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BRIEF REPORT

The Recombinant NS3 Protein a Potential Antigen for Dengue Vaccine Development

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Abstract

The NS3 protein is a multifunctional non-structural protein involved in dengue virus polyprotein processing. This protein is also target in the immune response against dengue virus infection. The predominance of cytotoxic T-cell lymphocytes epitopes in the NS3 structure suggests the participation of this protein in limiting virus replication and in the protection against dengue disease. A brief presentation on aspects related to antigenic characteristics and immunogenicity of the recombinant NS3 protein is reported in this work. A reduced number of studies have assessed the NS3 protein in a dengue vaccine formulation. Researches carried out in mice shown that DNA vaccines based on NS3 protein induced a protective response evaluated by their ability to produce IFNγ. Moreover, the incorporation of recombinant subunit NS3 in a purified inactivated vaccine significantly increased the immune response induced by this inactivated vaccine. Likewise, recent studies demonstrated that the combination of the recombinant modified-NS1 and NS3 proteins from dengue 2 virus induced higher immune responses and protection in mice. The immunological studies discussed herein support the possible inclusion of the NS3 protein in a dengue vaccine formulation.

Introduction

For decades, the efficacy of dengue vaccine has been associated with the induction of a balanced and serotype-specific of Neutralizing (Nt) Antibodies (Abs) response against the four Dengue Virus (DENV) serotypes [1,2]. Therefore, it is expected that there will be a correlation between Nt Abs titers and protection against DENV serotypes in developing vaccines [3,4]. However, Dengvaxia, the only licensed dengue vaccine so far, showed low efficacy in presence of high titers of Nt Abs [5,6]. The vaccine efficacy varied according to serotype, age and baseline dengue serostatus [7]. It is unclear the role of the age as factor associated with hospitalized and/or severe disease. It is hypothesized that age-related differences in physiology may predispose an individual to a higher risk of plasma leakage and severe disease [8]. Respect to how affect the baseline serostatus or pre-existing immunity to vaccine efficacy and safety, there is one hypothesis that explain Dengvaxia's® vaccination in seronegative individuals mimics a primary infection, setting up the individual for the higher risk of severe

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disease when individual experiences a subsequent sequential infection [9]. The low detected efficacy of Dengvaxia among seronegative individuals to dengue virus, the failure to elicit potent and broad cellular immune responses, as well as the fluctuations on Nt Abs titers after three doses administration, marks an urgent need to understand in depth the immune response after natural infection with dengue virus and to identify correlates of protection, taking into account pre-existing immunity to others flaviviruses and the relevant DENV genotypes [10,11].

Recently, World Health Organization's (WHO) Strategic Advisory Group of Experts (SAGE) on Immunization shared positive recommendations for use of QDENGA, a new tretavalent, live-attenuated dengue vaccine manufactured by Takeda Pharmaceuticals. This vaccine is currently available for children and adults in countries like Indonesia, Thailand, Argentina and Brazil. Despite these two vaccines and the promissory TV003/TV005 developed by National Institute of Allergy and Infectious Diseases (NIAID), there are no globally licensed vaccines that protect against all four of the DENV serotypes without any limitation in its use. The development of an efficacious vaccine against dengue virus faces many challenges such as: To achieve induce a balanced Nt Ab response against all four DENV serotypes in order to avoid the Antibody-Dependent Enhancement (ADE) phenomenon, to induce long-lasting protective immunity against all dengue serotypes, to characterize the dengue immunopeptidome for identifying T-cell critical epitopes in the generation of protective response, to define appropriate correlates or surrogates of protection for DENV infection, and to explore new animal models allow the validation of dengue vaccine efficacy and protection induced by the vaccine.

Immunological studies carried out in mice and humans suggest that T cells, particularly CD8 + cells, may be important mediators of the protection against DENV infection [12-15]. In that sense, the protective role for CD8+ T cells during primary DENV infection was demonstrated in mice by Yauch et al, 2009, who found that depletion of CD8+ T cells increased the viral load. They also showed the cytotoxic activity of DENV-specific CD8+ T cells (16) and that mice immunization with both dominant CD8+ and CD4+ T-cell epitopes led to enhanced viral clearance (12). In other study carried out by Zellweger et al, 2015 it was demonstrated that CD8+ T cells can mediate short-term protection against heterotypic DENV reinfection in mice [13]. Human studies have revealed that a DENV-specific CD4+ T cell subset can have directly cytolytic activity in a peptide-specific and MHC class II-restricted manner, so these cells may play a role in the control of dengue infection *in vivo* [15]. Altogether, these experiments corroborate the inclusion of biomolecules that immunostimulant the T cell response could be currently one promising strategy in dengue vaccine development based on recombinant subunits [17,18]. CD4+ and CD8+ T lymphocytes have been shown to play a critical role in other acute viral infections. While virus-specific CD8+ T cells are important for viral clearance, CD4+ T cells are required for the induction of protective antibody responses and for the generation of both B cell and CD8+ T cell memory responses [19]. Besides, they can directly kill virus-infected cells through expression of IFNγ and cytotoxic effector functions [20].

In dengue infection, the NS3 protein is considered the main target for CD4+ and CD8+ T cell responses and it could be involved in protection [21]. This protein present in its structure epitopes recognized by human and mouse CD8+ T cell lymphocytes clones [22,23]. A study using overlapped peptides covering the entire DENV polyprotein demonstrates that the largest ex vivo T cell response was directed to the region of the NS3 protein and mainly to the helicase domain [24]. Rivino L, et al. [25] stated that during DENV infection, the main targets of CD8+ T cells are NS3 and NS5 proteins; while for CD4+ T cells the preferential targets are the proteins recognized by B cells (E, C and NS1). In other study carried out in Vietnamese adults with secondary DENV infection was defined the relative antigenicity of peptides from multiple dengue viral antigens. NS3 was recognized by more than half of all Vietnamese adult patients, who identified 34 different antigenic peptides that potentially contained many novel T-cell epitopes [26]. Studies carried out in Macaccus rhesus allowed determining that the viral peptides capable of activating CD4+ and CD8+ T cells come primarily from the NS1, NS3 and NS5 proteins [27]. Furthermore, DENV-specific, cross-reactive cytotox ic T cells have been shown to recognize NS3 peptides [28].

