

KNOB-POSITIVE AND KNOB-NEGATIVE  
PLASMODIUM FALCIPARUM DIFFER IN EXPRESSION  
OF A STRAIN-SPECIFIC MALARIAL ANTIGEN ON  
THE SURFACE OF INFECTED ERYTHROCYTES

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Knob-like protrusions of the surface membrane of *Plasmodium falciparum*-infected erythrocytes are thought to account for the sequestration of trophozoite and schizont forms of this parasite in postcapillary venules (1, 2) and may be related to the pathology of cerebral malaria in which brain capillaries become occluded by parasitized erythrocytes (3). Knobless ( $K^-$ ) parasites have been derived from knobby ( $K^+$ ) isolates by continuous in vitro culture (4) and by passage in splenectomized monkeys (5). Erythrocytes infected with  $K^-$  parasites lack cytoadherence (5, 6). A comparison of the malarial proteins synthesized by  $K^+$  and  $K^-$  parasites identified a knob-associated protein that is exceptionally rich in histidine (histidine-rich protein, HisRP) (7, 8). This protein appears to be associated with the cytoskeleton of infected cells (9) and not to extend through the erythrocyte membrane (10; and Leech and Howard, unpublished results). Therefore, knob-associated HisRP cannot directly mediate cytoadherence. This is consistent with the observation that some  $K^+$  parasites that synthesize apparently normal knob-associated HisRP lack the cytoadherent property (11).

Recent studies by Leech and co-workers (12) identified a strain-specific malarial antigen ( $M_r$  250,000–300,000) on the surface of *P. falciparum*-infected erythrocytes that meets several criteria for the cytoadherent moiety. We examine the expression of this protein on the surface of  $K^+$ - and  $K^-$ -infected erythrocytes. The radioiodinated cell surface proteins were compared for  $K^+$  and  $K^-$  parasites from two different geographic regions (Malaysia and El Salvador). The strain-specific surface antigens could be radiolabeled only with  $K^+$  parasites.

#### Materials and Methods

*Parasites.* The Malayan camp (McK<sup>+</sup>) (14) and Santa Lucia (SLK<sup>+</sup>) (15) strains of *P. falciparum* were maintained by passage in *Aotus trivirgatus griseimembra* monkeys. Trophozoite and schizont stages of these strains induced knobs on infected erythrocytes ( $K^+$ ) and they sequestered in vivo and bound to melanoma cells in vitro.  $K^-$  variants no longer sequestered in vivo (5) or bound to amelanotic melanoma cells in vitro (5, 6). Cryopreserved blood (13, 14) with 10–30% parasitemia of ring stage parasites was thawed and cultured for 20–30 h by a modification (14) of the method of Trager and Jensen (15).

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**Lactoperoxidase-catalyzed Radioiodination.** Infected blood containing trophozoite stage parasites was radioiodinated using lactoperoxidase and  $H_2O_2$  (14).

**Separation of Infected Cells.** Radioiodinated, infected blood was layered onto a step gradient of Percoll (Pharmacia, Inc., Piscataway, NJ) made by diluting a stock solution of 90% Percoll in RPMI plus 25 mM HEPES and 6% sorbitol with Dulbecco's phosphate-buffered saline (PBS) plus 6% sorbitol. A typical gradient consisted of 2 ml 40%, 2 ml 60%, 2 ml 80%, and 4 ml 90% Percoll in a 15 ml Corex tube (Sorvall Instruments Div., Du Pont Co., Newton, CT). Gradients were centrifuged in an SS-34 rotor (Sorvall Instruments Div., Du Pont Co.) at 10,000 rpm for 20 min at 20°C. Very mature and damaged parasites found above the 40% layer were discarded. Intact infected cells (>95%) were collected from between the 80 and 40% layers of the gradient and washed with Dulbecco's PBS at room temperature. Uninfected erythrocytes from the bottom of the gradient were washed with PBS and taken through the extraction process for comparison.

