Journal of Immunological Methods 441 (2017) 24–30

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

NK cell degranulation as a marker for measuring antibody-dependent cytotoxicity in neutralizing and non-neutralizing human sera from dengue patients

Peifang Sun [a,*], Brian J. Morrison [b], Charmagne G. Beckett [c], Zhaodong Liang [a], Nishith Nagabhushana [a], An Li [d], Kevin R. Porter [b], Maya Williams [b]

[a] Henry Jackson Foundation, United States
[b] Naval Medical Research Center, United States
[c] Navy Bloodborne Infection Management Center, United States
[d] University of Maryland College Park, United States

ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form 26 October 2016
Accepted 12 November 2016
Available online 14 November 2016

Keywords:
Dengue virus
ADCC
NK cell degranulation
Dengue serum
CD16

ABSTRACT

The study assessed antibody-dependent NK cell degranulation, a biomarker relevant to antibody-dependent cell cytotoxicity (ADCC), to analyze dengue immune sera. We first determined binding intensity of patient sera to the surface of DENV-infected cells and examined the types of antigens expressed on infected cells. Antigens from pre-membrane (PreM) and envelope (E), but not from NS proteins were detected on the surface of infected cells. After adding NK cells to infected target cells previously treated with patient sera, rapid NK cell degranulation was observed. Non-neutralizing patient sera generated comparable NK cell degranulation as that of neutralizing sera, suggesting ADCC may be a protective mechanism apart from Ab neutralization. The level of NK cell degranulation varied dramatically among human individuals and was associated with the level of CD16 expression on NK cells, informing on the complexity of ADCC among human population.

1. Introduction

Antibody (Ab) neutralization is generally regarded as a protective mechanism against DENV infection. However, results from field clinical studies and from the tetravalent CYD phase 2b vaccine trial do not support a strong correlation between Ab neutralization and protection (Sabchareon et al., 2012). Meanwhile, Ab-neutralization to DENV is complicated by Ab-dependent enhancement (ADE). The theory of ADE suggests that low/non-neutralizing Ab can enhance DENV infection, which is responsible for the increased disease severity associated with secondary infection of DENV in the field. Unfortunately, there is no reliable method to differentiate a protective from an enhancing Ab response (Laoprasopwattana et al., 2005; Guzman et al., 2002).

Neutralizing Abs function by blocking or deactivating infectious viral particles to prevent viral infection. However, if a viral particle escapes from neutralizing Abs and establishes an infection inside a target cell, neutralizing Abs may no longer be effective; instead, the immune system may launch the cell-mediated immunity, i.e. T cell-mediated antiviral activity and Ab-dependent cell cytotoxicity (ADCC), to defend against the infection. ADCC begins with the binding of the viral-specific IgG to infected cells expressing viral antigens on the cell surface. These antigen-Ab immune complexes (IC) then interact with FcγR III (CD16) on a number of different effector cells, namely NK cells, monocytes, and neutrophils, leading to the activation of the effector cells to kill infected cells (ADCC).

Virus neutralization and ADCC activity can be mediated by the same Ab, but they are often mediated by Abs with different specificities. Very commonly, Abs without detective neutralizing activities can mediate ADCC. The role of ADCC has been extensively studied in HIV patients, and the presence of ADCC Abs appears to be more critical for controlling disease progression in HIV carriers than neutralizing Abs (Baum, 2010; Wren et al., 2013; Jegaskanda et al., 2013).

ADCC against DENV has been demonstrated in a few studies. NK cells can directly kill DENV infected cells in the presence of serum samples from confirmed dengue cases (Kurane et al., 1984). ADCC activities in pre-illness plasma from school children in Thailand appeared to correlate with neutralizing Ab titers and immune protection against secondary DENV-3 infection, but such a correlation was not seen with DENV-2 infection (Laoprasopwattana et al., 2007). The number of NK cells in the peripheral blood of dengue patients in Brazil rose earlier than $CD8^+$ T cells and seemed to be associated with mild disease (Azeredo et al., 2006). All these studies used a traditional ADCC assay with radioactive materials.

