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ANEMIA IN PARASITE- AND RECOMBINANT PROTEIN–IMMUNIZED *AOTUS* MONKEYS INFECTED WITH *PLASMODIUM FALCIPARUM*

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Abstract. *Plasmodium falciparum*–induced anemia was characterized in *Aotus* monkeys repeatedly immunized by infection with *P. falciparum* (FVO strain) parasites, then cross-challenged with CAMP strain, or in monkeys receiving blood stage challenges as part of malaria vaccine trials. In 4 studies, 25 (30.5%) of 82 monkeys had at least a 50% reduction in hematocrit; mean day of maximum parasitemia was 12.5, whereas the mean day of minimum hematocrit was 18.8 ($P < 0.0009$). Decreased hematocrit levels were not associated with reticulocytosis until parasite densities decreased significantly from peak levels. Direct antibody tests to detect IgG and C3d on the surface of erythrocytes were negative. Nonantibody/noncomplement-mediated lysis of uninfected erythrocytes seems to be the principal cause of the anemia, and it also seems that bone marrow suppression and lysis of infected erythrocytes contributed to the anemia. Partial immunity—whether induced by repeated immunization with whole parasites or with vaccine—seems important to the development of anemia.

INTRODUCTION

Anemia is an important contributor to malaria's morbidity and mortality. In humans, several factors contribute to the development of malarial anemia. There is evidence that both parasitized and nonparasitized erythrocytes are lysed, but what part autoimmune hemolysis or other immune-mediated events contribute to this lysis is unclear. Decreased erythrocyte production is also an important contributor to this anemia. Suppressive autologous serum factors, reduced erythropoietin synthesis, and alterations in cytokine production may be part of anemia development.[1] Not only does it seem that the anemia of malaria is caused by more than one factor, but there are also different forms of malarial anemia. In their study of anemia in young Gambian children, Abdalla and others[2] were able to divide the subjects into 3 groups (acute malaria, anemic acute malaria, and chronic anemic malaria), depending on factors such as history, parasitemia density, degree of illness, and hemoglobin concentration.

Aotus monkeys can be infected with *Plasmodium falciparum* and are thought to be an important model system in the development of blood-stage *P. falciparum* malaria vaccines.[3] The development of marked anemia in this model suggests that study of this vaccine in a primate model may yield information useful in understanding the anemia often seen in humans subjected to malaria transmission under natural conditions. In addition, the development of anemia during vaccine trials requires careful evaluation in order to rule out a specific immunogen as a contributor to the induction of anemia.

We report our evaluations of anemia observed in *Aotus* monkeys immunized to *P. falciparum* blood stage parasites of one strain (FVO) by repeated infection with that strain, then cross-challenged with another strain of *P. falciparum* (CAMP). We also noted cases of anemia in *Aotus* monkeys in 2 different *P. falciparum* vaccine efficacy trials. We have used these experimental opportunities to describe the natural history of *P. falciparum*–induced anemia in *Aotus* monkeys and to contribute to the understanding of its causes.

MATERIALS AND METHODS

Repeated infection of *Aotus lemurinus lemurinus* with homologous and heterologous strains of *P. falciparum*. *Aotus lemurinus lemurinus* monkeys (n = 8) are indigenous to Panama. They were housed at the Gorgas Memorial Institute, Panama City, Panama, and received their first *P. falciparum* (FVO strain) challenges between October 1994 and August 1995; they then received 6 more FVO challenges, each separated by ~ 5 months, until all the animals had received 7 FVO challenges. All animals received their seventh FVO challenge simultaneously. Five weeks after the seventh FVO challenge and 2 weeks before challenge with the heterologous CAMP, all animals were treated orally once a day for 5 days with 50 mg/kg quinine and 6 mg/kg doxycycline to eliminate any occult FVO parasitemia. All then received the heterologous *P. falciparum* (CAMP strain) challenge 10 days after treatment was concluded. Three naïve control animals were included in the *P. falciparum* (CAMP strain) challenge. All challenges described in this study were performed by intravenous injection via the saphenous vein of 10^4 parasitized *Aotus* erythrocytes.[4] The 11 animals were assessed daily for 35 days for parasitemia, hematocrit levels were assessed on days 10, 14, 17, 21, 24, 28, 31, and 35. Differences between the FVO and CAMP strains have been previously described.[4]

Vaccine trial 1 with challenge and rechallenge. A total of 34 male and female *Aotus nancymae* monkeys were used in this study. The monkeys, which are indigenous to Peru, were supplied by the Center for the Reproduction and Conservation of Nonhuman Primates, Iquitos, Peru. Male and female pairs were randomly divided into 8 groups of 4 monkeys. An additional 2 monkeys were maintained as parasite donors. As part of a DNA malaria vaccine trial, the monkeys were immunized with DNA and protein vaccines based on the 606 amino acid region II of the 175 kDa *P. falciparum* erythrocyte binding protein, EBA-175. The DNA and protein vaccines and the immunization regimens are completely described elsewhere.[5] Briefly, the monkeys received a total of 500 μg DNA encoding region II of *P. falciparum* EBA-175 or control plasmid not encoding *P. falciparum* antigen. Both were de-

livered with plasmid encoding *Aotus* granulocyte monocyte–colony stimulating factor. DNA vaccines were administered by intradermal inoculation while EBA-175 region II protein[6] or phosphate-buffered saline (PBS) control emulsified in Montanide ISA 720 were administered, half subcutaneously, half intramuscularly.[5]

Vaccine trial 1—first challenge and follow-up. On day 0, 28 days after the last dose of vaccine,[5] 1 mL of acid-citrate-dextrose (Red Cross, Rockville, MD) decoagulated blood was removed from a single donor monkey with a *P. falciparum* (FVO strain) parasitemia. The blood was diluted with RPMI 1640 medium at 37°C, and the 32 experimental monkeys were injected intravenously with 10^4 parasitized erythrocytes. The first challenge occurred in January 1999. Monkeys were monitored daily for parasites, starting on day 2. The daily blood films for parasite enumeration were prepared from blood taken from the saphenous vein. Parasitemia and hematocrit levels were evaluated daily. Sera were collected 2 and 4 weeks after challenge.

