Exoerythrocytic development of *Plasmodium gallinaceum* in primary fibroblast culture of chicken embryo

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Abstract

In this study we assessed the susceptibility of primary fibroblast culture of chicken embryo to infection of *P. gallinaceum* sporozoites as well as the initial development of exoerithrocytic stages. Fibroblasts were obtained from the chest muscles of chicken embryos and sporozoites were obtained from experimentally infected *Aedes fluviatilis* salivary glands. After 1h, 3h, 24h, 48h and 72h periods pos-infection, cell cultures were fixed and analyzed both by indirect immunofluorescent-antibody test with anti-circumsporozoite protein monoclonal antibodies and by transmission electron microscopy. Circumsporozoite protein was detected in all parasitic forms. The mean percentage of fibroblasts with adhered or penetrated sporozoites did not significantly increase proportionately to the concentration of parasites in the inoculum, and independently if fetal calf or normal chicken sera were used in the culture medium. It was noted that the longer the incubation time, higher the possibility of the sporozoites to adhere and penetrate to fibroblats. Spozoites were observed penetrating in the fibroblast after 3h incubation when 0.68% of the cells had adhered parasites. Differentiation and development of the exoerythrocytic forms was observed after 24h incubation, when an average of 0,14% of the parasites have already invaded the cells. Developing parasites were found until 72h, when only 0.04% of fibroblasts were infected. Fibroblast cell culture seems to be a valuable experimental tool for in vitro investigation of the exoerytrocytic cycle of *P. gallinaceum.*

Key words:
Avian malaria.
Exoerythrocytic cycle.
Fibroblast.
In vitro culture.

Introduction

The *in vitro* culture of exoerythrocytic forms (EEF) of malaria parasites was initially obtained in avian plasmodia, from primary cultures of bird tissues experimentally infected with blood forms.[1] Differently of mammalian species, EEF of avian plasmodia can be generated from both sporozoites and merozoites originated from both exoerythrocytic and erythrocytic cycles.

The capability of the EEF to propagate for long periods *in vitro* was demonstrated by the continuous culture of *P. gallinaceum* by Meyer and Mussacchio[2] and Oliveira and Meyer[3]**.** These authors initiated cultures by placing fragments of brain, spleen or liver from healthy chicken embryos in contact with infected fragments, whereby contamination of the former occurred by the parasites produced by the latter. The culture was propagated for several months in these conditions. The same authors[3] obtained infection of erythrocytes *in vitro* by placing blood in contact with the above-

mentioned EE culture forms. When Meyer[4] tried the opposite path – *i.e.* infecting fragments of healthy embryo tissue by placing them in contact with infected blood cells – only rare cells of the tissues became infected, despite the high parasitemia in the inoculum.

Many attempts to obtain *in vitro* infection of cell cultures directly with sporozoites were unsuccessful. Rodhain, Gavrilov and Cowez[5] were not able to obtain EEF from sporozoites of *P. gallinaceum* inoculated in culture cells of spleen, bone marrow and heart of chicken embryos. Later, numerous attempts were made with a view to standardizing the culture of the EEF of avian plasmodia.[5,6,7] The importance of the cell type for transformation of the sporozoite of the *P. gallinaceum* in EE forms and its latter multiplication was emphasized by Rocha et al.[7]. Their *in vitro* studies using cull lines (HEPG2-A16, VERO -monkey kidney epithelial cells, and SL-29 - chicken embryo fibroblast) have shown that the sporozoites invaded all three cells types but their development into exoerythrocytic forms occurred only for 48h in the SL-29 cells. Similarly, several other studies have demonstrated that the type of host cell is important for the development of the EE forms of human malaria parasites *in vitro*.[8,9,10] These studies suggest that the development of the parasite seems to depend more on the metabolism of each type of host cell than on specific receptors on it surface. Thus, while it is possible to grow EE stages of the human parasite *P.vivax* in human hepatoma cell line Hep-G2[11], *P. falciparum* will only grow in primary cultures of human hepatocytes[12].

