[Symposium: Cell electrophoresis—Present and future—]

Alterations of uninfected red blood cell membranes during
in vitro and *in vivo* parasite growth

Domagoj Sabolović* and Nobuya Hashimoto**

INTRODUCTION

Malaria remains one of the world-wide problem. According to WHO, 400 million of people suffer from the malaria disease resulting in over 2 millions annual death. The same problem face *Babesia*-infected animals, human malaria equivalent animal diseases.

Anemia is a major problem in both diseases caused by parasites-infected RBC.¹⁾ However there is disproportion between a small number of infected RBC and RBC destroyed during disease. Hosts versus parasite immunological reactions have been intensively studied and auto immune reactions suggested as a plausible explanation for anemia.²⁾

However only few experimental data considered a parasite versus host activity as an another possibility of host-parasite interactions.

In the present study, using cultures of *Plasmodium falciparum* parasitized RBC and unparasitized RBC separated by Millipore membrane, we have shown that, according to the intraerythrocytic developmental stage of parasite, parasite secrete factor(s) which modifies biophysical properties of RBC membranes of uninfected RBC. Therefore we extended our study to *in vivo* experiments using *Babesia canis* infected dogs. Our experimental results reaffirm the existence of parasite-mediated phenomena in both situations and suggest

the entirely new approach in the mechanism of host-parasite relationships.

MATERIALS AND METHODS

Cell cultures

Plasmodium falciparum infected human RBC, and/or *Babesia canis* infected dog red blood cells (DRBC) were added to the upper part of the Millicell membrane insert as shown on Fig. 1. The plates were incubated at 37°C in a candle jar for 54 h without medium changes. RBC samples were taken about every 6 h (in the case of *P. falciparum*) and about every hour (in the case of *Babesia canis*) cultures from uninfected compartments at the bottom of the wells. Control cultures were set on the same plates and in the same experimental conditions.

Animals

Beagle dogs were used for *in vivo* experiments. The animals were injected subcutaneously with *Babesia canis* infected RBC from cell culture.

Cell electrophoresis

The cell electrophoresis is made using laser Doppler spectrometer. Laser light from 5 mW He-Ne laser is scattered from erythrocytes moving in the electric field of a small electrophoretic chamber. The scattered light is frequency shifted according to the velocity of cells in the electric field. The electrophoretic mobility (*EM*) of individual parti-

*Domagoj Sabolović; INSERM Unit 313, Institut Sante et Developpement.

**橋本信也; Third Department of Internal Medicine, The Jikei University School of Medicine.

Correspondence address: Domagoj Sabolović, INSERM Unit 313, Institut Sante et Developpement, 15-21 rue de l'Ecole de Medecine, 75270 PARIS Cedex 06, France.

Symposium, the 42nd Spring Meeting of the Society of Electrophoresis.

Fig. 1. Millicell insert containing Milipore 0.22 μ membrane allows separation of parasitized RBC from uninfected RBC culture.

cles was calculated according to

$$EM = \frac{V_d}{E \frac{2n}{\lambda} \sin \frac{\theta}{2}}$$

cwhere V_d is the Doppler shift, E =electric field of 60 V; θ =scattering angle 15° ; λ =laser wavelength and n =index of refraction of the Medium. The output signal is processed by a spectrum analyzer using Fourier transformation method and presented in the form of histogram.³⁾ The mean EM is calculated from the half-width of histogram. About 10^4 cells were scored in about 30 min measuring period.

The results are expressed as percentage decrease of electrophoretic mobility of uninfected RBC versus RBC kept under the same experimental conditions and without infected RBC in the upper part of Millicell insert.

Linoleic acid lysis test

Linoleic acid (Sigma) was diluted in ethanol ant kept at -80°C . RBC were diluted with saline and incubated with the increasing concentrations of linoleic acid at 37 for 30 min. After centrifugation, the supermatant optical densities (D. O.) were measured at 543 nm in spectrometer.

