Point mutations in the *Plasmodium falciparum* cg2 gene, polymorphism of the kappa repeat region, and their relationship with chloroquine resistance

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Abstract

Based on the available DNA sequence data of the *Plasmodium falciparum* cg2 gene, we have hypothesized that 3 amino-acid substitutions, His275Gln, Gly281Ala, and His299Gln, may represent the key mutations that confer resistance to chloroquine. The presence of 14 tandemly repeated hexamer units in the kappa region has also been suggested to be indicative of chloroquine resistance. These 2 hypotheses were tested by determining the sequence of DNA fragments containing all 3 codons and kappa repetitive region (approximately 450-basespares) for 53 randomly selected clinical isolates (obtained in Cameroon in 1994–97) with known response in vitro and/or in vivo to chloroquine. The cg2 genotypes based on the 3 codons and the response in vitro to chloroquine, as well as the number of kappa repeat units and responses in vitro and in vivo to chloroquine, were associated (P < 0.05). cg2 gene mutations were more common in parasites from patients with failure in vitro. However, this difference did not achieve statistical significance (P = 0.055). The sensitivity and specificity of the 3 codons and kappa repeat region to predict the response in vitro to chloroquine ranged between 72% and 85%. The sensitivity and specificity of these genetic markers to predict the response in vitro to chloroquine were of lower values. The kappa repeat region of the clinical isolates is polymorphic but characterized by several conserved features.

Keywords: *Plasmodium falciparum*, drug resistance, chloroquine, genetic analysis, cg2, mutations, Cameroon

Introduction

The genetic basis of resistance to chloroquine in *Plasmodium falciparum* has not been fully established. The cg2 gene has been proposed to be one of the potential candidates for chloroquine resistance (Su et al., 1997). The cg2 gene is located in chromosome 7. The individual or collective expression of certain genes in the chloroquine-resistant phenotype has not been elucidated. Although the expression product of the cg2 gene has been hypothesized to be a Na+ -H+ antiporter on the basis of a consensus motif present in the deduced amino-acid sequence, the function of the protein coded by the cg2 gene remains unknown (Sánchez et al., 1998; Wemmels et al., 1998; Bray et al., 1999). In addition, the cg2 sequence does not have a clear homology with other genes. Despite the limited knowledge on this candidate gene for chloroquine resistance, preliminary studies on reference clones of *P. falciparum* have suggested that a distinct genetic profile characterized by 12 point mutations and 3 polymorphic patterns in repetitive regions is strongly associated with chloroquine resistance in vitro (Su et al., 1997). In our previous study, we confirmed the association between in-vitro and in-vivo chloroquine resistance and cg2 genetic profile in fresh clinical isolates obtained from Cameroonian patients treated with chloroquine (Basco & Ringwald, 1999a). In that study, the sequences of all 12 codons, as well as the polymorphisms in the κ, γ, φ, and δ regions and poly-asparagine tract, were characterized by DNA sequencing in 24 clinical isolates presenting concordant results for tests in vitro and in vivo of chloroquine resistance. This methodological approach is complete but time-consuming since several sequencing reactions are required for each isolate to determine the sequences in 12 codons that are widely separated over 6500 bp.

If key mutations exist in the cg2 gene, as in the case of single-point mutations in the dihydrofolate reductase–thymidylate synthase and dihydropteroate synthase genes which confer resistance to pyrimethamine and sulfadoxine, respectively (Sirawaraphorn et al., 1997; Triglia et al., 1997), correlational study between the genotype and phenotype will be more rapid to perform in a large number of field isolates. The codons 275, 281, and 299 of the cg2 gene seem to undergo mutation once the other 9 codons are mutated, and many chloroquine-sensitive parasites display mutant codons in the other 9 positions (Su et al., 1997; Basco & Ringwald, 1999a). The observed pattern of mutations occurring in 12 codons thus suggested that the most critical amino-acid substitutions leading to an abrupt change in chloroquine-sensitivity phenotype may be His275Gln, Gly281Ala, and His299Gln (Basco & Ringwald, 1999a). Based on these data, we designed a modified protocol that requires a single DNA sequencing reaction. The DNA fragment amplified by polymerase chain reaction (PCR) contains all 3 putative critical codons and the kappa repeat region, which was suggested to differentiate between the chloroquine-resistant parasites in the presence of 14 tandem repeat units and the chloroquine-sensitive parasites in the presence of ≤14 repeat units (Su et al., 1997; Basco & Ringwald, 1999a). To test our hypothesis, DNA sequences of 53 randomly selected Cameroonian isolates were analysed with the aim to determine whether the 3 putative key mutations and kappa repeat region are predictive of in-vitro and in-vivo chloroquine resistance determined in the field.

