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Case Report

Clinical *Trypanosoma cruzi* Disease after Cardiac Transplantation in a Cynomolgus Macaque (*Macaca fascicularis*)

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A cynomolgus macaque received a heterotopic cardiac allograft as part of a transplant study, with monoclonal antibodies targeted to specific immune costimulation molecules (CD154, CD28) but no traditional immunosuppressive therapy after surgery. Clinical anemia was detected on postoperative day (POD) 35 and had worsened (Hgb, 2.3 g/dL; Hct = 7.3%) by POD 47, despite type-matched whole-blood transfusions. After a total of 4 blood transfusions, hematologic parameters were improved (Hgb, 5.9 g/dL; Hct, 18.7%). On POD 50, a peripheral blood smear revealed trypomastigotes, and qualitative RT-PCR of whole blood identified the organism as *Trypanosoma cruzi*. Although clinically stable initially, the macaque soon developed sufficient weight loss to necessitate euthanasia on POD 64. The final diagnosis was clinical anemia due to *T. cruzi* infection. This study represents the first reported case of Chagas disease after heart transplant in a NHP.

Abbreviation: POD, postoperative day

The World Health Organization recognizes Chagas disease, which is caused by the kinetoplastid protozoan *Trypanosoma cruzi*, as "one of the world's 10 most neglected tropical diseases."[18] Primarily seen in Central and South America, *T. cruzi* is now recognized as endemic in various areas of the southern United States.[5] Transmission usually occurs through the bite of hematophagous triatomine bugs but can also occur through the oral route,[5] due to blood transfusion[4] or organ transplantation,[3,8] and through vertical transmission.[9]

Clinical signs of Chagas vary between the acute and chronic phases of disease. During the acute phase, symptoms can include swelling at the inoculation site, lymphoadenopathy, fever, myalgia, and rash. More rare severe acute disease can include meningoencephalitis,[12] acute myocarditis, and pericardial effusion.[15] In humans, the chronic phase usually occurs 10 to 30 years after the initial infection in untreated patients.[13] Symptoms include anemia; cardiomyopathy involving cardiac insufficiency, myocarditis, or cardiac arrhythmia; and digestive disease such as megacolon and megaesophagus.[18]

Case Report

History. A 5-y-old, male, research-naïve cynomolgus macaque (*Macaca fascicularis*) was enrolled in a cardiac allograft transplantation study under a protocol approved by the IACUC at the University of Maryland School of Medicine (Baltimore, MD). This macaque originated from a facility in the southern United States, where he was born, raised, and housed in indoor–outdoor group housing. The dam's origin was Chinese; sire's identity and origin were unknown. The heart donor was a 6-y-old, male cynomolgus macaque that was born and raised in China prior to importation into the United States; his parents were born and raised in China.

Upon arrival, both macaques underwent standard facility quarantine for 13 wk. During quarantine, each animal had a total of 6 consecutive negative tuberculosis tests at 2-wk intervals as well as 2 consecutive negative examinations for intestinal parasites and pathogenic intestinal bacteria (*Salmonella* spp., *Shigella* spp, *Campyolbacter* spp., and *Yersinia* spp.). After week 13, the macaques were released from quarantine for use by the investigator. For the duration of their stay at the School of Medicine, the animals were housed indoors and maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* [10] in our AAALAC-accredited facility. All procedures were approved by the IACUC of the University of Maryland School of Medicine (protocol #1013002).

The recipient macaque underwent surgical transplantation of a heterotopic cardiac allograft. Both the donor and the recipient macaques received heparin (200 IU/kg) intravenously prior to graft removal and implantation. Briefly, the donor heart was harvested by transection of the great vessels and by cutting the left atrial wall along the entrance of the pulmonary veins. The left atrium was closed after creation of an atrial septal defect by fossa ovalis resection. The donor heart was implanted into the recipient's abdomen by connecting the donor aorta to the recipient's infrarenal

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aorta and the donor pulmonary artery to the recipient's infrarenal vena cava. The recipient macaque recovered normally from surgery and received an immunosuppressive regimen comprising costimulation blockade with monoclonal antibodies specific for human CD154 (hu5c8 mouse–human chimeric antibody [recombinant], NHP Reagent Resource, Worcester, MA) and human CD28 (FR104, monovalent PEGylated Fab antibody antagonist, Effimune, Nantes, France).

