Journal of Structural Biology 162 (2008) 460–467

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

The avian malaria parasite *Plasmodium gallinaceum* causes marked structural changes on the surface of its host erythrocyte

Eriko Nagao [a,1], Takayuki Arie [a,*,1], David W. Dorward [b], Rick M. Fairhurst [a], James A. Dvorak [a,2]

[a] *Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[b] *Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA*

ARTICLE INFO

Article history:
Received 13 January 2008
Received in revised form 11 March 2008
Accepted 11 March 2008
Available online 21 March 2008

Keywords:
Plasmodium gallinaceum
Avian malaria
Erythrocyte
Surface topography
Atomic force microscopy

ABSTRACT

Using a combination of atomic force, scanning and transmission electron microscopy, we found that avian erythrocytes infected with the avian malaria parasite *Plasmodium gallinaceum* develop ~60 nm wide and ~430 nm long furrow-like structures on the surface. Furrows begin to appear during the early trophozoite stage of the parasite's development. They remain constant in size and density during the course of parasite maturation and are uniformly distributed in random orientations over the erythrocyte surface. In addition, the density of furrows is directly proportional to the number of parasites contained within the erythrocyte. These findings suggest that parasite-induced intraerythrocytic processes are involved in modifying the surface of host erythrocytes. These processes may be analogous to those of the human malaria parasite *P. falciparum*, which induces knob-like protrusions that mediate the pathogenic adherence of parasitized erythrocytes to microvessels. Although *P. gallinaceum*-infected erythrocytes do not seem to adhere to microvessels in the host chicken, the furrows might be involved in the pathogenesis of *P. gallinaceum* infections by some other mechanism involving host-pathogen interactions.

Published by Elsevier Inc.

1. Introduction

Historically, from the 1890s to the 1940s, avian malarias were commonly used as models for human malaria physiology and chemotherapy (e.g., see Slater, 2005). Although rodent malarias replaced avian malarias as the most productive model following World War II (e.g., see Aviado, 1969), the relative ease of maintenance of *Plasmodium gallinaceum*, an avian malaria parasite, has resulted in its continued use in basic malaria research. Its abilities to infect a variety of avian hosts and produce high parasitemias are important factors in its ongoing use in a wide range of cell biological and biochemical studies. Although *P. gallinaceum*-infected erythrocytes have been widely used as a research tool, very little is known about their surface structural characteristics.

While ultrathin sections and freeze-fracture replicas were used in the 1960s and 1970s to visualize by transmission electron microscopy (TEM) the fine structure of *P. gallinaceum*-infected erythrocytes (Ristic and Kreier, 1964; Seed et al., 1971; Lushbaugh et al., 1976), the structural details of infected erythrocyte surfaces were not reported. In a TEM study, Mackenstedt et al. (1989) described morphological alterations in infected erythrocytes induced by various parasite species including *P. gallinaceum*. In parasite-infected mammalian erythrocytes they described knobs, invaginations, and caveola-vesicle complexes on the erythrocyte surface, and clefts, microvesicles, and small vesicles in the erythrocyte cytoplasm. However, they did not describe or discuss changes in the surface of *P. gallinaceum*-infected erythrocytes although erythrocyte surface alterations appeared to be present (*c.f.* Fig. 18). They concluded that "*P. gallinaceum* induced only slight alterations in host cells; however, invaginations on the surface of the erythrocyte were not induced."

In contrast to *P. gallinaceum*, the human malaria parasite *P. falciparum* has been studied extensively using biochemical and biophysical methods including TEM, scanning electron microscopy (SEM) and atomic force microscopy (AFM) (Binnig et al., 1986). The mature stages of *P. falciparum* parasites may induce the formation of knob-like protrusions on the surface of their host erythrocyte (Trager et al., 1966). Knobs play a crucial role in the pathogenesis of malaria by mediating the sequestration of parasitized erythrocytes in the microvessels of the brain and other critical organs such as the kidney, lung, and liver, which may contribute to host death (MacPherson et al., 1985; Aikawa et al., 1990; Sherman et al., 2003). While *P. gallinaceum* is reported to be evolutionarily closely related to *P. falciparum* (Waters et al., 1991,1993a,b; McCutchan et al., 1996), detailed structural–biological comparisons between these mammalian and avian *Plasmodium* species

* Corresponding author. Fax: +1 301 480 1438.
E-mail address: TArie@niaid.nih.gov (T. Arie).
[1] These authors contributed equally to this work.
[2] Dedicated to the memory of the author, James A. Dvorak, who passed away in February, 2007.

1047-8477/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.jsb.2008.03.005

have not been described. In this report, we present the first study of the surface topography of *P. gallinaceum*-infected erythrocytes using a combination of SEM, AFM and TEM.

2. Materials and methods

2.1. Parasite cultivation

The 8A strain of *P. gallinaceum* was used for this study. The *P. falciparum* ITO/A4 clone (Roberts et al., 1992) was cultured in complete medium as described previously (Nagao et al., 2000) with the exception that 10% human serum (Type O+) was used in place of AlbuMAX I. Cultures were initiated with a mixture of freshly thawed, cryopreserved parasites and fresh erythrocytes at a 2% hematocrit in T75 culture flasks (Corning, Cambridge, MA) and incubated at 37 °C.

2.2. AFM sample preparation and imaging

Plasmodium gallinaceum-infected erythrocytes were diluted to 1×10^7 cells/ml with complete medium, and 200 μl portions were plated onto 22-mm diameter coverglasses (Becton–Dickinson Labware, Lincoln Park, NJ), previously coated with silane or poly-L-lysine (Sigma, St. Louis, MO). The erythrocytes were allowed to settle and attach to the surface of the coated coverglasses for 30 min at 37 °C, after which the culture medium was carefully removed. The erythrocytes were fixed with osmium tetroxide vapor for 2 min at room temperature, washed with 0.1 M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Sciences, Fort Washington, PA), post-fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer for 1 h at room temperature, washed and stored in 0.1 M sodium cacodylate buffer at 4 °C until processed for AFM imaging.

