

ASSOCIATION OF HAPTOGLOBIN LEVELS WITH AGE, PARASITE DENSITY, AND HAPTOGLOBIN GENOTYPE IN A MALARIA-ENDEMIC AREA OF GABON

FREYA J. I. FOWKES,† HEATHER IMRIE,† FLORENCE MIGOT-NABIAS, PASCAL MICHON, ANITA JUSTICE, PHILLIPE DELORON, ADRIAN J. F. LUTY, AND KAREN P. DAY*

Peter Medawar Building for Pathogen Research and Department of Zoology, University of Oxford, Oxford, United Kingdom; Institut de Recherche pour le Développement, Dakar, Senegal; Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea; Institut de Recherche pour le Développement, Unité de Recherche 10 Mother and Child in the Tropics, Faculté de Pharmacie, Paris, France; Human Parasitology, Institute for Tropical Medicine, University of Tübingen, Tübingen, Germany; Department of Medical Parasitology, New York University, New York

Abstract. Haptoglobin (Hp) levels were investigated in relation to host genotype in a malaria-endemic area in Gabon. A cross-sectional study of 1–12-year-old children was conducted in the rainy season, a period of high malaria transmission, to examine this relationship. Variables that influenced Hp levels were Hp genotype, location, and age interacting with parasite density. At low parasite densities, there was a negative correlation between Hp levels and age. At higher densities, there was a positive correlation with age. This suggests that in the presence of greater parasite-induced hemolysis, older children are capable of increased production of Hp. Sick cell trait and ABO blood group was not associated with Hp levels in this population.

INTRODUCTION

Haptoglobin (Hp) is an acute-phase protein that binds irreversibly to hemoglobin (Hb), enabling its safe and rapid clearance. Therefore, Hp has an important protective role in hemolytic disease because it greatly reduces the oxidative and peroxidative potential of free Hb.¹ Haptoglobin exists in three phenotypic forms: Hp 1-1, 2-1, and 2-2, which are encoded by two co-dominant alleles, Hp^1 and Hp^2 . The Hpl allele can be further sub-typed into HplF and HplS.² The ability to bind Hb is phenotype dependent and has been found in the order of 1-1 > 2-1 > 2-2, with the binding capacity reflecting the plasma levels of the various phenotypes.³ Functional differences between HplF and HplS are unknown. The Hp levels can be influenced by age, hemolysis, and particularly the acute-phase response.⁴

Hp is directly toxic to *Plasmodium falciparum* in vitro at concentrations that may occur during an acute-phase response.³ The exact mechanism of action is not known. Hp does not enter the infected erythrocyte but may act indirectly, disrupting normal parasite protein trafficking within the host cell.⁵ An *in vivo* model of malaria infection has demonstrated that parasite burdens and peak parasite densities were higher in Hp knockout mice.⁶ In addition, Hp is also antimicrobial; it makes iron unavailable to bacteria using Hb, (e.g., *Escherichia coli*) and causes agglutination of *Streptococcus pyogenes*.⁸ Levels of Hp are reduced by both chronic, low level parasitemia and possibly malaria-associated immune complex destruction of infected erythrocytes, as well as clinical malaria.^{9,10}

We recently demonstrated, in a malaria-endemic area of Papua New Guinea that individuals homozygous for a⁺-thalassemia had increased levels of Hp compared with heterozygotes when harboring chronic parasitemia (Imrie H. and others, unpublished data). We therefore decided to investigate whether the sickle cell trait influenced Hp levels in the face of malaria infection. Sickle cell Hb (HbS) is the most frequent abnormal Hb trait found in populations of African origin and is the result of an amino acid substitution on the (3 chain of Hb.¹¹ Individuals who are homozygous (HbSS) for

the HbS variant of Hb have severe consequences of sickle cell disease, whereas HbAS carriers have been shown to enjoy substantial protection against severe *P. falciparum* malaria compared with HbAA individuals.¹² The mechanism by which HbAS affords protection is yet to be elucidated. Studies on differences in hematologic parameters between normal Hb genotypes (HbAA) and sickle cell trait (HbAS) have been inconsistent.¹³

To date, Hp levels have not been directly measured in relation to sickle cell genotype in a malaria-endemic area. One previous study investigated the association between prevalence of ahaptoglobinemia and HbS and found no correlation.¹⁴ We conducted a cross-sectional study in children living in southeast Gabon to examine the relationship of host genotype and Hp levels in a malaria-endemic area. The prevalence rates of three genetic polymorphisms, Hp genotype, sickle cell trait, and ABO blood group, as well as parasitologic criteria, were determined. The relationships between these variables and median Hp levels are presented in this report.

