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Detection of 54-kDa protein overexpressed by chloroquine-resistant *Plasmodium berghei* ANKA strain in pyronaridine-resistant *P berghei* ANKA strain[1]

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AIM: A 54-kDa protein overexpressed by chloroquine-resistant (CR) *Plasmodium berghei* ANKA strain was first reported by us. This study is conducted to detect the protein in pyronaridine-resistant (PR) *P berghei* ANKA strain. **METHODS:** Immunoblotting analysis and immunoelectron microscopy were used. **RESULTS:** PR parasites, like CR parasites, mainly overexpressed 2 major bands of 37 (36—38) kDa and 16 (15—17) kDa which were considered to be 2 subunits of 54 (52—62) kDa protein. Three of 7 times of experiments showed a 54-kDa and a 96 (95—100) kDa bands. The proteins were localized to be mainly scattered in cytoplasm of trophozoites, schizonts, and merozoites of erythrocytic stage of *P berghei*. Some of them were distributed in cytoplasm of erythrocytes infected with parasites. **CONCLUSION:** Both PR and CR parasites overexpressed the same proteins.

KEY WORDS microbial drug-resistance; *Plasmodium berghei*; proteins; chloroquine; pyronaridine; immunoblotting; immunoelectron microscopy

The mechanisms of chloroquine resistance in malaria parasites are still controversial[1]. Based on our "specific antidrug substanc" hypothesis[2-3], we have identified a 54-kDa protein overexpressed by chloroquine-resistant (CR) *Plasmodium berghei* ANKA strain. Since the protein is probably of chloroquine-specific binding, it is considered to be the "specific antidrug substance or protein"[4]. Whether our hypothesis and findings can be extrapolated to explain other drug resistance in living organisms, it is necessary to detect the 54-kDa protein or analogues in other corresponding drug-resistant parasites. Pyronaridine is a new antimalarial first developed in China. Animal experiments and clinical trials proved it to be very efficient in treating CR malaria[5]. In this study, a pyronaridine-resistant *P berghei* ANKA strain was selected for the experiments.

MATERIALS AND METHODS

Parasites and mice We obtained drug-sensitive (DS) *Plasmodium berghei* ANKA strain from Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, and pyronaridine-resistant (PR) *P berghei* ANKA strain from Institute of Parasitic Disease, Chinese Academy of Preventive Medicine. The resistance level of the PR parasites remained about 10-fold when pyronaridine was given regularly. Chloroquine-resistant (CR) *P berghei* ANKA strain received from Laboratory for Antimalarial Drug Research, Second Military Medical University. Kunming-strain ♀ mice bred in Animal Center of Second Military Medical University were used.

Preparation of parasite lysates Erythrocytes infected with PR, CR, and DS parasites were separately washed thrice with phosphate-buffer saline (PBS,

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0.01 mol · L^{-1}, pH 7.2), and lysed with 0.15 % saponin. After 5 washes with PBS, the purified parasite pellets were added equal volume of PBS, and following resuspension of parasites, SDS-PAGE sample buffer without bromphenol blue was added, and then boiled for 5 min. After centrifugation at 10 000×g for 10 min, supernatant protein contents were determined with ultraviolet absorption method.

SDS-PAGE and immunoblotting The procedures for SDS-PAGE and electrophoretic transfer of protein to nitrocellulose sheets were similar to those of commonly-used methods[6,7]. Transfered nitrocellulose sheets were processed in PBS, and blocked with bovine serum albumin (BSA). Antiserum against 54-kDa protein[4] was used as first antibody. To visualize the target bands recognized by antiserum against 54-kDa protein, immunogold-silver staining (IGSS) was applied[8], and protein A-gold with a diameter of 13 nm was used as probe.

Immunoelectron microscopy[9] Erythrocytes infected with parasites were washed 5 times with phosphate buffer (0.1 mol · L^{-1}, pH 7.4), fixed with 0.25 % glutaral for 10 min, and then added NH_4Cl solution (0.05 mol · L^{-1} in phosphate buffer). After washed with the phosphate buffer, the samples were dehydrated in gradient ethanol solutions, then embedded in Poly/Bed 812, an embedding agent, and finally polymerized at 60 ℃ for 24 h. Thin sections were blocked with 1 % BSA in phosphate buffer (0.05 mol · L^{-1}, pH 7.4) containing 0.1 % Tween 20. Antiserum against 54-kDa protein was used as first antibody, and protein A-gold with a diameter of 10 nm used as probe. Following immunolabeling, sections were washed, stained with uranyl acetate, and examined with a JEM-100X electron microscope.

