TO COMPARE THE EFFECTIVENESS OF FRACTIONATED AND UNFRACTIONATED MALARIA INFECTED ERYTHROCYTES

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Abstract: In this study, the effectiveness of Unfractionated blood (% parasitemia was around 35%) as well as fractionated / treated/ purified infected erythrocytes (% parasitemia was around 80-90%) was compared. For this, the infected erythrocytes were separated from the blood by Percoll density gradient method. This infected cells rich fraction *i.e* Fractionated infected erythrocytes were having around 80% infected cells. The viability of fractionated infected erythrocytes was checked by dye exclusive test and were found to be 90%. For dose preparation, the volume of dose in case of unfractionated suspension was more as normal cells were also there (as % parasitemia was 35%). Where as in case of fractionated suspension, the % parasitemia was 80-85%, so the dose was mainly comprised of infected erythrocytes, thus the volume of dose was also comparatively less. Both the above preparations *i.e* unfractionated blood as well as fractionated infected erythrocytes (having $1x10^7$ infected erythrocytes each) were injected in two mice and noted the infection pattern till the death of the animal.The dose prepared by using fractionated erythrocytes was found to be equally effective.

Key words: Malaria, *P. berghei*, infected erythrocytes, parasitemia.

INTRODUCTION

The Disease: Malaria remains one of the most ravaging diseases of mankind and constitutes major public health problem for the large part of the world population. It still ranks high among the main causes of mortality and morbidity due to infectious diseases. The disease is quite prevalent in tropics. Unsuccessfulness of vector control programme and emergence of drug resistance towards various anti-malarial compounds have further compounded the problem.

Malaria in India Epidemiology of malaria varies considerably from country to country and even within the country due to variation in ecology, geography, movement of large number of people into endemic areas etc. (WHO, 1995).In India, resistance to malaria began in Assam in 1973. The resistance develops when *Plasmodium* encounters low concentration of drug which is not potent enough to kill it. In early 1960, malaria was almost eradicated from India (Sharma, 1996), as the magic bullet, DDT decimated the mosquito. The mortality and morbidity reduced considerably. In 1966-67 there was reemergence of malaria and cases of malaria in urban areas started multiplying, the use of malathion did not succeed and the number of malaria cases increased (Sharma, 1996). In 1976, National Malaria Eradication Programme (NMEP) recorded 6.4 million cases. The situation was improved for next 5-6 years and cases were reduced to 2 million (Sharma, 1996). In 1980s, there were regular malaria deaths and in 1990s, there was malaria epidemics.

Malaria parasite and erythrocyte stages of malaria infection

Malaria is caused by protozoan parasite of the genus *Plasmodium.* There are hundreds of species of *Plasmodium* which infect primates, rodents and birds. Few among those, causing malaria in humans and rodents are: Human malaria parasites: *P. vivax, P. falciparum, P. ovale and P. malariae*. Rodent malaria parasites: *P. berghei, P.yoelii, P. chabaudi and P. vinckei*. In human malaria, *P. falciparum* is considered to be the most dangerous form. It causes

malignant tertian malaria with symptoms: high fever, shivering, pain in joints and headache. The whole cycle in blood takes 48 h. If untreated, may lead to fatal cerebral malaria.

Murine parasites resembles closely with other mammalian malaria parasites (Aikawa *et al*., 1990). There are similarities in the *P. berghei* and *P. falciparum* parasite, regarding mechanism of drug resistance and conservation of housekeeping genes (Cowman *et al*., 1990). Murine parasites resembles closely with other mammalian malaria parasites. Hence, rodent parasites, being easy and safe to handle, are still considered to be a valuable models in malaria research, specifically for malaria vaccine development, drug testing etc. *P. berghei* has been used as a model of choice since long, to understand the basic biology of malaria parasite (Badell *et al*., 1995). The *in vivo* and *in vitro* drug susceptibility of *P. falciparum* and *P. berghei*, excluding the influence of host factors (Janse and Waters, 1995).It is also useful in determining the stage specific action of therapeutic agents (Janse and Waters, 1995). The *P. berghei* (rodent model) is also useful in the study of molecular biology and cell differentiation. The knowledge is limited in basic molecular processes involved in cell differentiation of *Plasmodium*.

In *P. berghei* infection, large number of sexual/asexual can be made available; the regulation of differential gene expression during sexual and asexual development has been studied in *P. berghei*. The infected cells can be separated by Percoll step gradient. Nevertheless, the poorly understood malaria pathogenicity, critical cell types and interleukins involved in the triggering of pathological events by the malaria parasite can be better analysed (Badell *et al*., 1995). Reprogramming of subpopulation of human blood neutrophils has also been done (Chakravarti, 2009). Percoll step gradient has been used successfully for the isolation of various cell types since long. Isolation of a mouse bone marrow population enriched in stem cell was done by Percoll gradient (Rosca and Burlacu, 2010). In the present study, the infected blood suspension was processed for Percoll Density Gradient Method to get infected cell enriched fraction.Unfractionated blood as well as fractionated infected erythrocytes (having $1x10^7$ infected erythrocytes each) were injected in two mice and noted the infection pattern till the death of the animal.

