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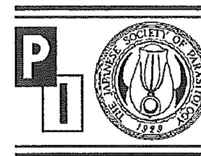

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Genetic diversity and kinetic properties of *Trypanosoma cruzi* dihydroorotate dehydrogenase isoforms☆

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Abstract

Dihydroorotate dehydrogenase (DHOD) is the fourth enzyme in the de novo pyrimidine biosynthetic pathway and is essential in *Trypanosoma cruzi*, the parasitic protist causing Chagas' disease. *T. cruzi* and human DHOD have different biochemical properties, including the electron acceptor capacities and cellular localization, suggesting that *T. cruzi* DHOD may be a potential chemotherapeutic target against Chagas' disease. Here, we report nucleotide sequence polymorphisms of *T. cruzi DHOD* genes and the kinetic properties of the recombinant enzymes. *T. cruzi* Tulahuen strain possesses three *DHOD* genes: *DHOD1* and *DHOD2*, involved in the pyrimidine biosynthetic (*pyr*) gene cluster on an 800 and a 1000 kb chromosomal DNA, respectively, and *DHOD3*, located on an 800 kb DNA. The open reading frames of all three *DHOD* genes are comprised of 942 bp, and encode proteins of 314 amino acids. The three *DHOD* genes differ by 26 nucleotides, resulting in replacement of 8 amino acid residues. In contrast, all residues critical for constituting the active site are conserved among the three proteins. Recombinant *T. cruzi* DHOD1 and DHOD2 expressed in *E. coli* possess similar enzymatic properties, including optimal pH, optimal temperature, V_{max}, and K_m for dihydroorotate and fumarate. In contrast, DHOD3 had a higher V_{max} and K_m for both substrates. Orotate competitively inhibited all three DHOD enzymes to a comparable level. These results suggest that, despite their genetic variations, kinetic properties of the three *T. cruzi* DHODs are conserved. Our findings facilitate further exploitation of *T. cruzi* DHOD inhibitors, as chemotherapeutic agents against Chagas' disease.

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Keywords: Trypanosoma cruzi; Dihydroorotate dehydrogenase; Pyrimidine–biosynthetic gene cluster; Genetic diversity; Drug target

1. Introduction

Chagas' disease is caused by infection with the parasitic protist, *Trypanosoma cruzi*. This disease affects about 17 million people in Latin America, with about 25% of the population estimated to be at risk [1]. Medication is usually effective when given during the acute phase, whereas, during the chronic phase, infected tissues become gradually impaired and the disease is no longer curable [2]. The chemotherapeutic drugs currently used are highly toxic and often lead to discontinuation of the therapy [1]. Thus, development of new drugs with low toxicity is needed.

Pyrimidine biosynthesis is an essential biological activity, which is conducted by both de novo and salvage pathways. The de novo pathway is comprised of six sequential enzymatic reactions. Dihydroorotate dehydrogenase (DHOD) is the fourth enzyme in the pathway catalyzing the oxidation of dihydroorotate (DHO) to orotate. Human DHOD, which is localized in the inner membrane of mitochondria, uses ubiquinone as electron acceptor [3,4]. In contrast, *T. cruzi* DHOD is a cytosolic protein that preferentially uses fumarate as electron acceptor, producing succinate [5]. Phylogenetic reconstruction of DHOD has shown that *T. cruzi* and human DHOD have the different origins, belonging to families 1A and 2, respectively

Abbreviations: DHOD, dihydroorotate dehydrogenase; ACT, aspartate carbamoyltransferase; *pyr*, de novo pyrimidine biosynthetic gene.

☆ Sequence availability: The sequences reported in this paper for the *Trypanosoma cruzi* dihydroorotate dehydrogenase (DHOD) gene loci *DHOD1*, *DHOD2*, and *DHOD3* have been placed into the GenBank, EMBL, and DDBJ databases under the accession numbers AB212955, AB212956, and AB212957, respectively.

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[6]. In conjunction with the unique properties of *T. cruzi* DHOD, we have previously reported that marine algae extracts inhibit the *T. cruzi* DHOD activity in a non-competitive manner, as well as inhibiting the growth and infection of *T. cruzi* in mammalian cells [7]. Thus, inhibitor(s) specific for *T. cruzi* DHOD may be promising candidate drugs in the treatment of Chagas' disease.

In *T. cruzi*, the *DHOD* gene is localized in the de novo pyrimidine biosynthetic (*pyr*) gene cluster, which contains five genes that encode all six enzymes in the de novo pyrimidine synthesis pathway [8]. Two complete sets of the *pyr* gene cluster have been found in all *T. cruzi* strains to date, along with amplification of parts of the cluster or individual *pyr* genes [9]. For example, three *DHOD* gene loci have been identified in the *T. cruzi* Tulahuen strain, two localized to the *pyr* gene clusters on an 800 and a 1000 kb chromosomal DNA. The third *DHOD* locus clusters only with the gene encoding the second enzyme in the pathway, aspartate carbamoyltransferase (ACT), on an 800 kb DNA (see Fig. 1). In addition, we observed extensive nucleotide and amino acid sequence polymorphisms among the *ACT* gene loci, suggesting that *T. cruzi DHOD* gene, as well as *ACT*, is polymorphic [9].

The presence of multiple *DHOD* gene loci and their possible genetic variation may indicate, however, that the encoded enzymes would not present drug targets. That is, a drug may inhibit one DHOD enzyme but not others, thus allowing parasite survival. Nevertheless, we have recently demonstrated that targeted disruption of at least two *DHOD* gene loci in *T. cruzi* Tulahuen resulted in loss of viability of the parasite even in the presence of pyrimidine substrates of the salvage pathway, suggesting that *T. cruzi* DHOD promises to be a drug target [10].

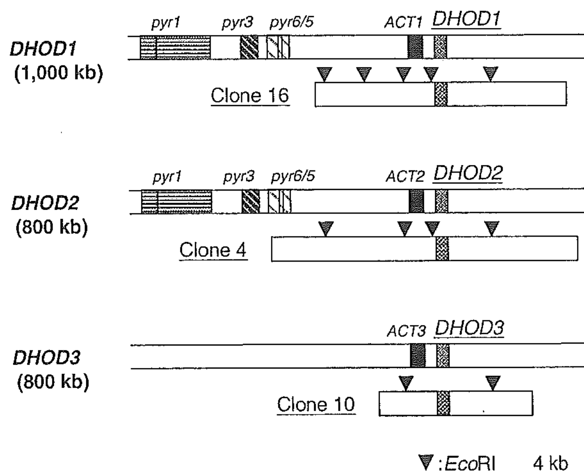

Fig. 1. Schematic illustration of the three dihydroorotate dehydrogenase (*DHOD*) gene loci in *Trypanosoma cruzi* Tulahuen strain. *DHOD1* is localized in the pyrimidine–biosynthetic (*pyr*) gene cluster on a 1000 kb chromosomal DNA. This cluster contains five genes of the de novo pyrimidine biosynthetic pathway: *pyr1*, encoding carbamoyl–phosphate synthetase II; *pyr3*, encoding dihydroorotase; *pyr6/5*, encoding orotidine-5′-monophosphate decarboxylase-orotate phosphoribosyltransferase; *ACT*, encoding aspartate carbamoyltransferase, and *DHOD*. *DHOD2* is localized in the *pyr* gene cluster on an 800 kb DNA. *DHOD3* is present on an 800 kb DNA and linked only to the *ACT3* gene. *DHOD1*, *DHODH2*, and *DHOD3* were isolated from phage clones, Nos. 16, 4, and 10 [9].

In the present study, we determined whether *T. cruzi DHOD* genes are polymorphic and, if so, whether polymorphisms would affect their enzymatic properties. We observed extensive nucleotide substitutions among the three *DHOD* loci, with replacement of 26 out of 945 nucleotides (2.75%), resulting in substitutions of 8 amino acids. Nevertheless, kinetic constants of the three recombinant DHODs were comparable. Our findings provide insight into *T. cruzi* DHOD as a drug target in the treatment of Chagas' disease.

2. Materials and methods

2.1. Nucleotide sequence determination of the DHOD loci in T. cruzi Tulahuen

We selected three phage clones, Nos. 16, 4, and 10, representing *DHOD1*, *DHOD2*, and *DHOD3*, respectively, that have been screened from a λEMBL3 genomic DNA library of *T. cruzi* Tulahuen strain [9]. *DHOD1* is present in the *pyr* gene cluster on a 1000 kb chromosomal DNA; *DHOD2* is in the *pyr* gene cluster on an 800 kb DNA; and *DHOD3* clusters partially with *ACT* gene on an 800 DNA (Fig. 1). Each phage DNA was digested with *Eco*RI, and the resulting DNA fragment, carrying a *DHOD* gene, was subcloned in the *Eco*RI site of pUC18. The open reading frames of *DHOD1*, *DHOD2*, and *DHOD3* were completely sequenced using synthetic oligonucleotide primers and an automated DNA sequencer (Model CEQ8000, Beckman Coulter Inc., Fullerton, CA, USA) under the conditions recommended by the manufacturer.

2.2. Bacterial expression of the recombinant T. cruzi DHODs

The open reading frame of each *DHOD* was PCR amplified using sense primers specific for *DHOD1* (5′-CACCATGATGCGTCTGAAACTCAA-3′), *DHOD2* (5′-CACCATGATGTGTCTGAAGCTCAA-3′), and *DHOD3* (5′-CACCATGACGTGTCTGAAGCTCAA-3′), a common antisense primer (5′-TCACTCAATTGTCTTGACAC-3′), and KOD plus DNA polymerase (High-fidelity type, Toyobo Co., Ltd., Osaka, Japan). The PCR products were cloned in pET100/D-TOPO® vector (Invitrogen, San Diego, CA, USA), which allows N-terminal fusion of a His_6-tag with the recombinant protein. Each cloned *DHOD* gene was sequenced and confirmed to be free of PCR-generated errors. The recombinant plasmids were used to transform BL21-CodonPlus® (DE3)-RP competent *E. coli* (Invitrogen), and expression of the recombinant *T. cruzi* DHOD was carried out as described, with minor modifications [7]. Briefly, expression was induced by incubating the cells in 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries, Tokyo) for 3 h. The cytosolic fraction of the bacteria was loaded onto a His•Bind® resin column (Novagen, EMD Biosciences, Inc., Madison, WI, USA) and the bound, His_6-tagged DHOD was eluted with 0.5M NaCl/0.5M imidazole/20 mM Tris, pH 7.4. This buffer in the protein eluate was

exchanged with phosphate-buffered saline (PBS, pH 7.2) using a PD-10 column (Amersham Biosciences Corp., Piscataway, NJ, USA). The final DHOD preparations were stored at −80 °C until use.

