ORIGINAL INVESTIGATION

Neeraj Chopra · Shukla Biswas · Beena Thomas
Leenu Sabhnni · D. N. Rao

Inducing protective antibodies against ring-infected erythrocyte surface peptide antigen of Plasmodium falciparum using immunostimulating complex (ISCOMs) delivery

Received: 10 February 2000

Abstract In the present study, synthetic peptides (EENVEHDA)_{2} [(oc)_{2}] and (DDEHVEEPTVA)_{2} [(un)_{2}] of ring-infected erythrocyte surface antigen (RESA) of Plasmodium falciparum were linked with palmitic acid and entrapped in immunostimulating complexes (ISCOMs). The immunogenicity of the peptide(s) and mixture of peptides were studied in mice with different genetic background. Peptide(s) entrapped in ISCOMs using a low-dose immunization strategy generated high-titer as well as high-affinity antibodies. Interestingly, no genetic restriction of the immune response was observed in any of the strains studied. The IgG subclass pattern with the peptide(s) showed predominately IgG2a/2b isotypes, while with the mixed peptide formulation, (un)_{2}-specific IgG1 and IgG2a/2b isotypes. These cytphilic antibodies inhibited the ring as well as schizont stage and total parasite growth during in vitro merozoite reinvasino inhibition study. In the mixed peptide preparation, the same pattern of immune response was achieved as that of individual peptide(s) using ISCOMs delivery. Therefore, the entrapment of otherwise poorly immunogenic synthetic peptides in ISCOMs resulted in increased immunogenicity followed by strong secondary response and can be adopted for developing subunit immunogen formulation against malarial parasite.

Keywords Ring-infected erythrocyte surface antigen peptides · Immunostimulating complexes · Inbred mice · Protective antibodies

Introduction

Immunomodulators and novel delivery systems are being increasingly used for the prevention, treatment and diagnosis of human disease. Various delivery systems like liposomes [7, 26], immunostimulating complexes (ISCOMs) [2, 29], proteosomes [19] and biodegradable microspheres [21, 24] are being tested for the efficient and controlled delivery of antigens, with lower antigen dose and shorter course of immunization, to increase the immunogenicity of antigen(s) with respect to antibody production.

ISCOMs are stable spherical particles in which amphipathic proteins or peptides are present as multimers in a matrix of glycoside adjuvant, i.e., Quil A, cholesterol and phospholipids [20]. Although components in Quil A have shown toxic effects, these side effects were almost absent when Quil A was incorporated into ISCOMs [14]. In addition, large scale purification of saponins yields an optimal mixture that allows preparation of highly immunogenic ISCOMs but does not have the side effects and, thus, are suitable for vaccine purposes [28]. ISCOMs are widely used in veterinary vaccines. The capacity of ISCOMs to induce strong antibody responses after administration via various routes is well documented. Incorporation of protein or peptide antigens into ISCOMs has been reported to enhance antibody responses to a large number of diseases [9, 15]. Immunization with ISCOMs has also been shown to induce a sustained humoral and cellular immune response against the incorporated antigens [16, 32].

Previous studies in our laboratory have established the significant role of immunomodulators like polytuftsin or the bioactive fragment of human IL-1ß in conjunction with liposomal delivery of synthetic peptides of ring-infected erythrocyte surface antigen (RESA) for developing effective subunit formulations against malaria [7, 17, 18, 33].

RESA antigen of the asexual erythrocyte stage of Plasmodium falciparum has been contemplated as one of
the candidate antigen for inclusion in a vaccine against malaria [10]. RESA is a non-glycosylated polypeptide of 155 kDa that is expressed on the inner side of the erythrocyte membrane shortly after merozoite invasion [34]. The antigen contains two extensive blocks of tandemly repeated amino acid sequences, one in the C-terminal region, comprising 5 repeats of the octamer EENVHEDA and about 35 repeats of the tetramer EENV. The second repeat region located in the center of the molecule comprises of seven 11-mer repeats of DDEHVEEPTVA [12]. Several lines of evidence suggest that immune response to RESA interferes with parasite growth. Seroepidemiological studies have shown correlation between antibody levels, particularly against the C-terminal repeat sequences and reduced parasitemia in individuals from different malarial areas [3, 4, 23, 27]. Furthermore, antibodies against RESA repeats are very efficient inhibitors of P. falciparum merozoite reinvasion of erythrocyte in vitro [35, 36]. The repeat sequences of malaria antigen(s) are genetically restricted and the immune response is varied among the diverse population. Various attempts have been made to overcome the MHC-linked immune unresponsiveness as well to increase the immunogenicity of these sequences by optimizing the adjuvant and delivery system.

