International Journal of Medicine and Medical Sciences Vol. 2(5), pp. 162-166, May 2010
Available online http://www.academicjournals.org/ijmms
ISSN 2006-9723 © 2010 Academic Journals

Full Length Research paper

Mouse mortality from a high *Plasmodium berghei* inoculum density may be due to immune suppression in the host

Chika K. Onwuamah[1]*, Phillip U. Agomo[2] and Peter G. C. Odeigah[3]

[1]Microbiology division, Nigerian Institute of Medical Research, 6 Edmund Crescent, P. M. B. 2013, Yaba 101212, Lagos, Nigeria.
[2]Biochemistry division, Nigerian Institute of Medical Research, 6 Edmund Crescent, P. M. B. 2013, Yaba 101212, Lagos, Nigeria.
[3]Department of Cell Biology and Genetics, Faculty of Science, University of Lagos, Akoka, Lagos.

Accepted 12th May, 2010

Patients' malaria parasite burden on hospital presentation has been implicated in human artemisinin monotherapy failure rather than any drug resistance-conferring genetic mutation in the infecting *Plasmodium* species. We investigated this observation using a mouse-*Plasmodium berghei* model at varying inocula abundance and monitored the pathogenesis without intervention. Three inoculums abundance levels were used: 10^7, 10^5 and 10^3 highly parasitized blood (≥ 4000 parasites/µl). All 10^7 inoculum mice died within 12.7 days. The 10^3 inoculum and the 10^5 inoculum mice cleared their malaria parasitaemia between days 12 - 14 and between days 34 - 36 post-inoculation respectively. The 10^3 inoculum and 10^5 inoculum mice had changes in baseline value of 1.71 and 2.02 in total white blood cells count between days 6 - 10 respectively, compared to 1.33 for the 10^7 inoculum mice. Monocytes counts (cells/mm^3) on day 6 was 223.3, 1254 and 40.7 for the 10^3 inoculum, 10^5 inoculum and 10^7 inoculum mice, while 152.5, 1073 and 477.5, respectively on day 10. Immunosuppression by the parasite might be the cause of the in appropriate immune response by 10^7 inoculum mice.

Key words: Malaria, mice, parasite density, immune response, immune suppression.

INTRODUCTION

Malaria has afflicted man and animals for over a century. Each year an estimated 300 – 500 million clinical cases of malaria occur and its direct and indirect economic costs can be tremendous, with an estimate of US$73.6 billion for 31 African countries from 1980 - 1995 (WHO, 1996; Sachs and Malaney, 2002).

Patients' malaria parasite burden on presentation at the hospital was implicated in human artemisinin mono-therapy failure rather than any drug resistance-conferring genetic mutation in the infecting *Plasmodium* species (Ittarat et al., 2003). Patients with high parasitaemia had a 9-fold likelihood of recrudescence compared with patients with lower parasitaemia (Ittarat et al., 2003). This suggests that high parasite challenge and not drug resistance predisposes the patient to recrudescence. It was proposed that the level of parasitaemia on admission may be useful in choosing therapeutic options (Ittarat et al., 2003).

From the immunological perspective, clinical and epidemiological studies suggest that the innate immune mechanisms contribute to protection from malaria and modulate adaptive immune responses (McGuinness et al., 2005; Stevenson and Riley, 2004). Riley (2002) proposed that the balance and timing of both innate and adaptive immune responses are the critical issues in our

*Corresponding author. E-mail: chikaonwuamah@yahoo.com. Tel: 234 - 803 719 1387.

Abbreviations: WBC, White blood cell; **RBC,** red blood cells; **MP,** malaria parasitaemia; **TWBC,** total white blood cell count; **DWBC,** differential white blood cell count.

understanding of the immune response to malaria. Parasitaemia burden / dose – dependent investigations are expedient in attempting to understand the clinical observation by Ittarat et al. (2003). Furthermore, the importance of the immune effector cells and different parasite abundance levels has never been evaluated concomitantly before. We thus investigated the differential white blood cell responses elicited by different inocula and observed the infection outcomes (death or clearance of parasitaemia) using a mouse- *Plasmodium berghei* model, while determining malaria parasitaemia, total and differential white blood cell (WBC) counts.

