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Characterization of humoral and cellular immune responses in cynomolgus macaques upon primary and subsequent heterologous infections with dengue viruses

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Abstract

Since studying the pathogenesis of dengue virus associated disease in humans has several limitations, an appropriate animal model is needed. Therefore, we investigated kinetics of viremia as well as humoral and cellular immune responses, after primary, secondary and tertiary heterologous dengue virus infections in cynomolgus macaques: these parameters were largely similar to those observed in natural human infection upon primary infection. Both antibody and T-cell responses measured were largely cross-reactive. Upon secondary infection with a heterologous virus serotype, T-cell responses specific for the primary infecting serotype were more pronounced, especially when the immune system was primed with dengue 1 virus. Measurement of transcription levels of pro- and anti-inflammatory cytokines in white blood cells upon primary and secondary infection generally showed a balanced response. In addition, a region of the NS2A protein of dengue viruses was identified that induces T-cell responses in macaques.

Keywords: Dengue virus; Cynomolgus macaque; Sequential infections

1. Introduction

The importance of specific B- and T-cell immune responses has been studied extensively in several acute and chronic viral infections. Activation of memory B and T cells that are able to cross-react with newly invading pathogens may either result in protective immunity or in predisposition for immune-mediated pathogenesis [1,2]. In dengue virus (DENV) infections such predisposition upon secondary infection with a heterologous serotype has been observed.

Infection with any one of the four antigenically distinct DENV serotypes usually results in mild flu-like illness (dengue fever; DF). DENV infection induces protective immunity against re-infection with the same serotype [3]. However, subsequent infections with heterologous DENV serotypes have been postulated to leave the individual at risk of developing more severe clinical manifestations, including hemorrhages and severe plasma leakage, a life-threatening syndrome collectively known as dengue hemorrhagic fever (DHF) which may lead to dengue shock syndrome (DSS) [4,5]. Several studies have proposed a role for (sub-) neutralizing antibodies and T-cells in the development of DHF, implicating cross-reactive B and T cells in the pathogenesis of enhanced disease seen in DHF and DSS [6,7]. Prospective studies in South East Asia offered the opportunity to analyze immune responses in small cohorts of children experiencing secondary DENV infections [8–10]. These studies, however, were limited in the number of subjects studied, the identification of the DENV involved in these infections and the measurement of the time span between sequential infections.

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Several monkey species are known to be susceptible to DENV infection although they do not present clinical signs. In this study we investigated the potential of cynomolgus macaques (*Macaca fascicularis*) as an animal model to study DENV infections. To this end, we experimentally infected cynomolgus macaques with DENV-1 or DENV-4. The effect of pre-immunity to DENV on the immune responses triggered by subsequent heterologous infections, was studied upon secondary DENV-3 and tertiary DENV-4 infections in these animals. Serotype specific and cross-reactive humoral and cellular immune responses were studied.

2. Materials and methods

2.1. Animals and viruses

The following viruses were kindly provided from Dr. V. Deubel (Pasteur Institute, Lyon, France): DENV-1 strain 40514, DENV-2 strain Jamaica 1982, DENV-3 PaH881 and DENV-4 strain 28128. Virus titers expressed as $TCID_{50}$/ml were determined by plaque assay on Vero E 6 cells as previously described [11].

Outbred population of *Macaca fascicularis* were infected subcutaneously with $10^{6.4}$ $TCID_{50}$ of DENV-1 (DENV-1 group, $n = 6$) or $10^{6.2}$ $TCID_{50}$ of DENV-4 (DENV-4 group, $n = 6$). Both groups were subsequently infected with $10^{4.8}$ $TCID_{50}$ of DENV-3 and a year later with $10^{5.8}$ $TCID_{50}$ of DENV-4. Peripheral blood samples were collected daily for 14 days after infection events and weekly or biweekly thereafter. Plasma was separated from cells by centrifugation and cells were collected either by red blood cell lysis (white blood cells) or standard Ficoll extraction density centrifugation (PBMCs). All experiments carried out on primates were approved by the animal ethical committee of the Erasmus MC, Rotterdam, The Netherlands.

2.2. Virus detection

Presence of plasma viral RNA was measured by means of real time RT–PCR as previously described [12].

2.3. Antibody responses

DENV specific IgM and IgG antibodies were measured using commercially available kits (Focus Diagnostics, Cypress, CA, USA) according to the manufacturer's recommendations [13]. Samples with a ratio >1 were considered positive.

DENV neutralizing antibodies (VN) were measured as follows: plasma was 3-log titrated, allowed to neutralize 100 $TCID_{50}$ of each DENV serotype for 1 h at 37 °C and transferred to a confluent monolayer of LLC-MK_2 cells. After incubation at 37 °C for 7 days, cells were fixed with absolute ethanol and developed with a fluorescent-based immunoassay using an in-house produced polyclonal DENV antibody and a rabbit anti-human IgG FITC-labeled conjugate (Dako, Glostrup, Denmark). The cut-off titer for VN antibodies was set at 1:50. Statistical analysis was preformed using one-way ANOVA test of the GraphPad Prism version 4 software (San Diego, CA, USA) and $P \leq 0.05$ values were considered significant.

2.4. T-cell responses against whole DENV

The frequency of DENV-specific interferon (IFN)-γ-secreting cells was determined in PBMCs using an ELIspot kit (U-Cytech) according to the manufacturer's recommendations. Autologous B cells were infected with the respective DENV serotypes and used as antigen presenting cells for PBMCs at a ratio of 1:5 (per 200,000 PBMCs). Cell suspensions were transferred on ELIspot plates, and developed according to the manufacturer's recommendations. Spots were enumerated with an automatic spot reader (Bioreader 3000, Bio-Sys GmbH). For statistical analysis and graphical representation, spot numbers were considered after subtraction of the background and the one-way ANOVA test was used (differences were considered significant when $P \leq 0.05$).

2.5. T-cell responses against DENV peptides

Short regions with high amino acid (aa) sequence diversity in the DENV polyprotein were selected for peptide design. A panel of 96 peptides were synthesized (PEPscreen© Sigma Genosys, The Woodlands, TX, USA), including some previously described human epitopes. The characteristics of the peptides are available from the authors upon request. PBMCs were enriched for DENV specific T cells and used in an IFN-γ ELIspot as described above with the following differences: Matrix peptide pools were prepared containing either 6 or 8 peptides per pool (each peptide at final concentration of 1 μM, and each peptide was present in two different pools). Peptides that were positive in both pools in which they were present, were subsequently tested individually at a concentration of 5 μM, in triplicate using 100,000 PBMCs per well. Reactive peptides were identified as follows: mean of spot numbers of the negative controls + 3 × S.D. (where negative controls were unstimulated PBMCs).

2.6. Measurement of expression of inflammation markers

Blood cells were separated from plasma by centrifugation and red blood cells were lysed (Roche Diagnostics, Almere, The Netherlands). Total RNA was isolated from white blood cells with phenol:chloroform (Fluka). An oligo dT primer (Invitrogen) was used for cDNA synthesis of mRNA using RT-superscript III (Invitrogen) according to the instructions of the manufacturer. Between 50 and 100 ng RNA was copied into cDNA. cDNA was used as a template for measuring expression of cytokines with real time PCR (primers available from the authors upon request) and a TaqMan Universal PCR kit (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) according to the instructions of the manufacturer. Transcript numbers were calculated relative to the internal control (GAPDH) with the following formula: $2^{-\Delta ct} * 10^5$, where