Materials and Methods

Patients

The patients were enrolled in randomized clinical trials conducted at the Nlongkak Catholic missionary dispensary in Yaoundé between 1994 and 1997 (Ringwald et al., 1996a, 1998). The following inclusion criteria were used for enrollment: age ≥5 years, fever at consultation (or history of fever within the previous 24 h), monoinfecion with *P. falciparum*, parasite density >5000 asexual parasites/μL of blood, easy access to the dispensary for daily monitoring, and no recent history of self-medication with antimalarial drugs, as confirmed by a negative Saker–Solomons urine test result (Mount et al., 1989). Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization (WHO, 1990), severe anaemia (haemoglobin <5.0 g/dL), or moderate and severe malnutrition were excluded. The clinical parameters and haematological and blood biochemistry do not differ significantly between the adult population (aged ≥15 years) and the paediatric population (aged 5–15 years) in Yaoundé.
Parasite DNA, PCR amplification, and sequencing

Clinical isolates of *P. falciparum* were obtained by venepuncture before treatment. Venous blood samples (5–10 mL of whole blood) were collected in a Vacutainer tube coated with an anticoagulant (EDTA) and washed 3 times in RPMI 1640 medium by centrifugation (2000g for 10 min) within 3 h after blood collection. An aliquot of 1.5–2 mL of red blood cell pellet was used to extract parasite DNA as previously described (BASCO & RINGWALD, 1999a).

Pifty-three clinical isolates of *P. falciparum* were randomly selected from our collection of parasites obtained between 1994 and 1997. The semi-nested CR was performed to obtain a sufficient quantity of DNA fragment for sequencing. For the primary amplification reaction, a 3'0-kilobasepair DNA fragment was amplified from a reaction mixture containing 200 ng of genomic DNA, 20 picomoles of primers CG-3 5'-GGTAATGGTATATATATACCTCTCAGGAG G-3' (forward primer, corresponding to nucleotides 517–737, HB3 numbering) and CG-6R 5'-TTGTTAAATCAGGACATTTGTTCTTTG3' (reverse primer, corresponding to nucleotides 3732–3703, HB3 numbering), buffer (50 mM KCl, 10 mM Tris, pH 8.3), 2.25 mM MgCl2, 500 μM dNTP, and 2–6 units of a mixture containing Taq and Poo DNA polymerases (Expand™ Long Template PCR System; Roche Diagnostics, Meylan, France) in a final volume of 50 μL. The PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) was programmed as follows: 94°C for 2 min for the first cycle and 1 min in subsequent cycles, 60°C for 1 min in subsequent cycles, and 72°C for 10 min in all cycles, with an additional extension step of 68°C for 15 min after the completion of 30 cycles.

