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Protection against Malaria Morbidity: Near-Fixation of the α-Thalassemia Gene in a Nepalese Population

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Summary

We have previously reported that the Tharu people of the Terai region in southern Nepal have an incidence of malaria about sevenfold lower than that of synpatric non-Tharu people. In order to find out whether this marked resistance against malaria has a genetic basis, we have now determined in these populations the prevalence of candidate protective genes and have performed in-vitro cultures of *Plasmodium falciparum* in both Tharu and non-Tharu red cells. We have found significant but relatively low and variable frequencies of β-thal, β^S, G6PD (−), and Duffy (a-b-) in different parts of the Terai region. The average in-vitro rate of invasion and of parasite multiplication did not differ significantly in red cells from Tharus versus those from non-Tharu controls. By contrast, the frequency of α-thalassemia is uniformly high in Tharus, with the majority of them having the homozygous α-/α-genotype and an overall α-thal gene (α-) frequency of .8. We suggest that holoendemic malaria has caused preferential survival of subjects with α-thal and that this genetic factor has enabled the Tharus as a population to survive for centuries in a malaria-holoendemic area. From our data we estimate that the α-thal homozygous state decreases morbidity from malaria by about 10-fold. This is an example of selection evolution toward fixation of an otherwise abnormal gene.

Introduction

It is widely accepted that malaria has played a major role as a selective factor in human evolution. In particular, various lines of evidence have been accumulating that support the notion that several genes expressed in red cells—such as β-thal, β^E, β^S *Gd–*, and *fy*—have reached high frequencies in highly endemic areas as a result of malaria selection (Luzzatto 1979; Pasvol and Wilson 1982; Livingstone 1985). More recently it has been shown that α-thal has also attained very high frequencies, positively correlated with the rates of past malarial morbidity, in Melanesia (Hill 1986). (Throughout the present paper we shall use the term "α-thal" to refer to the deletion of a single gene, commonly called "α-thal 2" or "α^+" and also designated by the symbol "α-." Thus, "α-thal heterozygote" denotes the genotype α-/αα, and "α-thal homozygote" denotes the genotype α-/α-.) Therefore it is convenient to refer to all of these genes as being "malaria related."

The Terai region in southern Nepal has been ill famed for a long time for being heavily affected by malaria. This generic notion was reported by nonprofessional epidemiologists—e.g., Temple (1887) and Tucci (1977)—who reported the then current notion that the Tharus, the only inhabitants of the region, had an "innate" resistance against malaria. Factual epidemiological data were first collected by J. Phillips in 1925 (quoted by Terrenato et al. 1988). In a survey

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1. Bruno Colombo died suddenly in November 1989. We dedicate this paper to his beloved memory.

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carried out in Chitwan, he found that virtually all children suffered from malaria and that in adolescents the spleen rate was "only" 85% (436/513).

In the 1950s an antimalarial campaign was started through the joint efforts of His Majesty's Government (HMG) of Nepal and the United States Agency for International Development (USAID), later supported also by the World Health Organization (WHO) (in 1958 a single organization, the Nepal Malaria Eradication Organisation [NMEO], resulting from the fusion of the previously mentioned projects, was established). First it was confirmed that the spleen was enlarged in about 80% of 2–9-year-old children. A full eradication project was then launched, and by the end of 1965 all of the Terai area of Nepal was under insecticide coverage, resulting in a period of almost complete eradication of malaria. Subsequently the malaria rate increased again until 1974, and then it remained stable at the present yearly rate of about 12,500 cases (see table 1). Over these years, a large and heterogeneous non-Tharu population migrated into the Terai region, which previously had been inhabited almost exclusively by the Tharus.

This unique demographic situation has enabled us to compare malaria susceptibility in a population previously exposed to the disease for many generations vis-à-vis that of other populations, living in the same environment and in comparable conditions, who had had little or no previous exposure to malaria. Indeed, we have recently reported that the incidence of both *Plasmodium falciparum* and *P. vivax* malaria is about sevenfold less in the Tharus of the Terai region than in synpatric, recently immigrated non-Tharus (Terrenato et al. 1988).

