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GENETIC COMPLEXITY OF *PLASMODIUM VIVAX* PARASITES IN INDIVIDUAL HUMAN INFECTIONS ANALYZED WITH MONOCLONAL ANTIBODIES AGAINST VARIANT EPITOPES ON A SINGLE PARASITE PROTEIN

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Abstract. Monoclonal antibodies against variant epitopes of a highly polymorphic protein (PV200) in schizonts of *Plasmodium vivax* have been used to analyze the variety of genetically distinct populations of parasites present in the peripheral blood of individual *P. vivax* infections in Sri Lanka. In 9 out of 10 isolates of freshly drawn *P. vivax* infected blood from different individuals, parasites of only 1 PV200 serotype was found within each individual infection, even though parasites were serotypically distinct between individuals. In 1 isolate parasite population, 3 distinct PV200 serotypes were identified. Thus, most *P. vivax* infections appeared to consist of a single genetically homogeneous population of parasites within the detection limits of the technique. The prevalence of *P. vivax* infections in an area of malaria transmission in southern Sri Lanka and the densities of oocysts in mosquitoes fed on *P. vivax* infected individuals indicated that parasite populations would be transmitted many times before encountering parasites of other origins, and that individual populations would tend to reduce to genetic homogeneity during transmission. These expectations are consistent with the high proportion of genetically homogeneous *P. vivax* isolates observed.

Populations of human malaria parasites are known to be genetically highly diverse. Variation has been shown for a wide variety of parasite properties, including, among others, the electrophoretic mobility of enzymes,[1–4] other proteins on 2-dimensional electrophoresis,[5,6] antigenic differences in specific protein antigens,[7–10] and chromosomal size polymorphism.[11,12] So many variant traits have now been defined among populations of *Plasmodium falciparum* and *P. vivax*, the 2 main human malaria species, that independently derived isolates may rarely, if ever, be characterized by the same combination of variants.

Since malaria parasites undergo fertilization between male and female gametes, potentially of different genetic composition, it is generally believed that cross-fertilization between genetically distinct populations of parasites and reassortment of genetic variation in the progeny of such hybridizations is a common occurrence during malaria transmission. However, the extent to which such cross-fertilization could take place would depend on the extent to which independently inoculated infections coincided with each other in the blood infection.

We have examined these questions in the context of *P. vivax* transmission in Sri Lanka. We have attempted to analyze the genetic complexity of individual *P. vivax* infections by studying the distribution of variants of a single *P. vivax* schizont protein, PV200,[13] using monoclonal antibodies against variant epitopes of PV200. We discuss the results in the context of epidemiological data on the incidence and duration of *P. vivax* infections in Sri Lanka and the number of fertilization events giving rise to viable zygotes when mosquitoes feed on *P. vivax* infections in humans in Sri Lanka.

MATERIALS AND METHODS

Parasites

Isolates of *P. vivax* were derived from acute malaria patients attending the General Hospital, Colombo (GHC). The GHC, as we have shown previously, provides an epidemiological sam-

pling pool for vivax malaria infections in Sri Lanka.[14] The transmission of malaria in Sri Lanka is "unstable," and malaria infections in both adults and children manifest as acute clinical disease. Malaria transmission does not occur in Colombo, and all the patients had acquired their infections following periods of travel outside Colombo. Patients were adults 22–48 years of age who had experienced 1–6 previous malaria attacks (except for 2, in whom this was the first malaria infection). Blood was drawn into sterile heparin and parasites were purified for preparation of slides for the indirect immunofluorescence test (IFT).[10] Only infections containing mature schizonts of *P. vivax* were used for this study.

