Published in final edited form as:

Exp Parasitol. 2009 July ; 122(3): 254–259. doi:10.1016/j.exppara.2009.04.003.

***Plasmodium yoelii*: Adverse Outcome of Non-lethal *P. yoelii* Malaria during Co-infection with *Schistosoma mansoni* in BALB/c Mouse Model**

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Abstract

Plasmodium yoelii and *Schistosoma mansoni* co-infections were studied in female BALB/c mice aged 4–6 weeks to determine the effect of time and stage of concomitant infections on malaria disease outcome. Patent *S. mansoni* infection in BALB/c mice increased malaria peak parasitemia and caused death from an otherwise non-lethal, self-resolving *P. yoelii* malaria infection. Exacerbation of malaria parasitemia occurred during both pre-patent and patent *S. mansoni* infection resulted in a delay of 4–8 days in malaria parasite resolution in co-infected mice. Praziquantel administered to mice with patent schistosome infection protected from fatal outcome during co-infection. However, this treatment did not completely clear the worm infestation, nor did it reduce the peak malaria parasitemia reached, which was nonetheless resolved completely. Hepatosplenomegaly was more marked in schistosome and malaria co-infected mice compared to either disease separately. The results suggest a complex relationship between schistosome co-infection and malaria disease outcome in which the timing of malaria infection in relation to schistosome acquisition is critical to disease outcome and pathology.

Index Descriptors

Schistosoma mansoni; *Plasmodium yoelii*; co-infection; patent infection; BALB/c mice; PZQ, praziquantel

1. Introduction

Helminth and malaria co-infections are known to occur commonly and hence polyparasitism is more of “the rule rather than the exception” in human populations living in malaria endemic areas (Buck, et al., 1978, Keusch and Migasena, 1982). However, little is known about how these concurrent infections affect disease outcome. Moreover, co-infection with helminths and malaria parasites can also modulate protective immune responses against each other.

The immune response to the intraerythrocytic stages of malaria parasite have been characterized in various rodent models and these studies have provided evidence that cell-mediated immunity and humoral immunity act in concert and sequentially to control and clear

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a blood stage malaria infection (Langhorne, et al., 1989, Stevenson and Tam, 1993, Taylor-Robinson, et al., 1993). On the other hand, experimental *S. mansoni* infections are known to induce a strong Th2 type response, as shown by occurrence of high IgE and eosinophil levels. At the time of patency or egg production, approximately 5 weeks post-infection, a Th2 response is thought to down-modulate the inflammatory response induced by egg deposition, mainly in the liver, that would cause granuloma formation and severe tissue damage (Brunet, et al., 1997, Flores-Villanueva, et al., 1996). In the context of Th1-Th2 paradigm, the majority of data from mice and humans have collectively categorized schistosomiasis as a predominantly Th2 modulated disease, implicating Th2 responses as the cause of morbidity and being detrimental to the host. However, recent data from rodent models have challenged the view that schistosome pathology can be clearly categorized as Th2 in nature, and more convincingly that evidence from infected humans implicate pro-inflammatory Th1 type responses as the cause of morbidity (Fallon, 2000).

In order to examine the underlying pathological and immunological mechanisms mediating concurrent helminth and malaria infections, rodent models offer many benefits that permit more appropriate experimental design and the ability to utilize an extremely well characterized rodent immune system (Druilhe, et al., 2002, Fallon, 2000). Drawbacks to the use of rodent models as opposed to actual clinical observations in humans include: differences between host species and parasite species, field versus laboratory conditions and presence of other confounding multiple infections as well as differences in response to drugs and varying drug pharmacokinetics. According to a review by Druilhe *et al.*, the pattern of infection is not a fixed characteristic for a given pathogen but depends - particularly for the parasite - on the host in which it develops: susceptibility, load of infection, time course and resistance all of which can vary greatly depending on host species or individuals (Druilhe, et al., 2002).

This study describes the outcome of infection by non-lethal *P. yoelii* malaria parasite during pre- and post-patent *S. mansoni* infection in terms of malaria disease severity, parasitemia, anemia or percent hematocrit, the extent of hepatosplenomegaly and mortality.

2. Materials and Methods

2.1. Animals

Female BALB/c mice (4-6 wk-old) were procured from the National Cancer Institute (Bethesda, MD, USA) and maintained in a clean micro-isolation facility. Experimental mice were determined to be free from a variety of common infections through testing of sentinel mice exposed to cage bedding of the experimental mice as well as occasional direct testing of selected experimental mice for murine hepatitis virus. Mice were supplied with food and water *ad libitum*.