MATERIALS AND METHODS

Animals: Animals taken for the study were inbreed female BALB/c mice in an age group of 8-10 weeks with an average body weights of 20 ± 2 g.

Preparation of *P. berghei* infected unfractionated and fractionated dose

The dose of 1×10^7 infected erythrocytes was inoculated intraperitoneally per mouse. Parasitemia was checked daily by Giemsa stained blood smear. Parasitemia reached upto 35-40% on 7^{th} day followed by death of the animal on 8^{th} or 9^{th} day post infection. Infected animal was anesthesized on day 7 post infection and the blood was collected. This was termed as Unfractionated blood.

Infected animal was anesthesized on day 7 post infection and the blood was collected through juglar vein in PBS solution, containing Heparin (25units of heparin per ml of PBS). The infected blood was washed with PBS and suspended in 20% v/v PBS. The suspension was processed for Percoll Density Gradient Method to get infected cell enriched fraction (Krungkrai *et al*.,1985 and Hansen *et al*., 2013)). This was termed as fractionated infected erythrocytes.

Both the above preparations *i.e* unfractionated blood as well as fractionated infected erythrocytes (having $1x10^7$ infected erythrocytes each) were injected in two mice and noted the infection pattern till the death of the animal.

Preparation of *P. berghei* infected erythrocyte rich membrane fraction

P. berghei infected erythrocyte membrane rich fraction was prepared by earlier described method (Joshi et al., 1987). Infected erythrocytes lysed with 10 volumes of 20 mM phosphate buffer, pH 7.6 containing 0.1 mM EDTA at 4°C. The cell lysate was centrifuged at 750 x g for 5 min. to separate parasite pellet was first centrifuged at 2250 x g for 7

min. at 4°C followed by another centrifugation of the supernatant at 20,000 x g for 45 min. at 4°C. The pellet of membrane rich fractions was used for immunizations.

RESULT AND DISCUSSION

P. berghei infection or Parasitemia reached upto 35-40% by 7th day followed by death of the animal on 8th or 9th day post infection. Parasitemia was checked daily by Giemsa stained blood smear (Fig. 1).

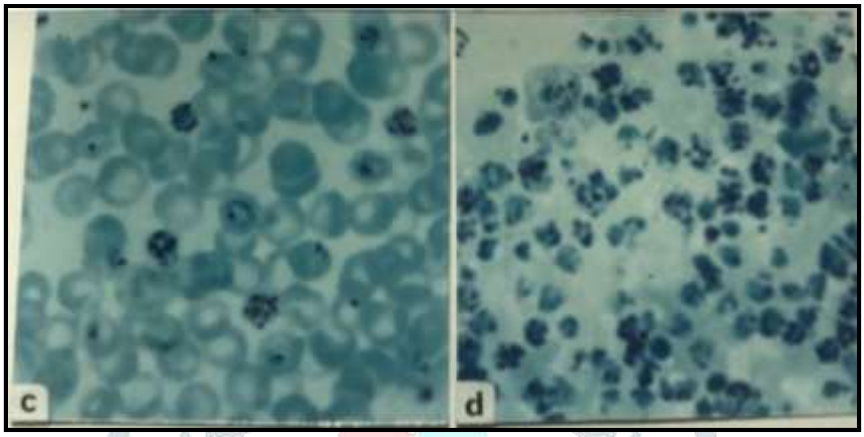

Fig 1. Micrograph showing Giemsa stained *P. berghei* infected erythrocytes (IRBC). c) Unfractionated blood. d) Fractionated/ Enriched infected erythrocytes

The infected erythrocytes were separated from uninfected erythrocytes by Percoll density gradient method. This infected cells rich fraction *i.e* Fractionated infected erythrocytes was having around 80% infected cells. The viability of fractionated infected erythrocytes was checked by dye exclusive test and were found to be 90% viable.
Unfractionated blood as well as fractionated infected erythrocytes (having 1x10^7 infected erythrocytes each) were injected in two mice and noted the infection pattern till the death of the animal. Both the doses were found to be equally effective showing that the fractionation of IRBC by Percoll Density Gradient does not affect the effectiveness and viability of infected erythrocytes (Fig. 2).