Vaccine trial 1—second challenge and follow-up. Nine months after the first challenge (October 1999) and 10 months after the last dose of vaccine, a second challenge was conducted identically to the first challenge, except that each monkey received 10^5 parasitized erythrocytes. Twenty-four of the original 32 monkeys were included in this challenge. Second challenge follow-up was the same as first challenge follow-up, except that a direct antibody test (DAT) was performed every third day and blood films for reticulocyte counts were prepared daily.

Vaccine trial 2 with challenge. A total of 18 male and female *Aotus nancymae* monkeys were used in this study. The monkeys were supplied by the Center for the Reproduction and Conservation of Nonhuman Primates, Iquitos, Peru. Male and female pairs were randomly divided into 3 groups of 6 monkeys. Briefly, the monkeys received a total of 500 μg DNA encoding region II of *P. falciparum* EBA-175, or control plasmid not encoding *P. falciparum* antigen. Both were delivered with plasmid-encoding *Aotus* granulocyte monocyte–colony stimulating factor. DNA vaccines were administered by intradermal inoculation. EBA-175 region II protein or PBS control emulsified in Montanide ISA 720 were administered half subcutaneously and half intramuscularly. Groups 1 (vaccine) and 2 (control) received 4 immunizations at weeks 0, 4, 8, and 24, whereas group 3 (vaccine) received its immunizations at weeks 12, 16, 20, and 24. All 3 groups were challenged simultaneously with 10^4 *P. falciparum* (FVO strain).

Vaccine trial 2—challenge and follow-up. On day 0, the experimental monkeys were infected from a single donor monkey with 10^4 parasitized erythrocytes in the same manner as the challenges in vaccine trial 1. The challenge occurred in July 2000. Monkeys were monitored daily beginning on day 2 for parasitemia, hematocrit level, and reticulocyte count. Modified DAT was performed every 4 days, and a complete blood count (CBC) was performed on days 2, 9, 17, and 24.

Treatment standards during vaccine trials 1 and 2. Infected monkeys were treated with mefloquine (20 mg/kg orally, single dose) if the parasitized erythrocytes rose to 300,000 parasites/μL (6% when erythrocyte count was 5×10^6/μL), if the hematocrit decreased by < 50% over preinfection values, or if other untoward clinical signs were observed, such as anorexia or reduced physical activity. The monkeys not requiring treatment during follow-up were treated on day 28 with mefloquine.

Hematologic, parasitology, and immunologic tests. *Parasite enumeration.* Parasites were counted by means of the Earle-Perez method.[7] Ten microliters of blood was dispensed onto a measured area of a microscope slide. A microscope with a known field of view diameter was used, and parasite densities were calculated and expressed as parasites per microliter of blood.

Hematocrit levels. Approximately 50 μL of blood was allowed to flow into a microcapillary tube containing heparin. The tube was then spun for 5 minutes in a dedicated microhematocrit centrifuge, and the percentage hematocrit was determined with an IEC microhematocrit reader.

Direct antibody technique. We tested for the presence of immunoglobulin (Ig) G on the surface of *Aotus* erythrocytes with a gamma-ReACT test kit (Gamma Biologicals Inc., Houston, TX) for detection of IgG on the surface of erythrocytes. Both protein A and protein G are covalently linked to an insoluble matrix in a microcolumn. *Aotus* blood samples are placed at the top of the microcolumn, then centrifuged. IgG on the surface of any erythrocytes is bound by the protein A and protein G, and these cells do not transit the microcolumn during centrifugation. When erythrocytes from 44 patients with positive tube agglutination DATs were tested in this affinity microcolumn DAT, 29 (66%) of 44 were positive.[8] This microcolumn technique has 2 important advantages over convention tube techniques: first, it uses only 25 μL of blood, an important issue with *Aotus* monkeys; and second, it does not rely on anti-human IgG, so there are no concerns about the degree of cross-reactivity between anti-human IgG and the *Aotus* IgG it is targeting.

Modified direct antibody technique. The protein A and G columns were modified by the addition of IgG directed against either human IgG or human complement C3d. Control studies indicated that the test did react with *Aotus* IgG.

Reticulocytes. New methylene blue–stained thin blood films were prepared, and the percentage reticulocytosis was determined microscopically on the basis of a count of 1,000 erythrocytes. Reticulocyte counts were corrected for anemia per Hutchison and Davy.[9]

Complete blood counts. Complete blood counts were performed with a Coulter Counter, model JT W/IR.

Enzyme-linked immunosorbent assay. Serum antibodies were assayed as previously described.[10] Purified baculovirus recombinant EBA-175 region II protein (1 μg/mL) in PBS–sodium azide buffer was used as capture antigen, The enzyme-linked immunosorbent assay results ae reported as the interpolated reciprocal dilution estimated to give an optical density of 0.5.

Statistical analysis. Statistical tests were performed by SPSS for Windows, version 8 (SPSS Inc., Chicago, IL).

RESULTS

***Aotus* immunized by repeated infection.** After the CAMP challenge, mean hematocrit levels for the 8 immunized monkeys decreased from 50.6% on day 10 after CAMP challenge to 36.5% on day 17, then stabilized, averaging 36.7% on day 21 and increasing to 48.5% by day 31 after CAMP challenge. Three of the 8 monkeys experienced reductions in hematocrit of > 50%. The hematocrit levels of the 3 naïve monkeys also

decreased to a low of 38.6 on day 17 after challenge, but then increased to 47.3% on day 21. All 3 of the naive monkeys were administered mefloquine on day 12 or 13 to treat elevated parasitemia; none experienced a 50% reduction in hematocrit. None of the immunized monkeys was treated. The 2 immune monkeys with the highest parasitemias during the CAMP challenge (monkey 12765 at 9,890 parasites/μL and monkey 12763 at 10,920 parasites/μL) also had the largest decreases in hematocrit referent to the day 10 baseline during the challenge (59.6% decrease and 51.8% decrease, respectively) (Table 1). In spite of these decreases, the hematocrit levels in both these animals returned to normal without treatment by day 35 (49% and 50%, respectively). Mean day of highest parasitemia among the 9 infected monkeys (immunized and controls combined) was day 12.3 versus day 18.8 for lowest hematocrit ($P = 0.001$, t-test). The 2 immunized monkeys that did not become infected had the least percentage decrease in hematocrit.

