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Original article

Microfluorometric assay for quantification of anti-erythrocytic antibody level in sera of *Plasmodium berghei* ANKA infected semi-immune mice

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Abstract: A simple, sensitive and reproducible fluorometry method was developed to quantify anti-erythrocytic auto-antibody in the sera of semi-immune mice. The level of anti-erythrocytic auto-antibody measured using fluorometry was significantly higher in sera of *Plasmodium berghei* ANKA infected mice than the uninfected, $p< 0.0001$. This result correlates significantly with the standard ELISA assay, $p<0.0001$, $r^2=0.7766$. Both methods show significant correlation with regard to extent of Hb loss, $p<0.006$ ($r^2=0.33$) (fluorometry method) and $p< 0.0001$, $r^2=0.30$ (ELISA). Thus the fluorometry method may serve as an alternative to standard ELISA assay and can be modified to suit similar objectives.

Key words: *Flourometry, ELISA, Plasmodium berghei ANKA, Malaria anaemia*

INTRODUCTION

Due to the consistent observation that severe malaria anaemia (SA) occurs at relatively low parasitaemia [1, 2], the autoimmune mediated destruction of uninfected red blood cells (uRBC) via auto-antibodies is thought to play an additional role in the RBC destruction at this low parasitaemia. Murine and human studies have demonstrated the binding of antibodies to erythrocytes by use of IFA, western blot and ELISA [2-4]. However, few studies have looked at the quantification of auto-antibodies or their correlation with the degree of anaemia during malaria infection [2-4]

Fluorometry is an analytical technique for identifying and characterizing minute amounts of a substance by excitation of the substance with a beam of ultraviolet light and detection and measurement of the characteristic wavelength of the fluorescent light emitted. This assay has been used to study the level of antibodies to DNA in systemic lupus erythematosus (SLE) [5]. In view of the fact that SLE is known to be an auto-immune disease and that the auto-immune mechanism has been implicated in uRBC destruction during malaria infection, it is possible that a fluorometric assay can also be used to estimate the level of anti-erythrocytic antibodies and its correlation with anaemia.

In the present study, we employed a fluorometric assay to assess/quantify anti-erythrocytic antibodies in the serum and to determine the correlation with levels of haemoglobin (Hb) in relation to degree of RBC destruction during malaria infection. One major problem associated with many fluorometric procedures is the inner filtering effect (IFE) [6], that is, the absorption of exciting and/or emitted radiation by dissolved species which can lead to decreased fluorescence intensity. Consequently, we evaluated this fluorometry method with the standard ELISA to confirm its reliability. Also, the use of non-radioactive materials and the easy analysis of large sample sizes are among the possible advantages of this method.

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MATERIALS AND METHODS

Mice, malaria infections, parasitaemia and haemoglobin measurement

Four strains of mice (BALB/c, C57BL/6, CBA and NZW) aged 8 weeks supplied by SLC laboratories, Japan, were injected intraperitoneally (i.p.) with 10^4 *PbANKA*-infected RBCs. Parasitaemia was monitored every 2 days by Giemsa-stained thin blood film and expressed as a percentage of more than 500 RBCs. Hb was measured in a 96-well plate at 570 nm on a Bio-Rad Model 3550 micro plate reader. Four microliters (4 µL) of tail-vein blood was suspended in 1 mL Drabkin reagent (Sigma, St Louis, MO), absorbance measured and is expressed as a percentage of baseline levels. The procedures were conducted according to the laboratory and animal practices of the animal center at the Institute of Tropical Medicine (NEKKEN), Nagasaki, after obtaining approval from the local ethics committee for animal care and research.

Generation of semi-immune mice and harvesting of serum

This modified method has been described elsewhere[1]. Four strains of infected mice were treated at day 6 after infection with chloroquine/ (10 mg/kg intraperitoneally) and pyrimethamine (10 mg/kg intraperitoneally) daily for 6 days. During subsequent rounds of infection, the mice were rested for 2 weeks before being rechallenged with 10^4 *PbANKA*, then monitored and drug-cured prior to parasitaemia reaching 5%. The mice underwent 7 to 8 cycles of drug -cured infection before finally being challenged with 10^4 *P. berghei ANKA* parasites. About 100µl of blood was collected *via* the eye vein during the 7^{th} and 8^{th} cycles of infection at low Hb. The sera were stored at -30 ℃ until use. The entire set of experiments was performed twice.

