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Platelet decline: An early predictive hematologic marker of simian immunodeficiency virus central nervous system disease

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As the prevalence of human immunodeficiency virus (HIV)-induced central nervous system (CNS) disease has increased with antiretroviral treatment, there is a critical need for identifying biomarkers that predict HIV CNS disease. To identify novel hematologic markers that precede and predict CNS disease, the authors examined longitudinal hematology data from 47 simian immunodeficiency virus (SIV)-infected macaques. This study demonstrated that the magnitude of decline in circulating platelet counts beginning at day 28 post infection, during asymptomatic SIV infection, predicted the eventual development of SIV encephalitis. Univariate analysis performed on platelet values obtained day 56 post inoculation demonstrated that SIV-infected macaques with the greatest decline in platelet numbers were 18 times more likely to develop SIV CNS disease than SIV-infected animals with minimal to no decline in circulating platelet counts. Decline in platelet number was a more robust marker than decline in hemoglobin levels, a previously identified marker of HIV CNS disease. The identification of an association between decline in platelets and the development of encephalitis demonstrates that monitoring platelet decline in HIV-infected individuals may serve as a predictive marker for clinical progression to HIV-induced CNS disease. Identifying those HIV-infected individuals at risk for CNS disease during asymptomatic stages of infection would promote early interventive, neuroprotective therapy to prevent neuronal damage and loss. *Journal of NeuroVirology* (2006) **12,** 25–33.

Keywords: encephalitis; hemoglobin; HIV; marker; platelet; SIV

Introduction

Acquired immunodeficiency syndrome (AIDS)-related neurologic disease is a devastating consequence of human immunodeficiency virus (HIV) infection. Although the incidence of HIV dementia has declined with the advent of highly active antiretroviral therapy (HAART), the resulting longer life span of treated HIV-infected individuals has paradoxically resulted in an increased overall prevalence of HIV-related central nervous system (CNS) complications (McArthur *et al*, 2003). Thus, as HIV-induced CNS disease remains common, there is a critical need for identifying biomarkers that predict HIV CNS disease. Although predictive cerebrospinal fluid (CSF) markers including monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, and CSF viral load have been reported in HIV-infected individuals and in simian immunodeficiency virus (SIV)-infected macaques, the identification of robust hematologic markers would reduce the need for obtaining serial CSF samples and could aid in elucidating pathogenic mechanisms common to both CNS and the periphery (Chang *et al*, 2004; Mankowski *et al*, 2004; Zink *et al*,

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1999, 2001). The objective of this study was to identify prognostic markers measured by routine hematology evaluation that predicted onset of CNS disease in a SIV macaque model of HIV CNS disease.

SIV infection of macaques is a well-established model that closely resembles HIV infection in humans, with development of parallel systemic and CNS disease outcomes (Letvin and King, 1990; Murray *et al*, 1992). Infection of pig-tailed macaques with a neurovirulent molecularly cloned virus, SIV/17E-Fr, and a virus swarm, SIV/DeltaB670, provides an accelerated and consistent model of HIV CNS disease (Mankowski *et al*, 2002; Zink *et al*, 1998). Examination of prognostic markers in a well-defined experimental animal model using standardized viral inoculums eliminates many potentially confounding factors including lifestyle choices, substance abuse, and variable antiretroviral treatment regimens.

Epidemiologic studies in humans have suggested that a decline in hemoglobin levels in asymptomatic HIV-infected individuals prior to development of AIDS is the most significant hematologic predictor of HIV-associated dementia; however, the basis for this relationship remains to be determined (McArthur *et al*, 1993). To identify novel hematologic biomarkers in a well-characterized SIV model, we examined clinical hematology data obtained longitudinally from 47 SIV-inoculated pig-tailed macaques, including 27 animals with SIV encephalitis and 20 infected animals with mild to no encephalitis. In addition to hemoglobin levels, we also examined numbers of circulating monocytes and platelets, both members of the bone marrow derived CD34+ cell lineage, to determine whether alterations in multiple CD34+ cell lineages were associated with HIV CNS disease.

