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Acute thrombocytopenia in patients treated with amiodarone is caused by antibodies specific for platelet membrane glycoproteins

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Summary

Amiodarone has been implicated as a cause of thrombocytopenia but the responsible mechanism is unknown. We performed studies in three patients to characterize the pathogenesis of this complication. No amiodarone-dependent, platelet-reactive antibodies were identified using conventional serological techniques. However, water-insoluble amiodarone solubilized in methanol and diluted to 1.0 mg/ml in aqueous buffer reproducibly promoted binding of IgG antibodies in patient serum to platelets. Solid phase assays identified drug-dependent antibodies specific for platelet glycoproteins (GP)Ia/IIa (integrin $\alpha_2\beta_1$) in each patient and a second antibody specific for GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin) in one patient. When studied by ion mobility analysis and transmission electron microscopy, the serologically active amiodarone preparation, a milky suspension, was found to consist of particles 2–30 nm in diameter, typical of a coacervate, a state characteristic of amiodarone in aqueous medium. The findings provide evidence that thrombocytopenia in the three patients studied was caused by drug-dependent antibodies specific for platelet glycoproteins GPIa/IIa and/or GPIIb/IIIa. We postulate that, *in vivo*, amiodarone may become incorporated into occult lipophilic domains in platelet glycoproteins, producing structural modifications that are immunogenic in some individuals, and that the resulting antibodies can cause platelet destruction in a person taking this drug.

Keywords

amiodarone (Cordarone®); immune thrombocytopenia; drug-induced thrombocytopenia

Drug-induced immune thrombocytopenia (DITP) is caused by drug-dependent, platelet-reactive antibodies (DDAbs) and can be a cause of life-threatening bleeding (Aster & Bougie, 2007; Aster *et al.*, 2009). Demonstration of a DDAb in a patient suspected of having DITP provides strong evidence that thrombocytopenia was caused by drug sensitivity, but it is not uncommon for test results to be negative (Aster & Bougie, 2007). One reason for this

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Authorship contributions

M. Sahud and R. Aster designed the study and wrote the paper; N. Clarke contributed the analytic chemistry analysis; M. Caulfield performed the ion mobility studies; D. Bougie performed the flow cytometry studies; and R. Koch performed and analysed transmission electron microscopic studies.

is that some drugs are virtually insoluble in water, making it very difficult to achieve concentrations of drug high enough to produce a positive test result.

Amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3, 5 diiodophenyl methanone), a benzofuran derivative virtually insoluble in water, was developed more than 30 years ago as an anti-anginal agent and is now widely used as an antiarrhythmic drug (Mason, 1987; Stevenson *et al*, 2002). Recognized adverse effects of amiodarone include pulmonary toxicity (Mason, 1987), hepatotoxicity (Rhodes *et al*, 1993) and thrombocytopenia (Weinberger *et al*, 1987) but DDABs specific for amiodarone have not been described. We encountered three patients who experienced severe thrombocytopenia while taking amiodarone. Here we describe the clinical aspects of these cases and show that, although conventional testing for amiodarone-dependent, platelet antibodies produced negative results, antibodies could be detected using a particulate suspension of amiodarone having the properties of a coacervate, i.e., microparticles consisting of hydrophobic organic molecules held together by hydration restraints (Sierra *et al*, 2006; Benedini *et al*, 2010) as the source of 'drug' in reaction mixtures. The findings provide evidence that thrombocytopenia in these patients was the result of an immune response to drug and suggest a possible means of detecting platelet-specific antibodies induced by other water-insoluble drugs.

Materials and methods

Case reports

Patient 1—A 49-year-old man presented with an acute myocardial infarction in cardiogenic shock. Clopidogrel, aspirin, and oral amiodarone (400 mg/d) were started and coronary angiography was performed, during which intravenous heparin was given. On day 10, he began to have uncontrolled oozing from the catheter insertion site in the groin. A coagulation screen was normal but his platelet count was $2.0 \times 10^9/l$. Heparin-induced thrombocytopenia (HIT) was considered, but the platelet factor 4 enzyme-linked immunosorbent assay (PF4 ELISA; GTI Diagnostics, Waukesha, WI) and ^{14}C serotonin release assays (SRA; Sheridan *et al*, 1986) for heparin-dependent antibodies were negative on two occasions. Clopidogrel, aspirin and amiodarone were discontinued and the patient was treated with intravenous gamma globulin 1 g/kg and intravenous prednisolone. Platelets remained low for 5 d but recovered to $338 \times 10^9/l$ 7 d after stopping amiodarone.