2.3. DHOD assays

DHOD activity was determined by incubating the enzyme in the presence of dihydroorotate (substrate) and fumarate (electron acceptor) in 50 mM potassium phosphate, and measuring orotate production by absorption at 300 nm ($\varepsilon = 3.30\ mM^{-1}\ cm^{-1}$). Optimal pH of each enzyme was determined by altering the pH of the potassium phosphate from 6.0 to 8.0. For kinetic analyses, each 100 μl reaction mixture, containing recombinant DHOD, dihydroorotate, fumarate, and 50 mM potassium phosphate, pH 7.0, was pre-incubated at 37 °C for 5 min and the reaction was started by addition of DHO. To evaluate orotate inhibition, the reaction mixture was incubated with orotate for 5 min before starting the reaction.

```
DHOD1         ATGATGCGTCTGAAACTCAACCTCCTCGACCATGTGTTCGCCAACCCCTTCATGAACGCC    60
DHOD2         ------T-------G---------------------------------------------
DHOD3         ----C-T-------G---------------------------------------------
AA rep            M->T
                    C->R

DHOD1         GCGGGTGTTCTCTGCAGCACCGAGGAAGACCTGCGCTGCATGACAGCCTCCTCCAGCGGC   120
DHOD2         -----A------------------------------------------------------
DHOD3         ------------------------------------------------------------
AA rep

DHOD1         GCACTTGTGTCGAAGAGCTGCACGAGTGCACCTCGCGATGGTAACCCCGAGCCGCGTTAC   180
DHOD2         ------------------------------------------------------------
DHOD3         ------------------------------------------------------------
AA rep

DHOD1         ATGGCCGTTCCACTTGGGAGCATCAATTCTATGGGGCTGCCTAACCTGGGCTTTGATTTC   240
DHOD2         ------T----------A------------------------------------------
DHOD3         -----------------A------------------------------------------
AA rep              F->V

DHOD1         TATTTGAAATACGCCATCGATCTGCACGATTACAGCAAGAAGCCGCTTTTTCTCTCCATT   300
DHOD2         ----------------G-------------------------------------------
DHOD3         ----------------G------------------------A---------G--------
AA rep                        S->I                               L->V

DHOD1         TCAGGTCTTTCCGTGGAGGAGAATGTGGCGATGGTGCGCCGCCTTGCCCCTGTGGCGCAG   360
DHOD2         ------------------------------------------------------------
DHOD3         ------------------------------------------------------------
AA rep

DHOD1         GAAAAAGGTGTATTGTTGGAGTTGAATCTTTCCTGCCCGAATGTGCCCGGCAAACCGCAG   420
DHOD2         ------------------------------------------------------------
DHOD3         ------------------------------------------------------------
AA rep

DHOD1         GTGGCCTATGACTTTGAGGCGATGCGCACTTACTTGCAGCAGGTGTCGTTGGCATACGGA   480
DHOD2         --------------------------------------------------------T---
DHOD3         --------------------------------------------------------T---
AA rep

DHOD1         TTGCCCTTTGGGGTGAAGATGCCGCCGTACTTTGATATTGCACACTTTGATACGGCTGCT   540
DHOD2         C----------C--------------------C---------------------------
DHOD3         C-C--------C--------------------C---------------------------
AA rep

DHOD1         GCTGTCTTGAATGAGTTCCCACTTGTCAAGTTTGTGACGTGTGTGAACAGTGTCGGCAAC   600
DHOD2         --------A--------------------------------------T------------
DHOD3         --------A--------------------------------------T------------
AA rep

DHOD1         GGCCTTGTTATTGATGCGGAGAGTGAGTCTGTTGTCATCAAACCCAAACAAGGGTTTGGT   660
DHOD2         ------------------------------------------------------------
DHOD3         ------------------------------------------------------------
AA rep

DHOD1         GGTATTGGCGGCAAGTATATCCTCCCTACAGCGCTGGCGAACGTGAATGCATTCTACCGC   720
DHOD2         ---C----------------------------AT-A------------------------
DHOD3         ---C----------------------------AT-A------------------------
AA rep           L->I

DHOD1         CGTTGTCCGGATAAGTTGGTCTTTGGCTGCGGCGGCGTTTACAGCGGCGAGGATGCCTTC   780
DHOD2         --C--C------------------------------------------------------
DHOD3         --C--C------------------------------------------------------
AA rep

DHOD1         TTGCATATACTTGCCGGTGCGTCGATGGTGCAAGTGGGAACTGCACTGCAGGAGGAGGGC   840
DHOD2         -----------------------------------------------------------G
DHOD3         -----------------------------------------------------------G
AA rep

DHOD1         CCCGGCATTTTTACGCGTCTTGAAGATGAGTTGCTGGAGATCATGGCACGGAAGGGGTAC   900
DHOD2         -----------------------------------------------G------------
DHOD3         -------------G---------------------------------G------------
AA rep                     T->R

DHOD1         AAGACTCTGGAGGAGTTCCGTGGACGTGTCAAGACAATTGAGTGA                  945
DHOD2         -G-------------------------------------------
DHOD3         ---------------------------------------------
AA rep         R->K
```

Fig. 2. Nucleotide sequences of the three *Trypanosoma cruzi* DHOD genes. Substitutions in the *DHOD2* and *DHOD3* nucleotide and deduced amino acid sequences are shown relative to those of *DHOD1*. Dashes indicate identical nucleotides.

3. Results

3.1. Nucleotide sequence polymorphism in the *T. cruzi DHOD* gene

To determine whether the three *DHOD* gene loci in *T. cruzi* Tulahuen strain are polymorphic, we subcloned the inserts of three *DHOD*-carrying phage clones, encoding DHOD1 (clone 16), DHOD2(clone 4), and DHOD3(clone 10) (Fig. 1). Each clone was found to have an open reading frame of 942 bp, without deletions or insertions, encoding 314 amino acids. We found that the three *DHOD* loci differed by 26 of the 942 nucleotides (2.75%), leading to replacement of 8 amino acids. Four of these amino acid replacements were not equivalent (Fig. 2). We calculated the molecular mass of DHOD1, DHO2, and DHOD3 as 34.2, 34.2, and 34.1 kDa, respectively, and their isoelectric points (pIs) were pH 5.6, 5.4, and 5.6, respectively.

We also found that the rate of nucleotide substitutions differed between each pair of *DHOD* gene loci. *DHOD1* differs from *DHOD2* and *DHOD3* by 21 (2.22%) and 23 (2.43%) nucleotides, respectively, resulting in 5 and 6 amino acid replacements, respectively. In contrast, *DHOD2* and *DHOD3* were found to differ by only 8 nucleotides (0.84%), resulting in replacement of 5 amino acids.

T. cruzi DHOD is classified as a family 1A enzyme [6], and, using *Lactococcus lactis* DHODA, the amino acid residues participating in the catalytic reaction have been well characterized [11–13]. The corresponding amino acids, Lys45, Asn69, Leu73, Asn129, Cys132, Asn134, Ser197 and Asn196, were all conserved among the three forms of *T. cruzi* DHOD.

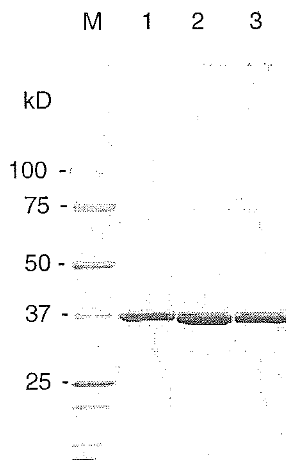

Fig. 3. Expression and purification of the recombinant *Trypanosoma cruzi* DHODs. Following expression in *E. coli* as His_6-tagged recombinant proteins and purification, 1 μg of purified DHOD1, DHOD2, and DHOD3 were loaded onto lanes 1–3, respectively, of a 10% SDS-polyacrylamide gel under denaturing conditions. Molecular weights of the protein standards (lane M) are indicated in kilodaltons (kD).

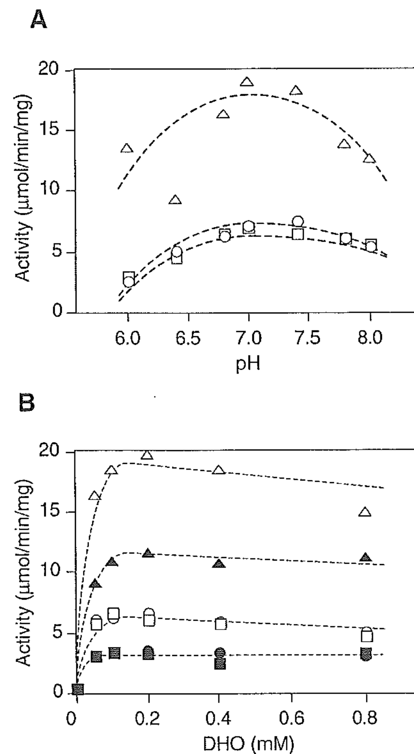

Fig. 4. Effects of pH (A) and temperature (B) on the activity of recombinant *Trypanosoma cruzi* DHODs. (A) Activity of DHOD1 (circle), DHOD2 (square), and DHOD3 (triangle) in the presence of 1 mM dihydroorotate (DHO) and 1 mM fumarate at 37 °C. (B). Activity in the presence of 50–800 μM DHO and 1 mM fumarate at 37 °C (open characters) and 25 °C (closed).

3.2. Expression of the recombinant *T. cruzi* DHODs

To determine the effect of the amino acid replacements, we assayed the kinetic properties of the three recombinant *T. cruzi* DHODs. Each recombinant *T. cruzi* DHOD with an N-terminal His_6-tag was expressed and purified by affinity chromatography as a single 37 kDa band (Fig. 3). Each of the purified DHODs was yellow, with absorption spectra having maxima at 375 and 459 nm, suggesting the presence of a flavin nucleotide, such as FMN, as a prosthetic group (data not shown).

Prior to kinetic analyses, we determined the optimal pH and temperature of each *T. cruzi* DHOD. We found that the optimal pH for all three was pH 7.0 (Fig. 4A). As *T. cruzi* parasitizes insects and mammals, we then measured the DHOD activity at 25 °C for the insect stage and at 37 °C for the mammalian

Table 1
Comparison of K_m values of *T. cruzi* DHODs

Enzyme	DHO (μM)[a]	Fumarate (μM)[b]
DHOD (native)[c]	25.9±5	53.4±3
DHOD1	30±3	30±8
DHOD2	30±3	35±6
DHOD[c]	24.3±4	43.9±2
DHOD3	71±6	67±3

In the presence of 1 mM fumarate[a] or 1 mM DHO[b].
[c]At 25°C [5].
All results are reported as mean±SD.

Table 2
Inhibition constants (K_i) for orotate against the recombinant *T. cruzi*

Enzyme	DHO (μM)[a]	Fumarate (μM)[b]
DHOD1	57.3±6.9	62.2±1
DHOD2	71.4±8.2	77.3±21.4
DHOD2[c]	20.5±3.6	15.6±4.32
DHOD3	42.4±17.4	49.0±17.8

In the presence of 1 mM fumarate[a] or 1 mM DHO[b].
[c]At 25°C [5].
All results are reported as mean±SD.

stage. All three *T. cruzi* DHODs showed a higher V_{max} at 37 °C than at 25 °C (Fig. 4B). These results can be correlated with findings that amastigotes replicating in mammalian cells depend on de novo synthesis of pyrimidines and are likely to require their accelerated biosynthesis [14]. Thus, all subsequent kinetic analyses of the *T. cruzi* DHODs were carried out at pH 7.0 and 37 °C.

We found that DHOD3 had the highest V_{max} at both 25 and 37 °C (Fig. 4B). This was unexpected, since none of the critical amino acids constituting the active site of the enzyme had been replaced.