Although platelet decline is a common hematologic finding in HIV infection, platelet decline has not been evaluated as a surrogate marker of HIV CNS disease. Thrombocytopenia is a common hematologic complication of HIV, occurring in 10% of HIV-infected individuals and in about a third of patients with AIDS (Scaradavou, 2002). SIV rhesus macaques similarly develop thrombocytopenia which affects 30% to 50% of infected animals (Dittmer *et al*, 1994). This study examined the hypothesis that alterations in levels of platelets during the asymptomatic phase of infection predicted later onset of SIV CNS disease. Identification of such predictive biomarkers in blood samples as an alternative to more invasive sampling, such as obtaining CSF, would contribute to HIV patient management and a better understanding of the pathogenesis of HIV CNS disease.

Results

SIV disease progression

Of the 47 macaques inoculated with SIV examined in this study, 27 (57%) developed moderate to severe lesions typical of SIV encephalitis whereas 20 animals (43%) had either mild or no CNS lesions. For analysis, animals were thus segregated into two groups based on CNS disease outcome: those with SIV CNS disease ($n = 27$) or those without SIV CNS disease ($n = 20$). Our previous studies have documented the value of segregating SIV-infected macaques into similar groups for analysis to identify predictive CSF markers (Mankowski *et al*, 2004; Zink *et al*, 2001). An additional group of uninfected macaques ($n = 9$) served as procedural controls. Longitudinal measurements of hematologic parameters were compared between the two groups of infected animals.

Hemoglobin levels

The mean change in hemoglobin levels in all SIV infected animals initially declined during acute infection (days 7 to 14 post infection [p.i.]; Figure 1). Levels recovered slightly between days 21 to 42 p.i. and then declined gradually until day 84 p.i. However, beginning at day 14 p.i., the group of macaques with SIV CNS disease consistently had greater mean decline in hemoglobin levels than the group without CNS disease, with statistically significant differences at days 56 and 70 p.i. ($P = .003$ and .008, respectively). Uninfected control animals demonstrated a stable trend in hemoglobin levels that remained near baseline from day 7 until day 84 p.i.

Monocyte levels

Increases in circulating monocytes have been detected before onset of neurologic symptoms in children infected with HIV-1 (Sanchez-Ramon *et al*, 2003). In addition, SIV-infected, CD8+-depleted rhesus macaques showed an early rise in the $CD14^{lo}\,CD16^{hi}$ monocyte subset corresponding temporally to detection of virus in the CNS (Kim *et al*, 2003). To determine whether alterations in levels of

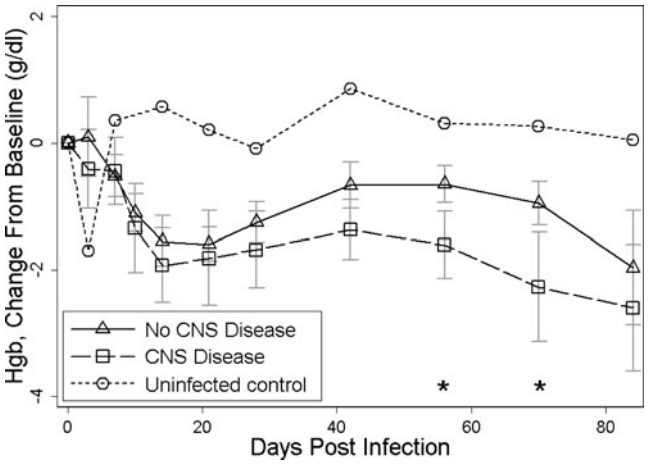

Figure 1 Mean decline from baseline of hemoglobin levels in SIV infected macaques (CNS disease and no CNS disease) and uninfected controls. Asterisks represent Wilcoxon rank-sum–based $P < .05$. Error bars represent 95% confidence intervals.

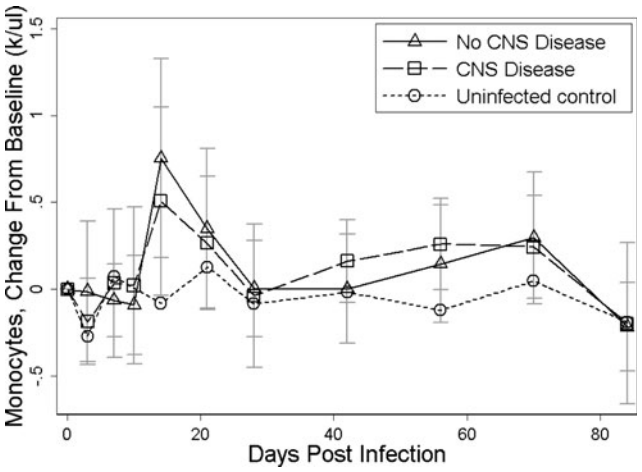

Figure 2 Mean change from baseline of monocyte counts in SIV infected macaques (CNS disease and no CNS disease) and uninfected controls. Error bars represent 95% confidence intervals.