In this study, we developed a non-radioactive assay to analyze serum Ab activity related to ADCC, a NK cell degranulation assay aimed to measure effector cell degranulation (CD107a expression). Testing patient serum samples using the degranulation assay, we

* Corresponding author.
E-mail address: peifang.sun2.ctr@mail.mil (P. Sun).

http://dx.doi.org/10.1016/j.jim.2016.11.005
0022-1759/

found that non-neutralizing sera had comparable NK cell activation Abs as that of neutralizing sera. Our study suggested that the non-neutralizing Abs maybe protective through ADCC mechanisms.

2. Materials and methods

2.1. Serum samples

The study protocol (NMRC2012.0012) was approved by the Navy Medical Research Center Institutional Review Board in complete compliance with all applicable federal regulations governing the protection of human subjects. De-identified human serum samples were used. All samples were tested for neutralizing Ab titers against all 4 serotypes of DENV using a method published previously (Sun et al., n.d.). Non-field samples were used as negative controls and tested negative for DENV Abs by an in-house ELISA.

2.2. Virus strains

DENV-1 West pac 74 and DENV-2S16803 cultivated on Vero cells were used for the study (Sun et al., 2006). The viruses were propagated in our lab and titrated on DC-SIGN Raji cells (NIH AIDS Repository).

2.3. Culture medium

The culture medium was RPMI 1640 w/o L-Glutamine supplemented with 1% of Penicillin 10,000 IU/ml and Streptomycin 10,000 μg/ml (Invitrogen, MA), 1% L-glutamine 200 mM in 0.85% NaCl, and 1% Non-essential Amino Acid (all from Mediatech, Corning, MA) and 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, UT).

2.4. Infection of DC-SIGN Raji cells with DENV

DC-SIGN Raji cells at 1×10^6/ml were pulsed with DENV at the ratio of 1 ml of cells + 1 ml of virus for 1 h at 37 °C and 5% CO_2. Experimental controls included cells pulsed with un-infected normal Vero cell supernatant or with complete medium alone. Cells were washed to remove cell-free virus and continuously cultured for the indicated time. Percentages of infected cells were determined at the end of the culturing period using a fluorescent monoclonal Ab (2H2) specific for DENV PreM. Cells were harvested, washed 2–3 times with RPMI 1640 (without FBS and any other additives) to remove free viral particles. The target cells were suspended in complete medium, counted and brought to 2×10^6/ml.

2.5. Binding of human serum Abs to infected target cells

Binding of human serum Abs to target cells was performed in 96 well plates. Briefly, DENV infected and un-infected target cells described above were pipetted into a 96 well U-bottom tissue culture plate (Costar, Corning Incorporated, NY) at 50 μl/ml (1×10^5 cells/well). An equal volume of complete medium containing or not containing serially diluted human sera was added to both the infected and uninfected target cells, and the plate was placed in the dark for 1.5 h on ice. After incubation, the cells were washed with PBS twice and were stained with a secondary Ab, anti-human IgG-PE (eBioscience, San Diego, CA) with 50 μl/well at the concentration of 5 μg/ml in PBS for 30 min on ice. The cells were then washed twice with PBS and run on a flow cytometer (FACS CANTO II, BD Bioscience, NJ). The mean fluorescent intensity (MFI) of PE represents the level of binding of human serum Abs to the cell surface. The serum-untreated cells serve as a baseline control for serum-treated cells. The serum-treated uninfected cells serve as a control for non-DENV-specific binding. DENV-specific binding is the value above the baseline and the non-DENV-specific binding which is obtained by subtracting the experimental MFI with the corresponding control MFIs.

