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P. FALCIPARUM INFECTED ERYTHROCYTES ARE CAPABLE OF ENDOCYTOSIS

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SUMMARY

P. falciparum, an intraerythrocytic parasite, obtains nourishment primarily through phagocytosis of the host cytosol but also through the incorporation of extracellular small molecules which enter through the parasitized red cell's membrane via pores. Normal mature erythrocytes are incapable of endocytosis. Several lines of evidence suggest that extracellular large molecules may be taken up when the mature red cell is parasitized by *P. falciparum*, but direct evidence has been lacking. We now report the use of ferritin, an electron dense protein, to demonstrate endocytosis in *P. falciparum* infected red cells.

Parasitized red cells incubated with ferritin internalize that macromolecule as demonstrated by electron microscopy. While normal red cells incubated with ferritin took up none of the tracer molecule, parasitized red cells internalized substantial amounts. In addition both ferritin and apoferritin inhibited the growth of *P. falciparum* in a dose dependent fashion, again indicating endocytosis of a macromolecule. These data indicate that *P. falciparum* can somehow stimulate the mature erythrocyte to engage in endocytosis. We also note that both infected and non-infected red cells in a culture in which *P. falciparum* is growing become abnormally sticky for ferritin. Moreover, parasitized red cells bind I^{125}-transferrin while non-parasitized erythrocytes do not. These observations suggest that a soluble parasite product alters the red cell membrane in a non-global manner, causing selective effects in relation to different proteins.

Key words: malaria; *P. falciparum*; endocytosis; ferritin.

INTRODUCTION

P. falciparum is an intracellular parasite that feeds by phagocytosing the surrounding host cytosol (16). Although most of the amino acids utilized for protein synthesis by the parasite come from digestion of the red cell's hemoglobin, some are imported (4). Other kinds of small molecules can also enter the parasitized red cell (7) most via pores but at least some by other routes. For example, the parasitized red cell, when incubated with radioactive L-glutamine, takes it up in a manner that increases the specific activity of the red cell cytosolic pool while allowing the parasite to maintain the constant specific activity of glutamine in the extracellular medium (5). This suggests that the parasite may be connected to the external milieu either through a channel or through endocytic vesicles. The latter is attractive because there is suggestive evidence for the uptake of at least one macromolecule, transferrin, by endocytosis.

P. falciparum parasitized red cells take up transferrin (13, 16), extract and retain the iron and degrade the protein (13). Transferrin initially binds non-specifically to the parasitized red cell. The binding is progressive with time, and is twofold greater at 37° than at 0° C (14). These observations are most easily explained by postulating endocytosis of the non specifically bound transferrin (20).

P. falciparum parasitizes mature red cells which are ordinarily not capable of endocytosis. We therefore wished to obtain direct evidence of endocytosis in the parasitized cell. We now report the use of ferritin, an iron binding, electron dense protein to demonstrate endocytosis in *P. falciparum* parasitized red cells. Ferritin is taken up by the parasitized red cell and can be visualized in vesicles found in the red cell cytosol. The protein accumulates both in the red cell cytosol and in the parasitophorous vacuole.

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Materials and Methods

P. falciparum (FCR-3 Gambian strain) was suspended in a 2.5% suspension of O+ erythrocytes collected in citrate phosphate dextrose adenine-one (CPDA-1). Cultures were maintained at 37° C using the Trager-Jensen candle jar technique (9,15,21) using RPMI-1640 medium supplemented with 20% O+ human plasma and other nutrients (18). Ferritin was dialyzed against 0.1 M NaCl 0.02 M Hepes, pH 7, and apoferritin was prepared by dialysis of ferritin against 0.1 M thioglycollic acid and 0.1 M sodium acetate (23).

