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CHEMICAL SYNTHESIS OF HISTIDINE RICH PROTEIN (HRP-2) ANTIGEN PEPTIDE FROM *PLASMODIUM FALCIPARUM* AND DETECTION OF ITS IMMUNOGENICITY

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ABSTRACT

Malaria is the one of the most prevalent human infections in the world. It causes significant morbidity & mortality worldwide, including countries with mainly imported malaria. Malaria parasite *Plasmodium falciparum* is the most common cause of severe & life threatening malaria & accounts over 1 million deaths every year. Microscopy of Giemsa-stained thick & thin films remains the current gold standard for diagnosis. Although it has good sensitivity & allows species diagnosis, it is time consuming, required equipments & skilled personnel. Antigen tests based on antigen capture assay are capable of detecting fewer parasites and of producing the results more quickly. Such antigen which is shown promising results is *plasmodium falciparum* histidine rich protein-2 (PfHRP-2). HRP-2 is contained tandem repeats of the sequences AHH & AHHAAD and has B-cells epitopes that allows its detection. In this study chemically HRP-2 antigen peptides were synthesized by solid phase technique & purified by HPLC gel chromatography. Microsphere (PLGA & PVA) entrapped peptides are injected to mice & rabbits subsequent booster doses. The high titre/affinity antibodies raised against these peptide antigens were estimated by indirect ELISA technique. The peptide specific peak absorbance varied from 0.6 to 2.6. The variation in the titre of antibody generated in mice & rabbits depend upon the individual immune responsive capacity. Incremental increase in antibody titre with microscope delivery may result in sustained release of the antigen.

Our studies show that chemically synthesized peptides of PfHRP-2 delivered in microsphere were able to generate immune response that can be quantified. This paves for development of tools for early diagnosis of malaria.

Keywords: HRP-2, LDH, Malaria, Microsphere, Poly lactic co glycolic acid (PLGA), Poly vinyl alcohol (PVA), *plasmodium falciparum*.

INTRODUCTION

Malaria is one of the most prevalent human infections world-wide. Over 40% of the world's population lives in the malaria endemic areas. An estimated 300-500 million cases and 2-3 million deaths are reported every year (Tomar D. et al). The estimated economic loss due to malaria in India from 1990-1993 is Rs. 286 million to Rs. 28594 million (Lema O.E. et al). Malaria is caused by the parasite *Plasmodium*, *P.falciparum*; *P.ovalae*; *P.malariae* and *P. vivax*.

P. falciparum causes malignant tertian malaria, which kills people through cerebral malaria or renal failure. *Plasmodium falciparum* is the most common cause of severe and life-threatening malaria and accounts for over one million deaths every year. The mortality rates in adults being higher than in children (Tomar D. et al).

Since the discovery of the malaria parasite over 100 years ago, the diagnosis of malaria still depends on examination of stained blood film. This allows species diagnosis, estimation of

parasitaemia, identification of stages of parasite development (Silamut and White, 1993), and assessment of prognosis (Field, 1949; Payne, 1988). The detection of parasite antigens or nucleic acid has been investigated for many years as a possible alternative to microscopy. Fast and simple immunochromatographic tests are commercially available for the diagnosis of malaria. Laboratory trials of the Parasight –F test have shown an overall average sensitivity of 77-98% and specificity of 83-98% when >100 parasites / μ l are present compared with microscopy (Beadle *et al*, 1994; Breier *et al*, 2000; Kilian *et al*, 1997). Several diagnostic techniques have been developed for the detection of malaria antigens (WHO, 1988; Tharavanji, 1990; Kawamoto, 1991) but none has been used widely because of their complexity, low sensitivity and the requirement for expensive equipment, reagents and skilled personnel. In the last few years, efforts to replace the traditional and tedious reading of blood smears have brought to techniques for the detection of

malaria parasites which yield sensitivity equivalent to or better than microscopy. Antigen tests are gaining popularity for diagnosing tropical diseases because antigen capture assay tests are capable of detecting fewer parasites and of producing the results more quickly. They do not require extensive training or equipment to perform or to interpret the results. Two such antigens, which have shown promising results both in the laboratory and in the field, are *Plasmodium falciparum* histidine rich protein-2 (HRP-2) and lactate dehydrogenase (LDH). HRP-2 contains 34% histidine, 37% alanine and 10% aspartic acid as deduced from cloned genomic DNA (Wellems & Howard *et al*, 1986) and is characterized by many tandem repeats of the sequences AHH and AHHAAD. As a result, HRP-2 has repetitive B cell epitopes that allow its detection using antigen capture assay (Torii & Ikawa, 1998). HRP-2 is water soluble protein synthesized throughout the asexual cycle and young gametocytes of *P. falciparum* (Rock *et al*, 1987).