2.6. Staining target cells with monoclonal anti-PreM and E and polyclonal anti-NS1, NS3 and NS5 Abs

To test the type of antigens expressed on the surface of DENV-infected target cells, the following monoclonal and polyclonal Abs were used: monoclonal Ab 2H2-FITC specific to DENV PreM (in house), monoclonal Ab 4G2 specific to DENV E protein (in house), rabbit polyclonal IgG Abs to NS1, NS3 and NS5 (GeneTex Inc. Irvine, CA). The 4G2 Ab was an unlabeled mouse Ab and a secondary goat-anti-mouse FITC (in house) was used to label the 4G2 Ab. Both 2H2 and 4G2 monoclonal Abs are DENV group-specific, capable of reacting to all 4 serotypes of DENV. The polyclonal rabbit Ab were all un-labeled Abs and a secondary PE-goat-anti-rabbit Ab (Imgenex Coporation, San Diego, CA) was used. All these polyclonal rabbit Abs were raised against DENV-2 according to the technical notes from the vendor. Briefly, DENV infected and uninfected target cells were stained with 2H2, 4G2, anti-NS1, NS3 and NS5 Abs for 30 min on ice. Cells stained with 2H2-FITC were directly acquired on FACS CANTO II. Cells stained with 4G2, NS1, NS3, and NS5 were further stained with the corresponding fluorescent secondary Abs for another 30 min on ice and then acquired on FACS CANTO II. Since we did not see binding of anti-NS1, NS3 and NS5 Abs to surface of DENV-2 infected cells, we performed an intracellular staining to see the intracellular expression of these proteins. Briefly, infected and uninfected target cells were fixed using Perm-Fix (BD Bioscience, NJ) and permeablized using Perm-Wash (BD Bioscience, NJ) according to the manufacturer's instruction. The cells were then stained with the polyclonal Abs and then stained with the fluorescent secondary Ab using the Perm-Wash buffer. We have used cells stained with the secondary Ab alone as controls for non-specific binding of the secondary Ab. For all the staining experiments, we have included un-infected cells as a control.

2.7. Effector cells

The effector cells for the NK cell assay were the non-adherent fraction of peripheral blood mononuclear cells (PBMCs) from random healthy donors. The preparation of non-adherent PBMCs has been described previously (Sun et al., 2006). Briefly, cryo-preserved whole PBMCs were thawed, washed and brought to 2×10^6 cells/ml in culture medium, and were placed into a 6 well plate (Becton Dickinson Multiwell™ Primaria™ 6 well, Becton Dickinson and Company, Franklin Lakes, NJ) at 3 ml/well. The plate was kept at 37° C for 2 h to allow monocytes to adhere to the plate. After adherence, non-adherent cells were gently collected from the plate into a 15-ml or a 50-ml conical tube. The non-adherent cells were then washed once and were brought to 1×10^6 cells/ml in culture medium. The cells were left in the 15-ml or 50-ml tube in a humidified 37 °C 5% CO_2 incubator overnight. Before adding the effectors to the target cells, they were washed once, counted and adjusted to 1×10^6/ml in culture medium.

2.8. Ab-dependent NK cell degranulation

Relevant ADCC activity was measured using a NK cell degranulation assay which was the expression of CD107a on effector cells using a modified method described by others (Chung et al., 2009). Briefly, DENV-infected and uninfected target cells were treated with serially diluted human sera in 96-well U bottom plates for 1.5 h on ice. The cells were washed twice with RPMI 1640 containing no additives to remove serum components in the supernatant. After the last wash, effector cells were added to the plate and cultures were kept at 37 °C, 5% CO_2 for 2 h or other specified time. The cells were then stained in 50 μl PBS with a cocktail of Abs containing 2.5 μl CD3-PerCP, 2.5 μl CD56-APC, 2.5 μl CD16-PE and 5 μl CD107a-FITC (all from BD Bioscience, NJ) at room temperature in the dark. After 30 min of staining, the cells were washed with PBS twice and acquired on a FACS CANTO II. The $CD3^-CD56^+$ NK cells were gated and the percentage of $CD107a^+$ cells