In one such attempt, two RESA sequences, (EENVHEDA)$_2$ [(oc)$_2$] and (DDEHVEEPTVA)$_2$ [(un)$_2$] were linked with palmitic acid to give an amphiphatic nature and entrapped/delivered in ISCOMs. Humoral response was studied in inbred mice of different genetic backgrounds using peptides alone or mixture of both peptides, and the protective efficacy of the generated antibodies were studied in vitro.

---

**Materials and methods**

**Mice**

Female inbred mice of different haplotypes, i.e., BALB/c (H-2$^b$), CBA/J (H-2$^a$), SJL/J (H-2$^b$) and FVB/J (H-2$^d$), of 6–8 weeks of age were procured from the breeding facilities of the National Institute of Immunology, New Delhi, India. Each experimental group consisted of four to six animals.

**Peptides**

Both (oc)$_2$ and (un)$_2$ were synthesized by a solid-phase method using T-Boc chemistry [22]. Coupling was carried out using the HOBT-DCC method. After cleavage and purification, the purity of the peptides was checked by HPLC and was found to be greater than 90% pure. Both peptides were palmitated for facilitating incorporation into ISCOMs.

**Preparation of ISCOMs**

ISCOMs were prepared according to our reported protocol [1]. Peptides were palmitated before entrapment into ISCOMs using the reagent N-palmitoyl oxysuccinimide [25]. The palmitated peptide, 5 mg in 50 mM TRIS pH 7.2 containing 100 mM NaCl (TM buffer), was treated with 200 µg each of cholesterol and phosphatidyl choline in 20 µl 20% MEGA-10 (w/v), 100 µl 20% MEGA-10 and 10 µl 10% Quil A (w/v) in double distilled water. This was sonicated for 15 min, followed by incubation at room temperature for 1 h and dialysis overnight at room temperature against TRIS NaCl buffer, pH 7.2. The ISCOMs were then pelleted by centrifugation on a sucrose gradient made by layering 500 µl of 8% sucrose containing 0.1% Triton X-100 onto 10 ml 15% sucrose for 4 h, at 10,000 g at 20 °C and then suspended in 500 µl PBS and stored at −20 °C till further use. A known amount of the ISCOMs preparation was lysed with 0.1% Triton X-100 and the released peptide content was estimated using Bradford’s method [6]. The entrapment efficiency was found to be in the range of 45–50%.

**Immunization protocol**

Mice were immunized with peptide(s) adsorbed on alum, peptide(s) entrapped in ISCOMs and mixture of peptides entrapped in ISCOMs. The optimum antigen dose for peptides adsorbed on alum and entrapped in ISCOMs preparation were standardized by dose kinetic studies and was found to be 50 µg and 5 µg, respectively. In case of the mixed peptide preparation, 5 µg each of both peptides was used for immunization. Mice were immunized in the footpad on day 0 and subsequently the same amount was given as a booster dose on days 21 and 35. Post-immunization bleeds were collected on days 28, 42 and 60 (third bleed was collected without booster immunization) from the retro-orbital plexus. Sera was separated and stored at −20 °C until use.

**Estimation of total anti-RESA antibodies by enzyme immunoassay**

For the enzyme immunoassay (EIA), 100 µl peptide (100 ng/well) in carbonate buffer was used to coat 96-well Immunon-2 microtiter plates (Dynatech, USA) and kept overnight at 4 °C. After washing and blocking with skimmed milk powder (3% in PBS, overnight at room temperature), individual mice sera were added at dilutions of 1:100 and 1:200 and incubated for 2 h at 37 °C. The antigen-antibody complex was detected by incubating with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution). Color was developed with H$_2$O$_2$/o-phenylenediamine and reaction terminated with 8 N H$_2$SO$_4$. The absorbance was read at 492 nm. Pre-immune sera was used as control.

**Estimation of end titers**

The same EIA protocol was used except that the mouse antisera (pooled from the same experimental group) were serially twofold diluted before incubating with the respective RESA peptides. End titers were expressed as the highest serum dilution giving an absorbance of ≥0.2 (i.e., + 4 SD from the mean of pre-immune sera).

**Estimation of IgG isotypes**

The same EIA protocol was used except that the individual mouse antisera was first incubated at a single dilution (1:100) for 90 min at 37 °C. The second antibody, i.e., goat anti-mouse IgG specific for each subclass (Sigma subtyping kit) was incubated (1:1000 dilution) with the above complex for 90 min at 37 °C. After the usual washings, the third antibody, horseradish peroxidase-conjugated rabbit anti-goat IgG (1:1000 dilution) was incubated for 60 min at 37 °C. After adding the substrate and stopping the reaction, the absorbance was read at 492 nm.

**Affinity measurement by EIA**

The binding affinities of antibodies raised against different antigen formulations were determined by measuring the $K_a$ value (dissociation constant) using the method of Friguet et al. [13]. In brief,