Vaccine trial 1—first challenge. During the 28-day follow-up period after the first *P. falciparum* challenge, 6 of the 32 monkeys under study required treatment for low hematocrit levels; another 21 were treated for parasitemia (Table 2). The mean day of treatment for monkeys treated for high parasitemia was day 10.9 (95% confidence limit [CL] = 10.5, 11.4), whereas the mean day of treatment for depressed hematocrit occurred on day 16.7 (95% CL = 12.8, 20.5; $P = 0.012$, 2-tailed t-test). Because monkeys treated for elevated parasitemia received drug before they had the opportunity to become anemic, the following comparison is restricted to those monkeys treated for decreased hematocrit versus those not treated (n = 5). For those monkeys treated for low hematocrit levels, the mean low hematocrit was 19.2% (95% CL = 16.6, 21.7), whereas the mean low hematocrit for those monkeys not treated (n = 5) was 36.6% (95% CL = 26.6, 46.6), $P = 0.007$ (2-tailed t-test).

We also looked for an association between an individual monkey's maximum parasitemia and its minimum hematocrit level by means of simple linear regression. In many monkeys treated for elevated parasitemia, hematocrit levels continued to decrease even after treatment. We therefore analyzed the data in 2 ways: first, maximum parasitemia versus minimum hematocrit recorded anytime during the 28-day follow-up, and second, maximum parasitemia versus minimum hematocrit recorded before treatment. Maximum parasitemia and minimum hematocrit recorded any time (case 1) were not correlated (case 1, $r^2 = 0.011$, $P = 0.25$). There was a modest association between maximum parasitemia and minimum hematocrit before treatment (case 2, $r^2 = 0.336$, $P < 0.0009$); high maximum parasitemia predicted high minimum hematocrit levels.

The minimum hematocrit levels in monkeys receiving EBA-175 in either or both forms (DNA vaccine or recombinant protein vaccine, n = 16, mean minimum hematocrit =

TABLE 1

Maximum parasitemia, minimum hematocrit levels, and the days on which they occurred in 8 *Aotus* monkeys challenged with 10^4 CAMP strain *Plasmodium falciparum*-infected *Aotus* erythrocytes*

Monkey	Max density	Max day	Min HCT	Min day	Decrease (%)
12749	0	–	45	21	15.1
12759	2,520	14	40	17	27.2
12739	0	–	31	24	11.4
12756	380	10	28	17	50
12757	2,980	9	27	17	35.7
12765	9,890	14	21	21	59.6
12763	10,920	14	26	26	51.8
12730	120	14	44	44	24.1
Mean†	3,351.3	12.5	32.8	23.4	34.4
Control 1	484,500	12‡	46	14	21.4
Control 2	519,000	12‡	29	14	40.8
Control 3	767,250	13‡	42	24	23.6
Mean§	590,250	12.3	39	17.3	28.6

* Monkeys were previously infected 7 times with FVO strain blood stage *P. falciparum*. The 3 control monkeys were naive. Max density = maximum parasite density (parasites/μL) observed during the follow-up period; max day = day of maximum parasitemia; min HCT = minimum hematocrit observed during follow-up period; min day = day of minimum parasitemia; decrease = percentage decrease in hematocrit levels from prestudy baseline.
† Mean of values from 8 experiment monkeys.
‡ Treated on that day with 20 mg/kg mefloquine as a result of parasitemia.
§ Mean of values from 3 control monkeys.

TABLE 2

Thirty-two *Aotus* monkeys, divided into 8 groups, immunized with varying regimens of *Plasmodium falciparum* EBA-175 region II DNA and protein, then challenged with 10^4 *P. falciparum*-infected *Aotus* erythrocytes*

Monkey	Pri-Boost	Max para (×10³)	Max day	Min HCT	Min day	Reason for treatment	Day of treatment
917	Dv_Dv	34	12	18	17	HCT	17
906	Dv_Dv	428	11	38	13	Para	11
918	Dv_Dv	485	11	32	16	Para	11
854	Dv_Dv	491	11	30	16	Para	11
Mean	Dv_Dv	359.5	11.3	29.5	15.5		12.5
913	Dv_Pv	112	11	27	21		NT
919	Dv_Pv	96	11	29	16		NT
909	Dv_Pv	314	11	44	12	Para	11
898	Dv_Pv	144	11	40	6		NT
Mean	Dv_Pv	166.5	11	35	13.8		11
899	Dv_DvPv	265	13	16	26	HCT	22
911	Dv_DvPv	347	13	23	16	Para	11
908	Dv_DvPv	553	11	29	16	Para	11
903	Dv_DvPv	752	11	23	17	Para	11
Mean	Dv_DvPv	479.3	12	22.8	18.8		13.8
912	Dc_Dc	750	11	32	15	Para	11
920	Dc_Dc	524	11	21	14	Para	11
944	Dc_Dc	545	11	30	26	Para	11
943	Dc_Dc	422	11	21	15	Para	11
Mean	Dc_Dc	560.3	11	26	17.5		11
948	Dc_DcPc	300	11	48	6	Para	11
950	Dc_DcPc	467	11	26	13	Para	11
954	Dc_DcPc	328	14	21	15	Para	14
931	Dc_DcPc	304	11	20	17	Para	11
Mean	Dc_DcPc	349.8	11.8	28.8	12.8		11.8
957	Pv_Pv	89	11	19	20	HCT	20
939	Pv_Pv	17	11	23	13	HCT	13
927	Pv_Pv	130	12	21	14	HCT	14
925	Pv_Pv	314	11	36	21	Para	11
Mean	Pv_Pv	137.5	11.3	24.75	17		14.5
916	Pc_Pc	275	11	43	23		NT
933	Pc_Pc	652	11	43	15	Para	11
921	Pc_Pc	193	9	44	13		NT
937	Pc_Pc	340	9	38	12	Para	9
Mean	Pc_Pc	365	10	42	15.7		10
935	n_n	880	11	42	14	Para	11
938	n_n	518	11	32	16	Para	11
946	n_n	294	12	18	16	HCT	14
949	n_n	319	9	39	14	Para	9
Mean	n_n	502.8	10.8	32.8	15		11.3
Mean	All	365	11.1	30.2	15.8		12.2