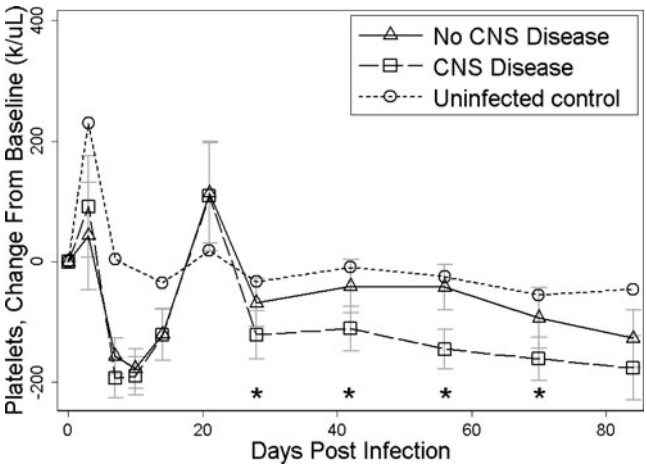

Figure 3 Mean decline from baseline of platelet levels in SIV-infected macaques (CNS disease and no CNS disease) and uninfected controls. Asterisks represent Wilcoxon rank-sum–based $P < .05$. Error bars represent 95% confidence intervals.

circulating monocytes in SIV-infected macaques corresponded with SIV encephalitis, longitudinal monocyte counts were examined. Macaques infected with SIV had an initial mean increase in monocyte counts that peaked at day 14 p.i., decreased to baseline, preinfection levels at day 28 p.i., and then subsequently rose slightly through day 70 p.i. before trending downward at day 84 (Figure 2). Groups with and without SIV CNS disease showed similar trends in monocyte levels throughout the course of infection. Uninfected control animals demonstrated a stable trend in monocyte counts that remained near baseline levels throughout all time points examined.

Platelet levels

The groups of macaques with and without SIV CNS disease demonstrated identical trends in change in platelet numbers during primary infection up to and including day 21 p.i. (Figure 3). Initially, a mean decline in platelet levels developed during acute infection (days 3 to 14 p.i.), with platelet counts then stabilizing slightly above pre-inoculation levels at day 21 p.i. From days 28 through 70 p.i., however, the group of animals with SIV encephalitis had a significantly greater mean decline in platelet levels as compared to the group of infected animals without SIV encephalitis (day 28 $P = .03$, day 42 $P = .01$, day 56 $P < .001$, day 70 $P = .02$). This difference was most pronounced at day 56 p.i. when the group of animals with SIV CNS disease had a mean decline in platelets of 145 k/μl, whereas the group of animals with no SIV CNS disease had a mean decline of 42 k/μl, for a difference in means of 103 k/μl between the two groups. Uninfected control animals demonstrated a stable trend in platelet levels, with counts remaining near baseline from day 7 until sacrifice. Absolute platelet levels for all groups did not drop to levels that would lead to clinically observable coagulation abnormalities.

A predictive model for SIV encephalitis

The multiple variable analysis for association with CNS disease was conducted for day 56 p.i.. This time point was chosen for modeling because it was the earliest point at which significant differences were detected between the groups with and without SIV CNS disease for both hemoglobin and platelet change from baseline (Figures 1 and 3). Mean uncorrected (i.e., absolute) platelet counts and hemoglobin levels differed between CNS disease groups at this time point as well ($P = .016$). This data, in addition to absolute platelet and hemoglobin levels at baseline, are presented in Table 1. As 2 of the 27 CNS diseased macaques did not have complete data at day 56 p.i., they were excluded from the analysis. Data and results from the univariate and multivariate analysis are presented in Table 2. In univariate models and in a model that simultaneously adjusted for hemoglobin levels, platelet levels, and treatment, a dose response for the odds of development of CNS disease was observed for both hemoglobin and platelets. On univariate analysis, macaques with moderate declines in hemoglobin had a 58% reduction in the odds of developing CNS disease when compared to macaques with large declines in hemoglobin ($P = .29$), whereas macaques in the category with minimal to no decline in hemoglobin had a 93% ($P = .003$) reduction. Conversely, macaques in the category with the greatest decline in hemoglobin were approximately 15 times more likely to develop SIV encephalitis when compared to macaques in the category with minimal/no decline in hemoglobin.