MATERIALS AND METHODS

Study preparation and animal husbandry

The study protocols were reviewed by the ethics committee of the Nigerian Institute of Medical Research (NIMR). The Swiss albino mice and the *Plasmodium berghei* NK65 strain were obtained from the Biochemistry Division, NIMR. The mice were maintained in fibre glass cages, with wood shavings as beddings and had access to water and food *ad libitum*.

Experimental design

This study was an observation of the pathogenesis of *Plasmodium berghei* infection without any chemotherapeutic intervention. *Plasmodium berghei* NK65 strain and Swiss albino mice aged 8 - 10 weeks were used. Each control and test group mice were age, sex and weight-matched, with three mice per group. Mice littermates formed the first control, the 10^3 inoculum and the 10^7 inoculum groups. Another set of littermates formed the second control and 10^5 inoculum group.

Innoculum preparation and dilution

Donor mice were previously infected through intra-peritoneal injection. Highly parasitized blood (≥ 4000 parasites/µl) was collected by retro-orbital procedure from them on the day of inoculation of the test groups (day 0) and three dilutions were used. A mature mouse (≥ 6 weeks old) has approximately 5 x 10^8 RBCs/ml (Agomo, 1980). A 1:5 dilution of the blood gave 10^8 infected RBCs/ml; from which 10^6 and 10^4 infected RBCs/ml dilutions were obtained by further 1:10 serial dilutions. Intra-peritoneal injection of 0.1 ml of each parasitized blood dilution (10^8, 10^7 and 10^4) into the appropriate mice groups delivered inocula of 10^7, 10^5 and 10^3 infected RBCs/ml respectively. The control mice were intra-peritoneally injected with 0.1 ml of sterile water.

Sampling and laboratory analysis

Baseline blood samples were obtained prior to injection with sterile water or parasite inoculum. Caudal blood was collected every other day for assaying malaria parasitaemia (MP), total white blood cells (TWBC) and the differential white blood cells (DWBC) counts in each mouse until test groups died or cleared parasitaemia in three consecutive samplings. MP and TWBC counts were performed in duplicates and the average taken using standard visual microscopic methods (Cheesbrough, 1998). DWBC counts were performed after Cheesbrough (2000). Briefly, thick and thin blood films were made for MP on the same pre-labelled slide with free-flowing whole blood directly from the mouse tail snips after the first drop was wiped off with cotton wool. The thin film was fixed with absolute methanol and air-dried. The dried blood films were then stained with 3% Giemsa stain solution for 30 min and air-dried. Dried slides were examined first using the x10 objective and then with the x100 objective. Each slide was examined by counting the number of asexual parasites and WBCs seen until ≥ 200 WBCs have been counted. Parasite density/µl was calculated as 'parasites counted' X 8000 / Number of WBCs counted.

For the total WBC counts, 20 µl of free-flowing caudal blood was added to 0.38 ml WBC diluting fluid and mixed by inversion. The improved Neubauer ruled chamber was used and cells in the four large corner squares were counted. After the assembly of the chamber, a 10 µl micropipette was used fill the chamber with the diluted blood sample. The chamber was left for 2 min for the WBCs to settle and then examined with the x10 objective. The number of WBCs counted was reported as cells/µl by multiplying the total count by 50. WBC counts were performed in duplicates and the average taken. The haemocytometer counts are acceptable if there is less than 20% difference (as percentage of the mean) between the two different chamber counts.

Free-flowing whole blood was used to make thin blood smear on pre-labelled microscope slides, directly from each mouse's tail snip, then air-dried and used for the DWBC counts. The dry smear was fixed with absolute methanol and stained with Leishman stain. The blood film was first covered with the undiluted stain for 2 min and then distilled water was added to dilute the stain on the slide, ensuring the water mixes well with the stain and allowed to stain for 8 – 10 min. Tap water was used to flood off the stain and the slide air-dried. The thin blood film was first examined using the x10 objective and then the monolayer portion examined with x100 (oil immersion) objective. The different white cells seen were counted until a total count of 100 was obtained. The absolute number of each WBC type was calculated by multiplying the number of each cell counted (expressed as a decimal fraction) by the TWBC count obtained for that sample.

Data analysis

Data obtained was analysed using Epi Info 2002 (CDC, Atlanta, USA). The Kruskal-Wallis test was performed to check for statistically significant variations between the groups with $p \leq 0.05$ regarded as being significant. Inter-group variations were determined for throughout the entire study period (days 0 -36) before host death or clearance of parasitaemia. Using the median and percentiles, the entire period was divided into four time frames (days '0 - 4', '6 - 10', '12 - 20' and '22 - 36') to show the actual time frame(s) where there were statistically significant variations. The sample size per group was small, being constrained by our efforts to reduce the influence of genetic variation by using litter-mates and matching them for sex and weight.

