DEVELOPING PEPTIDE BASED IMMUNOGEN AGAINST HUMAN MALARIA DELIVERED IN LIPOSOMES CONTAINING NON-TOXIC ADJUVANTS

D.N. RAO*, B. CHABA*, P. KUMAR*, W. HAQ** AND L. SABHNANI*

*Dept. of Biochemistry, AIIMS, New Delhi 110 029 **Division of Biopolymers, CDRI, Lucknow 226001

INTRODUCTION

Several malarial antigen(s) eliciting protective immune response has been characterized. One such asexual blood stage antigen, Ring infected erythrocyte surface antigen (RESA), deposited on the erythrocyte membrane has been considered an important vaccine candidate (1). RESA protein contains three tandemly repeated immunodominant B cell epitopes viz EENV, EENVEHDA and DDEHVEEPTVA. These sequences are found to be antigenically dominant and antibodies raised against them have been shown to inhibit merozoite invasion of RBCs.

Synthetic vaccines based on repeat sequences of malarial antigen(s) have shown poor protection or inconsistent results during clinical trials (2). This has been attributed to lack of efficient T cell help or genetic restriction of the immune response at the host level. To overcome these limitations and to increase the immune response to peptide antigens, chimeric immunogens containing well defined Th determinant (CS.T3) with B cell antigenic sequences (RESA peptides) made by covalent linkage were used. Two permissible and non toxic adjuvants viz nor muramyl dipeptide (nor MDP) or lauroyl tetrapeptides(LTP) have been coentrapped with the above peptide immunogens in the same liposome preparation to test the ability of the individual adjuvants in enhancing the immune response against the two RESA peptides. The immunogenicity of different antigenic formulations were studied in murine system of varying haplotypes.

Results indicate that liposomes with coentrapped nor MDP/LTP containing RESA peptide covalently linked to promiscuous T cell determinant (CS.T3) is found to be a superior immuno-

gen for producing high titre and affinity antibodies, cytophilic IgG1/2b isotypes antibodies are found to be protective in vitro, with no MHC linked unresponsiveness of the immune response.

MATERIALS AND METHODS

Mice : C57BL/6 H-2^b, SJL/j H-2^s, FVB/j H-2^a and Balb/c H-2^d mice were procured from the breeding facilities of National Institute of Immunology, New Delhi. Mice were 6-8 weeks old and all the experimental groups consisted of four animals.

Peptides : Peptide sequences chosen from the RESA

(octa)₂ = (EENVEHDA)₂

(UN)₂ = (DDEHVEEPTVA)₂

CS.T3 = ENDIEKKIAKMEKASSVFNV (378-398)

Adjuvants: nor MDP (84/246) and LTP (84/201) were the products of Central Drug Research Institute, Lucknow, India.

Coupling of RESA peptides to CS.T3 peptide:

The respective RESA peptides were chemically coupled to CS.T3 peptide using two step glutaraldehyde method (3).

Preparation of liposomes

Peptide entrapment in liposomes was carried out by the Freeze thaw procedure (4) which produces multilamellar vesicles. The amount of entrapment following solubilization of liposomes with 0.1% Triton x-100 was found to be 43.2 to 47.6% of the amount used.

Coupling of Peptides to BSA

The RESA peptides were coupled to Bovine serum albumin (BSA) was used as a capture antigen during EIA procedure.

Address for Correspondence :
Dr. D.N. Rao at above Address.

Estimation of total anti RESA peptide antibodies and IgG isotypes by EIA

A pool of sera collected from each group was titrated by ELISA, in case of anti RESA peptide antibodies while a single antisera dilution (1:50) was used for IgG isotype estimation

Affinity (Kd) measurement by EIA

The binding affinities of antibodies raised against different antigen formulations were determined by measuring the Kd value (dissociation constant) using the method of Friguet et al (5).