To identify the nature of malaria-resistance factors in Tharu people we have used two approaches. First, we have performed in-vitro cultures of *P. falciparum* in red cells from Tharu and non-Tharu subjects, matched for age and sex and living in the same or nearby villages. Second, we have determined in the Tharu people the frequency of several candidate protective genes. We found that almost all Tharus are either heterozygous or homozygous for an α-thalassemia gene and yield an estimated gene frequency of .8, one of the highest reported for any population.

Table 1

Changes in Malaria Endemicity in Terai (Chitwan District)

Year	Spleen Rate[a] (%)	Parasite Rate[a] (%)
1956[b]............	87	73
1958[b]............	80	89
1959[b]............	27	29
1970[c]............		.05
1975[d]............		.25

[a] The data until 1959 are for children aged 2–9 years; more recent data are for the general population.

[b] From unpublished records of NMEO.

[c] From Shrestha (1985).

[d] From Terrenato et al. (1988).

Subjects and Methods

Subjects

Blood samples were obtained from apparently healthy subjects of both sexes living in the Bardia or Banke districts of western Terai or in the Naval-Parasi or Chitwan districts of central Terai (see Terrenato et al. 1988, fig. 1). The classification of subjects as Tharus or non-Tharus was based on their designations for both parents. The non-Tharus were mostly Hindus of Indian origin; some were Chetris originating from hilly, malaria-free regions of Nepal. First-degree relatives were never included. However, because of the small size of some of the villages examined (from some hundreds to some thousands of inhabitants), more distant relationships could not be ruled out. All demographic information was obtained through an interpreter, who was usually an officer of the local NMEO section.

Blood samples were kept at 4°C and were carried refrigerated to Italy for the study of genetic markers within 3–4 d. Samples for in-vitro culture studies were carried refrigerated to Naples or to London, where they arrived within 30 h after collection. Samples from Tharus and non-Tharus were handled in strictly comparable conditions.

Duffy Phenotype and Hb Electrophoresis

The Duffy phenotype was determined by standard serological techniques. Hb electrophoresis was carried out on cellulose acetate (Beckham Microzone electrophoresis membranes) in a TRIS-EDTA-borate buffer at pH 8.6 (Smithies 1959), and all samples showing an electrophoretic band in the Hb S position were further analyzed by the dithionite solubility test in a concentrated phosphate buffer (Nalbandian et al. 1971). Hb A_2 was determined by the chromatographic method on DEAE cellulose according to the method of Efremov et al. (1974).

Southern Blot Analysis

Southern blot analysis of high-molecular-weight DNA extracted from mononuclear cells was carried out according to a method described by Di Rienzo et al. (1985). DNA samples were digested with *Bam*HI and hybridized to a 1.5-kb *Pst*I α-globin genomic probe cloned in pBR 322. *Apa*I digests were examined with the same probe to further characterize the α-globin gene deletions (Higgs et al. 1984) (These studies would not detect or rule out αα/--heterozygotes. However, on the basis of previous work, the deletion of both α-globin genes in cis must be extremely rare in India [Kar et al. 1986; Drysdale and Higgs 1988].) Two subjects showing a 1.9-kb *Apa* band were further characterized by *Bgl*II digestion in order to confirm the triplicated α-globin gene arrangement (Lie-Injo et al. 1981).

Culture Studies

Plasmodium falciparum culture studies were based on the system developed by Trager and Jensen (1976). We used the WL strain, originally from Nigeria and isolated by Dr. A. Reid (Richards and Maples 1979). Highly synchronous schizonts were prepared according to a method described elsewhere (Usanga and Luzzatto 1985) and were used as inoculum for the Tharu and non-Tharu control washed red cells obtained from blood specimens simultaneously collected in the same locale under identical conditions. Recipient red-cell suspensions had been previously analyzed in an electronic counter and then had all been adjusted to the same concentration in order to make sure that for each set of samples invasion rates could be accurately compared.