Patient 2—A 77 year-old Caucasian woman underwent aortic valve replacement for severe calcific aortic stenosis. The preoperative platelet count was $193 \times 10^9/l$. Oral amiodarone 400 mg/d was given for 3 d postoperatively, at which point re-exploration was required for cardiac tamponade. The patient recovered, but her platelet count fell to $24 \times 10^9/l$ on day 6. There had been no heparin exposure post-operatively or after re-exploration. A screen for disseminated intravascular coagulation was negative except for a borderline D-dimer result. PF4 ELISA and SRA for HIT antibodies were negative. Drugs to which the patient had been exposed included famotidine, furosemide, enalapril, ceftriaxone, lisinopril, and amiodarone. These were discontinued and propafenone (Rythmol) was substituted for amiodarone on day 8. The patient remained thrombocytopenic for 1 week but the platelet count rose to $105 \times 10^9/l$ on day 11 post-nadir and reached $255 \times 10^9/l$ on day 24.

Patient 3—A 63-year-old Caucasian woman was hospitalized with orthopnea and chest pain. Cardiac evaluation revealed critical aortic stenosis (aortic valve aperture of 0.64 cm^2) and left ventricular hypertrophy. She had been receiving glyburide and furosemide. Amiodarone (600 mg/d) had been given for 2 d prior to surgery. She underwent aortic valve replacement with exposure to heparin and did well until day 4, when she developed

alternating sinus bradycardia with paroxysmal atrial fibrillation. She was given 100 mg amiodarone intravenously and intermittent pacemaker support. On postoperative day 7, she was weak and lethargic and a blood count revealed Hb 89 g/l and platelets of $4.0 \times 10^9/l$. PF4 ELISA and SRA for HIT antibodies was negative. One unit of packed red cells was transfused. On postoperative day 12, the platelet count remained under $10 \times 10^9/l$ but rose to $31 \times 10^9/l$ 15 d after the last exposure to amiodarone and to $281 \times 10^9/l$ on day 18.

Methods

Reagents—Amiodarone HCL was purchased from Teva Novopharm (Toronto, ON, Canada) and was dissolved in methanol at 1.0 mg/ml for further studies. Amiodarone for injection was obtained as a 1.0 mg/ml solution in methanol from Cerilliant Inc, Round Rock, TX, USA. Protein A-Sepharose CL-4 beads, streptavidin-horseradish peroxidase (HRP) conjugate, and electrochemiluminescence (ECL) detection reagents were from Amersham Pharmacia (Piscataway, NJ, USA). Protease inhibitor cocktail tablets and polyvinylidene difluoride (PVDF) membranes were from Roche (Nutley, NJ, USA) and Millipore (Bedford, MA, USA), respectively. NHS-biotin, biotinylated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) broad range standard, and Laemmli sample buffer were from Bio-Rad (Hercules, CA, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies—Mouse monoclonal antibodies (mAbs) were of the IgG class affinity-purified using Staphylococcal protein A. Fluorescein isothiocyanate (FITC)-conjugated anti-human IgG F(ab')₂, clone P2 (anti-glycoprotein [GP]IIb/IIIa complex), clone Gi9 (anti-GPIa/IIa), clone SZ1 (anti-GPIX), and clone SZ2 (anti-GPIβ_a) were purchased from Beckman Coulter Inc. (Hialeah, FL, USA). Goat anti-mouse IgG (Fc specific), used in the Monoclonal Antibody Immobilization of Platelet Antigens Assay (MAIPA), was purchased from Sigma-Aldrich. Goat anti-human IgG with and without HRP-labelling, mouse anti-human clone GR-P and clone FMC-25 (anti-GPIX), and clone PM6/13 (anti-GPIIIa) was purchased from Serotec (Raleigh, NC, USA). The mAb 6F1 (anti-GPIa/IIa) was a gift from Dr. Barry Collier (Mount Sinai School of Medicine, NY).

