ISID Scientific Exchange Fellowship Report

Expression in *E.Coli* and Characterization of Dengue 2 Recombinant NS3 Protein.

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Rosa Ramírez Bartutis

Dr. Ramirez is a biochemist with a master degree in molecular biology and biotechnology. Currently, she is finishing her PhD program in Health Sciences. Her research focus on the expression in E.coli *and characterization of dengue virus proteins. Specifically, she is involved in the evaluation of the immunogenicity and protective capacity of NS3 protein in mice. She works at the Dengue Vaccine Lab, Institute of Tropical Medicine "Pedro Kourí."*

Background

Dengue is one of the most threatening mosquito-borne human viral diseases. It is caused by the four serotypes of dengue virus 1-4, members of the Flavivirus transmitted to humans by the bite of a domestic mosquito, *Aedes aegypti* (1, 2). Annually, an estimated of 50–100 million cases of dengue fever (DF) and several hundred thousand cases of dengue hemorrhagic fever (DHF) occur, depending on epidemic activity (3). The development of a tetravalent vaccine safe and effective against dengue viruses is necessary in order to eradicate the serious and threatening public health problem that constitute the dengue disease (4, 5). In this sense, a vaccine approach based on inducing dengue virus-specific cytotoxic T lymphocytes (CTL) and an appropriate humoral immunity should provide the best balance of protective dengue immune response. The identification of the target proteins of dengue virus-specific T cells should assist efforts to develop subunit vaccines against dengue virus (6).

The multifunctional NS3 protein has been identified as target of the cellular immune response. It has been demonstrated the predominance of cross-reactive CTL epitopes on dengue virus NS3 protein (7, 8). These CTL may play a protective role in limiting virus replication. In this study, the full length NS3 obtained from dengue 2 strain RNA isolated from patient serum infected during Cuba outbreak in 1997 with DHF was expressed with N-terminal histidine tag in *Escherichia coli (E.coli)* in order to characterize the NS3 protein immunized in BALB/c mice.

Methods

A cDNA fragment from DEN-2 (DEN-2) NS3 gene (1.8kb), obtained by RT-PCR with the RNA isolated from D2 58/97 2P C6/36 HT (Cuba outbreak in 1997) was cloned into a pQE-30 vector (Qiagen, USA). Different NS3 expression conditions in order to obtain a soluble NS3 protein with a histidine tag at the N terminus were evaluated in XL1-blue cells and compared with the protein expression pattern in *E.coli* cells transformed with a vector pQE30. The pellet after the centrifugation of the lysate was solubilized using urea 8M and then it was purified under denaturing conditions by passage through a His-Trap 5 mL column and Sephacryl S-200 HR 16/20 column using HPLC- AKTA purifier system. The samples were analyzed on 12% polyacrylamide gels and stained with Coomassie Blue and the inmunodetection of the NS3 was perfomed by Western Blot using anti-dengue 2 HMAF and anti-NS3 mAb. The purified and renatured NS3 protein was functional and antigenically characterized by enzymatic and immunological assays. Serine protease activity of the NS3 recombinant protein was conducted by a spectrophotometric assay, using the peptide substrate Ac-RTSKKR-pNA corresponding to pNA analogue of the 2A/2B cleavage site (9). The antigenic properties of the purified NS3 recombinant protein were analyzed by an amplified sandwich ELISA system using dengue-specific positive sera from mice. The protein was inoculated in Balb/c mice to evaluate the humoral immune response against DEN-2. Antibody titers against DEN-2 were determined by ELISA. Sera were also analysed by immunoblotting and immunofluorescence techniques.

Results

The cDNA fragment (1.8kb), containing the coding region for NS3 protein from DEN-2, was amplified from the viral genome by RT PCR. The amplified fragment was cloned into the pQE30 vector and completely sequenced. After the selection of the positive clones, three of them were choiced to evaluate the NS3 protein expression. The expressed protein was associated with insoluble inclusion bodies. Recombinant protein was recovered from the cell lysate by affinity chromatography on a His-Trap column. Because recovery of the recombinant protein was inefficient in native extraction buffer, lysis and purification were

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carried out under denaturing conditions (urea 8M), followed by a refolding process to obtain right protein conformation. Protein bands consistent of expected sizes were obtained (70kDa), accounting for 15% of total bacterial proteins when SDS-PAGE and Western Blot analysis were made. Sufficient purified DEN-2 NS3 protein was obtained to assess its proteolytic activity against the para-nitroanilide substrate. The refolded 6xHis-NS3 protein was found to cleave the chromogenic substrate. The enzymatic cleavage was dependent on both substrate and enzyme concentrations. However, when the activity of the NS3 protein was compared with the activity reported by the NS3 protein and NS2B protein together (protease complex), the NS3 protein showed much greater specific activity than the protease complex.

The NS3 recombinant protein showed its antigenicity against an anti-dengue 2 HMAF. Immunized mice showed a strong induction of anti NS3 IgG antibodies, as determined by ELISA and Western Blot analysis. All sera samples reacted with the native NS3 protein band at expected size and positive immunofluorescence on DEN-2 infected C636 cells was also observed.

Conclusions and Further Development

This study sponsored by the International Society for Infectious Diseases (ISID) yielded results that will allow evaluating the NS3 protein as a vaccine candidate. First, the study confirmed that the NS3 recombinant protein obtained was in a right conformation with enzymatic activity. Second, our results showed a good immunogenicity of the recombinant NS3 protein. Protection studies against viral challenge in mice are being conducted with NS3 protein alone and co-immunized with dengue 2 envelope domain protein in order to consider the DEN-2 NS3 protein as an immunoenhancer in a vaccine formulation for humans. Our work on the NS3 immunogenicity is being peer-reviewed for publication. Last, I would like to thank the ISID for supporting the research carried out in the Prof. Ronaldo Mohana-Borges lab at the UFRJ, Brazil. I found in this lab a highly motivated team dedicated to structural genomic in an excellent scientific and technical environment. I am also grateful to Dr. Ronaldo and his colleagues for their thoughtful advice and vast experience, which they generously shared to support this project. They made my stay enjoyable experience in a beautiful city. During my stay in Dr. Ronaldo Lab, I had the opportunity to write, and submit a collaboration project to the Brazilian governmental agencies CAPES in order to go on studies of molecular characterization on dengue nonstructural proteins. This project has been recently approved.

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