Preparation of red blood cell (RBC) white ghost membrane

The method used here was based on a previously described method [7] with some modifications. Briefly, heparinised blood (0.5 ml) from uninfected mice was washed with PBS and haemolysed in phosphate buffer (5 mM, pH 8.0). After vigorous shaking, the haemolysate was washed twice with an ample volume of phosphate buffer (5 mM, pH 8.0) for 20 minutes at 15,000rpm. The supernatant was removed by aspiration. The membranes were washed six times with the same haemolysate buffer until the pellet became white, and then washed 2-3 times with Tris-HCl (50 mM, pH 7.2) and finally in PBS. Antigen concentration was determined using a BCA protein assay kit.

Antibody to RBC membrane titer measurement in sera of semi-immune mice via ELISA:

This modified method has been described previously [8]. The RBC white ghost membrane was used as an antigen at a protein concentration of 2µg in 100µl of coating buffer per well to coat polystyrene plates (Nunc, Copenhagen, Denmark) at 4 ℃ overnight. The plates were washed thrice with 0.05% Tween-20-PBS, then optimum blocking conditions for non-specific binding were achieved using 300µl per well of 0.1% blocking reagent (Lot No. 13945300, Roche Diagnostics GmbH, Mannheim, Germany) -0.1% Tween-20/PBS, pH 7.2, and incubated for 1 hour at 37 ℃. Plates were washed thrice with PBS containing 0.05% Tween-20. To optimize the assay, sera titration was done from 1:10 to 1:5120, with the observation that 1: 40 gave a good signal for sera from all mice strains. The antigen in coated plates was then reacted with the serum samples obtained from non-infected (as negative control) and infected mice at 1/40 dilutions, in duplicates. After 3 hours incubation at 37 ℃, plates were washed 5 times with 0.05% Tween-20/PBS. Later, 100µl of horse radish peroxidise (HRP)-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) was added to each well and incubated for 1 hour at 37 ℃, then washed 5 times with 0.05% Tween-20/PBS. For color development, 3, 3', 5, 5'-tetramethylbenzidine (TMB, Catalog number SK-4400, Vector Laboratories, CA, USA) was used and prepared according to the manufacturer's instructions. The reaction was then interrupted at 30 minutes by the addition of 50µl 1N H_2SO_4. Absorbance was read at 450nm using an EIA-reader (Bio-Rad, Hercules, CA)

Antibody titer measurement using fluorometric method:

The white ghost RBC membranes prepared as described above were resuspended to make 5% suspension in PBS. (This was arrived at after a series of dilutions to estimate the best dilution for efficient signal, as shown in Figure 1). After stirring gently to ensure uniform mixture, 100 µl was pipetted into a 96-well optiplex plate (lot number 6651645/0606/12, PerkinElmer™ Life Sciences, The Netherlands), blocked with 200µl of goat serum, Chemicon International, CA (1:100) for 30 minutes, and washed twice at 780 *g* for 10 minutes. To optimize the assay, sera titration was done from 1:10 to 1:5120, with the observation that 1: 160 gave a good signal for all the mice strain sera. Subsequently, 30µl of sera (1:160) of the mice strains was added to the 100µl 5% RBC ghost antigen, incubated at RT for 3 hours and washed twice in PBS at 2,500rpm for 10 minutes. Fifteen microliters (15µl) of goat anti-mouse IgG-FITC (Sigma-Aldrich, St Loius, Missouri, USA) diluted 1:50 was added and incubated in the dark at RT for 1 hour, followed

