Transactions of the Royal Society of Tropical Medicine and Hygiene (2006) **100**, 395—400

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Genetic diversity of T-helper cell epitopic regions of circumsporozoite protein of *Plasmodium falciparum* isolates from India

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Received 27 January 2005; received in revised form 5 July 2005; accepted 5 July 2005
Available online 8 November 2005

KEYWORDS
Malaria;
Plasmodium falciparum;
T-helper cell epitope;
Circumsporozoite protein;
PCR;
India

Summary Genetic variation in the T-helper cell epitopic regions (Th2R and Th3R) of circumsporozoite protein of 135 *Plasmodium falciparum* isolates collected from different epidemic and endemic regions of India was studied. Variation in the Th2R and Th3R regions was found to exhibit restricted polymorphism and can be grouped. The variations were not regionally biased, as different isolates collected from different regions were found to belong to the same group. The Th2R and Th3R sequences were found to be linked in each isolate. Since the variations are regionally unbiased and restricted, the prototype variant from the groups could be included in a subunit polyvalent vaccine against sporozoites.

1. Introduction

The importance of T cells in immunity to malaria has been known for a long time. Mice devoid of B cells or antibody could be immunised to asexual stage parasites (Grun and Weidanz, 1981). There is good evidence that T cells in the absence of antibody can have a role in human malaria immunity (Brown et al., 1986; Jensen et al., 1983). Thus, knowledge of the activation and regulation of T cells in response to malaria antigens is important in understanding malaria immunity and subsequent vaccine development. A recent study (Stout et al., 1997) on the RTS,S vaccine formulation based on a section of circumsporozoite protein (CSP) including the T-cell epitope has claimed protection against homologous sporozoite challenge. This provides considerable incentive to study the T-helper cell epitope of CSP, which would provide important information as to which epitope should be included in a sporozoite vaccine. Two T-helper cell epitopes, Th2R and Th3R, have been identified at the C-terminal end of the CSP flanking the highly conserved RII region and spanning amino acid residues 326—343 (Th2R) and 361—380 (Th3R) (Good et al., 1988). The T-cell epitope domains could be included in a subunit polyvalent sporozoite vaccine to evoke natural boosting of antibody and cellular immune responses against sporozoites. The

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doi:10.1016/j.trstmh.2005.07.012

feasibility of such a strategy may largely depend on the extent of polymorphisms in these epitopes. Lockyer et al. (1989) found extensive polymorphism in the T-helper cell epitopic region. However, Yoshida et al. (1990) and, later, Doolan et al. (1992) showed restricted polymorphism. The latter studies raised hope for the development of a polyvalent vaccine and, as the variation appears to be finite, the combination of peptides necessary for inclusion in a polyvalent vaccine may be small. Data on the variation in T-cell epitopes are available from Africa, Brazil, Honduras, Malaysia, Papua New Guinea and The Netherlands (Doolan et al., 1992; Lockyer and Schwartz, 1987; Lockyer et al., 1989), but little information is available from India where the malaria situation is critical. A previous study (Bhattacharya, 1999) from India showed wide variation in the T-cell epitopic region of *Plasmodium falciparum*. However, the study was weakened by the fact that most of the *P. falciparum* were laboratory-adapted cultures that were not true representatives of naturally occurring parasites. Therefore, in this study 135 *P. falciparum* isolates from different epidemic and endemic regions of India, which represents broad geographic sampling, were studied to determine the extent of polymorphism in T-helper cell epitopic regions.

2. Materials and methods

2.1. Parasites

A total of 135 *P. falciparum* blood samples were collected from consenting subjects visiting malaria clinics or from those living in epidemic/endemic areas described elsewhere (Bhattacharya and Pillai, 1999).

2.2. Preparation of genomic DNA

Genomic DNA was prepared from parasitized blood samples by modification of a method described previously (Foley et al., 1992). In brief, 50 μl blood samples were washed three times, each time by vortexing in 1 ml ice-cold 5 mM Na_2HPO_4 buffer (pH 8.0) and then centrifugation (10 000 × *g*, 10 min). The final pellet was resuspended in 50 μl sterile water and then heated in a boiling water-bath for 10 min, cooled slowly at room temperature and centrifuged as before. Then, 40 μl of supernatant was taken and 5 μl of this supernatant was used in a 50 μl PCR (Saiki et al., 1998) mixture.

2.3. PCR

The oligonucleotide primers used for PCR to amplify the T-cell epitopic region correspond to nucleotides 1008—1028 and 1323—1347 in the sequence of the 7G8 clone (Lockyer et al., 1989). In addition to the genomic DNA preparation, each reaction mixture contained 50 pM each of the forward and reverse primers, 10 mM deoxynucleotide triphosphates and 2.5 U *Taq* polymerase (Bangalore Genei, Bangalore, India). Amplification consisted of denaturation at 95 °C for 2 min, annealing at 55 °C for 1 min, amplification at 72 °C for 3 min for 30 cycles and final extension at 72 °C for 10 min.

2.4. DNA sequencing

The PCR-amplified products, after purification by a PCR 'clean up' kit (Boehringer Mannheim, Mannheim, Germany), were directly sequenced by Sanger's dideoxy chain termination method (Sanger et al., 1997) using *Taq* DNA sequencing kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. Sequencing was carried out for each isolate from three independent PCR reactions.

3. Results

Plasmodium falciparum-infected blood samples were collected from malaria patients living in widely separated geographical regions of India, namely Delhi, Uttar Pradesh, Rajasthan, Assam, Orissa and Madhya Pradesh. The deduced amino acid sequences of Indian isolates were compared with 7G8 and LE5 sequences (Tables 1 and 2). Figure 1 shows the distribution of different alleles in different geographical regions of India. The Th2R sequences of the isolates were found to be more similar to LE5. The isolates could be categorised according to the deduced amino acid sequences into four groups (Tables 1 and 2). Variations were also found to be regionally unbiased, as similar variations were found in different regions. Of the 135 isolates studied, 25 isolates belonged to group I, 28 to group II, 37 to group III and 45 to group IV for both the Th2R and Th3R sequences. Thus, it appears that the allelic variations of group III and group IV of both Th2R and Th3R are more prevalent than the alleles of group I and group II. It is interesting to note that all the isolates in the present study that share identical Th2R sequence also share the identical Th3R sequence, e.g. the isolates belonging to group I (five from Delhi, four from Orissa, seven from Uttar Pradesh, five from Rajasthan, two from Assam and two from Madhya Pradesh) in Th2R sequence also belong to group I in Th3R sequence, i.e. sequence variation in Th2R and Th3 R are linked in each isolate.

The mutational changes observed mostly occurred at the first base of the codons and less frequently at the second and third base of the codons. However, all these base substitutions were found to be non-synonymous mutations resulting in amino acid changes; no synonymous mutations were observed. It is interesting to note that in codon 337 in the Th2R region, ACA has been substituted by CGA, and in the Th3R region codon 371, AAA has been substituted by CGA. As the variants are small in number, the prototype variants from the groups could be included into a subunit polyvalent vaccine against sporozoites.

4. Discussion

Several studies have demonstrated the importance of T-cell-mediated immunity to malaria (Brown et al., 1986; Grun and Weidanz, 1981; Jensen et al., 1983). CSP, which contains both B-cell and T-cell epitopes and is a potential candidate for vaccine development, was found to be a poor immunogen mostly owing to polymorphisms in the T-cell epitopic region, although the B-cell epitopes are largely invariant. However, the success of a T-cell epitope-based subunit polyvalent vaccine against sporozoites would largely depend on the extent of polymorphism in the T-cell epitopic region