Adult-onset, chronic, cyclic thrombocytopenia in a Rhesus macaque (

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Case Report: Adult-onset, chronic, cyclic thrombocytopenia in a Rhesus macaque (*Macaca mulatta*) after dengue virus vaccination and viral challenge

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Abstract

An 8-year old, male Rhesus macaque (*Macaca mulatta*) previously used for dengue virus vaccine research with viral challenge, presented with adult-onset, chronic, cyclic thrombocytopenia. Platelet number, morphology and function were evaluated by automated hematology, peripheral blood smears, electron microscopy, flow cytometry, and impedance aggregometry. Bone marrow was evaluated by cytology. Both, serum anti-dengue non-structural protein 1 (NS1) antibodies and anti-platelet antibodies were detected by ELISA. Platelet characterization showed a lack of aggregation to all agonists (ADP, ASP, and collagen), increased activation with increased expression of surface marker (HLA-ABC), and an absence of surface receptor GPIX during clinical episodes of petechiae and ecchymoses, even in the presence of normal platelet counts. Bone marrow aspirates identified potential mild megakaryocytic hypoplasia. All platelet functions and morphologic attributes were within normal limits during clinically normal phases. Presence of anti-dengue NS1 serum antibodies confirmed a positive dengue virus titer 8 years post-

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vaccination. Based on the history and clinical findings, a primary differential diagnosis for this chronic, cyclic platelet pathology was autoimmune platelet destruction with potential bone marrow involvement.

Keywords

Autoimmune; ITP; Platelets; nonhuman primate

Case Presentation

An 8-year old, intact male, Chinese-origin Rhesus macaque (*Macaca mulatta*), imported to the Massachusetts Institute of Technology (Cambridge, MA), presented with intermittent episodes of lethargy, hematochezia, and diffuse petechiae and ecchymoses for a period of 2 years (Figure 1). This monkey's prior research history included a dengue virus (DENV) vaccine trial and viral challenge at 3-4 years old at another institution, and a minimally invasive peripheral nerve study at the age of 5 years. These episodes occurred every 3-6 months, and typically lasted between 1-2 weeks. This macaque was off study during this 2-year period of clinical signs and had no documented history of coagulopathies or gastrointestinal disease. Abnormal physical examination findings during these episodes included a grade V/VI sternal systolic heart murmur (normally a grade II/VI), 5% dehydration, and a mild enlargement of the left testicle without associated erythema, edema, or pain. Thoracic and abdominal imaging revealed gas-distended descending colon and diffusely fluid-distended small intestines. Abdominal ultrasonographic examination revealed dilated and thickened irregular small intestinal walls. Repeat CBC and serum biochemistry profiles performed during these episodes showed mild to severe thrombocytopenia (12-271 $\times 10^6$ platelets/mL; RI 293-479 $\times 10^6$ platelets/mL), normal to moderate increase in mean platelet volume (MPV) (6.9-16.1 fL; RI 6.73-11.07 fL), normal to marked leukocytosis (5.28-25.1 K/uL; RI 5.16-14.28 K/uL), with an associated neutropenia or neutrophilia (0.381-193 K/uL; RI1.63-10.39 K/uL).[1] The MPV was generally normal at the beginning of an episode and then trended upwards over the following 2 weeks. Petechiae and ecchymoses were sometimes present prior to the onset of a peripheral thrombocytopenia. Peripheral blood smear analysis confirmed the thrombocytopenia with giant platelets, along with a 1-2+ toxic change, consisting of neutrophils with basophilic, vacuolated cytoplasm and Dohle bodies, and a left shift, indicative of an inflammatory reaction. A rebound thrombocytosis, 420-508 $\times 10^6$ platelets/mL, including large platelets with an MPV of 12.4-13.5 fL, was identified post-episodes (Figure 2A–C).

Basic coagulation testing through IDEXX Laboratories (North Grafton, MA) and the Comparative Coagulation Laboratory (Animal Health Diagnostic Center, Cornell University College of Veterinary Medicine, Ithaca, NY) was performed when the macaque had clinical signs with a platelet count ranging from 12-100 $\times 10^6$ platelets/mL (Table 1). Not all tests were performed during the same episode. Although there was a simultaneous prolongation of PT and APTT, suggestive of a potential consumptive coagulopathic state, these were not reproducible on multiple episodes. The serum lactate dehydrogenase (LDH) activity, and von Willebrand's factor (vWF) antigen and collagen binding assay were within normal

limits, while the fibrinogen and buccal mucosal bleeding time (BMBT) were not consistently elevated. The BMBT was performed when the monkey was clinically normal, with a platelet count of 271 × 10^6 platelets/mL and ranged from 1.4-4 minutes. The lack of schistocytes and spherocytes on the peripheral blood smears in addition to the normal LDH activity suggested absence of disseminated intravascular coagulation (DIC) and thrombotic thrombocytopenic purpura (TTP). The slight increase in fibrinogen could have been suggestive of an underlying acute phase response. Although the BMBT was not consistently prolonged[2], potentially due to the non-standardized methodology of the use of the BMBT in primates, the observed mild increase in BMBT was suggestive of a potential platelet-dysfunction based coagulopathy, although a normal vWF antigen level and collagen binding assay level ruled out most types of vWF-based disease.

As a primary platelet defect was suspected, platelet morphology and function were evaluated by flow cytometry, electrical impedance aggregometry (EIA), and electron microscopy (EM). Blood was collected from either the femoral or saphenous vein into two 2.9-mL sodium citrate (3.2%) (Sarstedt, Nümbrecht, Germany) using the 2-tube technique. The first sodium citrate tube was discarded to minimize the chance that endothelial and platelet activation from the initial venipuncture would result in spurious biomarker readings. Two control animals were age and sex-matched Rhesus macaques from the same room. Multiple platelet tests were performed on the same day, from the same blood draw when possible. The electrical impedance aggregometry analysis was performed within 4 hours of blood collection using a multiplate analyzer (Roche Diagnostics Limited, Switzerland). Briefly, a sample of whole blood was placed in a cuvette along with a platelet agonist (saline was used as a negative control). The electrical impedance of the sample was then recorded by the multiplate analyzer as a measurement of platelet aggregation. Due to time and sample volume constraints, each sample was run once, with the exception of the experimental subject, which was run in duplicate. Platelet function and activation testing was performed both during a clinical episode (petechiae +/− ecchymoses) and when the subject was clinically normal (absence of petechiae/ecchymoses). Care was taken to perform testing only when the platelet count was near normal limits, ≥ 263 and 300 × 10^6 platelets/mL, respectively, to eliminate the chance of thrombocytopenia causing false results.

Platelet function testing by EIA indicated a lack of aggregation of the affected macaque's platelets to all agonists (diluted in sterile water) during the clinical phase, including adenosine-5′-diphosphate (ADP, 2 μM), arachidonic acid (ASP, 50 μg/mL), and soluble calf skin Collagen (Type I, 190 μg/mL) (BIO/DATA Corp., Horsham, PA), while the control animal platelets and the platelets from the macaque during the non-clinical phase showed the expected reaction (Figure 3).