Detailed studies on the relationship between human parasites and host cell in the EE cycle were made possible only with the emergence of techniques that allow for maintaining hepatocytes in cultures and cultivation of EEF of human and rodent species of plasmodium.[10] It was demonstrated that the initial event in the interaction between sporozoite and hepatocyte is the recognition of the CS protein by proteoglycans present on the membrane of the hepatocyte.[13,14] The ligand for the receptor in the hepatocyte is located in region II of the CS protein, which is highly preserved among all species of parasites of mammalian malaria.[13] In this article, we attempted to obtain the culture of exoerythrocytic forms of *Plasmodium gallinaceum* and study the interaction of sporozoites with primary culture of chicken fibroblasts.

Material and Method

Primary culture and subculture of fibroblasts of chicken embryos

The primary culture of fibroblasts was obtained by dissection of the pectoral skeletal muscle of 11-14 days old chicken embryos. Eggs were opened cleaned with 96% alcohol and exposed for 15 min to UV and then opened in a laminar flow chamber where the embryo was transferred to PBS Ph 7.4. The pectoral skeletal muscles were cut into small pieces and subsequently digested by trypsin (2.5%) and EDTA (5%) in Dulbeco's Modified Eagle medium (DMEM) (Sigma, St Louis, MO, USA) without Ca^{++} and Mg^{++}, for 15 min at 37^0C. To stop digestion, DMEM with 10% fetal calf serum (FCS) was added, following 3 centrifugations (2,000rpm/10 min). The pellet was seeded in cell culture surface polystyrene bottles (Corning®, 25cm^3; 2x10^6 cells/bottle) previously coated inside with 0.01% gelatin (Sigma) and incubated at 37^0C in atmosphere with 5% CO_2 and 95% 0_2. After 2h, cultures were rinsed with PBS pH 7.4 to take away non adhered cells and a new DMEM+10% FCS was added. The subcultures were carried out for expansion and purification of the cells on day 2 or 3 using the same procedure. For transmission microscopy electron (TEM), the fibroblasts were plated on permanox slides (Lab-Tek® Chamber Slides Products, Miles Labs, Elkhart, IN, USA). For the indirect immunofluorescent-antibody test (IFA) fibroblast were plated on glass coverslips. (Lab –Tek®). The monolayer of fibroblasts

was ready to be inoculated after 48 h of incubation at 37°C.

Inoculation of the fibroblast cultures

Sporozoites of *P. gallinaceum* (strain 8A) were obtained from infected *Aedes fluviatilis* Lutz, females previously fed on 3 to 4-day old chicks with parasitemia between 1 and 10 %. After the infective blood meal, mosquitoes were kept at - 27 -$^{+}1^{0}$C and 70 – 98 % of relative humidity, provided daily with a sacarose-saturated solution. The maintenance of the mosquito colony and the infection of the mosquitoes were performed according to Consoli and Lourenço-de-Oliveira[15] and Camargo et al.[16], respectively. On the 14th day after feeding on the infected chicks, mosquitoes were immobilized on ice bath for 10 minutes and transferred to sterile processing cassette (Fisher Scientific, Pittsburg, PA, USA), where they were rinsed five times in disinfecting solutions in the following sequence: sodium hypochlorite 5%, alcohol 90%, PBS pH 7,2, DMEM containing fungizone (0,4 mL/100mL), gentamicin (250 μL/100mL), penicillin (140μL/100mL), streptomycin (140 μL/100mL) and garamycin (20 drops/100mL), and DMEM with the four above-mentioned antibiotics in the same concentrations, but fungizone. Sporozoites were obtained by centrifugation of mosquitoes in a glass–wool column[17]. Whereby head+thorax of the infected mosquitoes were centrifuged three times (5000rpm/5min) in 50μl of DMEM with 10% FCS or normal chicken serum (NCS). Sporozoites were counted in a Neubauer chamber, with 1:10 dilution, in phase contrast microscopy (MCF).

Before inoculating the cell cultures, the supernatant medium was removed and the sporozoites suspension added over the monolayer of fibroblasts in the following ratios (sporozoites/fibroblasts): 1:1; 2:1, 3:1 and 4:1. Three hours latter, the supernatant was removed and the monolayer of fibroblasts rinsed with PBS containing antibiotics and a new DMEM was added and replaced one or twice a day. For each experiment (ratio sporozoite/fibroblast and time of incubation) 5 replicates and one control (no parasite) were done. Mean and standard deviation of number of infected cells in replicates of each experiment and incubation time were calculated.