Antibodies

The monoclonal antibodies (Mabs) used were all against epitopes on the PV200 schizont surface antigen of *P. vivax*. Mabs A17 and A24 were derived as previously described.[10] Mabs 200/1, 200/2, 200/3, 200/4, 200/5, and 200/6 were made following immunization of a BALB/c mouse with a fusion protein representing PV200.[13] Mabs 200/1 and 200/5 appeared to react with the same epitope. The other Mabs reacted with apparently independent epitopes. All the epitopes were variants in the *P. vivax* population in Sri Lanka. Variation in an epitope is shown when a Mab fails to react with parasites expressing the PV200 antigen. This can only be demonstrated convincingly when 2 Mabs are reacted on the same preparation of parasites and 1 gives a positive reaction, demonstrating the presence of the PV200 antigen, and the other gives a negative reaction. This approach was used in the present study: 2 Mabs were reacted successively with the same parasite preparation, each being stained with a second antibody conjugated with a different fluorochrome (fluorescein or rhodamine). In this way, the reactivity of each Mab could be visualized independently on the same preparation by examining by UV light microscopy under appropriate alternative filters.

The fluorochrome conjugates used were FITC (fluorescein) conjugated rabbit IgG against either mouse IgG1, mouse IgM, or total mouse IgG, and TRITC (rhodamine) conjugated rabbit IgG against either mouse IgG2a or mouse IgG1. All reagents were supplied by Southern Biotechnology Associates, Inc., Birmingham, AL.

Monoclonal antibodies were used in the form of hybridoma culture supernatants; the fluorochrome conjugates were used at a dilution of 1:20 in PBS. The method of reaction of parasites with Mabs for IFT was as follows: air-dried, acetone-fixed blood smears were prepared from schizont infected blood cells of each isolate of *P. vivax*. The slides were incubated with anti-PV200 Mabs and isotype-specific fluorochrome-labeled second antibodies as follows: the slides were incubated with the first anti-PV200 Mab for 30 min, then washed with PBS; the slides were then incubated for 30 min with fluorescein conjugated antibody against the isotype of the first anti-PV200 mouse Mab and then again washed with PBS; the slides were incubated for 30 min with the second anti-PV200 Mab (against a different epitope than that recognized by the first anti-PV200 Mab) and washed with PBS; slides were then incubated for 30 min with rhodamine conjugated antibody against the isotype of the second anti-PV200 mouse Mab and washed with PBS.

The slides were then mounted with glycerol plus 10% PBS with DABCO (1,4-diazabicyclo [2,2,2,] octane) (Sigma), an anti-fade reagent, under a glass coverslip and examined on a Leitz Diaplan UV microscope. Each field was examined alternatively under 2 different filters. One, blue Leitz filter block I2/3, allowed visualization of parasites stained with fluorescein; the other, green Leitz filter block N2, allowed visualization of parasites stained with rhodamine. In this way, each schizont could be scored as reacting with 1 or the other of the 2 anti-PV200 Mabs or with both. For each combination of Mabs with a given *P. vivax* isolate, a minimum of 50 schizonts were examined; generally, 100 or more were counted.

Epidemiology

A comprehensive survey of malaria transmission was made over a 17 month period between November 1986 and March 1988 at Kataragama in the south of Sri Lanka.[15] This is a region of the dry zone in which malaria is endemic, with some seasonal fluctuations, throughout the year. The study population consisted of 3,625 people. At ~6 month intervals, 4 blood surveys were done on ~50% of the total population and the point prevalence of malaria positive blood infections recorded.

TABLE 1

Analysis of PV200 serotypes in 2 isolates of Plasmodium vivax *using double staining with pairs of anti-PV200 Mabs and labeling with fluorescein or rhodamine conjugated isotype-specific antibodies*