3.3. Kinetic properties of the recombinant T. cruzi DHODs

We have previously demonstrated that native DHOD purified from epimastigotes of *T. cruzi* Tulahuen and recombinant DHOD (DHOD2 without the His_6-tag) shared very similar kinetic properties [5]. Since the kinetic properties of recombinant DHOD2 are similar in the presence or absence of the His_6-tag (Tables 1 and 2), we analyzed the kinetics of the three DHODs with His_6-tag. We found that the K_m values of DHOD1 and DHOD2 were similar and consistent with those reported earlier (Table 1). In contrast, DHOD3 showed higher K_m values for both DHO and fumarate.

Orotate is a competitive inhibitor for family 1A DHOD, including *T. cruzi* DHOD [5]. We found that orotate competitively inhibited the activity of all three DHODs with DHO and fumarate at comparable levels (Table 2). In contrast, the K_i values were slightly higher than those reported previously, which may have been due to differences in the assay temperature (Table 2).

4. Discussion

Variations in the number of *DHOD* gene copies among *T. cruzi* strains and in the enzymatic properties of proteins expressed from the different loci may lead to difficulty in developing clinically useful DHOD-specific inhibitors. In the present study, we demonstrated genetic polymorphisms in the three *T. cruzi DHOD* gene loci and the kinetic profiles of the recombinant DHODs. We observed replacement of 2.75% of the nucleotides, a rate much higher than that of other *T. cruzi* genes, such as dihydrolipoamide dehydrogenase (0.2%), and regulatory-particle non-ATPase subunit 1 of the proteasome (0.8%) [13,15]. Recent whole-genome sequencing of *T. cruzi* CL Brener, a reference strain of *T. cruzi* genome project, clearly revealed the presence of three complete *pyr* gene clusters and one partial cluster including *ACT* and *DHOD* in its genome [16,17]. Analysis of the putative four *DHOD* genes in the CL Brener genome showed replacement of 3.5% of the nucleotides, consistent with our findings (data not shown). In addition, when the intraspecies variations of the dihydrofolate reductase–thymidylate synthase (1473 bp) gene were analyzed using 18 strains of *T. cruzi*, it was found that 25 (1.7%) of 1473 nucleotides were replaced, resulting in three amino acid substitutions [18]. In contrast, the rate of nucleotide substitutions in the *ACT* gene, which juxtaposed to all three *DHOD* gene loci in *T. cruzi* Tulahuen, is 3.0% [9]. Thus, it is likely that the rate of evolution of the *pyr* genes, including *ACT* and *DHOD*, is faster than that of other genes, despite the essential role of the former in de novo pyrimidine biosynthesis.

The genome of *T. cruzi* Tulahuen, as well as CL Brener, has a hybrid nature, in that it is comprised of two distantly related *T. cruzi* lineages [18]. We found higher rates of nucleotide substitutions for *DHOD1* versus *DHOD2* or *DHOD1* versus *DHOD3* than for *DHOD2* versus *DHOD3*, suggesting that *DHOD1* and *DHOD2* differ in evolutionary origin, whereas *DHOD3* was derived by gene duplication of *DHOD2*. These findings disagree with those obtained by comparing the three *ACT* gene loci. That is, the insertion of three nucleotides, encoding Ala265, was observed in *ACT2* and *ACT3*, while these two genes shared less similarity [9]. It is possible that *ACT3* and *DHOD3* were derived from partial duplication of the *pyr* gene cluster, including *ACT2* and *DHOD2*, but *ACT3* acquired more nucleotide substitutions than *DHOD3*. Further analyses are necessary to clarify the mechanisms of duplication and evolution of the *pyr* genes in *T. cruzi*.

Although expression profiles of the three DHODs should be examined in relation to parasite physiology, it is very difficult. We could not identify each *DHOD* transcript by single-stranded DNA conformation polymorphism (SSCP), nor could we isolate each of the DHOD isoforms, due to their very similar molecular weight and pI, from parasite extracts. Likewise, recent proteomic analyses in *T. cruzi* CL Brener failed to demonstrate presence of DHODs and their peptides in any developmental stages [19]. It is noteworthy that all three *ACTs* in *T. cruzi* Tulahuen are transcribed in all developmental stages at steady-state level [9]. Evidence of polycistronic transcription in *T. cruzi* makes it likely that *DHOD* and *ACT* genes are transcribed in the similar manner.

Despite the extensive nucleotide substitutions, the amino acids constituting the active site are completely conserved in all three *T. cruzi* DHODs. However, this does not necessarily mean that the enzymatic properties of the three DHODs are identical or very similar. We therefore determined the kinetic properties of the recombinant enzymes, all of which were expressed as flavoproteins and displayed fumarate-dependent DHOD activity, characteristic of family 1A DHOD. Interestingly, all three DHODs had a higher V_{max} at 37 °C than at 25 °C. Amastigotes rely on de novo pyrimidine synthesis, suggesting a requirement for accelerated de novo biosynthetic activity [14]. Thus, the preference of *T. cruzi* DHOD for 37 °C

may reflect evolution of the parasite DHOD to fit the environment present in its mammalian hosts.

The kinetic properties of the three DHODs were comparable, except that DHOD3 had a 2- to 3-fold higher V_{max} and 2-fold higher K_m values for DHO and fumarate. DHOD3 shows replacement of Met2, Leu98, and Arg285 (in DHOD 1 and DHOD2) with Thr2, Val98, and Thr285, respectively. Although these three amino acids are not crucial for catalytic activity, their replacement may lead to alteration of the three-dimensional structure of the protein.

Among DHODs in Tulahuen, the eight amino acids are polymorphic: Met/Thr2, Cys/Arg3, Phe/Val63, Ser/Ile86, Leu/Val98, Leu/Ile222, Thr/Arg285, and Lys/Arg301 (See Fig. 2). Interestingly, all of these amino acid polymorphisms are conserved in the putative DHODs in CL Brener, with two additional polymorphic sites at Gly/Ala223 and Leu/Phe228 [16,17]. Conservation of the amino acid polymorphisms in different *T. cruzi* strains suggests that the amino acid polymorphism in the *DHOD*gene loci have occurred mostly before diversification of *T. cruzi* and conserved during their evolution. It is important that none of the amino acids participating in the catalytic reaction is polymorphic in both Tulahuen and CL Brener. Therefore, it is likely that DHOD isoforms share similar enzymatic properties, despite the extensive nucleotide/amino acid sequence polymorphisms among the gene loci and among *T. cruzi* strains.

Although the *T. cruzi* DHOD gene is highly polymorphic, the gene products are less polymorphic in their kinetic properties. The latter findings are favorable for drug design and facilitate exploitation of drug development. DHOD promises to be a drug target not only for Chagas' disease, but also for malaria, *Pneumocystis carinii* pneumonia, cancers, and autoimmune diseases [20–23]. The DHOD inhibitors currently used and their derivatives should be used to test the three forms of *T. cruzi* DHOD. In addition, marine algae extracts contain a substance that inhibits *T. cruzi* DHOD activity [7]. Crystallographic analyses may determine the precise conformation of *T. cruzi* DHOD, leading to discovery of potent enzyme inhibitors that can be used clinically to treat Chagas' disease.

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References

[1] Urbina JA, Docampo R. Specific chemotherapy of Chagas disease: controversies and advances. Trends Parasitol 2003;19:495–501.
[2] Urbina JA. Chemotherapy of Chagas disease. Curr Pharm Des 2002; 8:287–95.
[3] Jones ME. Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. Annu Rev Biochem 1980;49:253–79.
[4] Hines V, Keys III LD, Johnston M. Purification and properties of the bovine liver mitochondrial dihydroorotate dehydrogenase. J Biol Chem 1986;261:11386–92.
[5] Takashima E, Inaoka DK, Osanai A, Nara T, Odaka M, Aoki T, et al. Characterization of the dihydroorotate dehydrogenase as a soluble fumarate reductase in *Trypanosoma cruzi*. Mol Biochem Parasitol 2002; 122:189–200.
[6] Nara T, Hashimoto T, Aoki T. Evolutionary implications of the mosaic pyrimidine-biosynthetic pathway in eukaryotes. Gene 2000;257:209–22.
[7] Nara T, Kamei Y, Tsubouchi A, Annoura T, Hirota K, Iizumi K, et al. Inhibitory action of marine algae extracts on the *Trypanosoma cruzi* dihydroorotate dehydrogenase activity and on the protozoan growth in mammalian cells. Parasitol Int 2005;54:59–64.
[8] Gao G, Nara T, Nakajima-Shimada J, Aoki T. Novel organization and sequences of five genes encoding all six enzymes for de novo pyrimidine biosynthesis in *Trypanosoma cruzi*. J Mol Biol 1999;285:149–61.
[9] Nara T, Hirayama-Noguchi Y, Gao G, Murai E, Annoura T, Aoki T. Diversity of aspartate carbamoyltransferase genes of *Trypanosoma cruzi*. Int J Parasitol 2003;33:845–52.
[10] Annoura T, Nara T, Makiuchi T, Hashimoto T, Aoki T. The origin of dihydroorotate dehydrogenase genes of kinetoplastids, with special reference to their biological significance and adaptation to anaerobic, parasitic conditions. J Mol Evol 2005;60:113–27.
[11] Björnberg O, Rowland P, Larsen S, Jensen KF. Active site of dihydroorotate dehydrogenase A from *Lactococcus lactis* investigated by chemical modification and mutagenesis. Biochemistry 1997;36: 16197–205.
[12] Rowland P, Björnberg O, Nielsen FS, Jensen KF, Larsen S. The crystal structure of *Lactococcus lactis* dihydroorotate dehydrogenase A complexed with the enzyme reaction product throws light on its enzymatic function. Protein Sci 1998;7:1269–79.
[13] Rowland P, Nielsen FS, Jensen KF, Larsen S. The crystal structure of the flavin containing enzyme dihydroorotate dehydrogenase A from *Lactococcus lactis*. Structure 1997;5:239–52.
[14] Gutteridge WE, Gaborak M. A re-examination of purine and pyrimidine synthesis in the three main forms of *Trypanosoma cruzi*. Int J Biochem 1979;10:415–22.
[15] Zou CB, Nakajima-Shimada J, Nara T, Aoki T. Cloning and functional expression of Rpn1, a regulatory-particle non-ATPase subunit 1, of proteasome from *Trypanosoma cruzi*. Mol Biochem Parasitol 2000; 110:323–31.
[16] El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 2005;309:409–15.
[17] http://www.genedb.org/genedb/tcruzi/.
[18] Machado CA, Ayala FJ. Nucleotide sequences provide evidence of genetic exchange among distantly related lineages of *Trypanosoma cruzi*. Proc Natl Acad Sci U S A 2001;98:7396–401.
[19] Atwood III JA, Weatherly DB, Minning TA, Bundy B, Cavola C, Opperdoes FR, et al. The *Trypanosoma cruzi* proteome. Science 2005; 309:473–6.
[20] Ittarat I, Asawamahasakda W, Bartlett MS, Smith JW, Meshnick SR. Effects of atovaquone and other inhibitors on *Pneumocystis carinii* dihydroorotate dehydrogenase. Antimicrob Agents Chemother 1995; 39:325–8.
[21] Chen SF, Ruben RL, Dexter DL. Mechanism of action of the novel anticancer agent 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt (NSC 368390): inhibition of de novo pyrimidine nucleotide biosynthesis. Cancer Res 1986;46:5014–9.
[22] Baldwin J, Farajallah AM, Malmquist NA, Rathod PK, Phillips MA. Malarial dihydroorotate dehydrogenase. Substrate and inhibitor specificity. J Biol Chem 2002;277:41827–34.
[23] Sanders S, Harisdangkul V. Leflunomide for the treatment of rheumatoid arthritis and autoimmunity. Am J Med Sci 2002;323:190–3.