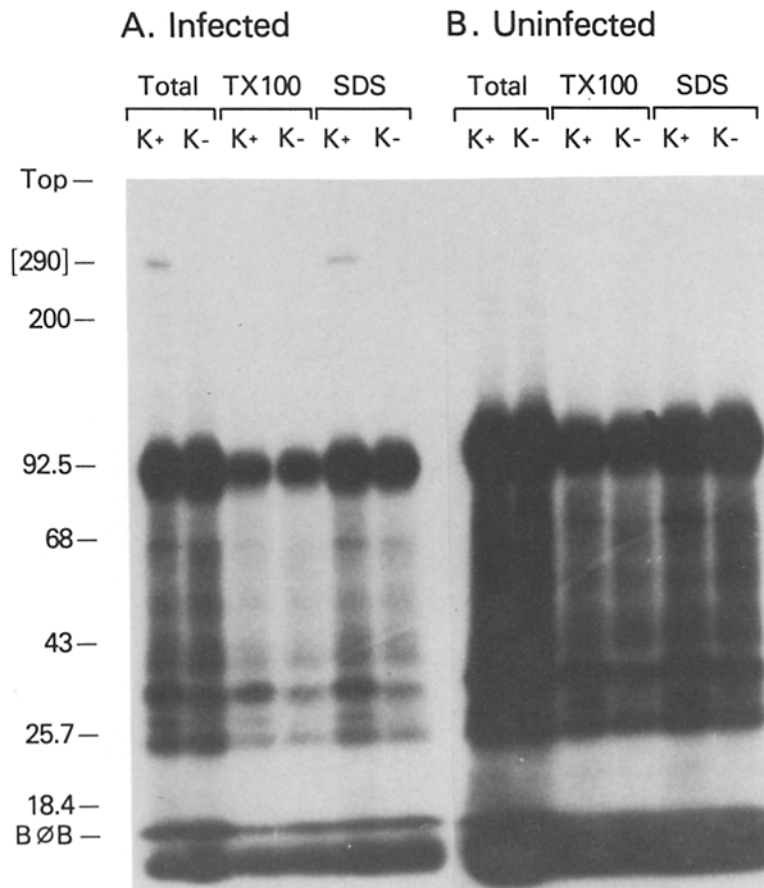
**Detergent Extraction of Radioiodinated Cells.** Radioiodinated, infected and uninfected erythrocytes were extracted sequentially with Triton X-100 and sodium dodecyl sulfate (SDS) as described previously (12, 16), except that only leupeptin (Boehringer Mannheim Biochemical, Indianapolis, IN) (5 µg/ml) was used as a proteinase inhibitor.

**Gel Electrophoresis.** Extracted proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) on 5–10% polyacrylamide gels as described previously (17), except that prestained standards of myosin (200,000 mol wt), phosphorylase B (92,500), bovine serum albumin (68,000), ovalbumin (43,000), alpha-chymotrypsin (25,700), beta-lactoglobulin (18,400), and cytochrome *c* (12,300) (Bethesda Research Laboratories, Gaithersburg, MD) were used as molecular weight markers.

## Results

**Comparison of  $MCK^+$ - and  $MCK^-$ -infected Erythrocytes.**  $MCK^+$ - and  $MCK^-$ -infected blood was radioiodinated by the lactoperoxidase method, and infected and uninfected cells were separated on Percoll gradients made hypertonic with sorbitol. We routinely obtained a fraction of 90–100% trophozoite and schizont-infected erythrocytes (*infected*; Fig. 1) and a fraction of <1% parasitized cells (*uninfected*, Fig. 1). Thus, a single culture could be used to identify  $^{125}I$ -labeled surface antigens while controlling for variation in uninfected erythrocytes. Radioiodinated proteins were extracted either directly into SDS or sequentially with Triton X-100 and SDS and were separated by SDS-PAGE. The strain-specific antigen on the surface of  $MCK^+$ -infected erythrocytes (12) was clearly visible as an  $M_r$  290,000 radioiodinated polypeptide after solubilization in SDS-PAGE sample buffer (Fig. 1A, *total K^+*). This polypeptide was not extracted by 1% Triton X-100 (Fig. 1A, *Triton K^+*) but was extracted from the insoluble residue of the Triton extraction by 2% SDS (Fig. 1A, *SDS K^+*). In contrast, there were no  $^{125}I$ -polypeptides of comparable size or detergent extractability with radioiodinated erythrocytes infected with  $MCK^-$  parasites (Fig. 1A, *K^-*). All other radioiodinated proteins were essentially the same on infected (Fig. 1A) or uninfected (Fig. 1B) erythrocytes.

