Differences in Binding of Nuclear Proteins from *P. berghei* Strains Differing by Chloroquine Resistance to Oligonucleotides Corresponding to *mdr1* Gene Regulatory Elements

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Proteins specifically reacting with AP1, MEF1, NF-IL6, and SP1 transcription factor binding sites were detected for the first time in nuclear extract of *P. berghei* (rodent malaria parasite) using gel retardation assay. *P. berghei* strains with different chloroquine resistance exhibited appreciable differences in the pattern of nuclear protein binding to the majority of the studied sites, which attests to changes in the plasmodial regulatory system during chloroquine resistance selection.

Key Words: *Plasmodium berghei; chloroquine resistance; transcription factors*

A characteristic feature of malaria agent (unicellular eukaryotic parasite of erythrocytes) is the capacity to rapid development of resistance to almost any drug. Appearance of strains with multiple drug resistance is responsible for increased incidence of malaria all over the world.

The malaria plasmodium dies because of formation of chloroquine complex with the hemoglobin toxic heme part in the plasmodium digestive vacuole [15]. Accumulation of chloroquine in the vacuole of a chloroquine-resistant parasite is reduced 40-50-fold [15], presumably due to increased activity of P-450-dependent microsomal monooxygenases (enzymes metabolizing and inactivating drugs, including chloroquine) [2,10,11]. Two more *P. berghei* proteins were identified: multiple drug resistance (mdr1) gene product, P-glycoprotein, and chloroquine-resistant transporter (crt) gene product [3,9,14].

The data on increased activity of cytochrome P-450 genes mdr1 and crt in connection with the development of chloroquine resistance indicate that treatment with this drug modifies the content or activity of factors serving as common regulators for all these genes. The main factors regulating the expression of eukaryotic genes are DNA-binding proteins (transcription factors) reacting with regulatory sites of the target genes. Regulatory domains of P-450 genes, plasmodium mdr1 and ctr, are not yet studied, but the regulatory sites of mammalian mdr1 genes are studied in detail. These areas contain AP1, MEF1, NF-IL6, and SP1 transcription factor binding sites stimulating the expression of these genes [4,5,7,12]. Since transcription factors are highly conservative in different species, it can be hypothesized that similar proteins stimulate the transcription of malaria parasite mdr1 gene. The aim of this study was to clear out whether malaria parasite contains these proteins and to study their role in the formation of chloroquine resistance.

MATERIALS AND METHODS

Laboratory strains of *Plasmodium berghei* (rodent malaria parasite) were obtained from Institute of

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Medical Parasitology and Tropical Medicine: chloroquine-sensitive strain N, LNK-65 strain with spontaneously 2-fold reduced chloroquine sensitivity, and LNK-65 ChlR strain selected for high chloroquine resistance [2,8]. *P. berghei* strains were maintained in 1.5-2-month-old outbred albino mice by intraperitoneal inoculations of infected blood. Chloroquine resistance of LNK-65 ChlR strain was maintained by oral administration of chloroquine (450 mg/kg) to mice with parasitemia. Parasitemia in infected animals was controlled by microscopic examination of Romanowskii—Giemsa stained fine blood smears.

In order to isolate *P. berghei* nuclei, the blood of mice with 40-60% parasitemia was purified from leukocytes by double filtration through a column packed with LK microcrystal cellulose (Chemapol). Purified erythrocytes were twice lyzed with 0.01% saponine. Nuclear proteins were isolated from precipitated parasites as described previously [6] with modifications.

Parasites ($3\text{-}9\times10^8$) were resuspended in 1 ml lyzing buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF), incubated for 15 min on ice, NP-40 was added to 0.65% and vortexed for 10 sec. The homogenate was centrifuged for 30 sec at 14,000*g* on the cold. Nuclear precipitate was resuspended in 50-100 µl extraction buffer (25 mM HEPES pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM PMSF). The samples were vigorously shaken on ice for 15 min and centrifuged for 5 min at 14,000*g*. Aliquots of nuclear extracts were stored at –70°C. Nuclear extracts from blood cells were prepared similarly. Microscopic control of parasite and nuclei purity was carried out during isolation of *P. berghei* nuclear extracts.