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IL4 Polymorphisms and IgE Levels on Malaria-Endemic Islands in Vanuatu

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Recent findings suggest that susceptibility to malaria is associated with genetic variants in the IL4 promoter region, resulting in the up-regulation of serum IgE. In this study, using a population-based approach, we investigated the mutant allele frequencies at positions −590 and +33 of the IL4 gene and total and *Plasmodium* (*P.*) *falciparum*-specific IgE levels on 3 islands with variable malaria endemicities in Vanuatu: Malakula (meso-endemic), Aneityum (meso-endemic with intervention), and Futuna (non-endemic). A total of 878 and 750 samples were typed for −590 and +33 positions, respectively. Variant allele frequencies varied from 0.27 to 0.39 for C−590T and from 0.39 to 0.48 for C+33T among 3 islands. There was a strong linkage disequilibrium between the 2 alleles ($p<0.001$). For both mutant alleles higher frequencies were detected in Aneityum than in Futuna ($p<0.05$). In Aneityum there was a significant association between the carriage of C+33T allele and increased levels of *P. falciparum*-specific IgE ($p<0.05$). However these relations were not observed in Malakula. This is the first report on IL4 polymorphisms in Melanesian populations. The observed mutant allele frequencies lay between higher values in Asian populations and rather lower values in Caucasians. The data suggest that IL4 promoter polymorphisms may be one of the genetic factors that explain relations between malaria disease and IgE.

Key words: malaria, IL4, IgE, polymorphism, Vanuatu

Introduction

The outcome of malaria infection is a reflection of host-parasite interactions, which in turn are dependent on their diversities. Ideal places to study these questions are isolated areas in Melanesia (the Southwest Pacific) with great variety of malaria endemic situations and also different ethnic populations. Our study area is the Vanuatu archipelago, which consists of 80 islands with 120 languages, confirming long time isolation. The islands of Vanuatu were settled less than 4,000 years ago during a rapid population expansion from Island Southeast Asia, that continued into western Polynesia[1)]. Malaria is endemic in most populated islands in Vanuatu while some small islands are apparently malaria-free[2)]. Malaria is unstable with apparently low mortality in Vanuatu[2)3)], while stable with high mortality in Papua New Guinea[4)]. This study represents part of the project to search for host genetic factors to explain these differences.

There is accumulating evidence that host genetic factors control malaria disease by regulating anti-malarial immune responses[5)]. A number of host genes have been identified, which seem to contribute to the protection against or susceptibility to malaria infection and disease. The geographical distri-

bution of certain genes in human populations may vary due to different selection pressures.

As example, the protection from malaria due to red cell abnormalities was documented in the sickle cell trait[6)7)], thalassaemias[8)] and G6PD deficiency[9)].

A number of other host genes have also been identified which may be related to acquisition of protective immunity against malaria disease, including polymorphisms in MHC class I genes[10)], regulating the production or expression of the inflammatory cytokine TNF[11)]. The previous studies showed that the class I antigen HLA-Bw53 and the class II haplotype DRB1*1302-DQB1*0501, associated with significant protection against severe malaria, are present in higher frequencies in people in Sub-Saharan Africa[10)12)] but rare in Caucasians[13)], while absent in Pacific islanders[14)]. A sib-pair linkage between the chromosome region 5q31-q33 and *Plasmodium* (*P.*) *falciparum* blood infection levels has been reported[15)]. This region is also linked to production of the cytokine IL4[16)] and to elevated serum levels of IgE[17)], which in turn may be related to pathogenesis.

IL4 is a multifunctional cytokine which serves as an important regulator in isotype switching from IgM/IgG to IgE[18)19)]. IL4 also regulates the differentiation of precursor T-helper cells into the Th2 subset that regulates humoral immunity and specific antibody production[20)]. Polymorphisms in the IL4 gene are known to play a significant role in allergic diseases such as atopic asthma[21)22)]. Recent investigations have revealed that genetic variants of the IL4 promoter regions lead to different transcription efficiency or dysregulation[23)], thereby causing variations in cytokine production.

In the IL4 gene, a single nucleotide polymorphism at 590 bp upstream of the transcriptional start site (C−590T) is known to have a higher luciferace reporter activity[24)], and this mutant allele has been found to modulate total serum IgE levels in asthma[22)]. An association between the C+33T mutant allele of the IL4 gene and increased levels of IgE was also documented in Japanese asthmatic patients[25)]. In this population, a strong linkage disequilibrium was found between the +33T allele and the −590T allele[26)], suggesting that both alleles may be acting on enhancement of the transcription and regulation of IgE production.

In *P. falciparum* infections, elevated levels of both total and malaria-specific IgE in sera of populations from malaria-endemic regions were documented[27)28)] and though the role of these IgEs are not particularly clear, IL4 seems to be involved in their excessive production. A recent study in Burkina Faso suggested that the mutant allele IL4 −590T is associated with elevated levels of anti-malarial antibody in the Fulani, thereby protecting the members of this ethnic group against malaria[29)]. Other studies also showed an association between genetic variants of the IL4 gene with increased total IgE levels and susceptibility to malaria[30)31)].

Sequestration of parasitized red blood cells in the endothelium of various organ vessels is an important mechanism in severe forms of malaria and this has been hypothesized to result from the up-regulation of TNF-α[32)]. A recent study also indicate the involvement of IL4 in inducing the high expression of the adhesion molecule V-CAM on endothelial cells[33)], a receptor for the *P. falciparum* erythrocyte membrane protein 1, a molecule implicated in sequestration.

In the IL4 gene, while high frequencies of the T mutant alleles (C−590T/C+33T) were found in Asian populations[25)34)], rather low frequencies were reported in Caucasians and populations from Sub-Saharan Africa[30)31)35)] except for the Fulani ethnic group where high frequencies were found[29)]. In this study, using a population-based approach, we investigated the mutant allele frequencies of the IL4 gene at positions −590 and +33 in Melanesian populations in Vanuatu and whether these polymorphisms were influenced by variable malaria endemicities. We also investigated the association between these polymorphisms and total and malaria-specific IgE production.

Since most of previous studies on the relation between IL4 polymorphism and malaria were conducted in Sub-Saharan Africa, this study in Melanesia provides a unique opportunity to compare results from this region with previous results from Sub-Saharan Africa.

Materials and Methods

Study areas

This study was designed as a component of malariometric surveys conducted on 3 islands of Vanuatu from 1997 to 1998. The details of these surveys were already reported elsewhere[36]. Briefly, Malakula is a meso-endemic island without any effective control measure. Aneityum is also potentially meso-endemic with a malaria-free situation since 1991, when an effective control measure was taken. Futuna is a non-endemic island due to the absence of vector *Anopheles* mosquito.

Filter paper blood sampling

During the above-mentioned surveys, finger-prick blood samples were also drawn into a 75 μl heparinized capillary tube (Drummond Scientific Company, USA) and transferred unto chromatographic filter paper (ET31CHR, Whatman Limited, England). After drying, the labeled filter paper samples were stored in separate small plastic bags at −20℃ until analyses performed on them.

DNA extraction and PCR

From each of randomly selected sub-samples, DNA was extracted from a quarter (19 μl) of one blood spot dried on filter paper using the method as previously described[37]. The IL4 +33 was typed by PCR amplification of a 200 bp fragment (forward primer 5'-TGCATCGTTAGCTTCTCCTG-3' and reverse primer 5'-biotin-GCTCTGTGAGGCTGTTCAAA-3') under the following conditions: denaturing at 95℃ for 10 min, amplification for 30 cycles at 95℃ for 15 sec, 60℃ for 15 sec and 72℃ for 30 sec and finally 72℃ for 10 min. The IL4 −590 position was typed by amplifying a 252 bp fragment (forward primers 5'-biotin-GCCTCACCTGATACGACCTG-3' and reverse primers 5'-GGGGCTCCTTCTCTGCAT-3') under the following conditions; denaturing at 95℃ for 10 min, amplification for 32 cycles at 95℃ for 15 sec, 62℃ for 15 sec, 72℃ for 30 sec and then finally extension at 72℃ for 10 min. All PCR products were run on 1% agarose gel to detect amplified fragments and visualized with UV illumination after staining with ethidium bromide. Template-free controls were included in each experiment.

Pyrosequencing

For both positions (IL4 +33, −590), the PCR products were immobilized on streptavidin-sepharose paramagnetic beads (Amersham Bioscience, Sweden) and the strands separated using 0.2 M NaOH. After separating the supernatant, 10 pmol of sequencing primer was added and annealed to the captured strands. The pyrosequencing primer sequences were 5'-TGTCCACGGACACA-3' and 5'-CCCAGCACTGGGG-3' for +33 and −590 positions respectively. The reactions were performed in a 96-well plate. The primed single-stranded DNA templates were then transferred to the microtitre plate-based PSQ™ Pyrosequencer, where real time sequencing of the IL4 gene was performed. Prior to this, an optimum dispensation order was determined and selected positions with mutations in the entire codon were detected after running the samples.

IgE antibody measurements

Levels of total IgE and *P. falciparum*-specific IgE were determined in study subjects from Aneityum and Malakula using ELISA as previously described[27]. A quarter (19 μl) of one blood spot dried on filter paper was extracted in phosphate-buffered saline with 0.5% bovine serum albumin (Fraction V, Boehringer Mannheim, Mannheim, Germany). For specific IgE, antigen was prepared from mature stages of the *P. falciparum* laboratory strain F32.

Statistical analysis

Allelic frequency distribution was tested according to the Hardy-Weinberg equilibrium by χ^2 test. The χ^2 test was also used to test for linkage disequilibrium of polymorphisms using observed and expected allele frequencies. The t-test was used to compare log-transformed IgE levels in different groups. The SPSS ver. 11 statistical package was used for the statistical analysis. P values less than 0.05 were considered significant.

Ethical considerations

The study was approved by the Department of Health, Vanuatu and the ethics committee of Tokyo Women's Medical University (No. 69). Study subjects were briefed on purposes and procedures of

Table 1 Polymorphisms in IL4 gene on 3 islands in Vanuatu: Malakula (meso-endemic), Aneityum (meso-endemic with intervention), and Futuna (non-endemic)

	IL4 −590					IL4 +33				
	n	CC	CT	TT	T allele frequency	n	CC	CT	TT	T allele frequency
Malakula	269	150 (56)	95 (35)	24 (9)	0.27	260	87 (34)	144 (55)	29 (11)	0.39
Aneityum	472	177 (37)	220 (47)	75 (16)	0.39	385	97 (25)	210 (55)	78 (20)	0.48
Futuna	137	74 (54)	48 (35)	15 (11)	0.28	105	44 (42)	41 (39)	20 (19)	0.39
Total	878	401 (45)	363 (42)	114 (13)	0.34	750	228 (30)	395 (53)	127 (17)	0.43

Percentages are in parentheses.