**Comparison of  $SLK^+$ - and  $SLK^-$ -infected Erythrocytes.** Radioiodinated erythrocytes infected with  $MCK^+$ ,  $SLK^+$ , and  $SLK^-$  parasites were separated as above and extracted with 1% Triton X-100. The Triton X-100-insoluble proteins were extracted with 2% SDS and separated by SDS-PAGE. (Fig. 2). The  $MCK^+$  strain-specific protein was again visible at  $M_r$  290,000 ( $MCK^+$ , Fig. 2A). The Santa Lucia strain-specific antigen (12) was present in  $SLK^+$ -infected cells at  $M_r$  265,000 (Fig. 2A; 265). As with the Camp strain protein, this polypeptide was not extracted with 1% Triton X-100 (data not shown). No comparable radioiodinated polypeptide was found in extracts of  $SLK^-$ -infected cells (Fig. 2A,  $SLK^-$ ) or in



**FIGURE 1.** Comparison of the surface-radioiodinated proteins on erythrocytes infected with K<sup>+</sup> or K<sup>-</sup> parasites of the Malayan camp strain. Washed erythrocytes from *Aotus* blood infected with either MCK<sup>+</sup> or MCK<sup>-</sup> parasites were radioiodinated and then separated into infected (>95% infected) (A) or uninfected (<1% infected) (B) cells. A portion of the cells was solubilized directly in SDS sample buffer (Total). The remaining cells were extracted with 2% Triton X-100 to obtain Triton X-100-soluble material (TX100). The residual material was extracted with 2% SDS (SDS). SDS sample buffer was added to the extracts for SDS-PAGE. The  $M_r$  290,000 Malayan camp strain-specific antigen (indicated in parentheses) was only on erythrocytes infected with MCK<sup>+</sup> strain parasites and could only be extracted with SDS-containing buffers. Molecular size markers are indicated on the left. BøB, bromphenol blue. Representative of six experiments.

uninfected cells isolated from the same cultures (Fig. 2B).

With both parasite strains, the normal erythrocyte proteins of K<sup>-</sup>-infected cells were radioiodinated to the same extent as or more than their counterparts on K<sup>+</sup>-infected cells. It is therefore unlikely that the lack of a radioiodinated strain-specific antigen on cells infected with K<sup>-</sup> parasites is due to selective inhibition of the lactoperoxidase reaction.

#### Discussion

In all strains of *P. falciparum* studied thus far, knobs are an absolute requirement for in vitro cytoadherence of infected erythrocytes to either human