Platelets—Normal platelets were isolated from healthy, non-medicated type O adult donors. Platelets from platelet-rich plasma anticoagulated with acid citrate dextrose (ACD) used in a quantity sufficient to produce pH about 6.5 were washed three times with Ringer's citrate dextrose (RCD), pH 6.5, containing 1% bovine serum albumin (BSA) and 50 ng/ml prostaglandin E₁ (PGE₁).

Flow cytometry—DDAb detection was performed by flow cytometric analysis as described previously (Curtis *et al*, 1994; Liu & Sahud, 2003). In brief, 1×10^7 platelets suspended in 25 µl RCD-1% BSA containing drug at the desired concentration were incubated with 25 µl of patient serum for 1 h at room temperature (RT). After incubation, the platelets were washed three times with RCD-1% BSA containing drug at the same concentration as in the primary mixture. Bound antibody was detected using FITC-labelled secondary antibody specific for IgG Fc using a PICS XL-MCL analyser (Beckman Coulter) with EXPO 32 software (Quest Diagnostics, San Juan Capistrano, CA, USA) or an LSRII flow cytometer (Becton-Dickinson, San Jose, CA, USA; Blood Center of Wisconsin, Milwaukee, WI, USA). A positive reaction was defined as one in which the median platelet fluorescence intensity (MFI) value obtained with patient serum in the presence of drug was at least twice the value obtained with patient serum in the absence of drug and with normal serum in the presence of drug. In studies with antibodies specific for drugs other than amiodarone, this value invariably exceeded the mean values obtained with patient serum in

the absence of drug and with normal serum in the presence of drug by 3.0 standard deviations (SD).

Monoclonal Antibody Immobilization of Platelet Antigens Assay—MAIPA used to identify glycoproteins recognized by platelet-reactive antibodies as previously described (Kiefel *et al*, 1987; Bettaieb *et al*, 1991; Kiefel, 1992). Briefly, 2×10^7 washed platelets suspended in 30 μ l of phosphate-buffered saline (PBS) and 1% BSA (PBS-BSA) were incubated with 30 μ l patient serum in the presence of drug (at a final concentration of 1 mg/ml in PBS) for 30 min at 37°C. After incubation, platelets were washed once in PBS-BSA containing drug and incubated with a glycoprotein-specific mAb (5 μ g/ml) in the presence of drug for another 30 min. Platelets were then washed three times in buffer and lysed in buffer containing 1.0% Triton X100). The lysate was then aliquoted into microtitre plate wells containing goat antibody specific for mouse IgG to capture the glycoprotein for which the mouse mAb was specific. Human antibody bound to the captured glycoprotein was then detected with HRP-labelled anti-human IgG (diluted at 1:3000) and ortho-phenylene-diamine substrate (OPD). Drug presence was maintained in all steps of the assay. A positive reaction was defined as mean absorbance (490 nm) in the presence of drug that exceeded the mean value obtained in the absence of drug by at least 3.0 SD.

Immunoprecipitation—The assay was performed as described previously (Smith *et al*, 1993) with minor modifications. Briefly, washed platelets suspended in PBS were surface-labelled with 1 mmol/l NHS-biotin, followed by three washes with PBS-BSA. An aliquot of biotinylated platelets (3×10^8) was incubated with 0.3 ml patient serum or normal serum with and without drug (1 mg/ml), and with mAb P2 (anti-GPIIb/IIIa) or Gi9 (anti-GPIa/IIa) at RT for 1 h. The sensitized platelets were washed three times in PBS-BSA containing drug at the same concentration and then solubilized at 4°C for 30 min in lysis buffer containing 25 mmol/l Tris-buffered saline (TBS), 1% Triton X-100, pH 7.8, a protease inhibitor cocktail (1:10 dilution), and 1 mg/ml drug. The lysate was centrifuged at 13 000 *g* for 30 min. A 100- μ l aliquot of pretreated protein A-Sepharose CL-4 beads uncoated (direct immunoprecipitation) or coated with goat anti-human IgG (indirect immunoprecipitation) was incubated with the platelet lysate at 4°C for 2–3 h with gentle agitation. The beads were then washed four times in lysis buffer containing 1 mg/ml drug and resuspended in Laemmli sample buffer. The eluates were subjected to a 6% SDS-PAGE under reducing conditions and blotted onto a PVDF membrane using a MiniProtein II system (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in TBST buffer (25 mmol/l TBS, 0.1% Tween-20, pH 7.4), incubated with streptavidin-HRP conjugate (diluted at 1:4000), and visualized with chemiluminescence substrate mixture.