2.2. *S. mansoni* infections

S. mansoni cercariae were maintained in *Biomphalaria glabrata* snails fed on a diet of lettuce. To obtain cercariae of both sexes, four infected snails were placed in 50 ml of spring water and exposed to bright light for 30 minutes to induce cercariae shedding. The snails were removed and the water stirred to mix well before a single drop was transferred to a glass slide, stained with Lugol's iodine, and the number of cercariae counted (three replicates). Suspensions were adjusted to obtain an average of five cercariae per drop. The required number of cercariae (~75) was added into a beaker and each mouse infected individually by dipping the tail for 30 minutes under anesthesia. After infection, a drop of Lugol's iodine was added into the beaker to stain cercariae in order to estimate the number of penetrating cercariae. Mice were found to be infected with *S. mansoni* cercariae at a level of ~70 cercariae per mouse. Fifty mice were randomly assigned into four experimental groups (A, B, C and D of ten mice each (Fig 1), and

a fifth control group (E). For each group five of the ten mice were infected with *S. mansoni* and the other five were mock-infected.

Patency of *S. mansoni* infection was confirmed by the miracidial hatching technique at six and seven weeks post schistosome infection. Briefly, fresh fecal pellets were collected in a small volume of saline solution in a 15 ml Pyrex tube. The tube was exposed to a strong beam of light and spring water added to cause the *S. mansoni* eggs to hatch by osmosis, releasing the phototactic miracidia, which then swam towards the light. The miracidia could be easily seen due to their swimming movement by use of a hand held magnifying lens within 30 minutes of initiation of hatching.

2.3. *P. yoelii* infections

At 14 days post schistosome infection (DPSI) (group A), 28 DPSI (group B) and 42 DPSI (groups C and D) mice were infected with 10^5 *P. yoelii* (Strain 17XNL –a non-lethal self-resolving malaria parasite) infected red blood cells (iRBCs) (Fig 1). Each of groups A, B, C and D of mice was then made up of two sub-groups i.e., schistosome and malaria co-infected (S+M) and malaria only infected (M). The control mice (group E) consisted of 5 mice infected with schistosomiasis only (S) and 5 uninfected (mock-infected) mice (N). In order to ensure uniformity of iRBC, parasite stabilates frozen from a single batch of *P. yoelii* were used for the infection of various groups. At each time of infection, frozen parasites were thawed and injected intraperitoneally into a single donor BALB/c mouse and parasitemia allowed to develop to 5-10% before infected blood from donor was taken and diluted to give a dose of 10^5 iRBCs per mouse. Malaria-free control groups received the same volume (200µl) of $1 \times$ PBS instead of iRBCs. Malaria infections were monitored by Giemsa-stained thin blood smears prepared from tail bleeds starting on the third day post-malaria infection and subsequently on every other day thereafter. Percent parasitemia was determined by examining at least 1000 RBCs on the slide and averaged for each group of mice.

2.4. Anthelmintic treatment

Mice in group D were treated perorally with praziquantel (PZQ) (Sigma, St Louis, Missouri) suspended in 2% cremophor EL (Sigma) at a dose of 500 mg/kg of body weight at 35 DPSI, when *S. mansoni* infection is considered to be patent (Fig 1). Fresh fecal pellets were tested by the miracidial hatching technique to check for worm clearance.

2.5. Other measurements

Total body mass was measured for each individual mouse on a weekly basis, whereas hematocrit (Hct) was measured after *P. yoelii* infection in individual mice at 1 and 2 weeks following malaria infection in all groups. Approximately 50 µl of blood were drawn via tail bleeds into heparinized microcapillary tubes (Fisher) and then centrifuged at 1200 rpm for 15 min. After 77 DPSI, all surviving mice were sacrificed, liver, spleen masses determined and compared among groups.

2.6. Data analysis

Mean parasitemia of schistosome and malaria co-infected (S+M) and malaria only infected (M) groups of mice were compared using a non-parametric test, the Mann Whitney test, whereas percent hematocrit, liver and spleen masses were compared based on standard errors of mean (SEM) differences. Differences in parasitemia between groups were considered significant when p value was less than 0.05.