Study of the parasite-fibroblast interaction, *in vitro*

Samples of cell cultures incubated for 3, 24, 48 and 72h were examined by IFA[6], using monoclonal antibodies against the circumsporozoite (CS) protein of *P. gallinaceum*[18]. Infected cultures were also fixed in glutaraldehyde (2,5%), diluted in a cacodylate sodium buffer 0,1M, pH 7.2, containing 3,5% sacarose during one hour, at 4° C, for analysis by TEM. After fixation, the infected cultures were rinsed 3 times with a cacodylate sodium buffer, in the same formulation as the above-mentioned, for 10 minutes each. Later, the material was post-fixed with 1% O_sO_4, diluted in a sodium cacodylate buffer 0,2M at a ratio of 1:1, during one hour at 4^{0} C. Subsequently, the culture samples were rinsed in the same buffer for 10 minutes and submitted to a series of baths with increasing concentrations of acetone or ethanol at 30%, 70% and 90% for 10 minutes each (we utilized acetone for the glass substrates and ethanol for the polyethylene substrates) and 3 baths with acetone or ethanol at 100% for 10 minutes each). Then, culture samples were kept in acetone 100% (for the glass slides) or ethanol 100% (for the polyethylene) and epoxy® resin (Epon) at the ratio of 1:1 for 12 hours, and polymerized in pure Epon, during 4 hours, at room temperature at 60^{0} C for 72 hours.

Results

In vitro* interaction between chicken embryo fibroblast and sporozoites of *Plasmodium gallinaceum

The mean percentage of fibroblasts with adhered or penetrated sporozoites did not significantly increase proportionately to the concentration of parasites in the

inoculum, and did not vary according to the use of different sera (FCS and NCS) in the culture medium (Table 1). The averages of adhered sporozoites per fibroblast 3 hours of incubation were similar when using FCS and NCS: 0.23 and 0.22, respectively. The highest percentage of fibroblasts with adhered parasites in the first hours of incubation was detected when we inoculated the ratio of 3 sporozoites/1 fibroblast. But during the following hours, both the percentage of infected fibroblasts and the mean number of parasites detected per slide were similar when concentrations of sporozoites:fibroblast in the inoculum were 1:1, 2:1 and 3;1. In a series of experiments when we used the ratio 1:1 sporozoite/ fibroblast (Table 2), the percentage of fibroblasts with adhered sporozoites 3 h after inoculation was 0.68% ± 0.26, while after 24, 48 and 72 h of incubation we detected respectively 0.14% ± 0.09, 0.05% ± 0.06 and 0.04% ± 0.09 fibroblasts were infected. It was ascertained that the development of the EE cycle was irregular among experiments carried out with identical procedures. For instance, in one of the experiment 1.14% of the inoculated sporozoites were adhered/penetrated, and 3.7% of the fibroblasts were parasitized in

Table 1 - Percentage of infected fibroblasts in cell culture inoculated with different concentration of sporozoites of *Plasmodium gallinaceum*. The mean number of parasites detected by indirect immunofluorescent-antibody test per inoculated cell culture is in parenthesis

Inoculated sporozoites/ fibroblast	Inoculation time			
	3h	24h	48h	72h
1:1	0.11(22.5)	0.03(6.5)	0.01(2.5)	0.02(4)
2:1	0.13(26.5)	0.02(3)	0.00(0.75)	0.01(2.9)
3:1	0.23(46)	0.04(7.8)	0.01(2.5)	0.01(2.5)
4:1	0.19(37.6)	0.04(7.1)	0.00(0.37)	0.01(1.3)

Table 2 - Percentage of fibroblast of chicken embryo with *Plasmodium gallinaceum* adhered or inside per experiment carried out the ratio of 1:1 sporozoite/fibroblast. The number of detected parasites is in parenthesis

Experiment	% of fibroblasts with parasites adhered or inside			
	3h	24h	48h	72h
1	0.65 (131)	0.17(34)	C	C
2	0.63(125)	0.17(34)	0.10(21)	C
3	1.01(201)	0.22(45)	0.14(29)	C
4	0.55(110)	0.13(27)	0.09(19)	0.09(19)
5	0.92(185)	0.25(50)	C	C
6	0.87(174)	ND	ND	0.28(57)
7	1.14(229)	0.24(48)	0.06(13)	C
8	0.66(133)	0.14(28)	0.01(2)	C
9	0.45(90)	0.01(2)	C	C
10	0.61(123)	C	C	C
11	0.33(67)	0.17(34)	C	C
12	0.3(60)	0.16(33)	0.16(32)	0.15(29)
Mean ± SD	0.68 ± 0.26	0.14 ± 0.09	0.05 ± 0.06	0.04 ± 0.09

C = Contamination; ND = Not done; Mean and standard deviation (SD) did not consider data from C and ND

3h of incubation.