P. vivax isolate	Anti-PV200 Mabs: Antibody conjugated with Fluorescein (green) 1st Mab (isotype)	Anti-PV200 Mabs: Antibody conjugated with Rhodamine (red) 2nd Mab (isotype)	*P. vivax* schizonts reacting with anti-PV200 Mabs: 1st Mab only (green only)	2nd Mab only (red only)	1st + 2nd Mabs (green + red)
AA 137	200/1 (IgG1)	A 17 (IgG2a)	0%	0%	100%
	200/2 (IgG1)	A 17 (IgG2a)	0%	100%	0%
	200/3 (IgG)	A 17 (IgG2a)	0%	0%	100%
	200/4 (IgG1)	A 17 (IgG2a)	0%	0%	100%
	200/5 (IgG1)	A 17 (IgG2a)	0%	0%	100%
	200/6 (IgG)	A 17 (IgG2a)	0%	0%	100%
	A 24 (IgM)	A 17 (IgG2a)	0%	100%	0%
AA 114	200/1 (IgG1)	A 17 (IgG2a)	77%	0%	23%
	200/2 (IgG1)	A 17 (IgG2a)	87%	5%	8%
	200/3 (IgG)	A 17 (IgG2a)	91%	0%	9%
	200/4 (IgG1)	A 17 (IgG2a)	87%	0%	13%
	200/5 (IgG1)	A 17 (IgG2a)	82%	3%	15%
	200/3 (IgG)	200/1 (IgG1)	0%	0%	100%
	200/3 (IgG)	200/2 (IgG1)	0%	0%	100%
	200/3 (IgG)	200/4 (IgG1)	0%	0%	100%
	200/3 (IgG)	200/5 (IgG1)	0%	0%	100%

Infectivity of P. vivax *to mosquitoes*

Small containers with ~50 female *Anopheles tessellatus* mosquitoes from a laboratory colony were allowed to feed on *P. vivax* patients attending the Kataragama Malaria Clinic (with the patients' consent). The mosquitoes were kept in an insectory at 27°C and 90% RH for 1 week and then dissected. Oocysts in the midguts were counted.

RESULTS

Genetic complexity of P. vivax *isolates*

An explanation of the interpretation of data is given using 2 of the 10 isolates as examples (presented in Table 1). For isolate AA137, 100% of schizonts reacted with both Mabs in mixtures of A17 with either 200/1, 200/3, 200/4, 200/5, or 200/6. Had a population of parasites been present which was negative for any one of these Mabs, this result could not have been obtained; some parasites would have been positive for 1 Mab and negative for the other (see isolate AA114). Therefore, isolate AA137 appears to contain a population of parasites which is uniformly positive for Mabs A17, 200/1, 200/3, 200/4, 200/5, and 200/6. In mixtures of A17 with 200/2 or A17 with A24, the parasites were positive only with A17 but were uniformly negative with 200/2 and A24. Thus, the combined results describe a single population of parasites in isolates AA137 whose PV200 serotype is shown in Table 2.

For isolate AA114, 3 populations of parasites can be distinguished. There is a predominant population (77–91%) which is negative for A17 and positive for all other Mabs tested; 8–23% of the parasites are positive for all Mabs tested, including A17. A minor population (~5%) also exists which is positive for A17 and negative for 200/2 and 200/5. Because of the small numbers involved, the status of this minor population with respect to other Mabs is uncertain. Thus, 3 populations of parasites, approximately 80%, 15%, and 5% of the total population, are identified in isolate AA114 as represented in Table 2.

A total of 10 isolates of *P. vivax* from patients reporting to the GHC were assayed in this way for heterogeneity of parasite populations as defined by Mabs against various epitopes of PV200 (Table 2). Within the limits of detection (each epitope was tested in each isolate on at least 50 schizonts and in most cases >100 were examined), only 1 of the 10 isolates was found to contain mixed populations of parasites. Since the probability of 2 unrelated populations of *P. vivax* having the same PV200 serotype for the panel of Mabs used is low (<1 in 20 [Table 2]), it is

TABLE 2
PV200 serotype composition in isolates of Plasmodium vivax

Mab no.	A 17	A 24	200/1	200/2	200/3	200/4	200/5	200/6	Parasites in isolates (%)
Isolate no.									
AA 122	+	−	+	+	−	+	+	−	100
AA 128	−	+	+	+	+	+	+	+	100
AA 84	+	−	+	+	+	+	+	−	100
AA 114	−	ND	+	+	+	+	+	ND	≈80
	+	ND	+	+	+	+	+	ND	≈15
	+	ND	(?)	−	(?)	(?)	−	ND	≈5
AA 115	−	+	+	+	+	+	+	+	100
AA 137	+	−	+	−	+	+	+	+	100
AA 138	+	+	+	−	+	−	+	−	100
AA 148	+	+	+	+	+	−	+	+	100
AA 149	+	+	+	−	+	−	+	−	100
AA 162	+	+	+	+	+	+	+	+	100
Frequency of epitope reactivity in *P. vivax* populations	66%	48%	90%	72%	80%	64%	84%	42%	

ND = not done; ? = not known.

likely that those populations in which a single serotype was identified are genetically homogeneous populations within the limits of detection. In most cases, minor poulations ≤5% of the total population would have been readily detected by this method. Therefore, in 9 out of 10 isolates, at least 95% of the parasites probably represented a genetically homogeneous population. In the remaining isolate, 3 distinct parasite populations of about 80%, 15%, and 5% of the total were identified.