RESULTS

Electrophoretic mobility test

In vitro results: The electrophoretic mobility of uninfected RBC collected from *Plasmodium falciparum* (Fig. 2A) or from *Babesia canis* infected RBC (Fig. 3A) cultures were determined during culture period that lasted 56 h for *Plasmodium falciparum* and 8 h for *Babesia canis* culture.

Fig. 2. A and B Show time-dependent decrease of EM of uninfected human RBC (at the bottom part of the well) correlated with developmental *P. falciparum* stages in the upper part of the well.

Fig. 3. A and B idem for *Babesia canis* and dog RBC.

This time-period corresponds to the one developmental intraerythrocytic parasite cycle. For *Plasmodium falciparum* in the following orders : ring stage, trophozoite stage, schizont 2 nuclei and schizont 4 nuclei developmental stages (Fig. 2B). For *Babesia canis* the cell cycle stages were: 1 pear,

Fig. 4. Decrease of *EM* of uninfected dog RBC at bottom part of the well plotted against percentage *Babesia canis* infected RBC in the upper part of the well during 14 h of culture.

2 pears, 4 pears, 8 pears and 16 pears (Fig. 3B). These stages were revealed and quantified under microscope on Gimsa stained slides of RBC collected from the upper part of Millipore membrane inserts.

The results show that decrease of *EM* of uninfected RBC was maximal at 36 h for *Plasmodium falciparum* culture and at 3.5 h for *Babesia canis* cultures. The percentages of decrease of *EM* were almost the same, ranging among 25 to 35% in five consecutive experiments. Figures 2B and 3B depict developmental stages of parasites. The maximal *EM* changes were found correlated to the highest percentages of *Plasmodium falciparum* found in a schizont 4 nucleus and *Babesia canis* in 4 pairs developmental stages.

The results of separated experiments, depicted on Fig. 4 show the whole life cycle of *Babesia canis* infected RBC cultures (upper compartment) during 14 h's observation period. The reduction of *EM* of uninfected RBC (lower compartment) peaked at 3 h of culture and disappeared 5 h later. The percentage of infected RBC increases progressively to the maximum on 6 h of culture and declined thereafter.

In vivo results: The electrophoretic mobility of RBC collected from experimentally *Babesia canis* infected dogs is shown on Fig. 5. Each curve represents individual dog and decreases of *EM* as

Fig. 5. Individual *EM* of experimentally infected dog's RBC and appearance of *Babesia canis* infected RBC on the peripheral blood smears.

compared to the healthy controls. All *Babesia canis* infected animal show a gradual decrease of *EM* reaching maximum on day 4 after infection. The observed modifications disappeared progressively as peripheral blood parasitaemia appeared on blood smears on day 5 and peaked on day 6.

Linoleic acid test

When fresh RBC were incubated with increasing concentration of linoleic acid they undergo a dose dependent lysis. When uninfected RBC from *Plasmodium falciparum* infected cultures were incubated with a single dose of linoleic acid they show the increased resistance to lysis (up to 5 fold) which peaked at 36 h of culture and disappears thereafter and RBC become rather more sensitive (Fig. 6). This resistance could be passively transferred to the fresh RBC following incubation of fresh RBC with supernatant collected at 36 h of infected culture as shown on Fig. 7.

DISCUSSION AND CONCLUSION

The *in vitro* experiments demonstrate a profound alteration of RBC membranes of uninfected RBC as measured by cell electrophoresis and by sensitivity of RBC to linoleic acid lysis. Both techniques clearly suggest that malaria parasite secretes some substance that reduces the net negative charge of uninfected RBC by 30% and at the

Fig. 6. Time dependent sensitivity and resistance to a single dose ($0.2 \mu\text{M}$) of linoleic acid of uninfected RBC (at the bottom of the well) tested on different hours of *Plasmodium falciparum* culture and compared to control RBC cultured in the same experimental conditions.

RBC lysis was expressed as optical densities' differences between experimental and control RBC supernatants.

same time increases resistance to LA lysis by 5 fold. The timing of a putative factor secretion was found to correlate to the specific developmental stages of parasites, i.e., 4 pairs or *Babesia canis* and schizont 4 nucleus for *Plasmodium falciparum*.