After simultaneous adjustment for platelet change category and treatment, an 89% ($P = .09$) and 98% ($P = .006$) reduction in odds of CNS disease development was noted for macaques in the moderate and minimal/no decline in hemoglobin categories, respectively. The trend for platelets was similar. Macaques with moderate declines in platelets had an

Table 1 Absolute platelet counts and hemoglobin levels at baseline and day 56 post infection according to CNS disease status

	CNS disease Baseline: n = 27; day 56: n = 25	*No CNS disease n = 20*	*Wilcoxon rank sum P value*
Baseline platelet count (95% CI), k/μl	418 (386.2–451.0)	388 (345.1–430.0)	.33
Day 56 platelet count (95% CI), k/μl	280 (247.2–312.0)	346 (308.1–383.1)	.016
Baseline hemoglobin level (95% CI), g/dl	10.1 (9.67–10.44)	9.9 (9.52–10.20)	.33
Day 56 hemoglobin level(95% CI), g/dl	8.5 (8.0–9.05)	9.2 (8.95–9.48)	.033

82% reduction in odds of developing CNS disease when compared to macaques with the greatest decline in platelet levels ($P = .06$), whereas macaques with minimal to no decline in platelets had a 94% reduction ($P = .003$). Alternatively, macaques in the category with the greatest decline in platelets were approximately 18 times more likely to develop SIV CNS disease when compared to those in the category with minimal to no decline in platelets. After simultaneous adjustment for hemoglobin change category and treatment, an 87% ($P = .08$) and 95% ($P = .02$) reduction in odds of CNS disease development was noted for macaques in the moderate and minimal/no decline in platelet level categories respectively. Summary statistics indicate that the multivariate model correctly classifies a macaque with respect to CNS disease outcome 80% of the time.

Mean platelet volume

Change in mean platelet volume (MPV) from baseline was examined as a marker of thrombopoiesis to determine whether platelet production was impaired at progressive stages of SIV infection (Figure 4). Both groups of SIV infected macaques demonstrated an initial mean increase in MPV during acute infection that peaked at days 10 to 14 p.i. and then returned to baseline values at day 21 p.i. Mean change in MPV again increased after day 21 p.i. The group of animals with SIV CNS disease experienced a greater increase in MPV compared to the group without SIV CNS disease. Trend lines for MPV diverged at day 21 p.i. and differences between groups were significantly different from days 28 to 56 p.i. (day 28 $P = .005$, day 42 $P = .01$, day 56 $P = .001$). The mean change in MPV converged for the two SIV infected groups at day 84 p.i. Interestingly, the SIV CNS disease group also had a significantly greater mean increase in MPV early in infection at day 7 p.i. ($P = .001$). Uninfected control animals demonstrated a stable trend in mean MPV that hovered about baseline from day 28 until sacrifice.

Mature megakaryocyte levels in bone marrow

To determine whether the observed decline in platelets during late stages of infection was the result of impaired platelet production, increased platelet loss, or a combination of these possibilities, bone marrow samples collected terminally were examined for differences in mature megakaryocyte numbers. Macaques with SIV CNS disease had significantly fewer mature megakaryocytes per high-power field when compared to animals without SIV CNS disease. The group of animals with SIV CNS disease had a median mature megakaryocyte count of 0.97 per 400× field compared to 1.38 mature megakaryocytes per 400× field in the group of macaques without SIV CNS disease ($P = .04$). Immunostaining of bone marrow with an anti-SIV glycoprotein 41 antibody revealed no evidence of SIV in mature megakaryocytes, although other cells in bone marrow were immunopositive for SIV, including lymphocytes and macrophages (Figure 5). Thus, productive infection of mature megakaryocytes by SIV is unlikely to be the cause of decreased megakaryocyte

Table 2 Univariate and multivariate logistic regression for associations between hematologic markers and odds of developing SIV CNS disease*

		CNS disease n = 25	*No CNS disease n = 20*	*Unadjusted OR*	*95% CI*	*Adjusted OR**	*95% CI*
Hemoglobin change from baseline (g/dl)							
−5.61 to −1.56	Large decline	12	3	1	Reference	1	Reference
−1.55 to −0.60	Moderate decline	10	6	0.42	0.08–2.11	0.11	0.01–1.44
−0.59 to 1.10	Minimal/no decline	3	11	0.07	0.01–0.41	0.02	0.001–0.32
Platelet change from baseline (k/μl)							
−287 to −140	Large decline	13	2	1	Reference	1	Reference
−1.39 to −49	Moderate decline	8	7	0.18	0.03–1.06	0.13	0.01–1.26
−48 to 176	Minimal/no decline	4	11	0.06	0.01–0.36	0.05	0.005–0.61

*Model includes hemoglobin and platelet change from baseline categories with adjustment for treatment category (none, minocycline, GPI-1485).