Glucose-6-Phosphate Dehydrogenase

The red-cell G6PD phenotype was determined as recommended by WHO (1967).

Results

Genetic Studies

Duffy and En^a.—Duffy was investigated because the lack of Fy antigens is known to confer a specific resistance against *Plasmodium vivax* (Miller et al. 1975). The phenotypic Fy^a and Fy^b distributions were all in agreement with Hardy-Weinberg's law (data not shown). Only four *fy/fy* subjects were found, and these were all Tharus of central Terai (table 2). Because of in-vitro culture data indicating decreased invasion of En^a (−) red cells by *P. falciparum* (Miller et al. 1977), Drs. Ruth Sanger and Patricia Tippett have tested 22 Tharu blood samples for this antigen; none was En^a(−).

G6PD.—The prevalence rate of G6PD deficiency was about 14% in the Tharu males of western Terai (table 2). Although quantitative assays were not carried out, we estimate that in the 20 cases classified as G6PD deficient the G6PD level was less than 10% of normal, since in 19 cases no enzyme-activity band was seen on electrophoresis and since in the remaining case a very weak band was visible in the G6PD B position. In addition, of 24 females tested in one village (Sonaha Patuwa) three were G6PD deficient (two of them probably homozygous and one heterozygous; our tests were not designed to detect all heterozygotes). The G6PD-deficient variant(s) was not further characterized. Only a single non-Tharu male was G6PD deficient.

Table 2

Frequencies of Malaria-related Genes in Terai

	Tharu				Non-Tharu			
	Western Terai		Central Terai		Western Terai		Central Terai	
Malaria-related Gene	N	q (mean ± SE)	N	q (mean ± SE)	N	q (mean ± SE)	N	q (mean ± SE)
fy	209	0 + .03	262	.12 ± .03	66	0 + .06		
Gd$^-$	143	.14 ± .03			36	.028 ± .028		
β^s	185	.05 ± .01	124	0 ± .004	49	0 + .01		
β-*thal*			124	.02 ± .01				
α-*thal*	18	.72 ± .08	18	.83 ± .06	17	.03 ± .03	17	.09 ± .05

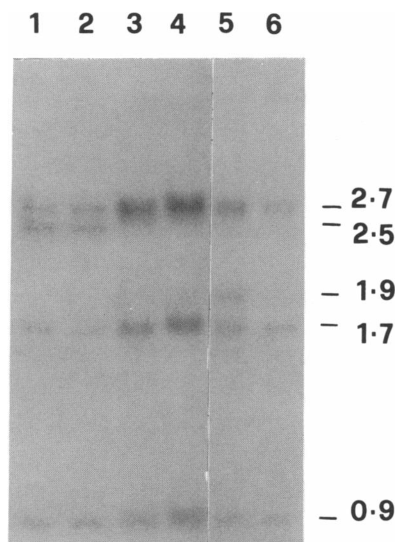

Figure 1 Examples of α-globin genotypes determined by Southern blot analysis. DNA samples were digested with *Apa*I, and blots were hybridized with an α-globin probe. Lane 1, Tharu $\alpha^{-3.7}$/αα (type I crossover). Lane 2, Hindu $\alpha^{-3.7}$/αα (type I crossover). Lanes 3 and 4, Hindu αα/αα. Lane 5, αα/$\alpha\alpha\alpha^{anti\text{-}3.7}$. Lane 6, Normal control.

Hb S.—AS heterozygotes have an appreciable frequency (about 10%) among Tharus of western Terai but not among Tharus of central Terai (table 2). This trait was never found in the non-Tharus we tested.

β-Thalassemia.—The heterozygote frequency was about 4% in the Tharus of central Terai (table 2). Elevated Hb A_2 was used as the sole diagnostic criterion because red-cell indexes are not likely to be reliable in a population who may have a high rate of parasite infestations and malnutrition.