For AFM studies, erythrocytes were further fixed with methanol, incubated with 1 U/μl DNase I, (RNase-free, Roche Molecular Biochemicals, Germany) in 50 mM Tris–HCl buffer (pH 7.5) containing 14 mM NaCl and 10 mM $MgCl_2$ for 2 h at 37 °C in a moist chamber, and subsequently stained with 2.5 μM YOYO-1 (Molecular Probes, Inc, Eugene, OR) in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The cells were washed with deionized (DI) water, serially dehydrated for 5 min each in ethanol/water mixtures (30–90%) and two changes of absolute ethanol, hexamethyldisilazane (HMDS) (Electron Microscopy Sciences) for at least 10 min, and then air-dried. In parallel light microscopy (LM) experiments, infected erythrocytes were smeared on 1″ × 3″ microscope slides, dried, fixed with methanol and stained with Giemsa.

Prior to AFM scanning, bright field (BF) and YOYO-1 fluorescence (FL) images of infected erythrocytes were collected with a Zeiss Plan APOCHROMAT 20 × /0.75 objective (Carl Zeiss, Thornwood, NY), a 4× projector and a chilled CCD video camera (Model C5985, Hamamatsu Photonic Systems, Bridgewater, NJ). All images were passed into a DT3155 video frame grabber (Data Translation, Marlboro, MA), and captured with Image-Pro Plus version 4 or 5.1 (Media Cybernetics, Silver Spring, MD).

We used a Bioscope AFM (Veeco Instruments, Santa Barbara, CA) mounted on a Zeiss Axiovert (Carl Zeiss) inverted LM and a Nano-Scope™ III controller (Veeco Instruments) as described previously (Nagao and Dvorak, 1998). A modified Bioscope AFM equipped with an nPoint X–Y stage (nPoint, Inc, Madison, WI) was used to collect some of the images. Silicon probes (Model TESP, Veeco Instruments) on single beam cantilevers (125 μm in length and 30–40 μm in width) with a nominal spring constant of 20–80 N/m and tuned to a resonant frequency of 250–320 kHz were used to image the cells. Topographic and amplitude (error signal) images were collected simultaneously at a tapping force (A_{sp}/A_0) of 0.8 (Magonov et al., 1997; Nagao and Dvorak, 1999) and scan rate of 0.5 Hz as 512 × 512 data arrays in the trace direction using tapping mode in air.

2.3. SEM and TEM sample preparation and imaging

Erythrocytes were plated onto 6 mm coverglasses and fixed as described for AFM. For SEM imaging, the cells were washed twice for 15 min each in 0.1 M sodium cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h, washed once in cacodylate buffer, twice in DI water and dehydrated in a graded series of ethanol (30–100%). The cells were dried through liquid carbon dioxide in a model CPD-30 critical point dryer (Bal-Tec, Balzers, Liechtenstein), lightly coated with chromium, and visualized in a Hitachi S5200 field emission scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA).

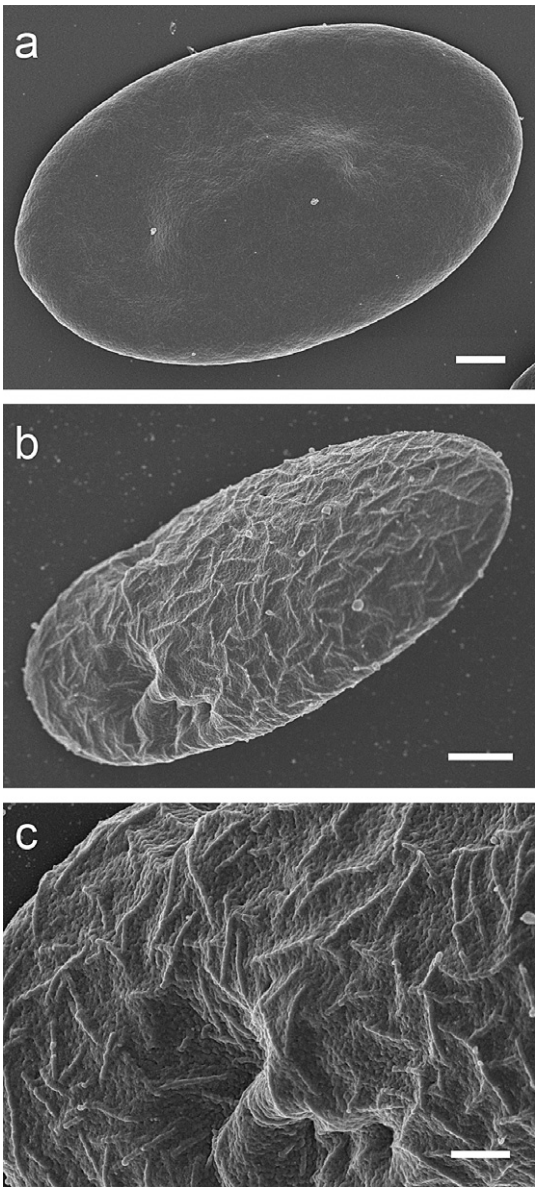

Fig. 1. Typical SEM images contrasting the surface topography of noninfected (a) and *P. gallinaceum*-infected erythrocytes (b, c). Noninfected erythrocytes have a smooth surface. In contrast, the furrow-like surface structures are seen on infected erythrocytes. Bars in (a) and (b) represent 1 μm, in (c) 200 nm.