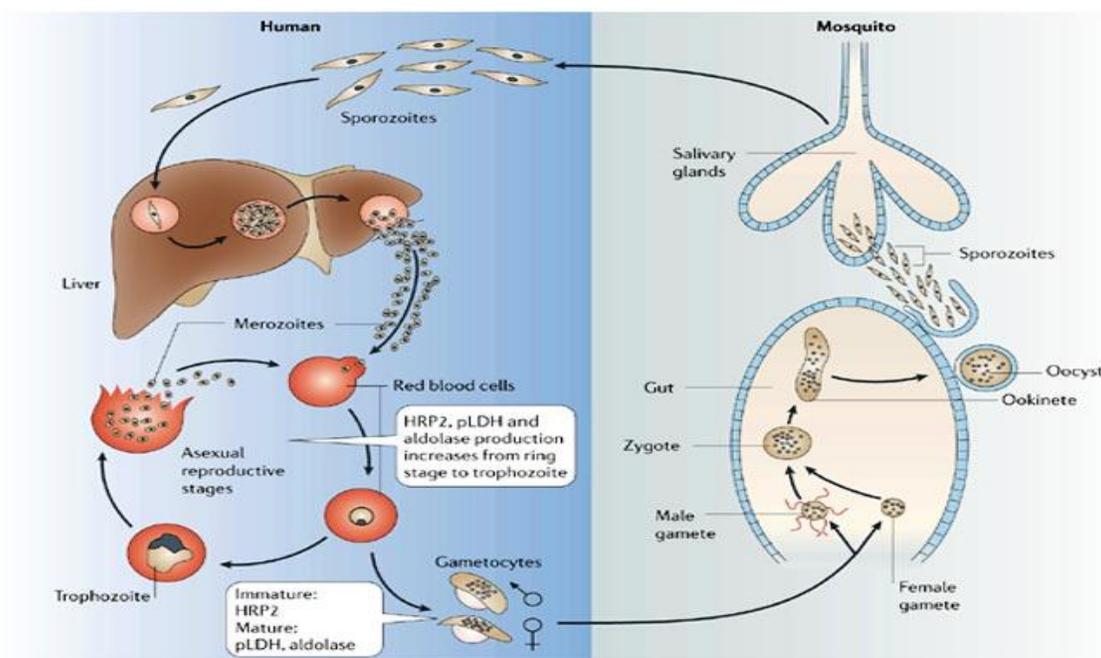


Figure:1- Stages of development of *Plasmodium falciparum* at which antigens detected by malaria rapid diagnostic tests are produced. (*Nature reviews microbiology*, Talman A. *et al*)

HRP-2 is expressed the RBC and is transported in concentration “ packets” through the red cells cytoplasm and released from intact infected cell into the culture medium (Howard et al,1986). It circulates in the blood of human host for up to 14 days post infection and its C-terminal half induces a partially protective response (Parra et al, 1991) in culture of *P. falciparum*, HRP-2 can be detected in the supernatant medium just 2-8 hrs after merozoite invasion, indicating its active secretion from infected erythrocytes (Howard et al, 1986). After shizont rupture in vitro, the amount of HRP-2 increases in the supernatant medium. HRP2 is also found in immature gametocytes and pLDH (and probably aldolase) is found in mature gametocytes (Howard et al, 1986).

In the present study, the two HRP-2 peptide sequences were chosen, which occur as tandem repetitive immunodominant epitopes in the HRP-2 protein. The microsphere delivery of immunization was attempted to generate high titre and high affinity antibodies. The aim of present study was to gain expertise in solid phase peptide synthesis and detecting its immunogenicity in rabbits and mice.

MATERIALS AND METHODS

The study was conducted in the department of Biochemistry, All India Institute of Medical sciences, New Delhi, India.