α-Thalassemia.—Both in western Terai and in central Terai the Tharus had an extremely high α-thal gene frequency. (Recently Dr. M. Ganczakowski [personal communication] independently tested DNA samples from Tharu subjects and found a similarly high frequency of α-thalassemia.) Indeed, in central Terai the commonest genotype was homozygous thal (α-/α-), and in both areas no "normal" people (αα/αα) were found. α-Thal was almost exclusively accounted for by the α- 3.7 I deletion (see fig. 1 and table 3). This same gene was also found among non-Tharu, but at much lower frequencies (3%–9%). Two non-Tharu subjects had the ααα (anti-3.7) haplotype.

In-Vitro Cultures of P. falciparum

We have measured *P. falciparum* invasion rates in Tharu and non-Tharu red cells by using a synchronized high-density inoculum and identical recipient red cell numbers. The values obtained were not significantly different, although an unexpected reduced invasion was seen in red cells from the older age group, both in Tharus and non-Tharus (table 4). We have also measured the multiplication factor, which results from invasion as well as from intraerythrocytic development and growth. Again, no statistically significant differences were found (table 5 and fig. 2).

Table 3

Frequencies of α-Thalassemia Genes in Terai

	No. of Genotypes				Estimated Haplotype Frequency (mean ± SE)			
People and Area in Terai (*n*)	αα/αα	αα/ααα	$\alpha\text{-}^{I\,a}$/αα	$\alpha\text{-}^{I\,a}/\alpha\text{-}^{b}$	αα	ααα	$\alpha\text{-}^{I\,a}$	$\alpha\text{-}^{II\,c}$
Tharu:								
Western[d] (18)	0	0	10	8[e]	.28 ± .08		.67 ± .08	.05 ± .04
Central[f] (18)	0	0	6	12[g]	.17 ± .06		.83	
Non-Tharu:								
Western[d] (17)	16	0	1	0	.97		.03 ± .03	
Central[h] (17)	12	2	3	0	.85 ± .06	.06 ± .04	.09 ± .05	

[a] Type I crossover (Higgs et al. 1984).
[b] Type I or type II crossover (Embury et al. 1980; Higgs et al. 1984).
[c] Type II crossover (Higgs et al. 1984).
[d] Banke district.
[e] Six subjects were ($\alpha\text{-}^{I}$)/($\alpha\text{-}^{I}$) (*Apa*I fragments 2.5 and 0.9), and two subjects were $\alpha\text{-}^{I}/\alpha\text{-}^{II}$ (*Apa*I fragments 2.5, 3.5, and 0.9).
[f] Chitwan district.
[g] All 12 subjects were ($\alpha\text{-}^{I}$)/($\alpha\text{-}^{I}$).
[h] Naval-Parasi district.

Table 4

Invasion Rate, by *Plasmodium falciparum* of Tharu and Non-Tharu Red Cells

		No. of Parasites/1,000 Red Cells[a]			
		Non-Tharu		Tharu	
Set	Age Range (years)	Individual Data	Mean ± SE	Individual Data	Mean ± SE
I	10–20	98, 83, 69, 85	83.8 ± 5.9	72, 88, 80, 78	79.5 ± 3.3
II	30–50	60, 51, 42, 58	52.8 ± 4.1	40, 49, 51, 56	49.0 ± 3.3

[a] Invasion rate values for local control samples were 75 and 70 for set I and set II, respectively.

Discussion

To investigate the Tharus' relative resistance to malaria, it was natural to determine first the frequency of malaria-related genes. All of these except for β^E (which is itself a mild β^+-thal gene; Orkin et al. 1982) were found in at least one of the Tharu groups tested. By contrast, the three malaria-related genes—β^s, *fy*, and Gd^-—were absent or rare in non-Tharus (table 2). We do not yet know (*a*) whether the malaria-related genes found among Tharus are autochthonous or imported or (*b*) why the frequencies of *fy* and β^S are quite different in the two groups of Tharus tested, who were otherwise relatively similar with respect to many other genetic markers (G. Modiano, unpublished data). The polymorphic frequency of the β^S gene (up to 10% heterozygotes) is of interest, because to the best of our knowledge it has never been reported in any population group regarded as Oriental in origin. At any rate, we think that these genes can only have played a minor role in the Tharus' resistance to malaria, because their frequencies are not uniformly high (e.g., β^S is not found in the Tharus of central Terai), whereas the Tharus' resistance to malaria is apparently homogeneous throughout the Terai region (Terrenato et al. 1988).

