

Evaluation of the inhibition of invasion *in vitro* of *P. yoelii* strain with different monoclonal antibodies raised against MSP-1₁₉

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SUMMARY

Objective. Antibodies are glycoproteins that can protect against diseases like malaria by binding and neutralizing parasites. The effect of antibodies on surface proteins is to block invasion. In this work, we developed an assay to evaluate the inhibition of invasion of *Plasmodium yoelii* mediated by the Merozoite Surface Protein 1 (MSP-1), which is one of the invasive blood stages of the malaria parasite, using several Monoclonal Antibodies (Mabs) raised against a 19 kDa fraction of MSP-1 (MSP-1₁₉).

Material and Methods. Mabs were previously evaluated in a passive transfer experiment which inhibited the growth of the parasite and were characterized. A panel of five of these Mabs (B10, B6, F5, D3 and D9) raised against the C-terminal fragment of MSP-1 from *P. yoelii* were used to evaluate the inhibition of invasion. An ANOVA test was used to compare the inhibition effect.

Results. The protective and partially protective Mabs were evaluated in a growth assay *in vitro*. The inhibition effect of invasion was determined as significant ($p < 0.05$) for Mab F5. The results suggest that Mab F5, an IgG3 which recognizes the first epidermal growth factor (EGF)-like module of the fragment C-terminal of MSP-1₁₉, can interfere with erythrocytes invasion *in vitro*, in the first 30 minutes of the invasion.

Conclusions. This suggests that the first module of MSP-1₁₉, which is recognized by Mab F5, is a

relevant epitope in the invasion process *in vitro* by the merozoite. This assay was a useful tool to evaluate *in vitro* protective Mabs implicated in the inhibition of invasion.

Key words: Malaria, *P. yoelii*, monoclonal antibodies, inhibit of invasion *in vitro*

RESUMEN

Evaluación de la inhibición de la invasión de *P. yoelii* *in vitro* por anticuerpos monoclonales contra la MSP-1₁₉

Objetivo. Los anticuerpos son glicoproteínas que pueden proteger de enfermedades como la malaria por su capacidad de unión a proteínas y consecuente neutralización de parásitos a través del bloqueo del proceso de invasión. En este trabajo, se ha desarrollado un ensayo para evaluar la inhibición de la invasión de merozoítos de *Plasmodium yoelii*, una de las etapas invasivas del parásito de la malaria, mediada por la Proteína de Superficie de Merozoíto 1 (MSP-1), usando Anticuerpos Monoclonales (Mabs) dirigidos contra un fragmento de 19 kDa de MSP-1 (MSP-1₁₉).

Material y Métodos. Los anticuerpos monoclonales (Mabs) fueron evaluados previamente en un ensayo de transferencia pasiva que inhibía el crecimiento del parásito y también fueron caracterizados. Para evaluar la inhibición de la invasión

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Recibido: el 25 de septiembre de 2007. **Aceptado para publicación:** el 14 de abril de 2008.

Este artículo está disponible en <http://www.revbiomed.uady.mx/pdf/rb081916.pdf>

Spencer *et al.*

se utilizó un panel de cinco de estos Mabs (B10, B6, F5, D3 and D9) hechos contra el fragmento C-terminal de la MSP-1₁₉ de *P. yoelii*. Para comparar el efecto de la inhibición se realizó una prueba ANOVA.

Resultados. Los Mabs protectores y parcialmente protectores fueron evaluados en un ensayo de crecimiento *in vitro*. El efecto de inhibición de la invasión fue significativo ($p < 0.05$) para Mab F5. Los resultados sugieren que Mab F5, una IgG3 que reconoce el primer dominio similar al factor de crecimiento epidérmico (EGF-like) del fragmento C-Terminal de la proteína (MSP-1₁₉), puede interferir con la invasión al eritrocito en un cultivo del parásito *in vitro*, en los primeros 30 minutos de la invasión.

Conclusiones. Esto sugiere que el primer dominio de MSP-1₁₉, reconocido por Mab F5, es un epítipo relevante en el proceso de invasión *in vitro* por el merozoíto. Este ensayo es útil para evaluar el efecto *in vitro* de Mabs protectores implicados en la inhibición de la invasión.