Infected cultures were fed at 72 hour intervals until parasitemias were in excess of 15%. Control cultures, consisting of red cells from the same donor and incubated under identical conditions were maintained in parallel. Horse spleen ferritin was added to one set of infected cultures at a final concentration of 0.6 mg/ml. A second set of infected cultures was left untreated. Control and ferritin treated cultures were incubated overnight, washed six times in RPMI, and fixed in 1.5% buffered gluteraldehyde. Specimens were embedded in plastic and ultrathin sections prepared for examination with a JEOL 100 CX electron microscope as described (1).

Ferritin was quantitated by planimetry using a clear acetate grid overlaid on micrographs taken at 50 000 times original magnification. Photographs of either red cell cytoplasm or parasitophorous vacuole devoid of any red cell outer membrane were given blinded code numbers and evaluated by an unbiased observer. The number of ferritin granules in each grid square was counted and the resulting number of granules per cm^2 calculated. The code was then broken and the mean number of granules per cm^2 of control and experimental red cell cytoplasm or parasite covered areas were compared using Student's t test for unpaired observations.

In another series of experiments, parasitized cultures were incubated under the above conditions with ^{125}I-transferrin, washed extensively and fixed in buffered formalin. After embedding in paraffin, thick sections were prepared for light microscopic autoradiography by