MATERIALS AND METHODS

Study area. The study was conducted in two villages (Dienga and Bakoumba) in southeast Gabon near the Congo border. Malaria is highly endemic with peaks of transmission at the end of the rainy seasons (September–December and March–June).¹⁵ A cross-sectional survey was conducted in May–June in 2000 in a cohort of 641 asymptomatic children 1–12 years of age. This study was initially designed to investigate antibody properties in relation to erythrocyte polymorphisms. Details on the study population and data collection procedures have been published elsewhere.¹⁶ Briefly, after obtaining informed consent from all individuals and/or their parents, venous blood was collected in tubes containing EDTA for parasitologic assessment, genotyping of the sickle cell trait, and determination of ABO blood group. Unsuccessful Hb phenotyping was due to an insufficient volume of sample rather than technical problems. The study was reviewed and approved by the ethics committee of the International Center for Medical Research of Franceville.

Parasitologic measurements. Parasite densities were counted per 500 leukocytes on Giemsa-stained thick blood smears, and were recorded as the number of parasites per

† These authors contributed equally to this work.

* Address correspondence to Karen P. Day, Department of Medical Parasitology, New York University School of Medicine, 341 East 25th Street, New York, NY 10010. E-mail: karen.day@med.nyu.edu

microliter of blood, assuming an average leukocyte count of 8,000/ μ L.¹⁷ Duplicate readings were made for a random 15% of smears to ensure quality control.

Plasma Hp levels. Plasma Hp levels were determined by an enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with rabbit anti-human Hp (H-8636; Sigma, St. Louis, MO) as capture antibody and monoclonal anti-human Hp (H-6395; Sigma) as detection antibody. Immunoplates (F96 maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with rabbit anti-human Hp (H-8636; Sigma) capture antibody diluted in phosphate-buffered saline (PBS) to a final concentration of 30 (μ g/mL (100 (μ L/well). Blank wells were coated with PBS alone. Wells were washed three times with 200 (μ L/well of PBS. Bovine serum albumin (BSA) (3%) in PBS was used to block the wells (200 (μ L/well for 1 hour at room temperature). Samples were diluted 1:10,000 in 3% BSA/PBS and Hp standards (100 ng/mL, 80 ng/mL, 60 ng/mL, 40 ng/mL, 20 ng/mL, and 0 ng/mL) prepared using pooled Hp (Sigma) in 3% BSA/PBS. Aliquots of 100 (μ L were added to wells in duplicate, incubated for 1 hour at room temperature, and washed three times in PBS-Tween 20 (subsequent incubations and washes were similar). Detection was made with monoclonal anti-Hp (H-6395; Sigma) diluted in 3% BSA/PBS (final concentration = 11.7 (μ g/mL), followed by sheep anti-mouse IgG alkaline phosphatase conjugate (A3563; Sigma) diluted 1:30,000 in 3% BSA/PBS. The substrate used was *p*-nitrophenyl phosphate (Sigma) 1 mg/mL in 10% diethanolamine, pH 9.8, containing 0.5 mM MgCl₂, 100 (μ L/well. Samples were incubated in the dark at room temperature and read at 405 nm. A standard curve was made from which sample levels were read and multiplied by the dilution factor, giving sample Hp concentrations of 0 mg/mL or within the range 0.01-1 mg/mL. Samples with > 1 mg/mL were diluted appropriately before repeating the ELISA.