Frequency of superinfection of P. vivax

A survey of *P. vivax* malaria was carried out in Kataragama, a typical area of transmission in the southern part of Sri Lanka. The point prevalence of *P. vivax* infections was available from 4 mass blood surveys of the Kataragama population (Table 3). In ~7,200 blood examinations, 51 *P. vivax* positive blood smears were identified. This corresponds to 1 individual in 142 carrying *P. vivax* parasites at any time. The chances that a second parasite infection would randomly coincide with a previous infection (superinfection) is about the same, i.e., ~1 in 100–200 infections. A mosquito will generally take <10 bloodmeals from which it will survive long enough to transmit an infection, should any bloodmeal have been on an infectious individual. Since only ~1 in 100–200 bloodmeals are infectious, the probability of a mosquito feeding twice on an infectious person is low. Thus, under these conditions, a population of parasites is likely to be transmitted many times through successive hosts before encountering another population of parasites.

Oocyst densities in P. vivax *infected mosquitoes*

The distribution of oocysts in mosquitoes fed on cases of *P. vivax* malaria reporting to the clinic is presented in Figure 1. The oocyst load was <10 oocysts/mosquito in almost half the cases and rarely exceeded 50 oocysts/mosquito. Since each oocyst is the product of a single fertilization event, most mosquito infections cannot represent more than a few tens of individual parasites from a blood infection. This in itself will rapidly eliminate minor populations of parasites from transmission. Thus, in any blood infection in which a single parasite population had become

TABLE 3
Plasmodium vivax *point prevalence*

MBS	Coverage of population*	No. positive for *P. vivax*
I	60%	22
II	50%	9
III	50%	9
IV	42%	11

* Total Kataragama population in the survey was 3,625.
Point prevalence of *P. vivax* infections by positive blood smear in 4 successive mass blood surveys (MBS) of the Kataragama population, November 1986–March 1988.

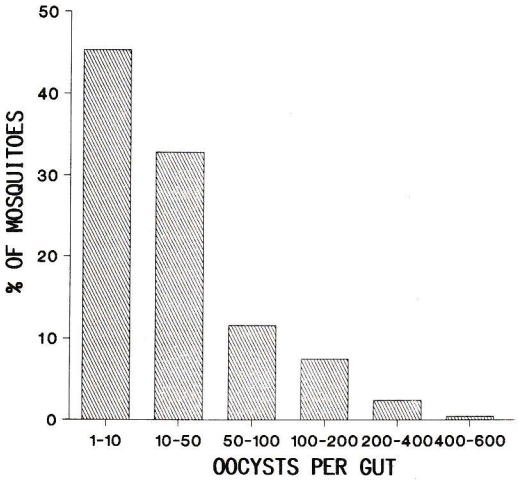

FIGURE 1. Distribution of oocysts in *A. tessellatus* mosquitoes fed on a random sample of gametocyte carriers among acute *P. vivax* patients in a malaria endemic area in Kataragama, Sri Lanka. Only infected mosquitoes were considered in this analysis.

predominant, mosquito transmission would tend to reduce the population to homogeneity for the major population(s) in subsequent infections.