Peptide Synthesis and Purification- The following peptide sequences were selected from the HRP-2 of *P. falciparum*. Solid phase peptide synthesis strategy (Merrifield R.B.et al) was used for the synthesis of following sequences using F-moc chemistry on C-terminal amino acid substituted on PAM resin (Caprino L.A. et al). The sequences were AHH(AHHAAD)₃ (HRP-2 peptide-I), (AHHAA)₃ (HRP-2 peptide-II). Kaiser test was done to confirm coupling of amino acids. The peptides prepared were passed through gel chromatography with sephadex G-25 column. The spectrophotometer reading was taken and

those fractions showing high absorbance were pooled and lyophilized, secondly those fractions showing identical Rf value during paper chromatography were also taken into criteria. Authenticity for the N terminal analysis, the peptide and the appropriate standard amino acid were first labeled with 1-Fluoro-2,4 dinitro benzene (FDNB) hydrolysed with HCl and the resultant hydrolysed samples were then spotted onto whatman paper 1. C-terminal amino acid identification of the peptide was done by carboxypeptidase A. This method is described by Bradshaw (Tomar D. et al,2006 & Sharma V.P. et al,1996)

Microsphere Protocol- Microparticles with entrapped peptides were prepared using poly lactic co glycolic acid (PLGA) & Poly vinyl alcohol (PVA) by water-in-oil-in-water (w/o/w) solvent evaporation technique (Smith et al, 1985). Antigen load in microspheres was estimated by double solvent method. The percentage peptide entrapment in the microparticles was determined by BCA (bicinchoninic acid) method (Sigma, st. Louis USA) following SDS alkaline dissolution of the microparticles (Tomar D. et al).

Animal species & Immunization- Outbred species of mice (4-6 weeks old) and rabbit (2-3 months old) were produced from the Experimental Animal Facility, AIIMS, New Delhi, India. All the animals were provided food and water *ad libitum*. Each experimental group consisted of 3 mice in the group of three & 2 rabbit in group of three. These animals were used for developing of IgG antibodies against synthetic HRP-2 peptides (HRP-I & HRP-II). All experiments were conducted in accordance with the guideline of Committee for Prevention and Supervision of Experiments on animals, for the care and use of laboratory animals (CPSEA), Ministry of Social justice, Government of India and adopted by the Ethics Committee on Animal Experimentation, AIIMS, New Delhi. Mice (3 in a group of three) and rabbits (2 in a group of three) were immunized with the

microspheres with entrapped antigen. The mice were given a dose of 30µg of microsphere/mice in HRP peptide I (via subcutaneous & intramuscular route) and 100µg for rabbits in HRP II (only subcutaneous route), on day zero. Then the booster doses of 30µg of microsphere/mice on 22nd day and 100 µg of microsphere/rabbit on 36th day were given on subsequent intervals. Mice & rabbits were bled from retro-orbital plexus and bleeds were collected on day 15, 28, 42. The sera were separated and kept at -20°C until use.

Elisa- Peptide specific antibody levels and peak titres were estimating using indirect ELISA protocol. Briefly 100ng/100µl of the peptide was coated per well (Peptide stock: 1mg/ml) and incubated with test sample (mice/rabbit sera) at a fixed dilution (1:100, serum:PBS 1X) for serum IgG levels. Absorbance was read at 492 nm.

RESULTS

HRP-2 is a water soluble protein synthesized throughout the asexual cycle and young gametocytes of the plasmodium falciparum. It is expressed on the RBC membrane and is released from intact infected cells.

The peptides were prepared by solid phase peptide synthesis, were passed through gel chromatography with sephadex G-25 column. The purity of each peptide was assessed by HPLC and gel column chromatography and was found to be >90%. Authenticity of the peptide were analysed by N and C terminal identification of peptide. Rf values of the DNP standard amino acid were compared with that of standard amino acid as shown in table 1. Amino acids sequence analysis were verified by method described by Bradshaw (Tomar D. et al,2006 & Sharma V.P. et al,1996) The microsphere were prepared and

checked for its antigen entrapment efficiency by BCA method – their entrapment comes around 70-75% which was adequate for immunogenicity. Prepared microspheres were sized using a diffraction spectrophotometer (size range- 5-10 µm). The microparticles morphology was studied by scanning electron microscopy (Phillips, CM10) (figure 2A and 2B). After Immunization the bleeds were collected and indirect ELISA was done for the estimation of IgG levels the serum. The peptide specific IgG levels were tested in each individual peptide antiserum of all the bleeds. Peptides entrapped in microsphere generated high peptide specific IgG levels in all the bleeds and the antibody levels were maintained till 42 days postimmunization with all the peptides of HRP-2 antigens.

In mice via subcutaneous route, there was not much difference in the first, second and third bleed sera but all these show much higher values as compared with preimmunized sera (Absorbance: 0.7) shown in table-2A (figure: 3A). There was marked increase in the IgG level on second bleed sera in mice via Intramuscular route on day 28th, after giving booster doses when it was compared with preimmunized sera (Absorbance: 0.7) and first bleed sera on day 15th. The second bleed was still higher when it was compared with last bleed sera on day 42nd as shown in table-2B(figure: 3B).

