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A 54-kDa protein overexpressed by chloroquine-resistant *Plasmodium berghei* ANKA strain

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ABSTRACT Using an insoluble chloroquine-adsorbent, a 54-kDa protein (with a range of 50-60 kDa) was extracted from serum of mice infected with chloroquine-resistant (CR) *Plasmodium berghei* ANKA strain. Immunoblotting assay with antiserum against the 54-kDa protein showed that the content of the protein was higher in serum of mice infected with the CR parasites than that of mice infected with chloroquine-sensitive (CS) *P berghei* ANKA strain, and that instead of the 54-kDa protein, a set of 15-, 16-, and 23-kDa proteins was found to be highly overexpressed in lysate of purified CR parasites in comparison with that of purified CS parasites, suggesting the 54-kDa protein probably to be composed of 3 subunits. These findings may bear great importance in probing mechanism of chloroquine resistance in malaria parasites.

KEY WORDS drug resistance; *Plasmodium berghei*; proteins; chloroquine; immunoblotting

The mechanism of chloroquine resistance in malaria parasites was supposed to be the same as in case of mammalian tumor cells of which the drug-resistant phenotype was associated with MDR gene encoding P-glycoprotein[1-3]. However, the MDR gene and P-glycoprotein were not essential for chloroquine resistance in malaria parasites[4,5].

We once proposed a hypothesis speculating that after a prolonged drug pressure, malaria parasites could produce an " active anti-drug substance" which binds specifically to antimalarials and therefore prevents the drugs from entering the body of malaria parasites or the site of drug action in the parasites[6]. Our previous work suggested chloroquine-resistant malaria parasites producing " specific anti-drug substance" has provided good evidence to support the hypothesis[7]. In order to identify the "anti-drug substance" in malaria parasites, we conducted this study.

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MATERIALS AND METHODS

Parasites and mice Chloroquine-sensitive (CS) *Plasmodium berghei* ANKA strain was obtained from Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, and chloroquine-resistant (CR) *P berghei* ANKA strain[8] was obtained from Laboratory for Antimalarial Drug Research, Second Military Medical University (SMMU). The level of chloroquine resistance of the CR parasites remained about 30-fold when the drug pressure was given regularly. Kunming-strain ♂ mice bred in Animal Center of SMMU were used.

Preparation and usage of insoluble BG-BSA-CQ adsorbent According to the method described by Rowell *et al*[9], 7-(2-aminoethylamino)-4-(4-diethylamino-1-methyl-butylamino) quinoline was made, and conjugated with bovine serum albumin (BSA). After passing through a Sephadex G25 column, the BSA-CQ conjugate was scanned with Du-68 spectrophotometer (Beckman) to examine the reliability. Finally, the BSA-CQ conjugate and BSA were linked to Bio-gel-p4 (Bio Rad product) with glutaraldehyde respectively. The prepared adsorbents were designated as BG-BSA-CQ and BG-BSA.

Mice were inoculated with CR *P berghei* ANKA strain. When parasitemia reached 20-30%, mouse blood was collected by cardiac puncture. The details for usage of the adsorbents to extract proteins from the serum were exactly the same as those of Boose[10].

Preparation of antiserum against 54-kDa

protein Based on the results of extracting proteins from serum of mice infected with CR parasites with BG–BSA–CQ adsorption method, a 54–kDa protein was identified probably to be CQ–binding and hence was used as an antigen to prepare the antiserum. The protein band was cut from gel of SDS–PAGE, and eluted in 0.1% SDS–PBS. Rabbits were immunized by the eluted protein together with Freund's complete adjuvant for the first time, and incomplete Freund's adjuvant for the second, and then by the protein alone for 2–3 times at a 2–wk interval.

SDS–PAGE and immunoblotting The procedures for SDS–PAGE was similar to those of Laemmli[11]. Electrophoretic transfer of protein to nitrocellulose sheets was conducted following the commonly–used method[12]. Transferred nitrocellulose sheets were processed in PBS (0.01 mol· L^{-1}, pH 7.2). Antiserum against 54–kDa protein was used as the first antibody at a dilution of 1 : 100, and horseradish peroxidase–conjugated anti–rabbit IgG was used as the second antibody. The peroxidase reaction was developed in a solution of freshly prepared diaminobenzidine and H_2O_2. Molecular weight markers, purchased from Shanghai Dong Feng Biochemical Technology Company, were used for identifying the molecular weight of unknown proteins.