Ion mobility analysis—Physical properties of opalescent aqueous solutions of amiodarone were characterized using ion mobility analysis (Caulfield *et al*, 2008). In brief, samples were introduced via an autosampler (LEAP HTC PAL, Eksigent, Dublin, CA, USA) connected to an electrospray generator (Model 3480, TSI, Shoreview, MN, USA) via methyl-deactivated silica capillary (50 μ m i.d., SGE, Austin, TX). Flow was introduced by nano-LC pumps (Eksigent) running a mobile phase of 25 mmol/l ammonium acetate, 0.5 mmol/l ammonium hydroxide, pH 7.4. The sample (10 μ l) was injected by means of an autosampler at 6 μ l/min into a transfer capillary (methyl-deactivated, 50 μ m, 33 cm long; SGE) by means of a capillary metal union (Upchurch Scientific, Oak Harbor, WA, USA). Samples were scanned at a flow rate of 200 nl/min.

Transmission electron microscopy—Two types of samples containing amiodarone were negatively stained and imaged with a Hitachi (H-7000) electron microscope at 75 keV. Aliquots of a clear amiodarone solution (1 mg/ml in absolute methanol) were diluted in

Tyrode's buffer to produce an opalescent solution that was applied to a carbon-coated grid (Ted Pella, Inc., Redding, CA, USA) for 5 min. Excess fluid was removed by wicking via wedges of filter paper and samples were allowed to air dry for 5 min. Then, droplets of 1% aqueous uranyl acetate were added to the amiodarone-covered grid, removed by wicking, and imaged by TEM. In separate studies, whole platelets were washed in Tyrode's buffer and resuspended in the same amiodarone preparation. After several minutes, the suspension was applied to carbon-coated grids and dried at room temperature for 5 min before excess solution was removed by wicking. Aliquots of 1% uranyl acetate were added to grids and allowed to stand for 5 min before the moisture was removed by wicking. Grids were air dried and imaged by TEM. Digitized images collected in this fashion were analysed for particle diameter using the length measurement tool in the National Institutes of Health (NIH) ImageJ 1.46r software. Statistical analysis was performed in Microsoft Excel 2011; two-tailed, Student *t*-test, unequal variance was used and significance was determined at the 95% confidence limit.

Results

Flow cytometry

In initial studies, attempts were made to detect DDABs specific for each medication to which the patients had been exposed using drugs dissolved in PBS, pH 7.2 for testing. All test results were negative (data not shown). The only drugs given to all three patients were heparin and amiodarone. Heparin-dependent antibodies were not detected by PF4 ELISA or SRA (see Case reports). Because negative results in conventional testing with amiodarone would be expected because of its water insolubility, further studies were undertaken that included the drug in a different formulation. Amiodarone is freely soluble in methanol. When the drug at 50 mg/ml in methanol was diluted 1:50 in PBS, pH 7.2, it immediately formed an opaque, milky solution. When flow cytometric studies were repeated using this material as the source of 'drug,' it was found that each of the three patient sera contained an IgG immunoglobulin that reacted with normal platelets when drug was present, but not in its absence (Fig. 1). Using the same conditions, no platelet-binding immunoglobulins were detected in serum from nine normal individuals. In titration studies, it was found that positive results were obtained with amiodarone at concentrations as low as 0.1 mg/ml.

MAIPA

In the MAIPA, platelets are sensitized with antibody in the presence of drug. After washing, a glycoprotein-specific mAb is added. The platelets are then washed and solubilized in detergent and the mAb and its target glycoprotein are captured in microtitre wells to which goat anti-mouse IgG has been fixed. ELISA is then used to detect human antibody bound to the captured glycoprotein. Drug presence was maintained at the same concentration at all stages of the assay. Serum from Patient 3 reacted with GPIIb/IIIa captured with mAb P2 (Fig. 2A) and serum from all three patients reacted with GPIa/IIa captured with mAb Gi9 (Fig. 2B). None of the patient antibodies reacted with GPIb/IX captured with mAb FMC-25 specific for GPIX (not shown).