Blood for flow cytometry analysis was mixed in a 2:1 ratio with PAMFix (Platelet Solutions Ltd, Nottingham, UK), a fixative solution that is optimized for the measurement of human platelet activation markers up to 9 days after blood collection, and whole blood collected in sodium citrate. Platelets in whole blood were then analyzed within 48 hours of collection.[3] Multicolor flow cytometry was performed on a BD FACS Calibur (BD Biosciences, San Jose, CA). Platelets were identified by forward (FSC) and side scatter (SSC) in PAMFixed whole blood and compensation completed with the aid of single color controls; all stained

samples were compared to unstained and IgG stained samples to set gates for positive populations. Analysis of Mean Fluorescence Intensity (MFI) using FlowJo software (FlowJo, LLC, Ashland, OR) revealed increased activation as evidenced by increased expression of platelet surface markers (using anti-human antibodies CD62P clone AC1.2, CD40L clone 24-31, PAC1 clone PAC-1, HLA-ABC clone G46-2.6, HLA-DR clone L243), as well as an absence of surface receptor GPIX (CD42a clone ALMA.16) during episodes, even in the presence of normal platelet counts (Figure 4) (all antibodies except CD40L from BD Biosciences; CD40L from BioLegend Inc., San Diego, CA).

Electron microscopy findings included distorted morphology with bulbar to tubule cytoskeletal extensions and pseudopod formation, an indiscernible open canalicular system, and decreased α-granule content, indicative of platelet activation (Figure 5).

Bone marrow aspirate cytology, along with peripheral blood smears (PBS), were submitted to IDEXX and evaluated by a board-certified clinical pathologist. Bone marrow cytology revealed an M:E ratio of 0.73:1.0, suggesting a mild increase in erythroid precursors with an orderly and complete erythroid series, indicating normal maturation. Myeloid precursors were present in adequate numbers with orderly and complete maturation. Despite a marked peripheral thrombocytopenia, the megakaryocyte numbers did not appear to be increased, there was no left shift and their morphology was unremarkable. It is important to note that this particular bone marrow sample had a low number of unit particles preventing a definitive interpretation. The CBC and peripheral smear revealed a marked thrombocytopenia (12×10^6 platelets/mL) and a mild anemia (HCT 30.3%) with occasional marked anisocytosis, low to moderate polychromatophilic cells, and rare nucleated red blood cells. Electrical impedance aggregometry and electron microscopy were performed once, and flow cytometry was performed twice when the animal was clinically normal, using the same negative control animals. All platelet function and morphologic attributes were within normal limits when the macaque was not having a clinical episode, as defined by a normal platelet count and no hematochezia or petechiae/ecchymoses.

Given the history of hematochezia in this animal, testing for pathologic gastrointestinal microbial and parasitic diseases was also performed to rule out infectious causes for the hematochezia and systemic coagulopathy. The macaque was negative for fecal parasites by Baermann sedimentation and flotation using both Sheather's and zinc sulfate solutions. The monkey was anesthetized and lower gastrointestinal (GI) endoscopy and biopsies revealed eosinophilic colitis. Fecal cultures were negative for common bacterial pathogens including *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Campylobacter* spp., and *Helicobacter* spp.. Enteropathogenic *Escherichia coli* (EPEC), serotype O-:H2, was identified based on isolation and an intimin Gene (*eae*)-positive shiga-like toxin-negative PCR profile (*E.* coli Reference Center, Penn State College of Agricultural Sciences, University Park, PA).[4] This infection was treated with enrofloxacin (5 mg/kg PO BID) and later with metronidazole (15 mg/kg PO BID) as diarrhea persisted. This episode of diarrhea did resolve with metronidazole treatment, although the monkey continued to have diarrhea during future clinical thrombopathy episodes. The macaque was subsequently negative for pathogenic *E. coli* on all fecal cultures. Subsequent lower GI biopsies showed a lymphocytic-plasmacytic ulcerative colitis. Upper GI endoscopy and biopsies demonstrated minor gastric ulcerations

with Helicobacter-like spiral organisms, which stained positive with silver stain. The 16S rRNA PCR and sequencing, using previously published 16S rRNA based primers, confirmed infection with *Helicobacter suis*.[5] Because *H. pylori*, the human gastric pathogen, has been linked to thrombocytopenia in select patients, the monkey was treated with a *H. pylori* eradication antibiotic therapy consisting of clarithromycin 15 mg/kg PO BID and amoxicillin 20 mg/kg PO BID for 10 days, and omeprazole 0.6 mg/kg PO SID for 21 days.[6] Antibiotic treatment for the *H. suis* infection did not resolve the coagulopathy and the episodes of petechiae +/− ecchymoses recurred. Although there was negative silver staining on gastric tissue collected during necropsy, 16S rRNA revealed that this antibiotic treatment did not completely eradicate the *Helicobacter* infection.

Due to the macaque's previous research history of exposure to DENV and the association between dengue infection and thrombocytopenia, the macaque was evaluated for the presence of dengue virus and anti-dengue antibody.[7] Serum anti-dengue non-structural protein 1 (NS1) IgG antibodies were detected by a commercially-available assay (GRF Diagnostica, Jilin, China; IBL America, Minneapolis, MN) during all 4 different episodes that were tested (Figure 6A), though PCR testing of serum for DENV was negative (Zoologix Inc., Chatsworth, CA). The presence of anti-dengue NS1 serum antibodies in absence of active infection confirmed a positive DENV titer 8 years post-vaccination. Additionally, an in-house ELISA was performed to detect anti-NS1 IgG specific to different serotypes of DENV recombinant NS1 antigen (Native Antigen, UK). A specific assay for anti-DENV1 NS1 antigen was also performed, as the monkey was vaccinated and challenged with a DENV1 variant. We hypothesized that the monkey would be positive for anti-DENV1 NS1 and potentially anti-DENV2-4 NS1 antibodies, as anti-DENV antibodies have been shown to cross-react with other DENV serotypes.[8] Briefly, 96-well plates were pre-coated with 100ng/mL recombinant NS1 from DENV serotypes 1-4. The samples were then incubated in the wells for one h at 37°C, washed, and then incubated with 100ng/mL biotinylated goat anti-Rhesus IgG (H+L) (SouthernBiotech, Birmingham, AL) for one h at 37°C. The plate was then washed and read with a standard Horse radish peroxidase (HRP) - conjugated streptavidin protocol. The positive control was serum from previously confirmed DENV-1 infected Rhesus macaques (provided by Dr. I. Bosch), and the negative controls were age- and sex-matched macaques from the subject's colony. This ELISA confirmed the presence of anti-DENV1 and DENV4 NS1 antibodies (Figure 6B). Although anti-DENV1 NS1 antibodies were present during both normal and clinical episodes, anti-DENV4 NS1 antibodies appeared to be higher during episodes. This could suggest that the anti-DENV4 NS1 antibody was related to the clinical bleeding episodes, with the antibody being cross-reactive to a native antigen, such as platelet surface antigens.

