Several recent studies have reviewed the pharmacologic and clinical effects of amiodarone. Since 1980, pulmonary toxicity, in the form of interstitial fibrosis, has occasionally been reported. The mechanism of the pulmonary fibrosis associated with amiodarone therapy is currently unknown, although possible mechanisms have been proposed.

We studied the postmortem tissue specimens from a patient who had received amiodarone. The lung tissue displayed severe, diffuse interstitial fibrosis without the development of a significant inflammatory reaction. The case was studied morphologically, immunologically, and biochemically. The patient’s serum was found to be capable of reacting with his own lung tissue. The reaction was detected by immunofluorescence, and the serum component was identified as an IgG class antibody. The antibody could be absorbed by the patient’s own lung tissue or by amiodarone. The patient’s serum failed to react with lung tissue obtained from normal postmortem lungs or lung tissue from patients with pulmonary fibrosis but no history of amiodarone therapy. Sera of two control patients who were under amiodarone therapy and who did not have pulmonary fibrosis failed to react with either the patient’s or normal postmortem lung tissue samples. Detergent (NP-40) phosphate buffer extracts of the patient’s lung tissue did not react with his own serum immunologically. Electrophoresis of NP-40 phosphate buffer protein extracts from the patient’s lung and normal postmortem lungs showed no qualitative differences. These findings imply that amiodarone fibrotic lungs contain NP-40 buffer extractable protein components qualitatively identical to normal lung tissue. The observed fibrosis, therefore, was presumably due to a quantitative increase of fibroblasts and fibro-collagen components rather than to a new synthesized protein. From the data to be presented, it is suggested that there is an immunologically mediated mechanism in the development of interstitial fibrosis associated with amiodarone therapy.

Case Report

This 62-year-old white man underwent coronary artery bypass grafting to three vessels in 1972. He reportedly fared well until December 1982, when he suffered a myocardial infarction. In the following two years, the patient experienced multiple transient episodes of dyspnea attributable to ventricular tachycardia which was verified by Holter monitor evaluation. The patient was begun on procainamide with little improvement in symptoms. Following an episode of full cardiac arrest, the patient underwent electrophysiologic studies in November 1984. Marked ventricular instability was demonstrated despite the procainamide therapy. Based on the results of these studies, the procainamide was discontinued and the patient was begun on amiodarone at a total daily dose of 1,000 mg. The patient showed no radiographic evidence of pulmonary disease at the time amiodarone was initiated. Due to gastrointestinal side effects, the patient’s antiarrhythmic regimen was changed to a combination of amiodarone, 600 mg per day plus propafenone, 600 mg per day. This regimen was tolerated well and the patient experienced acceptable control of his ventricular dysrhythmias.

In April 1985, the patient was admitted to the hospital with a history of progressive dyspnea. At the time, diffuse interstitial pulmonary infiltrates were noted radiographically. Subsequent examination failed to reveal evidence of congestive heart failure, pulmonary embolus, recurrent ventricular dysrhythmias, etc to account for the patient’s symptoms. Amiodarone was discontinued at
the time of admission with the recognition of possible drug-induced pulmonary toxicity. Despite maximal efforts, a rapidly deteriorating clinical course followed, resulting in refractory respiratory failure. The patient died on May 2, 1985.

Postmortem examination revealed severe, diffuse interstitial pulmonary fibrosis consistent with an amiodarone-associated pulmonary fibrosis process.

**Materials and Methods**

The postmortem lung tissue was processed for routine histopathologic examination. Portions of the left and right lungs were immediately frozen at –70°C. Frozen sections were prepared from the fresh postmortem lung tissue. Frozen sections were also made from lung tissue that was soaked and rinsed overnight in phosphate buffered saline solution, pH 7.4, at 4°C. A postmortem serum sample was obtained from the right ventricle. The serum was aliquoted and frozen at –70°C until use.

For the immunofluorescent examination, the frozen section tissue was incubated with various serum preparations at 25°C for 1 hour and then washed with phosphate buffered saline solution. Fluorescein isothiocyanate (FITC) conjugated rabbit anti-human gamma globulins (anti-IgA, anti-IgM, anti-IgD, anti-IgE, and anti-IgG) were applied to the tissue sections and reincubated for 30 minutes in a moisture chamber at 25°C. The tissue sections were washed and then examined with a Leitz fluorescent microscope.