Despite of the amount of attached sporozoites to fibroblasts in the first hours of incubation, only few parasites penetrated and developed. Three hours after incubation, approximately 0.36% of the inoculated sporozoites were adhered to the fibroblasts, and only 0.68% of the examined fibroblasts was about to be infected by the sporozoites or had parasite in the cytoplasm. There was a strong decrease in the mean percentage of plasmodia parasitizing fibroblasts after 3 hours of incubation. A day later, only 0.07% of parasites were still detectable in the fibroblasts, and in the following 48 and 72 h, the rates were 0.03% and 0.02%, respectively. In one experiment, a sample of the fibroblast cultures was incubated for only one hour with sporozoites, and then rinsed to remove the parasites not yet adhered to the host cell. We observed 16 adhered sporozoites and only 0.08% of the fibroblasts had parasites adhered, contrasting with the average of 0.68% of fibroblasts with parasites reported for 3h of incubation. This pointed to the fact that, in these conditions, the longer the incubation time, the higher the possibility of the sporozoites to adhere and penetrate the host cell in *vitro.*

Morphological aspects of the parasite culture process

The monoclonal antibody against the *P.gallinaceum* CS protein used in our experiments allowed to detect and follow the development of the parasite *in vitro* from adhered sporozoites to schizogony up to 72h (Figure 1). The sporozoites adhered to fibroblasts in 3 hour-incubation were in their peculiar elongated form (Figure 1). Subsequently, the detected parasites were rounded. These morphological changes followed the penetration of the host cell and precede schizogony. After 48 h (Figure 1), there was a considerable increase in the size of the parasites, which were round and located close to the nucleus of the fibroblasts. The cellular membrane of the parasites was delineated by IFA against the CS protein, still preserved and abundant at the beginning of schizogony. Indeed, all preparations revealed an uniform pattern of fluorescence surrounding the schizonts, which had a general rounded form, with regular and defined contours (Figure 1).

The plasmatic membrane of any part of the sporozoite was first seen adhered to that of the host cell (Figures 2a and 2b). Then, penetration was achieved when the anterior section of the parasite (conoid) attached to the fibroblast, with granular material being deposited on the host cell surface (Figures 2c and 2d). In the simple transversal section, besides the characteristic apical complex, rhoptries and micronemes, we observe several other organelles in the interior of the sporozoite, such as dense bodies, many granular structures and ribossomes surrounding the nucleus with a densely granulated nucleoplasm. Groups of microtubules were seen in the lateral section and cytostome found posteriorly. The cytoplasmatic matrix of the sporozoite contains few elements of endoplasm reticulum and numerous ribossomes. The nucleus of the parasite is spherical, surrounded by a double membrane, and located in the anterior third; mitochondria and numerous ribossomes were found close to the nucleus. Three hours after being inoculated in the cell cultures, the sporozoite still showed its elongated, semi-cylindrical shape, presented numerous and complex organelles and measured around 9μm length and 1.5μm diameter (Figure 2). After invasion, the sporozoites undergo a series of changes: 24 hours after incubation, young, rounded schizonts, measuring in average 2.6μm length and 1.81μm in diameter were detected in the host cells. The cytoplasm of infected fibroblasts contained a large number of vacuoles and structures with endoplasm reticulum and ribossomes close to and surrounding the schizont (Figure 3).