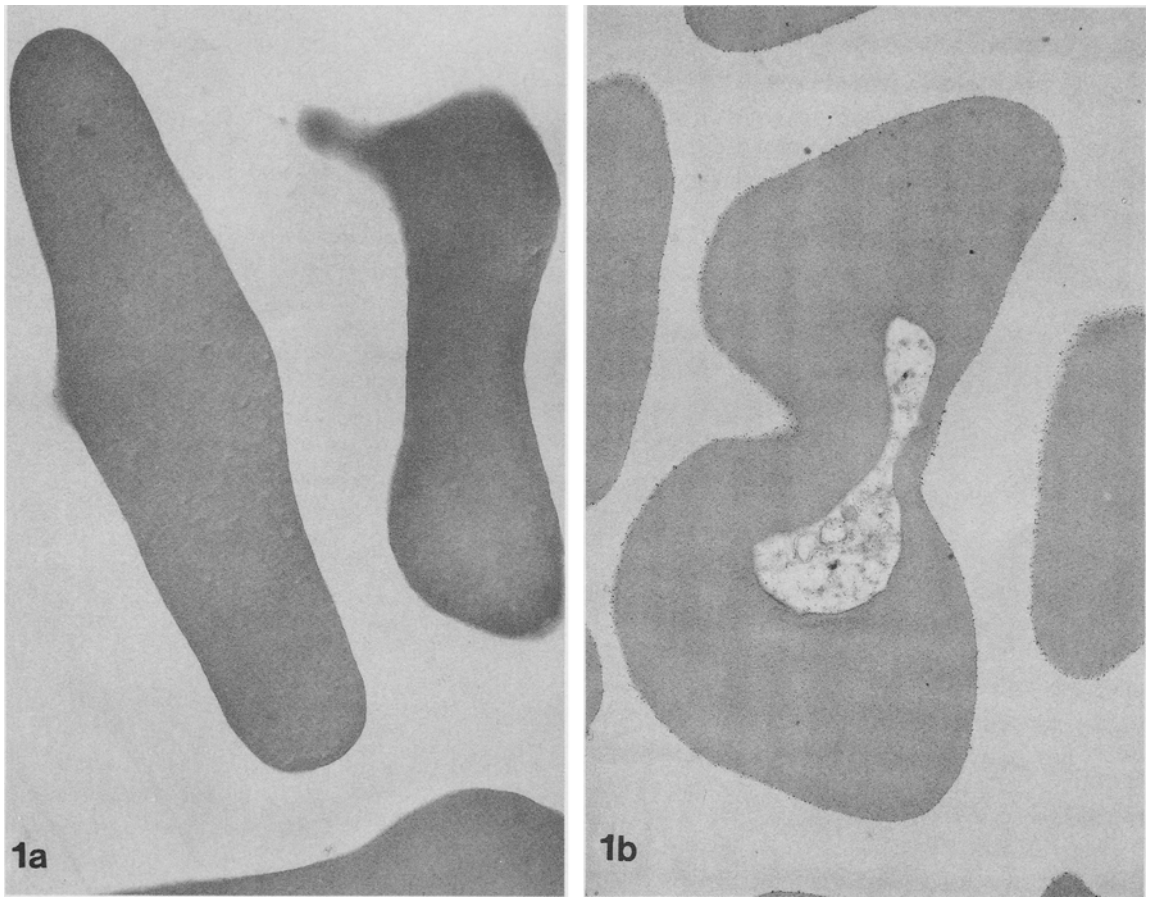

FIG. 1a and 1b: Figure la depicts control non-parasitized red cells incubated with ferritin and washed prior to fixation. No ferritin is seen. (Mag ×15 741). Figure 1b shows ferritin adherent to red cell membranes of all cells in culture dishes infected with *P. falciparum*. Note that ferritin adheres even to the non-parasitized cells of this culture. (Mag ×14 655).

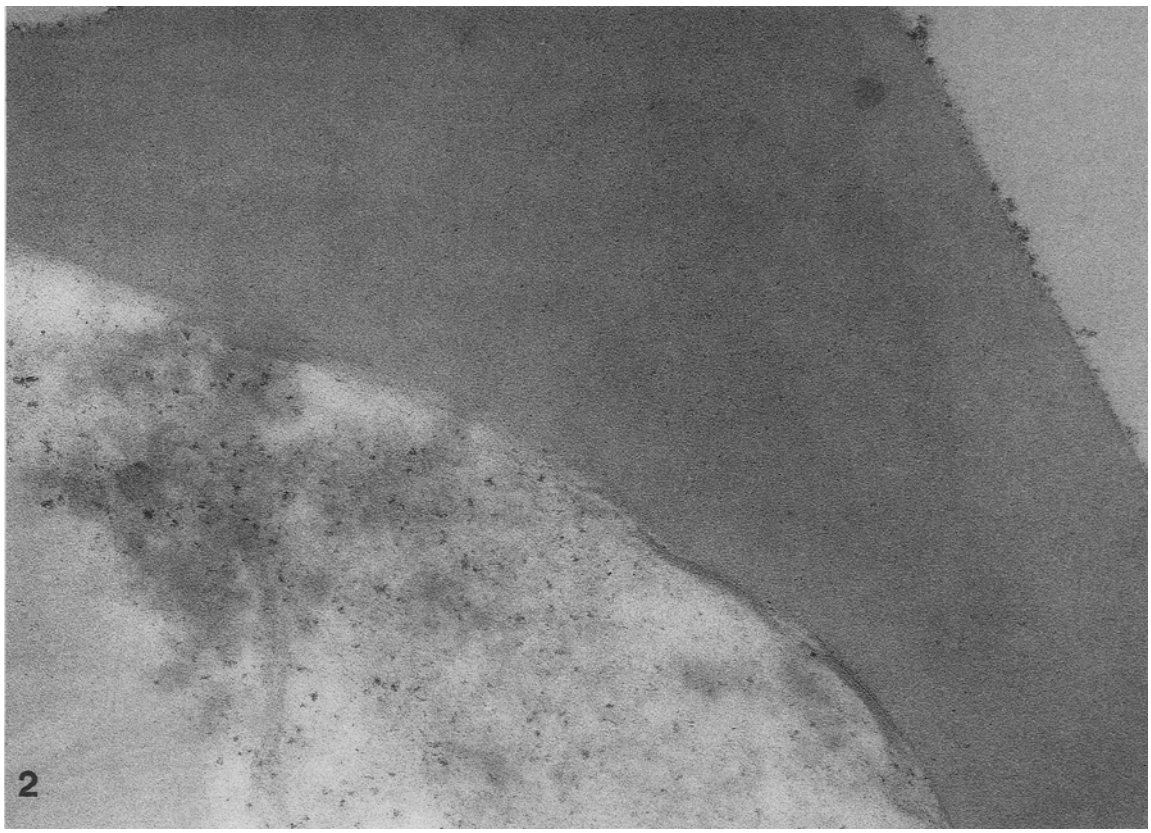

FIG. 2. Detail of P. falciparum infected erythrocyte showing ferritin within the parasitophorous vacuole and the red cell cytoplasm as well as adherent to the external red cell membrane. (Mag ×86 207).