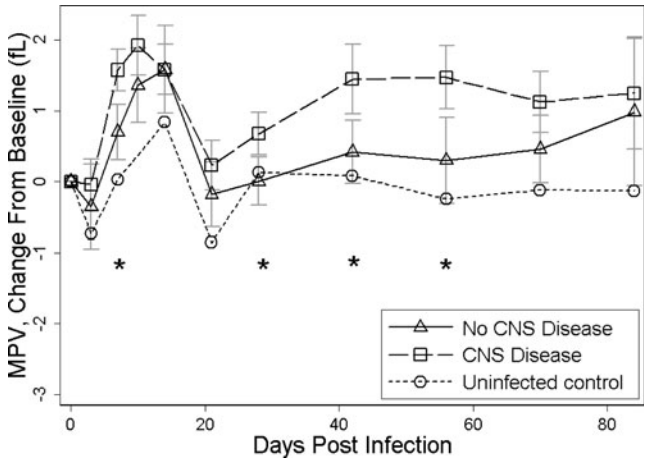

Figure 4 Mean change from baseline in mean platelet volume (MPV) in SIV infected macaques (CNS disease and no CNS disease) and uninfected controls. Asterisks represent Wilcoxon rank-sum–based $P < .05$. Error bars represent 95% confidence intervals.

numbers. It is possible that viral proteins as well as proinflammatory mediators produced by SIV-infected leukocytes could contribute to indirect damage of megakaryocytes. Alternately, disruption of homeostatic mechanisms, including expression of thrombopoietin, regulating megakaryocyte maturation, and platelet production, may be compromised in SIV infection.

Discussion

In this study, we demonstrated that the magnitude of decline in circulating platelet counts during asymptomatic SIV infection predicted the development of SIV encephalitis. SIV-infected macaques with moderate to severe CNS disease developed a greater decline from mean baseline platelet levels beginning at day 28 p.i. when compared to similarly infected macaques with no to mild CNS lesions. Univariate analysis performed on platelet values obtained day 56 p.i. demonstrated that SIV-infected macaques with the greatest decline in platelet numbers were 18 times more likely to develop SIV CNS disease than SIV-infected animals with minimal to no decline in circulating platelet counts. In this SIV/macaque model, animals typically do not develop encephalitis until after day 56 p.i. and exhibit no clinical signs until terminal infection. The identification of an association between decline in platelets and the development of encephalitis demonstrates that monitoring platelet decline in HIV-infected individuals may serve as a predictive marker for clinical progression to HIV-induced CNS disease. Identifying those HIV-infected individuals at risk for CNS disease during asymptomatic stages of infection could promote early interventive, neuroprotective treatments prior to extensive neuronal damage and loss.

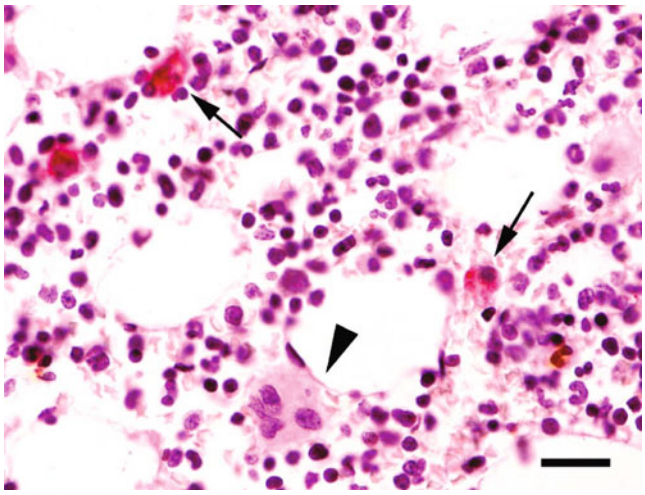

Figure 5 Representative photomicrograph of immunohistochemical staining for SIV glycoprotein 41 in bone marrow collected terminally from an SIV-infected macaque with SIV CNS disease demonstrates abundant SIV in mononuclear cells (*red, arrows*) but no SIV gp41 staining in mature megakaryocytes (*arrowhead*). Hematoxylin counterstain, Scale bar = 20 μm.