* Max para = maximum parasitemia; max day = day of maximum parasitemia (300,000 parasites/μL); min HCT = minimum hematocrit level; min day = day of minimum hematocrit level; Dv = P.f. EBA DNA vaccine; HCT = 50% decrease in hematocrit from pre-infection baseline; para = parasitemia; Pv = P.F. EBA protein vaccine; NT = not treated for either elevated parasitemia or decreased hematocrit, but treated as per protocol at the end of the 28 day follow-up period; Dc = DNA vaccine control; Pc = adjuvant control; n = nothing; P.f. = *Plasmodium falciparum*.

28) were compared with the hematocrit levels of monkeys not receiving EBA-175 (n = 16, mean minimum hematocrit = 32.4). No association between minimum hematocrit levels and receipt of EBA-175 vaccine was found ($P = 0.194$; power to resolve a true difference of 4.4 was 24%, whereas a true difference of 9.5 would yield a power of 80%).

One striking difference in the development of anemia was between the DNA prime-protein boost (Dv_Pv) and the recombinant protein prime and boost (Pv_Pv) groups. The Pv_Pv group had slightly higher levels of antibodies by enzyme-linked immunosorbent assay after the boost (geometric means, 520,651 versus 801,189, $P = 0.35$, *t*-test), and slightly lower peak parasitemias (166,492 versus 137,670, $P = 0.73$, *t*-test) than did the Dv_Pv group. One animal in each group reached the parasitemia threshold for treatment of 300,000 parasites/μL blood, but 3 of the 4 monkeys in the Pv_Pv group and none of the monkeys in the Dv_Pv group developed > 50% reduction in hematocrit. Among the monkeys that did not reach the parasitemia threshold, the minimum hematocrit levels in the 3 monkeys in the Pv_Pv group were significantly lower ($P = 0.037$, *t*-test on log-transformed values) than in the Dv_Pv group (19, 21, and 23 versus 27, 29, and 40, means of 21 and 32, respectively).

Vaccine trial 1—second challenge. Nine months after the first challenge, and 8 months after the last dose of mefloquine, the 24 monkeys (12 previously immunized with EBA-175, 12 previously controls) were rechallenged with 10^5 *P. falciparum*–parasitized erythrocytes. During the 28-day follow-up period, they were monitored daily for parasitemia, hematocrit levels, and reticulocyte count (Table 3), and they were monitored once every 3 days for IgG on the surface of their erythrocytes (DAT). All monkeys became parasitemic, but none required treatment for parasitemia exceeding 300,000 parasites/μL. However, 11 required treatment for reduced hematocrit levels; 7 of 12 previous controls and 4 of 12 previously immunized with EBA-175 vaccine ($P = 0.22$, chi-square test). Mean maximum parasitemia was 4,820 parasites/μL (95% CL = 1,899, 7,742). The mean day on which maximum parasitemia occurred was day 14.4 (95% CL = 12.9, 15.9). The mean minimum hematocrit level was 30.6% (95% CL = 27.3, 33.9), and the mean day of minimum hematocrit level was day 21.3 (95% CL = 20, 22.8). Mean maximum corrected reticulocyte count was 4.5% (95% CL = 3.8, 5.2), and the mean day of maximum corrected reticulocyte count was day 24.9 (95% CL = 22.7, 27). Direct antibody tests were performed on days 1, 3, 6, 9, 12, 16, 18, 21, 25, and 28. The DAT was not performed again after a monkey was treated. All DATs were negative for the presence of IgG on the surface of erythrocytes.

When daily parasitemia, hematocrit levels, and reticulocyte values were plotted for each monkey, a pattern emerged suggesting that regardless of hematocrit level, reticulocyte counts did not begin to increase until parasitemia density had fallen below ~ 100 parasites/μL. This was seen in 17 of 24 of the monkeys. Two examples, one from a monkey treated for depressed hematocrit and the other from an untreated monkey, are shown in Figure 1.

In order to establish a baseline corrected reticulocyte value, the mean of the daily reticulocyte counts from day 3 to day 6 was calculated for each monkey. Any reticulocyte count from day 7 to day 28 that was above that mean plus 2 standard deviations was designated as "elevated." For each monkey exhibiting elevated reticulocyte counts (15 of 24), we calculated the mean hematocrit level and the mean parasitemia for the day before the elevated reticulocyte counts. We also calculated the mean hematocrit and parasitemia counts for the days before each day with a nonelevated reticulocyte count. The mean hematocrit for days preceding an elevated reticulocyte count was 41.7% versus 46.7% for those days preceding a day with a nonelevated reticulocyte count ($P = 0.019$, paired *t*-test). Mean parasitemias for the same days were 118 parasites/μL (elevated reticulocyte count) versus 507 parasites/μL (nonelevated reticulocyte count) ($P = 0.018$, paired *t*-test).