Table 2 Age-specific geometric mean IgE levels on Aneityum and Malakula

Age group (years old)	Aneityum			Malakula		
	n	Total IgE (ng/ml)	*P. falciparum*-specific IgE (ng/ml)	n	Total IgE (ng/ml)	*P. falciparum*-specific IgE (ng/ml)
0-5	48	508	0.99	36	424	3.35
6-15	41	1,720	0.97	87	2,208	3.03
16-30	34	1,882	0.91	36	2,752	3.60
> 30	37	1,772	0.93	–	–	–

Table 3 Association between IL4 polymorphisms and IgE levels in subjects aged older than 5 years on Malakula and Aneityum

IgE (mean log ng/ml)	IL4 −590						IL4 +33					
	Malakula			Aneityum			Malakula			Aneityum		
	CC	CT/TT	p	CC	CT/TT	p	CC	CT/TT	p	CC	CT/TT	p
(n)	(45)	(31)		(18)	(36)		(19)	(57)		(15)	(39)	
Total	3.46	3.36	0.498	3.18	3.24	0.702	3.52	3.38	0.315	3.07	3.28	0.171
P. falciparum-specific	0.51	0.52	0.925	− 0.1	0	0.111	0.57	0.5	0.209	− 0.14	0	0.028*

*significant.

the survey and a written informed consent was obtained from each adult subject while in the case of children by their guardians who determined the child's participation.

Results

Genotypes and allele frequencies

Table 1 summarizes the distribution of genotypes and allele frequencies of the two IL4 polymorphic positions in the study populations. A total of 878 and 750 samples were typed for IL4 −590 and +33, respectively. In Malakula, Aneityum and Futuna, T allele frequencies were 0.27, 0.39 and 0.28 for IL4 −590 and 0.39, 0.48 and 0.39 for IL4 +33, respectively. Genotype distributions of both −590 and +33 positions were in agreement with the Hardy-Weinberg equilibrium ($p<0.05$). In all islands, the frequency of the homozygous TT allele was the lowest. Comparisons between islands for the genotype CC alone vs CT/TT revealed significantly higher frequencies of CT/TT genotypes in Aneityum than in Futuna for both −590 ($p<0.001$) and +33 positions ($p<0.01$). However there was no significant difference between Malakula and Futuna in these frequencies. There was a significant linkage disequilibrium between the −590 and +33 positions ($p<0.001$).

IgE levels

Table 2 summarizes age-specific distribution of geometric mean total and *P. falciparum*-specific IgE in subjects from Aneityum and Malakula. In both islands total IgE values are significantly higher in subjects >5 years old than those ≤5 years old.

Among age groups >5 years old, IgE levels were not significantly different. Hence the subsequent analysis on association between IL4 polymorphisms and IgE levels was conducted for subjects >5 years old. Age effect was not observed for specific IgE levels in both islands. In each age group mean specific IgE value was significantly higher in Malakula than in Aneityum.

Associations between IL4 polymorphisms and IgE levels

Table 3 summarizes associations between carriage of a T allele (CC vs CT/TT) and serum IgE levels (total and *P. falciparum*-specific) at the two polymorphic sites. We detected a significantly higher *P. falciparum*-specific IgE level in IL4 +33T allele carriers (CT/TT) than non carriers (CC) in Aneityum ($p<0.05$). This relation was not observed in subjects from Malakula.

Discussion

This is the first report to describe the degree of IL4 polymorphisms in Melanesian population and also to demonstrate an association between carriage of the mutant T allele and elevated levels of malaria-specific IgE.

The IL4 promoter mutant allele frequencies observed in Vanuatu were rather unique and different from those previously reported in other geographical populations. The observed mutant allele frequencies lay between higher values in Asian populations and rather lower values in Caucasian and Sub-Saharan populations. A study in Japanese subjects revealed that the frequency of the IL4 +33 mutant allele was 0.7 in asthmatic patients[25]. Another study in Taiwan depicted a similar high frequency of 0.78 of the IL4 −590T in patients with systemic lupus erythematosus[34]. In Caucasians lower frequencies (0.13 and 0.15) of the −590T allele were reported in asthmatic patients in Germany[35] and UK[38], respectively. Relatively low frequencies were also reported in populations from Sub-Saharan Africa with frequencies ranging from 0.22[36] to 0.28[31], except for the Fulani ethnic group where 0.45 mutant allele frequency was found[29].

As an example of ethnically specific genetic profiles of Vanuatu populations, we previously reported an unprecedentedly high prevalence of cytochrome P450 (CYP) 2C19-related poor metabolizer genotype individuals on the islands of Vanuatu[39]. In addition, there was substantial variation among populations of Vanuatu. Comparisons of genetic, linguistic and geographic patterns among populations suggest a strong geographic component to the current distribution of CYP2C19 alleles in Vanuatu[39]. Previous studies of patterns of malaria incidence and frequencies of alpha-thalassaemia and G6PD deficiency in the Papua New Guinea[8,40] and Vanuatu[2] have indicated selection on these loci by malaria.

The observed linkage disequilibrium between C−590T and C+33T in our study is consistent with the previous finding in a Japanese population[26]. The genetic association between single nucleotide polymorphisms (SNPs) in the IL4 promoter region and elevated levels of total serum IgE or IgG have been documented by several workers[25,29,35]. In addition, the clinical significance of IL4 polymorphisms in disease sequels has been reported in malaria[30,32,41] and in asthma[42].

Our study found a significant difference in frequencies of T allele carriers for both mutant alleles between meso-endemic Aneityum and non-endemic Futuna. Furthermore in Aneityum there was a significant association between the carriage of IL4 +33T allele and mean concentrations of *P. falciparum*-specific IgE. The observations in Aneityum suggest the role of IL4 promoter polymorphisms in up-regulating *P. falciparum*-specific IgE serum levels in the study subjects. A similar relation but with different repertoires was observed in a case-control study in West Africa, where the IL4 −590T allele frequency of 0.22 in one ethnic group[30] and a rather high frequency of 0.45 in another group (the Fulani) were found with association of elevated *P. falciparum*-specific IgG levels[29]. In the same region, the severity of malaria in children was also found to be associated with elevated levels of *P. falciparum*-specific IgE[43].

However in our study the mutant allele frequencies in another meso-endemic Malakula were similar to those in Futuna, suggesting selection towards IL4 mutation due to malaria endemicity is not uni-

versal.

Although we could not detect any significant association between the IL4 T allele carriage and total IgE, in earlier studies in Sub-Saharan Africa this association was found significant[30)31)]. Discrepancy between our study in Melanesia and the previous studies in Sub-Saharan Africa can be explained mainly by regional differences of environmental factors which contribute to total IgE levels, including repertoires of helminthes infections. There are also differences of degree of malaria disease severity in study subjects. While the study in Sub-Saharan Africa was conducted mainly on symptomatic cases including cerebral malaria, our study in Vanuatu was on asymptomatic parasite carriers.

The association between IL4 polymorphisms and specific IgE levels detected in Aneityum was not observed in Malakula, although both islands are potentially meso-endemic. This discrepancy may be explained by differences of malaria situations in the previous years. In Aneityum an integrated malaria control program was introduced in 1991 and a nearly malaria-free situation was maintained at the time of the survey, while in Malakula malaria transmission was continuous without any effective control measure. The observed difference of *P. falciparum*-specific IgE levels represents difference of transmission between two islands, while similar levels of total IgE are due to other common infections including intestinal helminthes. The difference of transmission might result in different immune response and persistence between Aneityum and Malakula, including *P. falciparum*-specific IgE levels.

In Aneityum we could not observe any difference of specific IgE level between subjects older than 5 years and those under 5 years, who have never been exposed to malaria, suggesting cross reactivity and limited role of specific IgE in asymptomatic subjects in our studies. It is obviously interesting to investigate relation between IL4 polymorphisms and IgE levels in severe malaria patients on these islands in future. In conclusion, this is the first report on the IL4 promoter polymorphisms in Melanesian populations. The observed mutant allele frequencies lay between higher values in Asian populations and rather lower values in Caucasians. The data suggest that IL4 promoter polymorphisms may be one of the genetic factors that explain association between malaria disease and IgE. However, further investigations are necessary to clarify the implications of these polymorphisms on malaria disease manifestations in Melanesia.

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References

1) **Diamond JM**: Express train to Polynesia. Nature **336**: 307–308, 1988
2) **Kaneko A, Taleo G, Kalkoa M et al**: Malaria epidemiology, G6PD deficiency and human settlement in the Vanuatu archipelago. Acta Trop **70**: 285–302, 1998
3) **Maitland K, Kyes S, Williams TN et al**: Genetic restriction of *Plasmodium falciparum* in an area of stable transmission: an example of island evolution? Parasitology **120**: 335–343, 2000
4) **Cattani JA, Tulloch JL, Vrbova H et al**: The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. Am J Trop Med Hyg **35**: 3–15, 1986
5) **Jepson AP, Banya WA, Sisay-Joof F et al**: Genetic regulation of fever in *Plasmodium falciparum* malaria in Gambian twin children. J Infect Dis **172**: 316–319, 1995
6) **Weatherall DJ**: Common genetic disorders of the red cell and the 'malaria hypothesis'. Ann Trop Med Parasitol **81**: 539–548, 1987
7) **Nagel RL, Fabry ME, Kaul DK et al**: Known and

potential sources for epistatic effects in sickle cell anemia. Ann N Y Acad Sci 565: 228–238, 1989