Given the association between platelet decline and SIV CNS disease, we examined the mechanisms contributing to platelet decline in SIV-infected macaques with and without SIV encephalitis. The observed decline in circulating platelets may result from (1) decreased thrombopoiesis, (2) accelerated destruction of platelets in the circulation, or (3) a combination of these processes. MPV was evaluated as a marker of thrombopoiesis because MPV rises with increased production of platelets stimulated by a decline in circulating platelet numbers. For example, increases in MPV have been demonstrated to provide sufficient specificity and sensitivity to distinguish between aplastic anemia, a platelet production deficit, and immune-mediated thrombocytopenia, characterized by increased platelet destruction (Kaito *et al*, 2005). Animals with SIV CNS disease demonstrated a greater mean increase in MPV beginning at day 28 p.i. when compared to infected macaques without SIV encephalitis. As the increase in MPV corresponded with the decline in platelet counts in macaques with SIV CNS disease, this finding suggested that platelet production was appropriately stimulated in response to a decline in circulating platelets. In HIV infection, immune-mediated peripheral platelet destruction contributes to thrombocytopenia (Ballem *et al*, 1992; Cole *et al*, 1998; Najean and Rain, 1994). Platelets of HIV-infected thrombocytopenic individuals have markedly increased platelet-associated and circulating complexes of IgG, IgM, and complement components C3 and C4 (Walsh *et al*, 1984), with IgG antibodies directed against the immunodominant epitope GPIIIa49-66 (Karpatkin *et al*, 1995; Nardi *et al*, 1997, 2001). We similarly suspect immune-mediated destruction of platelets leads to platelet

decline during asymptomatic SIV infection rather than deficits in thrombopoiesis.

We counted the number of mature megakaryocytes in the bone marrow collected terminally from SIV-infected macaques to determine whether loss of megakaryocytes also contributed to platelet decline at late stages of infection. Animals with moderate to severe encephalitis had reduced megakaryocyte numbers relative to macaques with no to mild encephalitis. This suggests that during terminal phases of SIV infection, a decline in platelet production also may develop and contribute to the observed decline in platelet levels in macaques with SIV CNS disease. This terminal decline is in keeping with the pathophysiology of thrombocytopenia in HIV/AIDS patients in which patients with advanced disease have been suggested to develop defects in platelet production (Najean and Rain, 1994). Whether a decrease in megakaryocytes contributes to decreased platelet production in HIV/AIDS patients is controversial and not well understood. Competing theories include a failure of compensatory megakaryocytopoeisis with reduced megakaryocytes/precursors or, alternatively, an ineffective delivery of platelets to circulation in the face of adequate numbers of megakaryocytes (Cole *et al*, 1998; Thiele *et al*, 1992; Zauli *et al*, 1991).

We were not able to detect SIV glycoprotein 41 in mature megakaryocytes via immunohistochemistry. Megakaryocytes express CD4, CXCR-4, and CCR-5, HIV infection of these cells has been demonstrated both *in vivo* and *in vitro*. The ability to infect megakaryocytes may be dependent on HIV strain (Chelucci *et al*, 1998; Dominguez *et al*, 1994; Kouri *et al,* 1993; Kowalska *et al*, 1999; Voulgaropoulou *et al*, 2000). Kitagawa *et al* examined the presence of SIV mRNA in bone marrow and were only able to detect infected megakaryocytes from one animal with a concurrent immunosuppressive simian type D retrovirus serotype-1 (SRV-1) infection (Kitagawa *et al*, 1991). In the absence of direct infection of mature megakaryocytes or precursors, megakaryocyte numbers and function may be affected by the cytokine milieu, release of factors such as viral proteins from adjacent infected cells in the marrow, or due to an inadequate hematopoietic support function of infected stromal cells (Banner *et al*, 1997; Chelucci *et al*, 1998).