RESULTS

Observations - physical and microscopic

All the three 10^7 inoculum group of mice died, on days 10, 12 and 16 post-inoculation. The 10^3 inoculum and the 10^5 inoculum groups cleared their malaria parasitaemia between days 12 - 14 and between days 34 - 36 post-inoculation respectively (Table 1). At the point of death,

Table 1. Initial and peak malaria parasite counts for each inoculums group.

Group	Sex	Ave. weight (g)	Infection outcome	Death or MP cleared day	First MP count (*Parasite density/ul*)	Day first MP seen	Peak MP count (*Parasite density/ul*)	Day peak MP seen
'10^3 inoculum'	Male	19.0	Cleared	13.3 (12,14,14)	435.5	2.7(4,2,2)	781.6	4.7(6,6,2)
'10^5 inoculum'	Female	22.3	Cleared	35.3 (34,36,36)	197.9	2(2,2,2)	1960.2	18(18,22,14)
'10^7 inoculum'	Female	17.5	Death	12.7(12,10,16)	333.3*	2*	464222.2	8*

*Blood from '10^7 inoculum' group was pooled before performing malaria parasitaemia counts. Numbers given in parentheses are the actual values.

10^7 inoculum mice had almost all RBCs parasitized and gametocytes and schizonts could be seen. Chromatin dots were seen bound to WBCs, particularly to lymphocytes and monocytes. This chromatin binding to WBCs was most prominent between days 24 - 26 amongst the 10^5 inoculum group of mice.

Analysis of test-control groups variation

There was significant variation at p = 0.5 between the control and the 10^3 inoculum group over the entire period in the parasite count. Within the time frames, there were significant variations between days 6 - 10 in their total WBC and lymphocyte counts (10^3 inoculum group > control values in both) and in their neutrophil counts between days 12 - 20 (control > 10^3 inoculum group). Except for the eosinophil counts, significant variation was recorded between the 10^5 inoculum group and its control in all assayed parameters over the entire period and for parasitaemia counts also within each time frame. Significant variation in the total WBC, neutrophil and monocyte counts (consistently '10^5 inoculum' group > control in all) from days 6 - 10 and the lymphocytes counts by days 22 - 36 (consistently 10^5 inoculum group > control) was obtained.

There was no significant variation between the 10^5 inoculum group and the control values between days 12 - 20 except in the parasitaemia counts. Compared to the controls, 10^7 inoculum mice had significantly different parasitaemia counts over the entire period and in each time frame. However, there was no significant variation in the total WBC, lymphocyte, neutrophil, monocyte and eosinophil counts over the entire period. Within the time frames, there was significant variation only in neutrophil counts between days 0 - 4 (consistently 10^7 inoculum group > control) and in monocyte counts between days 12 - 20 (control > 10^7 inoculum group).

Analysis of inter-group variation amongst test groups

Comparing 10^3 and 10^5 inoculum groups we observed significant differences in parasitaemia counts over the entire period, between the days 6 - 10 and between days 12 - 20. Significant differences in total WBC, lymphocyte and monocyte counts over the entire period, were observed at days 6 - 10 time frame with total 10^5 inoculum group values 2-fold that of the total 10^3 inoculum group values. Significant differences in the monocyte counts also occurred between days 12 - 20 (total 10^5 inoculum values 7-fold that of the total 10^3 inoculum values). When comparing 10^3 inoculum' and 10^7 inoculum groups, only the parasite counts showed significant variation over the entire period, and this was observed in days 6 - 10 and 12 - 20 time frames. Though there was no significant variation in neutrophil counts over the entire period [p = 0.210], the counts between days 0 - 4 differed significantly [p = 0.047] (10^7 inoculum group > 10^3 inoculum group).

