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The Evaluation of the Immune Response and the Effects of the Mixture of (Nt + Ct) Proteins of the Circumsporozoite (CSP) of *Plasmodium berghei* and *Plasmodium falciparum*

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Abstract: Malaria infection is initiated when an infected Anopheline mosquito injects sporozoites during a blood meal. The Circumsporozoite (CSP) protein is the major surface protein of plasmodium sporozoites and forms a dense coat on the parasite's surface. Although, the C-terminal (Ct) portion or side of the CSP protein has been extensively studied as a malaria candidate vaccine, little is known about the N-terminal (Nt) portion and the mixture of the 2 portions (Nt + Ct) of this protein on the immune response. Our data suggest that the mixture (Nt + Ct) peptides responded well and much more better inhibition of invasion of hepatocytes was given by the use of that mixture than when Ct or Nt was used separately. The 2 peptides seem to play an important inhibitory role against the invasion of hepatocytes by sporozoites.

Key words: Evaluation, immune response, circumsporozoite (CSP), *plasmodium berghei*, *plasmodium falciparum*

INTRODUCTION

Malaria is a parasitic disease caused by a protozoan, Plasmodium species that lives in tropical and subtropical regions (Gramiccia and Beales, 1988). Malaria constitutes a serious public health concern. The worldwide incidence of malaria is estimated by the World Health Organization to be approximately 300-500 million clinical cases annually, with >90% of these cases in Sub-Saharan. The majority of the estimated 3 million deaths from malaria occur in children <5 years of age (Elissa *et al*., 2005) and pregnant women. In fact, in pregnancy malaria is a major problem in sub-Saharan Africa, affecting an estimated 24 million pregnant women, especially primigravidae (Ayisi *et al*., 2003). Malaria is transmitted by an infected Anopheline mosquito, which injects sporozoites into the host during a blood meal (Menard *et al*., 1997). After entry the bloodstream, sporozoites are quickly transported to the liver where they extravagate and invade hepatocytes (Patrick *et al*., 2005).

The CSP (circumsporozoite) is the main surface protein of sporozoite, which forms a dense coat on the parasite's surface (Sinnis and Nardin, 2002). Studies have shown that CSP is responsible for the adhesion of sporozoites (Dharmendar Rathoret *et al*., 2002) to their arget cells (hepatocytes) and that its N-terminal portion (side) is proteolytically cleaved by a protease enzyme of the parasite of origin during the invasion of the hepatocytes. The cleavage of the N-terminal side is necessary for the invasion of hepaocytes by sporozoites (Alida Coppi *et al*., 2005) on the one hand. On the other hand, it was demonstrated that the protease system, responsible for the cleavage of CSP protein is inhibited by E-64 in *Plasmodium falciparum* and *Plasmodium berghei* as well and that mice treated with E-64 are completely protected from malaria even after the injection of sporozoites into the latter (Alida Coppi *et al*., 2005; Consuelo Pinzon-ort *et al*., 2001). May it be possible to inhibit the invasion of the target cells by sporozoites using antibodies against N-terminal? May this N-terminal region be more immunogenic than the C-terminal region frequently used as candidate vaccine against malaria?

The objective of this research is to:

- Evaluate the immunogenicity of N-terminal peptides of *P. berghei* and *P. falciparum*.
- Determine if the mixture of the N-terminal and C-terminal would give better response compared to that of N-terminal alone or that C-terminal alone.

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- Determine if the antibodies directed against Nt and (Nt + Ct) peptides can inhibit the invasion of the hepatocytes.

MATERIALS AND METHODS

Ten groups of mice have been evaluated with different synthetic peptides belonging to different CS protein fragments and this after several immunizations in the presence of montanide adjuvant.

Synthetic peptides: For each species of Plasmodium (*P. berghei* and *P. falciparum*), 3 synthetic peptides were used individually, 2 corresponding to fragment of different size belonging to the N-terminal region of the CSP and whereas, one of the C-terminal region; then the C-terminal peptide was added to the 2 different N-terminal fragments to obtain a mixture of peptides (Nt + Ct). Also, 10 groups of peptides were used for the immunization as seen below:

Nt peptides: PbCS Nt (21-110); PbCS Nt (59-91); PfCS Nt (22-110); PfCS Nt (65-110).