In rabbits, the mode of immunization was only subcutaneous. The booster dose was given on day 36th so the third bleed sera response was much higher than the first, second and preimmunized sera (Absorbance: 0.7) as shown in table:2C/figure: 3C.

There was a significant rise in the peptide specific IgG level with maximum at third bleed in Rabbits and second bleed in mice.

Table:1- Authenticity of the peptide were analyzed by N and C terminal identification of peptide

N-terminal analysis of DNP-amino acid by paper chromatograph				C-terminal analysis of amino acid by paper chromatography			
Peptide	Amino acid	standard Rf value	sample Rf value	Peptide	amino acid	standard Rf value	sample Rf value
HRP-I	Alanine	0.61	0.64	HRP-I	Alanine	0.61	0.64
HRP-II	Alanine	0.61	0.65	HRP-II	Alanine	0.61	0.65

Solvent system :- Acetic acid:Butanol:Water:Ethyl Acetate(1:1:1:1)v/v

Table:2 - Peptide specific IgG level against HRP-2 in mice and rabbits after immunization**Table 2A:-**

2A: HRP-2	Immunized mice/Group & Absorbance/O.D.		DAY-15	DAY-28	DAY-42
Peptide-I Subcutaneous Route (mice)	Mice(Group-1)-	Absorbance	1.9	2.2	2.0
	Mice(Group-2)-	Absorbance	2.2	2.3	2.1
	Mice(Group-3)-	Absorbance	2.0	2.3	1.8

Table 2B & 2C :-

2B: HRP-2	Immunized mice/Group & Absorbance/O.D.		DAY-15	DAY-28	DAY-42
Peptide-I Intramuscular Route (mice)	Mice (Group-1)-	Absorbance	1.0	1.5	1.3
	Mice (Group-2)-	Absorbance	1.8	2.7	1.5
	Mice (Group-3)-	Absorbance	0.9	1.6	0.7

2C: HRP-2	Immunized mice/Group & Absorbance/O.D.		DAY-15	DAY-28	DAY-42
Peptide-II Subcutaneous Route (rabbit)	Rabbit (Group-1)-	Absorbance	1.4	1.9	1.4
	Rabbit (Group-2)-	Absorbance	1.2	1.8	1.1
	Rabbit (Group-3)-	Absorbance	1.7	1.6	1.9

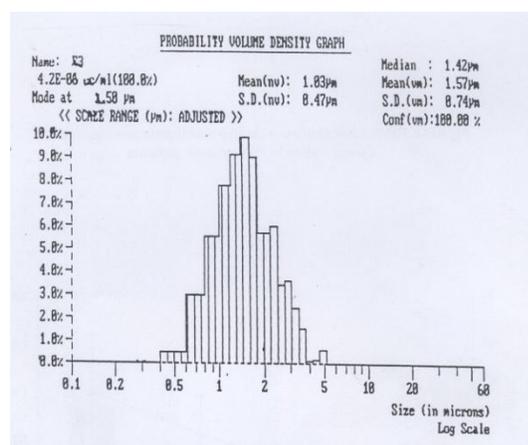
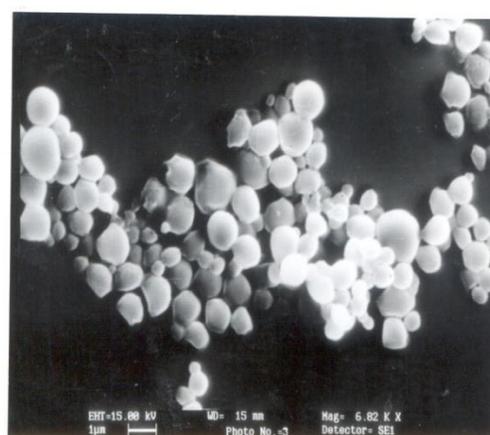
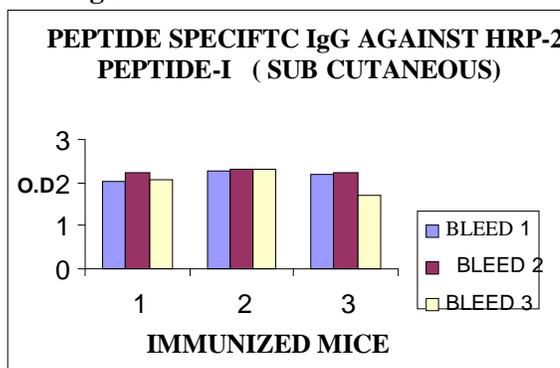
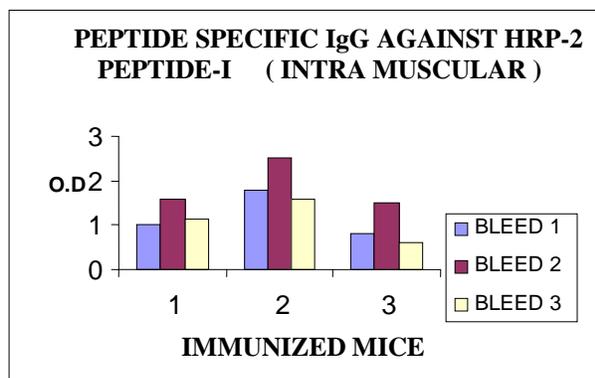
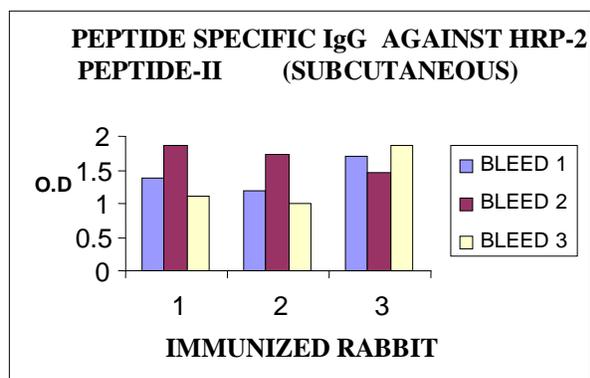
**Figure: 2A - Particle size distribution plot of microsphere preparation****Figure: 2B - Scanning electron micrograph of microsphere preparation**