In vitro merozoites reinvasion inhibition assay :

The in vitro merozoites reinvasion inhibition assay was performed as described earlier³.

RESULTS

Mice immunized with (OC)₂ peptide-CST3 conjugate containing nor MDP entrapped in liposomes showed the highest antibody titres in all the haplotypes in both primary and secondary immunizations. The (OC)₂ peptide alone entrapped in liposomes showed low antibody titres (1/1,600) in all the strains while inclusion of nor MDP/LTP enhanced the peak antibody titres four fold in mice bearing the haplotype H-2^{s&d} and two fold in H-2^{q&b} mice respectively. The antibody titres were further enhanced when (OC)₂ peptide CS.T3 conjugate containing nor MDP showed peak titres (1/12,800) in H-2^{s,d,b} and 1/6400 in H-2^q haplotypes respectively. Similarly when LTP is the adjuvant the peak titres were 1/12800 in mice of H-2^{s&d} and 1/6400 in H-2^{q&b} haplotypes respectively. The booster immunization induced a strong anamnestic response in each group. Mice immunized with (OC)₂-C.T3 conjugate containing either nor MDP or LTP showed the peak titres 1/51,200 in all the strains of mice. Mice immunized either with peptide alone or with the adjuvant showed peak titres 1/25,600. In most of the cases there was 2-4 fold increase in the antibody titres after the booster immunization. As seen with the (OC)₂ peptide inclusion of adjuvants (nor-MDP/LTP) increased immunogenicity of the (UN)₂ peptide with the peak titres 1/12,800 in the first bleed and a 2-4 fold increase in peak titres in the second bleed. The peak titres were always less either with peptide alone or containing the adjuvant.

Affinity of antibodies raised against different antigen preparations :

The results of Kd values of antibodies generated against different formulations, in both high responder strain H-2^s and low responder strain H-2^q was determined by EIA.

IgG subclass estimation :

The RESA peptide(s) alone in liposomes induced very low levels of IgG1 and IgG2b and barely detectable levels of IgG2a and IgG3. Inclusion of the adjuvants along with the RESA peptide(s) markedly enhanced the IgG1 and IgG2b isotype expression in all the four strains of mice. With the formulation containing CST3 sequence there is a rise in IgG2b isotype as compared to other isotypes.

Similarly, the (UN)₂ peptide alone in liposomes elicited very low levels of all the four levels of IgG1 and IgG2b; the proportion of IgG2b being greater than IgG1 in all the (OC)₂ peptide preparations and in both the bleeds. Chemical linkage of CS.T3 with the RESA peptides increased the proportion of IgG2b to IgG1 in all the (OC)₂ peptide preparations. In all the strains, the induction of IgG1 and IgG2b isotypes was seen in both the bleeds and their levels were significantly higher in the second bleed. Addition of adjuvants significantly enhanced the levels of IgG2b and IgG1 in all the strains of mice and in both the bleeds.

The antigen formulations containing the RESA peptide(s) alone showed 50-56% protection in vitro while the RESA peptides with the adjuvants showed 59-76% inhibition, while the RESA peptide(s)-CS.T3 conjugate with the adjuvants showed 70-97% inhibition in all the strains of mice, although inter strain variation was observed.

The sera raised against the (OC)₂-CS.T3 conjugate with nor MDP showed the highest percentage inhibition (80-97%) in all the haplotypes studied, whereas the sera raised against the RESA peptides alone in liposomes showed the lowest percentage inhibition.

DISCUSSION

The present study aims, to develop a suitable and safe delivery system with the builtin adjuvanticity to the peptide sequences of an asexual blood stage antigen, RESA. Liposomes

has been chosen in view of its diverse biological and immunological functions in enhancing both humoral and cellular responses to antigens (6). This resulted two-four fold increase in peptide specific IgG antibody end titres in all the strains when compared to peptide alone. The second booster immunization induced a strong anamnestic response in each group. When the binding affinities of antibodies raised against different formulations were studied, these are of high affinity with Kd value ranges from 0.9×10^{-9} M to 2.0×10^{-9} M.