Dengue virus infection has previously been associated with autoimmune thrombocytopenia.[9,10] To determine whether antibody- mediated platelet destruction was contributing to platelet decline during thrombocytopenia episodes, the auto-platelet antibody content of serum collected from the macaque during an episode was compared to normal controls. The presence of auto-platelet antibodies during one of the clinical episodes was detected with the PAK12G ELISA (Immucor Inc., Norcross, GA), while no auto-platelet antibodies were detected in the normal controls or during the normal clinical phase. The PAK12G ELISA is a commonly used assay in human medicine that has been validated for monkeys to detect the

presence of anti-platelet antibodies[11,12]; it has wells coated with many platelet antigens and their associated polymorphisms, including GPIIb/IIIa (HPA 1a/1a, 3a/3a, 4a; HPA 1b/1b, 3b/3b, 4a; HPA 1a/1a, 3b/3b, 4a), GPIa/IIa (HPA 5a/5a; HPA 5b/5b), GPIb/IX, and HLA. Briefly, plasma or serum is incubated in the wells coated with the platelet-antigens, the wells are then washed and anti-IgG antibodies are utilized to detect the presence of anti-platelet antibodies. The affected monkey did test positive for anti-GPIIb/IIIa (HPA-1a/1a, 3a/3a, 4a) platelet antibodies during the clinical episode tested. This finding suggests that there is a potential autoimmune component to this macaque's chronic recurring disease.

Over the next 3 years the macaque relapsed every 3-6 months; the clinical signs consisted of petechiae, ecchymoses, and thrombocytopenia. Although steroids and immunosuppressive therapy (prednisone, 1-2 mg/kg PO SID, and cyclosporine, 5 mg/kg PO SID) resulted in partial resolution of the cinical signs of the episodes, but did not prevent the episodes from recurring. Despite being on the therapies for up to 7 months at a time, a breakthrough clinical episode usually occurred within 3-6 months. Due to the poor prognosis, euthanasia was elected and performed. No significant pathologic findings were identified during necropsy with the exception of multi-focal ecchymoses on the trunk and inguinal region and mild left testicular enlargement. Terminal clinical pathology testing revealed a moderate peripheral thrombocytopenia (33×10^6 platelets/mL), and cytology of the bone marrow, based on a 500-cell count differential, showed an M:E ratio of 1.0:1.0. In the face of a marked peripheral thrombocytopenia, the megakaryocyte number was decreased with infrequent younger precursor stages and rare free megakaryocyte nuclei. The megakaryocyte morphology was unremarkable. The myelogram was interpreted as normal for the erythroid and the myeloid series, but with a megakaryocyte hypoplasia. Histopathology confirmed the persistence of a mild, multifocal, subacute, lymphoplasmacytic colitis. The testicular parenchyma appeared normal on histopathologic examination. Immunohistochemistry for the detection of flavivirus envelope antigen (sc-58128, Santa Cruz Biotechnology Inc., Dallas, TX) was performed at 1:100 for 1 hr at 37°C, on paraffin-embedded immune-privileged sites (the eye and testicle), as well as the bone marrow. Vero cells infected with dengue virus type 2 (DENV2) were used as positive controls. All samples were negative for flavivirus envelope antigen, indicating that there was no evidence of a current dengue virus infection. The clinical findings in this case were consistent with a tentative diagnosis of acquired, cyclic thrombocytopenia and platelet dysfunction – likely secondary to autoimmune platelet destruction, with reversible bone marrow involvement.

Discussion

Here we report a Rhesus macaque with adult-onset, chronic, cyclic potentially immune-mediated thrombocytopenia (ITP) with some associated platelet function deficits. Diagnosis of platelet-based bleeding diatheses is often complicated, with ITP often being a diagnosis of exclusion and identification of causative etiology frequently elusive. Reports of thrombocytopenia in macaques (*Macaca* spp.) are rare and characterization of the associated platelet pathologies challenging because of the need for highly specialized training and equipment, the lack of species-specific reagents, and the time-sensitive nature of platelet function testing. Immune-mediated thrombocytopenia has rarely been reported in the Rhesus macaque.[13,14] In this case, in- depth testing of the macaque's coagulation system and

platelet morphology and function revealed potential primary platelet pathology alongside the presence of auto-platelet antibodies.

Taken in combination, the functional and morphologic platelet abnormalities identified in this case are not consistent with any specific primary platelet disease. The lack of aggregation to all agonists during EIA testing along with a decrease of α-granules on electron microscopy imaging is consistent with a rare genetic abnormality called Gray Platelet Syndrome (GPS).[15] The lack of GPIX platelet surface receptors during clinical episodes was also a unique finding in this case. One differential diagnosis for decreased GPIX expression includes abnormal megakaryo- or thrombopoiesis, consistent with another rare genetic platelet disorder called Bernard Soulier's Syndrome (BSS).[16] Because this was an adult-onset case of chronic thrombocytopenia, the likelihood of a congenital etiology is highly unlikely. Another possible cause of these abnormal findings includes increased platelet activation prior to imaging and testing – this could occur either *in vitro* or *ex vivo*, during phlebotomy and sample processing; however, normal macaque controls run concurrently with the affected macaque's platelets did not display aberrant platelet activation.

It is important to take into account this macaque's research history, including both the experimental vaccination and live viral challenge with DENV. Dengue virus is a positive-stranded enveloped RNA flavivirus that is transmitted by *Aedes* mosquitoes.[17] It is endemic in sub-tropical and tropical climates, such as much of South America, Puerto Rico, the Caribbean Islands, Africa, and Southeast Asia, where the vector resides. Globally, the infection rate for this virus has been estimated to be > 50 million infected people per year, with the number of cases and geographic spread increasing presumably due to globalization and climate change, which can both lead to expansion of the viral vector, the *Aedes* mosquito.[18] Although DENV clinical infection is usually self-limiting and manifests with flu-like symptoms, some patients exhibit more severe clinical symptoms, resulting in Dengue hemorrhagic fever (DHF) or Dengue shock syndrome (DSS), which accounts for over 20,000 deaths per year.[19] The pathogenesis of DENV infection is under active investigation. Many hypotheses as to the cause of DHF and DSS have been proposed, including endothelial damage resulting in inflammation, complement activation, and increased vascular permeability, and bone-marrow suppression and auto-platelet antibodies, resulting in thrombocytopenia.[7,9,10,20]

The DENV vaccine development has made significant progress during the past 10 years, resulting in several vaccines entering clinical trials.[21] Importantly, the Rhesus macaque has been and continues to be one of the most commonly used models for DENV infection and vaccine development.[22] This macaque tested positive for anti-dengue NS1 antibodies 8 years post-vaccination and viral challenge. Nonstructural protein 1 antibodies have been shown to be cross-reactive with platelet surface antigens, and may contribute to thrombocytopenia and platelet dysfunction in DENV infection.[9,23] The virus has also inhibited megakaryopoiesis in mouse models of infection, resulting in thrombocytopenia.[24] An autoimmune mechanism has been speculated to play a role in the pathophysiology, and perhaps, a part of the chronic disease associated with DENV infection, including chronic ITP.[25,26] It is possible that in this case, ITP and the platelet pathology resulted from the development and persistence of the

anti-NS1 antibodies cross-reacting with platelets secondary to either vaccination or the DENV viral challenge.