DISCUSSION

In principle, the method used in this study for the analysis of genetically distinct populations of parasites in a single isolate of *P. vivax* allows detection of very minor populations of parasites and is limited only by the number of parasites counted in the analysis of an individual isolate. However, in the present study, populations representing <1–2% of the total would probably not have been detected. Our conclusions are made, therefore, in recognition of our inability to exclude the possible existence of such minor populations in any isolate. Within these limits, we have shown that in most infections of *P. vivax* acquired in Sri Lanka, the parasites are at least 95% homogeneous for a single serotype of the highly polymorphic antigen PV200. Only 1 out of 10 such isolates contained a mixture of PV200 serotypes. This is consistent with data from a previous study on chromosome polymorphism in isolates of *P. vivax* from Sri Lanka, which also indicated that most isolates consisted of a single genetically uniform population of parasites.[12] Parasites examined in this study were from infections that were contracted from different endemic regions of Sri Lanka and taken during the acute phase of infections. These parasite populations were thus likely to be fairly representative of malaria infections in Sri Lanka, many of which manifest as acute clinical disease, even in endemic populations, and last for no more than a few days.[15] We therefore conclude that these findings are likely to be of general applicability to *P. vivax* malaria transmission in Sri Lanka.

The extent to which parasites from different inoculations are likely to occur together in the same host (superinfection) or for a mosquito to become doubly infected has been estimated from an epidemiological survey of *P. vivax* infections in southern Sri Lanka. Such superinfections in either humans or mosquitoes are rare. Moreover, the numbers of oocysts resulting from mosquitoes feeding on *P. vivax* infections in Sri Lanka are sufficiently low that minor populations of parasites in the blood would be eliminated during transmission. Relapses in *P. vivax* contribute to about 17% of infections in Sri Lanka.[14] If each relapse results from the development of a single dormant hepatic stage parasite, as may well be the case, relapse populations would also give rise only to genetically homogeneous infections.

Thus, despite the high degree of antigenic polymorphism which we have shown to exist among natural parasite isolates of *P. vivax* in Sri Lanka,[10] the epidemiological circumstances of *P. vivax* transmission in the country would be expected to reduce the genetic diversity of parasites in individual infections and to maintain genetically homogeneous parasite populations through numerous successive mosquito transmissions. These expectations are consistent with the genetic homogeneity of parasites in most individual infections found in this and other studies on *P. vivax* from Sri Lanka.

Previous studies on the genetic complexity of malaria parasites in individual human blood infections have been done on *P. falciparum* under conditions of intense transmission, such as are found in tropical Africa. These conditions are very different from those prevailing in Sri Lanka. Individuals are constantly parasitemic, but also acquire clinical immunity from an early age. As a result, parasitic infections without symptoms persist untreated while reinoculations are frequent and multiple infections in the blood might be expected. However, an analysis of genetic complexity in such blood infections of *P. falci-*

parum in the Gambia, West Africa, using isoenzyme variant analysis indicated that most infections nevertheless consisted of a small number of genetically distinct parasite populations and many appeared to be genetically homogeneous.[1] A low proportion of genetically mixed parasite populations has also been found in *P. falciparum* blood infections from areas of relatively intense transmission in Malaysia (A. Foo, Institute of Medical Research, Penang, Malaysia, personal communication). Thus, even where superinfections are not uncommon the parasites maintain a relatively low degree of mixing of genetically distinct populations. In the Gambia and other areas of intense malaria transmission, such as Papua New Guinea, the oocyst load in wild-caught mosquitoes is very low. Most often, these mosquitoes have only 1 oocyst per gut, and the remainder have <3–4 (P. M. Graves, Queensland Institute of Medical Research, Brisbane, Australia, personal communication). This would have a strong tendency to reduce infections transmitted through mosquitoes to genetic homogeneity and could account for the low degree of genetic diversity in blood infections of *P. falciparum* even under conditions of intense transmission.

Under a wide range of transmission conditions, therefore, individual blood infections of malaria tend to contain parasite populations with a low degree of genetic diversity. Under conditions of low endemicity, this is probably achieved by the very low incidence of superinfections and the tendency for mosquito transmission to eliminate relatively minor populations of parasites. Under conditions of intense transmission, clinical immunity encourages the persistence of parasitemia without drug intervention, and superinfection is common. The very low number of oocysts found in mosquitoes under these conditions would reduce most mosquito inoculations to those parasites representing only the major population(s) in the blood infection from which they were derived.

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