The mechanistic link between change in platelet levels and SIV encephalitis remains to be determined. It is possible that up-regulation of hematopoietic growth factors in response to peripheral platelet destruction is a component of this link. Thrombopoietin (TPO) is the primary, lineage-specific cytokine involved in stimulating megakaryocyte growth and maturation *in vitro* and thrombopoiesis *in vivo*. TPO is constitutively produced by the liver with platelets autoregulating levels by binding TPO and removing it from circulation (Fielder *et al*, 1996; Kuter and Begley, 2002). Interestingly, TPO has recently been demonstrated to be strongly proapoptotic in the brain, leading to death of newly generated neurons (Ehrenreich *et al*, 2005). This cytokine may also prove to be important in the pathogenesis of SIV encephalitis.

SIV-infected macaques that developed moderate to severe CNS disease experienced a greater mean decline from baseline in hemoglobin values beginning at day 14 p.i. when compared to SIV-infected macaques with no to mild CNS lesions. Although differences in this parameter do not achieve statistical significance until day 56 p.i., making this marker a less robust predictor than platelet decline at early time points, these changes in hemoglobin parallel reports in HIV infection with pre-AIDS hemoglobin levels serving as a significant predictor of dementia in HIV patients (McArthur *et al*, 1993). In contrast to differences in platelets and hemoglobin levels, monocyte levels were similar between macaques with and without CNS disease. It is possible that the activation state of circulating monocytes rather than absolute monocyte numbers is of prognostic value in SIV infection (Kim *et al*, 2003).

Based on the observed trends in platelet and hemoglobin levels, we utilized a logistic regression model to assess the predictive value of these parameters for the development of CNS disease. A dose response was noted across categories of platelet and hemoglobin levels with both univariate and multivariate analyses. Those animals with moderate declines in platelets or hemoglobin demonstrated a reduction in odds of CNS disease relative to animals in the categories of highest decline. For animals in the categories of minimal to no decline compared to those with highest decline, this reduction in odds of disease was even greater. It should be noted, however, that the reduction in odds of CNS disease was not statistically significant for the category of moderate decline, although it is possible that with increased numbers of animals this trend would become statistically significant. The multivariate model included as covariates both the hemoglobin and platelet categories of change from baseline as well as the treatments (minocycline and GPI-1485) administered to a subset of animals. This model serves to demonstrate that adjustment for treatment did not impact the predictive value of platelets or hemoglobin. Multivariate models also enable the development of algorithms in which multiple biomarkers can be utilized to determine likelihood of developing encephalitis. In the proposed multivariate model, a macaque receiving no treatment and in the categories of greatest decline in both platelets and hemoglobin would have an estimated 99% probability of ultimately developing encephalitis. In contrast, a macaque receiving no treatment and having minimal decrease in hemoglobin and platelets would have an estimated 13% probability of developing encephalitis. The presence of small numbers of animals in each category of platelet and hemoglobin levels does contribute to extremes

in odds ratios but effect remains pertinent if an assessment is made of the conservative tail of the 95% confidence interval.

This study demonstrates a novel association between alterations in platelet count and the development of SIV CNS disease. As the decline in platelet levels occurred during asymptomatic infection and preceded the development of CNS disease, this hematologic parameter may be a predictive marker for CNS disease in HIV-infected individuals. The ease of measuring and monitoring platelet counts makes this an especially attractive marker to assess progression of infection. The development of multiparametric algorithms incorporating clinical measurements such as platelet counts and hemoglobin levels will improve patient management as well as contribute to an understanding of HIV CNS disease pathogenesis.

Materials and methods

Animal experiments and study population
A data set containing hematological parameters for 56 pig tailed macaques (*Maccaca nemestrina*) was compiled. Forty-seven animals were intravenously inoculated with SIV/DeltaB670 (50 AID_{50}) and SIV/17E-Fr (10,000 AID_{50}) as described (Zink *et al*, 1999). Of these 47, 12 SIV-inoculated animals were treated with oral minocycline and an additional 6 SIV-infected animals were treated orally with the neuroimmunophilin ligand GPI-1485 beginning day 21 and continuing until sacrifice (Zink *et al*, 2005). Nine additional animals served as uninfected, procedural controls. For all animals, acid citrate dextrose (ACD) anticoagulated blood samples were collected prior to infection and at days 3, 7, 10, 14, 21, 28 p.i., and then every 2 weeks thereafter. Occasionally the day of blood collection would vary by 2 to 3 days due to logistics of performing sample collection. Complete blood cell counts and differentials were determined on every blood sample using a Cell-Dyn 3500 hematology analyzer (Abbott, Abbott Park, IL). Values for platelet count, hemoglobin levels, monocyte count, and MPV were obtained from this complete blood cell count data. All animals were euthanized at day 84 or later based on presence of neurologic symptoms or decline in motor activity. All procedures were performed while monkeys were anesthetized with ketamine-HCl (Fort Dodge, Iowa). Animal procedures in this study were performed in accordance to the principles set forth by the Institutional Animal Care and Use Committee at Johns Hopkins University and the National Research Council's Guide for the care and use of laboratory animals.