Palabras clave: Malaria, *P. yoelii*, anticuerpos monoclonales, inhibición de invasión *in vitro*

INTRODUCTION

Malaria remains one of the priorities for improving public health in the world. The World Health Organization estimates that there are over 500 million clinical cases and approximately 3 million deaths are due to malaria per year. The development of a vaccine against malaria is urgently needed in countries where the disease is endemic (1).

One of the most important and well studied parasite surface proteins at the stage of the life cycle is merozoite surface protein-1 (MSP1). *Plasmodium* MSP-1 is the precursor to a complex of polypeptides on the merozoite's surface; all of the antibodies bound to this precursor and to a 42-kDa fragment (MSP-1₄₂) that is derived from the C-terminal of MSP-1. MSP-1₄₂ is further cleaved to an N-terminal 33-kDa polypeptide (MSP-1₃₃)

and a C-terminal 19-kDa polypeptide (MSP-1₁₉) comprised of two epidermal growth factor (EGF)-like modules (2).

Antibodies against *Plasmodium* have been implicated in the inhibition of merozoite invasion. The merozoite is shortly extracellular and must invade Red Blood Cells (RBCs) within minutes in order to ensure further development. Therefore, attempts to achieve protection have been focused toward the extracellular stages as sporozoite and merozoite, because is during these short periods that the parasite should become most vulnerable to immune attack.

Merozoite recognition and invasion of erythrocytes is a multi-step process and depends on the ability of the merozoite to recognize ligands on erythrocyte membrane. The initial contact of merozoite with erythrocyte is by adherence on any point of its surface but then re-orient itself to make contact with the erythrocyte by the apical end of the merozoite (3). Then, a distinctive junction zone is formed between the two cells. After this, the contents of two apical organelles (rhoptries and micronemes) are released. This is followed by the invasion of the red blood cell membrane, forming a parasitophorous vacuole membrane in which the parasite is internalized into the erythrocyte. Invasion proceeds only if the apical end of the merozoite binds to the erythrocyte surface (4,5).

In the invasion process at least one signal transduction event triggers organelle discharge. There are many proteins with cellular locations that may potentially involve them in this process, such as MSP-1 and MSP-2 together with rhoptry proteins located at the merozoite apex (6, 7). The presence of Epidermal Growth Factor EGF-like domain in MSP-1 in the 19 kDa fragment (MSP-1₁₉) and the fact that it is involved in receptor binding of many proteins, suggests that a possible function of the proteolytic processing of MSP-1 is to reveal these motifs during invasion (8,9).

Wilson (10) described a sequence of events that characterize the invasion of RBCs by the malaria parasite. These are recognition,

The inhibition of *P. yoelii* invasion *in vitro*

trigger, exocytosis, attachment, invasion and resealing. The first step of recognition consists of a specific erythrocyte ligand or molecule, such as glycophorin and Duffy glycoprotein, that interacts with merozoite surface-coat receptors. Trigger is the next event on the invasion process. A crosslinking of specific receptors activates Ca²⁺ channels, increases cAMP levels, or increases the turnover of phosphatidylinositol within the merozoite stimulating exocytosis of the contents of rhoptry organelles. In the exocytosis the rhoptry contents are released and this results in attachment of the merozoite to the erythrocyte membrane following apposition of the apical region by random movements (3).

Attachment is the process when fusion of rhoptry contents of parasite with host cell membrane forms a junction zone by insertion of parasite lipids and proteins into the bilayer.

The invasion step can be inhibited by cytochalasin, whence it is associated with proteolytic activity within the initial parasitophorous vacuole. Internalization of the parasite by merozoite penetration and the encapsulation of the merozoite within a membrane-vacuole is ATP-dependent. Finally, the erythrocyte membrane reseals by processes involving annular membrane fusion. This is called resealing (11).

One strategy for malaria vaccine development is to induce immune responses that prevent interaction of merozoite receptor with erythrocyte binding antigens. Antibodies specific for different *P. falciparum* asexual blood-stage, such as MSA-1 or MSP-1, Pf155/ring-infected erythrocyte antigen (RESA), Pf332 antigen and erythrocyte antigen (EBA)-175, reduce the number of new ring stages *in vitro* (4, 12-14). Experiments with FcγR knockout mice (where the FcγR receptors are nonfunctional) suggest that antibodies to the *P. yoelii* MSA1 alone are able to mediate protection in a murine malaria model (15).