Human genetic factors. Blood group was determined by serologic analysis and the sickle cell trait was detected by Hb electrophoresis.¹⁸ The Hp genotype was determined by a polymerase chain reaction (PCR) using a method previously described with modifications.¹⁹ The DNA was extracted from blood spots on filter paper using the QIAamp DNA Mini kit (Qiagen, Valencia, CA). Three PCRs were performed on each sample to genotype the Hp1S, 1F, and 2 alleles using forward primers C51, 5'-GCA ATG ATG TCA CGG ATA TC-3' and F3, 5'-CAG GAG TAT ACA CCT TAA ATG-3' and reverse primers C42, 5'-TTA CAC TGG TAG CGA ACC GA-3', C72, 5'-AAT TTA AAA TTG GCA TTT CGC C-3' and S2, 5'-TTA TCC ACT GCT TCT CAT TG-3'. The combination of F3 and C42 identified the Hp2 allele, C51 and S2 the Hp1S allele, and F3 and C72 the Hp 1F allele. The PCR was performed using 0.025 units/ μ L of HotStar Taq DNA polymerase (Qiagen) with supplied buffer, oligonucleotide primers (400 nM each), and dNTPs (200 (μ M each). The temperature cycles used were 94°C for 15 minutes, 35 cycles at 94°C for 40 seconds, 52°C for 1 minute for the C51-S2 and F3-C72 reactions and 58°C for 1 minute for the F3-C42 reactions, and 72°C for 2 minutes. Products of 1,400, 1,200, and 935 basepairs were obtained for the Hp1F, Hp1S, and Hp2 alleles, respectively.

Statistical analysis. Missing value analysis and frequency distribution analysis was assessed by chi-square tests. The association between categorical variables and parasite density was assessed using Kruskal-Wallis tests. A general linear

model was used to examine the effect of variables of interest on Hp levels. Since Hp levels showed heteroscedasticity (non-constancy of the variance), they were transformed before analysis using the transformation log (Hp + 0.0015). This was chosen by examining profile likelihoods.²⁰ The model was developed using R 1.6.2 (R Foundation for Statistical Computing, Vienna, Austria) and stepwise fitting with Akaike information criterion using a dataset that omitted all subjects for whom any data was missing.²⁰ The full model examined the effect of age (continuous or three categories [1-4, 5-9, and 10-12 years of age]), *Plasmodium* density (log(*Plasmodium* + 1) and 4 categories: 0, 1-99, 100-1,000, and >1000 parasites/ μ L), *P. malariae* and *P. falciparum* positivity (two categories), location, Hp genotype (three categories and six categories), sex, ABO blood groups together with sickle cell phenotype, and interactions between variables. Four hundred eighty-eight subjects from a cohort of 641 children 1-12 years of age were included in the final analysis. Exclusions included subjects who could not be phenotyped for sickle cell (n = 52), subjects whose Hp levels could not be determined (n = 78) missing values (n = 11), outliers (n = 8) and, due to small numbers, HbSS (n = 4). The final model was refitted to all data and variables with *P* values > 0.05 were removed. SPSS for Windows version 11.5 (SPSS, Inc., Chicago, IL) was used for initial examination of the data and for the final stage of the analysis.

RESULTS

This study investigated the association between host genotype and Hp levels together with parasitologic factors in a malaria-endemic area. A total of 641 asymptomatic children 1-12 years of age were recruited into the study. The Hp levels and sickle cell phenotype was successfully determined in 511 subjects. There was no statistical difference between the 511 children included in this analysis and those who were not included with respect to any of the other variables (*P* > 0.05). The genotypic, parasitologic, and other frequencies of variables of these 511 children are shown in Table 1.

The study population, which was composed of equal numbers of males and females, had only four children (0.8%) with the HbSS genotype; whereas 79.6% had the HbAA genotype. Approximately half (52.6%) the subjects were blood group O. These genetic frequencies are consistent with other studies done in the same area.¹⁸ Two hundred thirty-four children (45.8%) were slide negative for any species of *Plasmodium*. Of those who were positive, approximately half had greater than 100 parasites/ μ L of blood. The dominant species was *P. falciparum*, which was found in 51.7% of the cohort. The median parasite count (range) according to age group was as follows: 1-4 years of age, 0 (0-200,000) parasites/ μ L; 5-9 years of age, 107 (0-58,560) parasites/ μ L; and 10-12 years of age, 160 (0-14,400) parasites/ μ L. There was no significant association between age group and parasite prevalence (*P* = 0.13) or parasite density (*P* = 0.99). The median Hp level (range) of the 511 samples was 0.125 (0.0-3.35) mg/mL. Four individuals were ahaptoglobinemic. There was no association between any of the malarimetric indices and host genotype with respect to Hb phenotype (*P* > 0.1).

