Flow Cytometric Determination for Dengue Virus-Infected Cells: Its Application for Antibody-Dependent Enhancement Study

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Abstract

The theory of antibody-dependent enhancement plays an important role in the dengue virus infection. However, its molecular mechanism is not clearly studied partially due to lack of a sensitive assay to determine the dengue virus-infected cells. We developed a flow cytometric assay with anti-dengue antibody intracellular staining on dengue virus-infected cells. Both anti-E and anti-prM Abs could enhance the dengue virus infection. The anti-prM Ab not only enhanced the dengue virus infected cell mass, but also increased the dengue virus protein synthesis within the cells. The effect of anti-prM Ab-mediated enhancement on dengue virus infection is serotype-independent. We concluded that the target cell-based flow cytometry with anti-dengue antibody intracellular staining on dengue virus-infected cells is a sensitive assay to detect the dengue virus infected cells and to evaluate the effect of enhancing antibody on dengue virus infection on cell lines or human primary monocytes.

Keywords: Dengue, enhancing antibody, ADE, flow cytometry.

Introduction

Dengue is an acute infectious disease caused by the dengue virus, of which four serotypes have been identified. It is characterized by biphasic fever, headache, pain in various parts of the body, rash, lymphadenopathy and leukopenia[1,2]. In most cases, the disease of dengue fever is self-limited. However, there is a risk of progression to dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS), especially when cross-infection of different serotypes occurs. DHF is a severe febrile disease characterized by abnormalities of haemostasis and increased vascular permeability, which in some instances results in DSS. DSS is a form of hypovolemic shock that is associated clinically with haemoconcentration and frequently leads to death. We have reported the involvement of anti-platelet and anti-endothelial cell autoantibodies, in addition to immune deviation and cytokine over-production in the development of DHF[3-6]. An immunopathogenesis has been proposed to explain the progression of DF to DHF/DSS.[7-8] However, a major issue in the DHF/DSS pathogenesis is not

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solved. That is the antibody-dependent enhancement theory. Antibody-dependent enhancement (ADE) of infection has long been thought to play a central role in the dengue virus infection. The ADE theory has been formulated to explain the finding that the more severe manifestations of DHF/DSS seem to occur predominantly, although not exclusively, in children experiencing a second dengue virus infection that has a different serotype from the first one. It is reasoned that the non-neutralizing anti-dengue antibody bound to a dengue virion would enhance the virus entrance into target cells via the Fc receptor. However, the molecular mechanism of ADE has not been clearly illustrated, and is still a mystery since Halstead proposed it in the early 1970s\cite{9,10}. The traditional assay for ADE is done by plaque assay which is time-consuming and influenced by various parameters such as the virus strains, cell type and the multiplicity of infection. In this study we reported a sensitive flow cytometry to detect the dengue virus-infected cells and the ADE effect was evaluated on the target cell-based assay.

**Materials and Methods**

**Cells, viruses and reagents**

Cells of P388D1, K562, 206-35 and BHK were maintained in Dulbecco’s Modified Eagle (DME) medium supplemented with 10% fetal bovine serum (FBS). All four dengue virus serotype strains were obtained from the Center for Disease Control (CDC) in Taiwan. Unless otherwise specified, the DENV-2 strain PL046 was used as the source for most experiments. Viruses were propagated in mosquito C6/36 cell line which was incubated in Eagle’s minimal essential medium containing 2% heat-inactivated FBS at 28 °C for 5 days.\cite{11} The PL046 virus can reach a titre of 1x10^8 PFU/ml by standard quantification methods on BHK cells.

**Production of anti-dengue virus monoclonal antibody**

Breeder mice of BALB/c strain were purchased from either The Jackson Laboratory, Bar Harbor, ME, USA, or Charles River Japan, Inc. (Atsugi, Japan). They were maintained in the animal facility of the Medical College, National Cheng Kung University, Tainan, Taiwan. The animals were raised and cared for following the guidelines set up by the National Science Council of the Republic of China. Six- to twelve-week-old mice were used in all experiments. Groups of BALB/c mice were inoculated intravenously with DENV-2 PL046 (1 x 10^8 PFU)\cite{11}. The mice were sensitized intraperitoneally 3 or 4 times at two-week interval with 1 x 10^7 PFU. Before the fusion, the mouse was boosted intravenously with 1 x 10^6 PFU for three days. The splenocytes were then fused with FO myeloma using 1% PEG as described previously\cite{12}. Several methods including cellular ELISA, immunohistochemical staining and flow cytometric assay on dengue virus-infected BHK cells were used to screen the antibodies. The isotypes of each monoclonal antibody were identified using mouse monoAb ID kit (Zymed Laboratories, San Francisco, CA). The antigenicity of each clone was further characterized by Western blot or immunoprecipitation of dengue-infected C6/36 total cell lysate.