Antibody-dependent enhancement (ADE) is currently one of the leading hypotheses on the pathogenesis of DHF and DSS. In ADE, preexisting anti-DENV antibodies from an initial dengue infection bind to the antigens of a subsequent dengue infection of a different virus serotype. The primary antibodies are incapable of neutralizing the new viral infection and, instead, the antibody-virus complexes bind to monocytes, allowing for increased efficiency of monocyte infection. This results in increased viral replication and a higher risk of a severe dengue infection, accompanied by DHF and DSS.[14] Although this monkey was not infected twice with 2 different dengue serotypes, the monkey was exposed to a DENV1 mutant and then challenged with the wild-type DENV1 virus. There is the possibility that the mutation in the DENV1 virus could make the 2 exposures sufficiently diverse antigenically to result in an antibody-dependent enhancement of platelet clearance. Although there is no definitive proof at this time of the anti-DENV IgG cross-reacting with platelets in this monkey, this possibility should be considered in future vaccine development and design of these vaccines for proposed clinical trials.

Additionally, long-term persistence of clinical symptoms and immunologic disorders in people has been documented subsequent to human DENV infection. Clinical manifestations include general malaise, arthropathy, palpitations, rash, diarrhea, vaginal bleeding, and gingivorrhagia.[27] Many times, symptoms of autoimmune disease are not limited to one organ. It is important to note that this macaque did have chronic lymphocytic-plasmacytic colitis, despite the clearance of the pathogenic *E. coli* infection. Additionally, an enlargement of the left testicle was detected during many of the episodes, although no abnormalities were detected on histopathology. Infection with DENV has been associated with acute scrotal edema in human patients, although this has not been reported as a chronic condition.[28,29]

In this case, a presumptive etio-pathologic basis for this chronic, cyclic platelet dysfunction is immune-mediated platelet destruction with reversible bone marrow involvement. Based on this macaque's history, the platelet auto-antibodies and associated platelet pathology may be associated with DENV NS1 antigen cross-reactive antibodies or it may be idiopathic; if it is associated with the DENV NS1, we have not determined whether it is secondary to the dengue vaccine or the viral challenge. Clinically, this immune-mediated condition may be controlled but not cured, and the etiology of the cyclic nature of this condition is not well understood. To our knowledge, this is the first reported case of chronic, cyclic, platelet-dysfunction of this type in the Rhesus macaque; and the first reported case of chronic platelet dysfunction after DENV vaccination and wild virus challenge in both the veterinary and human literature.

References

1. Didier ES, Sugimoto C, Bowers LC, Khan IA, Kuroda MJ. Immune correlates of aging in outdoor-housed captive rhesus macaques (*Macaca mulatta*). Immunity & ageing : I & A. 2012; 9(1):25. [PubMed: 23151307]

2. Lewis, JH. Comparative Hemostasis in Vertebrates. New York, NY: Plenum Press; 1996. The Primates; p. 154-166.
3. Meyers, KWJ. Platelets and Coagulation. In: SM, C., editor. Comparative Transfusion Medicine. San Diego, CA: Academic Press; 1991. p. 87-136.
4. Swennes AG, Buckley EM, Madden CM, et al. Enteropathogenic Escherichia coli prevalence in laboratory rabbits. Vet Microbiol. May 3; 2013 163(3–4):395–398. [PubMed: 23391439]
5. Esmail MY, Bacon R, Swennes AG, et al. Helicobacter Species Identified in Captive Sooty Mangabeys (Cercocebus atys) with Metastatic Gastric Adenocarcinoma. Helicobacter. Oct 19.2015
6. Frydman GH, Davis N, Beck PL, Fox JG. Helicobacter pylori Eradication in Patients with Immune Thrombocytopenic Purpura: A Review and the Role of Biogeography. Helicobacter. Aug; 2015 20(4):239–251. [PubMed: 25728540]
7. Zapata JC, Cox D, Salvato MS. The role of platelets in the pathogenesis of viral hemorrhagic fevers. PLoS Negl Trop Dis. Jun.2014 8(6):e2858. [PubMed: 24921924]
8. Falconar AK, Young PR. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1. The Journal of general virology. Apr; 1991 72(Pt 4):961–965. [PubMed: 1849979]
9. Cheng HJ, Lei HY, Lin CF, et al. Anti-dengue virus nonstructural protein 1 antibodies recognize protein disulfide isomerase on platelets and inhibit platelet aggregation. Mol Immunol. Dec; 2009 47(2–3):398–406. [PubMed: 19822367]
10. Lin CF, Lei HY, Liu CC, et al. Generation of IgM anti-platelet autoantibody in dengue patients. J Med Virol. Feb; 2001 63(2):143–149. [PubMed: 11170051]
11. Pate KAM, Lyons CE, Dorsey JL, et al. Platelet Activation and Platelet-Monocyte Aggregate Formation Contribute to Decreased Platelet Count During Acute Simian Immunodeficiency Virus Infection in Pig-tailed Macaques. J Infect Dis. Sep 15; 2013 208(6):874–883. [PubMed: 23852120]
12. Kuwata T, Nishimura Y, Whitted S, et al. Association of Progressive CD4(+) T Cell Decline in SIV Infection with the Induction of Autoreactive Antibodies. Plos Pathog. Apr.2009 5(4)
13. Halliday LC, Fortman JD. Severe thrombocytopenia in aged rhesus macaques (Macaca mulatta) infected with simian varicella virus. J Am Assoc Lab Anim Sci. Jan; 2011 50(1):109–113. [PubMed: 21333173]
14. Hanley PW, Baze WB, McArthur MJ, Bernacky BJ, Wilkerson GK, Barnhart KF. Acquired amegakaryocytic thrombocytopenia purpura in a Rhesus macaque (Macaca mulatta). Comp Med. Jun; 2012 62(3):229–233. [PubMed: 22776057]
15. Nurden AT, Nurden P. The gray platelet syndrome: clinical spectrum of the disease. Blood Rev. Jan; 2007 21(1):21–36. [PubMed: 16442192]
16. Lanza F. Bernard-Soulier syndrome (hemorrhagiparous thrombocytic dystrophy). Orphanet J Rare Dis. 2006; 1:46. [PubMed: 17109744]
17. Halstead SB. Dengue virus-mosquito interactions. Annu Rev Entomol. 2008; 53:273–291. [PubMed: 17803458]
18. Guzman MG, Halstead SB, Artsob H, et al. Dengue: a continuing global threat. Nat Rev Microbiol. Dec; 2010 8(12 Suppl):S7–16. [PubMed: 21079655]
19. Murray NE, Quam MB, Wilder-Smith A. Epidemiology of dengue: past, present and future prospects. Clin Epidemiol. 2013; 5:299–309. [PubMed: 23990732]
20. Avirutnan P, Hauhart RE, Somnuke P, Blom AM, Diamond MS, Atkinson JP. Binding of flavivirus nonstructural protein NS1 to C4b binding protein modulates complement activation. J Immunol. Jul 1; 2011 187(1):424–433. [PubMed: 21642539]
21. Wan SW, Lin CF, Wang S, et al. Current progress in dengue vaccines. J Biomed Sci. 2013; 20:37. [PubMed: 23758699]
22. Zompi S, Harris E. Animal models of dengue virus infection. Viruses. Jan; 2012 4(1):62–82. [PubMed: 22355452]
23. Chen MC, Lin CF, Lei HY, et al. Deletion of the C-terminal region of dengue virus nonstructural protein 1 (NS1) abolishes anti-NS1-mediated platelet dysfunction and bleeding tendency. J Immunol. Aug 1; 2009 183(3):1797–1803. [PubMed: 19592650]