Protection against infectious diseases frequently depends on the stimulations of an appropriate antibody isotype. The formulations containing the RESA peptide(s) alone in liposomes elicited low levels of IgG subclass antibodies. All the antigen formulations containing the adjuvant, produced predominantly IgG1 and IgG2b isotype responses in all the strains which is known to confer better protection against infectious agents than comparable antibodies of other isotypes in murine system. This provides an indirect evidence for the induction of both TH1 and TH2 responses.

The central role of CD4⁺ T cells in malaria require the recruitment of CD4⁺ memory cells crucial for boosting the response to natural infection, which is the case in the present study when the antigen contains B & T cell determinants with a strong adjuvant, administered in liposomes, considered to be safe for humans. In most of the formulations containing the RESA peptides(s) and

the adjuvant with the inclusion of T cell epitope increased the proportion of IgG2b to IgG1. The presence of CS.T3 sequence is known to activate CD4⁺ Th1 subsets leading to increased rIFN production. Thus play a major role in switching the IgG subclass towards IgG2a/2b. In murine system, IgG2a/2b isotypes are known to have a better protection than other isotypes.

Lastly, when ability of the antibodies to prevent the entry of the parasite into RBCs was analysed in vitro for different formulations, the pattern of inhibition/protection observed correlate well with the peak Ab titres and affinity, that was achieved through liposomal preparations. This could apparently be possible as a result of liposome acting as a depot thereby constantly supplying Ag to APCs such as macrophage at rates favouring its efficient processing by the cells and presentation and partly through liposome migration to the lymph nodes.

Finally, the present study highlights the importance of appropriate immuno modulators like, derivative of MDP (norMDP) and lipopeptide (LTP) along with a universal T-cell epitope CS.T3 in enhancing the immunogenicity and overcoming the MHC linked genetic restriction of the immune response to B-cell epitopes of the RESA peptides using liposomes as a delivery system.

ACKNOWLEDGEMENT

Authors are grateful to CSIR and ICMR for financial assistance.

REFERENCES

1. Coppel, R.L., Cowman, A.F., Anders, R.F., Bianco, A.E., Saint, R.B., Lingelbach, K.R., Kemp, D.J. and Brown, G.V. (1984) Immune sera recognize on erythrocytes a *Plasmodium falciparum* antigen composed of repeated amino acid sequences. *Nature* 310, 789-791.
2. Collins, W.E., Pappaioanou, M., Anders, R. F., Campbell, G.H., Brown, G.B. and Kemp, O.J. (1983) Immunization trials with the ring infected erythrocyte surface antigen of *Plasmodium falciparum* in owl monkey & (*Aotus vociferans*). *Am. J. Trop. Med. Hyg.* 38, 268-282.
3. Pawan, K., Kabilan, L., Ivanov, B.B. and Rao, D.N. (1994) Construction of synthetic immunogens : Use of natural immunomodulator polytuftsin in malaria vaccine against RESA antigen of *P. falciparum*. *Vaccine* 16(9), 819-824.
4. Turanek, J., Toman, J., Novak, J., Krehnak, V. & Horavova, P. (1994) Adjuvant effect of liposomes and adamantylamide dipeptide on antigenicity of entrapped synthetic peptide derived from HIV-1 transmembrane region glycoprotein gp41. *Immunol. Letts.* 39, 157- 161.
5. Friguet, B., Chaffote, A.F., Djavadi-Ohanian, L. & Goldberg, M.E. (1995) Measurement of the true affinity constant in solution of antigen- antibody complexes by enzyme linked immunosorbent assay. *J. Immunol. Meth.* 77, 305-319.
6. Alving, C.R. Liposomes as carriers of antigens and adjuvants. *J. Immunol. Meth.* (1991) 140, 1013.