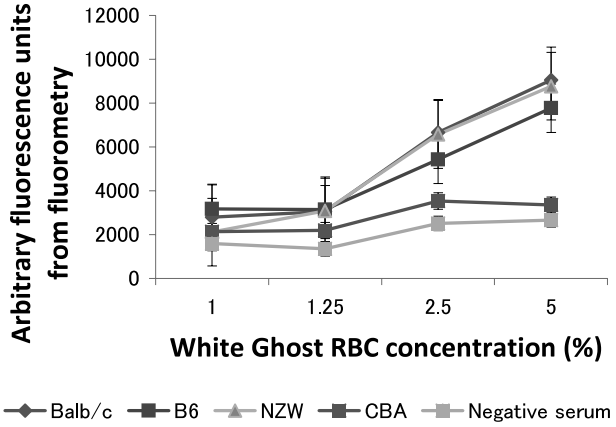

Figure 1: Intensity for varying RBC concentration in the sera of semi-immune mice

Ghost RBC was prepared as described (see materials and methods) and was titrated against sera (1:160) from the semi-immune mice as well as controls. This was to select an appropriate Ghost RBC concentration for effective signal determination. Values are means of duplicate experiments, and error bars show standard error. The total number of mice number was 4 (1 per strain).

by washing and centrifuging twice at 780 *g* for 10mins per wash. During washing, the supernatant was carefully and gently aspirated. Next, a hundred microliters (100µl) of PBS was added and read immediately at 485nm/525nm for 1.0 second in the fluorometry instrument (Perkin Elmer[precisely] 1420 Multilabel Counter, Finland). All experiments were done in duplicate.

Data Analysis:

Graphpad prism version 4.0 was used to analyze the data. Student t test was used to compare the results between the 2 groups. Fluorescent intensity values and ELISA OD were logarithmically transformed (unless otherwise stated) to achieve normal distribution. Linear regression analysis was used to compare association between variables. Values were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

With the flourometry method we measured the anti-erythrocytic antibodies in sera of both uninfected and infected mice and observed a statistical difference between them, $p<0.001$ (Table 1). A much greater number of anti-erythrocytic antibodies was also observed in the sera of highly anaemic mice due to *Plasmodium berghei* ANKA infection, with a statistically significant positive correlation, $r^2=0.33$, $p<0.006$, Figure 2a. ELISA values also correlated significantly with the extent of Hb loss, $r^2=0.30$, $p<0.0001$ (Figure 2b). To further evaluate the reliability of the fluorometry assay developed here, we compared fluorometry fluorescence intensity with the standard ELISA and found a strong positive correlation between the two ($r^2=0.7766$, $p<0.0001$), Figure 3. The measurement and comparison of anti-erythrocytic antibodies by means of ELISA and fluorometry was also analyzed in the sera of mice at recovery, and a positive, statistically significant correlation was observed between them, $p<0.0001$, $r^2=0.56$ (data not shown).

The results presented here show that the quantification of anti-erythrocytic antibodies can be successfully per-

Table 1: Anti-erythrocytic antibody level in uninfected and infected semi-immune mice

Mouse type	n	*Anti-erythrocytic antibody level, Mean (SD)
Uninfected	8	3054(1568)
Infected	22	16827(9371)
P value	-	<0.0001

*Anti-erythrocytic antibody level was determined by fluorometry as described in Materials and Methods. SD= standard deviation.

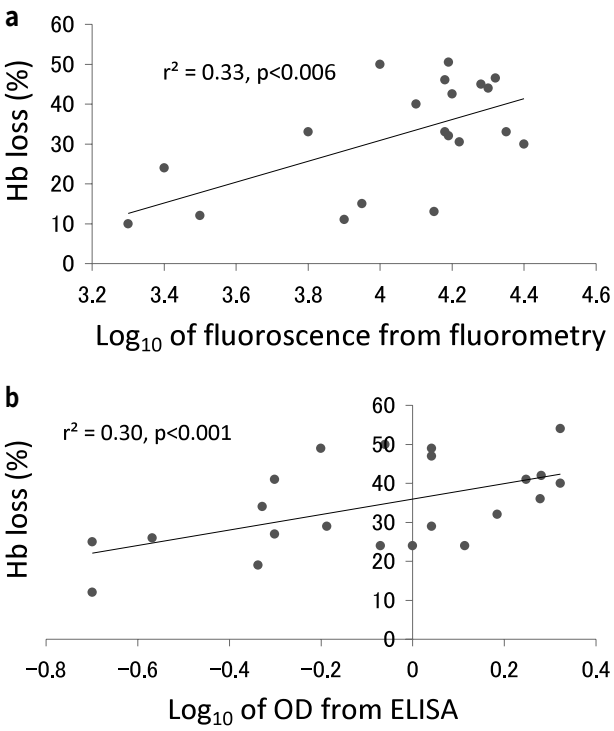

Figure 2:

a. Relationship between haemoglobin loss and fluorescence intensity

b: Relationship between haemoglobin loss and ELISA values

Sera were obtained from all the semi-immune mice (Balb/c, B6, CBA, NZW), n=20. Anti-erythrocytic antibodies were measured using fluorometry (see Materials and Methods for procedure). Fluorometry fluorescence intensities were compared with Hb. Hb loss was the difference between Hb at infection when mice were sacrificed, and Hb before the first cycle of infection. Hb, haemoglobin.

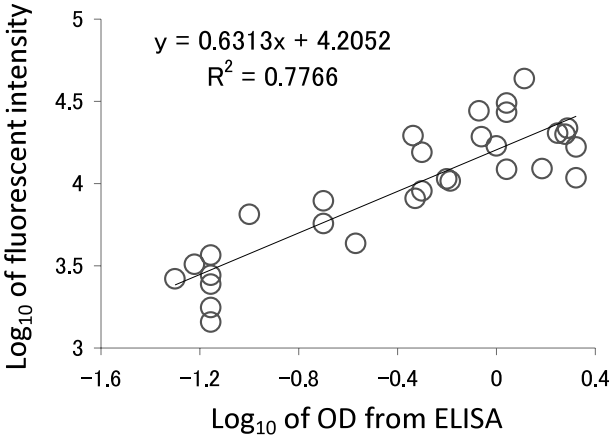

Figure 3: Correlation between ELISA and fluorometry

Sera were obtained from all semi-immune *P berghei* ANKA infected mice (Balb/c, B6, NZW and CBA, n=21) and uninfected mice (Balb/c, B 6, NZW and CBA, n=9), and anti-erythrocytic antibodies measured using ELISA and fluorometry (see Materials and Methods for procedure). The ODs from ELISA and the fluorometry fluorescence intensities were compared. OD, Optical density.

formed in malaria anaemic individuals by the use of fluorometry as well as ELISA. The significant correlation of the ELISA and fluorometry results reported here is consistent with data from a study on SLE, an auto-immune disease [5]. The clearly different fluorescence intensity between the uninfected and the *PbANKA* infected mice shows the important role of these anti-erythrocytic antibodies, which could therefore be employed in the quantitative flourometric approach to estimating immunological events related to the pathogenesis of SA.

To our knowledge, our use of fluorometry to assess anti-erythrocytic antibodies in malaria infection is the first to date. Most fluorometric studies such as the Farr assay, are centered on estimating circulating levels of anti-DNA antibodies [5], especially for SLE, because the assessment of anti-DNA has become a standard clinical procedure in assessing these patients [9]. The use of radioactive materials is one of the disadvantages of the Farr assay. As a result, Bjorkman et al. [5] have used Pico green as a dye for modification. However, we used FITC in the fluorometric assay reported here, not radioactive material, thus making it very safe, sensitive and friendly to use. IFE is undoubtedly involved here, but we did not take measures to control it in this study. Increase in temperature and considerations of linearity in Stern-Volmer plots have been recommended in resolving these issues [6]. The use of fluorometric assay as described here has some other advantages, such as (1) the speed using a microplate reader; (2) no need for overnight coating; (3) no need to determine protein concentration; (4) screening/analyzes of large sample sizes; (5) safe, simple, and free from difficult techniques.

In conclusion, the results of the present study confirm the fact that binding of antibody to erythrocytes can be quantified by fluorometry and that this assay can be modified and perfected for other similar objectives as an alternative to ELISA.

COMPETING INTEREST

None of the authors of the manuscript have any competing interest due to commercial or other affiliations.

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