Figure:3 - IgG level in mice and rabbits after immunization**Figure: 3A****Figure: 3B****Figure: 3C**

DISCUSSION

The aim of the present study was synthesis of solid phase peptide and to generate the high titre/affinity antibody in the mice and rabbits using antigen capture assay and detect its immunogenicity. Delivery of HRP-2 in microspheres not only generated high titre/affinity antibodies in mice and rabbits, but also maintained the peak titre over long duration without booster immunization. Sera that showed high titre and affinity were selected for developing the immunoassay (Tomar D. et al).

Antigen capture assay is sensitive and specific easy to perform, does not require specialized equipment and requires a small amount of unprocessed whole blood. Minimal training is required to perform the assay (Braunwald et al). Our studies show that the mice immunized with

antigen entrapped microsphere through subcutaneous route of administration gives better response in comparison to that of intramuscular route as it shows high titre antibody early and late response following the initial and booster doses. In rabbits only subcutaneous route was used for administration of antigen which gives high titre values. Incremental increase in antibody titre seen with microsphere delivery may result in sustained release of the antigen. Microsphere presents the small amount of antigen continuously to the immune system to stimulate the correct repertoire of B cells (i.e. secreting antibodies of the high affinity), also a very critical point in view of the antibodies generation was that only a single dose of the peptide antigen in microsphere was enough to generate long lasting and high titre. (Tomar D. et al, 2006)

In future, on the basis of the present study, for developing an efficient vaccine or immunoassay, not only the titre, but also the affinities of the antibody do have remarkable influences for its sensitivity. Thus high affinity antibodies are important in control of parasitaemia so that even very small amounts can be detected and neutralized. This paves way for development of tools for early diagnosis of malaria.

CONCLUSION

The aim of the present study was to gain expertise in solid phase peptide synthesis and immunological techniques. To achieve the above goal, initially unique peptide sequence of 22 and 15 amino acid long of HRP antigen from *Plasmodium falciparum* is synthesized using F-moc Chemistry. After synthesis, the peptides were purified using Gel filtration chromatography and were found to about 90% pure. The N and C terminal of the peptide was analyzed for the authenticity of the peptide. Antipeptide antibodies were raised in the mice using microspheres as the novel delivery vector for the peptide HRP. Peptide specific IgG levels and peak titre were measured by indirect ELISA.

Humoral Response: - Peptide in microsphere elicited high specific IgG level. A very critical point in view of the antibody generation was that, only a single dose of the peptide antigen in microsphere was enough to generate long lasting high titre.

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those articles, journals and books from where the literature for this article has been reviewed and discussed.

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