24. Sridharan A, Chen Q, Tang KF, Ooi EE, Hibberd ML, Chen J. Inhibition of megakaryocyte development in the bone marrow underlies dengue virus-induced thrombocytopenia in humanized mice. J Virol. Nov; 2013 87(21):11648–11658. [PubMed: 23966397]

25. Morel Z, Ramirez A. Autoimmune response in children with dengue. Case reports. Reumatol Clin. Jul-Aug;2014 10(4):257–259. [PubMed: 24666813]

26. de Souza LJNC, Bastos DA, da Silva Siqueira EW, Ribeiro Noqueira RM, da Costa Carneiro R, Nogueira FPBD, Pereira LV, Gouveia TS. Dengue and Immune Thrombocytopenic Purpura. Dengue Bulletin. 2005; 29:6.

27. Garcia G, Gonzalez N, Perez AB, et al. Long-term persistence of clinical symptoms in dengue-infected persons and its association with immunological disorders. Int J Infect Dis. Jan; 2011 15(1):e38–43. [PubMed: 21112804]

28. Shamim M, Naqvi SZ. Dengue fever associated with acute scrotal oedema: two case reports. J Pak Med Assoc. Jun; 2011 61(6):601–603. [PubMed: 22204221]

29. Chen TC, Lu PL, Chen YH, Tsai JJ, Chen TP. Dengue hemorrhagic fever complicated with acute idiopathic scrotal edema and polyneuropathy. Am J Trop Med Hyg. Jan; 2008 78(1):8–10. [PubMed: 18187776]

30. Frydman GH, Bendapudi PK, Marini RP, Vanderburg CR, Tompkins RG, Fox JG. Coagulation Biomarkers in Healthy Chinese-Origin Rhesus Macaques (*Macaca mulatta*). J Am Assoc Lab Anim. May; 2016 55(3):252–259.

31. Patterson MM, Jackson LR, Brooks MB, Catalfamo JL. Type-3 von Willebrand's disease in a rhesus monkey (*Macaca mulatta*). Comparative Med. Aug; 2002 52(4):368–371.

32. IDEXX. IDEXX Reference Laboratories Manual. Grafton; MA: 2013.

33. Quest Diagnostics. Quest Diagnostics Reference Ranges. Madisone; NJ:

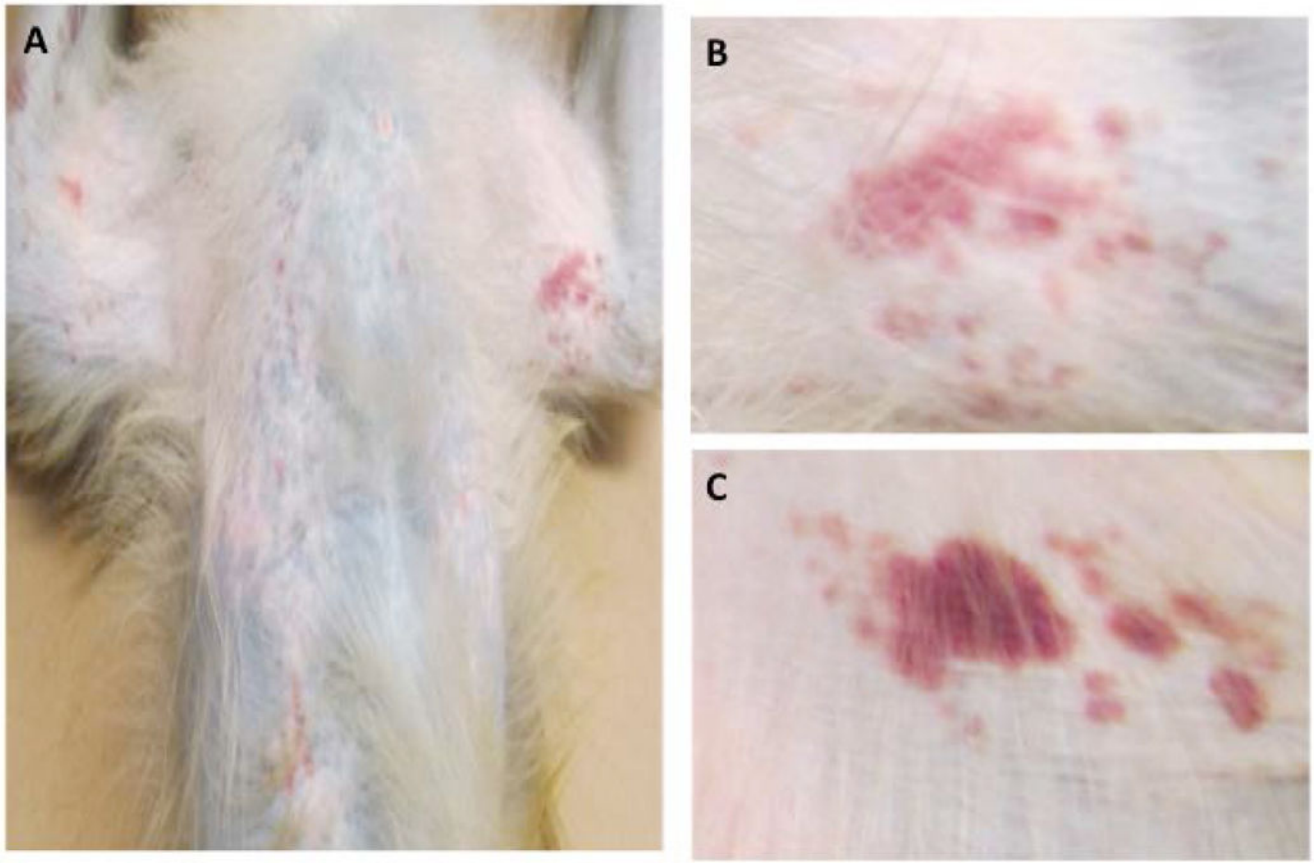


Figure 1.