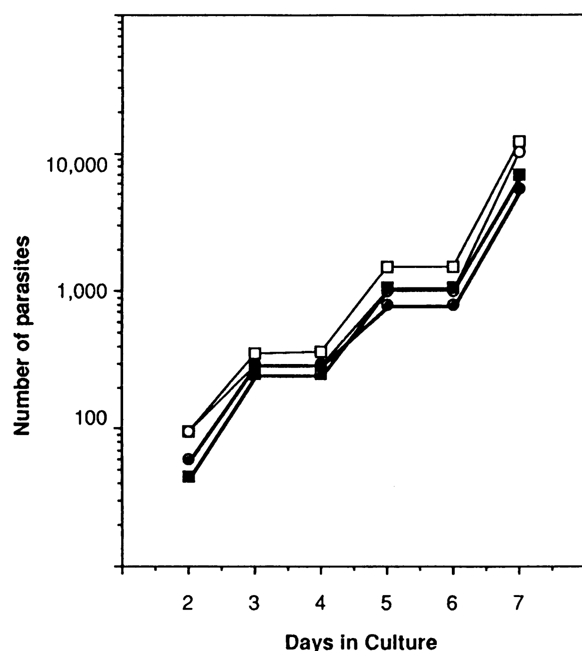

Figure 2 Growth pattern of *Plasmodium falciparum* in Tharu and non-Tharu red cells. Highly synchronous cultures (see Subjects and Methods) were set up on day 0 and were diluted fivefold with fresh red cells on day 3. The dilution factor was taken into account in calculating the total number of parasites on subsequent days. Blackened symbols denote non-Tharus; open symbols denote Tharus. The mounting portions of the graph reflect the multiplication factor from one schizont to the next generation rings. There is no apparent difference between Tharus and non-Tharus.

Like others (Ifediba et al. 1985) who have tested α-thalassemia red cells (α-/αα and α-/α-), we have not observed a statistically significant impairment of either invasion or growth of *Plasmodium falciparum* when Tharu red cells were infected in vitro (tables 4 and 5 and fig. 2). This may mean that protection is effected in vivo by an extraerythrocytic mechanism. For instance, it is possible that the spleen is more effective in removing parasitized α-thal red cells than parasitized normal red cells. Along these lines, Yuthavong et al. (1988) have observed increased susceptibility to phagocytosis of malaria-infected α-thal red cells; and Luzzi et al. (1989) have shown very recently that a

Table 5

Growth Rate of *Plasmodium falciparum* in Tharu and Non-Tharu Red Cells

Experimental Series and Subject	Local Controls[a]				Non-Tharu				Tharu			
	No. of Parasites/ 1,000 Red Cells		Ratio (cycle II/cycle I)[b]		No. of Parasites/ 1,000 Red Cells		Ratio (cycle II/cycle I)[b]		No. of Parasites/ 1,000 Red Cells		Ratio (cycle II/cycle I)[b]	
	Cycle I	Cycle II	Individual Data	Mean ± SE	Cycle I	Cycle II	Individual Data	Mean ± SE	Cycle I	Cycle II	Individual Data	Mean ± SE
I:[c]												
1	10	60	6.0		11	52	4.7		2	11	5.5	
2	13	77	5.9	5.9 ± .05	12	53	4.4	3.8 ± .79	11	24	2.2	4.6 ± 1.19
3					11	24	2.2		10	60	6.0	
II:[c]												
1	20	84	4.2		20	83	4.2		20	66	3.3	
2	18	95	5.3	4.8 ± .55	14	87	6.2	5.1 ± .59	9	27	3.0	3.6 ± .46
3					22	105	4.8		22	100	4.5	
III:[c]												
1	14	71	5.1		10	70	7.0		8	45	5.6	
2	16	100	6.3	5.7 ± .60	20	82	4.1	5.7 ± .86	14	74	5.3	6.1 ± .66
3					21	128	6.1		16	119	7.4	
IV:[d]												
1					10	82	8.2		8	56	7.0	
2					10	71	7.1	7.7 ± .24	10	79	7.9	7.2 ± .27
3					9	68	7.6		7.5	55	7.3	
4					8	63	7.9		8	53	6.6	

[a] From staff of laboratory where cultures were done.