**Monoclonal antibody biotinylation**

Various monoclonal antibodies were purified from mouse ascites by Montage Prosep-G kit (Millipore, Bedford, MA). Purified antibodies were dialysed against 0.1 M phosphate buffer saline (PBS), pH7.4 and their concentration determined by measuring the absorbance at 280 nm. A 1 mg/ml solution will have an absorbance of 1.4 in a cuvet with a 1 cm path length. 2 mg/ml antibody solution was reacted with NHS-LC-Biotin (Pierce, Rockford, IL) and then incubated for 30 minutes at room temperature.
Excessive or un-reacted biotin reagents were removed by extensively dialysing against PBS.

**Infection and preparation of virus-antibody complexes**

The infection was performed by co-incubating cells with dengue viruses at the multiplicity of infection (moi) of 1~10 for 90 min at 37 °C. For antibody-enhancing experiments, the virus-antibody complexes were prepared by mixing monoclonal antibodies (10 μg/ml) with viruses for 30 minutes at 37 °C before addition to the cell suspension (2 x 10^5/sample) for infection. After infection, cells were washed twice and resuspended in complete medium for further culture. At various time points, cells were assayed for virus infection by flow cytometric analysis, and the supernatants were assayed for virus production by plaque assay on BHK cells.

**Flow cytometric detection of dengue virus-infected cells**

Various dengue virus-infected cells were harvested and fixed with 2% paraformaldehyde in PBS for 20 minutes on ice. After fixation, they were washed with PBS twice and then stained with appropriate amounts of different biotinylated anti-dengue monoclonal antibodies (anti-E, anti-prM, anti-NS1 and anti-Core antibodies) in the permeabilization buffer (2% FBS, 0.1% saponin, 0.1% sodium azide in PBS) for 30 minutes on ice. These cells were then washed with permeabilization buffer twice and stained with streptavidin-FITC in the same buffer for another 30 minutes. Finally, these cells were washed again with the permeabilization buffer and resuspended in staining buffer (2% FBS, 0.1% sodium azide in PBS) for flow cytometric analysis by FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Flow cytometric data were re-analysed using the software WinMDI version 2.8.

**Results**

A flow cytometry to detect dengue virus-infected cells

To study the mechanism of enhancing antibody on dengue virus infection, a flow cytometric analysis can be employed with anti-dengue antibody intracellular staining, in which the percentage of dengue virus-infected cell mass can be quantitated. Using the monoclonal anti-dengue E, NS-1, or core antibody, the intracellular dengue protein can be stained per cell. Either the adherent BHK cells (Figure 1A) or suspension K562 cells (Figure 1B), the intracellular staining of the dengue virus envelope, prM, NS-1 or core antigen with biotinylated anti-NS1, anti-E, anti-prM or anti-core monoclonal antibodies followed by streptavidin-FITC can be detected. The proportion of dengue virus-infected cells was determined. Forty-one percentage of cells have detectable E protein at moi of 1 at 24 h post-infection on BHK cells. The proportion of cells expressing NS-1 or prM is equivalent to that of E protein, but less cells expressed core protein. For K562 cells, a similar observation was found, twenty-seven percentages, twenty-four, and twenty-five percentages of cells expressing E, NS-1, and prM proteins, respectively. The low percentage of core protein positive cells comparing with that of the E/NS1/prM-expressing cells was probable due to the low affinity of the anti-core antibody used. By testing on macrophage-like P388D1 cell lines, we found that different monoclonal anti-E and prM antibodies had different effects on the enhancement of dengue virus infection. Anti-prM Ab was found to enhance dengue virus-infected cell mass. Some anti-E antibodies, for example 185-10, enhanced the dengue virus-infected cell percentage, but anti-E Ab 137-22 abolished the DENV-2 infection (Figure 2A).