Discussion and Conclusions

In vivo, adhesion and penetration of sporozoites depend on the recognition of specific receptors on the target host cell

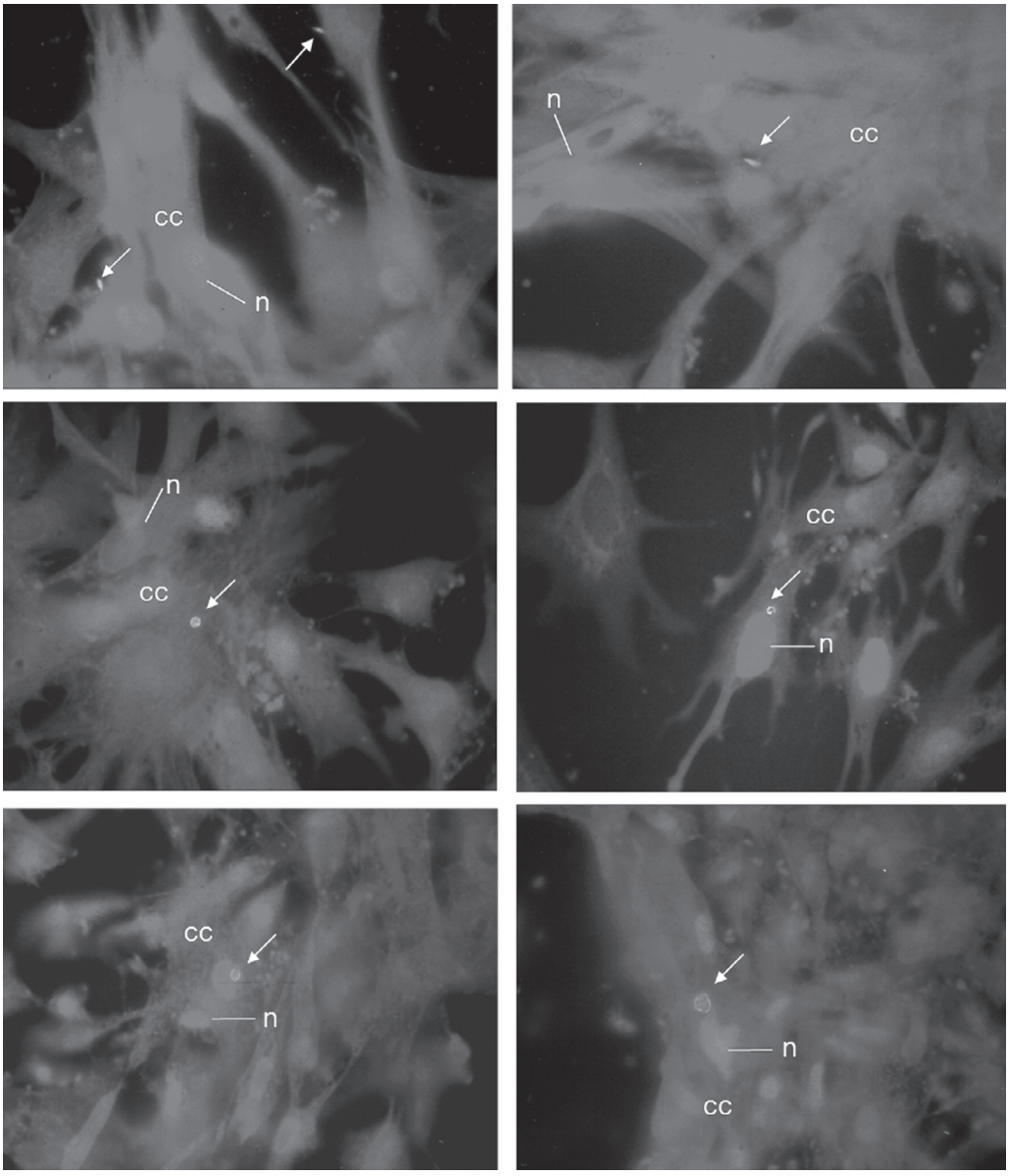

Figure 1 - Indirect immunofluorescent-antibody test with anti-circumsporozoite protein monoclonal antibodies of *Plasmodium gallinaceum* in cultures of fibroblast of chicken embryos inoculated with sporozoites. a –b: sporozoites adhered to the host cell membrane (3h incubation); c – d: rounded parasites in the fibroblast cytoplasm (24h); e – f: developing parasites close to the host cell nucleus (48 h). Cell cytoplasm (cc); nucleus (n). Bar = 10μm

surface, which is facilitated *in vitro* where sporozoites may invade many types of cells.[19,20] There is evidence that interactions between the target cell and the sporozoite occur at the region II-plus of the circumsporozoite protein (CS), which is present throughout the surface of the sporozoite, and of the trombospondin-related anonymous protein/sporozoite surface protein 2"(TRAP/SSP2), which plays a critical role at least in the first stage of cell recognition.[13,14] It is believed that the recognition and adhesion of the sporozoite to the surface of the target cell is directly associated to the region II-plus of protein CS and the glycosaminoglycan (GAG) chain.[21]