[b] Cycle II rate (no. of parasites/1,000 red cells) divided by cycle I rate (no of parasites/1,000 red cells).

[c] Experiments performed in 1983.

[d] Experiments performed in 1986.

malaria-specific antibody binds preferentially to malaria-infected α-thal red cells. By contrast, a more active Tharu immune response against malaria parasites is unlikely to be an important mechanism in the Tharus' decreased morbidity, because under the present epidemiological circumstances immune stimulation must be very low. On the other hand, it is of course still possible that a small impairment in invasion or growth rate, an impairment not detected by our culture technique, could play a protective role in vivo by being exponentially amplified through successive schizogonic cycles.

At any rate, the distribution of α-thal fulfills three prerequisites for playing the major role in the Tharus' increased malaria resistance vis-à-vis other synpatric populations (table 3). (1) α-thal is so frequent among Tharus that only very few of them are expected to be homozygous for the "normal" haplotype. (2) α-thal frequency is uniformly high in both the Tharu subsamples analyzed. (3) α-thal genes are much less prevalent among the non-Tharus whom we have tested in the same region of Terai. The major role here ascribed to α-thalassemia is in good agreement with evidence accruing from the impressive geographic analysis carried out by the Oxford group in Papua New Guinea and in Melanesia (Hill 1986). In the Terai region, selection has operated on a broad scale and seems to have produced a uniformly high frequency of this gene. Indeed, this frequency (similar to that reported recently for some tribal groups in India; Fodde et al. 1988; Labie et al. 1989) strongly suggests that, were it not for genetic admixture, the α-thal gene would have reached fixation in the Tharus. This in turn suggests that in the case of α-thal the best-fitted genotype is the homozygote. Thus, whereas β^S and β-thal are typical examples of stable balanced polymorphism, α-thal may be an example of transient polymorphism evolving toward fixation. Of course this is made possible by the fact that the α-globin gene is duplicated, so that in the α-thal homozygotes protection against malaria is reconciled with the synthesis of an adequate amount of normal hemoglobin.

Because we have shown elsewhere that, compared with the rates in other presently synpatric ethnic groups, the rate of malaria infection in Tharus is decreased by a similar factor with respect to both *P. falciparum* and *P. vivax,* our data suggest directly for the first time that α-thalassemia may be protective against both of these parasites. With respect to *P. vivax,* the important protective effect must be against morbidity rather than against mortality. In other words, we think that *P. falciparum* has been by far the most important selective agent for the α-thal gene, and resistance to *P. vivax* may be just an epiphenomenon.

Previous estimates of the extra fitness conferred by malaria-protective genes have been based in general on the assumption that a balanced-polymorphism situation is at equilibrium (Cavalli-Sforza and Bodmer 1971; Luzzatto 1979). In the present work we have been able instead to compare frequencies of malaria-related genes in two populations whom we already know (from direct malariological data) to have different susceptibility to malaria. A quantitative estimate of the protective effect of the α-thal gene can be obtained, therefore, in terms of decreased susceptibility (see Terrenato et al. 1988). We could not prove directly that α-thal homozygotes are more malaria resistant than heterozygotes, but we assume this is so because the α-thal gene is nearing fixation (see above). Since with about 60% of homozygotes (see table 3) we found a level of resistance of sixfold to sevenfold vis-à-vis non-Tharu controls (see Terrenato et al. 1988), we surmise that if the entire population was homozygous the protection would be about 10-fold.

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