The recipient macaque was doing well until postoperative day (POD) 35, when CBC analysis revealed mild anemia (Table 1). Subsequent CBC panels showed progression of the anemia, and type-matched whole blood transfusions were started on POD 47. The macaque was bright and alert at this time. A total of 4 blood transfusions on POD 47, 49, 50, and 56 were provided for clinical support according to the approved IACUC protocol. After these 4 blood transfusions, the animal's anemia showed mild regeneration as evidenced by anisocytosis and reticulocytosis on blood smears. Throughout this time, the animal was bright initially but became progressively lethargic and weak. Because the macaque's weight subsequently dropped below the euthanasia criteria of 20% weight loss, he was euthanized on POD 64. The transplanted graft had normal function at the time of euthanasia. A necropsy was performed, and tissues were sent for histopathology.

Hematology and PCR testing. Immune monitoring of the recipient included standard hematologic and flow cytometric analysis of peripheral blood samples at regular intervals. Absolute cell counts with differentials were obtained (Antech Diagnostics, Irvine, CA). In addition, blood samples (50 µL) were mixed with the fluorescently labeled antibodies CD45–PerCP (clone D058-1283), CD2–FITC (clone RPA-2.10), CD3–BV450 (clone SP34-2), and CD20–PE (clone 2H7; all labeled antibodies were obtained from BD Biosciences, San Jose, CA) in specialized tubes (BD Trucount, BD Biosciences). The absolute count of each cell population was calculated as the number of positive cell events divided by the number of bead events and then multiplied by the bead concentration. For phenotypic analysis, 50 µL of blood was stained with CD3–BV450 (clone SP34-2, BD Biosciences, San Jose, CA), CD4–BV500 (clone L200, BD Biosciences, San Jose, CA), or CD8–PerCP (clone SK1, BD Biosciences Pharmingen, San Diego, CA), and the CD4:CD8 ratio of the $CD3^+$ population was calculated. All samples were evaluated by flow cytometry (Verse, BD Biosciences), and results were analyzed by using Flowjo (version 0.6; Tree Star, Ashland OR).

Because of the macaque's persistent anemia and our previous experience with simian parvovirus as a cause of posttransplantation anemia,[19] POD 50 blood was submitted for RT-PCR testing for simian parvovirus (Zoologix, Chatsworth, CA); the assay results were negative for this virus. In these studies, mild anemia is common and routinely addressed with iron dextran injections, but this animal's anemia was persistent and not responsive to transfusion. In addition, after the second blood transfusion, the reference laboratory measuring CBC values noted few to moderate numbers of trypomastigotes (most closely resembling *T. cruzi*) on blood smears (Figure 1). At this time, whole blood sent for RT-PCR testing was positive for *T, cruzi*.

In an attempt to identify the source of the *T. cruzi*, whole-blood samples from all colony animals that were transfusion donors for the anemic transplant recipient were tested by RT-PCR (Zoologix) for the presence of the organism. In addition, samples from the organ donor, including archived serum, plasma, and snap-frozen tissue (colon, kidney, spleen, liver) were sent for testing. All samples from blood donors and from the organ donor were negative for *T. cruzi* by RT-PCR.

Pathologic findings. Spleen, lymph node and esophageal samples from the recipient animal were free of noteworthy gross and histologic lesions. On necropsy, multiple firm, nodular lung lesions were noted that were approximately 0.5 to 1 cm in diameter with circumferential erythema. These lesions corresponded to focal areas of moderate to severe chronic pneumonia on histopathology. All other tissues were grossly normal on necropsy. Mild myeloid hyperplasia of the bone marrow, consistent with a mildly regenerative anemia, was present. The liver demonstrated evidence of mild chronic hepatitis, cholestasis, and hemosiderosis. The colon and skeletal muscle showed histologic inflammation, presenting with chronic serositis and with chronic myositis and cellulitis, respectively. Samples of the native heart had moderate myocarditis with lymphocytic and monocytic infiltrates. The most striking finding involved the transplanted heart, which demonstrated severe myocarditis and epicarditis with moderate fibrosis, and numerous protozoa consistent with *T. cruzi* in multiple tissue sections (Figure 2).