The serum samples used in the study were prepared as follows: 1) 1:50 dilution with phosphate buffered saline solution, and 2) 1:50 dilution of serum after absorption with amiodarone. Due to the insolubility of the amiodarone in water, it was first dissolved in 50 percent ethanol. (The amiodarone was the generous gift of Dr. J. K. Bissett.) The alcohol content of the solution was subsequently diluted to 0.5 percent with PBS, approximately 1 ml diluted serum to 1 mg amiodarone, and the diluted serum samples were then incubated with the amiodarone solution. The mixture was incubated at 4°C for 24 hours, then centrifuged at 15,000 rpm for 5 minutes in a Sorvall centrifuge with an SS-34 rotor. The supernatant was collected for use in the experiments. Alternatively, it was found that undissolved amiodarone could be used directly to absorb the antibody in the serum without first being dissolved in alcohol.

For the lung absorption experiments, the lung tissue was minced with scissors into minute fragments. The minced lung was washed with PBS and incubated with a 1:50 dilution of the postmortem serum for 24 hours at 4°C. The sample was centrifuged at 5,000 rpm and the supernatant collected for the experiments.

For the detergent extraction experiments, the postmortem lung sample was washed and rinsed extensively in PBS at 4°C. It was then homogenized by Polytron in buffer (0.14 M NaCl, 1 x 10−3 M Tris-HCl, 1 x 10−3 M MgCl 3, containing 0.5 percent Nonidet P-40). After homogenization, the suspension was placed in ice water for 50 minutes and then centrifuged at 15,000 rpm. The supernatant was collected and aliquoted for use in the experiments.

For electron microscopic examination, the postmortem tissue was processed routinely and examined by a transmission electron microscope.

For SDS-acrylamide gel electrophoresis, the procedure was essentially that previously described by Laemmli. Approximately 100 μg protein was applied onto each gel column. After electrophoresis, the gel was stained with either Coomasie brilliant blue R or electrophoretically transblotted onto a nitrocellulose membrane filter (Bio Rad Laboratories, Richmond, CA) by the procedure described by Towbin et al. The transblot buffer was 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20 percent methanol (v/v) with 1 percent SDS. The gel and filter were transblotted for 6 hours at constant voltage. The membrane filter was then incubated against the serum samples, 1:2,000 dilution, for 2 hours in a moisture chamber. After incubation, the filter was washed with PBS and reincubated with horseradish peroxidase conjugated rabbit anti-human IgG antiserum for 2 hours. The filter was then developed with a standard substrate solution containing 3,3′-diamino-benzidine and hydrogen peroxide. Controls of rabbit anti-human albumin were subjected to identical electrophoresis and transblotting. The immunodiffusion study was the traditional 1 percent agarose in barbital buffer immunoplate method.

For in vitro microcomplement-fixation procedure, the method employed was that previously described. Guinea pig complements and sheep RBCs were commercially obtained (Grand Island Biologicals, Grand Island, NY). For immunofluorescent evaluation of the lung tissue for complement deposition, the frozen sectioned lung tissue was first incubated with rabbit anti-human C1 complex or C3b and after washing, reincubated with FITC-conjugated goat anti-rabbit IgG (antisera obtained from Miles Laboratories, Elkhart, IN). After subsequent washing with PBS, the sections were examined under a fluorescent microscope.

**Results**

Gross examination of the lungs postmortem showed a relatively diffuse interstitial fibrosis without fibrotic nodularities. The left lung weighed 1,120 g. The right lung weighed 1,350 g. There was no significant edema fluid noted when the lungs were sectioned. Furthermore, acute inflammatory exudates were not detected on gross examination. Patchy myocardial fibrosis was noted in the left ventricle of the heart. Arteriosclerotic changes were noted in both the native coronary arteries and the bypass grafts. Moderate cellular degeneration was found in the liver and the consistency was softer than normal. The kidneys grossly were unremarkable.

Microscopically, the H and E- and trichrome-stained lung sections showed a diffuse interstitial fibrosis with widening of the alveolar septae. There were very few acute or chronic inflammatory exudates, such as neutrophils, lymphocytes, plasma cells, or cellular debris. Occasionally, some pneumocytes displayed hyperplastic changes and foamy macrophages were seen in the distorted alveolar spaces. The interstitial fibrosis could be dramatically demonstrated by use of fluorescent microscopy of the H and E-stained sections of lung tissue (Fig 1).