TABLE 3

Maxima and minima for recorded blood values of *Aotus nancymae* previously infected with *Plasmodium falciparum**

Monkey	Group	Max para	Max day	Min HCT	Min day	Max retic	Day retic	Day of treatment
917	Dv_Dv	156	7	34	12	7.0	26	NT
906	Dv_Dv	130	5	43	21	5.0	27	NT
854	Dv_Dv	1176	14	19	21	4.9	27	19
Mean	Dv_Dv	487.3	8.7	32	18	5.6	26.7	19
919	Dv_Pv	1265	12	31	24	2.2	26	NT
909	Dv_Pv	11174	14	32	24	2.4	28	NT
898	Dv_Pv	2060	17	23	27	2.0	6	20
Mean	Dv_Pv	4833	14.3	28.7	25	2.2	20	20
899	Dv_DvPv	1723	12	25	18	6.7	27	18
908	Dv_DvPv	6428	14	16	19	5.1	25	15
903	Dv_DvPv	1419	14	35	19	7.1	27	NT
Mean	Dv_DvPv	3190	13.3	25.3	18.7	6.3	26.3	16.5
912	Dc_Dc	952	12	37	19	2.9	24	NT
920	Dc_Dc	4929	15	28	23	2.7	26	23
944	Dc_Dc	3506	15	34	23	4.3	27	NT
Mean	Dc_Dc	3129	14	33	21.7	3.3	25.7	23
954	Dc_PcDc	200	18	27	21	4.6	26	21
931	Dc_PcDc	624	16	27	20	4.5	27	20
Mean	Dc_PcDc	412	17	27	20.5	4.6	26.5	20.5
957	Pv_Pv	315	20	39	25	2.6	27	NT
927	Pv_Pv	1359	14	31	20	4.5	27	NT
925	Pv_Pv	2112	17	48	26	4.9	26	NT
Mean	Pv_Pv	1262	17	39.3	23.7	4	26.7	–
916	Pc_Pc	3406	16	27	23	2.9	12	21
933	Pc_Pc	20368	18	23	22	2.8	26	20
921	Pc_Pc	7072	21	39	26	4.9	27	NT
Mean	Pc_Pc	10282	18.3	29.7	23.7	3.5	21.7	20.5
935	n_n	23948	15	23	22	5.9	27	20
938	n_n	894	11	26	18	4.8	22	NT
946	n_n	878	14	28	20	7.0	27	20
949	n_n	19604	14	40	20	6.8	27	NT
Mean	n_n	11331	13.5	29.3	20	6.1	25.8	20
Mean	All	4820.8	14.4	30.6	21.4	4.5	24.9	19.7

* *Aotus nancymae* previously infected once with 10^4 *P. falciparum* were rechallenged with 10^5 *P. falciparum*–parasitized *Aotus* erythrocytes 9 months after the first challenge, and they were followed for 28 days. The first entry identifies immunogen used in first 3 immunizations, and the second entry identifies the immunogen or immunogens used in the fourth immunization. All monkeys treated before the end of the 28-day follow-up period were treated for a decrease in hematocrit levels of > 50% of baseline, and none were treated for elevated parasitemia. max para = maximum parasitemia observed during the 28-day follow-up period; max day = day of maximum parasitemia; min HCT = minimum hematocrit observed during the 28-day follow-up period; min day = day of minimum hematocrit; max retic = maximum reticulocyte count during the 28-day follow-up period; day retic = day of maximum reticulocyte count; day of treatment = day of treatment for anemia; Dv = P.f. EBA DNA vaccine; NT = not treated for either elevated parasitemia or decreased hematocrit level, but treated per protocol at the end of the 28-day follow-up period; Pv = P.f. EBA protein vaccine; Dc = DNA vaccine control; Pc = adjuvant control; n = nothing; P.f. = *P. falciparum*.

Vaccine trial 2—challenge. During the 28-day follow-up period after *P. falciparum* challenge, 6 of the 18 monkeys required treatment for low hematocrit levels, and another 11 were treated for elevated parasitemia (Table 4). The mean day for treatment for elevated parasitemia was day 11.9 (95% CL = 14.3, 9.5), and the mean day of treatment for decreased hematocrit was day 20.8 (95% CL = 23.4, 18.3; $P = 0.001$,

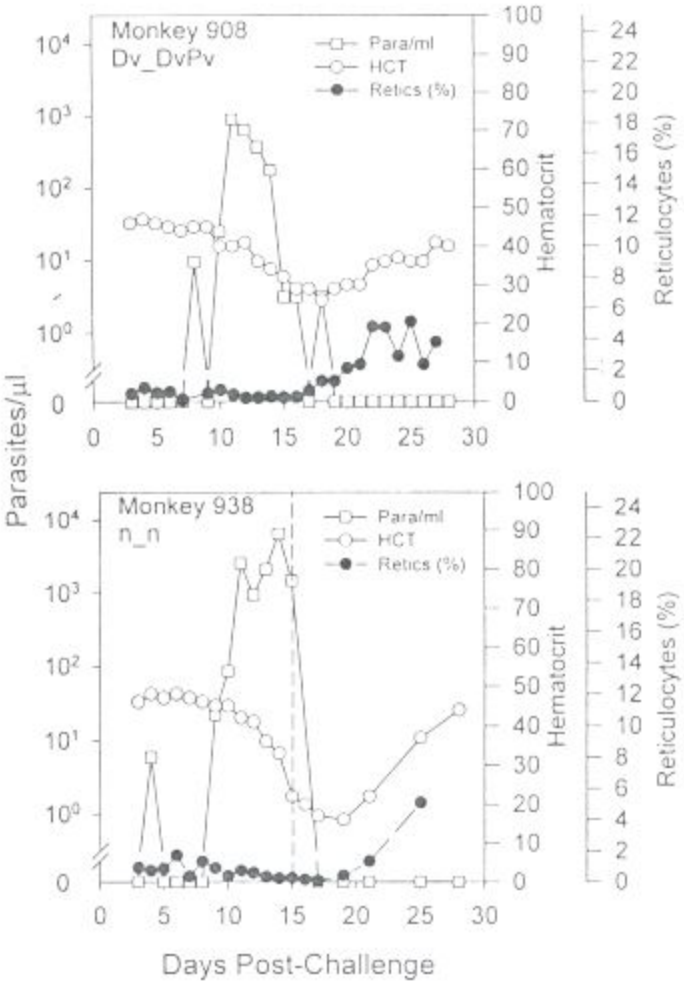

FIGURE 1. The values for parasitemia density, hematocrit levels, and reticulocyte count for 2 monkeys, one untreated **(top)** and one treated with mefloquine for low hematocrit **(bottom)**, are shown for the 28-day period after challenge with 10^5 *Plasmodium falciparum*–infected *Aotus* erythrocytes. The vertical dashed line in the bottom panel indicates the day of mefloquine treatment. In the upper left of each panel is the monkey number and the prime-boost immunization regimen it received.