The anti-prM Ab 70-21 also enhanced the DENV-2 infection. The anti-dengue NS1 or anti-core antibodies did not have any effect on
Figure 1. **Flow cytometric assay to quantitate the dengue virus-infected BHK (A) or K562 cells (B)**

[BHK (A) and K562 (B) cells were infected by dengue virus at moi = 1 and 0.1, respectively. After 24 hours post-infection, dengue virus-infected cells were determined by intracellular antibody staining. The antibodies used included biotinylated anti-dengue monoclonal antibodies (anti-core, anti-prM, anti-E, or anti-NS1 antibody) followed by streptavidin-FITC. The percentage of dengue antigen positive cells was shown. Mock infection was used as the negative control.]
Anti-dengue Antibody Staining on Dengue Virus-Infected Cells

Figure 2. The enhancement of dengue virus infection by anti-E or anti-prM antibodies
(P388D1 cells were infected by dengue virus at the moi = 1 in the presence of monoclonal anti-prM Ab 70-21, anti-E Ab 185-10 and 137-22 (10 µg/ml). The dengue virus infected cells were determined by intracellular antibody staining on flow cytometry. The effect of anti-prM or anti-E Ab on dengue virion production was shown in the bottom. At 24 or 48 hours after dengue virus infection on P388D1 cells, the virus production in the culture supernatant was determined by the standard quantification methods on BHK cells. An asterisk (*) denotes the significant difference from virus control group.)

Dengue virus infection (data not shown). This flow cytometric assay of dengue virus-infected cells was compared with the traditional plaque assay (Figure 2B). The anti-E Ab 185-10 and anti-prM Ab 70-21 enhanced the DENV-2 virion production, but anti-E Ab 137-22 neutralized the DENV-2 infection at both 24 and 48 h post-infection.

We went further to check the enhancement by anti-prM Ab on various moi of dengue virus infection. As shown in Figure 3A, the anti-prM Ab enhanced the DENV-2-infected cell mass was observed on moi from 0.001 to 1; the lower the moi, the higher the fold of enhancement. When the moi is high as 10, no enhancement was observed. Furthermore, the mean fluorescent intensity of the NS-1 protein per infected cell was also higher in the anti-prM Ab-treated group than non-treated control, suggesting that the viral protein synthesis in the DENV-2-infected cells was also enhanced by the anti-prM Ab (Figure 3B). Although the flow cytometric method was demonstrated to be comparable to the traditional plaque assay, it has the advantages of being simple, fast, sensitive, time-saving and target cell-based. The flow cytometric assay not only provides the information on the percentage of dengue virus-infected cells, but also the relative amount of synthesized dengue virus protein per cells, whereas the plaque-forming unit determined by the plaque assay is a combination of increased infected mass and virus replication.

Antibody-dependent enhancement of dengue virus infection on various cells

To further understand the role of antibody in the dengue virus infection, a hybridoma cell line (206-35) and primary cells were tested. Anti-prM Ab 70-21 enhanced the dengue virus infection at the doses of 1—40 µg/ml, the anti-E Ab 185-10 enhanced the dengue virus infection at doses of 10—40 µg/ml (Figure 4A). The enhancement seems to be dose-
For the human peripheral blood monocytes, anti-prM Ab 70-21 and anti-E Ab 185-10 could enhance the DENV-2-infected CD14 positive cells (Figure 4B). Furthermore, the monoclonal anti-prM Ab 70-21 was derived from DENV-2-infected mice, it cross-reacts with four serotypes of dengue virus by immunohistochemical staining of dengue-infected cells (data not shown). This anti-prM Ab 70-21 could enhance the dengue virus infection of all four serotypes (Figure 5). Based on the above results, we concluded that this anti-prM Ab 70-21 could enhance the dengue virus infection of all four serotypes.
target cell-based flow cytometry with anti-dengue antibody intracellular staining in dengue virus-infected cells was a sensitive assay to detect the dengue virus infection and could be used for the evaluation of antibody-dependent enhancement on dengue virus infection.