8) Flint J, Hill AV, Bowden DK et al: High frequencies of alpha-thalassaemia are the result of natural selection by malaria. Nature 321: 744–750, 1986
9) Ruwendu C, Hill A: Glucose-6-phosphate dehydrogenase deficiency and malaria. J Mol Med 76: 581–588, 1998
10) Hill AVS, Allsopp CEM, Kwiatkowski D et al: Commom West African HLA antigens are associated with protection from severe malaria. Nature 352: 595–600, 1991
11) Fernandez-Reyes D, Craig AD, Kyes SA et al: A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya. Hum Mol Genet 6: 1357–1360, 1997
12) Okoye RC, Williams E, Alonso A et al: HLA polymorphisms in Nigerians. Tissue Antigens 25: 142–155, 1985
13) Baur MP, Neugebauer M, Deppe H et al: Population analysis on the basis of deduced haplotypes from random families. *In* Histocompatibility Testing (Albert ED, Baur MP, Mayr WR eds), pp333–341, Springer-Verlag, Berlin・Heidelberg・New York・Tokyo (1984)
14) Serjeantson SW, Hill AVS: The colonization of the Pacific: The genetic evidence. *In* The Colonization of the Pacific: a Genetic Trait (Hill AVS, Serjeantson SW eds), pp120–173, Oxford University Press, Oxford (1989)
15) Rihet P, Traore Y, Abel L et al: Malaria in humans: *Plasmodium falciparum* blood infection levels are linked to chromosome 5q31-q33 chromosome region. Am J Hum Genet 63: 498–505, 1998
16) Keegan AD, Nelms K, Wang LM et al: Interleukin 4 receptor: signaling mechanisms. Immunol Today 15: 423–432, 1994
17) Marsh DG, Neely JD, Breazeale DR et al: Total serum IgE levels and chromosome 5q. Clin Exp Allergy 25: 79–83, 1995
18) Vitetta ES, Brooks K, Chen YW et al: T-cell-derived lymphokines that induce IgM and IgG secretion in activated murine B cells. Immunol Rev 78: 137–157, 1985
19) Nelms K, Keegan AD, Zamorano J et al: The IL4 receptor: signaling mechanisms and biologic function. Annu Rev Immunol 17: 701–737, 1999
20) Romagnani S: Biology of human Th1 and Th2 cells. J Clin Immunol 15: 121–129, 1995
21) Ober C, Tsalenko A, Parry R et al: A second-generation genomewide screen for asthma susceptibility alleles in a founder population. Am J Hum Genet 67: 1154–1162, 2000
22) Rosenwasser LJ, Klemm DJ, Dresback JK et al: Promoter polymorphism in the chromosome 5 gene cluster in asthma and atopy. Clin Exp Allergy 25: 74–78, 1995
23) Anderson LC, Beaty JS, Nettles JW et al: Allelic polymorphism in transcriptional regulatory regions of HLA-DQB genes. J Exp Med 173: 181–192, 1991
24) Borish L, Mascali JJ, Klinnert M et al: SSC polymorphism in interleukin genes. Hum Mol Genet 3: 1710, 1994
25) Suzuki I, Hizawa N, Yamaguchi E et al: Association between a C+33 polymorphism in the IL-4 promoter region and total serum IgE levels. Clin Exp Allergy 30: 1746–1749, 2000
26) Suzuki I, Yamaguchi E, Hizawa N et al: A new polymorphism in the 5' flanking region of the human interleukin (IL)-4 gene. Immunogenetics 49: 738–739, 1999
27) Perlmann H, Helmby H, Hagstedt M et al: IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum*: association of high IgE levels with cerebral malaria. Clin Exp Immunol 97: 284–292, 1994
28) Nyakeriga MA, Troye-Blomberg M, Bereczky S et al: Immunoglobulin E (IgE) containing complexes induce IL4 production in human basophils: effect on Th1-Th2 balance in malaria. Acta Trop 86: 55–62, 2003
29) Luoni G, Verra F, Arca B et al: Antimalarial antibody levels and IL4 polymorphism in the Fulani of West Africa. Hum Hered 2: 411–414, 2001
30) Verra F, Luoni G, Calissano C et al: IL4-589/T polymorphism and IgE levels in severe malaria. Acta Trop 90: 205–209, 2004
31) Gyan BA, Goka B, Cvetkovics JT et al: Allelic polymorphisms in the repeat and promoter regions of the interleukin-4 gene and malaria severity in Ghanaian children. Clin Exp Immunol 138: 145–150, 2004
32) Kwiatkowski D, Bate CAW, Scragg IG et al: The malarial fever response-pathogenesis, polymorphism and prospects for intervention. Ann Trop Med Parasitol 91: 533–542, 1997
33) Schleimer RP, Sterbinsky SA, Kaiser J et al: IL-4 induces adherence of human eosinophils and basophils but not neutrophils and endothelium: association with expression of VCAM-1. J Immunol 148: 1086–1092, 1992
34) Wu M-C, Huang C-M, Tsai JJP et al: Polymorphisms of the interleukin-4 gene in Chinese patients with systemic lupus erythematosus in Taiwan. Lupus 12: 21–25, 2003
35) Kabesch M, Tzotcheva I, Carr D et al: A complete screening of the IL4 gene: novel polymorphisms and their association with and IgE in childhood. J Allergy Clin Immunol 112: 893–898, 2003
36) Kaneko A, Taleo G, Kalkoa M et al: Malaria eradication on islands. Lancet 356: 1560–1564, 2000
37) Uchida JY, Kasahara T, Bobogare A et al: The prevalence of falciparum malaria in the Solomon islands investigated by a filter paper disk-PCR method. Jpn J Parasitol 44: 119–127, 1995
38) Marshall SE, McLaren AJ, McKinney EF et al: Donor cytokine genotype influences the development of acute rejection after renal transplantation. Transplantation 71: 469–476, 2001
39) Kaneko A, Lum JK, Yaviong J et al: High and variable frequencies of *CYP2C19* mutations: medi-

cal consequences of poor drug metabolism in Vanuatu and other Pacific islands. Pharmacogenetics 9: 581–590, 1999

40) Ganczakowski M, Town M, Bowden DK et al: Multiple glucose 6-phosphate dehydrogenase-deficient variants correlate with malaria endemicity in the Vanuatu Archipelago (Southwestern Pacific). Am J Hum Genet 56: 294–301, 1995

41) McGuire W, Hill AVS, Allsopp CEM et al: Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria. Nature 371: 509–511, 1994

42) Noguchi E, Shibasaki M, Arinami T et al: Association of asthma and the interleukin-4 promoter gene in Japanese. Clin Exp Allergy 28: 449–453, 1998

43) Calissano C, Modiano D, Sirima BS et al: IgE antibodies to *Plasmodium falciparum* and severity of malaria in children of one ethnic group living in Burkina Faso. Am J Trop Med Hyg 69: 31–35, 2003

ヴァヌアツのマラリア流行島嶼における IL4 多型と IgE 濃度

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血中 IgE 上昇に帰結する IL4 プロモーターの遺伝的変異が，マラリアに対する感受性と相関することが最近の研究によって示唆されてきている．本研究においては集団遺伝学的方法を用いヴァヌアツにおけるマラリア流行度が異なる 3 島嶼において，IL4 −590 および +33 塩基における変異対立遺伝子頻度，血中総 IgE および熱帯熱マラリア原虫特異的 IgE 濃度を調べた．3 島嶼は中等度の流行が続く Malakula，中等度の流行だが対策が功を奏している Aneityum およびマラリア流行がない Futuna である．これらの島嶼住民より採取した血液サンプルより IL4 −590 および +33 についてそれぞれ計 878 および 750 サンプルの解析を行った．変異対立遺伝子頻度はこれら 3 島嶼間において C−590T が 0.27〜0.39，C+33T が 0.39〜0.48 の範囲で変動した．両対立遺伝子間には顕著な連鎖不均衡が認められた ($p<0.001$)．これら両変異対立遺伝子とも Aneityum においては Futuna より高い頻度で認められた ($p<0.05$)．Aneityum においては IL4 +33 位における変異対立遺伝子の存在する群での血中熱帯熱マラリア原虫特異的 IgE 濃度は有意に上昇していた ($p<0.05$)．しかしながら，これらの関係は Malakula においては認められなかった．本研究はメラネシア住民集団において当該変異遺伝子頻度に関する最初の報告である．見出された変異対立遺伝子頻度はこれまで報告されている，より高いアジア住民集団とより低いヨーロッパ住民集団の中間の値であった．さらに IL4 多型が特異的 IgE とマラリア病形の関係に関る遺伝的因子の一つであることが示唆された．

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Original Article

Microsatellite Polymorphism in the Heme Oxygenase-1 Gene Promoter Is Associated with Susceptibility to Cerebral Malaria in Myanmar

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SUMMARY: Cerebral malaria (CM) is a serious complication of *Plasmodium falciparum* malaria, and its pathogenesis leading to coma remains unknown. Heme oxygenase-1 (HO-1) catalyzes heme breakdown, eventually generating bilirubin, iron and carbon monoxide. The HO-1 gene promoter contains a polymorphic $(GT)_n$ repeat which may influence the expression level of HO-1. To explore the correlation between this $(GT)_n$ polymorphism and susceptibility to CM, we analyzed the frequencies of the $(GT)_n$ alleles in 120 Myanmarese patients with uncomplicated malaria (UM) and 30 patients with CM. The frequency of homozygotes for the short $(GT)_n$ alleles (<28 repeats) in CM patients was significantly higher than those in UM patinets ($P < 0.008$, OR = 3.14). Thus, short $(GT)_n$ alleles represent a genetic risk factor for CM.

INTRODUCTION

Cerebral malaria (CM) is the most serious complication of malaria caused by *Plasmodium falciparum*, manifesting as coma. CM may be related to the sequestration of infected red blood cells (RBCs) to the cerebral microvasculature (1,2). The adherence of parasitized RBCs to the vascular endothelium may result in local ischemia and hypoxia, microhemorrhage and the release of hemoglobin after hemolysis of parasitized erythrocytes (1-3), leading to CM in certain susceptible individuals. However, in addition to the effects of direct parasite sequestration, the possible role of host and parasite metabolic processes in inducing coma has begun to attract considerable interest.

Patients with malaria must cope with excess amounts of cellular and plasma hemoglobin, because a large number of infected and uninfected RBCs are destroyed in the spleen or rupture in the blood stream. Free hemoglobin can serve as a biological Fenton's reagent to provide iron for the generation of hydroxyl radicals (4,5), and is therefore quickly removed from circulation by haptoglobin and taken up by macrophages. Within a cell, hemoglobin is dissociated into heme and globin, and the heme moiety is cleaved to generate iron, carbon monoxide (CO) and biliverdin by heme oxygenase-1 (HO-1), a microsomal inducible enzyme (6). Biliverdin is subsequently reduced to bilirubin, a potent antioxidant (7). The potential beneficial and harmful effects of heme breakdown products in falciparum malaria have been reviewed in a previous study (8).

In the present paper, we analyzed $(GT)_n$ repeat polymorphism in the promoter region of the inducible HO-1 of patients with CM and with uncomplicated malaria (UM), finding a significant association between homozygotes of short alleles and CM.

MATERIALS AND METHODS

Subjects: One hundred and twenty Karen patients with UM (mean age, 23.4 ± 8.1; mean hemoglobin, 11.9 ± 2.4 g/dl) from the Mae Sot Malaria Clinic and 30 patients with CM (mean age, 23.4 ± 8.1; mean hemoglobin, 11.8 ± 3.0 g/dl) admitted to the Mae Sot General Hospital, Mae Sot, Thailand were recruited for this study between 1996 and 1997. UM was defined by a positive smear without any of the following criteria of severe malaria (3): high parasitemia (>100,000 parasites/μl), severe anemia (hematocrit <20%, or hemoglobin <7.0 g/dl) or coma., CM was diagnosed by coma without any other recognized cause of altered consciousness. The protocol, including the informed consent form, was approved by the ethical review committee of the Ministry of Health of Thailand and by the institutional review board in Nagasaki. All procedures were appropriately explained to patients in their own language.

Extraction of genomic DNA: Genomic DNA was extracted from 1 ml of peripheral venous blood with 0.05 M EDTA using a DNA extractor kit (Wako Pure Chemicals, Osaka, Japan).

Microsatellite polymorphism: To amplify the $(GT)_n$ microsatellite located at position -270 of the HO-1 gene (9), a fluorescein-conjugated 5′ primer (HeOp-1/6-FAM 5′-

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agagcctgcagcttctcaga-3′) and an unlabeled 3′ primer (HeOp-1/R 5′-acaaagtctggccataggac-3′) were used for PCR amplification with 35 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 3 min. An amplicon of at least 148 bp from positions -165 to -312 of the HO-1 gene was obtained (10). The size of the PCR products was analyzed with a laser-based automated DNA sequencer, ABI GeneScan GS-310 (Applied Biosystems, Foster City, Calif., USA), with five cloned alleles that were labeled with different colors as size markers. The repeat numbers of these cloned alleles used as size markers were 21, 23, 25, 28 and 30.

Statistical analysis: The associations between disease groups and specific classes of alleles, and between disease groups and genotype groups were analyzed for significance by the chi-square test of independence by 2×2 contingency table and the P values were corrected using Bonferroni's method. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative disease risk conferred by a particular allele and genotype. The age and sex ratios of subjects with UM and CM were matched in the present study. Furthermore, all subject were Myanmarese, who represent a distinct ethnic population.