A complete profile of blood cell populations is shown in Figure 3. WBC, neutrophil, and lymphocyte counts remained quite stable throughout the posttransplantation and infection periods. The CD4/CD8 ratio decreased, probably as a result of the alloimmune response. However, blood eosinophils increased (2- to 3-fold) during the weeks after transplantation, suddenly decreased on POD 35, and then remained very low until euthanasia.

Discussion

The literature contains several reports of asymptomatic (but serologic or PCR-positive) *T. cruzi* in NHP. Reports of symptomatic clinical disease include sudden death in a chimpanzee (*P. troglodytes*),[6] encephalitis in a Celebes macaque (*M. nigra*),[15] natural Chagas infection in baboons,[20] and reactivation of *T. cruzi* after experimental infection with SIV.[11] What makes the presented case unique is that this animal succumbed to *T. cruzi* after treatment with an alternative immunosuppressive regimen in which no glucocorticoids or calcineurin inhibitors were used. Specifically, costimulation blockade consists of the selective inhibition of receptor–ligand interactions involved in the activation of T and B cells. The CD28–B7 and CD40–CD154 receptor–ligand pairs play major roles in the initiation of immune responses, and inhibition of one or both of these pathways (by using antibodies or fusion proteins) is associated with significantly decreased responses to pathogens, vaccines, and transplant antigens.[2,16]

Although we were unable to determine the source of the disease, 2 scenarios might explain the occurrence. The most likely case is that the donor animal was the source of the *T. cruzi*. Given that histopathologic changes were present only in the donor heart, the immunomodulation provided by costimulation blockade likely reactivated latent disease in the transplanted heart, which then shed organisms that were seen on peripheral blood smears and ultimately led to widespread Chagas disease. The other possibility is that the recipient animal was actually previously infected with *T. cruzi* but was asymptomatic, such that immunotherapy led to reactivation of the disease and clinical Chagas disease.

Table 1. Results of CBC analyses

POD	Hgb (g/dL)	Hct (%)	WBC ($\times 10^3$ cells/μL)	RBC ($\times 10^6$ cells/mL)	Neutrophils (cells/μL)	Lymphocytes (cells/μL)	Eosinophils (cells/μL)	Platelets ($\times 10^3$ cells/μL)
35	9.0	24.4	5.3	3.82	2968	2067	53	849
47	2.3	7.3	4.6	0.96	1380	2898	0	859
50	5.6	17.8	9.0	2.27	3780	4860	180	385
54	5.9	18.7	8.3	2.26	3071	4814	0	451
56	4.5	14.1	11	1.90	—	—	0	—
64	5.1	14.4	9.9	1.77	2871	6237	0	903

POD, postoperative day
All CBC results are from ANTECH Diagnostics (Irvine, CA), except for those on POD 56, which were obtained inhouse.
Normal reference ranges: Hgb, 9.6–13.3 g/dL; Hct, 24%–41%; WBC, 4.5–18.3 $\times 10^3$ cells/μL; RBC, 3.5–6.9 $\times 10^6$ cells/mL; neutrophils, 4800–12,000 cells/μL; lymphocytes, 999–10,551 cells/μL; eosinophils, (80–800 cells/μL)

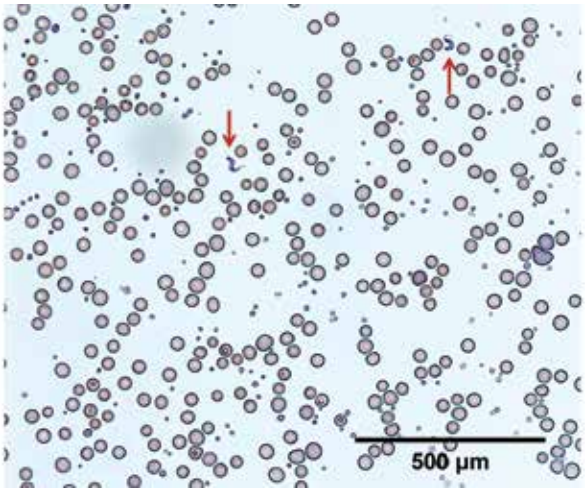

Figure 1. This peripheral blood smear from POD 50 contains trypomastigotes (red arrows), with anisocytosis and polychromasia due to the poorly regenerative anemia