RESULTS

Detection of proteins Immunoblotting analysis showed that PR parasites mainly overexpressed 2 major bands of 37 (36—38) kDa and 16 (15—17) kDa (Fig 1). Of 7 times of experiments, 3 showed a 96 (95—100) kDa and a 54 (52—62) kDa bands. Comparison of immunoblotting analysis of PR, CR, and DS parasite lysates verified that both PR and CR parasites mainly overexpressed 37-kDa and

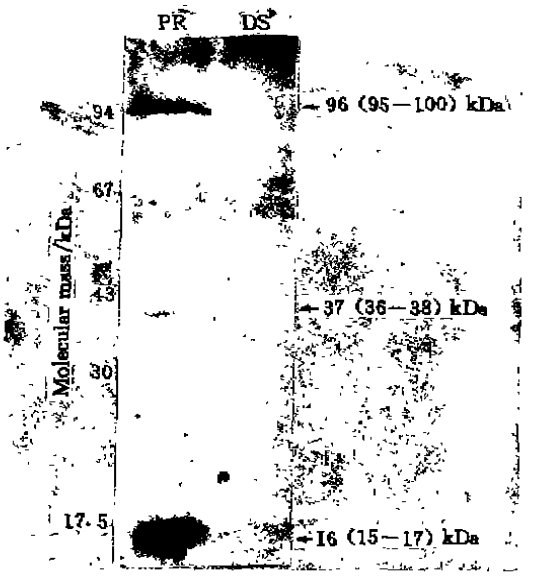

Fig 1. Immunoblotting analysis of PR and DS parasite lysates. Loaded about 50 μg of protein each. Arrows showing bands recognized by antiserum against 54-kDa protein.

16-kDa bands and that 96-kDa and 54-kDa bands were occasionally found (Fig 2 a, b).

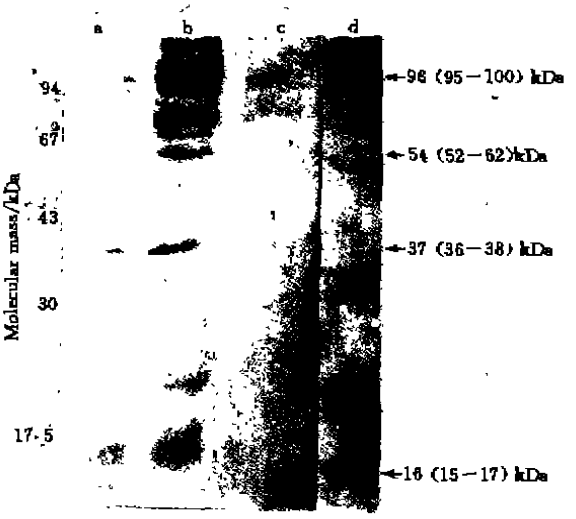

Fig 2. Immunoblotting analysis of PR (a), CR (b) and DS (c) parasite lysates. Lane d loaded with PR parasite lysate which lost mercaptoethanol, each at about 50 μg of protein. Arrows showing bands recognized by antiserum against 54-kDa protein.

After reductant (mercaptoethanol) in the PR parasite lysate was to be vaporized out, the mercaptoethanol-lost sample was reused for immunoblotting analysis which showed that a 54-kDa band appeared instead of 37-kDa band (Fig 2 d).

Localization of proteins Immunoeletron microscopy exhibited that the proteins recognized by antiserum against 54-kDa protein were mainly scattered in cytoplasm of trophozoites (Fig 3 A, D, Plate 1), schizonts, and merozoites (Fig 3 B, Plate 1) at erythrocytic stage of *P berghei* ANKA strain. Some of them were found in cytoplasm of the infected erythrocytes (Fig 3 A, Plate 1). Other labeled gold particles were seen around nuclei of parasites (Fig 3 C, D, Plate 1). Rhoptries of merozoites were also labeled with a small number of gold particles, indicating that at least some of the protein were released from the apical pore of merozoites.