Immunoprecipitation

To further define the target specificities of the patient antibodies, biotin-labelled platelets were incubated with the three patient sera and with normal serum in the presence of drug and then washed. MAbs Gi9 (anti-GPIa/IIa) and P2 (anti-GPIIb/IIIa) were then added. Following washing and detergent lysis, IgG was captured with Staphylococcal protein A-labelled beads and biotinylated platelet proteins bound to the captured antibody were separated by gel electrophoresis and detected with streptavidin-HRP. Figure 3 shows that mAb Gi9 precipitated bands of estimated molecular weights 150 and 130 kDa corresponding

to GPIa and GPIIa, respectively, and that mAb P2 precipitated bands of estimated molecular weights 125 and 110 kD, corresponding to GPIIb and GPIIIa in a reduced gel. Each of the three patient sera precipitated bands corresponded to GPIa and GPIIa; serum from Patient 3 also precipitated bands corresponding to GPIIb and GPIIIa. No bands were precipitated using normal serum in the presence of amiodarone or patient serum in the absence of amiodarone (not shown).

Ion mobility analysis

Further studies were done to characterize the physical properties of the amiodarone preparation that promoted binding of the patient antibodies to platelet glycoproteins. For this purpose, amiodarone solubilized in absolute methanol was diluted to 1.0 mg/ml using 25 mmol/l ammonium acetate, pH 7.4, producing a milky, opalescent mixture. Further dilutions were made in Tyrode's buffer. The samples were centrifuged to remove any potential precipitate but none was identified. Particle distribution in pre- and post-centrifugation samples was analysed using ion mobility analysis (Caulfield *et al*, 2008) in which particles are separated on the basis of their gas-phase electrophoretic mobility. This method is routinely used for particle size distribution analysis in the aerosol sciences and for lipoprotein particle analysis (Caulfield *et al*, 2008). Analysis of solutions prepared at three different concentrations of amiodarone (0.25, 0.5 and 1.0 mg/ml) is shown in Fig. 4A. A prominent peak consisting of particles with diameter averaging about 5.8 nm was observed at the highest concentration, which decreased to about 4.75 and 3.2 nm, respectively, at the lower concentrations. The profile of particles in the 1.0 mg/ml solution was unchanged by centrifugation (Fig. 4B).

TEM

To visualize the particles identified in the ion mobility studies, amiodarone dissolved in absolute methanol was diluted in Tyrode's buffer to produce an opalescent suspension that was dried, negatively stained and analysed by TEM. A typical image contained spheroidal aggregates of 15–30 nm diameter in a field of smaller spherical structures (Fig. 5). The larger aggregates appeared to be made up of smaller particles 1.35 ± 0.23 nm (SD) ($n = 58$) in diameter. Numerous smaller particles were present in the field external to the large aggregates that were similar in size 1.44 ± 0.24 nm ($\pm$ SD, $n = 107$). The two populations of smaller particles were not significantly different from each other ($P > 0.05$) and, taken together, had an average diameter of 1.41 ± 0.24 nm ($\pm$ SD, $n = 165$) (unpaired Student's *t*-test).

To visualize interactions of the amiodarone particles with platelets, aliquots of whole platelets were exposed briefly to amiodarone (0.5 mg/ml in Tyrode's buffer), washed gently in buffer and dried onto carbon-coated grids, negatively stained, and examined by TEM. This treatment led to the collapse of whole platelets onto the carbon film coating the grids and the collection of negative staining solution on and around the platelet surfaces as evidenced by the electron dense regions on the boundary and in surface pockets of the collapsed cell (Fig. 6). As shown in Fig. 6 (insert), particles averaging about 2.26 ± 0.39 nm ($\pm$ SD, $n = 51$) in diameter were found to be clustered on the surface of stained platelet membranes. These particles were not significantly different in diameter ($P > 0.05$) from those seen in amiodarone-only samples (Fig. 5).