Immunogold–silver staining (IGSS) Mouse erythrocytes infected with CR and CS parasites were separately washed thrice in PBS. The washed cells were reconstituted to a 50% suspension in 1% BSA–PBS. Thin smears were prepared, and fixed in methanol for a few seconds. The fixed smears were processed in TBS (0.05 mol· L^{-1}, pH 7.2) using ovalbumin as a blocking agent. Antiserum against 54–kDa protein was used as the first antibody at a dilution of 1 : 200 (vol/vol), and protein A conjugated colloidal gold with a diameter of 13 nm was used as a probe. The details for IGSS were essentially the same as those described by Holgate *et al*[13].

Purification of malaria parasites and their lysates Erythrocytes infected with CR and CS parasites were separately washed thrice in PBS, and passed through CF–11 cellulose powder (Whatman product) column to remove the leukocytes, and then lysed with 0.15% saponin. After 3 washes in PBS, the purified parasites were obtained. The parasite lysates were prepared by repeated freeze–thaw lysis of the purified parasites.

RESULTS

Scanning of BSA–CQ conjugate The scanning graph showed the CQ–specific double peaks within 330–360 nm and the protein peak of 280 nm (Fig 1), suggesting that CQ was indeed conjugated with BSA.

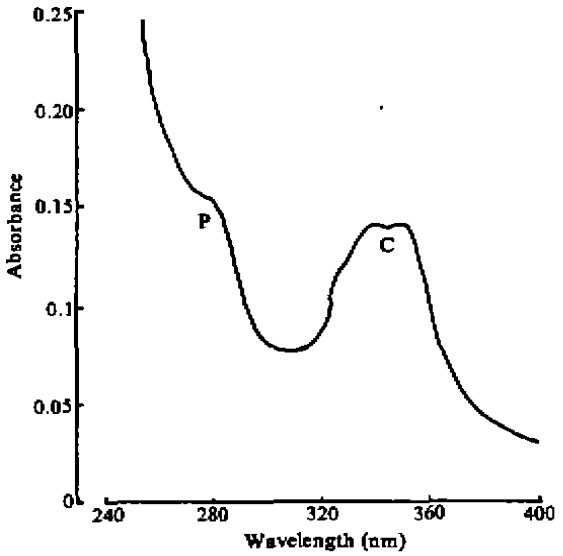

Fig 1. Scanning of BSA–CQ conjugate. P: protein peak. C: chloroquine–specific peaks.

Proteins isolated with BG–BSA–CQ and BG–BSA adsorbents Several protein bands were found in the gel of SDS–PAGE, but a clear 54–kDa protein band (with a range of 50–60 kDa) was found only in the eluate of BG–BSA–CQ adsorbent, not in that of BG–BSA adsorbent (Fig 2), indicating that the protein may be of CQ–binding.

Immunoblotting assay of 54–kDa protein in mouse sera and parasite lysates Antiserum against 54–kDa protein was used as the first antibody to detect the 54–kDa protein in 3 sera and 2 malaria parasite lysates. The

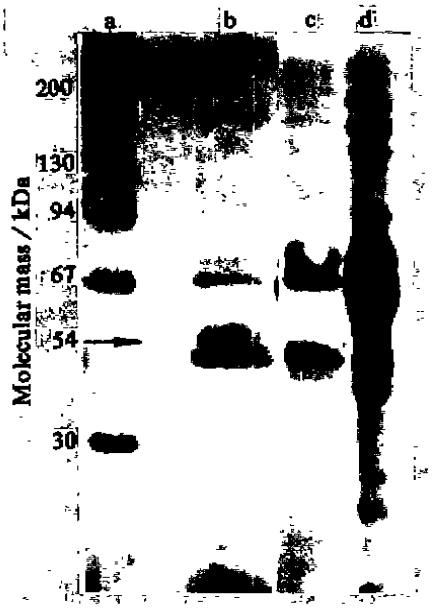

Fig 2. SDS–PAGE–separated and Coomassie blue–stained proteins in eluates of BG–BSA–CQ and BG–BSA adsorbents. Lane a: molecular mass marker; Lane b: eluate of BG–BSA–CQ adsorbent loaded with 20 μg protein; Lane c: eluate of BG–BSA adsorbent loaded with 20 μg protein; Lane d: serum of mice infected with CR *P berghei* ANKA strain, 20 μl of 20–fold diluted serum loaded.

higher content of 54–kDa protein was found in serum of mice infected with CR parasites. Instead of the 54–kDa protein, a set of 15–, 16–, and 23–kDa proteins was highly overexpressed in the lysate of purified CR parasites (Fig 3), suggesting that the 54–kDa protein is probably composed of 3 subunites.