Results

3.1. Increased susceptibility of pre-patently *S. mansoni*-infected mice to malaria

To determine if pre-patent schistosome infection altered mouse susceptibility to malaria, mice in groups A (14 DPSI) and B (28 DPSI) and their age-matched worm-free counterparts respectively, were injected intraperitoneally with the same dose of 10^5 iRBCs of non-lethal *P. yoelii* strain (17XNL). Overall, pre-patently co-infected (S+M) mice developed higher group mean malaria parasitemia compared to malaria-only infected (M) mice though this difference was not statistically significant by Mann Whitney test (p values were 0.15 and 0.18) for groups A and B respectively. In other words the median parasitemia values of S+M and M mice were not different. Fig 2 (panels A and B) show time course of the malaria parasitemia exhibited by the co-infected groups and the malaria only infected mice. Mean peak parasitemia in the M group reached 20% at 11 days post malaria infection, whilst the peak parasitemia of S+M groups was higher reaching 42% at about the same time of 11-12 days after malaria infection. Additionally, co-infected mice displayed a longer course of *P. yoelii* malaria patency with mice remaining parasitemic for an average of 4 days longer than the mice in the malaria only groups (Fig 2, panels A and B). An interesting observation was that there was no mortality associated with the high parasitemia during co-infection with schistosomes at 14 and 28 DPSI even though some individual mouse parasitemias rose as high as 72% before complete resolution.

3.2. Increased susceptibility and mortality of patently *S. mansoni*-infected mice to malaria

Fig 2, panel C shows time course of malaria parasitemia in mice infected with malaria after schistosome patency (S+M) and that of malaria only infected mice (M). Patently schistosome-infected mice in group C (S+M) developed significantly higher mean parasitemia overall than the age-matched malaria only infected mice (M) ($p=0.025$). On day 42 following schistosome infection, the mice in group C did not show any overt disease symptoms (looked as healthy as the controls) prior to malaria infection. More importantly, while the malaria only infected mice, as expected, resolved their malaria infection, all (5/5) co-infected mice died at peak malaria parasitemia or soon thereafter. The results shown are from one of two independent experiments (Fig 2, panel C).

3.3. Reduction of mortality by PZQ treatment

Mice in group D presumed to be patently infected on 35DPSI (S+M) and M were treated with 500 mg/kg body weight of PZQ one week prior to *P. yoelii* infection to treat for schistosomiasis. The treatment group was included to investigate the impact of PZQ treatment on the time course, extent of parasitemia, anemia outcome as well as mortality of co-infected mice. From Fig 2, panel D, the malaria parasite clearance kinetics of the treated group (S+M) were almost similar to those of the untreated group Fig 2, panel C, but with one major difference: only one mouse out of the five mice died on the 17th day post malaria infection compared to all five mice dying in the untreated co-infected group by day 17 (Fig 2, panel C). More importantly, the difference in parasitemia between S+M and M infected groups did not reach statistical significance ($p=0.056$). Though PZQ treatment had no observable effect on malaria parasite clearance (as observed in the similar time course of malaria patency in the in PZQ treated and untreated mouse groups), it appeared to alleviate fatality resulting from co-infection probably through the modification of other host factors. However, group D mice with patent *S. mansoni* infection (S+M), and treated with PZQ, displayed a longer *P. yoelii* malaria patency, remaining parasitemic for more than one week longer than the parasite clearance of age-matched, malaria only controls also given PZQ treatment (Fig 2, panel D). Malaria only infected mice (M) in group D showed normal malaria parasite clearance kinetics even after PZQ treatment.

3.4. Miracidial hatching test results

In order to demonstrate *S. mansoni* infection patency and evaluate the treatment efficacy of PZQ we carried out the miracidial-hatching test with fecal samples from mice in groups C, D and control group (S) at 7 and 8 weeks post schistosome infection. At 7 weeks post schistosome infection (or 2 weeks after PZQ treatment) 60% (3/5) of untreated mice and 40% (2/5) of the PZQ-treated mice tested positive for viable eggs. When repeated one week later, 80% (4/5) from both groups tested positive by the miracidial hatching test. These results demonstrate that PZQ treatment of mice from group D was not effective in clearing the worms as they may have received drug before all worms had reached maturity (the drug is only effective against adult worms) and left immature forms that may have proceeded to maturity and to shed eggs by week 8 after schistosome infection. Alternatively, the eggs may have remained viable in the tissues well after the death of adult worms that had shed them.