2-tailed *t*-test). Only one monkey (monkey 1098, an immunized animal) completed the 28-day follow-up without requiring treatment for either elevated parasitemia or decreased hematocrit. Maximum parasitemia and minimum hematocrit recorded any time during the 28-day follow-up period were correlated ($r^2 = 0.436$, $P = 0.002$). The correlation strengthened when maximum parasitemia and minimum hematocrit recorded before treatment were evaluated ($r^2 = 0.681$, $P < 0.0009$). As in the first trial, high maximum parasitemia predicted high minimum hematocrit levels. The minimum hematocrit levels of the monkeys receiving EBA-175 (31.7, 95% CL 36.6, 26.7) versus those not receiving it (37.8, 95% CL 43.7, 31.9) were not different ($P = 0.158$, 2-tailed *t*-test).

The parasitemia, hematocrit level, reticulocyte count, and platelet count plots for each monkey revealed that in 12 of 18 monkeys, reticulocyte count did not begin to rise until after the parasitemia had fallen to ~ 100 parasites/µL. These findings were consistent with those of vaccine trial 1—second challenge.

Blood counts were performed on days 2, 9, 17, and 24 of the postchallenge follow-up period (Table 5). Lymphocyte and monocyte counts decreased 44% and 42%, respectively, between day 2 and day 9 but rebounded to day 2 levels by day 17. Granulocyte and platelet counts decreased more slowly, but more markedly. By day 17, granulocyte and platelet counts dropped to 25% and 15% of day 2 values and did not rebound by day 24. Because CBCs were performed less frequently than were other hematologic tests, it was not clear whether granulocyte or platelet counts were associated with other measures, but they did not seem closely related to changes in parasitemia.

As we described above (vaccine trial 1, second challenge), we established a baseline corrected reticulocyte value by means of the mean daily reticulocyte counts from day 1 to day 6. As before, any reticulocyte count from day 7 to day 28 that was above that mean plus 2 standard deviations was arbitrarily designated as "elevated." The mean hematocrit for days preceding an elevated reticulocyte count was 40.2% versus 45.3% for those days after a day with a nonelevated reticulocyte count ($P = 0.08$, paired *t*-test). Mean parasitemia for the same days were 5,968 parasites/µL (elevated reticulocyte count) versus 38,927 parasites/µL (nonelevated reticulocyte count) ($P = 0.006$, paired *t*-test).

Modified DATs for IgG and complement component C3d were performed on days 1, 4, 8,13, 16, 20, 24, and 28 of the 28-day follow-up period; all tests in all monkeys were negative.

DISCUSSION

Anemia is a frequent and often serious complication in *P. falciparum* malaria.[11] This malaria anemia could be caused by any of 3 mechanisms: lysis of infected erythrocytes, lysis or sequestration of noninfected erythrocytes, and suppression of hematopoiesis. Work by others has made it clear that more than one of these mechanisms is involved.[12-14]

It is immediately clear from the data obtained in the first study (repeated infections) that lysis of infected erythrocytes alone cannot account for the often marked decreases in hematocrit. For example, in the *Aotus* subjected to repeated infection, 3 animals with maximum parasite densities never exceeding 11,000 parasites/µL experienced decreases in hematocrit levels of between 50% and 60%. This means ~ 0.2% of circulating erythrocytes were parasitized, ~ 250 times too few erythrocytes to explain the removal from circulation of more than half these animals' erythrocytes. Minimum hematocrit levels were, on average, reached between days 17 and 21. Such a rapid onset of anemia indicates that suppression of bone marrow alone could not produce these dramatic decreases in hematocrit. If all erythropoietic activity ceased on day 0 of the challenge, by day 20, the monkey should have lost only ~ 20% of its erythrocytes—clearly not enough to explain a 50% decrease in the hematocrit. Because dyserythropoiesis cannot account for the entire decrease in hematocrit does not mean, however, that it is not a contributing factor. Because neither lysis of parasitized erythrocytes nor dyserythropoiesis seems able to account for the entire decrease in hematocrit levels, lysis or removal of noninfected erythrocytes is invoked as the major contributor to the anemia.

In 3 subsequent trials with *P. falciparum* blood stage challenges, we evaluated the following: (1) whether immune-mediated lysis of the noninfected erythrocytes could be the

TABLE 4

Maxima and minima for recorded blood values for *Aotus nancymae* challenged with 10^4 *Plasmodium falciparum* and followed daily for 28 days