RESULTS

The number of $(GT)_n$ repeats in the HO-1 gene was between 14 and 38 in all subjects. The distribution of the numbers of $(GT)_n$ repeats was trimodal, with peaks at 23, 30 and 36 repeats (Figure 1). We therefore grouped the alleles into three subclasses: short (S) alleles with 14-27 repeats, intermediate (M) with 28-33 repeats and long (L) with 34-38 repeats, as described in the analysis by McGinnis and Spielman (11). The frequencies of the L, M and S allele groups were 0.11, 0.45 and 0.44, respectively, in UM patients and 0.05, 0.32 and 0.63 in CM patients. The allele frequencies in CM and UM were not significantly different.

The frequencies of the genotypes (LL, ML, MM, SL, SM, SS) of $(GT)_n$ repeats were 0.0, 12.5, 16.7, 9.2, 44.2 and 17.5%, respectively, in UM cases (n = 120), and 0.0, 0.0, 13.3, 10.0, 36.7 and 40.0% in CM cases (n = 30) (Table 1a). The frequency of homozygotes for S alleles in the CM group (40%) was significantly higher than that in the UM group (17.5%) after Bonferroni correction (P < 0.008; OR = 3.14; 95% CI: 1.32-7.49) (Table 1b).

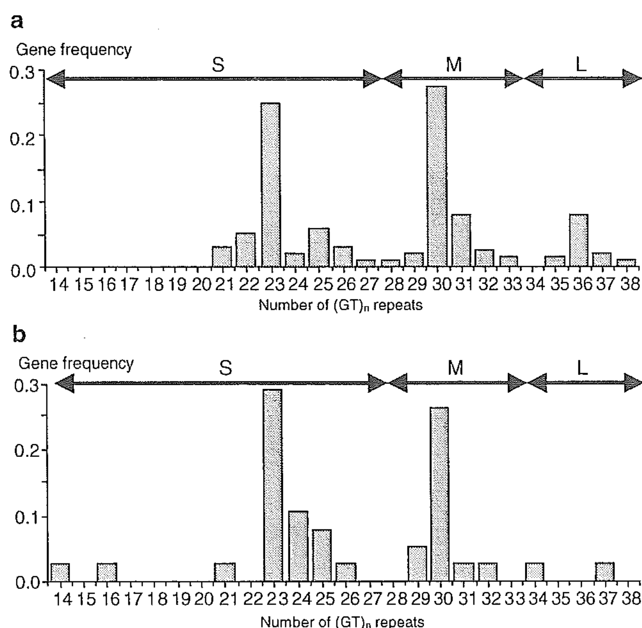

Fig. 1. Gene frequency of the $(GT)_n$ alleles in each group.
a, Uncomplicated malaria patients (n = 120); b, Cerebral malaria patients (n = 30).

Table 1. Comparison of the genotype frequencies between uncomplicated and cerebral malaria groups

a. Genotype frequencies

Genotype	UM n = 120 (%)	CM n = 30 (%)
M/L	15 (12.5)	0 (0.0)
M/M	20 (16.7)	4 (13.3)
S/L	11 (9.2)	3 (10.0)
S/M	53 (44.2)	11 (36.7)
S/S	21 (17.5)	12 (40.0)

b. Chi-square test for S/S homozygotes in the two patients groups

Genotype	UM n = 120 (%)	CM n = 30 (%)	P value	OR	95% CI
S/S	21 (17.5)	12 (40.0)	0.008	3.14	1.32-7.49

The frequencies of the S/M/L HO-1 promoter $(GT)_n$ genotypes between cerebral malaria (CM) and uncomplicated malaria (UM).
Alleles were determined by their GT repeat numbers. L, 34-38 repeat; M, 28-33; and S, 14-27. Genotype was determined by individual combination of the alleles. SS genotype frequencies were significantly deviated between two groups.

DISCUSSION

The pathogenesis of CM is complex and multifactorial. Previous studies indicate that tumor necrosis factor (TNF)-α promoter polymorphism is one of the most frequently reported candidate host factors associated with CM in Africa as well as in Asia (12-14). In addition to TNF-α, many other genes have been postulated to be involved in the pathogenesis of CM (15-18). The present study is the first to suggest an association between the length of the $(GT)_n$ repeat in the HO-1 gene promoter and clinical presentation of malaria. Individuals with genes carrying the short repeat were found to be more likely to present with CM, while those carrying the longer repeats were marginally more likely to fall into the UM category. Chen et al. (19) reported that subjects with the short allele containing $(GT)_n$ = 22 showed 8 times higher transcriptional activity than those with the $(GT)_n$ = 30 allele sequence, as determined by luciferase assay using rat aortic smooth muscle cells. Therefore, a short $(GT)_n$ in the promoter may directly enhance the transcription of HO-1 in malaria patients and the resultant reaction products, CO, iron and bilirubin, may increase in the brain lesion. Recently, it was reported that HO-1 protein levels are increased in Durck's granuloma, a typical lesion of advanced CM (20). Clark et al. (21) also reported a more detailed immuno-histochemical study on the expression of inducible HO-1 protein using many CM autopsy cases in which monocytes or macrophages in the brain and lung and the Kupffer cells in the liver systemically expressed HO-1. It is not clear what induced HO-1 or how it relates to pathology, however, excess amounts of catalytic product CO might impair neuronal activity as a toxic gas or might disrupt the complex neuronal network as a neural messenger (23,24). Additionally, there have been a number of reports suggesting the toxic effect of free iron in patients with CM. Gordeuk et al. reported that iron chelation

therapy enhances recovery from deep coma in CM (24) and that increased transferrin saturation may be associated with delayed recovery from coma during standard therapy (25). These findings suggest that the excess free iron directly contributes to the pathogenesis of CM.

In chronic inflammatory diseases, short alleles are less associated with severe pathology, while in the present study they were significantly associated with CM, a severe manifestation of infection. Yamada et al. reported that short $(GT)_n$ alleles are protective against smoking-induced emphysema (26) and restenosis after percutaneous transluminal angioplasty in arterial occlusive diseases (27). They showed that long $(GT)_n$ repeats reduced the level of HO-1 induction by oxidative stress in cultured cells and that short $(GT)_n$ alleles are likely to provide anti-inflammatory activities of heme breakdown products (26). It is therefore conceivable that short $(GT)_n$ alleles might provide older individuals with protection against chronic inflammatory processes. In malaria, it has not yet been determined how HO-1 is involved in the pathogenesis of CM and what tissues are involved in this HO-1 related event. Furthermore, regulation of promoter activity by the length of a microsatellite may differ between tissues. This may explain why the resistant short allele in lung and vascular diseases is susceptible to CM, (26,27) though there exists the possibility that unknown causative single nucleotide polymorphisms (SNPs) are present in linkage disequilibrium with this microsatellite allele.

Transcription of the HO-1 gene is under the regulation of the fine tuning system that includes the Bach-1 repressor (28) and $(GT)_n$ spacer polymorphism. To confirm our findings, further studies are needed, using larger populations and different ethnic groups, and examining the linkage between genotype and phenotype. Nevertheless, we can say here that the repression of HO-1 expression by pharmacological means, such as competitive inhibitors of HO-1, might provide a useful adjunct to the treatment of CM (29).

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REFERENCES

1. Aikawa, M. (1988): Human cerebral malaria. Am. J. Trop. Med. Hyg., 39, 3-10.
2. MacPherson, G. G., Warrell, M. J., Looareesuwan, S. and Warrell, D. A. (1985): Human cerebral malaria: a quantitative ultrastructural analysis of parasitised erythrocyte sequestration. Am. J. Pathol., 119, 385-401.
3. World Health Organization, Communicable Diseases Cluster (2000): Severe falciparum malaria. Trans. R. Soc. Trop. Med. Hyg., 94 (Suppl. 1), S1-90.
4. Sadrzadeh, S. M. H., Anderson, O. K., Panter, S. S., Hallaway, P. E. and Eaton, J. W. (1987): Hemoglobin potentiates central nervous system damage. J. Clin. Invest., 79, 662-664.
5. Sadrzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E. and Eaton, J. W. (1984): Hemoglobin: a biologic Fenton reagent. J. Biol. Chem., 259, 14354-14356.
6. Tenhunen, R., Marver, H. S. and Schmid, R. (1970): The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. J. Lab. Clin. Med., 75, 410-421.
7. Stocker. R., Yamamoto, Y., McDonagh, A. F., Glazer, A. M. and Ames, B. N. (1987): Bilirubin is an antioxidant of possible physiological significance. Science, 235, 1043-1046.
8. Shibahara, S., Kitamuro, T. and Takahashi, K. (2002): Heme degradation and human disease: diversity is the soul of life. Antiox. Redo. Signal., 4, 593-602.
9. Shibahara, S., Sato, M., Muller, R. M. and Yoshida, T. (1989): Structural organization of the human heme oxygenase gene and the function of its promoter. Eur. J. Biochem., 179, 557-563.
10. Kimpara, T., Takeda, A., Watanabe, K., Itoyama, Y., Ikawa, S., Watanabe, M., Arai, H., Sasaki, H., Higuchi, S., Okita, N., Takase, S., Saito, H., Takahashi, K. and Shibahara, S. (1997): Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson's disease. Hum. Genet., 100, 145-147.
11. McGinnis, R. E. and Spielman, R. S. (1995): Insulin gene 5′ flanking polymorphism length of class 1 alleles in number of repeat units. Diabetes, 44, 1296-1302.
12. Knight, J. C., Udalova, I., Hill, A. V. S., Greenwood, B. M., Peshu, N., Marsh, K. and Kwiatkowski, D. (1999): A polymorphism that affect OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat. Genet., 22, 145-150.
13. McGuire, W., Hill, A. V. S., Allsopp, C. E. M., Greenwood, B. M. and Kwiatkowski, D. (1994): Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria. Nature, 371, 508-511.
14. Ubalee, R., Suzuki, F., Kikuchi, M., Tasanor, O., Wattanagoon, Y., Ruangweerayut, R., Na-Bangchang, K., Karbwang, J., Kimura, A., Itoh, K., Kanda, T. and Hirayama, K. (2001): Strong association of a tumor necrosis factor-α promoter allele with cerebral malaria in Myanmar. Tissue Antigens, 58, 408-410.
15. Quaye, K. E., Ekuban, F. A., Goka, B. Q., Adabayeri, V., Kurtzhals, J. A. L., Gyan, B., Ankrah, N-A., Hviid, L. and Akanmori, B. D. (2000): Haptoglobin1-1 is associated with susceptibility to severe *Plasmodium falciparum* malaria. Trans. R. Soc. Trop. Med. Hyg., 94, 216-219.
16. Aitman, T. J., Cooper, L. D., Norsworthy, P. J., Wahid, F. N., Gray, J. K., Curtis, B. R., Mckeigue, P. M., Kwiatkowski, D., Greenwood, B. M., Snow, R.W., Hill, A. V. and Scott, J. (2000): Malaria susceptibility and CD36 mutation. Nature, 405, 1015-1016.
17. Kwiatkowski, D. (2000): Genetic susceptibility to malaria getting complex. Curr. Opin. Genet. Develop., 10, 320-324.
18. Kikuchi, M., Looareesuwan, S., Ubalee, R., Tasanor, O., Suzuki, F., Wattanagoon, Y., Na-Bangchang, K., Kimura, A., Aikawa, M. and Hirayama, K. (2001): Association of adhesion molecule PECAM-1/CD31 polymorphism with susceptibility to cerebral malaria in Thais. Parasitol.