IGSS of parasite–infected erythrocytes

The CR parasite–infected erythrocytes were covered by silver precipitate, and the CS parasite–infected erythrocytes were not stained (Fig 4, Plate 1), showing that the overexpressed 54–kDa protein existed in CR parasite–infected erythrocytes.

DISCUSSION

In this paper, a 54–kDa protein (with a range of 50–60 kDa) of malaria parasites is reported. Since the protein was found to be overexpressed in CR malaria parasites, and may be of chloroquine–binding, we speculate that the protein probably belonged to the "specific anti–drug substance" suggested in our previous paper[7], and could serve as a further evidence to support our hypothesis concerning the mechanism of chloroquine resistance in malaria parasites[6].

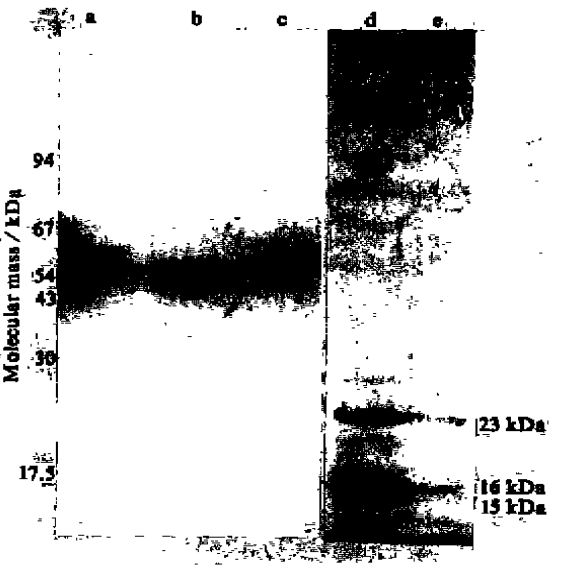

Fig 3. Immunoblotting assay of 54–kDa protein in mouse sera and parasite lysates. Lanes a, b, and c loaded with serum of mice infected with CS parasites, serum of mice infected with CR parasites, and serum of normal mice at 10 μl of 20–fold dilution, respectively. Lanes d and e loaded with purified CR and CS parasite lysate at about 60 μg of protein, respectively.

Immunoblotting assay showed that instead of the 54–kDa protein, a set of 15–, 16–, and 23–kDa protein bands appeared in parasite lysates (Fig 3), suggesting the 54–kDa protein to be composed of 3 subunits. The reason why the 54–kDa protein in serum of mice infected with malaria parasites did not decomposed into subunits in

the same assay was unknown and needs research.

Since no way was capable of distinguishing the CR malaria parasite-infected erythrocytes from CS ones, our IGSS results will serve as a pioneer to show a possibility of using immunomethod to do so.

In conclusion, the discovery of the overexpressed 54-kDa protein and its subunits in CR malaria parasites is of great importance in understanding the biochemical basis of mechanism of chloroquine resistance in malaria parasites. The chemical nature of the protein and the details in its association with CR phenotype of malaria parasites merit further research.

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抗氯喹伯氏疟原虫 ANKA 株过度表达 54-kDa 蛋白质

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摘要 用不溶性氯喹吸附剂自抗氯喹(CR)伯氏疟原虫 ANKA 株感染鼠血清中提取了一种 54-kDa 蛋白质. 用针对该蛋白抗血清进行免疫印迹检测, 发现 CR 原虫感染鼠血清中该蛋白含量明显高于氯喹敏感(CS)原虫感染鼠血清; 与 CS 原虫相比, CR 原虫溶解液中发现一组 15-、16-和 23-kDa 蛋白过度表达, 提示 54-kDa 蛋白可能由 3 个亚单位组成.

关键词 抗药性; 伯氏疟原虫; 蛋白质; 氯喹; 免疫印迹