Monkey	Group*	Max para ($\times 10^{-3}$)	Max day	Min HCT	Min Day	Max retic	Day retic	Day treatment	Treatment
1061	Dv_Pv	36	14	24	21	3.220	28	21	HCT
1062	Dv_Pv	378	13	42	15	4.522	20	13	Para
1063	Dv_Pv	425	11	41	11	3.255	24	11	Para
1064	Dv_Pv	390	13	29	20	4.086	28	13	Para
1054	Dv_Pv	276	14	29	20	3.418	28	20	HCT
1065	Dv_Pv	185	15	23	17	4.970	24	17	HCT
Mean	Dv_Pv	281.7	13.3	31.3	17.3	3.9	25.3	15.8	–
1049	Dc_Pc	455	12	39	28	3.575	24	12	Para
1067	Dc_Pc	309	11	30	24	2.574	28	11	Para
1072	Dc_Pc	152	11	26	28	2.118	9	25	HCT
1085	Dc_Pc	302	10	41	13	3.177	28	10	Para
1031	Dc_Pc	525	11	39	20	2.354	24	11	Para
1043	Dc_Pc	351	12	47	20	3.459	24	12	Para
Mean	Dc_Pc	349	11.2	37	22.2	2.9	22.8	13.5	–
1077	Dv_Pv	348	13	39	28	3.089	1	13	Para
1074	Dv_Pv	138	14	25	20	3.604	28	18	HCT
1071	Dv_Pv	372	13	25	20	1.370	6	13	Para
1092	Dv_Pv	380	12	49	17	2.699	16	12	Para
1056	Dv_Pv	142	14	24	24	3.139	11	24	HCT
1098	Dv_Pv	148	11	29	22	4.073	27	28	NT
Mean	Dv_Pv	254.7	12.8	31.8	21.8	3	14.8	18	–
Mean	All	295.1	12.4	33.4	20.4	3.3	21	15.8	–

* The first entry identifies the immunogen used in first 3 immunizations, and the second entry identifies immunogen or immunogens used in the fourth immunization. max para = maximum parasitemia observed during the 28-day follow-up period; max day = day of maximum parasitemia; min HCT = minimum hematocrit levels observed during the 28-day follow-up period; max retic = maximum reticulocyte count during the 28-day follow-up period; day retic = day of maximum reticulocyte count; Dv = P.f. EBA DNA vaccine; Pv = P.f. EBA protein vaccine; HCT = hematocrit > 50% below baseline; Para = parasitemia > 300,000 parasites/μL; Dc = DNA vaccine control; Pc = adjuvant control; n = nothing; NT = not treated for either elevated parasitemia or decreased hematocrit levels, but treated per protocol at the end of the 28-day follow-up period; P.f. = *P. falciparum*; Day HCT = day of minimum hematocrit.

mechanism driving the severe decreases in hematocrit in the *Aotus*, (2) any association between parasitemia density and hematocrit; (3) any association among parasitemia, hematocrit level, and reticulocytosis; (4) and association of parasitemia and anemia on other blood cell types.

By use of fresh whole blood collected during the challenge phase of the rechallenge of vaccine trial 1, we tested for the presence of IgG on erythrocyte surfaces with a protein A/protein G column system. All samples from all animals were negative. During the challenge in vaccine trial 2, we tested the erythrocytes for both IgG and complement fragment C3d by using specific antibody. In all cases, the tests were negative for both. DAT studies of human volunteers in malarious areas have not been entirely consistent. Facer and others[15] studied 162 Gambian children with variable malaria histories and found that ~ 50% of those with past or current malaria infection had C3d, IgG, or both on their erythrocytes. There was no correlation between DAT positivity and parasitemia density at the time they sought care, but they did observe a significant association between a positive DAT and a hematocrit level < 30%. Abdalla and Weatherall[16] studied 134 Gambian children and found that 52 had positive DATs (twice as many as a result of C3 as IgG). They also found, however, that hemoglobin levels and reticulocyte counts were the same in these children whether they had positive or negative DATs. Merry and others[17] studied 83 *P. falciparum* malaria patients in Thailand. There was no difference in the number of IgG molecules per erythrocyte between uncomplicated malaria cases and healthy controls. The authors thought that this low level of coating would not have caused accelerated destruction of these cells. They also found no association between the presence of IgG on the surface of erythrocytes and anemia.

TABLE 5

Mean blood cell values from 18 monkeys in vaccine trail 2*

Postchallenge day	White blood cells	Lymphocytes	Monocytes	Granulocytes	Platelets
2	11.3 (2.5)	7.0 (1.6)	1.2 (0.4)	3.2 (1.5)	406 (82)
9	6.8 (2.1)	3.1 (1.4)	0.5 (0.4)	3.2 (1.4)	315 (66)
17	8.5 (3.1)	6.6 (2.2)	1.1 (0.6)	0.8 (0.6)	64 (62)
24	12 (5.3)	9.8 (4.8)	1.3 (0.6)	0.9 (0.7)	117 (90)

* Blood for complete blood counts was drawn on days 2, 9, 17, and 24 during the 28-day postchallenge follow-up period. Values are cells/μL × 10^3 with standard deviations in parentheses.

In their study of anemia in young Gambian children, Abdalla and others[2] were able to divide the subjects into 3 groups: acute malaria (brief history, high parasitemia, hemoglobin ≥ 8 g/dL), anemic acute malaria (brief history, high parasitemia, hemoglobin < 8 g/dL), and chronic malaria (no history of acute illness, low parasitemia, and hemoglobin < 6 g/dL). The experimental malaria induced in the *Aotus* in this study most closely parallels the first group. Monkeys being challenged for the first time become parasitemic rapidly, and most required drug therapy by day 12 as a result of parasitemias exceeding 300,000 parasites/μL. In those animals not receiving treatment for elevated parasitemia, the lowest hematocrit level recorded occurred ~ 6 to 9 days later than the maximum parasitemias. Simple linear regression analysis suggested that monkeys with high maximum parasitemias tended to have less anemia, but this assessment is badly confounded by the fact that monkeys with high parasitemias were treated, whereas those with low parasitemias were not. This means that only animals with low parasitemias remain parasitemic after approximately day 12, and this may account for the differences in incidence and intensity of anemia. Carvalho and others[18] also had a case of severe anemia among 5 *Aotus infulatus* infected with *P. falciparum*. Three of the animals

were intact, and 2 were splenectomized. The 2 splenectomized animals rapidly developed high parasitemias (> 5%) by day 10 and required treatment; they did not become anemic. The one intact animal that controlled its parasitemia such that it did not require treatment developed a profound anemia (hematocrit of 13%).