Indirect immunofluorescent examination of the postmortem lung tissue showed a significant positive reaction if the patient’s lung was incubated with his own serum and subsequently reacted with FITC conjugated rabbit anti-human IgG antibody (Fig 2a). No reaction was detected from serum specimens of control subjects with pulmonary fibrosis but without history of amiodarone therapy. Similarly, negative findings were noted in patients receiving amiodarone therapy but without pulmonary fibrosis (Fig 2b). Furthermore, when the patient’s lung tissue was incubated separately with rabbit anti-human IgA, IgD, IgM, or IgE, there was no evidence of reaction noted. These findings indicated that these four classes of immunoglobulins were neither involved with nor adhered to the washed lung tissue. This finding further
washed postmortem pathology.

amiodarone could be suggested that the detected presence of IgG in the washed lung tissue was specific.

The reactive IgG antibody in the patient’s serum could be absorbed in vitro by his own lung tissue or by amiodarone compound. After the absorption process, the absorbed serum samples would no longer exhibit a positive reaction against the patient’s own lung tissue. The patient’s serum, absorbed or not absorbed, displayed no evidence of reaction against normal postmortem lung or the lung tissue obtained from patients with interstitial fibrosis but without a history of amiodarone therapy.

SDS-gel electrophoresis of the detergent buffer extracts from the patient’s lung tissue revealed a general electrophoretic protein pattern quite compatible with that of normal postmortem lung extracts (Fig 3). These findings indicated that no qualitative differences were noted between the patient’s lung and normal buffer extractable protein components.

When the standard Western blot method was used to detect an antibody-antigen reaction on the membrane filters transblotted from the SDS-acrylamide gel, as mediated by an alkaline phosphatase detection system, no evidence to indicate the presence of antigen (protein components) was noted. This finding indicated that the antigen which was present in the patient’s lung tissue was not extractable by the NP-40 phosphate buffer, or the antigenic component was not a protein component. Moreover, by traditional agarose
immunodiffusion, there was no detectable precipitation line noted between the patient's serum and the detergent lung extract solution. Attempts to perform agarose immunodiffusion between amiodarone compound and serum were not successful due to the insolubility of amiodarone in aqueous buffer. If ethanol was used, the amiodarone would dissolve, but serum proteins were denatured. It was found that dilution of the amiodarone-ethanol solution could absorb the antibody in the patient's serum.

The \textit{in vitro} microcomplement fixation procedure, using the patient's serum as a source of the antibody activity and the patient's lung tissue NP-40 PBS extract as the antigen source, also failed to demonstrate a positive antigen-antibody type reaction. This was indicated by the failure to inhibit the lysis of sensitized RBCs at all antigen or antibody dilution ranges (data not presented). However, when amiodarone powder was used in undissolved form as antigen source, hemolysis was noted to be inhibited by a concentration of 10 \(\mu\)g per 6 ml reaction mixture (w/v). These findings indicated an antigen-antibody reaction with complement fixation had occurred between the patient's serum and the amiodarone compound.

However, by immunofluorescent method, positive reaction was noted on the patient's lung tissue in the deposition of complements, as detected by both rabbit anti-human C'1 complex and C'3b. This finding was more or less expected, since most \textit{in vitro} antibody-antigen reactions would lead to complement activation or deposition.

The transmitting electron microscopic examination of the postmortem lung tissue showed the presence of numerous so-called lysosomal lamellar inclusion bodies in the interstitial cells (Fig. 4). Morphologically, these were similar to those previously described inclusion bodies associated with amiodarone treatment.\textsuperscript{14}

There was no evidence of morphologically abnormal collagen fibers, such as the long spaced collagen fibers, noted in the patient's lung tissue. It should, however, be stated that it has been our experience that the ultrastructural changes seen, which resembled lysosomal lamellar inclusion figures, occasionally can be found in a variety of mammalian tissues which were never exposed to amiodarone.

\textbf{Discussion}

As described, this 62-year-old white man with life-threatening ventricular dysrhythmias was begun on therapy with amiodarone in November 1984 when electrophysiologic studies revealed significant ventricular irritability despite procainamide therapy. The patient subsequently developed pulmonary fibrosis (Fig 5).