Photographs of a macaque with multiple recurring thrompocytopenic and thrombopathic episodes displaying diffuse petechiae and ecchymoses on the trunk and forelimbs (A). Close-ups of the lesions on the medial aspect of the left brachium (B) and the medial aspect of the right inguinal region (C).

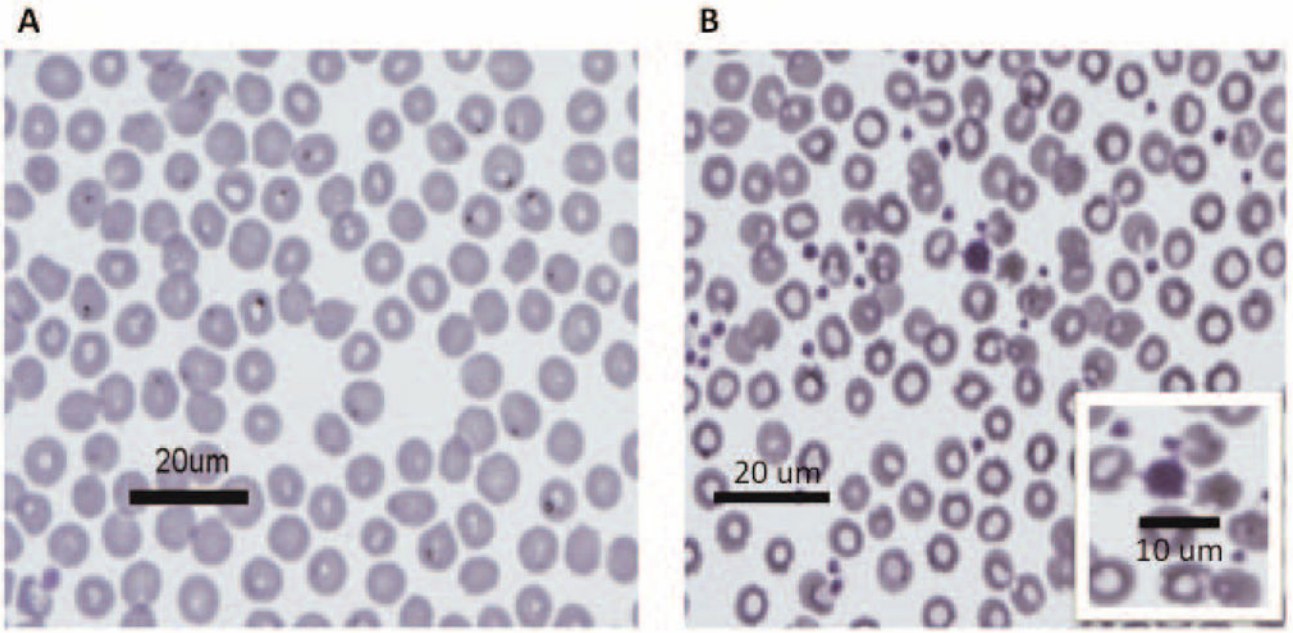


Figure 2.

Platelet morphology analysis of a macaque during a thrombocytopenic and -pathic episode. Peripheral blood smear during thrombocytopenic episode showing lack of platelets (20×) (A), and 3 weeks after episode showing a rebound thrombocytosis (20×) (B), and a giant platelet (40×) (B, insert).

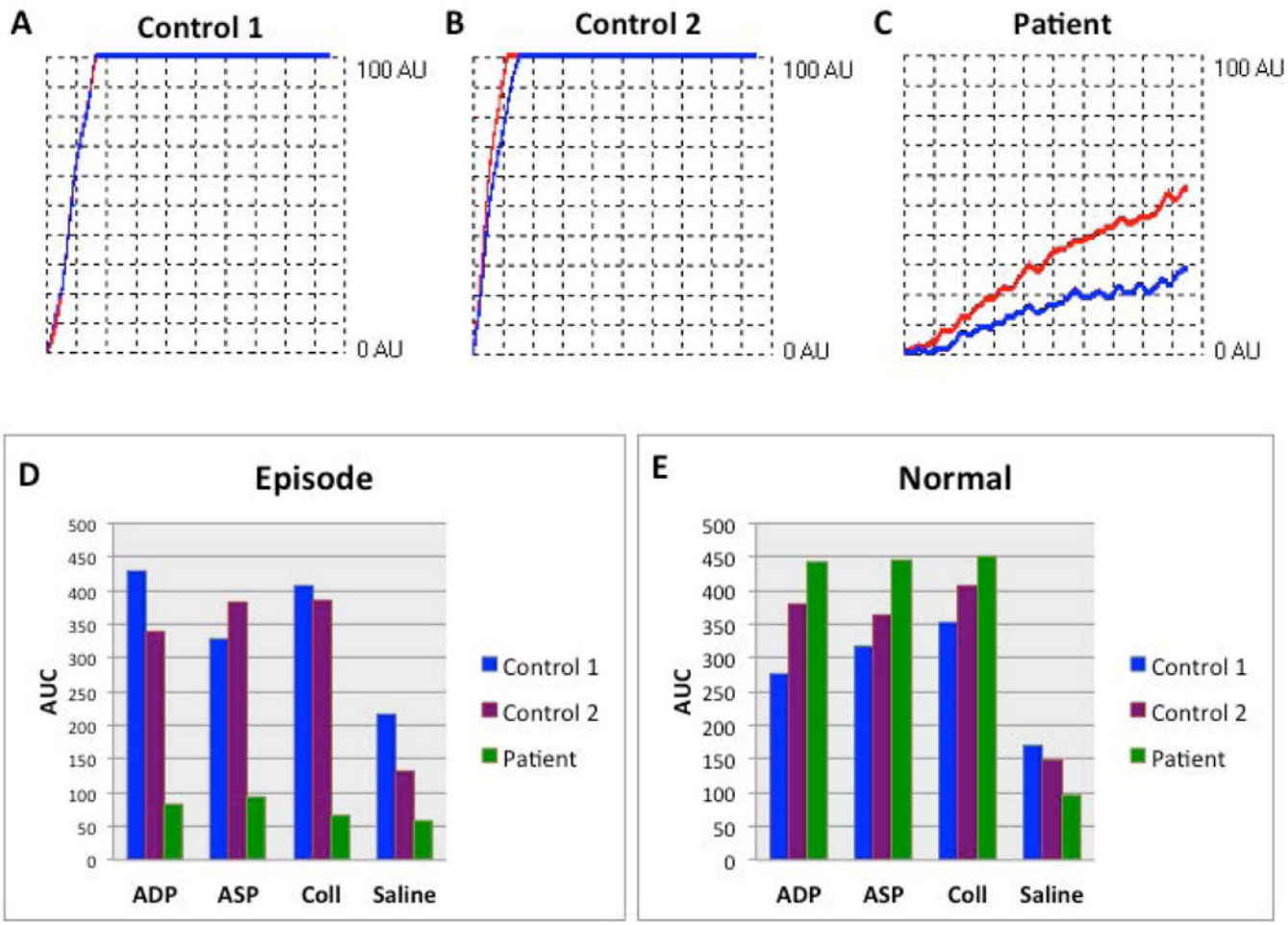
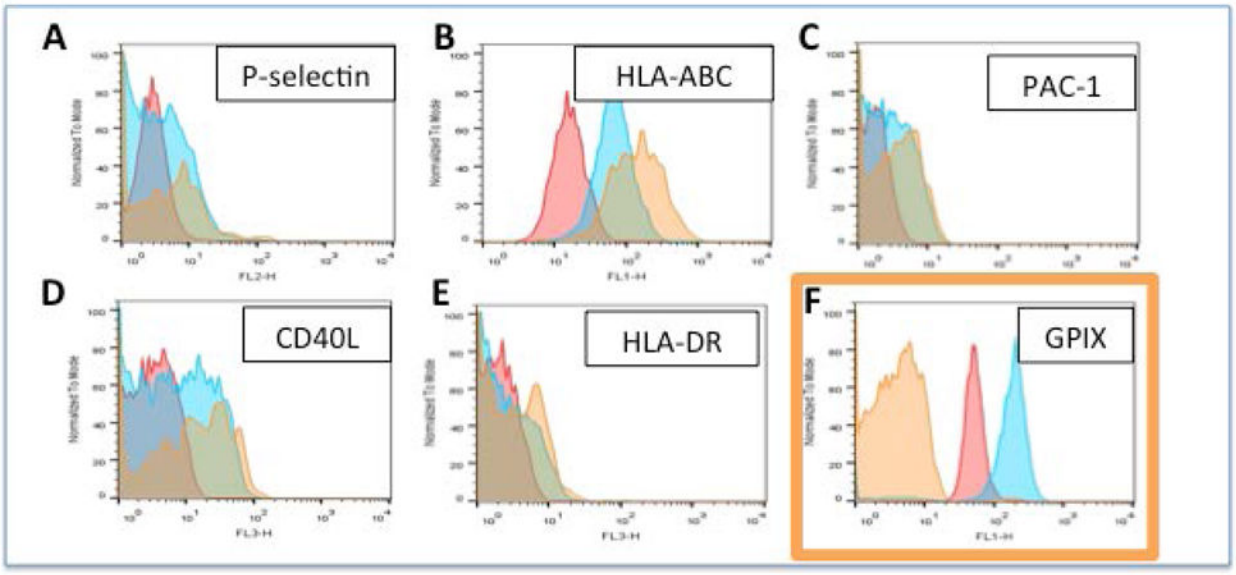