Significant variations between 10^5 inoculum and 10^7 inoculum groups occurred in the parasite count (p = 0.001), total WBC (p = 0.000) and monocyte (p = 0.000) counts over the entire period during days 6 - 10 and 12 - 20 time frames. The 10^7 inoculum group parasite counts between days 6 - 10 was 433-fold that of the 10^5 inoculum group parasite count. However, 10^5 inoculum group's total WBC, lymphocyte, and monocyte counts were 2 times, 2-fold that of and 4-fold that of the corresponding 10^7 inoculum group values respectively. Between days 12 - 14, average 10^7 inoculum group parasite count was 158-fold that of the 10^5 inoculum group value and the monocyte counts was 2-fold that of the 10^5 inoculum group values. For the other parameters, the 10^5 inoculum group values were still 2 times greater than the 10^7 inoculum group values. There was no significant variation between 10^5 and '10^7 inoculum groups during days 0 - 4.

Profile of changes in leukocytes counts

Increase or decrease rates in the assayed parameters were obtained by comparing the average values in every time frame against their baseline values (Table 2). These could not be calculated for monocytes in the 10^7 inoculum group because of their zero baseline value. The changes in haematological parameters over time , particularly in

Table 2. Changes in baseline haematological values with time.

Groups →	**Control 1**				**10^3 inoculum**			
Time (Days) frames→ Parameter (Cells/mm^3)	Baseline	2-4	6-10	12-20	Baseline	2-4	6-10	12-20
Total WBCs	4583.0	2.05	1.62	2.29	7117.0	0.91	1.71	1.70
Lymphocytes	3358.8	2.44	1.70	2.30	6052.3	0.80	1.49	1.83
Neutrophils	1046.7	0.99	1.43	2.15	659.8	2.29	3.39	1.27
Monocytes	82.0	1.31	2.07	5.76	265.3	0.60	1.48	0.58

Groups →	**Control 1**				**10^7 inoculum**			
Time (Days) frames→	Baseline	2-4	6-10	12-20	Baseline	2-4	6-10	12-20
Total WBCs	4583.0	2.05	1.62	2.29	7817.0	1.21	1.33	1.00
Lymphocytes	3358.8	2.44	1.70	2.30	5814.7	1.27	1.42	0.93
Neutrophils	1046.7	0.99	1.43	2.15	2002.0	0.91	0.92	1.07
Monocytes	82.0	1.31	2.07	5.76	0.0	-	-	-

Groups →	**Control 2**					**10^5 inoculum**				
Time (Days) frames→	Baseline	2-4	6-10	12-20	22-36	Base-line	2-4	6-10	12-20	22-36
Total WBCs	15720.0	0.39	1.09	1.00	0.87	11470.0	0.60	2.02	1.67	1.94
Lymphocytes	13416.0	0.38	1.14	1.02	0.91	9718.0	0.57	2.01	1.73	2.04
Neutrophils	1977.7	0.45	0.71	0.66	0.63	1576.2	0.79	1.85	0.87	1.20
Monocytes	323.0	0.39	1.16	2.16	0.43	172.5	0.76	5.90	6.47	2.88

the total WBC, monocytes and lymphocytes were negligible for 10^3 inoculum and '10^7 inoculum' groups ($0.60 \geq$ fold change ≤ 1.83) while significant fold changes were observed in the 10^5 inoculum group ($0.57 \geq$ fold change ≤ 6.47).

DISCUSSION

Low immune response by the 10^3 inoculum group is suggested by the lack of variation between it and its control group. We postulate that the low parasite inoculum density gave rise to low malaria parasitaemia that seems to have elicited low cellular immune response. Significant variation in the total WBC, lymphocyte, neutrophil and monocyte counts with the 10^5 inoculum values consistently greater than its control values suggest an adequate cellular immune response. The late lymphocyte response observed in the 10^5 inoculum group could have enhanced the parasitaemia clearance times and is consistent with sequential synergistic action by innate and adaptive immune systems reported by Perlmann et al. (1995) and Troye-Blomberg et al. (1999). In all the parameters assayed, little or no significant variation was seen between the 10^7 inoculum group and its control and suggests an inadequate cellular immune response. Monocyte response was almost non-existent in the 10^7 inoculum group and would have contributed to its infection outcome. High neutrophil counts early in the course of infection amongst the 10^7 inoculum group may have also contributed to the severe infection outcome through the inflammatory response (Chen and Sendo, 2001).

A comparison of the variations amongst the test groups over the study period reveal that some cell types were inadequate. We postulate the 10^5 inoculum group exhibited an appropriate immune response while both 10^3 inoculum and 10^7 inoculum groups exhibited little or no cellular immune response and expectedly there was no significant variation between them. However, significant variations occurred between the 10^3 inoculum and 10^5 inoculum groups and between the 10^5 inoculum and 10^7 inoculum groups in the total WBC, lymphocyte and monocyte counts. This indicates the total WBC count, lymphocytes and monocytes as the particular cells / parameters inadequate. Deficiency in total WBC counts in 10^3 inoculum and 10^7 inoculum groups may indicate inadequate volume response while deficiency in lymphocytes and monocytes show the particular cells implicated. Thus an inadequate immune response by the 10^7 inoculum mice, in volume and type, is suggested.