Discussion

The three patients studied experienced severe thrombocytopenia after being exposed to several different medications and recovered after these drugs were discontinued. Because multiple drugs were being taken and because no challenge was attempted to determine

whether thrombocytopenia could be re-induced, on clinical grounds, the diagnosis of DITP would be considered 'possible' rather than 'probable' or 'confirmed' using the diagnostic criteria defined by George *et al* (1998). In such cases, demonstration of a DDAB that reacts with platelets in the presence of one of the implicated drugs can provide support for a diagnosis of DITP (Aster *et al*, 2009; Reese *et al*, 2010). In the three patients studied, negative tests for DDABs were obtained using each of the drugs to which they had been exposed. Negative results were expected with amiodarone because it is virtually insoluble in water. We then dissolved amiodarone at 50 mg/ml in methanol and diluted it to 1.0 mg/ml in aqueous buffer, which resulted in formation of a milky, opalescent solution. Unexpectedly, this material caused significant quantities of IgG immunoglobulins to bind to target platelets when serum from the three patients was present in the reaction mixture, but not when serum from nine different normal subjects was used (Fig. 1) or when patient serum was tested in the absence of amiodarone. When the opalescent amiodarone solution was used as the source of 'drug' in two independent assays, the MAIPA (Fig. 2) and direct precipitation of biotin-labelled membrane glycoproteins (Fig. 3), it was found that each patient had an antibody that recognized the platelet GPIa/IIa complex ($\alpha_2\beta_1$ integrin) when amiodarone was present and that Patient 3 also had a DDAB specific for GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin). Together, these findings provide evidence for drug-dependent antibodies reactive with platelet-specific glycoproteins in these patients and support the likelihood that amiodarone sensitivity was the cause of their thrombocytopenia. The slow recovery of platelet levels after discontinuation of amiodarone (one week in Patients 1 and 2 despite administration of intravenous IgG to Patient 1 and more than 2 weeks in Patient 3) is atypical (Aster & Bougie, 2007) and could be related to the known, slow clearance of this lipophilic drug from body tissues (Mason, 1987).

As to our knowledge there is no precedent for using a drug preparation of this type for serological testing to detect a drug-dependent platelet antibody, we performed additional studies to characterize the physical nature of the amiodarone preparation that was serologically active. Using ion mobility analysis, a tool that permits particle size distribution to be characterized on the basis of gas phase electrophoretic mobility (Caulfield *et al*, 2008), we found that the amiodarone used in testing consisted of particles with an average diameter that increased from about 3 to 6 nm as amiodarone concentration was increased from 0.25 to 1.0 mg/ml (Fig. 4A). TEM studies showed that an amiodarone suspension similar to the one that promoted antibody binding to platelets in flow cytometric studies (Fig. 1) consisted of relatively large particles averaging 15–30 nm in diameter and many smaller particles with average diameters in the range of 1.5–2.5 nm (Fig. 5). When washed platelets were present, the smaller particles tended to cluster on the plasma membrane (Fig. 6).

The properties of amiodarone in aqueous media were recently studied by Benedini *et al* (2010). They found that, at concentrations ranging from 1.0 to 3.0 mg/ml at 25°C, amiodarone molecules coalesced to produce an opaque solution consisting of various-sized molecular aggregates. TEM studies showed the suspensions consisted of larger spherical particles averaging about 18 nm in diameter and many smaller particles with average diameter about 2.5 nm. Steady state fluorescence experiments using pyrene as a luminescence probe and hexadecylpyridinium chloride as a static quencher provided evidence that the smallest aggregates contained about 4 amiodarone molecules (Benedini *et al*, 2010). Related physical chemical studies provided evidence that the particles were not micelles but, rather, were in the form of a coacervate, i.e., clusters of hydrophobic organic molecules held together by hydration constraints (Benedini *et al*, 2010; Veis, 2011). Our analyses of aqueous suspensions of amiodarone using ion mobility analysis (Fig. 4) and TEM (Figs 5 and 6) demonstrated larger particles averaging 15–30 nm diameter and many smaller ones averaging 2–6 nm in diameter. It is apparent that the particles identified using these techniques closely resemble those described by Benedini *et al* (2010).