It is important to show out that *P. vivax* and *P. yoelii* share more than 50% similarity in their amino acid sequences (16). Therefore, the

rodent parasite *P. yoelii* represents a good malaria experimental model to understand the processes involved in the invasion of *P. vivax*, a human malaria parasite. In this study, we have tried to demonstrate the inhibition of invasion with a panel of Mabs against MSP-1 of *P. yoelii*, which were protective in passive transference (17).

MATERIAL AND METHODS

Experimental inhibition of invasion. A group of 10 female Balb/c mice were infected with 5×10^3 parasitized red blood cells (RBCs) of *Plasmodium yoelii yoelii* 17XL strain intraperitoneally and when the parasitaemia reached 40%, the trophozoites and schizonts were isolated by a discontinuous percoll column. Parasites were then added at 10% of haematocrits to flasks containing 10 ml of culture medium (RPMI with 0.5% albumax and 1% glutamine) with parasitized RBCs, pregassed with 7% O₂, 5% CO₂ and 88% N₂ and incubated at 37°C. Parasite development was followed in different fractions (at 15, 30, 60, 120 and 180 minutes) when samples of cultures supernatants were removed by centrifugation at 13,000 g for 3 minutes and Giemsa stained smears were examined by light microscopy (100X).

Malaria parasite. *P. yoelii yoelii*, lethal strain 17XL, was used in all experiments. It was maintained as a frozen stabilate and routinely passaged by intra peritoneal inoculation of adult female Balb/c mice.

In the inhibition of invasion experiments, the parasitaemias were determined by Giemsa stain of thin smears. In the onset of parasitaemia where fields counted over 200 RBC, the number of infected erythrocytes per 100 RBCs was determined (percentage of parasitaemia). Animals were considered negative if no parasites could be detected on thin smears by 25 days after all of the mice cleared the parasitaemia (17).

Production and characterization of Mabs. In previous work, the Mabs were produced against *Plasmodium yoelii* merozoite surface protein 1 (MSP-1) and were assessed their ability to sup-

press blood stage parasitemia by passive immunization. Six immunoglobulin G antibodies were characterized in detail: three (B6, D3, and F5) were effective in suppressing a lethal blood stage challenge infection, two (B10 and G3) were partially effective, and one (D9) was ineffective. To locate the epitopes recognized by the MAbs, the ability of the antibodies to bind to recombinant proteins expressed from parts of the *msh-1* gene was investigated by Western blotting. Mab D3 reacted with MSP-1₄₂ but not with either of the constituents MSP-1₃₃ and MSP-1₁₉, D9 recognized an epitope within the N terminus of MSP-1₃₃, and B6, B10, F5, and G3 bound to MSP-1₁₉. Mabs B10 and G3 bound to epitopes that required both C-terminal EGF-like modules for their formation, whereas B6 and F5 bound to epitopes in the first EGF-like module. These results indicate that at least three distinct epitopes on *P. yoelii* MSP-1 are recognized by antibodies that suppress parasitemia *in vivo*. Also, Mabs present different isotypes such as IgG1 to Mab G3, IgG2a to Mab D3, IgG2b to Mab B10 and D9, IgG 3 to Mab F5 and B6 (17).

Parasites growth inhibition was assessed by decreased numbers of ring stages. The asynchronicity of some of the rodent malaria strains is a specific characteristic that may be confusing in the analysis of experimental results as it is parasites growth inhibition. Therefore, synchronized infection of *P. yoelii* and other malaria rodent parasites is very helpful when using the percoll-glucose gradient centrifugation, which results in the very efficient fractionation of the developmental stages of *P. yoelii* (18).

Infected RBCs from *P. yoelii* 17XL strain were isolated as described in the experimental inhibition of invasion section and synchronized parasites were obtained by percoll columns. Parasites growth inhibition was assessed by decreased numbers of ring stages. They were diluted in RPMI medium with 10% of normal mice serum (NMS). Parasites (late trophozoites and schizonts) were washed twice in medium by centrifugation for 5 minutes at 250 x g. They were

diluted in medium supplemented with 10% NMS to 1 % parasitaemia and 10% hematocrit (13).