Ct peptides: PbCS Ct (242-310); PfCS Ct (282-383).

Mixture of peptides (Nt + Ct): PbCS (Nt + Ct) with PbCS Nt (21-91); PbCS (Nt + Ct) with PbCSNt (59-91); PfCS (Nt + Ct) with PfCSNt (22-110); PfCS (Nt + Ct) with PfCSNt (65-110).

The animals and parasites: Ten groups of mice, each group having 4 mice were used for the experiments. The 6 weeks old female mice, CB6F1 (H2-Kdb) strains constituted our animals samples. The parasites of *Plasmodium berghei*, ANKA strains were maintained by the parasite life cycle transmission in the mosquito (Anopheline stephensis) and the mouse (Balb C strains-M2Kdb). This was carried out in the institute of Biochemistry, university of Lausanne, Switzerland. Once, the mosquitoes were infected, they were dissected under a stereoscope using a sterile needle in the DMEM medium in order to isolate the salivary glands. Once isolated, the salivary glands were homogenized, centrifuged at 500 rpm for 3 min and the supernatant containing sporozoites was collected and sporozoites numerated for further use for different experiments (IFAT, Western blot, invasion inhibition, etc.).

Immunizations: Injections were done by cutaneous road. For each mouse, 3 injections were given with an interval of 3 weeks between 2 injections. Total 10 days after, each injection, mice were bled and serum was collected after centrifugation and was kept al -20°C to be used for different experiments (ELISA, IFAT).

ELISA, IFAT and inhibition of invasion of hepatocytes: ELISA was carried out by reading the absorbance at 405 nm using an ELISA reader. IFAT was done by microscopic examination of slides using a fluorescent microscope. The in vitro inhibition of invasion was realized on HepG2 cells that were maintained in culture in a small flask and were observed microscopically using a fluorescent microscope.

RESULTS

Evaluating the antibody production in *P. berghei*: A progressive increase was noticed for all the peptides of *P. berghei* used after each immunization. Responses obtained with PbCSNt (21-91) (Fig. 1) were slightly lesser than those obtained with PbCS (242-310) (Fig. 2). Responses obtained with PbCS (Nt + Ct) mixture for Nt (21-91) practically followed the same pattern as those realized by Nt (21-91) or Ct (242-310) alone according to the peptide against, which the antibody mixture was tested. There was more less production of antibodies with PbCSNt (59-91) peptide than that obtained with the longest PbCSNt (21-91) fragment (Fig. 3).

Evaluating the production of antibodies in *P. falciparum*: Like in *P.berghei*, a progressive increase for all the peptides used after each immunization. PfCSNt (22-110) (Fig. 4) gave slightly lesser responses than PfCSCt (282-383) (Fig. 5). Responses obtained with PfCS (Nt + Ct) mixture for Nt (21-110) followed the same pattern as those realized by Nt (21-110) or Ct (282-383) alone according to, the peptide against, which the mixture was tested.

PfCSNt (65-110) did not yield significant antibody production until the third immunization trial (Fig. 6). Similarly, there was no significant response with PfCS (Nt+ Ct) mixture when this was tested against the short

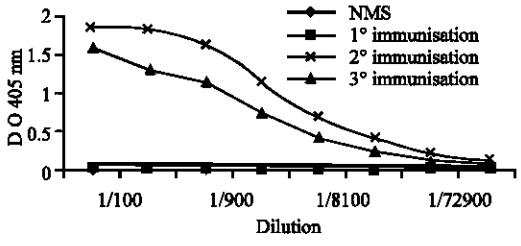


Fig. 1: Antibodies response against PbCSNt (21-91)

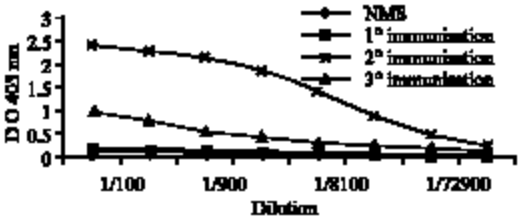


Fig. 2: Antibodies response to PbCSt (242-310)