For the secondary reaction, CG-3 primer was phosphorylated at its 5'-end by T4 polynucleotide kinase. The phosphorylation reaction was performed by incubating a mixture of 1 nanomole of primer, 0.5 mM ATP, buffer (10 mM MgCl2, 5 mM dithiothreitol, 70 mM Tris–HCl, pH 7.6), and 20 units of T4 polynucleotide kinase in a final volume of 100 μL at 37°C for 15 min. The kinase was heat-inactivated at 65°C for 20 min. A semi-nested PCR was performed under the following conditions: primary amplification product diluted to 1/1000 (2–4 μL), 15 picomoles of the phosphorylated CG-3 forward primer and CG-4R reverse primer 5'-CGCTATATCCGAAATCTCGTCTACGAG G-3' (corresponding to nucleotides 1210–1181, HB3 numbering), buffer (50 mM KCl, 10 mM Tris, pH 8.3, and 1.5 mM MgCl2), 200 μM dNTP, and one unit of Taq DNA polymerase in a 50 μL reaction. The thermal cycler was programmed as follows: 94°C for 2 min for the first cycle and 30 s in subsequent cycles, 60°C for 1 min for the first cycle and 30 s in subsequent cycles, and 72°C for 1 min for all cycles, for a total of 30 cycles, followed by a 10 min extension step at 72°C. The amplified DNA fragments were resolved by electrophoresis in a 1–9% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination. The nucleotide sequences were determined by a single-stranded, dyeoxy chain termination reaction protocol (AUSUBEL et al., 1995).

Drug response

Infected erythrocytes were washed 3 times in RPMI 1640 medium and suspended in RPMI 1640 plus 10% human serum, 25 mM HEPES, and 25 mM NaHCO3 at a haematocrit of 15% and an initial parasitaemia ranging between 0.2% and 1.0%. If the blood sample had a parasitaemia of >1%-0%, fresh, uninfected type A+ erythrocytes were added to adjust the parasitaemia to 0-6%. The isotopic microtest used in this study was previously described (DESJARDINS et al., 1996b). The 50% inhibitory concentration (IC50), defined as the drug concentration corresponding to 50% of the uptake of [3H]hypoxanthine measured in the drug-free control wells, was determined by non-linear regression analysis of logarithm of concentrations plotted against growth inhibition. The threshold IC50 for resistance in vitro to chloroquine was estimated to be ≥100 nM (RINGWALD et al., 1996b).

The 14-day test of therapeutic efficacy, developed by WHO in 1996, was used to assess the response in vitro to chloroquine (WHO, 1996). The patients assigned to the chloroquine treatment group were treated with the standard dose of chloroquine (25 mg base/kg body-weight in 3 divided daily doses) under supervision. The clinical conditions and parasitaemia were monitored on days 0, 1, 2, 3, 4, 7, and 14 on an outpatient basis. The responses in vitro were classified as early treatment failure, late treatment failure, or adequate clinical response, as defined by the new classification scheme (WHO, 1996).

Data interpretation

The isolates analysed in this study were randomly selected from the list of patients who completed the 14-day follow-up. Twenty-four isolates that were selected in our earlier study, based on concordant responses in vitro and in vivo, were withdrawn from our list before the isolates were randomly selected (BASCO & RINGWALD, 1999a). Responses in vitro were classified as 'sensitive' if the patient responded with an adequate clinical response and 'resistant' if the patient responded with late or early treatment failure. Responses in vitro were classified as 'sensitive' for chloroquine IC50 < 100 nM and 'resistant' for chloroquine IC50 ≥ 100 nM. Because of the possibility of reinfection with a new set of parasite populations during the follow-up period, acquired immunity, and inter-individual variations in pharmacokinetics, responses in vitro in vivo may be discordant (RINGWALD & BASCO, 1999). However, although reinfection may occur after chloroquine treatment, even in a hypendemic area, it is less frequent, and inter-individual variations (due to the same parasite populations as the pre-treatment parasites) occur after day 14 (BASCO & RINGWALD, 2001).