Using 10^5 inoculum group fold changes as an arbitrary standard, a 6-fold increase in monocytes and a 2-fold increase in both total WBCs and lymphocytes (particularly by the 4th day post-infection) seem to indicate an adequate cellular immune response and result in the clearance of parasitaemia. This precludes parasitaemia clearance when the inoculum parasite density is not

sufficient to establish an infection as seen in the 10^3 inoculum group. There was an indication that the 10^7 inoculum mice failed to mount an adequate immune response. Leucocytes changes pattern/rate confirms this, being the group with the least fold changes and the control group even had more monocytes between days 12 - 20. These suggest that the cellular immune response in the 10^7 inoculum mice was suppressed by the parasite. The malarial parasite is known to interfere with the maturation and presentation of antigens to T cells, turning off the dendritic cells such that the immune system cannot recruit T cells for effective immune response (Urban, 2002). There is also evidence that children with malaria are more vulnerable to other infections and that they may also have reduced immune response to vaccines (Urban, 2002).

Conclusions

Immune suppression by the abundant parasites may be the cause of the inadequate and inappropriate immune response by 10^7 inoculum mice. High parasitaemia enhance the parasite evasion mechanisms and host immunosuppression. The influence of the malaria parasitaemia burden or differing malaria endemicity zones needs to be considered in malaria vaccine development efforts and in monitoring drug resistance in view of the potential for the malaria parasite to suppress the host immune responses. However, given the small sample size used in this study, further follow-up investigation on this phenomenon are necessary.

ACKNOWLEDGEMENTS

We acknowledge the excellent technical help of Mr Samuel Akindele. We also acknowledge Dr O. Aina for providing the parasites and for proof-reading the manuscript. Mr Chimere Agomo also proof-read the manuscript and is acknowledged.

REFERENCES

Agomo PU (1980). Murine malaria – evaluation of cytotoxic mechanisms *in vitro*. PhD Thesis. University of London.

Cheesbrough M (1998). District laboratory practice in tropical countries – part 1. Low-priced edition. Cambridge, Cambridge University Press.

Cheesbrough M (2000). District laboratory practice in tropical countries – part 2. Low-priced edition. Cambridge, Cambridge University Press.

Chen L and Sendo F (2001). Cytokine and chemokine mRNA expression in neutrophils from CBA/NSlc mice infected with *Plasmodium berghei* ANKA that induces experimental cerebral malaria. Parasitol. Int., 50: 139-143.

Ittarat W, Pickard AL, Rattanasinganchan P, Wilairatana P, Looareesuwan S, Emery K, Low J, Udomsangpetch R and Meshnick SR (2003). Recrudescence in artesunate-treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. Am. J. Trop. Med. Hygiene, 68: 147-152.

McGuinness DH, Dehal PK and Pleass RJ (2005). Pattern recognition molecules and innate immunity to parasites. Trends Parasitol., 19: 312-319.

Perlmann H, Kumar S, Vinetz JM, Kullberg M, Miller LH and Perlmann P (1995). Cellular mechanism in the immune response to malaria in *Plasmodium vinckei* – infected mice. Infection Immunity, 63: 3987-3993.

Riley E (2002). Body battleground. In Wellcome News Supplement 6, Research directions in malaria. The Wellcome Trust, London, pp. 14-15.

Sachs J, Malaney P (2002). The economic and social burden of malaria. Nature, 415: 680-685.

Stevenson MM, Riley EM (2004). Innate immunity to malaria. Nature Rev. Immunol. 4: 169-180.

Troye-Blomberg M, Perlmann P, Nilsson M and Perlmann H (1999). Immune regulation of protection and pathogenesis in *Plasmodium falciparum* malaria. Parassitologia, 41: 131-138.

Urban B (2002). Survival instincts. In Wellcome News Supplement 6, Research directions in malaria. The Wellcome Trust, London, pp. 23.

World Health Organisation (1996). World malaria situation in 1993, part 1. Weekly Epidemiological Record. 71: 17-22.