In order to analyze the effect of Mabs to *P. yoelii* antigen MSP-1 on erythrocyte cycle of the parasite, a panel of Mabs (B10, F5, B6, D3 and D9) was evaluated. Mabs were added (5µg/ml) to late stage cultures at a parasitaemia of 1% and final hematocrit of 10% and the cultures were incubated for 3 hours. The cultures were subsequently Giemsa-stained using thin smears of blood which were obtained every 30 minutes. The distribution of different developmental stages was examined by ordinary light microscope (14).

Kinetic of inhibition. The use of discontinuous percoll gradients to concentrate and synchronize rodent malaria parasites *in vivo* should provide a useful tool for studying the mechanisms regulating the biological rhythms. The percoll-glucose gradient fractionation has the advantage of concentrating a large number of infected cells containing viable parasites (18). Thereby, it provides for each parasite stage to settle at the appropriate density and thus allows their fractionation. For the assay, a population of schizonts was seeded to evaluate the kinetic of inhibition. To study the inhibition effect of Mabs on the schizont development by the apparition of ring stage of parasite, Mabs were added (1µg/ml) to synchronized parasite cultures predominantly in trophozoite and schizont stages and the initial parasitaemia was 1% in all groups. Samples to evaluate the inhibition from cultures were made films from each flask at 15, 30, 60, 90 and 120 minutes by spinning and 100µl of suspension down through a small volume (13,000 g for 3 minutes), discarding the supernatant and spreading the pellet on slide.

Statistical analysis. Each value was presented as mean ± standard deviation with three replicates. Mean differences of parasitaemia between Mabs and the control groups in difference times were evaluated by analysis of variance one-way (ANOVA) taking $\alpha=5\%$ ($p<0.05$) as significant, followed by Dunnett's *post hoc* tests using the 13.0 MINITAB.

The inhibition of *P. yoelii* invasion *in vitro*

RESULTS

Inhibitory action of Mabs on merozoite invasion. The reduction of newly infection ring stage is a parameter commonly measured in parasite growth inhibition assays. In order to analyze the inhibitory effect of Mabs to *P. yoelii* C-terminal antigens of erythrocytes cycles of the parasite, a panel of Mabs at 1 µg/ml were added to late cultures at 2% parasitaemia. Protective Mabs (F5, B6 and D3) in passive transfer were assessed in previous studies. Mab D9 was non-protective in passive transfer experiments (17). After 30 minutes incubation, the distribution of different stages was examined. The cultures without Mab were used as negative controls.

Figure 1 presents the mean values of three independent experiments are shown to Mabs B10, G3, F5, B6, D3, D9 and without Mab, respectively. At 180 minutes, the number of schizonts had an increase in all the cases where Mabs had been assessed, but in the culture without Mab the increase of parasitaemia was evident (more of 5% of parasitaemia). Normally, when the strain is synchronous, the major increase in parasitaemia is

at 3-6 hours after schizogony, when the majority of merozoites have penetrated into erythrocytes and the predominant stages is ring or young trophozoites (18). This observation was achieved in assays *in vitro*. The increase observed at 180 minutes in these assays can be explained by the parasites synchrony.

After 180 minutes (3 hrs), the parasites that infected the RBCs presented degeneration, possibly due to their death. In addition, after 120 minutes the erythrocytes infected and non-infected presented disintegration, probably because of the non adaptation in *P. yoelii* rodent parasite cultures.

DISCUSSION

The fact that antibodies did not have any inhibitory effect as in *P. falciparum*, is related to the fact that the culture has never been maintained for more than 3 hours. This was showed in the different cultures with Mabs as a consequence that it does not exist as a continuous culture *in vitro* for the murine model and the humoral immunity remains poorly investigated in this kind of system.