According to SU et al. (1997), chloroquine-resistant reference clones and strains have mutations in all 12 codons. Three mutant codons in the DNA fragment that we studied are Gin-275, Ala-281, and Gin-299. Chloroquine-sensitive laboratory-adapted strains and clinical isolates are characterized by various qg2 alleles that may be either wild-type or combinations of up to 11 mutant alleles (SU et al., 1997; BASCO & RINGWALD, 1999a). In the DNA fragment analysed in this study, there may be 1 or 2, but not 3 mutations for chloroquine-sensitive parasites. In addition, chloroquine-resistant parasites have 14 repeat units in the kappa polymorphic region. The kappa repetitive region of the chloroquine-sensitive parasites has <14, but not 14 tandem repeat units. Mixed qg2 genotypes (n = 13) were considered to represent chloroquine-resistant allele only if mixed codons were detected at all 3 positions. Otherwise, mixed genotypes were grouped with chloroquine-sensitive alleles. Mixed kappa polymorphisms were considered to represent chloroquine resistance if a 14-tandem repeat unit was also present in mixed infections. The IC50 values of a group of isolates were expressed as the geometric mean.
and range. Qualitative variables were presented on a 2 × 2 contingency table and analysed by Yates’ continuity corrected χ² test. Quantitative variables were compared by the unpaired t-test.

**Results**

Forty three of 53 patients were treated with chloroquine. The other patients were treated with either amodiaquine (n = 3) or sulfadoxine–pyrimethamine (n = 7). The in-vitro drug sensitivity phenotype was successfully characterized for 47 isolates. The failure of in-vitro assays for 6 isolates was due to bacterial contamination or inadequate parasite growth. DNA sequencing showed 5 different alleles (based on 3 unmixed, or ‘pure’ codons) and 6 different combinations of mixed alleles. The pure wild-type allele (His-275/Gly-281/His-299) and the pure triple mutant allele (Gln-275/Ala-281/Gln-299) represented 11 (21%) of 53 and 22 (42%) of 53 of the alleles, respectively. Mixed alleles were present in 13 (25%) of 53 of the isolates. The remaining isolates had intermediate-type of alleles with 1 or 2 mutations.

The chloroquine-sensitive cg2 alleles, including pure and mixed alleles, were observed in 15 patients with an adequate clinical response (3 of these patients had an asymptomatic parasitaemia on day 14) and 4 patients with treatment failure (Table 1). The chloroquine-resistant alleles (18 pure and 3 mixed) were found in isolates obtained from 11 patients with an adequate clinical response (1 patient had an asymptomatic parasitaemia on day 14) and 10 patients with treatment failure. There was no association (P = 0-083) between the response in vivo and cg2 genotypes. The in-vitro assay results showed that the chloroquine-sensitive cg2 alleles were found in 17 chloroquine-sensitive isolates and 5 chloroquine-resistant isolates. The chloroquine-resistant allele was observed in 4 chloroquine-sensitive parasites and 21 chloroquine-resistant parasites (Fig. 1). The cg2 genotype was statistically associated with the in-vitro chloroquine sensitivity/resistance pattern (P < 0-0001).

The triple mutant allele, Gln-275/Ala-281/Gln-299, was strongly associated with the presence of 14 kappa repeat units (20 of 22 isolates with the mutant allele). Likewise, all isolates displaying pure wild-type allele His-275, 281, and 299 in comparison with the alternative amino-acid residues His (H), Gln (Q), and Ala (A) at positions 275, 281, and 299. The alternative amino acids are wild-type His (H) and mutant Gln (Q) at position 275, wild-type Gly (G) and mutant Ala (A) at position 281, and wild-type His (H) and mutant Gln (Q) at position 299. S, sensitive type; R, resistant type.

Table 1. *P. falciparum* cg2 genotypes defined by the amino-acid residues 275, 281, and 299 in comparison with responses *in vivo* and *in vitro* to chloroquine (Cameroon, isolates obtained 1994–97)

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>In vivoB</th>
<th>In vivoC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Wild-type allele H–G–H (S)</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Single mutation H–G–Q (S)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Single mutation H–A–H (S)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Double mutation Q–A–H (S)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Triple mutation Q–A–Q (R)</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Mixed alleles</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>H–G–Q/H</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>H–A–G–Q/H</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Q/H–A–H</td>
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</tr>
<tr>
<td>Q/H–A–Q</td>
<td>0</td>
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<tr>
<td>Q–A–G–Q/H</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Q/H–A–G–Q/H</td>
<td>0</td>
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</tr>
</tbody>
</table>