The NS3 protein is localized exclusively in DENV-infected cells, but due to lysis by a viral cytopathic effect or by cell-mediated lysis it may be accessible for binding to the B cell receptor [29]. Using immunolocalization techniques, NS3 was found to co-localize in membrane structures identified as vesicle packets synonymous with smooth membrane vesicles

[30]. Interestedly, DENV NS3 antigen was detected by immunohistochemistry assay in macrophages inside the placental villus, and in endothelium and macrophages in the umbilical cord, suggesting a possible DENV vertical transmission [31].

The reactivity of a recombinant NS3 protein with the pools of polyclonal Hyperimmune Mouse Ascitic Fluids (HMAFs) to the four DENV was demonstrated by ELISA. Besides, sera from recombinant NS3-immunized mice recognized the native viral NS3 protein by immunofluorescence in C6/36 HT cells and Western Blot [32].

It is reported that specific Abs against the NS3 protein of DENV 1 are capable of increasing the survival time of mice challenged with lethal doses of the homologous serotype, although the mechanism involved is not yet defined [33]. Some authors propose that in DENV infections, anti-NS3 Abs are present in acute phase samples from primary and secondary cases [34,35]. Valdes K, et al. [36] demonstrated that a specific response of anti-NS3 Abs is detected mainly in secondary cases of dengue, which is significantly depending on the infecting serotype. It opens the possibility of implementing a diagnostic assay with high sensitivity for the detection of the NS3 antigen in samples of dengue and severe dengue patients [37]. In this same line, Álvarez-Rodriguez LM, et al. [38] developed a reliable "in house" serological system for the diagnosis of dengue infection based on the recombinant NS3 proteins from each serotype. This diagnosis system was field-tested and showed very good results even comparable to antigen-detection NS1 Kit commercially available. These results suggested that the use of ELISA with recombinant NS3 protein may be an alternative method for serological analysis of dengue virus in the acute phase. On the other hand, the diagnostic potential as well as immunogenicity of a recombinant NS3 protein of dengue virus 1-4 was also studied [38].

Few studies have evaluated the use of the NS3 protein as a protective antigen against DENV. In 2011, a first study investigated the protective efficacy of DNA vaccines based on the NS3 protein from DENV2 in mice [39]. Different plasmids were constructed encoding the whole DENV2 NS3 protein or only its functional domains (protease or helicase). Mice were immunized with these DNA vaccines and challenged with a lethal dose of DENV2. The protection results showed that animals immunized with plasmids encoding the protease domain were not protected after challenge, while those immunized with vaccines based on the helicase domain or the full-length NS3 protein survived virus infection. This latter group was also able to produce IFNγ by CD8+ T cells. Five year later, Hurtado-Melgoza ML, et al. [40] evaluated the potential of the NS3 protease domain as a protective antigen by comparing the administration of a recombinant protein versus a DNA vaccine in mice. They showed that immunization of pcDNA3/NS3-DENV3 in mice induces a favorable response in the activation of T lymphocytes with low production of specific antibodies against DENV3 NS3-protease domain, meanwhile cells from mice immunized with the recombinant protein were not able. The results confirmed that the most T cell epitopes on NS3 protein are located in the helicase region, which could be responsible of inducing a cell-mediate immunity in mice. On the other hand, Simmons M, et al. [41] evaluated the T-cell responses induced by DENV2 inactivated vaccines combined with the recombinant NS3 proteins, representing protease and helicase domains. Mice were immunized by intramuscular inoculation in a scheme of three doses spaced 15 days among them. By using an IFN-γ ELISPOT assay and overlapping NS3 peptides, it was detected the highest levels of IFN-γ secreting T cells in the groups that received the helicase protein and the inactivated vaccine-helicase combination. In addition, T-cell depletion analysis showed that the observed IFN-γ secretion is due to CD4+ T cells. These results indicate that a purified recombinant NS3 helicase protein may be included in the dengue virus 2 inactivated vaccines in order to induce more potent and effective immune response.

IPK dengue group produced a full-length NS3 protein in E.coli cells and the antigenicity and immunogenicity of this protein was also evaluated in mice [32]. Results demonstrated that there was a T cell response to recombinant antigen upon *in vitro* stimulation in splenocytes obtained from DENV2-immunized mice. A Th1 response was induced with an IFNγ/TNFγ ratio in favor of IFNγ. It also detected low levels of IL-10 (Th2 cytokine) produced by these T cells upon NS3 stimulation. This purified protein was able to stimulate a Th-1-type response in mice which suggests that it may be incorporated in future dengue vaccine candidates. High IgG antibodies titers against the recombinant NS3 protein was also obtained. These results demonstrated the antigenic potential of this protein in order to include in a dengue vaccine formulation. The results shown of antigenic

characterization and immunogenicity studies on the NS3 protein allow recommending the inclusion of the recombinant NS3 protein as a dengue subunit vaccine. Further experiments are in progress by IPK dengue group in order to evaluate the immune response induced by and the immunopotentiating capacity of the recombinant NS3 protein in the Cuban vaccine formulations, monovalent and tetravalent Domain III-Capsid.

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