The maximum factor activities were situated before parasitized-erythrocyte rupture and spreading of newly formed parasites to other RBC occur. LA resistance and the reduction of the net negative charges (not shown) could be transferred to the other related RBC or non-related RBC by using culture supernatant at its maximum activity. This indicates a factor's non specific nature. LA lysis resistance should be considered in the lights of frequently described membrane's lipid composition changes during parasitic infections.^{4,5} Decrease in deformability and increase in rigidity of RBC in malaria was responsible for severe hemolysis, endothelial cell damage, cerebral dysfunction and anemia.^{6,7} Recently it was described that uninfected RBC may contain up to 40% of linoleic acid⁸) in their membranes making them fragile target to free radical peroxidation. What is the exact relationship between parasite-secreted factor, membrane al-

Fig. 7. Experimentally transmitted linoleic acid lysis resistance to fresh RBC using supernatant (PTX) from 36 h *Plasmodium falciparum* infected culture.

terations and linoleic acid lysis resistance, remains to be determined.

The *in vivo* experiments using *Babesia canis*, malaria equivalent animal disease show that 3 days after experimental infection of dogs there was uniform ± 30 percentage reductions of RBC negative charges as detected by cell electrophoresis. These alterations undoubtedly concern uninfected RBC because they preceded at least 24 h peripheral RBC parasitaemia. These alterations disappeared when peripheral parasitaemia reaches its maximal value. *Babesia canis* infected dogs undergo severe anemia on the following days and if untreated die 8 to 10 days later.

Thus, our *in vivo* results correlate well with *in vitro* observations. They are the first experimental evidence of uninfected RBC membranes' alterations induced by developing parasite. These alterations were produced before peripheral spreading of parasite and without any host-mediated immunological reaction. We postulate that these modifications are due to parasite secreted factor as detected in *Babesia canis* and *Plasmodium falciparum* cultures. The results suggest also that parasite-secreted factor is short lived and non-specific and indicate that other biological membranes excepting RBC could be affected as well. He might be implicated in the impaired RBC function leading to anemia during malaria infections.

REFERENCES

- 1) Weathrall DJ, Abdalla S, Pippard MJ. The anemia in *Plasmodium falciparum* malaria. In: Evered D, Whelan J, editors. Malaria and the red cell. Ciba Foundation Symposium 94. London: Pitman, 1983: 74-97.
- 2) Wright IG, Goodger BV, Clark IA. Immunopathophysiology of *Babesia bovis* and *Plasmodium falciparum* infections. Parasitology Today 1988; 4: 214-8.
- 3) Kaufmann R, Steiner R, Hoffmann W. Lacypher—a new laser cytopherometer for measuring the electrophoretic mobility of particles and cells. In: Preece AW, Sabolović D, editors. Cell electrophoresis: clinical applications and methodology. Elsevier/North-Holland Biomedical Press, 1979: 435-44.
- 4) Moll, GN, Vialh J, Bevers EM, Ancelin ML, Roelofsen B, Comfurius P, Slotboom AJ, Zwaal RF, Opden kamp JA, Van Deenen LL. Phospholipid asymetry in the plasma membrane of malaria infected erythrocytes. Biochem Cell Biol 1990; 68: 579-85.
- 5) Othmane A, Bitbol M, Snabre P, Mills P. Influence of altered phospholipid composition of the membrane outer layer on red cell aggregation: Relation to shape changes and glyco-calyx structure. Eur Biophys J 1990; 18: 93-9.
- 6) Macpherson GG, Warrell MJ, White NJ, Warrell S, Looareesuwan DA. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol 1985; 119: 385-401.
- 7) Aikawa M, Miller LH. Structural alterations of the erythrocyte membrane during malaria parasite invasion and intraerythrocytic development. In: Evered D, Whelan J, editors. Malaria and the red cell. Ciba Foundation Symposium 94, London: Pitman, 1983: 45-63.
- 8) Beaumelle B, Vial HJ. Uninfected red cells from malaria-infected blood: Alterations of ty acid composition involving a serum protein: fatan *in vitro* and *in vivo* study. In Vitro Cell Dev Biol 1988; 24: 711-8.