coating slide mounted sections with Kodak NBT2 emulsion and developing the slides in the dark for four months. The number of developed grains per parasitized and non-parasitized red cell was calculated.

RESULTS

All of the red cells from parasitized dishes were coated with ferritin (Fig 1) while those of uninfected dishes were not, despite their incubation with ferritin. Since the parasitemia in these experiments did not exceed 15% it is clear that even non-parasitized red cells adsorb ferritin in the parasitized dish. Ferritin was also found in the red cell cytosol and parasitophorous vacuoles of parasitized cells (Fig 2). Based on the numeration of 23 431 granules we observed (Table 1) that the cytoplasm of control cells contained a mean of 1.4 ± 0.7 granules per cm^2, while the ferritin treated specimens had 5.3 ± 1.8 granules ($p = 0.0012$). In addition, a mean of 2.4 ± 0.9 granules were found in sections of control parasitophorous vacuoles versus 6.5 ± 3.0 granules in the ferritin treated specimens($p = 0.0038$). The background granules of the control specimens have been noted previously (8) and may be

TABLE 1

COMPARISON OF CONCENTRATIONS OF INTRACELLULAR FERRITIN

	Total # Grains	Total Surface Area, (cm^2)	Mean Grains/cm^2	p value
Control Parasitophorous Vacuole	2,211	1,348	2.36 ± 0.9	0.004
Ferritin Parasitophorous Vacuole	4,757	816	6.54 ± 3.0	
Control Cytosol	1,471	820	1.42 ± 0.7	0.001
Ferritin Cytosol	14,992	2,868	5.28 ± 1.8	

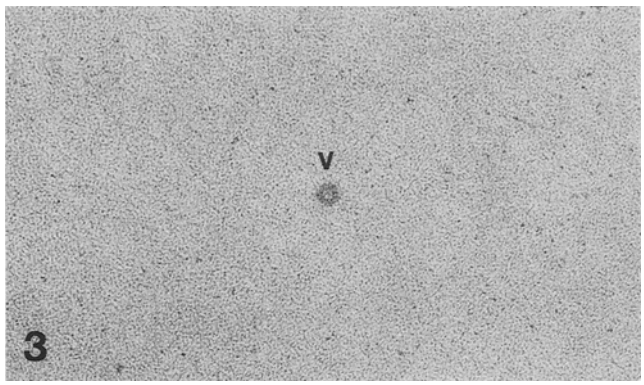

FIG. 3. Detail of a portion of the red cell cytoplasm of a parasitized cell demonstrating endocytic vesicle(V). Unstained section (Mag ×130 000).

endogenously produced ferritin since this iron storage protein is found in a large variety of organisms (6).

The large ferritin molecule must have traversed the red cell membrane as a result of endocytosis occurring as a consequence of some action of the parasite. This was supported by the rare observation of ferritin containing vesicles in parasitized cells (Fig 3).

Because of the observed diminution in the parasitemia of ferritin treated cultures we investigated the effect of increasing concentrations of ferritin on developing parasitemia. At a dose as low as 0.25 mg/ml, ferritin inhibited the growth of *P. falciparum* (Fig 4). The inhibitory activity appeared to be due to the protein moiety rather than the iron since apoferritin given in equimolar doses was equally toxic (Fig 4). These results are similar to those showing toxicity of lactoferrin and apolactoferritin (6). The effect presumably is due to endocytosis of these proteins which, once internalized, upset the iron balance of the parasite.