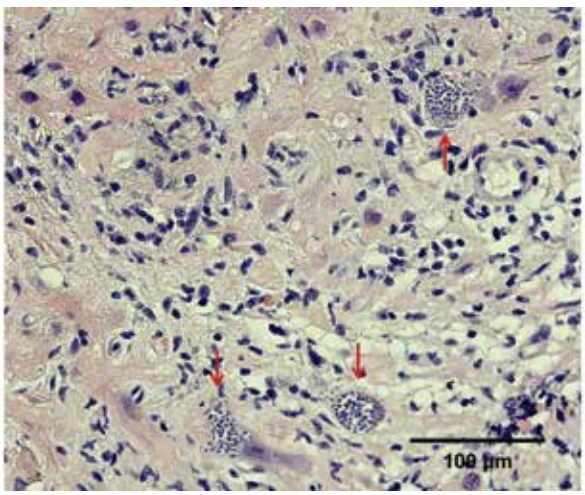

Figure 2. Transplanted heart, right ventricle. Clusters of protozoa, consistent with *T. cruzi* (red arrows), severe myocarditis, and fibrosis are present.

Although pharmacologic therapies for *T. cruzi* infections in humans exist, few resources for treatment of NHP are available. Medications used to treat humans include benznidazole, which has the highest efficacy, and nifurtinox.[18] Both of these drugs can have serious side effects, are not fully effective, and are only available through the Centers for Disease Control and Prevention in the United States. In humans, these drugs are used only during acute infection (which is usually not recognized) or reactivation; other than organ transplant in the face of organ failure, there is no specific treatment for the end-organ damage caused by infection. Drug unavailability and humane experimental endpoints precluded treatment of this animal after diagnosis. Because treatment is usually prolonged (60 to 90 d) and is most effective during acute or reactivated phases, therapy is not a viable option in NHP used for research. Finally, the efficacy of treatment against *T. cruzi* has not been validated in the context of immunosuppression[7]. Therefore, careful screening is the key to prevent use of NHP that may have been infected with this organism.

Routine screening testing should be performed on all NHP entering research facilities when the animals have originated from a geographic location where Chagas is known to be endemic. This is particularly true of NHP that will be used in cardiac, gastrointestinal, or immunosuppressive studies, such as those involving transplantation or myelosuppressive irradiation. Testing methods for *T. cruzi* detection include RT-PCR, dipstick assay (ELISA, rapid immunochromotographic strip assay), and peripheral blood smears. Although PCR testing is typically considered the 'gold standard,'[14] even this method can miss animals that have been infected with *T. cruzi*, especially chronically ill animals. In a recent study, 23% of macaques that were IgG ELISA-positive tested negative by RT-PCR, and of these animals, 70% had previously been positive for *T. cruzi* by PCR assay.[17] Given the inconsistency of these tests, multimodal testing by PCR analysis and ELISA and multiple testing of quarantined animals may be the most accurate method for detecting prior or active infection with this organism. Because of the associated cost, we recommend this approach only for animals from endemic areas and their first-generation offspring and when *T. cruzi* would likely have a significant effect on the study to be performed.

Eosinophils play an important role in fighting parasitic infections, when their numbers first increase and then decrease due to migration from peripheral blood to the site infected by the parasite.[13] In the animal we describe here, eosinophil numbers were increased during the first weeks after transplantation and then decreased abruptly on POD 35. In control transplant recipients treated with the same regimen, eosinophil numbers either remained stable or typically increased during the weeks after transplantation. This increase might reflect the inhibition