A general linear model was used to examine the effect of variables of interest on transformed Hp levels in samples from the cohort. The resulting fitted model predicts the me-

TABLE 1

Genetic, demographic, and parasitologic characteristics of 511 children successfully genotyped for hemoglobin S*

Variable	
Haptoglobin, median (range), mg/mL	0.125 (0.0-3.35)
Sickle cell genotype	
A/A	407 (79.6)
A/S	100 (19.6)
S/S	4 (0.8)
ABO blood group†	
A	132 (25.8)
B	87 (17.0)
AB	15 (2.9)
O	269 (52.6)
Haptoglobin genotype	
1-1	84 (16.4)
1F-1F	83 (16.2)
1S-1S	20 (3.9)
2-1	168 (32.9)
1F-2	75 (14.7)
1S-2	81 (15.9)
2-2	81 (15.9)
Sex	
Male	265 (51.9)
Age groups, years†	
1-4.9	147 (28.8)
5-9.9	295 (57.7)
10-12	58 (11.4)
Location	
Dienga	33 (6.5)
Bakoumba	478 (93.5)
<i>Plasmodium</i> density, [xL]†	
0	234 (45.8)
1-99	27 (5.3)
100-999	121 (23.7)
> 1,000	126 (24.7)
<i>P. falciparum</i> positive	264 (51.7)
<i>P. malariae</i> positive	24 (4.7)

* Values are no. (%) unless otherwise indicated.

† Some values are missing.

dian Hp level for the population controlling for relevant variables. After exclusions, 488 subjects were included in the final model. The final model chosen included location, Hp genotype, and parasite density interacting with age. The Hp levels did not significantly vary with sickle cell phenotype ($P > 0.05$). Median levels (95% confidence interval [CI]) in children with the HbAA phenotype were 0.122 (0.105, 0.141) mg/mL compared with 0.128 (0.095, 0.172) mg/mL in HbAS children. There was no significant difference in median Hp levels between ABO blood group, sex, or parasite positivity ($P > 0.05$).

The Hp levels were higher in samples from Bakoumba compared with those from Dienga ($P < 0.001$). The model also predicts a difference in median Hp level between the different Hp genotypes with higher levels in 1-1 (0.115 [95% CI = 0.085, 0.157] mg/mL) compared with 2-1 (0.057 [0.042, 0.077] mg/mL) and 2-2 (0.058 [0.039, 0.085] mg/mL) (overall $P < 0.001$). There was no significant difference between the different 1F and 1S subtypes of 1-1 and 2-1 ($P > 0.3$). The Hp level decreased with increasing parasite density ($P < 0.001$). There was no significant effect of age as a main effect in the model ($P = 0.21$). However, there was evidence of an interaction between age and parasite density ($P = 0.001$). Figure 1 shows that in low level infection (0 and 1-99 parasites/xL), Hp levels decrease with age ($r = -0.57$ and $r = -0.76$, respectively). In high parasitemic infections (100-999 and > 1,000 parasites/xL), the Hp level increased with age ($r = 0.61$ and $r = 0.65$, respectively).

DISCUSSION

In malaria-endemic areas, low levels of Hp reflect recent parasitemia and malaria-induced hemolysis, as well as transmission intensity.²¹ Levels of Hp in this study were low compared with Caucasians and Africans without malaria but comparable to those of asymptomatic malaria-infected populations in Africa.^{10,22-24} The prevalence of ahaptoglobinemia was low compared to other studies, where prevalences of 20-50% were found.^{9,14,21,25-34} This discrepancy can be explained by the different methodologies used to define ahaptoglobinemia. In our study, the sensitivity of the ELISA was high and we were able to detect Hp levels as low as 0.01 mg/mL. Earlier studies used either protein electrophoresis to determine phenotype or an ELISA in which the lower limit of detection was 0.05 mg/mL. A total of 34.2% of our population had Hp levels < 0.05 mg/mL, which is consistent with the aforementioned studies.