*The cg2 genotype was defined by the combination of 3 amino-acid residues corresponding to positions 275, 281, and 299. The alternative amino acids are wild-type His (H) and mutant Gln (Q) at position 275, wild-type Gly (G) and mutant Ala (A) at position 281, and wild-type His (H) and mutant Gln (Q) at position 299. S, sensitive type; R, resistant type.*

*Data are expressed as the number of patients. The terms ‘sensitive’ and ‘resistant’ response *in vivo* refer to adequate clinical response and treatment failure (either early or late), respectively. Sensitivity = 84% (71% including mixed alleles), specificity = 50% (62% including mixed alleles). Sensitivity = 82% (81% including mixed alleles), specificity = 80% (81% including mixed alleles).
Fig. 2. Structure and polymorphisms of the kappa repetitive region of the Plasmodium falciparum cg2 gene. The kappa repetitive region was composed of 5 types of hexamer repeat units, according to the deduced amino-acid residues: GSNKSY, GINKSY, GNKNSY, GNNNSY, and GSNNSY. The first 2 variants (GSNKSY and GINKSY) occurred at the first 2 positions invariably and occupied the central region in various alternative patterns. The variants GNNKSY and GNNNSY occurred at the last 2 positions. The variant GSNNNSY occurred in a single isolate. The first of two 14-unit alleles represented the dominant polymorphism in almost all isolates with 11 kappa units. Repeat unit alignment was performed based on the dominant 14-unit allele. Dot denotes identical hexamer unit. Dashes were introduced for alignment. The complete nucleotide sequences of unmixed isolates are available from the GenBank (accession numbers AF297573 to AF297605).

Table 2. Size polymorphism in the kappa repeat region and responses in vivo and in vitro of P. falciparum Cameroonian isolates to chloroquine

<table>
<thead>
<tr>
<th>No. of kappa repeat units</th>
<th>Sensitive</th>
<th>Resistant</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 (S)</td>
<td>8</td>
<td>1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>12 (S)</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>13 (S)</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>14 (R)</td>
<td>9</td>
<td>9</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Mixed (13 and 14) (R)</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*a*The kappa polymorphic region consists of <14 repeat units in the chloroquine-sensitive strains (S) and 14 repeat units in the chloroquine-resistant reference strains (R).

*b*Data are expressed as the number of patients with an adequate clinical response (sensitive) or treatment failure (resistant). Sensitivity and specificity = 69%.

*c*Data are expressed as the number of P. falciparum isolates responding with IC50 values <100 nM (sensitive) or >100 nM (resistant). Sensitivity = 75%, specificity = 85%.

chloroquine-resistant isolates (IC50 ≈ 100 nM) (Fig. 3). Six isolates were resistant in vitro but carried <14 repeat units. The presence of 14 kappa repeat units was statistically associated with chloroquine treatment failure (P = 0.048) and in-vitro chloroquine resistance (P = 0.0002). The sensitivity and specificity of the 2 genetic markers, the putative key codons and kappa repetitive units, to determine the in-vitro chloroquine sensitivity/resistance varied between 75% and 85%.

There were 37 paired in-vivo/in-vitro responses to chloroquine. Seven mixed alleles were excluded from the following analysis. Fifteen of 17 mutant isolates were characterized by the triple mutation, 14 kappa repeat units, and a geometric mean IC50 of 238 nM (range 88-575 nM, 1 isolate with IC50 <100 nM). The corresponding patients responded with an adequate clinical response (n = 9), late treatment failure (n = 5), or early treatment failure (n = 1). Two of 17 mutant isolates had the triple mutation and 11 or 13 kappa repeat units. The overall geometric mean IC50 for isolates characterized by the chloroquine-sensitive cg2 alleles and 11 to 13 kappa repeat units was 63 ± 1 nM (n = 13; range 9-8-376 nM, 4 isolates with IC50 > 100 nM). Among these patients, there were 9 with an adequate clinical response (2 with asymptomatic parasitaemia on day 14) and 4 with late treatment failure.