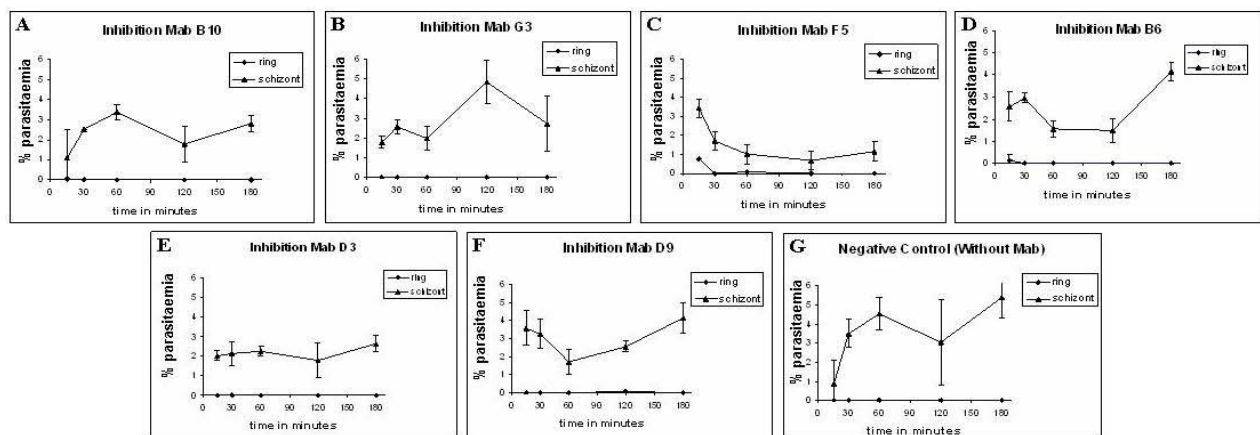


Figure 1. The lines display the distribution of different developmental stages expressed as percentage parasitaemia. Each point of the line represents the mean values of three independent assays, error bars indicate standard distribution within each sample. The figure shows the effect of Mab B10 on ring and schizont development (A), the effect of Mab G3 on the ring and schizont development (B), the effect of Mab F5 on the ring and schizont development (C), the effect of Mab B6 on the ring and schizont development (D), the effect of Mab D3 on the ring and schizont development (E), the effect of Mab D9 on the ring and schizont development (F), the effect of negative control (without Mab) on the ring and schizont development (G).

In order to know if the reduction of parasitaemia to Mab F5 with respect to the negative control was statistically significant, an ANOVA test was used to 30 (F= 8.65; d.f. = 1,4; p = 0.042), 60 (F= 41.4; d.f. = 1,4; p = 0.003) and 180 minutes (F= 28.41; d.f. = 1,4; p = 0.006). In consequence, the difference is significant for Mab F5 at 30, 60 and 180 minutes.

However, the effect of the reduction of parasitaemia can be with respect to Mab D9 (not protective Mab) and negative control (without antibody) in **Figure 1**.

In the panels F and G where the schizont stages around 5 % at 180 minutes in contrast to the protective Mabs (F5, B6 and D3 were 1%, 4% and 3% respectively). Also, the partially protective Mabs (B10 and G3) in passive transfer experiments presented reductions of parasitaemia, which were of nearly 6% at 180 minutes.

The inhibition of invasion assay with Mab F5 had an effect inhibiting the red blood cell invasion by *P. yoelii* in contrast with the other antibodies. Until 1% of parasitaemia at 120 minutes (See **Figure 1**). This result had a correlation with passive transfer experiment in Spencer *et al.* (17).

From the five murine Mabs which are specific for MSP-1 and which are protective in passive transfer experiment of *P. yoelii*, only Mab F5 inhibited the growth of the parasite *in vitro*. However, B6 is the same isotype and recognized the same epitope on MSP-1 by western blot (17). But, a difference in the amino acid sequence could be the reason for the inhibition *in vitro*.

This suggests that at least in the rodent malaria, the epitope recognized by Mab F5 on MSP-1₁₉ protein in *P. yoelii* has provided very significant inhibition of invasion and protection against challenge infection. It is possible that the epitope on the first module of MSP-1 could be the most relevant part of the protein implicated in the inhibition of the invasion process.