3.5. Levels of anemia as reflected in percent hematocrit values

In order to determine levels of anemia induced in different groups of mice we measured hematocrit percentage by measuring packed cell volume (PCV) at 1 and 2 weeks post malaria infection. Table 1 shows results of PCV values at 2 weeks after malaria infection in groups A, B, C and D respectively and group E (mice infected with schistosomiasis alone (S) or none (N)). Similar hematocrit values were obtained one week after malaria infection (not shown). For group A mice (malaria-infected at 14 DPSI), only the S+M co-infected group exhibited a higher mean level of anemia compared to the control group N with a 30% drop in PCV. The S and M only groups had similar values to that of the controls, i. e., were not anemic (Table 1). Group B mice (malaria-infected at 28 DPSI), the S+M group displayed lower mean PCV (25% less than control group) and thus were more anemic than the controls at both one and two weeks after malaria infection, whereas M group was more anemic (mean PCV lower than controls by 20%) one week after malaria infection but had recovered by two weeks post *P. yoelii* infection. For Groups C and D (malaria-infected after schistosome patency), the S+M and M infected groups had mean PCV values that were lower (at least 35%) than the controls. The (M)-only infected group exhibited even lower PCV (higher anemia level) than the co-infected group. The results suggest that at these levels of infection and after patency of schistosomiasis, malaria, and not schistosome infection, is the primary cause of anemia. However, before schistosomiasis patency, co-infection results in greater levels of anemia in mice (Table 1).

3.6. Hepatosplenomegaly among groups with different infections

In order to determine trends in hepatosplenomegaly in relation to infection types, timing, and anemia trends, the masses of liver and spleen were measured for each individual mouse upon death or sacrifice at 77 DPSI (Table 1). Compared to the uninfected control sub-group (N), the malaria-only infected groups (M) showed 25-50% increase in liver mass. On the other hand, schistosome infected groups generally showed much higher increase in hepatic mass (80-150%) than control sub-group (N). Group C (S+M) mice had slightly lower levels of organomegaly probably due to less time for its development as a result of early death (at least 3 weeks earlier than the time of sacrifice compared to other groups). Malaria infection by itself caused an approximate doubling of spleen size and mass whereas schistosome-only (S) infection increased spleen size about 5 times. However, S+M co-infection resulted in up to nine-fold increase in liver size and mass. Generally, hepatosplenomegaly was much higher in the context of schistosome-malaria co-infection compared to either infection separately (Table 1).

4. Discussion

Concurrent infection with diverse pathogens is a common phenomenon in many endemic areas. In this study, we investigated the effect of an intestinal tissue digenetic trematode (blood fluke) *S. mansoni* infection on a non-lethal malaria infection, using a rodent model of co-infection. We observed a significant impact of *S. mansoni* infection upon subsequent infection with *P. yoelii* resulting in notably increased malaria parasitemia, increased duration of patent malaria, and a dramatic increase in malaria case fatality rate following schistosoma infection patency, which was ameliorated by administration of PZQ. Other groups have made similar observations on increased malaria parasitemia (Helmby, et al., 1998, Legesse, et al., 2004, Noland, et al., 2005, Su, et al., 2005) albeit with lower case fatalities of 5-10%. An increased parasitemia may imply more severe disease and complications especially in naïve populations, whereas increased malaria patency may lead to the emergence of drug resistant malaria parasites. Increased malaria patency may also translate to increased transmission potential. Thus, such interactions among co-infecting parasites may have profound implications for disease epidemiology and control in humans.

There was no difference in body weight gain between the control sub-groups or schistosoma-only infected (S) and non-infected (N) (data not shown). From Table 1, trends in percent hematocrit values show similarity between sub-groups N and S. These results suggest no hematological differences between uninfected and schistosoma-only infected mice, which could possibly explain the observed differences in parasitemia trends following malaria infection. In similar work with *Echinostoma caproni* and *P. yoelii* co-infection, Noland *et al.* found no hematological differences between co-infected and malaria-only infected mice in spite of the exacerbation of parasitemia during co-infection (Noland, et al., 2005). In Thailand among cerebral malaria patients Nacher *et al.*, found reduced reticulocytopenia and increased anemia in patients co-infected with *Ascaris* compared to those patients infected with malaria only (Nacher, et al., 2001, Nacher, et al., 2002). Exacerbation of *P. berghei*, *P. yoelii* and *P. chabaudi* malaria parasitemia has been demonstrated in *S. mansoni* co-infected mice (Helmby, et al., 1998, Lwin, et al., 1982, Yoshida, et al., 2000). However, to our knowledge, it is the first time a similar exacerbation has been demonstrated in pre-patent *S. mansoni* co-infection (Fig 2, panels A and B) in which malaria infection was initiated at 14 and 28 DPSI, respectively.