In the autoradiographic studies the parasitized cells had a mean(±S.E.M.) silver grain count of 0.83 ± 0.05 grains per cell(N = 500) versus 0.51 ± 0.04 grains in non-parasitized cells located at least 3 cells away from a parasitized cell(N = 500, $p < 0.0001$).The mean background count of non-parasitized cells located in areas remote from any

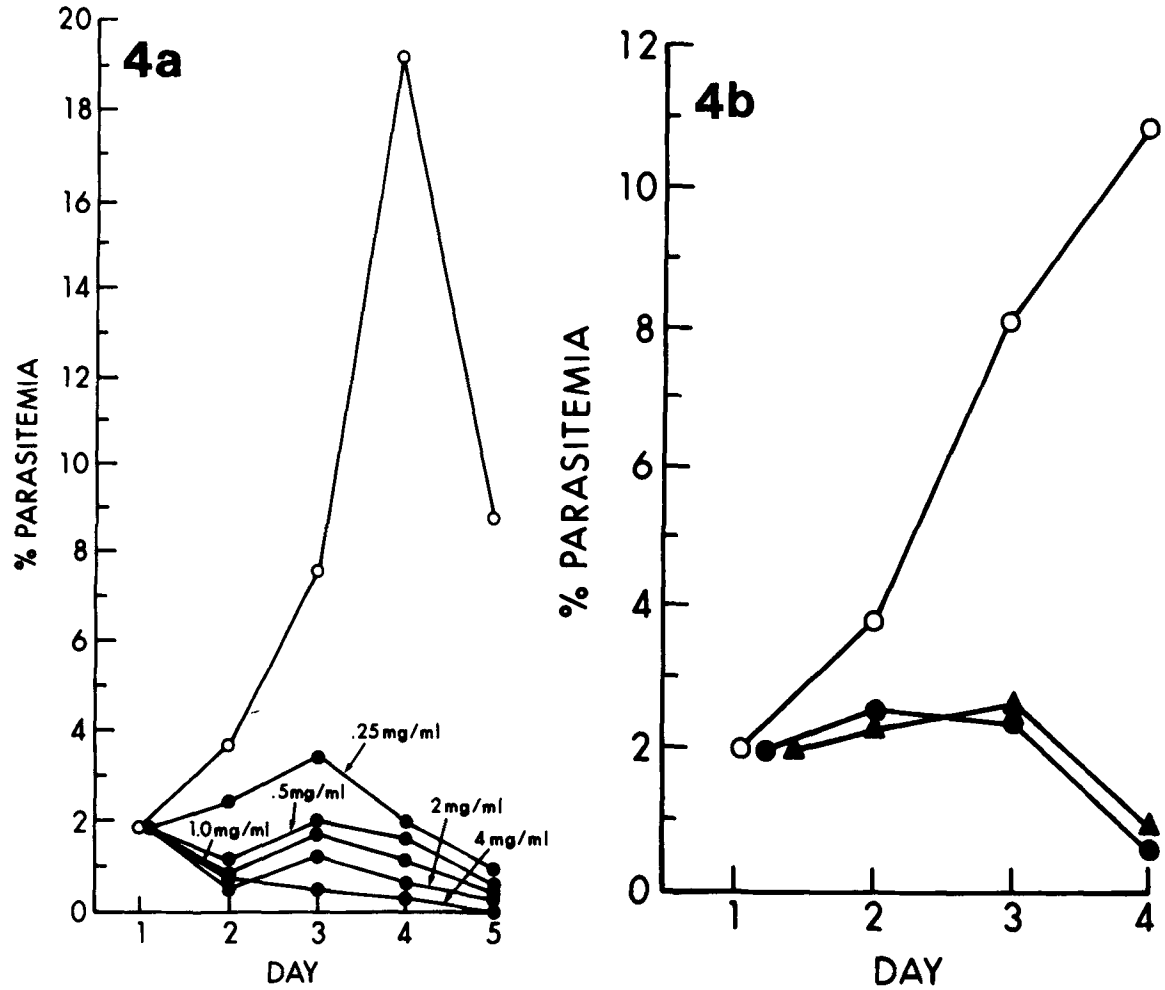

FIG. 4a and 4b: 4a shows effect on parasitemia of the addition of ferritin at final concentrations of 0.25 to 4.0 mg/dl. (○ = control, • = ferritin) 4b depicts the differing effects of adding ferritin or apoferritin at a concentration of 0.6 mg/dl on parasitemia. (○ = control, • = ferritin, ▲ = apoferritin).