CNS and bone marrow histopathology
At euthanasia, macaques were perfused with sterile saline to remove blood from vasculature prior to fixation. Hematoxylin and eosin–stained sections of frontal and parietal cortex, basal ganglia, thalamus, midbrain, and cerebellum were examined in a blinded fashion to establish severity of encephalitis. Severity of encephalitis was based on presence of perivascular macrophage cuffs and was designated as none to mild, moderate, or severe based on previously described scoring criteria. If sections contained more than 30 perivascular macrophage-rich cuffs on average, the animals were classified as having severe encephalitis. Animals averaging 10 to 30 perivascular cuffs were scored as moderate encephalitis, and those with less than 10 perivascular cuffs were considered to have mild encephalitis (Mankowski *et al*, 2004; Zink *et al,* 1999). Mature megakaryocytes were counted by microscopic examination of hematoxylin and eosin–stained paraffin-embedded sections of bone marrow collected at euthanasia. A total of 50 adjacent 250 μm^2 fields with comparable cell density were scored for each animal.

Immunohistochemisty
To detect SIV-infected cells in bone marrow, kk41, a monoclonal antibody directed against the SIV transmembrane portion of the SIV envelope, was used (diluted 1:400; NIH AIDS Reagent Program, Bethesda, MD). Streck-fixed, paraffin-embedded sections of bone marrow were deparaffinized, rehydrated, and then post-fixed in Streck tissue fixative (Streck Laboratories, Omaha, NE) for 20 min. After rinsing in water, tissues were heated in a microwave in sodium citrate buffer (0.01 M, pH 6.0) for 8 min to retrieve antigen. Endogenous peroxidase was quenched with 3% H_2O_2 for 10 min and then sections were blocked with buffered casein for 10 min. Primary antibody was applied to tissue sections for 60 min at room temperature, the tissues were washed in buffer, and then secondary biotinylated multilink antibody (Biogenex, San Ramon, CA) was added for 20 min. After washing, streptavidin-horseradish peroxidase was applied for 20 min, followed by diaminobenzidene tetrahydrochloride in buffer containing H_2O_2 for 10 min. Sections were then washed, dehydrated, and mounted.

Statistical methods
For statistical analyses, macaques with moderate or severe encephalitis were classified as having SIV CNS disease whereas macaques with none to mild encephalitis were designated as having no SIV CNS disease. Values from uninfected control animals are presented for reference. Absolute monocyte counts, platelet counts, hemoglobin levels, and MPV were examined as an absolute change from baseline. The change from baseline per animal was calculated by subtracting the value of each blood parameter at baseline from the subsequent values at each measurement p.i. Although normal platelet counts are maintained within a tight range, platelet counts will vary widely between individuals (Kuter, 1996). Change from baseline was used to correct

for this expected physiologic interanimal variability. Further reference to mean change or mean decline/increase in hematologic parameters refers to the animal group means taken at each time point p.i. for this change from baseline. Means and 95% confidence intervals of changes in markers since baseline were calculated and plotted over time (i.e., days p.i.). The Wilcoxon rank-sum test was utilized to determine differences, if any, in hematologic markers' distributions between the two infected groups at each time point p.i. A multiple logistic regression model was fit for day 56 p.i. data to assess predictive value of change in hemoglobin levels, platelet levels, and development of CNS disease. For regression models, the platelet and hemoglobin changes were grouped into categories based on tertiles. The prognostic value of each categorical variable was evaluated after adjustment for the other and for treatment category (no treatment, minocycline, and GPI-1485). Summary statistics (sensitivity, specificity, negative predictive value, positive predictive value, percent correctly classified) for the multivariate model were assessed for a probability cutoff of 50% (i.e., a macaque was considered "positive" for likely development of CNS disease when the regression model predicted a probability of CNS disease of 50% or greater). All analyses were performed using Stata statistical software (version 8.0; Stata Press, College Station, TX) (StataCorp, 2003).

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