In this population, Hp levels were associated with Hp genotype, location, and age interacting with parasite density. As expected, the Hp levels were higher in the 1-1 genotype compared with 2-1 and 2-2.² Children from the Dienga region had lower Hp levels than children in Bakoumba, which reflected differences in transmission. During the rainy season, the entomologic inoculation rates in Bakoumba and Dienga are 0.83 and 0.91 infective bites/person/night, respectively.^{15,35} The Hp levels were significantly negatively correlated with parasite density. This finding is consistent with a study performed in Tanzania,¹⁰ although another study in The Gambia showed no association between parasite density and Hp levels.³⁶

The finding of most interest in our study was the interaction between parasite density and age. At negative or low parasitemias (< 100 parasites/jxL), Hp levels decreased with age. In contrast, at higher parasitemias (> 100 parasites/jxL), Hp levels increased with age. All age groups experienced the full range of parasite densities, so it is unlikely that age distribution within each parasite category biased the results.

The inverse relationship between age and Hp in non-infected subjects is similar to that seen in non-malarious populations of the same age, although the reason for this is unknown.^{37,38} In parasitized subjects, it is known that a positive correlation exists between interleukin-6 (IL-6)-dependent C-reactive protein (CRP) and parasite density.^{10,23,36,39} An interaction between age and parasite density is yet to be investigated with respect to CRP or other acute-phase proteins.

The interaction in our data indicate that Hp production is activated relative to the level of malaria-induced hemolysis. Older children may have an enhanced response to this hemolysis by increasing production of Hp induced by IL-6.⁴⁰ It is possible that the improvement of the acquired immune response, together with the acute-phase response with age, enables Hp production to be enhanced during the mass hemolysis that occurs during high-density infections. The interaction between acquired and innate immunity could provide an explanation for the interaction between parasite density and age.

This study also examined the affect of the HbAS phenotype on median levels of Hp. It might be expected that this trait alters Hp levels due to its effects on levels of hemolysis. This may occur by increased erythrocyte turnover in the HbAS genotype because of altered Hb structure or decreased turnover of erythrocytes because of reduced levels of para-