parasitized cells was 0.32 ± 03 grains/cell (N = 500, p = 0.002). Since most of the grains in the non-parasitized cells were located on red cells adjacent to parasitized cells, it is surmised that these represent stray isotopic rather than true transferrin binding and/or internalization.

DISCUSSION

We have demonstrated that *P. falciparum* infected red cells are capable of endocytosis. The evidence derives from the morphologic finding of increased ferritin in infected cells treated with ferritin and growth inhibition in similarly treated cells. It is likely that the endocytosis of large molecules is a generalized process and is not limited to iron binding proteins inasmuch as the parasite will not grow when supporting serum is deprived of its nondialyzable components (12) and these cannot be replaced by the simple addition of transferrin (Pollack S:Unpublished observations).

The means by which the parasite reprograms the mature red cell to resume endocytosis, a function present in the reticulocyte but lost with maturation, warrants consideration. Several hypotheses have been suggested to explain the poorly understood mechanics of endocytosis. One is that intercalation of molecules into the membrane may cause it to bend inward. Another suggests that contractile proteins may pull the membrane inward (2). It has also been suggested that spectrin, the scaffold like protein which lies beneath the red cell membrane is in opposition to these forces and stops endocytosis in the mature red cell (22). Malaria rearranges the lipids of the red cell membrane, intercalates proteins into the red cell cytosol lying beneath the membrane (10), and can destroy spectrin (24). These may work individually or in concert to allow endocytosis. Nevertheless, not every parasite directed reprogramming event is involved in or required for endocytosis. The generation of knobs is one such example as evidenced by the fact that the strain used in these studies lacks knobs.

A tangential observation of particular note was the finding that ferritin bound to the red cell membrane of non-parasitized cells in infected cultures. This observation is all the more intriguing inasmuch as the autoradiographic studies suggested that labeled transferrin was selectively bound to the parasitized cells exclusively. It is possible that the parasite elaborates some soluble product which coats all of the surrounding red cells. If so, then this substance appears to have a selective affinity for specific proteins and may be responsible for the inherent stickiness of *P. falciparum* infected red cells.

It must also be noted that the present experiments do not exclude the possibility that non-parasitized red cells in the infected culture not only bind feritin but also engage in endocytosis. In the analysis of thin section electron micrographs it is not possible to conclude with certainty that an individual cell is not parasitized since the plane of section might not pass through the parasite. However, it can be said that adsorption of ferritin per se probably does not trigger endocytosis. Previous work using concanavalin A labelled with ferritin demonstrated that this adsorbed complex was not endocytosed by mature red cells (25).