There may be more than one type of first contact between the sporozoite and the target cell (Figure 2). It is believed that, in spite of the initial process of adhesion of sporozoites to the host cell, penetration occurs only after directives, by means of chemical mechanisms, in which the TRAP protein is essential for the gliding motility of the sporozoite that will direct the conoid in order to penetrate the host cell.[22] Indeed, we

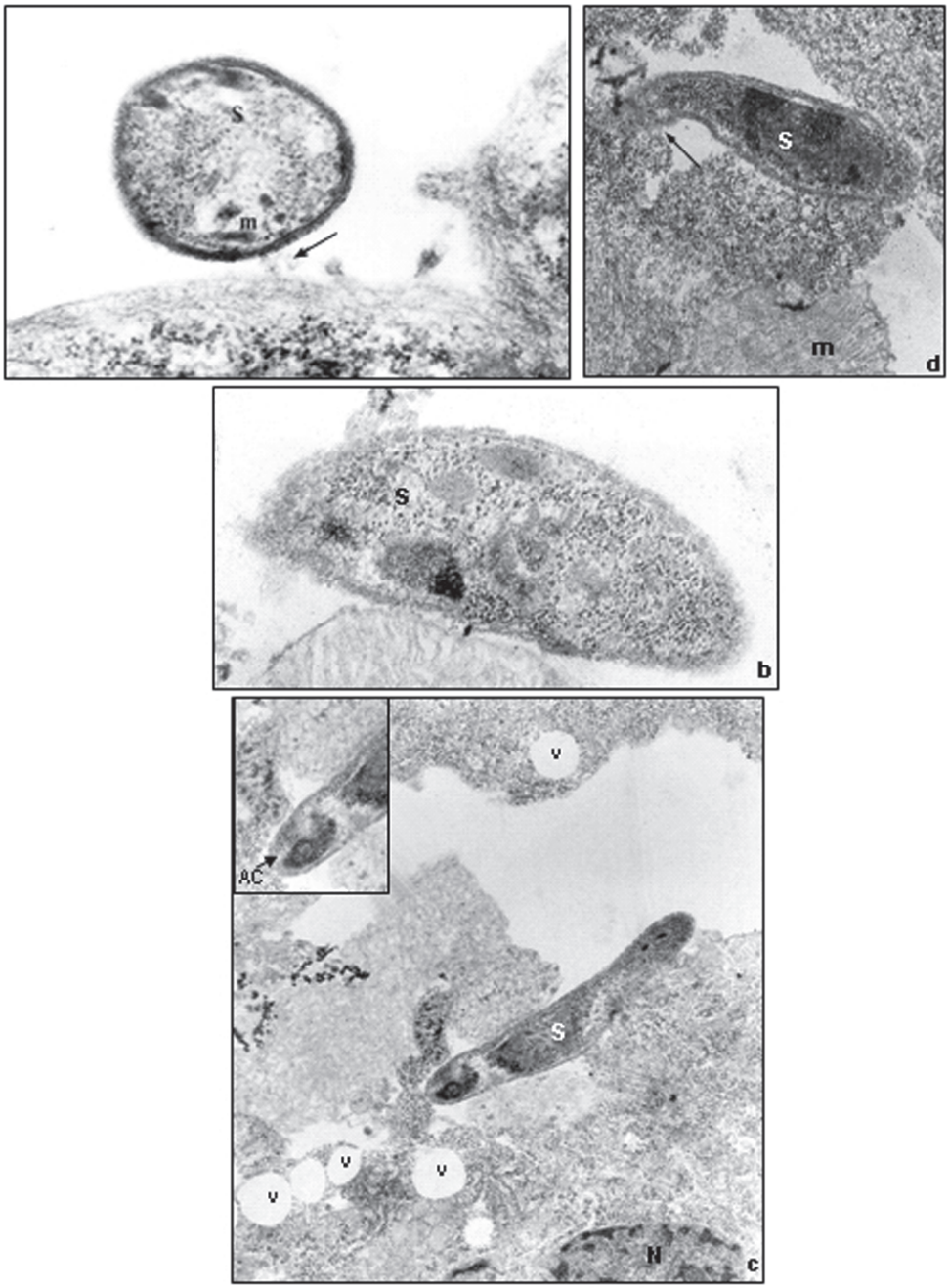

Figure 2 - Electron microscopy transmission of in vitro cultures of fibroblast of chicken embryos inoculated with sporozoites of *Plasmodium gallinaceum* (3 hour of incubation). a (16,500x), b (26,000x): sporozoites adhered to the host cell membrane; c (15,500x),d (10,150x): sporozoites penetrating the fibroblast with the conoid directed against the host cell membrane. AC: apical complex, S: sporozoite, m: microtubules, N: nucleus, v: vacuole

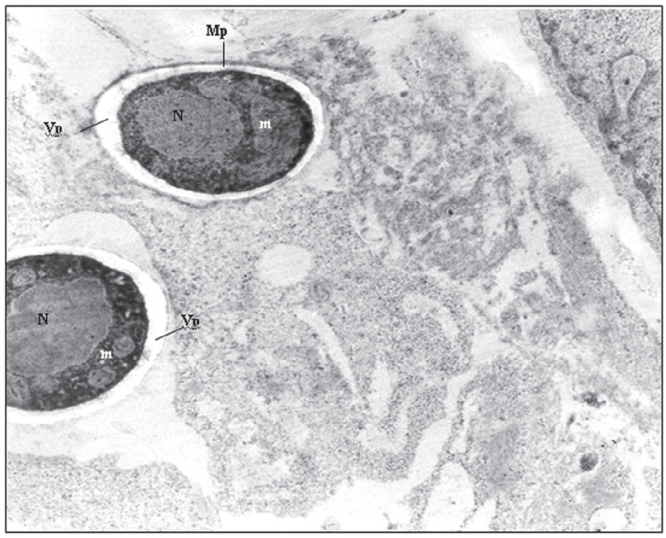

Figure 3 - Electron microscopy transmission of fibroblast of chicken embryos inoculated with sporozoites of *Plasmodium gallinaceum* (24 hour of incubation). Two rounded parasites in parasitophorous vacuole (Vp) in the host cell. (17,500X). Parasite: m: mitocondria, MP: plasmatic membrane of parasite, N: nucleus