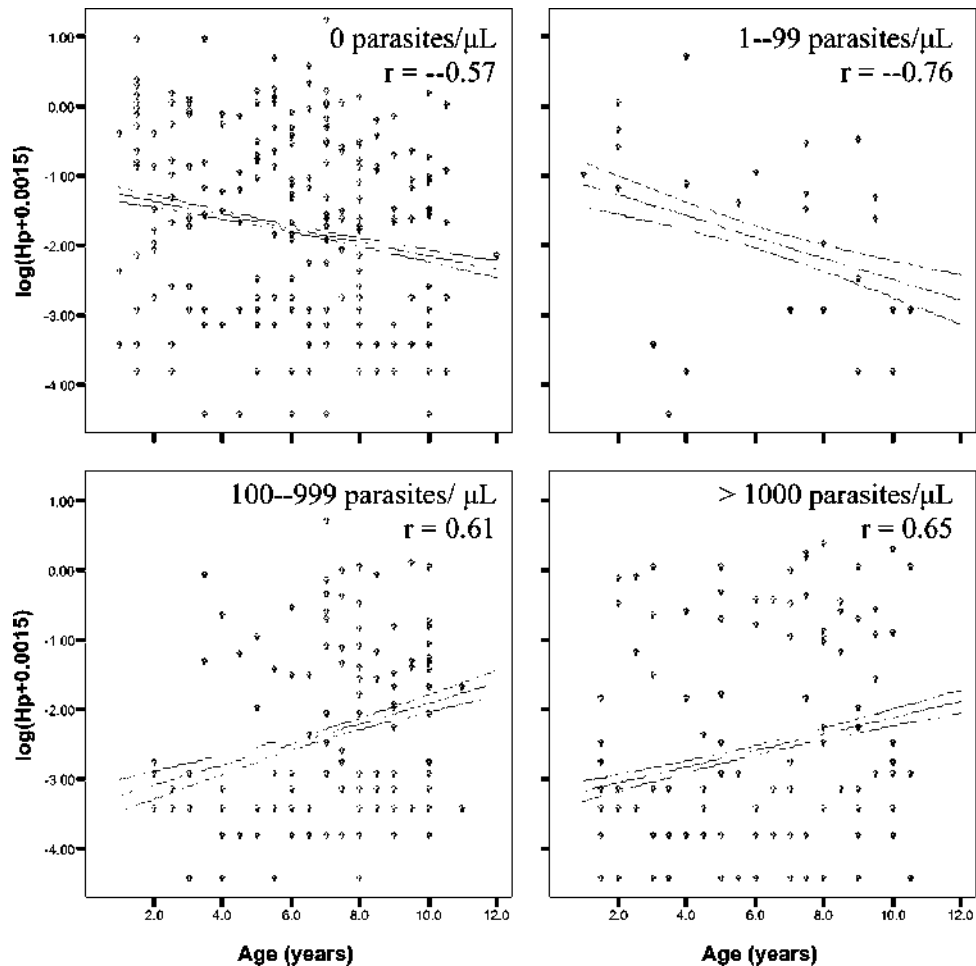


FIGURE 1. General linear model showing evidence of interaction between age and parasite density ($P = 0.001$). Haptoglobin (Hp) levels are associated with parasite density and age. At low parasite densities (< 100 parasites/ μL), Hp (transformed data) is negatively correlated with age, but at high parasite densities (> 100 parasites/ μL), Hp is positively correlated with age. Lines represent predicted Hp values with 95% confidence interval from modeled data, i.e., controlling for relevant variables. Dots represent unmodeled data.

sitemia in this protective genotype.⁴¹ This study did not demonstrate a significant difference between median Hp levels and HbS phenotype. This result is also consistent with our finding of no evidence of increased parasitemia in either HbAA or HbAS phenotype and provides further evidence of indistinguishable hematologic parameters between these two phenotypes.

Collection of samples took place during the wet season when parasite burdens are high. High parasitemias could mean that all subjects, regardless of Hb genotype, have very low levels of Hp due to high levels of hemolysis and clearance of the Hp-Hb complex. To fully investigate the effect of Hb genotype on Hp levels, and its potential role in malaria, repeating the survey would be required during a period of low transmission or in another country where malaria transmission is strictly seasonal. The interaction of parasite density with age warrants further investigation into the role of Hp and other acute phase proteins in the protection against malaria.

Received January 18, 2005. Accepted for publication August 29, 2005.

Acknowledgments: We are grateful to the children and their families of Bakoumba and Dienga for their willing participation in the study. We thank Justice Mayombo, Faustin Lekoulou, and Hubert Mou-

kana for excellent technical assistance and Dr. René Nabias for determination of ABO blood groups and hemoglobin phenotypes. We also thank Jean Bourgeois, (Societe d'Exploitation des Produits Alimentaires) for his logistical support in Bakoumba.

Financial support: This study was supported by The Wellcome Trust and the European Union INCO Programme (contract no. IC18-CT98-0359).

Authors' addresses: Freya J. I. Fowkes and Karen P. Day, Department of Medical Parasitology, New York University School of Medicine, 341 East 25th Street, New York, NY 10010, Telephone: 212-263-6800, Fax: 212-263-8116, E-mails: freya.fowkes@med.nyu.edu and karen.day@med.nyu.edu. Heather Imrie and Anita Justice, Peter Medawar Building for Pathogen Research, Department of Zoology, South Parks Road, Oxford University, Oxford, OX1 3SY, United Kingdom, E-mails: heather.imrie@medawar.ox.ac.uk and neatsey@hotmail.com. Florence Migot-Nabias, Institut de Recherche pour le Développement, Unité de Recherche 10, BP 1386, CP 18524 Dakar, Senegal, Telephone: 221-849-36-11, Fax: 221-832-43-07, E-mail: migot@ndakaru.ird.sn. Pascal Michon, Papua New Guinea Institute of Medical Research, P.O. Box 378, Madang 511, Papua New Guinea, Telephone: 675-852-2909, Fax: 675-852-3289, E-mail: pmichon@datec.net.pg. Philippe Deloron, Institut de Recherche pour le Développement, Unité de Recherche 10 Mother and Child in the Tropics, Faculté de Pharmacie, 4 Avenue de l'Observatoire, 75006 Paris, France, Telephone: 33-1-53-73-96-22, Fax: 33-1-53-73-96-17, E-mail: philippe.deloron@ird.fr. Adrian J. F. Luty, Medical Parasitology, De-

partment of Medical Microbiology, University Medical Centre, St. Radboud, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands, E-mail: a.luty@mmb.umcn.nl.

Reprint requests: Karen P. Day, Department of Medical Parasitology, New York University School of Medicine, 341 East 25th Street, New York, NY 10010.

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