Figure 3.

Electrical impedance aggregometry (EIA) of platelets from a macaque with multiple recurring thrompocytopenic and thrombopathic episodes. Representative aggregometry response tracings to stimulation by ADP of 2 normal control macaques (A and B) and the affected macaque (C) during an episode. The red and blue lines in figures A–C represent dual electrode measurements and are internal duplicates. Bar graphs of area under the curve (AUC) for EIA responses to various agonists during an episode (D) show that the thrombocytes of the affected macaque exhibit a decreased response to all agonists. Bar graphs of AUC for EIA responses to various agonists during a clinically normal phase (E) show a normal to increased response to all agonists (adenosine-5′-diphosphate [ADP, 2 μM]; arachidonic acid [ASP, 50 μg/mL]; and soluble calf skin Collagen Type I[Coll,190 μg/mL]). Saline was used as a negative control for establishment of baseline activation.



Color	Clinical Signs	Platelet Count (x10⁶/mL)
Red	No	348
Blue	Yes	181
Yellow	Yes	12

Figure 4.

Representative images of platelet flow cytometry of various surface receptors in thrombocytes of a macaque with multiple recurring thrompocytopenic and thrombopathic episodes. The chart below shows the color-coding of the flow cytometry graphs and the presence of clinical signs and the coinciding platelets count. (A, P-selectin; B, HLA-ABC; C, PAC-1; D, CD40L; E, HLA-DR; F, GPIX). These graphs demonstrate a progressive increase in surface protein receptor expression of HLA- ABC activation coinciding with the presence of clinical signs and a decreasing platelet count (A-E), while GPIX receptor decreased during the clinical episode (F).