Our laboratory studies suggest that amiodarone particles produced in an aqueous solution, by virtue of their lipophilic nature, become incorporated into platelet membranes or into normally occult lipophilic domains of surface glycoproteins and modify glycoproteins in such a way that they are recognized by antibodies of the type identified in the three patients described here. The large aggregates (15–30 nm) observed *in vitro* would not be expected to form *in vivo*, where amiodarone becomes widely distributed through the body, especially in lipid-rich tissues (Latini *et al*, 1984; Haffajee, 1987; Mason, 1987) and where, in the circulation, it is almost entirely bound to plasma protein (Mason, 1987). It seems possible that, *in vivo*, the drug may localize in glycoproteins of megakaryocytes and platelets and produce structural changes that are immunogenic in some individuals and that, *in vitro*, incorporation of amiodarone particles into platelets mimics this process, enabling amiodarone-induced antibodies to be detected. Drugs other than amiodarone that are sparingly soluble in water are also known to cause DITP (Aster *et al*, 2009; Reese *et al*, 2010). The possibility that the approach employed here to detect amiodarone-dependent antibodies could be useful for antibody detection in patients with thrombocytopenia induced by other water-insoluble drugs deserves investigation.

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Fig. 1.

Flow cytometry. Serum from three patients with amiodarone-associated thrombocytopenia contained IgG antibodies that reacted with normal platelets when amiodarone was present (solid curves) but not in its absence (dashed curves). Histograms shown are representative of three or more independent studies done with each patient. No amiodarone-dependent antibodies were identified in any of nine normal serum samples tested (representative control shown).

Fig. 2.

Monoclonal antibody immobilization of platelet antigens (MAIPA). (A) Serum from all three patients reacted with GPIa/IIa immobilized by monoclonal Gi9 when amiodarone was present (filled bars), but not when it was absent (unfilled bars). (B) Serum from Patient 3 reacted with GPIIb/IIIa immobilized from detergent-solubilized platelets by monoclonal P2 when amiodarone was present (filled bars) but not when it was absent (unfilled bars). Reaction of serum from Patients 1 and 2 did not differ significantly from normal donor serum (ND) (** $P < 0.001$). OD, optical density; Pt, Patient.

Fig. 3.

Immunoprecipitation. Serum from all three patients (Lanes 2–4) precipitated bands corresponding to those recognized by monoclonal Gi9 specific for GPIa/IIa (Lane 5). Serum from Patient 3 (Lane 4) also precipitated bands corresponding to those recognized by monoclonal P2 specific for GPIIb/IIIa (Lane 6). No bands were precipitated by normal serum (Lane 1). Platelet glycoproteins were labelled with biotin and precipitated proteins were detected by enzyme-linked immunosorbent assay as described in Methods.

Fig. 4. Ion mobility studies. (A) A stock solution of amiodarone (10 mg/dl in methanol) was diluted to 1.0 (black), 0.5 (blue) or 0.25 mg/ml (red) in 25 mmol/l ammonium acetate, pH 7.4. Samples were analysed in a gas-phase electrophoretic analyser (ion mobility) that separated particles based on their size and counted them using a condensation particle counter. The profile shown is for 2 to 12 nm. All 3 dilutions were scanned up to 100 nm but no other peaks at other particle diameters were detected (data not shown). (B) Size distribution of particles in the 1.0 mg/ml preparation was unaffected by centrifugation at 16 000 *g*.

Fig. 5.

Transmission electron microscopy. Transmission electron microphotography of amiodarone in aqueous solution, 1.0 mg/ml, using Hitachi H-7000 at 75 Kev with 2% uranyl acetate, dried and plated on a Formvar ultrathin carbon grid (Ted Pella Inc.). The suspension contained spheroidal particles averaging 15–30 nm in diameter and larger numbers of smaller particles of diameter about 2 nm. The larger particles appeared to consist of aggregates of the smaller particles.

Fig. 6. Transmission electron microscopy. Aliquots of washed platelets were exposed to amiodarone (0.5 mg/ml in Tyrode's buffer), processed as described in Methods and examined by transmission electron microscopy. As shown in this representative field, particles about the size of the smaller particles identified in Figure 5 accumulated on platelet membranes (arrows).