REFERENCES

1. Aikawa, M., Uni, Y., Andrutis, A. T., Howard, R. J. Membrane associated electron-dense material of the asexual stages of plasmodium falciparum:evidence for the movement from the intracellular parasite to the erythrocyte membrane. Am J Trop Med Hyg. 35:30-36; 1986.
2. Anderson, R. G. W., Kaplan, J. Receptor mediated endocytosis. Modern Cell Biol. 1:1-52;1983.
3. Burns, E. R., Zucker-Franklin, D. Pathologic effects of plasma from patients with thrombocytopenic purpura and cultured vasular endothelial cells. Blood 60:1030-1037;1982.
4. Divo, A. A., Geary, T. G., Davis, N. L. et al. Nutritional requirements of plasmodium falciparum in culture I. Exogenously supplied dialyzable components necessary for continuous growth. J. Protozool. 32:59-64; 1980.
5. Elford, B. C., Haynes, J. D., Chulay, J. D., et al. Selective stage-specific changes in the permeability to small hydrophillic solutes of human erythrocytes infected with Plasmodium Falciparum. Molec. Bioch Parasitol. 16:43-60; 1985.
6. Fristch, G., Sawatski, G., Treumer, J., et al. Plasmodium falciparum inhibition in vitro with lactoferrin, desferriferrithiocin and desferricrocin. Experimental Parasitol. 63:1-9; 1987.
7. Ginsburg, H., Kutner, S., Krugliak, M., et al. Characterization of permeation pathways appearing in the host membrane of plasmodium falciparum infected red cells. Molec Biochem Parasitol. 14:313-322; 1985.
8. Jacobs, A., Worwood,. Iron in Biochemistry and Medicine. London:Academic Press; 1980.
9. Jensen, J. B., Trager, W. Plasmodium falciparum in culture. Use of outdated erythrocytes and description of the candle jar method. J. Parasitol. 63:883-886; 1977.
10. Kilejian, A. The biosynthesis of the knob protein and 65 000 dalton histidine rich polypeptide of plasmodium falciparum. Mol. Biochem. Parasitol. 12:185-194;1984.
11. Langreth, S. G., Jemsen, S. B., Reese, R. T., et al. Fine structure of human malaria in vitro. J. Protozool. 25:443-452;1978.
12. Nivet, C., Pereira, D. S. Serum factors involved in the development of plasmodium falciparum in red blood cells in vitro. In: Israel, L., Lagrenge, P., Salamon, J. C., eds. Les Colloques de l'INSERM 97:329-339; 1980.
13. Pollack, S., Fleming, J. P. falciparum takes up iron from transferrin. Br. J. Haematol. 58:289-293; 1984.
14. Pollack, S., Schnelle, V. Inability to detect transferrin receptors on P. falciparum parasitized red cells. Br. J. Haematol. 68:125-129; 1988.
15. Reber-Liske, R. A labour saving method for the in vitro culture of plasmodium falciparum. Acta Tropica 40:39-43;1983.
16. Rodriguez, M. H., Jungery, M. H. A protein on Plasmodium falciparum infected erythrocytes functions as a transferrin receptor. Nature 324:388-391; 1986.
17. Rudzinska, M., Trager, W. The fine structure of trophozoites and gametocytes in Plasmodium Coatneyi. J. Protozool. 15:73-88; 1968.

18. Schnelle, V., Pollack, S. Plasmodium falciparum:improved method for continuous in vitro cultivation without daily medium replacement. Ann Trop Med Parasitol. 81:63-64;1987.

19. Schwartz, R. S., Olson, J. A., Raventos-Suarez, et al. Altered membrane phospholipid organization in Plasmodium falciparum infected human erythrocytes. Blood 69:401-407; 1987.

20. Silverstein, S. C., Steinman, R. M., Cohn, Z. A. Endocytosis. Ann Rev Biochem. 46:669-722;1977.

21. Trager, W., Jensen, J. B. Human malaria parasites in continuous culture. Science 193:673-675;1976.

22. Tokuyasu, K. Y., Schekman, R., Singer, S. J. Domains of receptor mobility and endocytosis in the membranes of neonatal human erythrocytes are deficient in spectrin. J. Cell Biol. 80:481-486;1979.

23. Treffry, A.; Hamson, P. M. The binding of ferric iron to ferritin. Biochem J. 181:709-716;1979.

24. Yonguth, Y., Prapon, W., et al. Alteration in membrane proteins of mouse erythrocytes infected with different species and strains of malaria parasites. Comp. Biochem, Physiol. 63B:83-85; 1979.

25. Zweig, S., Singer, S. J. Concanavalin A induced endocytosis in rabbit reticulocytes and its decrease with reticulocyte maturation. J. Cell Biol 80:487-491, 1979.

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