The prevailing hypothesis is that helminth infections induce a Th2 type immune response (Helmby, et al., 1998, Nacher, et al., 2002) and this might explain the increased malaria parasitemia after the schistosoma infection has reached the patency stage (Grzych, et al., 1991, Legesse, et al., 2004, Lwin, et al., 1982). However, the observation of increased parasitemia in mice during pre-patent schistosoma infection seems counter-intuitive, especially given that this stage of worm infection also promotes a Th1 response in the host as reviewed by Fallon (Fallon, 2000). In such a scenario, it would be expected that the Th1 environment would favor control of primary malaria parasitemia. On the other hand, such an environment may suppress a switch from Th1 to the Th2 cytokine milieu, which is required for the antibody mediated final parasite clearance. This may offer an explanation for the increased parasitemia and delayed clearance of malaria parasite observed in the co-infected mice in groups A and B. Future studies to investigate the cytokine milieu in each of these groups might help shed light on the possible mechanisms leading to the exacerbation of malaria in both pre-patent and patent schistosomiasis infections as well as increased case fatality in the latter. It would also be of interest to carry out similar studies to evaluate spleen cells for response to malaria antigens in order to determine the cytokines produced and how they differ between co-infected and malaria-only infected groups of mice.

Our data demonstrate that mice with a patent *S. mansoni* infection (groups C) developed significantly higher malaria parasitemia, following infection with blood-stage *P. yoelii* and

died during an infection that would otherwise be non-lethal and self-resolving. Praziquantel treatment against schistosomes greatly reduced mortality (group D). The findings from co-infection outcome in the mouse model provide the impetus for assessing similar infection outcomes in areas where schistosomiasis and malaria are co-endemic and super-infections occur in endemic human populations.

Acknowledgments

The Fogarty International Center Malaria Research and Training Program in Zimbabwe funded this study. Animal handling procedures were observed and carried out under the Johns Hopkins School of Public Health Protocol number M007H4. None of the authors has a conflict of interest in this work. Authors thank Dr Fred Lewis, Biomedical Research Institute, Bethesda, MD for providing the schistosome infected snails used in this study.

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Fig 1. Schematic of the study design starting from day zero (day 0) or the day of *S. mansoni* infection proceeding to day 77 the day on which survivors were sacrificed

Schematic of the study design. Mice were infected with *S. mansoni* (Sm) on day 0. *P. yoelii* (Py) infection was initiated on day 14 (Group A), day 28 (Group B), day 42 (Group C and D). Mice in Group D were treated with praziquantel (PZQ) on day 35. All the surviving mice were sacrificed on day 77. Each group had schistosome and malaria (S+M) co-infected and malaria only (M) infected sub-groups of mice represented by the two horizontal arrows. The control group (E) had schistosome only infected (S) and uninfected (N) sub-groups of mice. (All groups were subjected to same treatment by mock-infection).

Fig 2. Time course of malaria parasitemia in groups A, B, C and D

Time course of malaria parasitemia in malaria only-infected mice (M) or schistosomiasis and malaria co-infected mice (S+M) in groups A, B, C and D. Error bars represent standard errors of mean for 5 mice in each sub-group. Each arrow indicates the death of a single mouse in the various sub-groups. The Mann Whitney test was used to compare S+M and M infected group percent malaria parasitemia (* indicates p value of 0.01 up to 0.05).

* Significant p value of 0.01-0.05

Effect of malaria infection only or malaria-schistosome co-infection on hematocrit and hepatosplenomegaly
Mean percent hematocrit (% Hct) values were measured at 2 weeks post *P. yoelii* infection. Mean Liver and spleen masses were obtained at sacrifice or death. SEM represents standard errors of mean values from the five mice per sub-group in each case.

Subgroup	Group E			Group A			Group B			Group C			Group D		
	N	S		M	S+M		M	S+M		M	S+M		M	S+M	
% Hct	69.8	67.0		65.8	42.2		63.6	50.0		35.4	38.7		34.6	45.2	
SEM	1.7	1.1		7.1	5.0		1.9	4.6		5.9	4.0		2.7	4.9	
Liver Mass (g)	1.02	1.85		1.29	2.21		1.31	2.52		1.33	1.48		1.38	2.17	
SEM	0.08	0.32		0.04	0.28		0.06	0.14		0.05	0.08		0.09	0.24	
Spleen Mass (g)	0.12	0.54		0.25	0.87		0.23	0.86		0.21	0.65		0.26	0.57	
SEM	0.01	0.18		0.02	0.17		0.01	0.15		0.003	0.14		0.01	0.11	

N-Control sub-group with no infection
S-Control sub-group with schistosome-only infection
M- Sub-group with Malaria-only infection
S+M-Schistosome and Malaria co-infected sub-group