DISCUSSION

In order to prevent decomposition of proteins in process of preparing parasite lysates, SDS-PAGE sample buffer containing mercaptoethanol was used to treat purified parasites, which is somewhat different from our previous method, and of course gives results somewhat different from our previous report[4]. The results showed that PR parasites, like CR parasites highly overexpressed 2 major bands of 37 kDa and 16 kDa, but once reported 23-kDa band[4] may be a decomposed fragment of 37-kDa band. A 54-kDa and 96-kDa bands were occasionally detected in several experiments only. How to explain the relationship among all these proteins or peptides recognized by antiserum against 54-kDa protein seems to be a must in discussing present results. There are 3 reasons for considering 37-kDa and 16-kDa bands as 2 subunits of 54-kDa protein. First of all, both 37-kDa and 16-kDa bands could be recognized by antiserum against the 54-kDa protein. Secondly, the total molecular weight of the 2 bands was approximately 54 kDa. Finally, when reductant in the parasite lysate was removed, a 54-kDa band appeared while 37-kDa band disappeared. The result suggested that 37-kDa and 16-kDa peptides could probably combined together by disulfide bond to form the 54-kDa protein. As for the relationship between 96-kDa and 54-kDa bands, we suppose that the 96-kDa protein may be formed by 2 54-kDa molecules. In another word, the 96-kDa protein is the real and complete "specific antidrug protein" (SAP) suggested in our previous papers[3,4], while the 54-kDa band may serve as a monomer of the 96-kDa protein. Since disulfide bonds may take part in the formation of 54-kDa and 96-kDa peptide or protein, therefore, the 54-kDa and 96-kDa bands would occasionally appear if reductant in sample buffer did not disrupt disulfide bonds completely.

If the above speculation is reasonable, there is a composition similarity between SAP and IgG antibody. The molecular weight of IgG is about 150 kDa. The half of an IgG molecule contains a heavy chain of 53 kDa and a light chain of 23 kDa. The ratio of 53 kDa to 23 kDa is about 2.30, and 150 kDa is about 2 times of 76 (53+23) kDa. The half of SAP molecule contains a 37-kDa and a 16-kDa chains. The ratio of 37 to 16 is about 2.31, and 96 kDa is also about 2 times of 53 (37+16) kDa. This interesting comparison results implicate that SAP may be a very important protein in cell's defence system.

The distribution of proteins or peptides recognized by antiserum against 54 kDa protein overexpressed by CR parasites was similar to that found in CR parasites[10]. Some of the proteins or peptides were located around nuclei of parasites, implicating an association

between the proteins or peptides and nucleus functions.

In conclusion, our proposed "specific antidrug substance" hypothesis was based on comparison of some features of drug-resistant malaria parasites and those of acquired immunity in human beings[2,3], which coincides well with the present results suggesting a possible composition similarity between SAP and IgG. Theoretically, acquired drug resistance in malaria parasites is actually a kind of cell's acquired defence or immunity response against foreign harmful substances. Therefore the results presented in this paper would be very important not only in revealing mechanisms of drug resistance in malaria parasites, but also in illustrating evolution of acquired immunity in higher living organisms[1]

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在抗咯萘啶伯氏疟原虫 ANKA 株中检测抗氯喹伯氏疟原虫 ANKA 株过度表达的 54-kDa 蛋白[1]

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目的：我们曾报道了抗氯喹伯氏疟原虫过度表达 54-kDa 蛋白，本研究旨在探讨抗咯萘啶伯氏疟原虫是否也过度表达这种蛋白. **方法**：使用免疫印迹分析及免疫电子显微镜检查. **结果**：抗咯萘啶疟原虫和抗氯喹疟原虫一样主要过度表达 37 (36—38) kDa 和 16 (15—17) kDa 两条带；在 7 次实验中有 3 次检测到 54 (52—62) kDa 和 96 (95—100) kDa 条带. 这些蛋白主要散在分布于红内期疟原虫滋养体、裂殖体及裂殖子的胞质中，疟原虫寄生的红细胞中也有少量分布. **结论**：抗咯萘啶疟原虫和抗氯喹疟原虫都过度表达同一类蛋白质.

关键词 微生物抗药性；伯氏疟原虫；蛋白质；氯喹；咯萘啶；免疫印迹；免疫电子显微镜检查