Int., 50, 235-239.
19. Chen, Y. H., Lin, S. J., Lin, M. W., Tsai, H. L., Kuo, S. S., Chen, J. W., Charng, M. J., Wu, T. C., Chen, L. C., Ding, P. Y., Pan, W. H., Jou, Y. S. and Chau, L. Y. (2002): Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum. Genet., 111, 1-8.
20. Schluesener, H. J., Kremsner, P. G. and Meyermann, R. (2001): Heme oxygenase-1 in lesions of human cerebral malaria. Acta Neuropathol., 101, 65-68.
21. Clark, I. A., Awburn, M. M., Harper, C. G., Liomba, N. G. and Molyneux, M. E. (2003): Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. Malaria J., 2, 41-54.
22. Stevens, C. F. and Wang, Y. (1993): Reversal of long-term potentiation by inhibitors of heme oxygenase. Nature, 364, 147-149.
23. Zhuo, M., Small, S. A., Kandel, E. R. and Hawkins, R. D. (1993): Nitric oxideand carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. Science, 260, 1946-1950.
24. Gordeuk, V., Thuma, P., Brittenham, G., McLaren, C., Parry, D., Backenstose, A., Biemba, G., Msiska, R., Holmes, L., McKinley, E., Vargas, L., Gilkeson, R. and Poltera, A. A. (1992): Effect of iron chelation therapy on recovery from deep coma in children with cerebral malaria. New Engl. J. Med., 327, 1473-1477.
25. Gordeuk, V. R., Thumam P. E., McLarenm, C. E., Biemba, G., Zulu, S., Poltera, A. A., Askin, J. E. and Brittenham, G. M. (1995): Transferrin saturation and recovery from coma in cerebral malaria. Blood, 85, 3297-3301.
26. Yamada, N., Yamaya, M., Okinaga, S., Nakayama, K., Sekizawa, K., Shibahara, S. and Sasaki, H. (2000): Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am. J. Hum. Genet., 66, 187-195.
27. Exner, M., Schillinger, M., Minar, E., Mlekusch, W., Schlerka, G., Haumer, M., Mannhalter, C. and Wagner, O. (2001): Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty. J. Endovasc. Ther., 8, 433-440.
28. Kitamuro, T., Takahashi, K., Ogawa, K., Udono-Fujimori, R., Takeda, K., Furuyama, K., Nakayama, M., Sun, J., Fujita, H., Hida, W., Hattori, T., Shirato, K., Igarashi, K. and Shibahara, S. (2003): Bach1 functions as a hypoxia-inducible represser for the heme oxygenase-1 gene in human cells. J. Biol. Chem., 278, 9125-9133.
29. Sassa, S. (1997): Inhibition of carbon monoxide production by tin-protoporphyrin. J. Pediatr. Gastroenterol. Nutr. 6, 167-171.

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Associations between frequencies of a susceptible TNF-α promoter allele and protective α-thalassaemias and malaria parasite incidence in Vanuatu

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Summary Tumour necrosis factor-alpha (TNF-α) is one of the key cytokines that influence the pathology of microbial infections. The genetic susceptibility to severe forms of falciparum malaria is differentially associated with TNF-α promoter gene polymorphisms (TNFP alleles). In a previous study, we identified a TNFP-allele characterized by a C to T transition at position −857 (TNFP-D allele) as a marker for susceptibility to cerebral malaria in Myanmar. The frequencies of TNFP alleles on six islands of Vanuatu, Melanesia (South-west Pacific) were estimated to investigate whether malaria selection pressure on this susceptibility marker has influenced its prevalence. Within the archipelago of Vanuatu there is a decreasing cline of parasite incidence from North to South. Of the four alleles of the TNFP gene detected in Vanuatu, the TNFP-D allele frequencies were inversely correlated with the parasite incidence of islands; TNFP-D varied from 0.55 on the island with the lowest parasite incidence to 0.26 on the island with the highest parasite incidence ($r = -0.855$, $P = 0.03$). We also observed a significant correlation between the frequencies of α-thalassaemia alleles, thought to protect against malaria and parasite incidence in the same populations. These data are consistent with a previously reported correspondence between the frequencies of glucose 6-phosphate dehydrogenase (G6PD) deficiency and parasite incidences on the islands of Vanuatu (Kaneko *et al.* 1998) and indicate that the degree of malaria endemicity has influenced the allele frequencies of at least three loci that confer both susceptibility (TNFP-D) and protection (α-thalassaemias and G6PD deficiency).

keywords malaria, selection, TNF-α, α-thalassaemia, Melanesia

Introduction

A number of studies have indicated that parasite incidence has influenced the frequencies of susceptible or resistant genes within populations. The loci thought to confer resistance to malaria include haemoglobin alleles such as sickle cell trait in Africa (Allison 1954), α-thalassaemias in the Mediterranean and in Asia (Beaven *et al.* 1961), and ovalocytosis in South-east Asia (Baer *et al.* 1976). In Melanesia, α-thalassaemia allele frequencies were significantly correlated with altitude and latitude indicating long-term malaria selection pressure (Hill *et al.* 1985; Flint *et al.* 1986; Roberts-Thomson *et al.* 1996). Genetic variants of immunity-related genes (e.g. major histocompatibility complex, immunoglobulin, cytokine, chemokine, adhesion molecule, and complements) have also been postulated to influence susceptibility to malaria (Gimenez *et al.* 2003). Of these immunity-related molecules, tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that plays an essential role in the protection against many infectious diseases, but is also fatal in excess (Beutler *et al.* 1993; Knight *et al.* 1999). An association between single nucleotide polymorphisms (SNPs) in the TNF-α promoter region (TNFP) and susceptibility to malaria has been observed in several studies. In Africa, the severe form of malaria was associated with the TNFP −238A allele (McGuire *et al.* 1999) whereas cerebral malaria (CM) was associated with the TNFP −308A allele in the same population (McGuire *et al.* 1994). In Myanmar patients, we reported a strong association between CM and TNFP-D (Ubalee *et al.* 2001). TNFP-D is characterized by a C to T transition at position −857, the location of an OCT-1 binding site adjacent to a NF-κB binding site (position −863) (van Heel et al. 2002). The frequencies of glucose 6-phosphate dehydrogenase (G6PD) deficiency of islands of Vanuatu were significantly correlated with parasite incidence (Kaneko *et al.* 1998). In this study, we investigated whether the frequencies of the TNFP-D allele

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associated with susceptibility to CM in Myanmar and α-thalassaemias thought to protect against malaria also reflects parasite incidences of six islands of Vanuatu.

Materials and methods

Field study

We performed malariometric surveys of children under 15 years of age from 1997 to 2001. As part of the surveys, blood samples were collected from all examinees with informed consent from their parents. Finger-prick blood samples were drawn into heparinized capillary tubes (Drummond Scientific Company, USA) and then transferred onto filter paper strips (ET31CHR, Whatman Ltd, UK). The strips were air dried and then kept in the plastic bags at −20 °C prior to analysis. A total of 1074 DNA samples from individuals living on six islands of Vanuatu with varying malaria endemicity [Malakula (MLK), Pentecost (PEN), Espiritu Santo (SAN), Gaua (GAU), Erromango (ERO), and Aneityum (ANT) (Figure 1)] were extracted using the QIAamp® DNA Mini Kit (Qiagen GmbH, Germany). The average age of the participants from each island was 6.3–7.0 years, and the sex ratio was unbiased. The study was approved by the Department of Health of the Republic of Vanuatu and Institutional Ethical Review Board of Japan.

Genotyping of TNF-α 5′-flanking region polymorphisms

A 1042-bp fragment of the 5′-flanking region (position −66 to −1107) of the TNF-α gene was amplified by polymerase chain reaction (PCR). The PCR reactions and dot-blot hybridization with sequence-specific oligonucleotide probes were performed as described previously (Higuchi *et al.* 1998). To determine the five SNPs combination haplotypes, we first identified homozygotes (TNFP-A, B, C and D) that were then evaluated to resolve heterozygotes (Ackerman *et al.* 2003). We then confirmed the four extrapolated haplotypes by cloning and sequencing as previously described (Ubalee *et al.* 2001).

Genotyping of α-thalassaemias

Two α-globin gene deletions of 3.7 kb ($-\alpha^{3.7}$) and 4.2 kb ($-\alpha^{4.2}$) that are common in Pacific populations (Hill *et al.* 1985; Roberts-Thomson *et al.* 1996), were detected by the multiplex PCR method of Chong *et al.* (2000) while the non-deleted allele was confirmed via PCR as described previously (Sakai *et al.* 2000). The $-\alpha^{3.7}$ subtypes ($-\alpha^{3.7}$ I, II and III) were then inferred from *Apa*I restriction polymorphism patterns (Dodé *et al.* 1993).

Statistical analysis

The chi-squared test was used to evaluate whether the allele frequencies of the populations were in Hardy–Weinberg equilibrium. The correlations between TNFP and α-thalassaemia allele frequencies and parasite incidences estimated as the log mean Annual Parasite Incidence (API) of the study islands from malariometric surveys conducted from 1985 to 1990 were evaluated by Pearson's correlation coefficient test using the SPSS statistical analysis software package.

Results

The four alleles of the 5′-flanking region of TNF-α detected in Vanuatu are shown in Table 1. These alleles (TNFP-A, B, C and D) were the same four that were previously found in Japan (Higuchi *et al.*

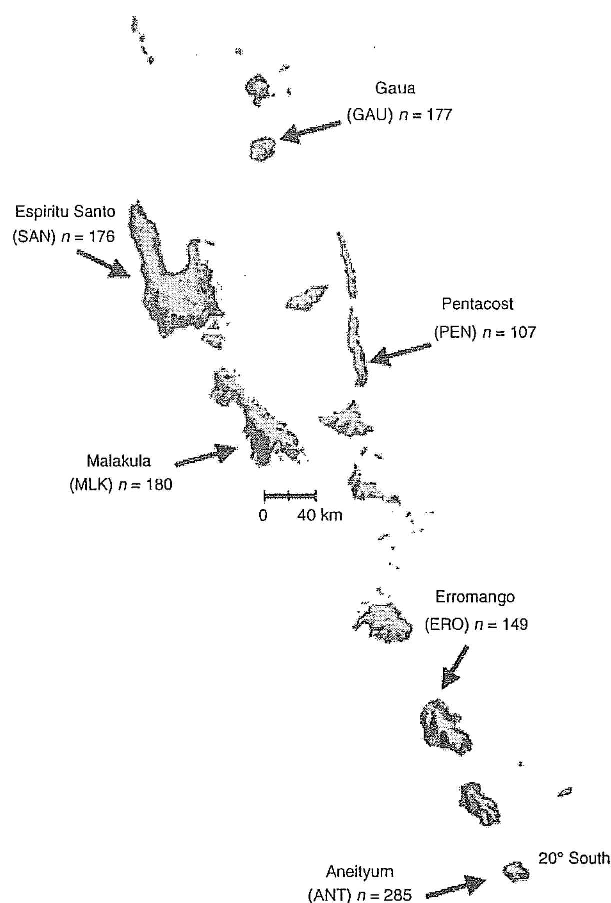

Figure 1 Number of individuals and location of the islands.

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