observed that initially the adhesion take place through the bind of the parasite's membrane to that of the fibroblast, and the subsequent ensuing invasion occurs when the apical portion of the sporozoite - the conoid – is directed against the host cell (Figure 2c). At this point, an invagination of the host cell membrane was observed, which progressively expands to turn into the parasitophorous vacuole.

Most of our observations on the interaction of *P. gallinaceum* sporozoites-fibroblast were done after 3 h of inoculation. We noticed that the percentage of adhered parasites after 1h of incubation was far lower than at 3 h (0.08% and 0.68%, respectively). The highest rate of sporozoites adhered to the fibroblasts in the experiments was 1.14%, decreasing after 24 hours (when the highest rate was 0.25%) and subsequent incubation times. However, the infection rates are similar to or higher than the average of those obtained in other studies with *P. gallinaceum*[7] and other plasmodia *in vitro*.[8,10]

After 24 hours of incubation, all detected parasites were rounded and found in the interior of the host cells. That is, those that had not adhered in the first hours and then penetrated will not do so afterwards, being probably removed during the replacement of the culture medium. Hollingdale et al.[19] verified that the sporozoites of *P. berghei* essentially penetrate the host cells during the first hours of interaction, although penetration may also take place at 18 and 20 hours. In the preparations we examined 48 and 72 hours of incubation, the parasites were of different sizes and located close to the nucleus of the fibroblasts (Figure 1).