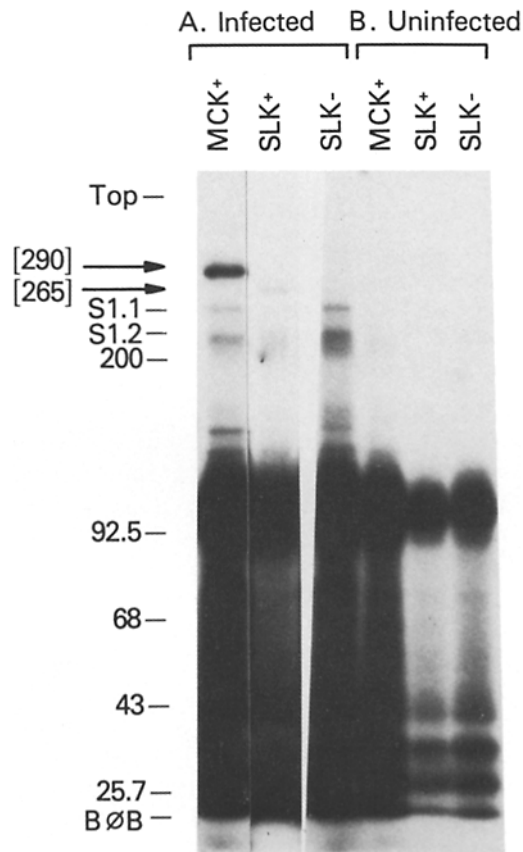


FIGURE 2. Comparison of the surface-iodinated proteins on erythrocytes infected with  $K^+$  and  $K^-$  parasites of the Santa Lucia strain. Washed erythrocytes from infected *Aotus* blood were radioiodinated and separated into (A) erythrocytes  $>95\%$  infected with trophozoites or schizonts, and (B) erythrocytes  $<1\%$  infected with trophozoites. Each sample was extracted with 2% Triton X-100 and the residual material extracted with 2% SDS. SDS sample buffer was then added to the SDS extracts and the proteins separated by SDS-PAGE. The strain-specific antigen of  $SLK^+$  was clearly of different  $M_r$  (265,000) than the comparable protein on  $MCK^+$  (290,000). This protein was not identified either on uninfected erythrocytes or on erythrocytes infected with the  $K^-$  variant of  $SLK^+$  strain. The  $^{125}I$ -labeled proteins S1.1 and S1.2 co-migrate with the spectrin bands of *Aotus* erythrocytes and represent a small degree of intracellular radiolabeling that often occurs with erythrocytes infected with mature parasites. Molecular size markers are indicated on the left. *BøB*, bromphenol blue. Representative of four experiments.

endothelial cells (5) or amelanotic melanoma cells (6). To date, the only biochemical correlate of the  $K^+$  phenotype has been a HisRP of variable size, insoluble in nondenaturing detergents, and presumably associated with the cytoskeleton of infected erythrocytes (12, 18). The presence of knobs is not sufficient for binding, as  $K^+$  strains of *P. falciparum* that do not bind in vitro have been recently identified (11). Leech and co-workers recently described a strain-specific antigen on the surface of *P. falciparum*-infected erythrocytes of variable  $M_r$ , readily labeled by  $^{125}I$  and lactoperoxidase, and insoluble in nondenaturing detergents (12). The proteinase sensitivity and antigenic specificity of these proteins associated them with the capacity of infected cells to bind to melanoma cells in vitro

(12). These proteins have been suggested as possible binding ligands, associated with the submembrane knob structure and the cell surface (18).

K<sup>-</sup> variants of *P. falciparum* strains neither exhibit cytoadherence (5, 6) nor synthesize knob-associated HisRP (7, 8). The surface antigens on erythrocytes infected with these K<sup>-</sup> variants, particularly in regard to the strain-specific antigens described by Leech and co-workers (12), have not been previously investigated. The strain-specific antigens of MCK<sup>+</sup> and SLK<sup>+</sup> were readily identified by surface iodination and had detergent solubility properties and *M<sub>r</sub>* corresponding to published values (12). Uninfected cells from the same cultures did not display these proteins. K<sup>-</sup> variants from both strains lacked a <sup>125</sup>I-labeled surface protein with the size and solubility properties of the proteins identified on K<sup>+</sup>-infected cells. No other parasite-dependent proteins were identified after radioiodination of K<sup>-</sup>-infected cells.

While the association of knobs and strain-specific surface antigen is evident in parasites derived from two widely separate geographical locations, the nature of this association remains uncertain. Because of similarity in detergent solubility and cell location between knob-associated HisRP and strain-specific surface antigen, it is tempting to suggest a direct link between these two knob-associated proteins. Knobs and the submembrane electron-dense material could act to cluster the strain-specific antigen for the cytoadherent function (18). We do not yet know at what step the surface expression of the strain-specific antigen is blocked in these K<sup>-</sup> parasites. The K<sup>-</sup> variants may be unable to synthesize the strain-specific antigen, as appears to be the case for the knob-associated HisRP. Alternatively, this antigen may be synthesized but either not transported to the erythrocyte surface or not inserted correctly through the erythrocyte membrane.

### Summary

We have investigated the expression of a strain-specific malarial antigen on the surface of erythrocytes infected with knobless (K<sup>-</sup>) variants of knob-positive (K<sup>+</sup>) strains of *Plasmodium falciparum*. *Aotus* blood infected with K<sup>+</sup> or K<sup>-</sup> parasites derived from two independent geographical isolates (Malayan camp and Santa Lucia) was surface iodinated by the lactoperoxidase method. Infected and uninfected erythrocytes were then separated by a new procedure involving equilibrium density sedimentation on a Percoll gradient containing sorbitol. Strain-specific antigens were readily identified on the surface of erythrocytes infected with either of the K<sup>+</sup> strains by their characteristic size and detergent solubility. These proteins were not detected on the surface of erythrocytes infected with either of the K<sup>-</sup> variants nor on uninfected erythrocytes isolated from K<sup>+</sup>- or K<sup>-</sup>-infected blood. These results are consistent with a role for the strain-specific surface antigen in cytoadherence of *P. falciparum*-infected erythrocytes. Our findings represent the second biochemical difference (with the knob-associated histidine-rich protein) between K<sup>+</sup> and K<sup>-</sup> *P. falciparum*.