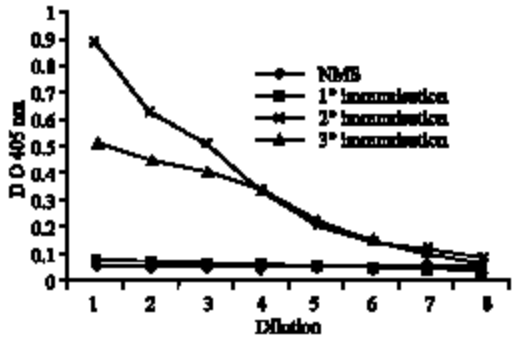


Fig. 3: Antibodies response to PbCSNt (59-91)

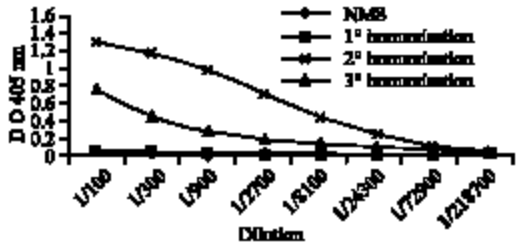


Fig. 4: Antibodies response to PfCSNt (22-110)

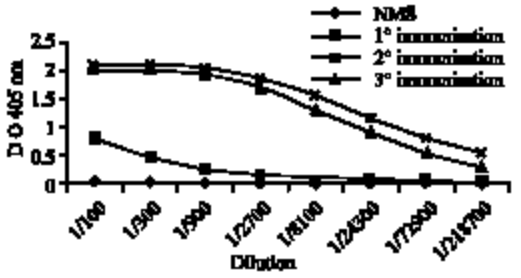


Fig. 5: Antibodies response to PfCSNt (282-383)

Nt (65-110) peptide during the course of the 3 immunizations. However, with PfCS (Nt + Ct) mixture tested against the PfCSCt (282-383) peptide, a significant production of antibodies as easily as the first immunization trial. Response obtained with the mixture was practically similar to that obtained with the PfCSCt (282-383) peptide alone as well as that realized by PfCS (Nt+Ct) mixture containing the longer peptide Nt(22-110).

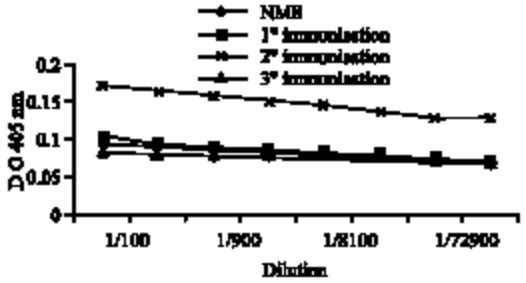


Fig. 6: Antibodies response to PfCSNt (65-110)

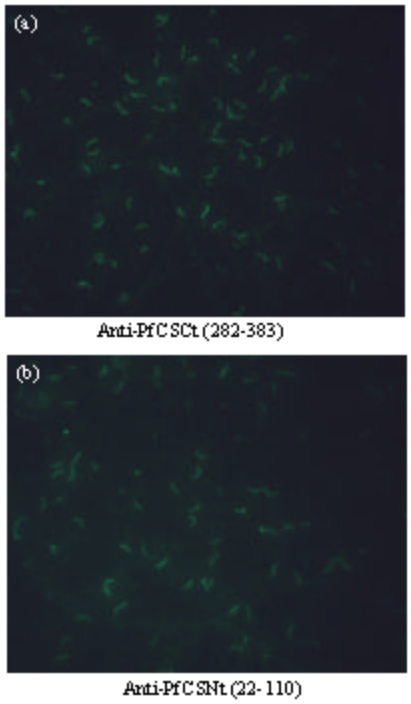
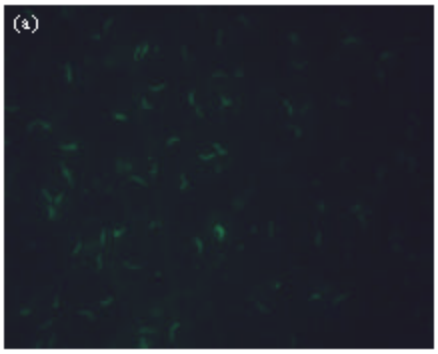