Splenic enlargement, often associated with nonacute parasitemias, may itself play a part in the induction of anemia. Looareesuwan and others[19] showed that clearance of noninfected, radiolabeled erythrocytes was increased in *P. falciparum*–infected humans only when splenomegaly was present. Infected humans free of splenomegaly had normal clearance rates unless treated with antimalarial drugs, and then clearance of noninfected cells also increased.

Reticulocyte counts in vaccine trial 1—second challenge were associated statistically with both hematocrit and parasitemia, particularly the latter. The mean hematocrit level calculated for days with normal reticulocyte counts was about 5 percentage points higher than the mean hematocrit calculated from days with elevated reticulocyte counts (46.7 versus 41.7 and 45.3 versus 40.2 in the 2 studies). In both studies in which reticulocyte count data were collected, the mean parasitemia on days with elevated reticulocyte counts was much lower (4.3- or 6.5-fold) than on days with nonelevated reticulocytes. In addition, in 29 (69%) of 42 monkeys across 2 different studies, reticulocyte counts did not rise above normal until the parasitemia had dropped below ~ 100 parasites/μL, irrespective of hematocrit levels. The most obvious explanation for this is that a high parasitemia suppresses either the production or the release of reticulocytes in spite of the presence of an anemia driving reticulocyte release.

The appearance of the reticulocytosis was similar to that documented by Abdalla and others.[2] Those patients they classified as having "acute malaria" (short history and high parasitemia) experienced a drop in hemoglobin when parasites disappeared as a result of drug treatment. They also observed that reticulocytosis developed 5 days after treatment—that is, after parasitemia had fallen. They also studied the bone marrow of the patients with acute malaria and found the relative number of erythroid precursors to be normal or low, and erythropoiesis to be normoblastic. Bone marrow from patients with chronic malaria, on the other hand, showed significant evidence of dyserythropoiesis.

CBC data, collected at 4 points during the 28-day follow-up period in vaccine trial 2, showed changes in white blood cell populations. Lymphocytes and monocyte counts dropped modestly between days 2 and 9, but they recovered by day 17. Granulocyte and platelet levels, on the other hand, showed marked drops through day 17, with no or incomplete recovery by day 24. Kakoma and others[20] studied changes in blood cell values in *Aotus nancymai* monkeys infected with *P. falciparum*. They tested the animals on day 0, 7, and 14 after challenge, and we tested on days 2, 9, 17, and 24. They also noted severe thrombocytopenia, particularly on day 14. This is similar to our finding of significant thrombocytopenia on day 17 after challenge. They noted, as did we, that lymphocyte numbers decreased earlier than platelets and rebounded by 14 or 17 days. In a study of radiolabeled platelet distribution in humans, Aster[21] found that a significant fraction of the platelets can pool in the spleen. In cases of hypersplenism (*Aotus* experience enlarged spleens when infected with *P. falciparum*), that fraction can increase to 50% or even 90%.

Twenty-five (30.5%) of 82 of the *P. falciparum* infections in *Aotus* described in this study resulted in a > 50% decrease in hematocrit. Lysis of infected erythrocytes is insufficient to explain the removal of so many erythrocytes from circulation. In fact, 18 of the 32 monkeys experiencing ≥ 50% decreases in hematocrit had parasitemia that never exceeded 100,000 parasites/μL or ~ 2% of erythrocytes infected. This is ~ 1.5 orders of magnitude too few to explain the removal of 50% of circulating erythrocytes. In the repeated-infection experiment, 3 of 8 monkeys experienced a > 50% decrease in hematocrit levels, yet no maximum parasitemia exceeded 11,000 parasites/μL, 2.5 orders of magnitude too few infected erythrocytes to explain the anemia. Although the mean day of maximum parasitemia for the 82 infections was day 12.5 (95% CL = 11.9, 13.1), the mean day of minimum hematocrit occurred later at day 18.8 (95% CL = 17.8, 19.9). This is too soon for marrow suppression to explain the observed loss of erythrocytes.

Because neither lysis of infected erythrocytes nor marrow suppression alone can explain the anemia, we suggest that lysis of uninfected erythrocytes is the major contributor to the anemia. The failure to find either antibody or complement on the surface of erythrocytes harvested while the monkeys were infected indicates that an antibody-mediated or C3d-mediated lysis was not occurring. Although lysis of infected and noninfected erythrocytes seems to be the major contributor to the anemia, there is evidence of marrow suppression. The appearance of a reticulocytosis that correlated only with low, and not high, parasite densities, indicating that high parasite densities suppress the development or release of reticulocytes from the marrow. This suppression may also explain the thrombocytopenia seen in some of the monkeys. The dramatic decrease in platelets may also be caused by pooling in the enlarged spleens often seen in *P. falciparum*–infected *Aotus* monkeys.

Because most of the *P. falciparum* infections occurred in the context of EBA-175 vaccine trials, we looked for an association between this specific vaccine immunogen and anemia, but we did not find one. In addition, an efficacy trial in *Aotus* of another vaccine immunogen (merozoite surface protein 1) showed similar instances of marked anemia.[22] Also, some of the cases of anemia reported here were seen in monkeys not immunized with a specific vaccine immunogen, but rather in animals subjected only to repeated infection with *P. falciparum*. It is our view that anemia onset is related to the acquisition by the monkey of sufficient immunity to partially control its parasitemia. We believe that this partial control results in a continuing low-level parasitemia that is the proximate cause of the anemia. This would mean that the anemia could be induced by any antigen or agent capable of inducing a modicum of immunity and that the anemia is not associated with one or another specific immunogen. The mechanism we propose here may be a major contributor to the anemia seen in children in Africa at the end of the intense transmission season.[23] If this is the case, it will be extremely important to monitor hemoglobin status during trials of malaria vaccines that are designed not to prevent blood stage infection, but rather to limit it.[24]

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Disclaimer: The experiments reported here were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The opinions and assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy or the U.S. Department of Defense.

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