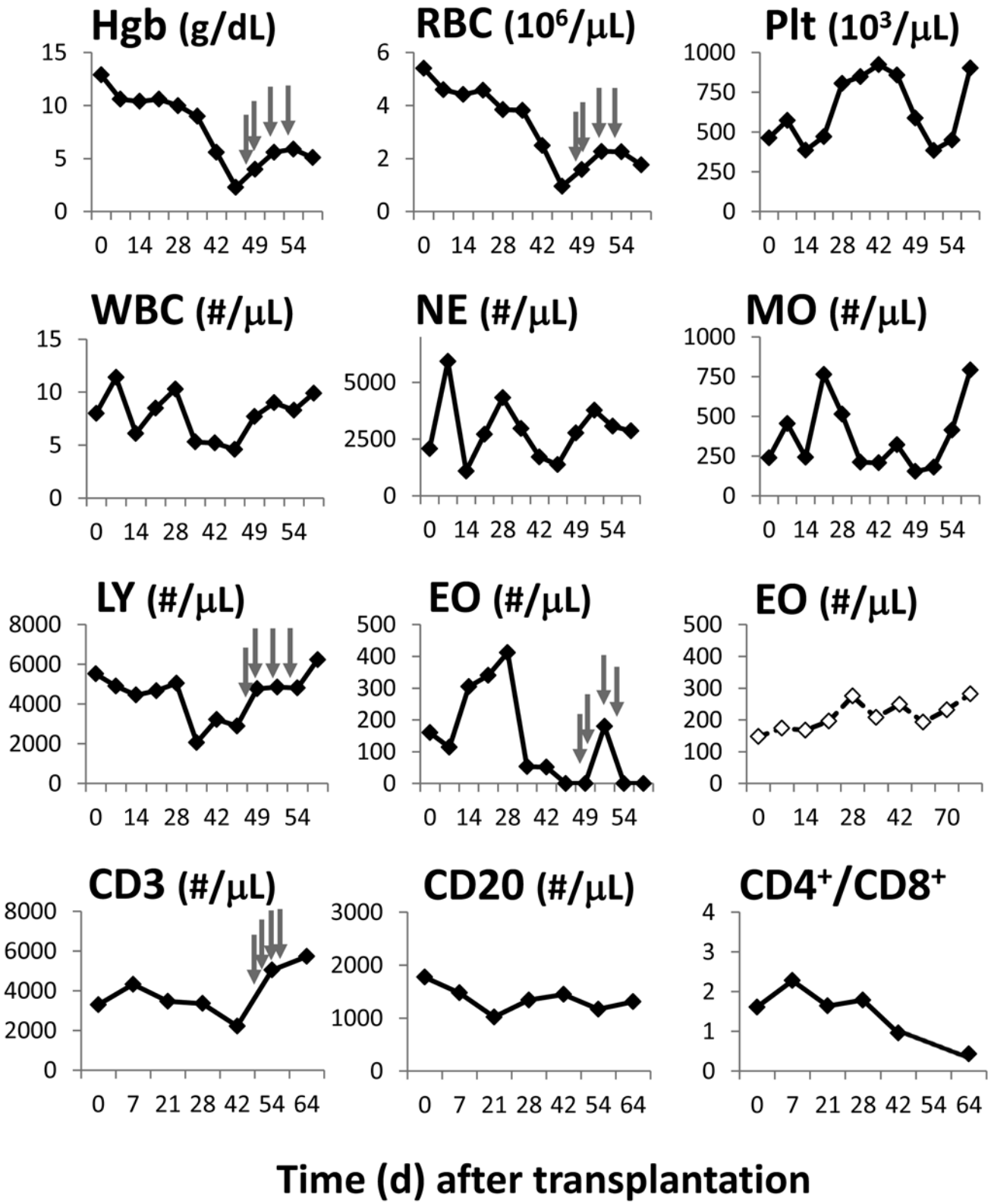

Figure 3. Hematology and flow cytometric analysis. Solid symbols, *T. cruzi*-infected recipient; open symbols, average eosinophil counts from 5 control transplant recipients treated with the same regimen. Gray arrows indicate blood transfusions. Plt, platelets; NE, neutrophils, MO, monocytes; LY, lymphocytes; EO, eosinophils; $CD3^+$, T cells; $CD20^+$, B cells.

of Th1 responses by costimulation blockade-based immunomodulation, allowing ongoing alloimmune responses to favor an immune shift toward Th2 responses. Therefore, the initial increase in eosinophils in our macaque may be related to the immunosuppression and transplantation events rather than to the parasitic infection. However, uninfected control animals did not demonstrate a decrease in eosinophil counts (Figure 3, open symbols). Therefore we infer that the dramatic decrease in peripheral blood eosinophils might be an early marker of infection with *T. cruzi* (or other parasites), and we therefore recommend initiating specific diagnostic tests in response, especially when this observation is combined with decreased RBC numbers and anemia in NHP.

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