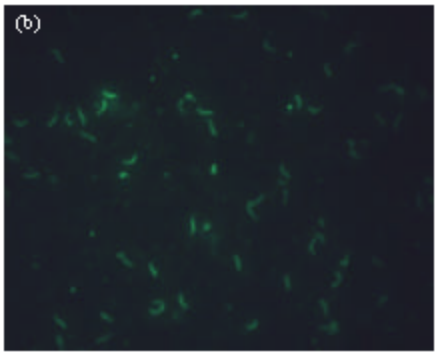
Fig. 7: Immunofluorescence on slides of *P. falciparum*

Immunofluorescence (IFAT): Specificity of antibodies for the native CS proteins by immunofluorescence: The ability of antibodies generated by the peptides to react with the native CS proteins on the surface of the sporozoite cells (sporozoites of *P. berghei* and *P. falciparum*) was analyzed by viewing the slides mounted on an immunofluorescence microscope. Results showed that the mode of recognition of antibodies by different peptides PbCSNt (21-91); PfCSNt (22-110); PbCSCt (242-310); PfCSCt (282-383) and the mixtures PbCS (Nt + Ct); PfCS (Nt + Ct); was the same for sporozoites and the synthesized peptides used for the immunization as well (Fig. 7 and 8).

Inhibition of invasion of hepatocytes: Results also showed that the antibodies induced by the synthesized peptides were able to inhibit the invasion of the hepatocytes by



Anti-PbCSCt (242-310)



Anti-PbCSNt (21-91)

Fig. 8: Immunofluorescence on slides of *P. berghei*

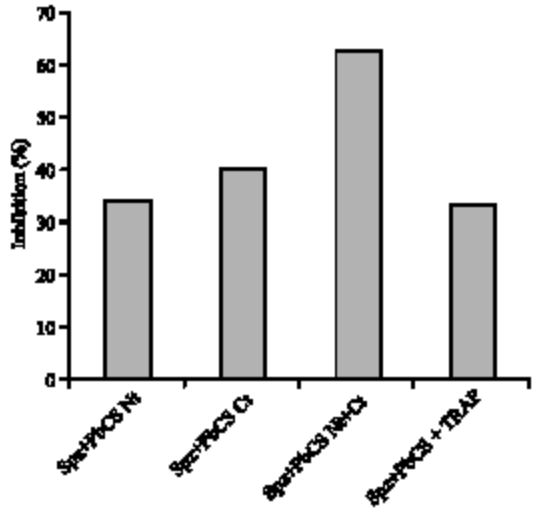


Fig. 9: Percentage of inhibition of *P. berghei* SPZ invasion of HepG2 cells with different sera

sporozoites. This experiment was realized to test the capacity of antibodies generated with PbCS to inhibit the invasion of hepatocytes (HepG2) by the sporozoites of *P. berghei* due to the failure of transgenic sporozoites, this experiment was not realized with the antibodies of *P. falciparum*.

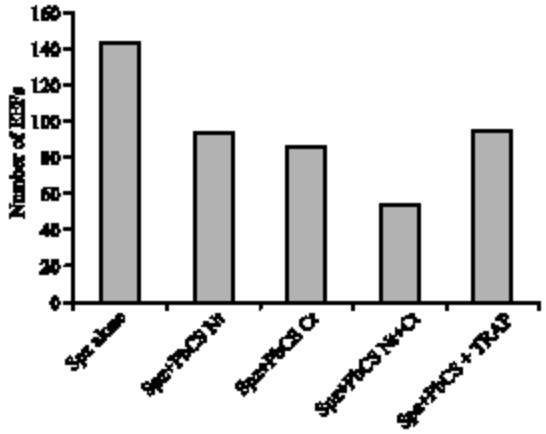


Fig. 10: Inhibition of *P. bergheispz* invasion of HepG2 cells with different sera

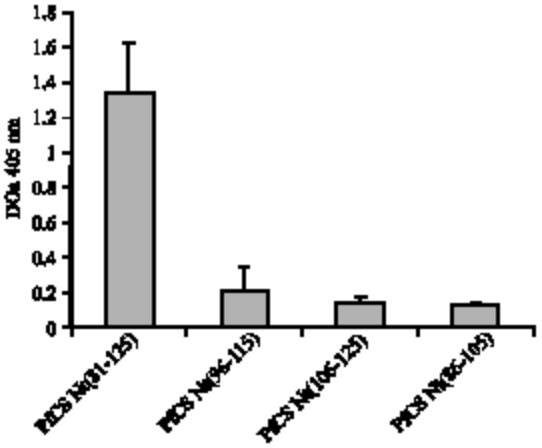


Fig. 11: Indentification of immunogenic segments in the Nt region of the CS protien of *P. falciparum* against sera of mice immunized with PfCSNt (22-110)

The rate of inhibition (in percentage) induced by the antibodies generated by PbCSNt (21-91) was slightly lower than that of antibodies generated by PbCSCt (242-310) (Fig. 9). A grater rate of inhibition was obtained with PbCS (Nt + Ct) mixture than that induced by PbCSNt or PbCSCt alone (Fig. 10).