In our experiments, an average of 0.36% of the inoculated sporozoites adhered to the fibroblasts in the first 3 hours of interaction, when around 0.68% of the fibroblasts were found with adhered and/or penetrated parasites. Subsequently, the percentage of invaded fibroblasts decreased, corresponding to 0.14%, 0.05% and 0.04% respectively at 24, 48 and 72 hours of incubation. The number of parasites found developing in the fibroblasts after 24h of incubation was very small in comparison with the number of inoculated sporozoites: it corresponded to less than 0.07%. Microbial contamination and/or detachment of host cells rather than the cellular type and physiology is the best explanation for this gradual decrease in infection rates. Controls have never exhibited contamination or cell detachment. Actually, decreasing of the

infection rates throughout incubation times have also been reported by authors working with other plasmodia [9]. Studies carried out on the development *in vitro* of EE forms of *P.gallinaceum* from sporozoites in a culture of macrophage cells, obtained from the spleen of chicken embryos, showed that many of the parasites developed into schizonts in the first 48 h of incubation [23]. However, after 72 hours, the number of parasitized cells had diminished considerably with regard to the 48-hour incubation. Rocha et al.[7] demonstrated that one cell line SL-29, derived from chicken embryo fibroblast, allows the development of this parasite up to multinucleated schizonts only up to 48h after inoculation of sporozoites. Using primary culture of chicken embryo fibroblast we found parasites schizonts up 72h.

The asynchronous development of *P.gallinaceum* in the *in vitro* EE cycle in chicken embryo fibroblasts observed in the present study is in accordance with the findings *in vivo*.[24] In our study, schizonts of the first EE generation were observed at different stages of development in one same fibroblast. Some authors believe that the development of EE forms *in vitro* seems to be slower than *in vivo* in avian malaria. [9, 24]

The circumsporozoite protein plays an important role in the development of the parasite in the vertebrate host. Studies carried out *in vivo* and *in vitro* with mammalian plasmodia demonstrated that the EE stages have antigens common both to sporozoites as well as to erythrocyte forms.[25,26,27] We demonstrated by IFA that the CS protein of *P.gallinaceum* remains on the surface of the sporozoites that have just invaded the host cell as well as in the schizonts (Figure 1). Some authors demonstrated that the CS protein persists during the whole development of the EE cycle.[26,27]

P. gallinaceum-fibroblast cell culture system showed to be a valuable experiment model for the study of biology of the development of the exoerytrocytic cycle of plasmodium. Remarkable phenotypic and physiological changes may be observed between primary cells culture and cell lines culture that may or may not affect the sporozoite invasion and its differentiation into exoerythrocytic forms. Primary culture of fibroblast is ease to obtain and can be maintained for many months by serial passages. These cells represent a system closed related to the in vivo system and probably mimic all required conditions and elements to the parasite differentiation and development in vitro.

Desenvolvimento exoeritrocítico de *Plasmodium gallinaceum* em cultura primária de fibroblasto de embrião de galinha.

Resumo

No presente estudo, avaliamos a susceptibilidade de cultura primária de fibroblastos de embrião de galinha à infecção por esporozoítas de *P. gallinaceum*, assim como o desenvolvimento de estágios do ciclo exoeritrocítico. Fibroblastos foram obtidos a partir da musculatura do peito de embriões de galinha e esporozoítas foram obtidos de glândulas salivares de *Aedes fluviatilis* experimentalmente infectados. Após períodos de 1h, 3h, 24h, 48h e 72h após a infecção, culturas de células foram fixadas e analisadas através de imunofluorescência indireta empregando-se anticorpos monoclonais contra a proteína circum-esporozoíta e microscopia eletrônica de transmissão. Proteína circum-esporozoíta foi detectada em todas as formas parasitárias. O percentual médio de fibroblastos com esporozoítas aderidos ou já penetrados não aumentou proporcionalmente com a concentração de parasitos no inóculo e independeu se o soro utilizado no cultivo celular era soro bovino fetal ou soro de galinha normal. Foi observado que,

Palavras-chave:
Malária aviária.
Ciclo exoeritrocítico.
Fibroblasto.
Cultura *in vitro*.

quando maior é o período de incubação, maior é a possibilidade dos esporozoítas aderirem e penetrarem nos fibroblastos. Esporozoítas foram observados penetrando em fibroblastos depois de 3h de incubação, quando 0,68% das células tinham parasitos aderidos. A diferenciação e o desenvolvimento das formas exoeritrocíticas foram observados após 24h de incubação, quando somente 0.04% dos fibroblastos achavam-se infectados. A cultura primária de fibroblastos de galinha parece ser um valioso modelo experimental para a investigação *in vitro* do ciclo exoeritrocítico do *P. gallinaceum*.

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