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### References

1. Rudzinska, M. A., and W. Trager. 1968. The fine structure of trophozoites and gametocytes in *Plasmodium coatneyi*. *J. Protozool.* 15:73.

2. Luse, S. A., and L. H. Miller. 1971. *Plasmodium falciparum* malaria: ultrastructure of parasitized erythrocytes in cardiac vessels. *Am. J. Trop. Med. Hyg.* 20:655.
3. Spitz, S. 1946. The pathology of acute falciparum malaria. *Mil. Surg.* 99:555.
4. Langreth, S. A., R. T. Reese, M. R. Motyl, and W. Trager. 1979. *Plasmodium falciparum*: loss of knobs on the infected erythrocyte surface after long-term cultivation. *Exp. Parasitol.* 48:213.
5. Barnwell, J. W., R. J. Howard, and L. H. Miller. 1983. Influence of the spleen on the expression of surface antigens on parasitized erythrocytes. *Ciba Found. Symp.* 94:117.
6. Schmidt, J. A., I. J. Udeinya, J. H. Leech, R. J. Hay, M. Aikawa, J. Barnwell, I. Green, and L. H. Miller. 1982. *Plasmodium falciparum* malaria. An amelanotic melanoma cell line bears receptors for the knob ligand on infected erythrocytes. *J. Clin. Invest.* 70:379.
7. Kilejian, A. 1979. Characterization of a protein connected with the production of knob-like protrusions on membranes of erythrocytes infected with *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA.* 76:4650.
8. Hadley, T. J., J. H. Leech, T. J. Green, W. A. Daniel, M. Wahlgren, L. H. Miller, and R. J. Howard. 1983. *Plasmodium falciparum*: a comparison on knobby (K<sup>+</sup>) and knobless (K<sup>-</sup>) parasites of two strains. *Mol. Biochem. Parasitol.* 9:271.
9. Leech, J. H., J. W. Barnwell, M. Aikawa, L. H. Miller, and R. J. Howard. 1984. *Plasmodium falciparum* malaria: association of knobs on the surface of infected erythrocytes with a histidine-rich protein and the erythrocyte skeleton. *J. Cell Biol.* 98:1256.
10. Kilejian, A. 1981. Alterations of human erythrocyte membranes due to infection with *Plasmodium falciparum*. In *The Biochemistry of Parasites*. G. M. Slutsky, editor. Pergamon Press, Ltd., Oxford. 68-73.
11. Udeinya, I. J., P. M. Graves, R. Carter, M. Aikawa, and L. H. Miller. 1983. *Plasmodium falciparum*: effect of time in continuous culture on binding to human endothelial cells and amelanoma cells. *Exp. Parasitol.* 56:207.
12. Leech, J. H., J. W. Barnwell, L. H. Miller, and R. J. Howard. 1984. Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. *J. Exp. Med.* 159:1567.
13. Meryman, H. T., and M. Hornblower. 1972. A method for freezing and washing red blood cells using a high glycerol concentration. *Transfusion (Phila.)* 12:145.
14. Aley, S. B., J. W. Barnwell, W. A. Daniel, and R. J. Howard. 1984. Identification of parasite proteins in a membrane preparation enriched for the surface membrane of erythrocytes infected with *Plasmodium knowlesi*. *Mol. Biochem. Parasitol.* 12:69.
15. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science (Wash. DC)* 193:673.
16. Howard, R. J., and J. W. Barnwell. 1984. The detergent solubility properties of a malarial (*Plasmodium knowlesi*) variant antigen expressed on the surface of infected erythrocytes. *J. Cell Biochem.* 24:297.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
18. Leech, J. H., S. B. Aley, L. H. Miller, and R. J. Howard. 1984. *Plasmodium falciparum* malaria: cytoadherence of infected erythrocytes to endothelial cells and associated changes in the erythrocyte membrane. In *Malaria and the Red Cell*. Alan R. Liss, Inc., New York. 63-77.