Discussion

The initial study on reference strains and clones of P. falciparum has strongly suggested that a distinct cg2 genotype may be associated with chloroquine-resistant phenotype (SU et al., 1997). These findings were confirmed in 24 Cameroonian clinical isolates that were fully characterized by DNA sequencing (BASCO & RINGWALD, 1999b). However, in our subsequent study based on the size polymorphism of the omega repeat region as the resistance marker, it was shown that the relationship between the chloroquine-resistant cg2 genotype and phenotype may not be absolute (BASCO & RINGWALD, 1999b). In that study, the sensitivity and specificity of the
The presence of 14 kappa repeat units was highly associated with the triple mutation Gln-275/Ala-281/Gln-299. Of the total of 23 isolates carrying 14 repeat units, 20 were associated with the triple mutation, 2 with mixed alleles at all 3 codons, and 1 with double mutation (Gln-275/Ala-281/His-299). The genotype defined by the triple mutation Gln-275/Ala-281/Gln-299 and 14 kappa repeat units was not strongly predictive of the response in vivo to chloroquine in our clinical setting, but it was definitely associated with the response in vitro to chloroquine. Part of the explanation lies in the relatively small sample size. The discrepancy between responses in vivo and in vitro to chloroquine is probably due to several confounding factors that are inherent in host–parasite relationship and independent of drug resistance. When patients clear asexual parasitaemia after chloroquine therapy, despite the fact that they are infected with parasites that are characterized to be chloroquine-resistant by in-vitro assay and cg2 genotype analysis, one of the most probable underlying reasons may be related to the efficacy of acquired immunity. Other possible explanations include self-medication with other drugs after or during chloroquine therapy. Conversely, in patients who are infected with chloroquine-sensitive parasites, but fail to clear asexual parasitaemia, pharmacodynamic and pharmacokinetic factors, unreported vomiting, re-infection, and multilocal infections may account for treatment failure (RINGWALD & BASCO, 1999; BASCO & RINGWALD, 2001).

Another possibility that may explain some cases of discrepancies between responses in vivo and in vitro is related to the reliability of genetic markers for chloroquine resistance. In the study conducted by SU et al. (1997), 1 Sudanese chloroquine-resistant strain displayed the chloroquine-resistant cg2 allele. In the present study, some fresh clinical isolates with the wild-type cg2 allele were resistant in vivo, while others with triple mutation at the putative key codons were sensitive in vitro. Furthermore, using the kappa and omega repetitive regions as genetic markers, we have found that, in some clinical isolates, the response in vitro to chloroquine does not correspond to the criteria based on the number of repeat units, as confirmed in another study on various African isolates (BASCO & RINGWALD, 1999b; DURAND et al., 1999). These discrepancies imply that the cg2 gene is not directly involved or may not be the only gene required for the expression of chloroquine-resistant phenotype. In a recent transfection experiment involving a homologous recombination to replace chloroquine-resistant cg2 allele with chloroquine-sensitive cg2 allele (FIDOCK et al., 2000), the level of chloroquine response in vitro remained unchanged in transformed parasites. These findings suggest that cg2 is physically linked to a neighbouring gene that is more directly involved in chloroquine resistance. Further investigations on these candidate genes, in parallel with tests of resistance in vivo and in vitro, need to be conducted in various epidemiological settings to elucidate the genetic mechanism of chloroquine resistance.

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References


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**Announcements**

**ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE**

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The Chalmers Medal is awarded each year in recognition of research contributing to the knowledge of tropical medicine or tropical hygiene. Only persons of 45 years or under on 1 June of the year of the award shall be eligible.

The Donald Mackay Medal is awarded for outstanding work in tropical health, especially relating to improvement in the health of rural or urban workers in the tropics.

Nominations may be made by any Fellow of the Society, on forms available from Manson House. Completed forms should be sent to the Honorary Secretaries by 1 September 2001. For full details please refer to the Yearbook.

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**ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE**

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