Oligonucleotides corresponding to both chains of transcription factor binding sites (small letters denote the protruding terminals):

AP1 — 5'cagtAGCTTGATGAGTCAGCCGGATC-3' [1]; MEF1 — 5'cagtATCAGCATTCAGTCAATCCGGGCC-3' [7]; NF-IL6 — 5'-cagtTTCAACCTGTTTCGCAGTTTCTCGAGGAATTCA-3' [4]; SP1 — 5'cagtCGACTCTAGGCGGGGTTAAAGTTCT-3', and SRE element (5'-cagtACAGGATGTCCATATTAGGACATCTGCGT-3' [1] were synthesized on ACM-102U (Biosset) by the H-phosphate method [1]. After annealing oligonucleotides were labeled with (γ-^{32}P)ATP using T4 polynucleotide kinase. The reaction mixture (14-20 µl) contained 25 mM HEPES (pH 7.6), 50-80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 2 ng labeled oligonucleotide, and 6-15 µg nuclear extract protein. After 10-min incubation at 20°C the mixture was analyzed by electrophoresis in 5% PAAG in 0.5×TBE buffer.

RESULTS

We hypothesized that *P. berghei* strains with different chloroquine resistance differ by the content or activity of nuclear protein factors involved in the regulation of transcription of genes responsible for drug resistance. AP1 proteins are known to be essential for the regulation of mammalian mdr1 gene transcription. Human mdr1 gene promoter contains a TCAGTCA (-121-115) sequence recognized by transcription factors of this family, the DNA-binding activity of these factors strictly correlates with drug resistance [5]. Transcription MEF1 (multidrug enhancing factor) detected in nuclear extracts of only vincristine-resistant myeloid cell strain [7] reacts with human mdr1 gene sequence GTCAAT

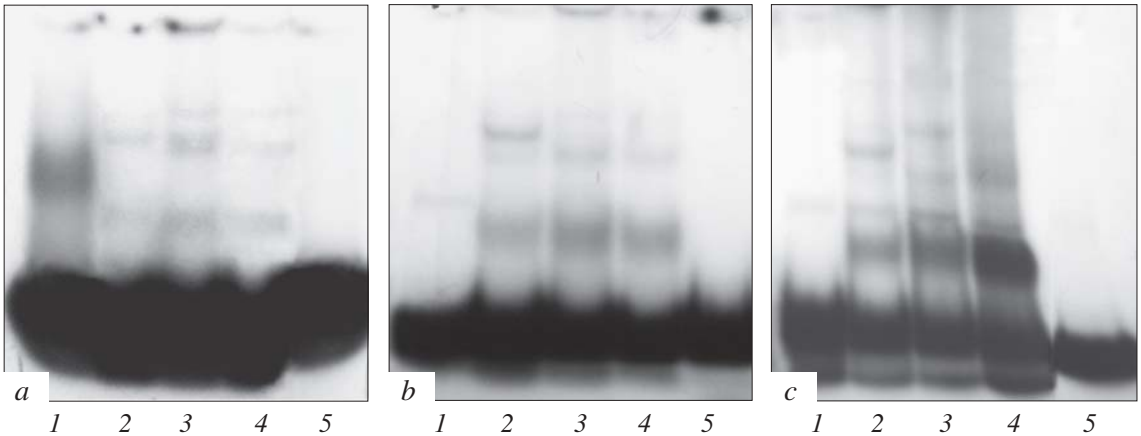

Fig. 1. DNA binding activity of AP1, MEF1, and NF-IL6 transcription factors in nuclear extracts of *P. berghei* strains with different chloroquine resistance. *a*) AP1; *b*) MEF1; *c*) NF-IL6. *1*) nuclear extract of mouse blood cells, 8 µg; *2*) nuclear extract of strain N, 8 µg; *3*) nuclear extract of LNK-65 strain, 8 µg; *4*) nuclear extract of LNK-65 ChlR strain, 8 µg; *5*) free probe mobility.