The postmortem lung tissue was studied immunologically, morphologically, and biochemically. A humoral IgG class antibody, which reacted with the patient's own lung tissue, was identified in the patient's serum by immunofluorescent technique. No similar antibody was present in the serum of four control patients undergoing amiodarone therapy but with no evidence of pulmonary disease. This antibody could be removed by incubating the patient's serum with his own lung tissue or with amiodarone compound or its alcohol solution. There was no reaction noted between the patient's serum with either normal postmortem lung or fibrotic lung from patients with no history of amiodarone therapy. These findings suggest that the antigen-antibody relationship in this case consisted of an "amiodarone-anti-amiodarone" reaction.

Attempts to extract the antigen from the patient's lung tissue by detergent buffer with sonification of the postmortem lung tissue failed to reveal an immunologically detectable antigen within the aqueous buffer extract. This suggests that the amiodarone served as a hapten which was bound to a native

\begin{figure}
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\includegraphics[width=\textwidth]{Figure4.png}
\caption{Electron microscope morphology. The interstitial cell of the amiodarone-associated pulmonary fibrosis lung postmortem showed numerous lamellar inclusions. Morphologically, these structures resembled the lysosomal lamellar inclusions reportedly associated with amiodarone toxicity. (TEM original magnification, X 7500; inset: X 15,000.)}
\end{figure}
pulmonary protein carrier \textit{in vivo}. This hapten-protein complex was capable of initiating a humoral antibody response. However, it appears that this hapten-protein interaction does not involve covalent bonding since lung detergent extracts failed to display an antigen-antibody reaction by either Western-blot technique or the complement fixation procedure.

Acrylamide gel electrophoresis revealed no qualitative differences between the patient's washed lung extract and postmortem lung tissue from patients with no pulmonary disease. Specifically, no abnormal protein species could be identified, at least in the buffer extractable form.

Routine histologic sections revealed an advanced degree of interstitial fibrosis with generalized paucity of accompanying inflammatory reaction. The only significant accompanying feature was the presence of occasional focal collections of vacuolated histiocytes within the alveolar spaces. The lack of inflammatory cellular reaction suggests that the observed interstitial fibrosis was not secondary to acute or chronic inflammation and the subsequent reparative process. Electron microscopic examination of the fibrotic lung demonstrated multiple lamellated lysosomal inclusions which have previously been reported as a characteristic finding of amiodarone therapy.

In addition to amiodarone, the patient had also received propafenone. To our knowledge the form of pulmonary toxicity observed in this case has not been associated with propafenone therapy.

The exact mechanism of amiodarone-associated pulmonary fibrosis is not presently known. The possibility of binding of the drug to phospholipids resulting in inhibition of their normal enzymatic degradation and subsequent lysosomal accumulation has been considered. Also, evidence of a drug-induced autoimmune mechanism has been reported. Our findings are consistent with an IgG-mediated immune response initiated \textit{in vivo} against amiodarone bound to a native pulmonary component, which is presumably a protein molecule. We hypothesize that the antigen-antibody reaction induces a proliferative fibroblastic response with enhanced fibro-collagen deposition, resulting in pulmonary interstitial fibrosis. This possibility was discussed in a previous study in which we presented evidence of a humoral antibody-mediated pathogenesis for idiopathic pulmonary interstitial fibrosis.

It appears that a similar, although more specific, mechanism is also operative in the amiodarone-associated form of pulmonary interstitial fibrosis.

However, in view of the fact that only approximately 6 percent of patients receiving amiodarone therapy develop pulmonary fibrosis or pulmonary toxicity, it is possible that the amiodarone-lung protein complex or the amiodarone compound itself is a weak antigen or lacks strong antigenicity in a majority of patients. Alternatively, the binding of amiodarone compound to lung tissue may not be a first order kinetic reaction. Other factors, such as MHC antigens in individual patients may also play a role in the pathogenesis of amiodarone-associated pulmonary fibrosis. It would be interesting to study the MHC antigens in a larger series of patients who received amiodarone and developed clinically recognizable pulmonary fibrosis. Furthermore, whether the antibody crossreacts with the major amiodarone metabolite, desethylamiodarone, or whether the metabolite itself is capable of initiating a humoral response within the parental compound therapeutic dosage also needs further study.
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REFERENCES

Amiodarone-associated Pulmonary Fibrosis (Fan et al)