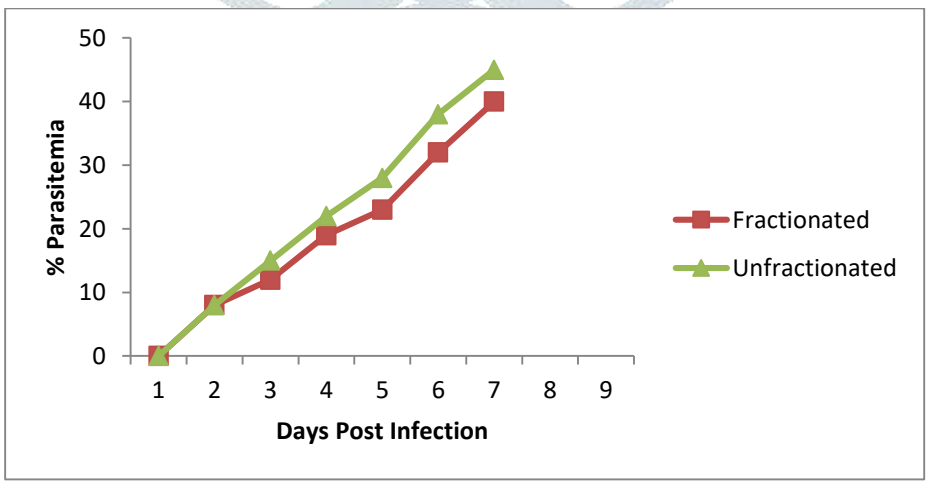

Fig 2. *In-vivo* infectivity pattern of unfractionated blood and fractionated IRBC showing % parasitemia. Values represent mean of triplicates.

For dose preparation, the volume of dose in case of unfractionated suspension was more as for injected $1X10^7$ infected cells, normal cells were also there as % parasitemia was 35%. Where as in case of fractionated suspension, the % parasitemia was 80-85% as dose was mainly comprised of infected erythrocytes so the volume of dose was also comparatively less. The dose prepared by using fractionated erythrocytes was found to be equally effective. Main precaution which should be taken is that the Percoll step gradient method should be performed quickly and by maintaining the temperature of 4°C.

Moreover, when Immunofluorescence assay (IFA) and Enzyme Linked Immunosorbant Assay (ELISA) were performed using standard protocol, to see the reactivity of antiserum prepared by injecting *P. berghei* infected erythrocyte membrane rich fraction, the antiserum showed reactivity with infected erythrocytes up to 1:400 dilution of antisera (Fig. 3) as well as with parasite lysate at dilution 1:800 (Table 1). Thus it can be concluded that the fractionated infected erythrocytes were found to be equally effective as unfractionated blood.

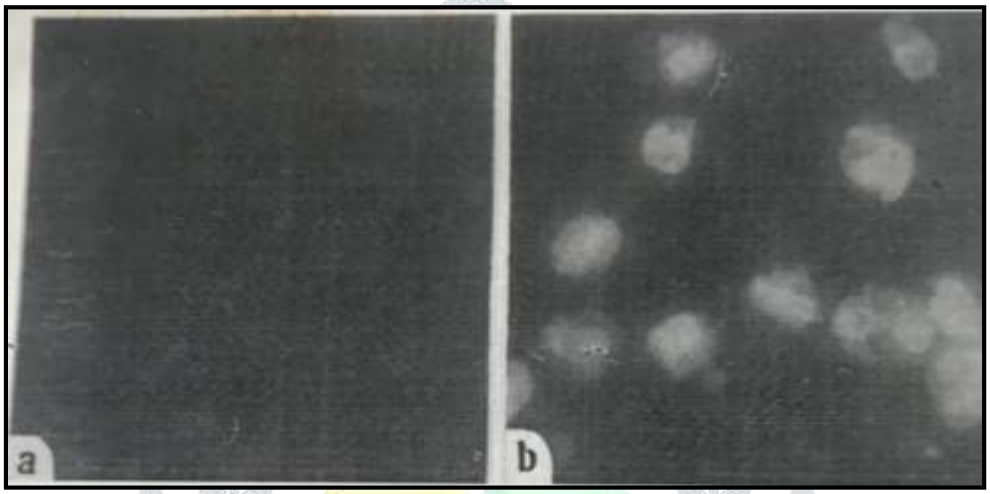

Fig 3. Fluorescence micrograph showing the reactivity of anti *P. berghei* antiserum raised by injecting infected erythrocyte rich membrane fraction. a) With normal RBCs b) With infected RBCs. The air dried monolayer of cells was incubated with serum followed by incubation with FITC conjugated secondary antibody

TABLE 3: Enzyme linked immunosorbent assay (ELISA) to see the reactivity of anti *P. berghei* antiserum raised by injecting infected erythrocyte rich membrane fraction with parasite lysate.

Sr. No	Parasite lysate	Test/Control serum	Absorbance at different dilutions (405 nm)a					
			1:100	1:200	1:400	1:800	1:1600	1:3200
1.	*P. berghei*	Normal Serum	0.201±0.00	0.104±0.00	0.067±0.00	0.039±0.00	0.006±0.00	0.001±0.00
		Raised Antiserum	1.109±0.02	0.950±0.01	0.802±0.00	0.675±0.00	0.173±0.00	0.015±0.00

Values represent mean ±S.D.

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