Epitope mapping: This experiment that is just an ELISA technique was carried out to test the ability of short fragments of N-terminal peptides to be recognized by antibodies present in the serum that was obtained after immunization of long N-terminal fragments (Fig. 11 and 12).

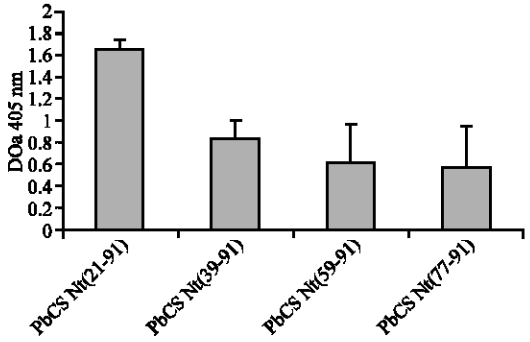


Fig. 12: Indentification of immunogenic segments in the Nt region of the CS protien of *P. berghei* against sera of mice immunized with PfCSNt (21-91)

ELISA test with the serum from the Burkina Faso populations: PfCS Nt (65-110) PfCS Nt (65-110): This experiment was carried out to determine whether there could be an correlation between protection of the population against malaria and the presence of the antibodies directed against PfCSNt (22-110) and PfCSNt (65-110).

DISCUSSION

Malaria is initiated when a female anopheles mosquito injects into somebody sporozoites during a blood meal. The evaluation of the humoral immune response specific to the used peptides clearly indicates the specificity of the humoral immune response to those peptides at different group levels.

In fact, results obtained from ELISA test show that the C-terminal peptides for the two sp. (*P. berghei* and *P. falciparum*) responded better than the long N-terminal peptides. The short N-terminal peptide of *P. falciparum* failed to respond where as (Nt + Ct) responded well, thi is on one hand.

On the other hand, better inhibition was realized with Ct than Nt; however much were better inhibition was given by the use of (Nt + Ct) mixture than when Ct or Nt was used separately. These results enable us to conclude that Nt and Ct peptides do not exert influence on each other, that in there is no dominance of one peptide on another but the two peptides play an important inhibitory role against the invasion of hepatocytes by sporozoites. As a result, it would be preferable to use them collectively than to use each peptide singly for a better protection against malaria parasites; and more over, it would not be beneficial to focus on one or other of the two peptides in the research for an efficient candidate vaccine.

The epitope mapping experiments were carried out to better target the nearest immunogenic N-terminal fragments to the cleavage region of Cs protein. Results also showed that serum PfCSNt (22-110) only recognized the short peptide PfCSNt (81-125) whereas serum PbCSNt (21-91) only recognized the short peptide PbCSNt (59-91) and PbCSNt (77-91). On the other hand, the long peptides PbCSNt and PfCSNt were recognized only by the peptides nearest to the region I identified as the cleavage site of the CS protein. The more one gets away from the cleavage site the less is the immune response. Certain persons living in the endemic areas develop resistance to malaria infection.

In order to determine whether there could be a correlation between protection of people against malaria and the presence of antibodies serum of individuals directed against PfCSNt (22-110) and PfCSNt (65-110), ELISA test was conducted using the sera of people from 3 regions of Burkina Faso (results not presented there).

Those results showed that some individuals produced antibodies recognized by the peptides PfCSNt (22-110) and PfCSNt (65-110). It is therefore suggested that similar experimented should be carried out on the population of the other endemic areas in order to verify if the persons whose sera recognize peptides PfCSNt (22-110) and PfCSNt (65-110) do not really develop the disease. This approach would permit a better understanding of the role of the N-terminal region of CS protein in malaria infection. Targeted study of this region may lead to the development of new prophylactic agents against malaria.

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