CONCLUSION

The protective and partially protective Mabs (F5, B6, D3, B10 and G3) obtained in previous work, which provided passive protection against challenge infection of *P. yoelii* are evidence that antibodies play a very important role in the immune response to this rodent parasite. The results of this study showed that Mab F5 was the most protective antibody in the inhibition of invasion experiments *in vitro*. Therefore, this suggests that

Mab F5 could be the more relevant protective antibody to inhibit invasion and that this assay is a useful tool to evaluate the potential protective effect of an antibody as targets of vaccine design.

ACKNOWLEDGMENTS. We thank Dr. Anthony Holder for the monoclonal antibodies, Dr. Karem Noris for the culture room and Lic Beatriz Pernia for the statistical analysis. This investigation received partial financial support from the DID-USB (Decanato de Investigación de la Universidad Simón Bolívar).

REFERENCES

1. **Maurice J, O'Brien C.** Time to put malaria control on the global agenda. *Nature* 1997; 386: 535-543.
2. **Blackman MJ, Holder AA.** Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP1₃₃ as a noncovalently associated complex with other fragments of the MSP1. *Mol Biochem Parasitol* 1992; 50:307-15.
3. **Blackman MJ.** Proteases in host cell invasion by the malaria parasite [Review]. *Cell Microbiol* 2004; 6(10):893-903.
4. **Sim BK, Orlandi PA, Haynes JD, Klotz FW, Carter JM, Camus D, et al.** Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. *J Cell Biol* 1990;111(5 Pt 1):1877-84.
5. **Chitnis CE, Blackman MJ.** Host cell invasion by malaria parasites [Review]. *Parasitol Today* 2000; 16(10):411-5.
6. **Coppel RL.** Malaria-revealing the ties that bind [Discussion 411]. *Parasitol Today* 1992; 8(12):393-4.
7. **Baum J, Maier AG, Good RT, Simpson KM, Cowman AF.** Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. *PLoS Pathog* 2005; 1(4): e37. 229-309.
8. **Braun BC, Pereira LH.** Malaria proteases and red blood cell invasion. *Parasitol Today* 1993; 9(3): 92-6.
9. **Ling IT, Ogun SA, Holder AA.** Immunization against malaria with a recombinant protein. *Parasite Immunology* 1994; 16:63-67.
10. **Wilson RJ.** Biochemistry of red cell invasion [Discussion 253-6]. *Blood Cells* 1990; 16(2-3):237-52.
11. **Cowman AF, Crabb BS.** Invasion of red blood cells by malaria parasites [Review]. *Cell* 2006;124(4):755-66.
12. **Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA.** A single fragment of a malaria merozoite surface protein remains on the parasite

- during red cell invasion and is the target of invasion-inhibiting antibodies. *J Exp Med* 1990;172(1):379-82.
13. **Wahlin B, Wahlgren M, Perlmann H, Berzins K, Bjorkman A**, Patarroyo ME, Perlmann P. Human antibodies to a Mr 155,000 *Plasmodium falciparum* antigen efficiently inhibit merozoite invasion. *Proc Natl Acad Sci* 1984; 81(24):7912-6.
 14. **Ahlborg N, Andersson R, Perlmann P, Berzins K**. Immune responses in congenic mice to multiple antigen peptides based on defined epitopes from the malaria antigen Pf332. *Immunology* 1996; 88(4):630-5.
 15. **Ramasamy R, Ramasamy M, Yasawardema S**. Antibodies and *Plasmodium falciparum* merozoites. *Trends in parasitology* 2001; 17 (4): 194- 97.
 16. **Del Portillo H. A, Longacre S, Khouri E, David P.H**. Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species. *Proc. Natl. Acad. Sci.* 1991; 88: 4030-34.
 17. **Spencer-Valero LM, Ogun SA, Fleck SL, Ling IT, Scott-Finnigan TJ, Blackman MJ, et al**. Passive immunization with antibodies against three distinct epitopes on *Plasmodium yoelii* merozoite surface protein 1 suppresses parasitemia. *Infect Immun* 1998; 66(8):3925-30.
 18. **Deharo E, Gautret P, Ginsburg H, Chabaud AG, Landau I**. Synchronization of *Plasmodium yoelii nigeriensis* and *P. y. killicki* infection in the mouse by means of Percoll-glucose gradient stage fractionation: determination of the duration of the schizogonic cycle. *Parasitol Res* 1994; 80(2):159-64.