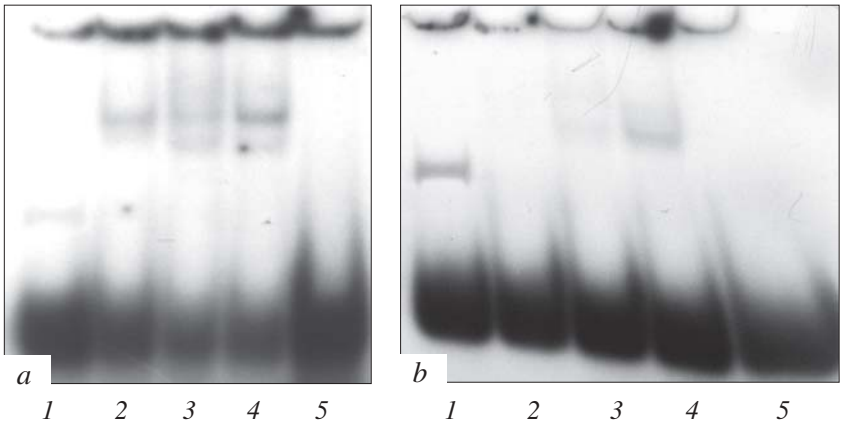

Fig. 2. DNA-binding activity of SP1 transcription factor and SRE element in nuclear extracts of *P. berghei* strains with different chloroquine sensitivity. *a*) SP1; *b*) SRE. *1*) nuclear extract of mouse blood cells, 8 μg; *2*) nuclear extract of strain N, 15 μg; *3*) nuclear extract of LNK-65 strain 15 μg; *4*) nuclear extract of LNK-65 ChlR strain, 15 μg; *5*) free probe mobility.

CC(-118-111). Moreover, the level of mdr1 gene expression is enhanced by C/EBPβ transcription factor (NF-IL6). TTTCCCAGT(-148-140) sequence, recognized by this factor, was also detected in human mdr1 gene promoter region [4]. SP1 transcription factor binding sites were detected in -61-43, -78-70, and -110-103 regions of human mdr1 gene 5'-flanking area and stimulated the activity of this gene [12]. We evaluated DNA binding activity of transcription factors in nuclear extracts isolated from *P. berghei* strains with different chloroquine resistance using gel retardation assay.

Nuclear proteins specifically binding to AP1, MEF1, and NF-IL6 transcription factor binding sites were detected using DNA probes corresponding to these binding sites (Fig. 1). This binding is not a result of contamination of the plasmodium nuclear proteins with nuclear proteins of host cells, because binding of proteins from blood cells to all DNA probes used in this study principally differs binding of *P. berghei* nuclear proteins (Fig. 1, 2).

These differences in the binding of nuclear extract proteins were most demonstrative for NF-IL6 binding site (Fig. 1, *c*). One of the main retardation band formed by chloroquine-sensitive strain N nuclear protein is absent in LNK-65 and LNK-65 ChlR strains, but two new retention bands appear, with different intensity for LNK-65 and LNK-65 ChlR, which attested to difference in the chloroquine resistance mechanisms in the strains with natural and induced resistance. Both resistant strains differ from chloroquine resistant strain N by the set of nuclear proteins reacting with oligonucleotides reproducing AP1 and MEF1 binding sites (Fig. 1, *a*, *b*). Binding of nuclear extract proteins to SRE element responsible for blood serum stimulation was also studied [13]. Proteins reacting with SRE were not detected in strain N, but were present in strain LNK-65, and much more pronounced in LNK-65 ChlR strain highly resistant to chloroquine (Fig. 2, *b*). These strains did not differ by SP1 DNA binding activity (Fig. 2, *a*).

These data indicate profound changes in the plasmodium regulatory system as a result of selection by chloroquine resistance, which was seen from changes in the spectrum of DNA binding proteins of *P. berghei* strains. Presumably, these changes in the set of DNA binding proteins are essential for the expression of genes linked with chloroquine resistance.

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