**Discussion**

Dengue disease is an acute infection caused by the dengue virus that has four different serotypes. Most dengue infections are subclinical or very mild, as manifested by undifferentiated fever with or without rash that are seen in infants and young children. On the other hand, older children and adults may develop dengue fever (DF). In general, DF is self-limited, but there is a risk of progressive development to DHF or DSS. Viral replication plays an important role in the disease pathogenesis. Severe DHF is associated with a higher virus load than mild DF\(^{13}\). ADE is a central hypothesis to explain the increased dengue-infected cell mass\(^{9,10}\). The ADE theory proposes that non-neutralizing antibodies from a previous infection would trigger DHF/DSS in secondary infection if it involves a different serotype of the virus. Newborn babies less than 1 year of age with maternal anti-dengue IgG antibodies were known to be susceptible to DHF/DSS post primary infection\(^{14,15}\). The enhancing antibody is previously presumed to act against the dengue virus envelope protein. Using a sensitive flow cytometry for the detection of the dengue virus-infected cells, we reported in this study that anti-prM antibody could also work as an enhancing antibody. It enhanced the dengue
Anti-dengue Antibody Staining on Dengue Virus-Infected Cells

Dengue virus is icosahedral, ~500 A in diameter[20-22]. It contains three structural proteins: an anchored capsid consisting of 113 residues, a pre-membrane consisting of 166 residues and a glycoprotein consisting of 495 residues. There is a maturation step for an immature prM dengue particle to become a mature infectious virion. The prM-containing dengue particle has 60 icosahedrally organized trimeric spikes on the particle surface. Each spike consists of three prM:E heterodimers. The cleavage of the prM glycoprotein by furin or a furin-like protease creates an N-terminal pre-peptide 91 amino acid long and an M protein 75 residues long. This cleavage leads to the dissociation of prM:E heterodimers and the formation of E:E homodimers. The glycosylated pre-peptide is released from the maturing particle, altering the accessibility of domain II of the E glycoprotein to fusion. The infectivity is increased 100-fold in the prM-containing tick-borne encephalitis virus after furin treatment[23]. The pre-peptide components of the prM protein in each spike cover the fusion peptide to prevent the E protein from prematurely undergoing conformational changes that trigger fusion in endosomal vesicles. The mature virion has a smooth surface, which is different from the immature virion that had prM-containing spikes. Our anti-prM antibody can bind to the dengue virion produced in the mosquito C6/36 cell line. The immunoprecipitated dengue virion by anti-prM Ab showed E (50 kD) and prM (20 kD) (unpublished data) by Western blot, indicating that there are immature prM particles in our dengue virus preparation. Currently, we are determining the epitopes recognized by our enhancing antibodies. The possibility that anti-prM Ab binds to a prM-containing particle, causing a conformation change that increases the exposure of a fusion peptide on domain II of E, thereby causing it to become fusogenic to target cells, and hence resulting in increased infection, is not excluded.

ADE is known as an in vitro observation when a non-neutralizing antibody or sub-neutralization amount of antibody is present in the culture with the virus. The virus will enter the cells through the Fc receptor and the replication of the virus in the cells will be enhanced. Takada and Kawaoka have reviewed the possibility that ADE can involve the complement C3 fragment receptor, C1q and its receptor C1qR, in addition to the Fc receptor[16]. Antibody bound to a receptor-binding site of the viral protein induces a conformational change, which facilitates membrane fusion with HIV. Viral replication via ADE entry to suppress cellular anti-viral gene response occurs in Ross River virus[17]. For dengue virus infection of Fc receptor-bearing macrophages or B cells, ADE is mediated primarily by the Fc receptor pathway. Complement CR and C1qR were not involved because we used heat-inactivated FCS to culture the target cells. Since the receptor for dengue virus is not clearly identified, whether anti-prM antibody binds to dengue virion to cause the conformational change and increase the entry of dengue virus requires the demonstration of a dengue virus receptor. Although DC-SIGN has been reported as a dengue receptor, no ADE phenomenon was observed on immature dendritic cells[18]. The possibility of IL-10 production post dengue virus infection via FcR signalling to suppress the further dengue virus replication is intriguing. We have found that FcRII was involved because anti-FcRII Ab blocked the dengue virion binding and infection on K562 cells (unpublished observation). The immune complex binding to FcRII was reported to enhance the IL-10 production[19]. We are currently testing this possibility.
References