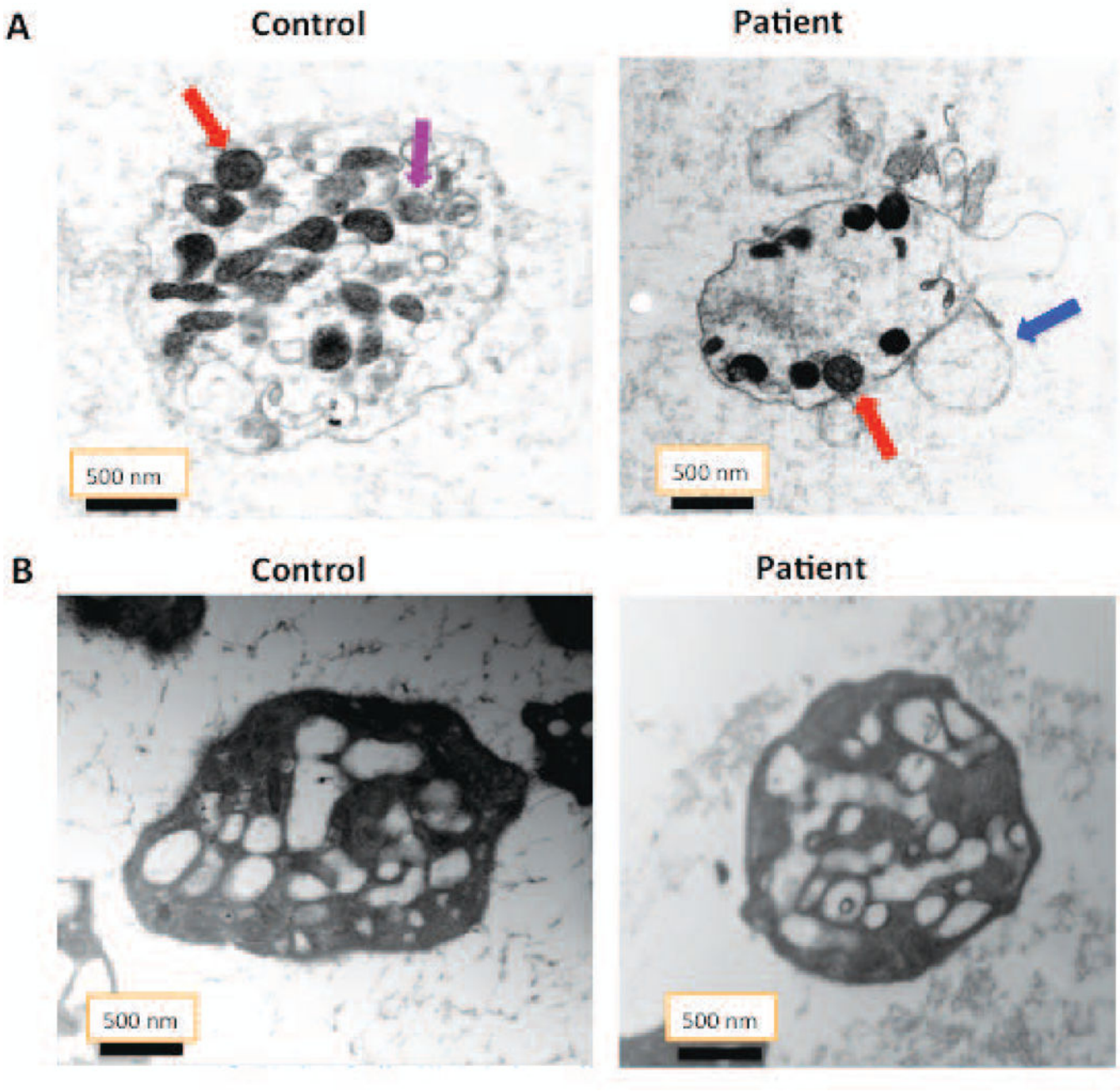


Figure 5.

Electron microscopy images of thrombocytes of a macaque with multiple recurring thrompocytopenic and thrombopathic episodes during a clinical episode (D) and a normal clinical phase (E). The control is on the left panel and the affected monkey on the right panel. Blue arrow, dilated open canalicular system; red arrow, dense granule; purple arrow, alpha granule.

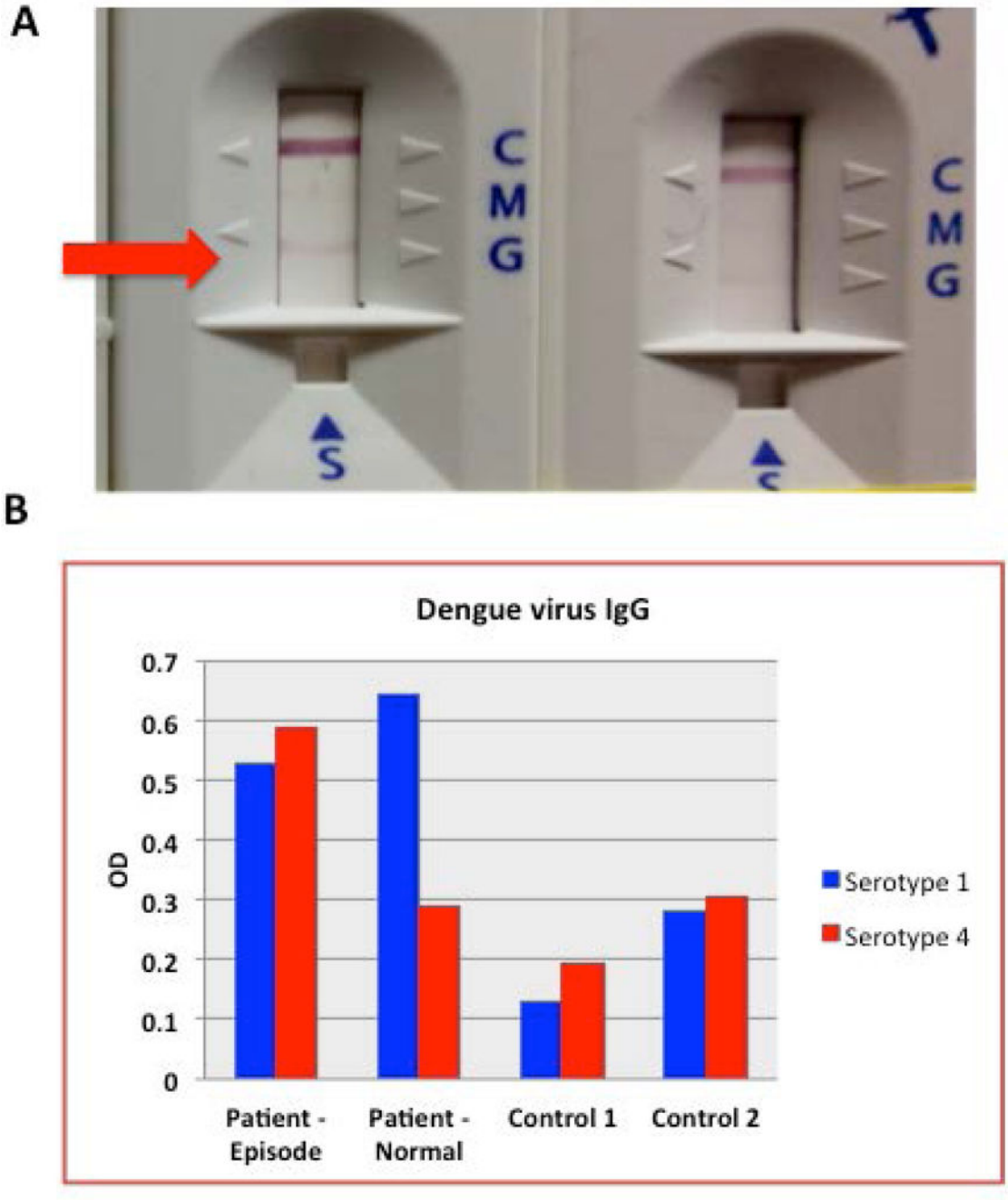


Figure 6.

Anti-dengue virus non-structural protein 1 specific-Elisa results with recombinant antigen from 4 different dengue virus (DENV) serotypes in the serum of the affected macaque during 2 thrombocytopenic episodes (slightly positive IgG titer, red arrow) and a normal clinical phase. Data indicate the presence of anti-DENV1 NS1 and anti-DENV4 NS1 IgG (B), but not anti-DENV2 or DENV3. The control macaques were negative.

Table 1

Coagulation profile in a macaque vaccinated against and challenged with Dengue virus, and exhibiting clinical episodes of chronic recurring thrombocytopenia and thrombopathy.

Coagulation Test	Published Reference Range	Episode 1	Episode 2	Episode 3
Platelet count[30] (× 10^6/mL)	289-475	15	11	126
PT (sec)[31]	11.3-13.0	15.2	13.0	12.4
APTT (sec)[31]	20.2-23.8	26.0	22.3	26.7
Fibrinogen (mg/dL)[32]	100-300		542	241
LDH (IU/L)[32]	173-275			258
vWF Collagen	45–198%			129%
Binding Assay[33]	>0.5			0.84
vWF Antigen[33]	55-200%			108%

vWF indicates van Willebrand factor

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